Anal. Chem. **2007,** *79,* 4066−4073

Spatiotemporal Micropatterning of Cells on Arbitrary Substrates

Vinay V. Abhyankar and David J. Beebe*

Department of Biomedical Engineering, University of Wisconsin—Madison, Madison, Wisconsin 53706

This paper presents a readily accessible patterning platform—based upon geometric constraints, discrete cell suspension droplets, and controlled cell settling—that provides both temporal and spatial patterning capabilities. As a demonstration, single-cell (and bead) suspensions as well as multicellular human embryonic stem cell colonies are spatiotemporally patterned onto arbitrary substrates. These substrates include tissue culture surfaces, cell monolayers, protein-coated surfaces, and 3D gel matrices. The generation of soluble factor gradients is also demonstrated. This method is completely passive and does not require external power sources. Spatiotemporal patterning provides a foundation for future biological studies that explore the time-dependent relationships between cell−cell signaling and cellular responses.

The in vivo environment is a complex yet structured system where cellular positioning is highly regulated and soluble factor signaling plays an important role in maintaining, or inducing, appropriate cellular responses. Microfluidic approaches represent excellent platforms for studying biological interactions because these methods provide precise control over both exogenous cues and cellular positioning in a culture. The predictability and control over cellular-scale domain interrogations, plug flow pulse treatments, and concentration/temperature gradients[1−4] have facilitated quantitative correlations between environmental factors and resulting cellular responses.[5−7]

The ability to control the position of cells (spatial micropatterning) in a culture is particularly useful because it allows physiologically relevant cells to be cultured together in an organized fashion in vitro in an attempt to recapitulate the in vivo behavior of the target cell population. Organizational control over a culture also provides a convenient way to investigate cell−cell communication; the separation distance between cell populations can be used to probe the degree of soluble factor interactions.[8−10] The importance of spatial micropatterning in biological assays has led to the development of numerous techniques[11−13] that effectively position cells in a culture. The most common techniques include microcontact printing and stencil based methods.

Microcontact printing transfers a desired film onto a surface using a precoated, geometrically defined polymeric stamp.[14,15] Complex patterns can be created by strategically positioning both adhesion promoting and limiting regions onto the patterning surface. Stencil-based methods rely on physical, rather than chemical, means to define pattern cells. A polymeric membrane containing defined through holes is applied to the patterning surface and physically prevents cells from attaching anywhere other than the areas defined by the through holes.[16−18] Thus, the location and geometry of adherent cell patterns on the surface is defined by the size and location of the through holes in the stencil.[16−18] Adjacently placed microchannels can similarly be used to pattern cells; the geometry of the patterned regions is defined by the channel width, and the separation distance between the cell populations is defined by the distance between the microchannels.[19] The addition of microfluidic functionality to an organized culture requires the alignment of microchannels over the patterned regions.[20,21]

Soluble factor interactions are typically studied by culturing cells with a medium removed from another culture (i.e., a conditioned medium) or by creating co-culture environments that

* To whom correspondence should be addressed. E-mail: djbeebe@wisc.edu.

(1) Jeon, N. L.; Dertinger, S. K. W.; Chiu, D. T.; Choi, I. S.; Stroock, A. D.; Whitesides, G. M. *Langmuir* **2000**, *16* (22), 8311−8316.
(2) Takayama, S.; Ostuni, E.; LeDuc, P.; Naruse, K.; Ingber, D. E.; Whitesides, G. M. *Chem. Biol.* **2003**, *10* (2), 123−130.
(3) Jeon, N. L.; Baskaran, H.; Dertinger, S. K. W.; Whitesides, G. M.; Van De Water, L.; Toner, M. *Nat. Biotechnol.* **2002**, *20*, 826−830.
(4) Mao, H.; Yang, T.; Cremer, P. S. *J. Am. Chem. Soc.* **2002**, *124* (16), 4432−4435.
(5) Taylor, A. M.; Blurton-Jones, M.; Rhee, S. W.; Cribbs, D. H.; Cotman, C. W.; Jeon, N. L. *Nat. Methods* **2005**, *2* (8), 599−605.
(6) Flaim, C. J.; Chien, S.; Bhatia, S. N. *Nat. Methods* **2005**, *2* (2), 119−125.
(7) Lucchetta, E. M.; Lee, J. H.; Fu, L. A.; Patel, N. H.; Ismagilov, R. F. *Nature* **2005**, *434* (7037), 1134−1138.
(8) Atencia, J.; Beebe, D. J. *Nature* **2005**, *437* (7059), 648−655.
(9) Bhatia, S. N.; Balis, U. J.; Yarmush, M. L.; Toner, M. *Biotechnol. Prog.* **1998**, *14* (3), 378−387.
(10) Bhatia, S. N.; Balis, U. J.; Yarmush, M. L.; Toner, M. *J. Biomater. Sci., Polym. Ed.* **1998**, *9* (11), 1137−1160.
(11) Sia, S. K.; Whitesides, G. M. *Electrophoresis* **2003,** *24* (21), 3563−3576.
(12) Chen, C. S.; Jiang, X. Y.; Whitesides, G. M. *MRS Bull.* **2005**, *30* (3), 194−201.
(13) Whitesides, G. M.; Ostuni, E.; Takayama, S.; Jiang, X. Y.; Ingber, D. E. *Annu. Rev. Biomed. Eng.* **2001**, *3*, 335−373.
(14) Whitesides, G. M.; Stroock, A. D. *Phys. Today* **2001**, *54* (6), 42−48.
(15) Tien, J.; Nelson, C. M.; Chen, C. S. *Proc. Natl. Acad Sci. U.S.A.* **2002**, *99* (4), 1758−1762.
(16) Ostuni, E.; Kane, R.; Chen, C. S.; Ingber, D. E.; Whitesides, G. M. *Langmuir* **2000**, *16*, 7811−7819.
(17) Folch, A.; Jo, B. H.; Hurtado, O.; Beebe, D. J.; Toner, M. *J. Biomed. Mater. Res.* **2000**, *52* (2), 346−353.
(18) Folch, A.; Ayon, A.; Hurtado, O.; Schmidt, M. A.; Toner, M. *J. Biomech. Eng.* **1999**, *121* (1), 28−34.
(19) Takayama, S.; Ostuni, E.; LeDuc, P.; Naruse, K.; Ingber, D. E.; Whitesides, G. M. *Nature* **2001**, *411* (6841), 1016.
(20) Khademhosseini, A.; Yeh, J.; Eng, G.; Karp, J.; Kaji, H.; Borenstein, J.; Farokhzad, O. C.; Langer, R. *Lab Chip* **2005**, *5* (12), 1380−1386.
(21) Khademhosseini, A.; Suh, K. Y.; Jon, S.; Eng, G.; Yeh, J.; Chen, G. J.; Langer, R. *Anal. Chem.* **2004**, *76* (13), 3675−3681.

10.1021/ac062371p CCC: $37.00 © 2007 American Chemical Society
Published on Web 04/28/2007

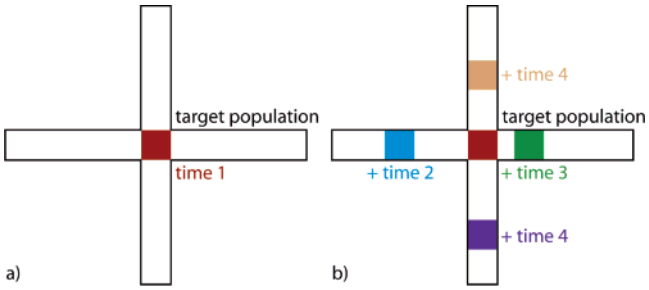


Figure 1. (a) Target cell population patterned at time 1. (b) Additional cell populations can be added to the culture at different locations and at different time points. Temporally patterned cell populations may help to determine the combinations of cell types (and the soluble factors they produce) that result in a desired behavior. The method describe here is an extension of the commonly used co-culture assay that is used to probe for autocrine/paracrine interactions between cell types. The extension allows different types of cells to be added to discrete locations of a culture at different time points in an attempt to isolate the timing windows critical for soluble factor interactions.

contain multiple cell types. While these approaches have shown utility in biological studies, they are not able to probe the temporal aspects of the signaling process. The method presented here allows cells to be added to a culture with both spatial and temporal resolution. The advantage of this approach is that it provides an additional variable with which to manipulate the environment and help study complex soluble factor interactions.

Using spatiotemporal patterning, all of the cells in the culture do not have to be added simultaneously and a co-culture environment involving cell types with different growth rates becomes possible simply by adding the cells to the culture at different times. Additionally, in traditional co-culture assays, it is difficult to observe localized effects of soluble factor interactions. Spatiotemporal patterning allows these localized effects to be investigated by adding a new cell population to a defined location of the culture and observing the responses of the surrounding cells. The addition of cells at different time points can then be used to isolate a temporal window that can help to determine when to add new cells in order to yield the desired result from a target population (see Figure 1).

Conditioned medium is also widely used to study soluble factor interactions. However, since conditioned medium is gathered from a culture after a given amount of time and the soluble factors present in the medium represent an aggregate of the factors produced by a culture where cell age and position in the cell cycle are not homogeneous, it is difficult to determine when the critical soluble factors are produced. Adding cells to discrete locations of a culture at different time points can help to determine when (i.e., day one, two, etc.) important the factors are produced by observing the behavior of the target population. Adding cells rather than soluble factors is beneficial when the identity of the factor and timing of the interaction is not known or when the half-life of the soluble factor is short.

We present a readily accessible microfluidic platform—based upon geometric constraints, discrete cell suspension drops, and controlled cell settling—that provides both spatial and temporal patterning capabilities (spatiotemporal patterning). This method does not require external power sources, connective tubing, chemical surface modification, or fluid flow. This method can be used to pattern single-cell suspensions or multicellular cell colonies onto arbitrary patterning substrates. We have demonstrated spatiotemporal patterning of human embryonic stem (hES) cell colonies onto mouse embryonic fibroblast feeder (MEF) layers and 3D Matrigel matrices as well as single bead and cell suspensions onto untreated polystyrene substrates. We also demonstrate the ability to create exogenous factor gradients in the system to treat patterned cell populations. Spatiotemporal patterning provides a foundation for future biological studies that explore the time-dependent relationships between cell−cell signaling and cellular responses.

THEORY

The design of the device guides cell settling into defined patterning corridors by using appropriately scaled fluidic reservoirs as shown in Figure 2. The design concept is analogous to a small, slowly flowing stream entering a large stagnant lake; the disparity in volume significantly decreases the flow velocity[23] and deposits sediment carried by the stream into the lake. Our system represents the limiting case where the input stream is stagnant (in the form of a cell laden drop) and provides a point source of sediment (cells) into the lake (microwell reservoir).

In the microfluidic analogy, the second layer creates a large-volume microwell above the patterning corridor (lake), and the aperture template allows discrete, small-volume cell suspension drops (stagnant stream) to be placed in contact with the liquid column of the microwell. Figure 2b demonstrates the patterning procedure. The contents of the drop settle through the reservoir and into the defined patterning corridor of the assay channel. The geometry of the patterning corridor limits the region where cells are able to enter the assay channel.

METHODS AND MATERIALS

Device Fabrication. Soft lithography using the polymer poly(dimethylsiloxane) (PDMS) was used to create a multilayer microfluidic platform.[24,25] As shown schematically in Figure 2a, patterning corridors are defined in the ceiling of the assay channel using two-layer photolithography.[26] A PDMS layer, containing large-volume microwells and an aperture template (containing 100-μm-diameter access holes) are aligned using a microscope and reversibly bonded (via conformal contact) to the assay channel. The assembled device is then reversibly bonded to the desired substrate. For the demonstrations shown here, the assay channel is 1 cm in length, 1 mm in width, and 25 μm in height. The patterning corridors are 1 mm by 1 mm squares defined in the ceiling of the assay channel, and the microwell layer contains the same geometry as the patterning corridors and is 1 mm in thickness.

Each layer is sterilized with UV light for 1 h before being reversibly bonded to the other layers. In our experience, reversible

(22) Rudy-Reil, D.; Lough, J. *Circ. Res.* **2004**, *94* (12), e107−116.
(23) Bird, R.; Stewart, W.; Lightfood, E. *Transport Phenomena*; John Wiley and Sons: New York, 2001.
(24) McDonald, J. C.; Duffy, D. C.; Anderson, J. R.; Chiu, D. T.; Wu, H. K.; Schueller, O. J. A.; Whitesides, G. M. *Electrophoresis* **2000**, *21* (1), 27−40.
(25) Jo, B.-H.; Van Lerberghe, L. M.; Motsegood, K. M.; Beebe, D. J. *J. Microelectromech. Syst.* **2000**, *9* (1), 76−81.
(26) Anderson, J. R.; Chiu, D. T.; Jackman, R. J.; Cherniavskaya, O.; McDonald, J. C.; Wu, H. K.; Whitesides, S. H.; Whitesides, G. M. *Anal. Chem.* **2000**, *72* (14), 3158−3164.

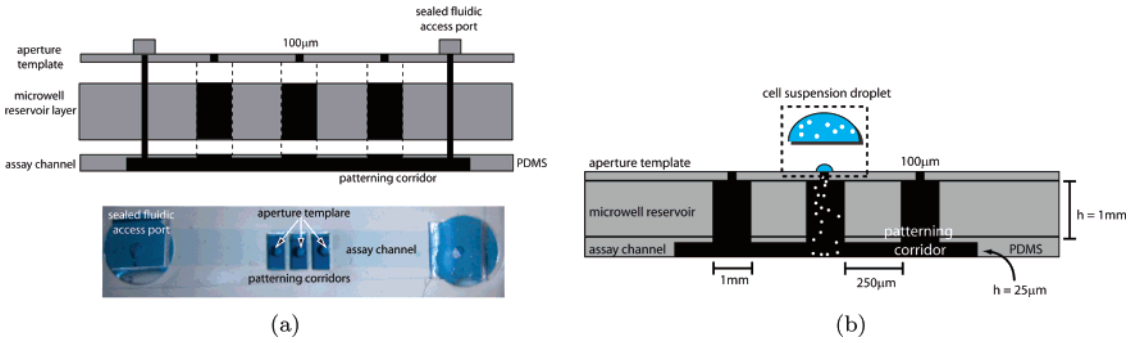


Figure 2. (a) Schematic representation the patterning device. Defined patterning corridors are created in the roof of the assay channel (layer 1) using multilayer lithography. The microwell reservoirs (layer 2) and the template aperture layers (layer 3) are aligned on top of the assay channel. (b) A small volume drop containing cells is placed on the access port of the aperture template. The drop makes fluidic contact with the microwell reservoir and deposits cells into the patterning corridors defined in the assay channel. Temporal patterning is possible because the individually addressable apertures allow cells to be added at different times.

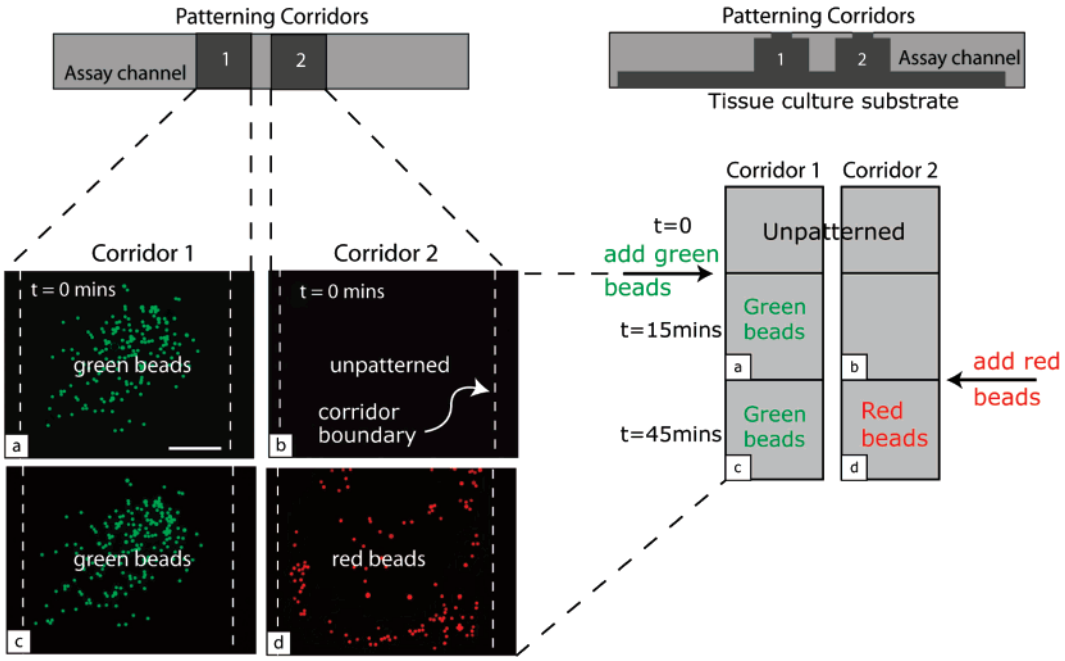


Figure 3. Red and green fluorescent beads used to demonstrate the spatiotemporal patterning of single cell/bead suspensions on a polycarbonate substrate. Both patterning corridors are unpatterned at $t = 0$. (a) Green beads are added into corridor 1 at $t = 15$ min while (b) corridor 2 is unpatterned. At $t = 45$ min, the (c) distribution of green beads remains undisturbed after (d) red beads are added to corridor 2. The dashed lines denote the corridor boundaries. Corridor−corridor spacing is 250 μm and scale bar is 250 μm.

bonding is acceptable when passive systems are used where fluid is not driven through the channels with a syringe pump. Irreversible bonding via oxygen plasma treatment may be required for long-term experiments (longer than one week) to prevent delamination and leakage between the layers over time. The channels are loaded through the fluidic access ports, which are then sealed. The open architecture (i.e., the open patterning ports) allows the system to be filled with media without bubble formation. Medium is changed by blocking off the holes of the aperture template with a PDMS slab and gently flushing out the assay channel with a syringe. The frequency of medium change depends upon the metabolic activity of the cultured cells. Cells are added to the system by placing cell-laden drops onto the aperture template covering the desired patterning corridor. The cells settle through the microwell reservoir and into the patterning corridor. Each corridor is individually addressable, and cells can be added to the corridors at different time points.

Cell Culture. Human embryonic stem cells and mouse embryonic fibroblasts were cultured and maintained as described in ref 27. The assay channel was first filled with a 0.1% gelatin solution and incubated overnight at 37 °C. The channels were then rinsed, filled with a suspension of irradiated MEF cells, and incubated. The channel was gently flushed to remove nonadherent MEF cells before hES cells were patterned into the desired corridor. Matrigel (BD Biosciences) was loaded into the assay channel at stock concentration and allowed to solidify at room temperature using the protocol described in ref 28. NMuMG cells were cultured and maintained as described in ref 29.

(27) Thomson, J. A.; Itskovitz-Eldor, J.; Shapiro, S. S.; Waknitz, M. A.; Swiergiel, J. J.; Marshall, V. S.; Jones, J. M. *Science* **1998**, *282* (5391), 1145−1147.
(28) Xu, C.; Inokuma, M.; Denham, J.; Golds, K.; Kundu, P.; Gold, J.; Carpenter, M. *Nat. Biotechnol.* **2001**, *19*, 971−974.
(29) Paguirigan, A.; Beebe, D. J. *Lab Chip* **2006**, *6* (3), 407−13.

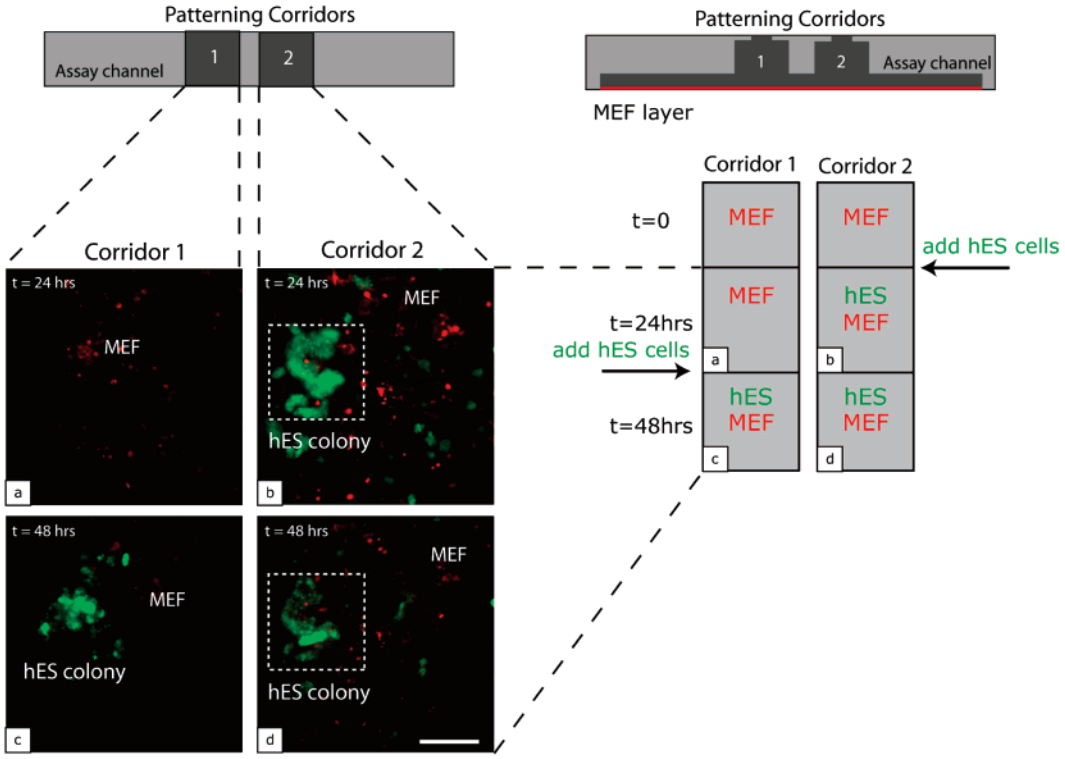


Figure 4. hES colonies spatiotemporally patterned in adjacent corridors on an MEF feeder layer. At $t = 0$ h, the corridors contain only MEF cells. hES colonies are then added to corridor 2 while corridor 1 remains unpatterned. (a) shows the unpatterned corridor 1, and (b) shows the hES colony in corridor 2 24 h after addition. hES colonies are then added to corridor 1. (c) shows the hES colony in corridor 1 at 48 h, while (d) shows the hES colony in corridor 1. Red and green fluorescent CellTracker dyes are used to label the MEF and hES cells. Scale bar, 150 μm.

Imaging and Staining. Red and green fluorescent 15-μm-diameter polystyrene beads were purchased from Molecular Probes. Red and green fluorescent CellTracker dyes (Molecular Probes) were used to label MEF and hES cells. The dyes were diluted to 15 μM concentrations and applied to each cell population for 30 min prior to the experiment. The unconjugated fluorophore Alexa 488 (Molecular Probes) was used to create the gradient that is shown in Figure 6. Fluorescence images were taken using an inverted microscope (Nikon IX70) and captured using Metamorph (Molecular Devices). ImageJ (version 1.33i) was used for image analysis.

RESULTS

Polystyrene Beads on Tissue Culture Substrate. Fifteen-micrometer-diameter green and red fluorescent polystyrene beads are patterned in adjacent patterning corridors at different time points. As shown in Figure 3, the green fluorescent beads are first patterned in corridor 1 while corridor 2 remains unpatterned. Thirty minutes later, red fluorescent beads are added into corridor 2. The distribution of beads in corridor 1 is unchanged when the beads are added to corridor 2, and no crossover of beads between the two corridors is observed.

Multicellular Colonies on a Cell Monolayer. Figure 4 demonstrates the spatiotemporal patterning of multicellular hES colonies on an MEF monolayer. Green and red fluorescent CellTracker dyes (Molecular Probes) are used to label the cells. The hES colonies are first added to corridor 2, while corridor 1 contains only MEF cells. Twenty-four hours later, hES colonies are added to corridor 1. The separation between patterning corridors is 250 μm. These cell types were chosen because undifferentiated hES cell culture was first demonstrated using a co-culture of hES cells and MEFs in 1998.[27]

Multicellular Colonies on on 3D Gel Substrate. hES cells can also be maintained in an undifferentiated state by culturing them on a Matrigel substrate and using a MEF conditioned medium.[28] In this demonstration, hES cells are spatiotemporally patterned on a 3D Matrigel substrate. Cell tracker dyes are again used to label the cells (Figure 5).

The assay channel is filled with Matrigel creating a 3D substrate (the thickness of the gel is determined by the height of the assay channel), and hES colonies are spatiotemporally patterned on the surface of the Matrigel. We have previously demonstrated that hES cells are viable in static microchannel culture for one week.[30]

Gradient Generation. The generation of a soluble factor gradient is demonstrated by sandwiching a 0.2-μm-pore diameter membrane between the assay channel and microwell layers. As shown in Figure 6, normal murine mammary gland (NMuMG) epithelial cells are first patterned in corridor 2 and the fluorophore Alexa 488 (Molecular Probes) is added (via a drop) to the microwell containing the porous membrane.

The fluorophore was added before the NMuMG cells attached to the substrate to demonstrate that the introduction of the fluorophore did not flush the cells from the downstream patterning corridor. Modeling of the diffusive front predicts that the gradient reaches its steady state (along the 250-μm distance between

(30) Abhyankar, V.; Beebe, D. In *Lab-on-Chips for Cellomics: Micro and Nanotechnologies for Life Science*; Andersson, H., van den Berg, A., Eds.; Kluwer: Dordrecht, The Netherlands, 2004; pp 257−272.

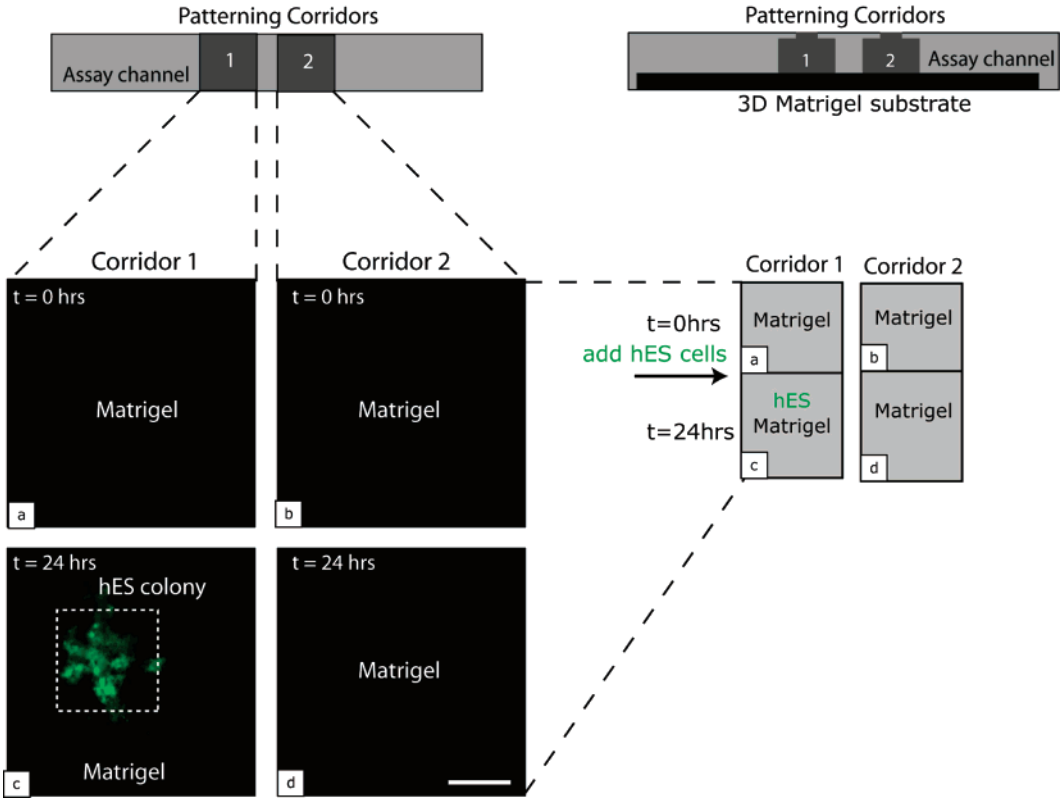


Figure 5. hES colonies spatiotemporally patterned in adjacent corridors on a 3D Matrigel substrate. At $t = 0$ h, the corridors (a) 1 and (b) 2 contain only Matrigel. hES colonies are then added to corridor 1 and (c) imaged after 24 h while (d) corridor 2 remains unpatterned. Green fluorescent CellTracker dye is used to label the hES cells. Scale bar, 150 μm.

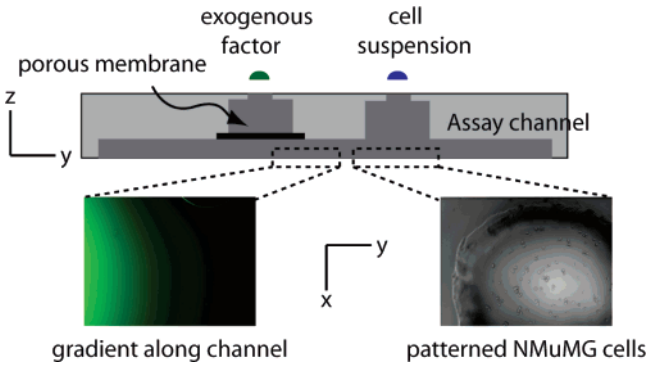


Figure 6. NMuMG cells are patterned in one corridor, and a gradient of the fluorophore Alexa 488 is created in the assay channel. A porous membrane is incorporated in the microwell to increase the fluidic resistance of the system, and the fluorophore is added to the microwell. The molecules diffuse through the membrane and create a gradient in the channel.

corridors) in 5 min. We have discussed the generation of stable chemical gradients further in ref 31.

DISCUSSION

Time-dependent signaling between cells (i.e., autocrine/paracrine signaling) and the extracellular matrix (ECM) helps to regulate cellular responses in vivo. Embryogenesis and early development exemplify the importance of time-dependent signaling; temporal waves of signaling factors are thought to help orient, reorganize, and differentiate groups of cells to form biochemically and organizationally complex structures.[32] In our system, soluble factors are added by sequentially adding new cell populations into the system or by creating exogenous factor gradients. Coupling spatiotemporal patterning of cell populations in a culture may improve the ability to conduct studies that delve into the effects of time-dependent signaling in vitro.

Design Parameters. The system is characterized by defined patterning corridors, large-volume microwell reservoirs, and an aperture template. The ratio between the input volume and the system volume, the sizes of the patterned particles are important parameters in the operation of the device.

The introduction of the drop displaces a volume of fluid in the system proportional to the volume of the input drop (e.g., the addition of a 0.1-μL drop displaces 0.1 μL of fluid in the system). The fluidic disturbance to the system can be limited by reducing the ratio of input drop volume to the system volume. The aperture template creates a rigid dosing region and provides a small fluidic contact area for the dispensed drop. The small surface area of the aperture template helps to decrease fluidic disturbance associated with the addition of a liquid drop onto a large liquid interface.

The relationship between gravitational settling and diffusion of a particle can be estimated by finding the ratio β between the diffusive and settling time scales. By applying the Stokes−Einstein relation for the diffusion coefficient and defining the Boltzmann

(31) Abhyankar, V. V.; Lokuta, M. A.; Huttenlocher, A.; Beebe, D. J. *Lab Chip* **2006**, *6* (3), 389−393.

(32) Ridley, A. J.; Schwartz, M. A.; Burridge, K.; Firtel, R. A.; Ginsberg, M. H.; Borisy, G.; Parsons, J. T.; Horwitz, A. R. *Science* **2003**, *302* (5651), 1704−1709.

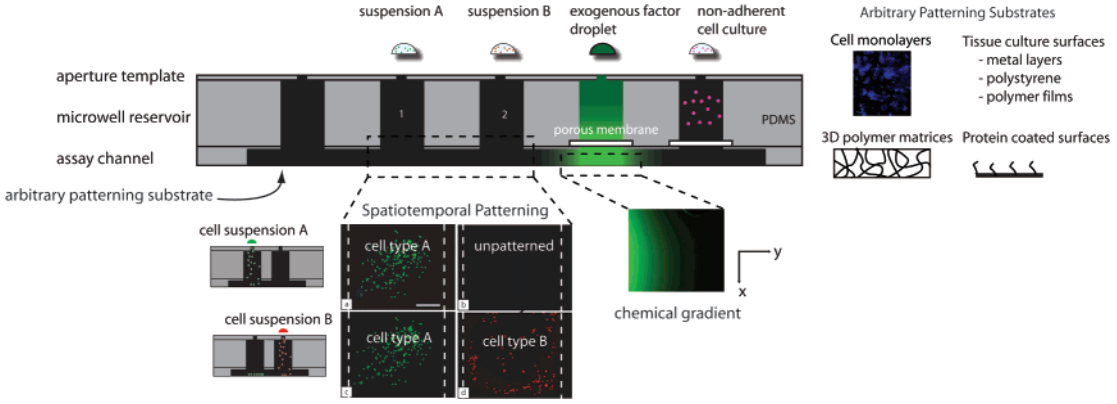


Figure 7. Schematic representation of the general capabilities of the platform. Spatiotemporal patterning is possible because each corridor is individually addressable. Multiple cell types can be sequentially introduced to create multiple unique co-culture environments on the same chip. Both the desired patterning geometries and the patterning substrates are arbitrary. Porous membranes increase the fluidic resistance of the system and facilitate soluble factor gradient generation. Nonadherent cells can be cultured in membrane-containing microwells. The droplet-based deposition can be incorporated into a robotic liquid handling system for high-throughput patterning.

characteristic length,[33] ($L_B = k_BT/(\mathrm{vol}_s\Delta\rho)$) and characteristic system length L, β represents the relation between the kinetic and the potential energy of the particle. When $\beta = 4L_B/L \ll 1$, sedimentation dominates (as is generally the case of particle above 1 μm in diameter).[34] The ratio illustrates why small molecules tend to diffuse rather than sediment. As a consequence, a small molecule or protein treatment must be applied to the entire assay channel, but multiple co-culture environments can created in the assay channel by sequentially adding cell populations to the desired corridor.

The ability of a patterned cell to remain within the footprint of the patterning corridor is explained using a Hjulstrom diagram. A Hjulstrom diagram is used by hydrologists to determine the relationship between fluid velocity and the size of particulate that can be transported by the stream.[35] According to the curve, a 10-μm-diameter particle (i.e., diameter of a small cell) requires a minimum stream velocity of 1 mm s^{-1} to maintain the particle in suspension (corresponding to a microchannel flow rate of 72 μL h^{-1}). The maximum flow rate that is possible in the system (as a result of a 1-mm pressure head) is 2.9 μL h^{-1}. The comparison between the flow rates above explains why the patterned cells are not redistributed along the channel after initial seeding as a result of small pressure differences. Cell redistribution as a result of pressure differences was not observed in our experiments.

Soluble Factor Addition. Laminar flow and diffusion-based microfluidic platforms allow micropatterned cell populations to be treated with soluble factor pulses and complex shaped gradients. Laminar flow facilitates rapid switching and precise control of a chemical microenvironment, but the continuous flow required to maintain these treatments disrupts important soluble factor interactions. Additionally, the development of an appropriate soluble factor cocktail for use in diffusion-based systems is problematic because the complex interplay between cell−cell, cell−ECM, and cell−soluble factor interactions makes it difficult to determine the precise sequence and identity of the released factors.

In this system, soluble factors are either introduced by adding new cell populations (adherent or nonadherent) at different time points or by creating soluble factor gradients. The sequential addition of new cell populations exposes the existing cells to the signaling factors produced by the newly added cells. Temporally presented endogenous signaling coupled with exogenous soluble factor gradients may help promote synergistic interactions where cells can autonomously control the release of subsequent factors as a function of their microenvironment.

The microwell above the membrane can also be used to culture nonadherent or suspension cultured cells by using membranes with pore diameters smaller than the cell diameter. The inclusion of these membranes allows suspension and adherent cells to be cultured together in the same device. The nonadherent cells can also present signaling factors to the adherent cells in the assay channel.

Exogenous factor gradients can be created in this platform by incorporating small-pore (0.2-μm pore diameter) microporous membranes into the design. The desired patterning corridor is covered with a small-pore-diameter membrane and then sandwiched between the first and second layers during fabrication. The membrane increases the fluidic resistance of the system and provides a source for an exogenous stimulus. A gradient is developed in the assay channel as the stimulus drop added to the microwell aperture diffuses through the membrane and into the channel. A detailed description of gradient stability using this design is discussed in ref 31. In addition to chemical stimuli, fixing and lysing agents can also be similarly introduced to the system through membranes.

Gradients can also be created by adding beads coated with an exogenous factor or drug-loaded degradable polymer beads into the desired corridor.[36,37] The gradient is developed as the factor is released from the bead and diffuses down the channel. The gradient characteristics are easily modeled by using bead/factor conjugates with known release kinetics. Figure 7 generalizes the functionalities provided by the platform.

(33) Faucheux, L. P.; Libchaber, A. J. *Phys. Rev. E: Stat. Phys., Plasmas, Fluids, Relat. Interdiscip. Top.* **1994**, *49* (6), 5158−5163.
(34) Berthier, J.; Silberzan, P. *Microfluidics for Biotechnology*; Artech House, Inc.: Norwood, MA, 2006.
(35) Chow, V.; Maidment, D.; Mays, L. *Applied Hydrology*; McGraw-Hill: New York, 1988.
(36) Langer, R.; Cleland, J. L.; Hanes, J. *Adv. Drug Delivery Rev.* **1997**, *28* (1), 97−119.
(37) Kohane, D. S.; Tse, J. Y.; Yeo, Y.; Padera, R.; Shubina, M.; Langer, R. *J. Biomed Mater Res. A* **2006**, *77* (2), 351−361.

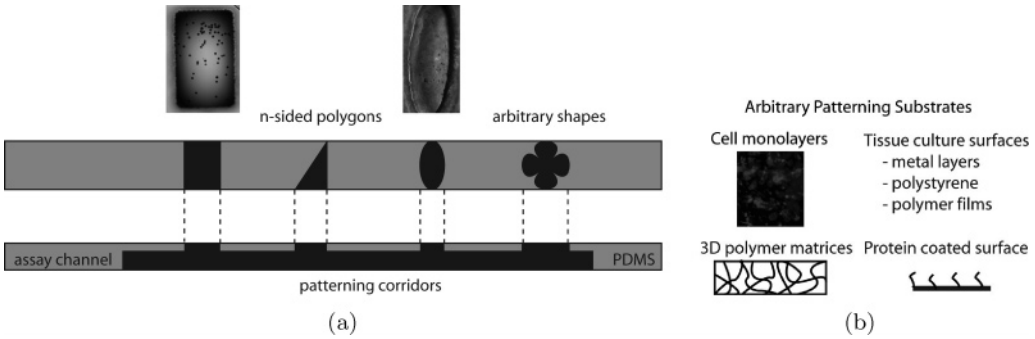


Figure 8. (a) Schematic representation of the patterns as defined in the roof of the assay channel. In static systems, the cells remain contained within the footprint of the defined shapes. (b) Defines the arbitrary patterning substrates (e.g., cell monolayers, 3D gels, metals, tissue culture surfaces, protein-coated substrates) that can be used the system.

Spatiotemporal Patterning. Temporal patterning is possible using other microfluidic methods but requires the combination of complex channel arrays with adhesion promoting patches and multiple channel alignment steps. Additionally, the available patterning surfaces are limited to those where the polymeric patterning elements (channels, stencils, or stamps) make a strong conformal seal with the surface. For example, hydrated gel surfaces are difficult to patten using these methods. Co-culture environments can be created by flowing new cell populations over the patterned populations but results in a loss in the overall spatial resolution of the culture.

The method presented here allows patterning of single-cell suspensions or multicellular colonies onto arbitrary substrates, and the individually addressable and geometrically defined corridors provide both spatial and temporal resolution (see Figure 8). Single-cell-sized particles sediment very quickly (e.g., 15-μm-diameter beads settle in 5 min), and a video showing beads settling into a patterning corridors is included as Supporting Information. As shown in Figure 3, with beads positioned on untreated polystyrene, surface modification is not required to pattern effectively. This functionality can be advantageous because the patterned cells can continue to spread after initial positioning and are not confined to an adhesive patch.[38,39]

The distance between corridors was chosen to be 250 μm for the demonstration purposes of this paper. We have achieved 50-μm resolution using high-resolution transparency masks, and the resolution between features can be increased to tens of micrometers by using high-resolution chrome masks and clean room equipment. Corridor−corridor spacing of 250 μm is not prohibitively large for cell−cell communication studies because soluble growth factors produced by cells are often small molecules with diffusion coefficients on the order of 10^{-5}−10^{-6} cm^2 s^{-1}.[40] There is recent evidence in the literature that soluble factor interactions can take place rapidly (on the order of hours) for cells in microchannel culture.[41−43.] Therefore, resolution on the order of hundreds of micrometers between corridors is acceptable for cell−cell communication studies that study phenotypical changes (on the order of days). Biological phenomena that require more immediate soluble factor communication require corridors patterned closer together.

The droplet-based deposition demonstrated here allows cells to be positioned directly into the desired location of the channel, and postpatterning microchannel alignment, multiple rinsing (or blocking) steps are not required. As a result, unwanted adhesion outside the patterning corridor is drastically decreased and assays involving primary cells (that are often difficult to isolate and are available in small numbers) become feasible. Droplet-based cell deposition is also easily integrated into robotic liquid handling systems for use in high-throughput assays.[44]

CONCLUSIONS

The platform presented here combines spatial and temporal patterning functionalities. This methods allows multiple co-culture environments to be created on a microfluidic chip by sequentially introducing cell population into the desired corridor of culture. The ability to spatiotemporally pattern cells onto 3D gels, cell layers, tissue culture surfaces, and protein-coated surfaces creates a robust and flexible platform. These capabilities provide a unique platform to study the relationship between cell−cell interactions, exogenous treatments, and downstream cellular responses.

The field of tissue engineering has long used three-dimensional ECM protein or polymer matrix constructs to promote the development of 3D cellular scaffolds in vitro.[45,46] Our method provides the ability to easily pattern cells on the top surface of gel constructs (e.g., Matrigel, collagen) in a spatiotemporal fashion to investigate temporal invasion into the matrix. For example, if cell type A is patterned and allowed to invade the matrix, biologically appropriate cells populations (B and C) can be added to adjacent corridors at different time points to investigate matrix invasion, hierarchal organization, and cellular branching.

The approach presented here may also provide insight into the effect of time-dependent signaling on 2D substrates. If cell type A exhibits a particular phenotype when exposed to cell type

(38) Chen, C. S.; Mrksich, M.; Huang, S.; Whitesides, G. M.; Ingber, D. E. *Biotechnol. Prog.* **1998**, *14* (3), 356−363.
(39) Chen, C. S.; Mrksich, M.; Huang, S.; Whitesides, G. M.; Ingber, D. E. *Science* **1997**, *276* (5317), 1425−1428.
(40) Alberts, B.; Johnson, A.; Lewis, J.; Martin Raff, M.; Roberts, K.; Walter, P. *Molecular Biology of the Cell*, 4th ed.; Garland Science: New York, 2002.
(41) Yu, H.; Meyvantsson, I.; Shkel, I. A.; Beebe, D. J. *Lab Chip* **2005**, *5* (10), 1089−95.
(42) Yu, H.; Alexander, C. M.; Beebe, D. J. *Lab Chip.* In press. DOI: 10.1039/b618793e.
(43) Yu, H.; Alexander, C. M.; Beebe, D. J. *Lab Chip* **2007**, *7* (3), 388−391.
(44) Meyvantsson, I.; Beebe, D. J. Submitted.
(45) Lavik, E.; Teng, Y. D.; Snyder, E.; Langer, R. *Methods Mol. Biol.* **2002**, *198*, 89−97.
(46) Levenberg, S.; Huang, N. F.; Lavik, E.; Rogers, A. B.; Itskovitz-Eldor, J.; Langer, R. *Proc. Natl. Acad Sci. U.S.A.* **2003**, *100* (22), 12741−12746.

B after 1 day in culture, but does not behave similarly if cell B type is added after the second day in culture, the assay has provided a temporal window that can be further investigated. The medium removed during the window can be analyzed using mass spectrometry to determine the identity of the factors.

The spatiotemporal functionality of this device coupled with exogenous factor treatments has potential applications in studying the time-dependent signaling involved in stem cell differentiation and cancer metastasis. Spatiotemporal patterning may be used in the future to determine whether undifferentiated human embryonic stem cells can be coaxed toward a particular lineage by adding appropriate cell populations into the culture at different times and different positions.

ACKNOWLEDGMENT

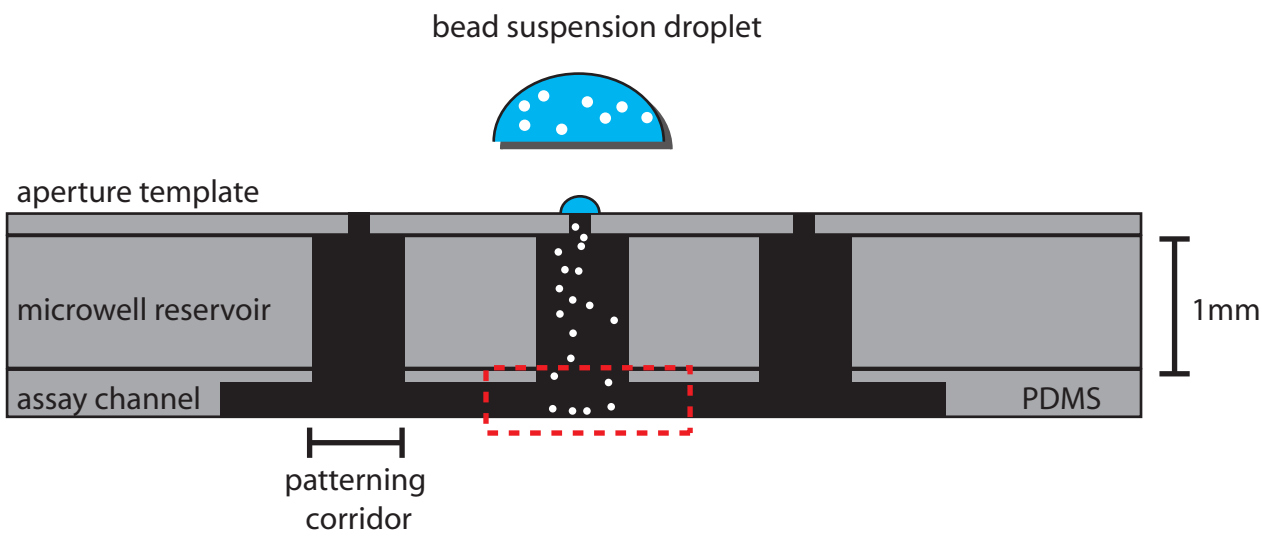
The authors thank Prof. Bill Murphy and the MMB research group for helpful discussions and WiCell Research Institute for kindly allowing the use of their facilities. This work was supported by NIH Grant No. K25-CA104162-02.

SUPPORTING INFORMATION AVAILABLE

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

Received for review December 14, 2006. Accepted March 26, 2007.

AC062371P



The supplementary video shows a top down view into the patterning corridor as highlighted by the red box. Fifteen micron diameter beads are added to the aperture template (via a droplet). The video shows the sedimentation of the beads into the patterning corridor. The beads take five minutes to sediment and settle within the geometic footprint of the patterning corridor.