Fabrication of a modular tissue construct in a microfluidic chip†

Derek A. Bruzewicz,‡ Alison P. McGuigan‡ and George M. Whitesides*

Received 4th January 2008, Accepted 5th March 2008
First published as an Advance Article on the web 20th March 2008
DOI: 10.1039/b719806j

By combining microfluidics and soft-lithographic molding of gels containing mammalian cells, a device for three-dimensional (3D) culture of mammalian cells in microchannels was developed. Native components of the extracellular matrix, including collagen or Matrigel™, made up the matrix of each molded piece (module) of cell-containing gel. Each module had at least one dimension below ~300 μm; in modules of these sizes, the flux of oxygen, nutrients, and metabolic products into and out of the modules was sufficient to allow cells in the modules to proliferate to densities comparable to those of native tissue (10^8–10^9 cells cm^{-3}). Packing modules loosely into microfluidic channels and chambers yielded structures permeated with a network of pores through which cell culture medium could flow to feed the encapsulated cells. The order in the packed assemblies increased as the width of the microchannels approached the width of the modules. Multiple cell types could be spatially organized in the small microfluidic channels. Recovery and analysis of modules after 24 h under constant flow of medium (200 μL h^{-1}) showed that over 99% of encapsulated cells survived this interval in the microfluidic chamber.

Introduction

Experimental systems that make it possible to study cells in a three-dimensional (3D) environment, at tissue-like cell densities (cells per unit volume), would be useful (i) in understanding the differences between cell behavior *in vitro* and *in vivo*,[1,2] (ii) in generating realistic *in vitro* models of disease,[3] and (iii) in testing drugs.[4] Although engineered tissues now provide experimental 3D systems for cell culture, and may eventually have clinical uses,[5,6] they are currently not ideal for high-throughput studies, such as those possible using microfluidic systems.[4] This paper describes work that combines the control and high-throughput potential offered by microfluidic devices with the versatility of modular tissue engineering[7] to develop a chip-based tool for studying the biology of mammalian cells in 3D environments at high densities of cells. We describe a method to organize modules (molded pieces of collagen that have controlled dimensions, and that contain cells[7–9]) in a microfluidic channel, and to use these organized modules to create an artificial "tissue construct" that can incorporate multiple cell types in 3D environments at densities of cells that approach the densities of real tissues (10^8–10^9 cells cm^{-3}). This system offers advantages over most 2D microfluidic systems, in which cells are cultured as a monolayer,[4] and over 3D systems limited to low densities of cells.[1,8] We demonstrate that 3T3 cells and HepG2 cells encapsulated in modules remain alive, and appear to metabolize normally in the assembled "tissue construct," when perfused with culture medium for at least 24 h.

Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA, 02138, USA.
E-mail: gwhitesides@gmwgroup.harvard.edu; Fax: +1-617-495-9857; Tel: +1-617-495-9430

† Electronic supplementary information (ESI) available: Cell culturing and multi-color labeling. See DOI: 10.1039/b719806j

‡ These authors contributed equally to this work.

Using this approach, we organized distinct populations of cell-containing collagen modules (distinguished by color or by type of cells) in a microfluidic channel. We also generated different patterns of assembled gel modules by varying the width of the microfluidic channel relative to the dimensions of the modules.

Background

Culturing cells on 2D substrates is often convenient, but many cell types behave differently in 2D and 3D.[10,11] Cells respond to cues in their environment, including exposure to soluble signals,[12] contact with neighboring cells,[13–15] and the mechanics and dynamics of the surrounding extracellular matrix (ECM).[16,17] Cellular signaling and response in tissues *in vivo* is therefore often different from that in cells cultured on a flat, rigid 2D substrate.[3,18,19] The technology for culturing cells in 2D is so familiar and standard that a 3D system must offer considerable advantages before investigators will adopt it.[10,11,20] Some desirable characteristics in a 3D culture system are (i) control over the position and shape of gradients in nutrients, oxygen, growth factors, and signaling molecules; (ii) control over the density and relative location of different cell types, and over the magnitude of physical forces such as fluid shear; (iii) an optically transparent container that allows easy characterization of the cells by microscopy;[20] (iv) reconfigurability of the system, so users can assemble, dismantle, and adapt the system to answer a range of biological questions; (v) ease of fabrication and use; (vi) the potential for high-throughput experiments, like those possible in 2D with 96-well plates; and (vii) available choices of extracellular matrix and maximum density of healthy cells that match those found in native tissue. Whether a particular *in vitro* system can mimic the *in vivo* behavior of interest often determines the choice of a system for cell culture, whether in 2D or in 3D. Thus, investigators would often prefer 3D systems that

create environments like those of native tissue to 2D systems, if appropriate technologies for creating 3D systems were available.

Seeding cells in polymer scaffolds or hydrogels is a popular method for generating engineered tissues.[1,8,9,21–28] Engineered tissues offer an interesting and controllable alternative to other 3D culture methods, such as cells aggregated into spheroids,[29–32] for studying cells in 3D. The main drawback to this approach is that most engineering methods focus on the production of one, relatively large (mm- to cm-scale), section of tissue. This is not ideal because (i) transport of nutrients and waste to and from the center of the artificial tissue limits the maximum possible density of healthy cells in tissues with dimensions greater than ~200 μm[33] and (ii) large tissue sections are not ideal for biological studies that often require many experimental treatment groups to be run in parallel. A method to generate, and culture in parallel, a large numbers of identical 3D engineered tissues with smaller volumes (and, presumably, at higher cell densities) would enable engineered tissues to be utilized as 3D culture systems for investigating 3D cell biology.

Recently, McGuigan and Sefton proposed a method to engineer artificial tissues by assembling cells encapsulated in molded collagen particles—or "modules"—with sub-mm dimensions (see ESI Fig. S1†).[7,34] Loosely packing tens-to-hundreds of these small modules, at random, into a tube yields a porous assembly permeated by random, irregular channels. When the diameter of the module is less than ~200 μm, every cell in the assembly is within ~200 μm of one of these porous channels. By perfusing cell culture medium through the network of interstitial channels, this strategy, in principle, overcomes the limitations of diffusion on nutrient transport to cells in the center of the artificial tissue. Growing cells in assemblies of modules offers many advantages over other methods for cell culture in 3D: (i) the sizes of the modules and the densities of cells in them are uniform and controllable. (ii) Batches of modules can be fabricated separately from different gel matrixes, or from different types of cells; modules from different batches can then pack together to form an assembly. (iii) The assembly of gel modules containing cells is reversible, and dissolution of the matrix protein can recover the individual cells for analysis at any time during the experiment. (iv) Cells in modules are easy to image and, after dissolution of the matrix, easy to characterize by light microscopy or flow cytometry.

The random packing of modules in an assembly leads to two significant disadvantages of modular assemblies: (i) little control over the microenvironment experienced by cells in the modules, and (ii) the inability to make ordered arrays of modules, instead of random mixtures. Microfluidics offers an attractive solution to these limitations[35,36] because it provides (i) optical transparency for easy characterization of the samples, and (ii) precise control over the microenvironment of the cells and over the dimensions of the microfluidic channel in which the modules pack together. Solid objects packed into a narrow microchannel form an ordered array,[37] in contrast to the random assemblies discussed above. Microfluidics also provides the potential for processing multiple samples in parallel. In this paper, we combine the attractive qualities of modular tissue engineering with the control offered by microfluidic systems to generate an on-chip device for 3D cell culture at high densities of cells (Fig. 1) for use in investigative studies.

Experimental

Experimental design

We wanted to develop a system that allowed controlled assembly of modules into a non-random artificial tissue, and organization of modules with different cell types into a single assembly. The experiments reported here demonstrate (i) fabrication of cell-containing gel modules of various sizes and with various types of cells; (ii) assembly of a modular artificial tissue by packing the modules into a microfluidic channel; (iii) continuous 3D cell culture of several modular artificial tissues in parallel by perfusion with cell culture medium for 24 h; (iv) recovery of the modules and subsequent biochemical assays on the constituent cells; and (v) organization in a single microfluidic chamber of modules that contain multiple types of cells. We used 3T3 fibroblasts and HepG2 mouse liver cells, because both types of cells are well characterized, and because HepG2 cells respond to the presence of nearby fibroblasts in several ways, one of which is an increase in the production of albumin.[38] A mixture of 3T3 cells and HepG2 cells on a chip therefore serves as a proof-of-principle demonstration that we can fabricate a 3D "tissue-on-a-chip" with multiple, interacting types of cells.[18] To show that modules containing cells from different populations can be combined, we built modular artificial tissues containing (i) modules made from either 3T3 cells and modules made from HepG2 cells; and (ii) modules made from one of three different populations of fluorescently labeled 3T3 cells.

Photolithography

Transparency masks were designed in Freehand[39] (Macromedia, Inc.) and printed by Pageworks.com (Cambridge, MA, USA). We spin-coated SU-8 100 (Microchem Corp., Newton, MA, USA) to form masters for channels up to 250 μm in height according to the manufacturer's instructions. To fabricate 500 μm high masters, we first oxidized a 7.5 cm silicon wafer in oxygen plasma for 5 min at 100 W. We then poured 3 g of SU-8 50 in the middle of the wafer and spin-coated the wafer at 500 rpm to form an even coating (no photoresist was allowed to spin off of the wafer). We baked the photoresist for 2 min at 40 °C, and then increased the temperature to 110 °C at 80 °C h^{-1}. After baking 1 h 45 min at 110 °C, the wafer cooled to room temperature. We exposed the baked photoresist for 100 s through a photomask (see above) at 50 mJ cm^{-2} (AB-M mask aligner) The bake after exposure required 1 min at 40 °C, then an increase in temperature to 110 °C at 80 °C h^{-1}. After baking for 20 min at 110 °C, the wafer cooled to room temperature. Developing the photoresist required ~5 min in propylene glycol methyl ether acetate (PGMEA) with sonication, followed by drying under a stream of nitrogen. The procedure for masters with a 1000 μm height was the same as the procedure for 500 μm high masters, except in the following respects: we used 6.5 g of SU-8 50, pre-baked the resist for 12 h at 110 °C (after the same gradual increase in temperature from 40 °C) before allowing it to cool to room temperature, exposed the resist for 300 s through the photomask (see above) at 50 mJ cm^{-2} (AB-M mask aligner), post-baked the resist for 80 min at 110 °C (after the same gradual post-bake increase in temperature described above), and

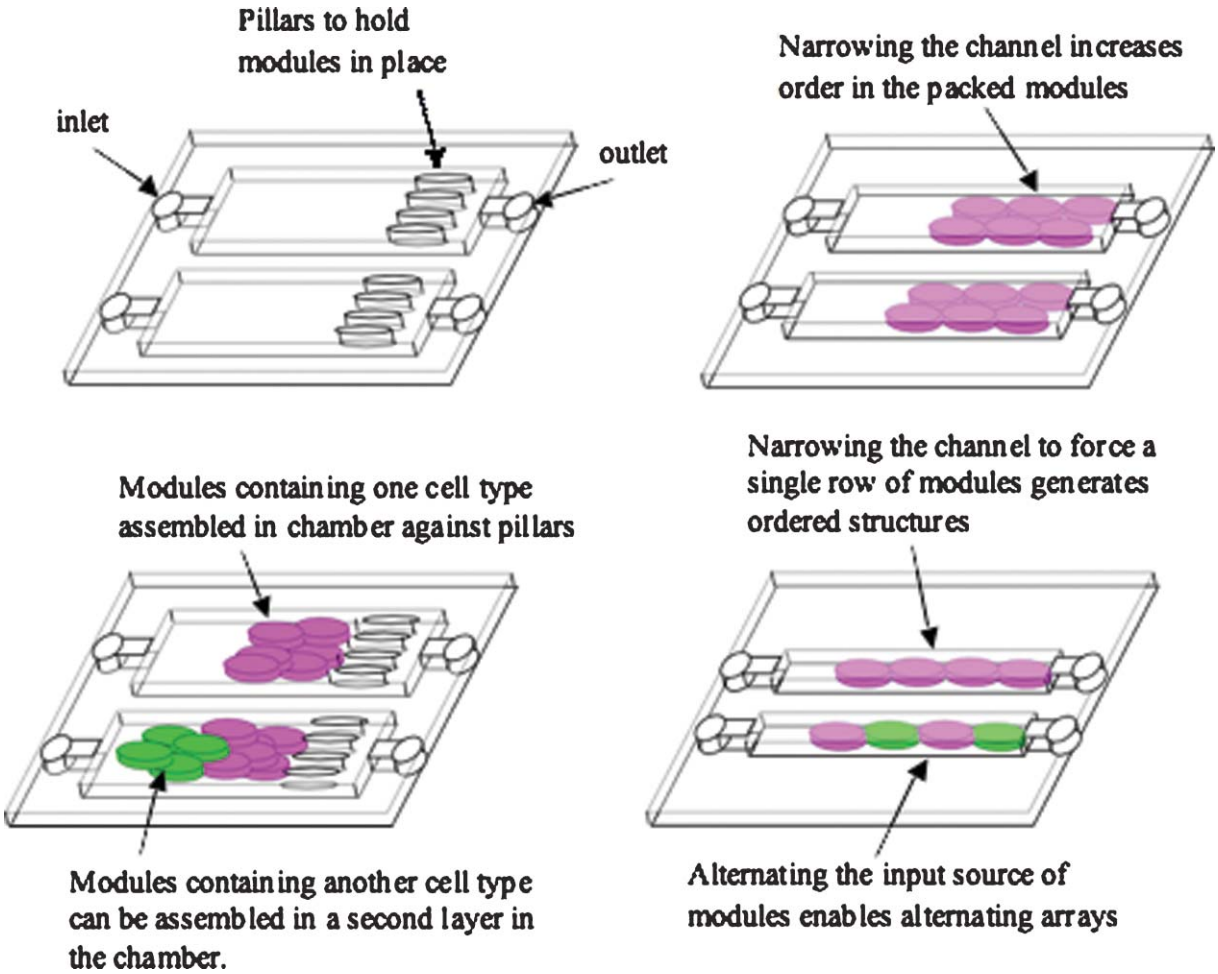

Fig. 1 Schematic suggesting the assembly of modules in a microfluidic chip.

developed the resist ~15 min in propylene glycol methyl ether acetate (PGMEA) with sonication.

Fabrication and cleaning of the membranes

We used a soft-lithographic method described elsewhere to generate poly(dimethyl siloxane) (PDMS) membranes for molding gel modules.[39,40] Photolithography (described above) generated an array of posts (25–800 μm high, 40–1000 μm wide) on silicon. After photolithography, masters were kept in a dessicator at 20 Torr with a few drops of tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane (United Chemical Technologies, Haverhill, PA, USA) for three hours. This treatment left a monolayer of fluorinated molecules on the surface of the master, and rendered the surface "non-stick" to most materials, including PDMS. We then spin-coated a degassed mixture of 10 : 1 Sylgard 184 base : catalyst over the posts for 20 s; faster spin times yielded thinner membranes. We wiped the tops of the posts with a slab of cured PDMS and cured the membrane at 70 °C for one hour.

Before filling the membranes with gel, we oxidized the membranes in air plasma at 1 Torr (SPI Plasma Prep II) and submerged them in Millipore water at 20 Torr to remove air from the PDMS and to keep the surfaces of the membrane hydrophilic. We cleaned the membranes with 2% Micro-90 detergent (International Product Corporation) in water, and then with ethanol. We repeated the plasma oxidation and then autoclaved the membranes in Millipore water to sterilize them.[41]

Fabrication of the modules

Fabrication of the modules followed McGuigan *et al.*;[40] we mixed NIH 3T3 cells (10^6 cells cm^{-3} of a 3 mg cm^{-3} solution of collagen (Inamed Biomaterials, Fremont, CA, USA) with 10X minimum essential medium (Gibco/Invitrogen, USA) and neutralized the pH with 1 N sodium bicarbonate. Dipping a clean, hydrophilic PDMS membrane in the mixture of gel and cells filled the holes in the membrane. Wiping the faces of the membrane on the edge of a Petri dish removed excess gel but did not affect gel already in the holes. Suspending the membrane on the edge of a Petri dish for 45–60 min in an incubator at 37 °C caused the gel to solidify. Shaking the membrane under cell culture medium released the modules.

Viability of cells in modules

We released the cells from the modules by digesting the gel in 100 μL of a solution (~50 000 caseinolytic units per cm^3) of dispase (BD Biosciences) for 45 min at 37 °C, followed by 100 μL of a solution of 1X trypsin for 15 min at 37 °C. After mixing the

suspension of single cells with Trypan Blue, we counted living (colorless) and dead (blue) cells.

Fabrication of the microfluidic chambers

After photolithography, masters were fluorinated as described above. Replica molding proceeded by pouring a degassed 10 : 1 w/w mixture of Sylgard 184 PDMS prepolymer and catalyst on the masters and curing the polymer at 70 °C for three hours. After curing, the replicas were peeled from the masters and 12G needles were used to punch holes for the inlets and outlets of each channel. A clean glass slide and the PDMS replica were oxidized in air plasma for five minutes and one minute, respectively. We laid the PDMS slab on the glass slide, so that the oxidized side (which bore the channels) made contact with the glass. After baking 20 min at 70 °C, irreversible bonding occurred between the glass and the PDMS.

To preserve the hydrophilicity of the PDMS, we filled the channels with water or phosphate-buffered saline (PBS) within 30 min of the plasma oxidation. Air bubbles did not form during subsequent experiments if the walls of the chamber were kept hydrophilic. For perfusion experiments (longer than 3 h), we stored the PDMS under water in a glass jar, overnight. Keeping the PDMS in water removed air from the polymer, and thus prevented the formation of air bubbles during perfusion.

Assembly of the modular construct in the chip

We used a filed and blunted 20G needle to punch an inlet and outlet in the ends of the chamber. We then inserted PE-60 polyethylene tubing into the inlet, and submerged the other end of the tubing in a 15 mL conical tube of culture medium (see above). To draw liquid through the channel, we inserted another section of tubing into the outlet of each chamber and connected the tubing to an empty 10 mL syringe *via* a 20G needle. We used suction to fill the microfluidic device with medium by withdrawing the plunger of the syringe before adding modules or other particles.

For the 3 mm wide channels, we assembled a layer of glass beads against the pillars in the microchannels by (i) submerging the inlet tubing in a suspension of glass beads, and then (ii) withdrawing the syringe connected to the outlet tubing. The layer of glass beads prevented modules from deforming and escaping the chamber during the flow of medium. To add modules to the chambers, we pipetted a sample of modules contained in cell culture medium into a conical tube and allowed the modules to settle. We moved the inlet tubing to the conical tube that contained the modules. Withdrawing the syringe at the outlet channel pulled modules into the channel to form a modular assembly. We pulled the modules into the chamber instead of injecting them through a needle to avoid tearing of the gel modules by the rough inside of the metal needle. For modules over 100 μm in size, it was possible to count modules in the inlet tubing before they packed together on the chip.

Construct perfusion

We perfused medium through the microfluidic chip containing the construct by connecting a syringe filled with culture medium, to the inlet, and regulating perfusion of the assembly with a syringe pump at a rate of 200 μL per hour. During perfusion, the entire microfluidic chip was submerged in PBS to prevent the formation of gas bubbles in the channels. A 50 mL conical tube collected waste medium from the tubing at the outlet.

Results and discussion

Fabrication of the modules

We fabricated cylindrical collagen modules (750 μm diameter, 500 μm length) containing 3T3 cells using a method described elsewhere.[40] After fabrication, we cultured a suspension of the modules in medium for seven days, during which time the cells proliferated in the gel, shrunk the modules (to an average diameter of 280 ± 7 μm and an average length of 210 ± 6 μm for 41 modules after seven days), and grew to a density near that of native tissue ($8.4 \pm 0.5 \times 10^7$ cells cm^{-3}, compared to 10^8–10^9 cells cm^{-3} for native tissue). The density of cells in the modules was calculated from measurements of the number of cells per module and measurements module dimensions by optical microscopy, as reported elsewhere.[40] The modules were denser, and easier to handle, after seven days in culture than immediately after fabrication, and we therefore only loaded seven-day-old modules into microfluidic chambers and channels (see below). Fig. 2A shows a light microscopy image of a module after seven days in culture. Dimensions of the modules were highly reproducible within and across batches.[40] Confocal

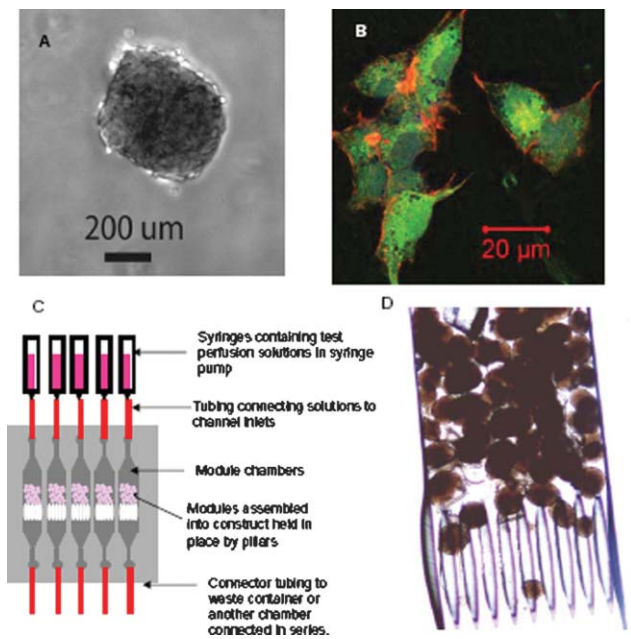

Fig. 2 Cell-containing gel modules in a microfluidic chamber. (A) Light-micrograph of a 750 μm collagen module containing 3T3 cells after five days in cell culture. (B) Confocal image of HepG2 cells in a collagen module (seeded at 10^6 cells cm^{-3} 17 h earlier). The nucleus was stained with DAPI (blue); the cytoskeleton was labeled with CFSE (green), and actin was labeled with phalloidin (red). (C) Schematic diagram of parallel microfluidic chambers for the assembly and perfusion of a porous assembly of modules (a "construct"). (D) Light-micrograph of a construct of modules in the channel. The long pillars of PDMS held the modules in place.

imaging of the modules revealed highly detailed 3D cell–cell interactions and cellular organization (Fig. 2B).

Fabrication of the chip

We used soft lithography[39] to generate two types of channels to form modular artificial tissues in microfluidic chips: a large (3 mm wide) chamber that yielded poorly organized modules, and a narrow (250 μm wide) channel that constrained the positioning of the modules. To hold the modules in place in the 3 mm wide chamber, we partially blocked one end of the chamber with an array of 250 μm wide, 2 mm long PDMS pillars at one end. The pillars were incorporated into the design (see Experimental), and prevented modules from exiting the chamber under the pressure of culture medium flowing at low to moderate rates. To prevent modules from deforming and exiting the chamber under the pressure of medium flowing faster than $\sim$100 μL h^{-1}, we loaded a layer of 260 μm wide glass beads into the microfluidic chamber before loading the modules into the chamber: to load the beads, we connected polyethylene tubing to both ends of the chamber and submerged the tubing at the inlet in a suspension of glass beads, then manually applied suction at the outlet with a 10 mL syringe.

We expected the packing of modules in narrow channels to be more organized than their packing in the 3 mm wide chambers. We fabricated narrow channels (150–500 μm wide) with narrow "bottlenecks" (40 μm wide) before the outlet. Convective mixing did not occur at the corners of channels wider than 500 μm that had this design.

Perfusion of the assembly and retrieval of the modules

Seven days before loading the microfluidic chambers, we fabricated collagen modules with encapsulated 3T3 cells (see Fabrication of modules, above) and suspended them in cell culture medium. To load the modules, we connected polyethylene tubing to the inlets of five parallel 3 mm wide microfluidic chambers (containing a layer of glass beads; see above). We submerged the polyethylene tubing in the suspension of modules. We then applied gentle suction at each outlet to load modules into the chip. Fig. 2C and 2D show a schematic drawing that represents modules packed on a chip, and a corresponding image taken using light microscopy. Passing the modules through the inlet tubing and into the channel (for assembly) or *vice versa* (to retrieve the modules from the device) did not visibly damage them.

Approximately one metre of polyethylene (PE-60) tubing connected each inlet to a 10 mL syringe of cell culture medium mounted on a syringe pump. One more metre of tubing connected the outlets to a single tube for waste medium. The long tubing allowed the medium (at the inlet) to be kept outside of the cell incubator at room temperature while the chip remained inside. The medium passed through approximately 80 cm of tubing inside of the incubators, so the medium reached 37 °C before flowing through the chip. We perfused the modular construct assemblies on the chip with medium at 200 μL h^{-1} for 24 h. The system is entirely compatible with longer periods of perfusion, but we believe that 24 h is sufficient to show that cells can be cultured successfully in 3D in the chip. Under high rates of flow, the collagen modules deformed, passed through the array of pillars, and exited the device; choosing a lower rate of flow allowed us to avoid deformation of the modules. Clogging of the device did not occur at any rate of flow used here. Deformation of the modules limited the flow rates that could be used. A flow rate of 200 μL h^{-1} was selected because this did not produce module deformation. Furthermore, this rate corresponded to 4.8 mL of medium over a 24 h period, and that amount of medium is sufficient to maintain the viability of an 75 cm^2 flask, containing $\sim 10^6$ 3T3 cells, for 24 h . Since the number of cells in one module was $\sim 10^3$, we expected all the cells on the chip to survive for at least 24 h under flow at 200 μL h^{-1}, even with 1000 modules in the chip.

After perfusion, we retrieved the modules from each channel by reversing the direction of flow and collecting the modules from the original inlet. The construct easily separated into individual modules after gentle pipetting. To verify that the cells inside the modular assembly remained healthy during perfusion, we digested the collagen component of the modules by incubating them in a solution of dispase and trypsin for 30 min, and pipetted the suspension up and down to break up the gel. We stained the digested solutions with Trypan Blue, which is excluded from living cells whose membranes are intact. Cell counts revealed that less than 1% of the total number of cells in perfused modules died after 24 h in the chambers.

Assembly of multiple cell types in 3D on a chip

To demonstrate the versatility of systems based on cell-containing gel modules for 3D cell culture in microfluidic chips, we built two types of mixed construct assemblies: (i) a semi-ordered mixture of modules containing 3T3 fibroblasts and modules containing HepG2 liver cells, and (ii) a highly ordered assembly of three fluorescently labeled populations of 3T3 cells. Both systems exploited the fact that narrow channels template the packing of objects that fill the channel.[37] The packing behavior of soft, cylindrical modules differs, however, from that of rigid glass spheres (Fig 3, see ESI† for a more detailed discussion of these differences).

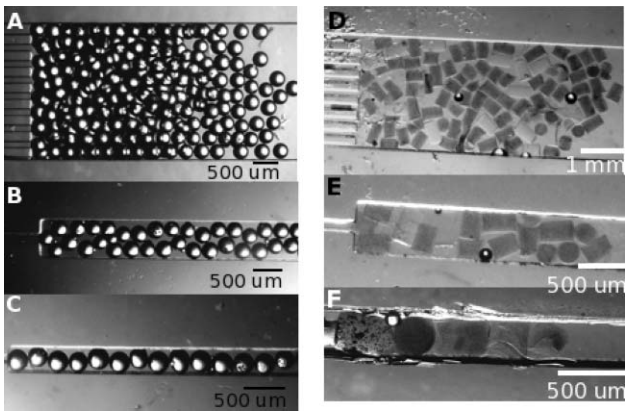

Fig. 3 Templated packing of hard and soft objects in microchannels. Glass beads (260 μm wide) in microchannels (A) 2 mm, (B) 500 μm, and (C) 250 μm wide. The beads were most ordered when confined to the narrowest channels. Agarose cylinders (150 μm diameter, 300 μm long) in microchannels (D) 2 mm, (E) 500 μm, and (F) 150 μm wide also showed more order in narrow channels than in wide ones.

To demonstrate that it is possible to create a construct of multiple cell types, we generated 750 μm long, 500 μm wide modules that contained either 3T3 cells or HepG2 hepatocytes, and additional 3 mm wide microfluidic chambers. As above, we cultured the modules in suspension for seven days and loaded a layer of glass beads into the devices before adding modules. We also incubated each type of module with a different color of fluorescent probe. The modules containing HepG2 cells were visibly larger than those containing 3T3 cells, because HepG2 cells do not pull on the collagen and contract the gel as their population increases.[7,42–44]

Both types of modules loaded easily into the chamber, either separately (Fig. 4A, modules containing 3T3 cells only and Fig. 4B, HepG2 only) or in a random mixture (Fig. 4C). By alternating layers of modules containing 3T3 cells with layers containing HepG2 cells, we generated assemblies for semi-organized co-culture (Fig. 4D–F). These layered assemblies were more ordered than the random mixture, and including a layer of glass beads between layers of different types of modules (Fig. 4) allowed even greater order and control over the minimum distance between types of cells. In all of these layered assemblies, some glass beads did occasionally mix into the layers of modules during loading, but the beads did not appear to harm the cells or damage the modules. The soft HepG2 modules deformed more under the pressure of flow than did modules containing 3T3 cells, so the layered assemblies were limited to ~5 mm in length. The largest assemblies with multiple types of cells were approximately the same size as gel assemblies used elsewhere in modular tissue engineering.[7]

To demonstrate that templated assembly in microfluidic channels can yield well ordered arrays of modules, we formed a modular assembly that contained three differently labeled populations of 3T3 cells encapsulated in collagen gel. As described above, we fabricated collagen modules that contained 3T3 cells, and allowed the cells to proliferate in the modules for seven days. We then divided the modules into three groups, and incubated each group with a different color of fluorescent probe (see ESI†), and then loaded modules from each group sequentially into a 250 μm wide microchannel by moving the tubing at the channel inlet from one group of modules to another. Fig. 5 shows a light microscopy image, and the corresponding fluorescent image, of three different types of modules assembled in a microfluidic channel.

Previously, some groups have attempted to generate microfluidic systems for cells in 3D. Tang and co-workers have demonstrated 3D cell culture in microfluidic channels by using microtransfer molding,[45] and Tan *et al.* used micromolding in capillaries to generate isolated and interconnected collagen structures with encapsulated fibroblasts.[46,47] It is unclear how well such systems simulate the cellular microenvironment of native tissues, since most cell culture in channels has involved culturing the cells at low densities to avoid distortion of the molded gel inside of the channels; this distortion occurs as the gel is populated and remodeled by proliferating cells.[42–44,48] Separating the culture of the cells in 3D (by culturing the modules in suspension) from the assembly of the 3D system in the microfluidic chip alleviates this problem in the system discussed here.

After building the devices and loading them with modules, a major challenge of this work was demonstrating that some

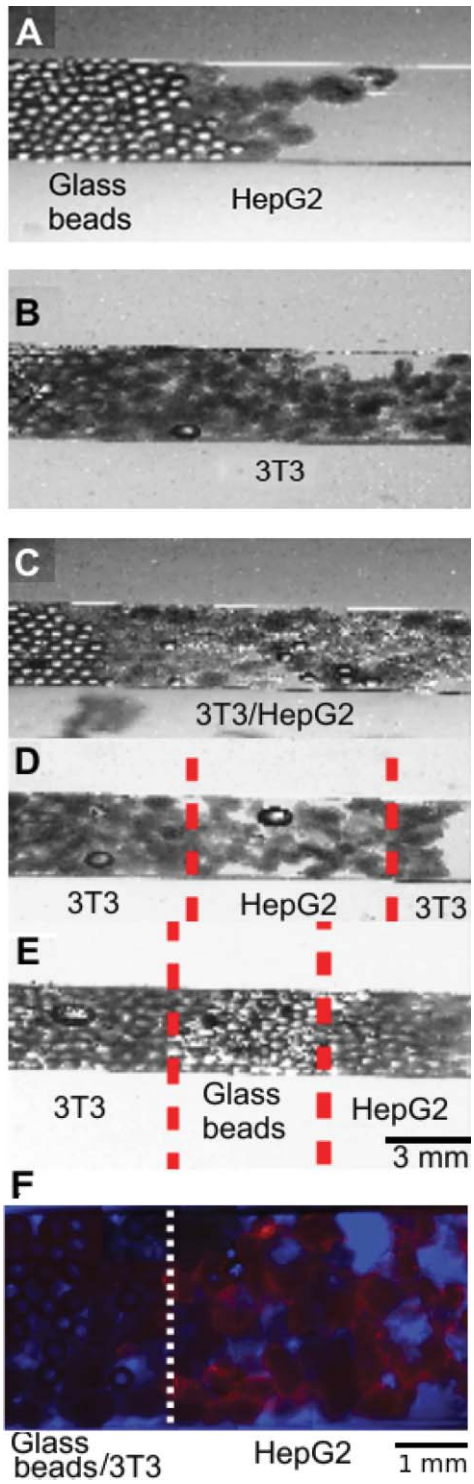

Fig. 4 Mixed modular assemblies on microfluidic chips. (A) Light-micrograph of an assembly of modules containing HepG2 cells. (B) Light-micrograph of an assembly of modules containing 3T3 cells. (C) Light-micrograph of an assembly of a mixture of modules containing HepG2 or 3T3 cells. (D) Light-micrograph of an assembly containing a mixture of types of modules. (E) Layered assembly of modules containing 3T3 cells, glass beads, and modules containing HepG2 cells. (F) Image of construct formed by layering 3T3 (un-colored) and HepG2 modules (red). The image was formed by combining the fluorescent channel and phase channel images showing only the top layer of modules contain the red HepG2 cells.

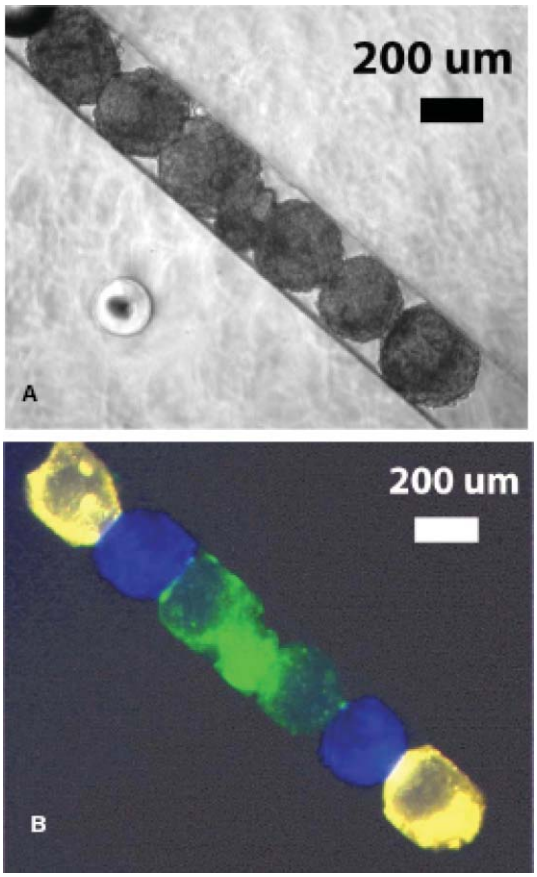

Fig. 5 Templated assembly of gel modules containing three differently labeled groups of cells. Three batches of modules were fabricated, and each batch contained 3T3 cells labeled with a different fluorescent dye. Sequentially loading a narrow microfluidic chamber with modules from each batch generated an organized array of modules. (A) Light micrograph of a sequence of modules in a templated assembly in a microchannel. (B) Fluorescent image of (A).

rate of fluid flow through the modular assembly is (i) sufficiently rapid that all cells in the modules can receive enough nutrients to avoid death, but (ii) slow enough that pressure does not build and deform (or destroy) the gel modules. In the 3 mm wide channels, over 99% of 3T3 cells encapsulated in modules survived for 24 h at flow rates up to 200 μL h^{-1} (~1 mL h^{-1}), and the modules did not deform or suffer damage during perfusion or removal from the chamber. Narrow channels or soft modules (*e.g.*, modules that contain HepG2 cells) require low rates of perfusion to avoid deformation of the modules. Since collagen is a weak hydrogel,[49] our modular devices were rather simple, but they demonstrated that it is possible to culture 3D gel modules containing cells inside of a microfluidic chip. More rigid hydrogels that have been developed for the encapsulation of cells[50] may allow the generation of much more complicated modules and assemblies.

In addition to forming randomly assembled "tissues" in microfluidic channels, we wanted to develop a method to generate greater organization of the modules on the chip for studies with multiple types of cells. Drawing on previous work in our group,[37] we used the channel to template the assembly of modules into an ordered structure; in the current work, the objects to be organized were soft cylinders instead of rigid spheres. In channels much wider than the modules, random packing occurred. In channels approximately twice the width of the modules, the soft modules became noticeably more organized (soft components did not, however, pack into arrays as highly ordered as did glass beads). Channels with widths comparable to the dimensions of the modules resulted in the greatest degree of organization. The ratio of the dimensions of the channels to the dimensions of the modules therefore determines the extent of ordering in the packed assembly.

Modules are soft, deformable, and less regularly shaped than, for example, glass beads; modules therefore packed into somewhat disordered assemblies, except when the channels were approximately the same diameter as the modules (Fig. 3). Gel modules, unlike glass beads, deformed under the shear force of flowing fluid; this effect limited the length of even our well ordered assemblies (Fig. 5). To minimize disorder and damage caused by shear, we perfused our microfluidic systems at rates of flow that did not noticeably deform the modules. (An alternative option is to develop more rigid material for the encapsulation of cells.[50])

One benefit of this modular system is the ability to encapsulate different types of cells, either in the same module, or in different ones; mixing different types of modules then forms an assembly that contains multiple types of cells. We believe that the ability to culture cells in 3D, and then further to organize the gel objects that contain cells, into a 3D array will allow hierarchical structures for the study of 3D cell–cell interactions and signaling. Here we generated two artificial tissues with different structures containing HepG2 cells and 3T3 fibroblasts: a random mixture of modules, and layered structures (Fig. 4C–4E). It was particularly easy to assemble a random mixture of multiple cell types, and this method of assembly will find use in experiments where precise organization of the different types of cells is not critical—for example, when intercellular signaling takes place over a length-scale comparable to the dimensions of the modules. The mechanical strength of the gel still limits the maximum size of the assembly, however, since the softest modules will deform first under shear in a mixture of modules.

Generating modular tissues in microfluidic chips offers several advantages over either 2D cell culture in microfluidic channels or 3D culture in large blocks of gel or other matrices: (i) easy control over the conditions of cell culture, (*e.g.*, rate of fluid flow and composition of the medium); (ii) parallel fabrication and experimentation on multiple chambers on a single device (to date, most tissue-engineered constructs for use as culture models have been cumbersome and have not allowed multiple experiments to be performed in parallel); (iii) increased sensitivity to rare analytes, due to the small volumes of sample used in microfluidics; (iv) easy imaging of cells in modules by standard tools such as confocal and light microscopy. Microfluidics allow far more control over the cellular microenvironment than does culture of modules in suspension, and we expect future work to exploit this control in analysis and other applications.

Conclusions

We have generated a simple technique for assembling ~200 μm sized gel modules that contain cells at densities near that of

native tissue (10^8–10^9 cells cm^{-3}) in a microfluidic chamber. With this technique, it is possible to design the composition and architecture of an artificial modular tissue by assembling multiple cell types in different modules, and then organizing the modules in a confined chamber. By allowing placement of cell-containing gel modules with sub-mm precision, this system provides a 3D cellular microenvironment that is better controlled and more complex than that provided by most other methods of 3D cell culture, yet is simple enough to be accessible and attractive to biologists. Building the system on a transparent microfluidic chip allows real-time imaging of the assembled artificial tissue, and allows the investigator to alter the inputs to or monitor the outputs from the chamber. Removing the individual modules from the chip at any time allows standard biochemical analysis of the cells. We believe that combining 3D cell culture in gel modules with the control provided by a microfluidic device will allow new studies of the differences between cell behavior in 2D and in 3D environments.

Developing realistic and versatile systems to study the behavior of cells in 3D is important to improve our understanding of *in vivo* tissues under normal and pathological conditions. In order to be widely adopted, a new system for cell culture must offer easy control over a variety of experimental variables, including density and organization of cells, and must also enable the creation of a realistic cellular microenvironment. We believe that engineered modular "tissues" assembled from particles of gel that encapsulate cells will be useful for studying the behavior of cells in 3D. Engineered tissues provide greater control over the cellular microenvironment than do other methods of 3D cell culture, and can therefore simulate *in vivo* tissue more accurately than, for example, culturing cells in spheroids.[29–32,51] Microfluidics provides extensive control over many experimental variables in cell culture,[4,35,41,52,53] but most of the microfluidic systems used to study cells have examined 2D cell culture at the bottom of microfluidic channels.[54–56] The work presented in this paper combines the advantages offered by modular tissue engineering—an approach that enables 3D cell culture at high density of cells—with the geometric control provided by microfluidic devices.

Acknowledgements

The authors wish to thank NIH EHS grant GM 065364, the Harvard University NSEC/MRSEC facilities, the Harvard Center for Nanoscale Science, Manish Butte for technical assistance and advice with the confocal microscope, and Cliff Brangwynne and Professor David Weitz for valuable discussions and help with equipment.

References

1 J. E. Barralet, M. Lawson, J. T. Triffitt, P. R. Cooper and R. M. Shelton, *J. Mater. Sci. Mater. Med.*, 2005, **16**, 515–519.
2 H. Liu and K. Roy, *Tissue Eng.*, 2005, **11**, 319–330.
3 V. M. Weaver, O. W. Petersen, F. Wang, C. A. Larabell, P. Briand, C. Damsky and M. J. Bissell, *J. Cell Biol.*, 1997, **137**, 231–245.
4 P. J. Hung, P. J. Lee, P. Sabounchi, R. Lin and L. P. Lee, *Biotechnol. Bioeng.*, 2005, **89**, 1–8.
5 H. C. Fiegel, P. M. Kaufmann, H. Bruns, D. Kluth, R. E. Horch, J. P. Vacanti and U. Kneser, *J. Cell. Mol. Med.*, 2007, 18021311.
6 H. Jawad, N. N. Ali, A. R. Lyon, Q. Z. Chen, S. E. Harding and A. R. Boccaccini, *J. Tissue Eng. Regen. Med.*, 2007, **1**, 327–42.
7 A. P. McGuigan and M. V. Sefton, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 11461–11466.
8 A. Khademhosseini and R. Langer, *Biomaterials*, 2007, **28**, 5087–5092.
9 A. P. McGuigan and M. V. Sefton, *Tissue Eng.*, 2007, **13**, 1069–1078.
10 M. A. Wozniak, K. Modzelewska, L. Kwong and P. J. Keely, *Biochim. Biophys. Acta*, 2004, **1692**, 109–119.
11 M. J. Blewitt and R. K. Willits, *Annu. Rev. Biomed. Eng.*, 2007, **35**, 2159–2167.
12 S. M. Schwartz, R. L. Heimark and M. W. Majesky, *Physiol. Rev.*, 1990, **70**, 1177.
13 P. L. Hordijk, E. Anthony, F. P. Mul, R. Rientsma, L. C. Oomen and D. Roos, *J. Cell Sci.*, 1999, **112**, 1915.
14 M. J. Wheelock and K. R. Johnson, *Curr. Opin. Cell Biol.*, 2003, **15**, 509–514.
15 G. Fenteany, P. A. Janmey and T. P. Stossel, *Curr. Biol.*, 2000, **10**, 831–838.
16 D. E. Ingber, *Circ. Res.*, 2002, **91**, 877.
17 J. L. Tan, J. Tien, D. M. Pirone, D. S. Gray, K. Bhadriraju and C. S. Chen, *Proc. Nat. Acad. Sci. U. S. A.*, 2003, **100**, 1484–1489.
18 D. R. Albrecht, G. H. Underhill, T. B. Wassermann, R. L. Sah and S. N. Bhatia, *Nat. Methods*, 2006, **3**, 369–375.
19 G. N. Li, L. L. Livi, C. M. Gourd, E. S. Deweerd and D. Hoffman-Kim, *Tissue Eng.*, 2007, **13**, 1035–1047.
20 K. W. Ng, D. T. Leong and D. W. Hutmacher, *Tissue Eng.*, 2005, **11**, 182–191.
21 M. Radisic, H. Park, H. Shing, T. Consi, F. J. Schoen, R. Langer, L. E. Freed and G. Vunjak-Novakovic, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 18129–18134.
22 M. Radisic, H. Park, F. Chen, J. E. Salazar-Lazzaro, Y. Wang, R. Dennis, R. Langer, L. E. Freed and G. Vunjak-Novakovic, *Tissue Eng.*, 2006, **12**, 2077–2091.
23 X. Wu, E. Rabkin-Aikawa, K. J. Guleserian, T. E. Perrry, Y. Masuda, F. W. Sutherland, F. J. Schoen, J. E. Mayer and J. Bischoff, *Am. J. Physiol.: Heart Circ. Physiol.*, 2004, **287**, H450.
24 *Scaffolding in Tissue Engineering*, ed. P. X. Ma, and J. Elisseeff, CRC, Boca Raton, FL, 2005.
25 D. R. Albrecht, V. L. Tsang, R. L. Sah and S. N. Bhatia, *Lab Chip*, 2005, **5**, 111–118.
26 V. Liu Tsang, A. A. Chen, L. M. Cho, K. D. Jadin, R. L. Sha, S. DeLong, J. L. West and S. N. Bhatia, *FASEB J.*, 2007, **21**, 790–801.
27 J. Yeh, Y. Ling, J. M. Karp, J. Gantz, A. Chandawarkar, G. Eng, J. III, Blumling, R. Langer and A. Khademhosseini, *Biomaterials*, 2006, **27**, 5391–5398.
28 F. Lim and A. M. Sun, *Science*, 1980, **210**, 908–910.
29 A. Lazar, H. J. Mann, R. P. Remmel, R. A. Shatford and F. B. Cerra, *In Vitro Cell. Dev. Biol.: Anim.*, 1995, **31**, 340–346.
30 A. Lazar, M. V. Peshwa, F. J. Wu, C. M. Chi and F. B. Cerra, *Cell Transplant.*, 1995, **4**, 259–268.
31 J. Fukuda, A. Khaddemhosseinib, Y. Yeoa, X. Yanga, J. Yeha, G. Enga, J. Blumlinga, C.-F. Wanga, D. S. Kohaned and R. Langer, *Biomaterials*, 2006, **27**, 5259–5267.
32 S. F. Abu-Absi, J. R. Friend, L. K. Hansen and W. S. Hu, *Exp. Cell Res.*, 2002, **274**, 56–67.
33 E. S. Avgoustiniatos, and C. K. Colton, in *Principles of Tissue Engineering*, R. G. Landes, Co, Austin, TX, 1997pp. 333–346.
34 A. P. McGuigan, B. Leung and M. V. Sefton, *Nat. Protoc.*, 2007, **1**, 2963–2969.
35 D. B. Weibel and G. M. Whitesides, *Curr. Opin. Chem. Biol.*, 2006, **10**, 584–591.
36 T. H. Park and M. L. Shuler, *Biotechnol. Prog.*, 2006, **19**, 243–235.
37 H. Wu, V. R. Thalladi, S. Whitesides and G. M. Whitesides, *J. Am. Chem. Soc.*, 2002, **124**, 14495–14502.
38 J. Y. Lee, C. Jones, M. A. Zern and A. Revzin, *Anal. Chem.*, 2006, **78**, 8305–8312.
39 Y. Xia and G. M. Whitesides, *Angew. Chem., Int. Ed.*, 1998, **37**, 550–575.
40 A. P. McGuigan, D. A. Bruzewicz, A. Glavan, M. Butte, and G. M. Whitesides, submitted.
41 J. C. McDonald, D. C. Duffy, J. R. Anderson, D. T. Chiu, H. Wu, O. J. Schueller and G. M. Whitesides, *Electrophoresis*, 2000, **21**, 27–40.
42 T. H. Finesmith, K. N. Broadley and J. M. Davidson, *J. Cell Physiol.*, 1990, **144**, 99–107.

43 E. Bell, B. Ivarsson and C. Merrill, *Proc. Natl. Acad. Sci. U. S. A.*, 1979, **76**, 1274–1278.
44 H. P. Ehrlich and T. Rittenberg, *J. Cell Physiol.*, 2000, **185**, 432–439.
45 M. D. Tang, A. P. Golden and J. Tien, *J. Am. Chem. Soc.*, 2003, **125**, 12988–12989.
46 W. Tan and T. A. Desai, *Biomaterials*, 2004, **25**, 1355–1364.
47 W. Tan and T. A. Desai, *Biomed. Microdev.*, 2003, **5**, 235–244.
48 F. Kamamoto, A. O. Paggiaro, A. Rodas, M. R. Herson, M. B. Mathor and M. C. Ferreira, *Artif. Organs*, 2003, **27**, 701–705.
49 S. L. Mitchell and L. E. Niklason, *Cardiovasc. Pathol.*, 2002, **12**, 59.
50 A. Sosnik and M. V. Sefton, *Biomaterials*, 2005, **26**, 7425–7435.
51 C. Soranzo, G. Della Torre and A. Ingrosso, *Tumori*, 1986, **72**, 459–467.
52 Y. Li, B. Yuan, H. Ji, D. Han, S. Chen, F. Tian and X. Jiang, *Angew. Chem., Int. Ed.*, 2007, **119**, 1112–1114.
53 S. K. Sia and G. M. Whitesides, *Electrophoresis*, 2003, **24**, 3563–3576.
54 D. T. Chiu, N. L. Jeon, S. Huang, R. S. Kane, C. J. Wargo, I. S. Choi, D. E. Ingber and G. M. Whitesides, *Proc. Nat. Acad. Sci. U. S. A.*, 2000, **97**, 2408–2413.
55 N. Li Jeon, H. Baskaran, S. K. Deringer, G. M. Whitesides, L. Van de Water and M. Toner, *Nat. Biotechnol.*, 2002, **20**, 826–830.
56 R. Gomez-Sjoberg, A. A. Leyrat, D. M. Pirone, C. S. Chen and S. R. Quake, *Anal. Chem.*, 2007, **79**, 8557–8563.