Trap and corral: a two-step approach for constructing and constraining dynamic cell contact events in differentiating progenitor cell populations

# Trap and corral: a two-step approach for constructing and constraining dynamic cell contact events in differentiating progenitor cell populations

**S Chen[1], N Patel[1], D Schaffer[1,2] and M M Maharbiz[3]**

[1] Department of Bioengineering, UC-Berkeley, Berkeley, CA 94720, USA
[2] Department of Chemical Engineering, UC-Berkeley, Berkeley, CA 92720, USA
[3] Department of Electrical Engineering, UC-Berkeley, Berkeley, CA 94720, USA

E-mail: sisichen@berkeley.edu

## Abstract

Cells are constantly subjected to a host of external signals which can influence their state, phenotype and behavior. Mammalian cells are dependent on signals from surrounding cells to maintain viability, proliferate and coordinate their actions. During developmental and regenerative processes, these lateral signals between cells provide instructive cues informing stem cells how, when and where to differentiate. Moreover, differentiating cells often experience cell–cell contact events interspersed with bouts of motility, process extension and multi-cell agglomeration (Gage 2000 *Science* **287** 1433–8) processes which are not easily recapitulated in existing cell capture devices. Here, we present a two-step process involving microwells to trap cells with high efficiency followed by the alignment of a PDMS mesh around the cells to corral them after the trapping. The microwells trap single cells and paired cells with up to 90% and 80% efficiencies, respectively. After seeding, the PDMS mesh is aligned with the seeded wells to create a 150 $\mu$m × 150 $\mu$m corral around each trap, allowing cells to interact in a larger arena. The corralling must be done in liquid after seeding because the seeding requires high cell densities to achieve near-full occupancy in the wells. Low-density seeding of the PDMS corrals alone can result in two cells being trapped in each well, but in those conditions, the two cells often engage in very little contact or none at all (and seeding obeys a non-desirable Poisson distribution). Interestingly, trapping cells in proximity and then corralling them elicits much higher contact times than simply seeding into corrals.

(Some figures in this article are in colour only in the electronic version)

## Introduction

Cells are constantly subjected to a host of external signals which can influence their state, and thus phenotype and behavior. Mammalian cells are dependent on signals from surrounding cells to maintain viability, proliferate, and coordinate their actions. During developmental and regenerative processes, these lateral signals between cells provide instructive cues informing stem cells how, when and where to differentiate.

Localized intercellular cues can formally be categorized into paracrine and juxtacrine signals. In paracrine signaling, the signaling molecules are diffusible ligands, which are secreted by the sender cell. The docking of ligands to the receptors of the target cell triggers the signal transduction cascade that eventually regulates the gene transcription of the target cell and drives differentiation. In juxtacrine signaling, direct cell-to-cell contact is required for the signaling to take place because both ligands and receptors are membrane-bound. Many canonical juxtacrine signaling pathways, such

as the Notch pathway [1], the Wnt pathway, and the Eph-Ephrin pathway [2, 3], play crucial roles in specifying fates during early development and regenerative processes during adulthood [4].

To study these juxtacrine signaling processes *in vitro*, it is desirable to be able to controllably place individual cells into contact. Most methods to create small-scale cell assemblies are based on microfabrication technologies (although cellular self-assembly using DNA-conjugated surface proteins has also been reported [5]). In general, two types of approaches have been used to bring discrete numbers of cells together. In one approach, surface micropatterning is used to create cytophobic and cytophilic regions. Surface patterning is typically done using self-assembled monolayers (SAMs) of thiols on gold [6–8], selectively masked vapor deposition of metals [9], laser ablation [10], direct-write processes [11, 12], or photolithographic processes [13, 14]. The other approach is to constrain cells mechanically using 3D structures such as microwells [15, 16] and microfluidic traps [17, 18] to arrange cells into spatial proximity.

SAMs patterned in bowtie shapes have been used successfully to study the effect of cell–cell contact in endothelial cell proliferation [7]. However, thiol-patterning techniques generally suffer from low yield and degrade over time [19]. Similar approaches using poly-ethyleneglycol (PEG) hydrogels resist degradation but still suffer from poor efficiency in their ability to pair cells [8, 16]. Typically, the distribution for each type of pattern follows a Poisson distribution with lambda equal to the desired number of cells. Thus, the best reported efficiencies for capturing two cells peak at around 35–40% [7, 8]. Microscopy-based *in situ* photolithography [13] appears to have good capture efficiencies but the exact numbers are not reported.

Three-dimensional structures are more successful at capturing well-defined numbers of cells. Skelley *et al* achieved up to 70% pairing efficiencies using a microfluidic design for high-yield electrofusion. However, the patterning is not preserved after long-term culture in the device (up to 3 days) [18].

While these microdevices and substrates can place cells in proximity, cell contact is generally difficult to constrain for long periods *in vitro* without influencing viability or artificially altering cell state, especially for durations of time that are likely necessary to bias fate decisions. Often, markers of differentiation are not detectable by mRNA screening methods or immunocytochemistry until 2–5 days after the initial stimulus to differentiate. Moreover, in both *in vivo* and unconstrained *in vitro* experiments, differentiating cells often experience cell–cell contact events interspersed with bouts of motility, process extension and multi-cell aggregation [1] which are not recapitulated in simple cell capture devices.

Specifically, we are interested in understanding how cell–cell contact events bias differentiation in adult neural stem cells of various population sizes prior to the emergence of neuronal, glial and oligodendrocytic precursors. We address this problem by using a two-step process involving microwells to trap cells with high efficiency followed by the alignment of a PDMS mesh around the cells to corral them after the

trapping. The microwells trap single cells and paired cells with the highest reported efficiencies, up to 90% and 80%, respectively. After seeding, the PDMS mesh is brought down using an alignment jig to create a 150 $\mu$m × 150 $\mu$m corral around each trap so that when cells migrate out of the well, they cannot make contact with cells from neighboring traps. The corralling must be done in liquid after seeding because the seeding requires high cell densities to achieve near-full occupancy in the wells. Low-density seeding of the PDMS corrals alone can result in two cells being trapped in each well, but in those conditions, the two cells often engage in very little contact or none at all (and seeding obeys a non-desirable Poisson distribution). By contrast, trapping cells and then corralling them proves to elicit much higher contact times.

## Materials and methods

### Polystyrene microwell fabrication

Polystyrene (PS) microwells are fabricated according to a previously published hot embossing technique [20]. The technique uses PDMS pillars as a mold, because PDMS is elastically deformable and does not melt at the high temperatures necessary to emboss PS. During this process, the PS must be heated to 180 °C (above the ~100 °C [21, 22] glass transition temperature but below its melting point of 240 °C).

An inexpensive benchtop press is constructed using a laboratory hot plate (Thermo Scientific, Cimarec) and two flat blocks of aluminum. The bottom block is milled to create a 2 cm × 2 cm × 0.3 cm indent for holding a glass slide cut to size. The glass slide is clean and ensures that the bottom surface of the PS will be flat and optically clear after embossing. Four holes are drilled in the corners of the aluminum blocks and metal dowels are inserted as guide rails. The hot plate is heated to 250–270 °C, so that the surface temperature on the bottom block is 180 °C (measured with a thermocouple).

PS coupons are cut to 1.5 cm × 1.5 cm and washed in isopropanol (IPA) and water. A clean glass slide is inserted into the indent and a PS coupon is quickly placed in the center of the slide. A 0.28 mm thick PDMS mold that has been plasma bonded to a large glass slide is then inverted on top of the PS coupon. The top block of aluminum is aligned on top using the guide rails and gently brought down onto the assembly. A 1 lb free weight is then placed on top, resulting in a final pressure of 32 kPa. After 2 min, two more 1 lb weights are placed on top, resulting in a final pressure of 72 kPa. After 5 min, the weights are removed and the entire assembly is quickly taken off the heated aluminum block. After cooling, the embossed chip is removed and cut to size by scoring the edges and breaking the edges carefully with a pair of pliers. The PDMS mold can be reused >30 times.

The PDMS pillars used for embossing the PS coupons are made using standard soft lithography protocols. Briefly, an SU-8 mold is made by spinning SU-8 2015 to a thickness of 10 $\mu$m on a silicon test wafer using a Headway spinner. The wafer is then soft-baked and exposed on a Karl Suss MA6

Mask Aligner. After post-baking the wafer is developed in SU-8 developer and washed with IPA and water. The wafer is hard-baked at 150 °C for 10–30 min to anneal thermal cracks and to improve adhesion to the substrate. The wafer is then coated with trichloro(1H,1H,2H,2H-perfluorooctyl)silane (Sigma) by vapor deposition under a vacuum-trapped house vacuum line for 1 h. This step is integral because if the wafer is incompletely coated, PDMS does not release from the mold, resulting in defective pillars and a permanently damaged SU-8 mold. PDMS is cured to a height of 0.28 mm on the wafer in a convection oven at 60 °C for 1 h.

### Alignment jig fabrication

The alignment jig is designed in AutoCAD Student 2010 and rapidly prototyped in an aluminum alloy (First Cut). Because the alloy contains reactive metals that form salt precipitates with the anions that are typically present in any cell culture medium, the entire jig is coated with parylene, a chemically inert and biologically compatible polymer. The jig is sonicated in IPA for 30 min, and washed three times in DI water, before coating with 10 $\mu$m of parylene C in the Parylene Deposition System 2010 LabCoter 2.

The mold for the PDMS corralling mesh is also fabricated using standard photolithography techniques as described above. For thicker layers of SU-8, the resist formulations have much higher viscosities. Thus, the only modification to the technique is that a thin layer of low-viscosity SU-8 2002 is spun onto the bare wafer first, and then soft-baked. This layer of SU-8 makes the high viscosity SU-8 spread more evenly. To create the mesh, 24 $\mu$L of 10:1 PDMS is deposited onto the edge of the developed SU-8 mesh. PDMS wicks into the features by capillary forces to create a mesh with square through-holes that are 150 $\mu$m $\times$ 150 $\mu$m. Although the SU-8 features are fabricated to a height of 120 $\mu$m, the thickness of the resulting PDMS film is measured to be 85–95 $\mu$m. The top part of the alignment jig is aligned onto the mesh within a 100 $\mu$m wide square of SU-8 that has been patterned to match the size of the ridge on the underside of the top piece. Then the entire assembly is cured at 60 °C. The top piece of the alignment jig is then peeled off the SU-8 mold carefully, bringing the PDMS mesh with it.

### Alignment jig assembly

The PS microwells are aligned under the mesh using a stereomicroscope (Zeiss) and held in place by conformal contact. PDMS is applied to a small ring around the viewing hole on the bottom piece and the two pieces are then brought together. The assembly is then cured in the convection oven at 60 °C for 1 h. When the top part of the jig is removed, the PS microwells remain adhered to the bottom piece of the jig.

### Cell culture

Adult rat hippocampal progenitor cells are originally isolated from the subgranular zone of the rat hippocampus. They are maintained in a cell culture incubator in DMEM/F-12 media (Gibco), supplemented with N2 (Gibco) and 20 ng mL$^{-1}$ FGF

(Peprotech). Cells are kept in a tissue culture incubator at 37 °C and 5% $CO_2$. The media are changed every 2 days. Cells are used at passage number 30–38.

Mixed differentiation media containing 1% fetal bovine serum (FBS), 1 $\mu$M retinoic acid (RA) and 1% penicillin/streptomycin are prepared fresh from stocks on each day they are used. RA is prepared in dimethyl sulfoxide (DMSO) to a stock concentration of 1 mM and stored frozen at $-20$ °C in aliquots until use. FBS is also aliquoted and stored frozen at $-20$ °C until use.

### Experimental procedure

For all experiments, the PS microwells must be coated with extracellular matrix (ECM) to promote cell adhesion. First, the top surface of the embossed PS microwells is blocked using 10 mg mL$^{-1}$ BSA (Sigma) for 30 min at 37 °C. The BSA solution does not enter the microwells due to surface-tension-mediated liquid pinning. Then the microwells are washed three times in PBS and a 10 $\mu$g mL$^{-1}$ laminin solution in PBS is added. The wells are then vacuumed for 2 min so that the laminin solution can fill the wells. The bubbles that remain on the surface are knocked off with gentle pipetting. Then the microwells are incubated in the cell culture incubator overnight.

For experiments without the corrals, the microwells are anchored to a 3.5 cm dish or 12-well plate using PDMS and UV-sterilized before coating. To count the capture efficiencies of the microwells, the cells are stained with Hoechst in PBS for 10 min, and washed before imaging. Progenitor cells are dissociated from the dish by replacing the media with Accutase (Innovative Cell Tech.) at 37 °C for about 2–3 min and spun down at 1000 rpm for 2 min. They are then resuspended in the media to a high density, passed through a 40 $\mu$m nylon cell filter (BD Falcon) to ensure a single-cell suspension, and counted using a hemocytometer.

The cells are then seeded onto the microwells at an areal density of 300 000 cells cm$^{-2}$. They are incubated for 10 min at 37 °C in the incubator and then triturated gently to disrupt cell adhesion to the top surface. This incubation/trituration sequence is repeated two to three times until the wells are filled and there is minimal cell adhesion to the top surface of the PS. Then the cells are washed five times in PBS.

The top piece of the alignment jig is then placed face-down onto a sterile glass slide and plasma oxidized (Harrick Plasma) at high power (30 W). This prevents the bottom surface of the PDMS from being made hydrophilic. The top piece is then snapped into the bottom piece using sterilized plastic push-in fasteners (MicroPlastics). Then the PBS in the device is replaced with the mixed differentiation media (1% FBS, 1 $\mu$m RA, 1% penicillin/streptomycin prepared in DMEM/F12) and then taken to the imager. An overview of the experimental scheme using the alignment jig is shown in figures 1(e), (f).

Some cells are seeded into PDMS meshes without the microwells. In these experiments, the PDMS meshes are conformally sealed to tissue culture PS dishes and seeded with cells at low density.
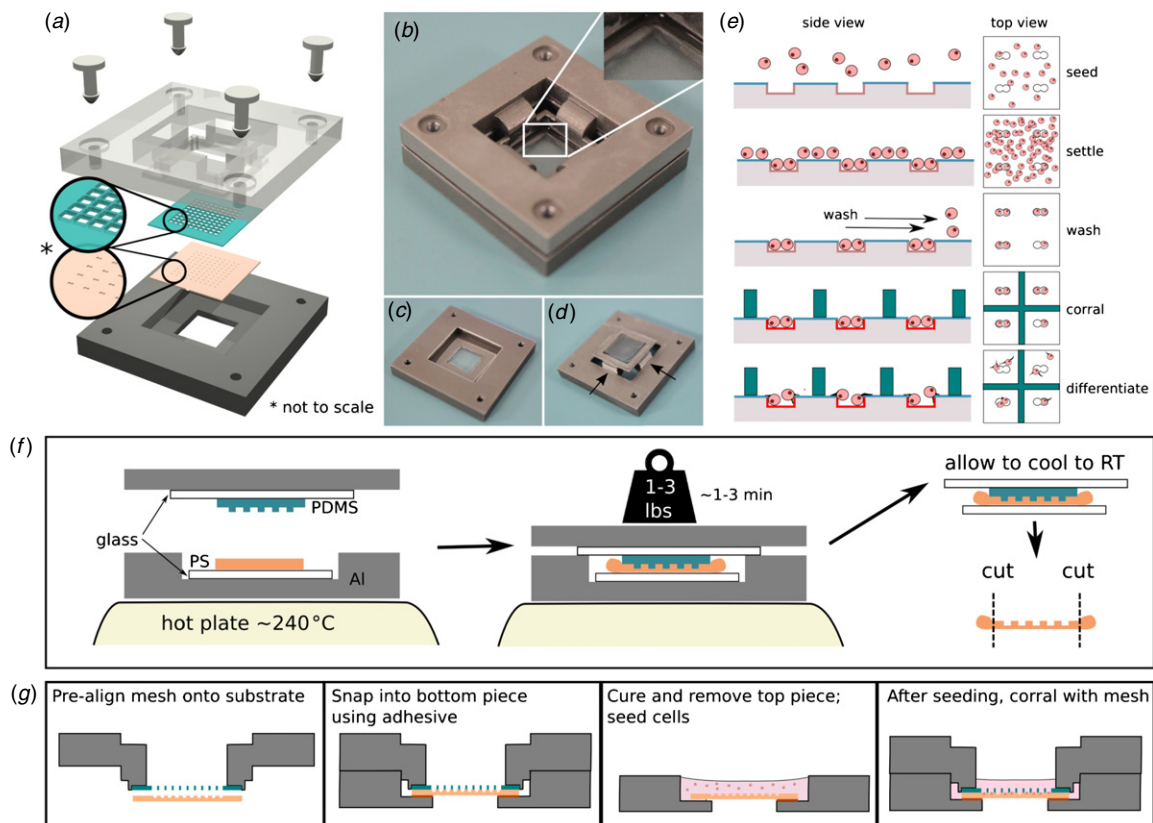
**Figure 1.** Trap and corral alignment jig and experimental scheme. (*a*) 3D rendering of the alignment jig. The top piece is rendered in a transparent material to show the layers below. A PDMS mesh is cured onto the bottom of the top piece (blue) and aligned onto a PS substrate (pink), which has been affixed to the bottom of the jig. Plastic push-in fasteners are used to keep the assembly together. (*b*) Macro photographs of the assembled jig with a blowup of the mesh as the inset. (*c*) The bottom piece of the jig, with the PS microwells affixed. (*d*) The top piece of the jig has a ridge for aligning onto the SU-8 mold for the PDMS mesh. The experimental scheme is given in (*e*), showing that cells are seeded onto the microwells at high density before washing and corralled. Differentiating these cells in the mixed differentiation medium results in outward migration (shown in the bottom panel) over the course of several days. (*f*) The fabrication of microwells in PS is done by hot embossing a PDMS master onto PS. The assembly is sandwiched between a set of custom-milled aluminum blocks, on which free weights are placed to apply pressure. (*g*) Schematic of the device assembly before cell seeding.

*Timelapse microscopy*

The cells are imaged for up to 2 days on a Zeiss AxioObserver Z1 in a humidity-, temperature- and $CO_2$-controlled live imaging chamber. For microwell experiments without the corrals, the cells are imaged using 10× (35 $\mu$m spacing) or 5× objectives (90 $\mu$m spacing). The PlasDIC objective filter is used for enhanced contrast. Images are taken on a QImaging 5MPix Micropublisher camera every 10 min.

*Data processing*

Well occupancy data are tabulated by hand into a spreadsheet. In this analysis, we are primarily concerned with how well the initial contact state is maintained. When cells leave the well, the occupancy of the well is reduced by the number of cells that leave. When cells migrating along the top surface make contact with the cells in the microwell, the effective cell count in that well is reduced to 0, reflecting that the initial state has been disturbed. Cells migrating into empty wells do not increase the count for that well. This occupancy data are then parsed into residence times using a custom Matlab script implementing the aforementioned rubric.

*Scanning electron microscopy*

The samples are fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2 for 1 h and then rinsed three times for 15 min in the buffer. After post-fixing in 1% osmium tetroxide for 1 h, they are rinsed again three times in the 0.1 M sodium cacodylate buffer. The samples are dehydrated in a succession of ethanol rinses (35%, 50%, 70%, 80%, 95%, 100%, 100%), each for 10 min. The samples are then dried in a critical point dryer and mounted onto stubs. Gold is sputtered onto the sample to a thickness of 35 nm and they are then scanned in Hitachi S-5000.

*Immunostaining*

In preparation for immunostaining, the cells are cultured in the corrals for 4 additional days after the imaging, with half-media changes every day. The cells are fixed in 4% paraformaldehyde in PBS at room temperature for 10 min. They are washed three times and then blocked with 5% donkey serum (Sigma) in tris-buffered saline (TBS) at pH 7.4, with 0.3% Triton X-100 (Sigma) for permeabilization. After blocking for 1 h at room temperature on a shaker, the cells are then washed

three times in the buffer and incubated overnight at 4 °C with primary antibody—chicken anti-GFAP (Abcam)—at 1:2000 dilution. The next day, the sample is washed three times and then incubated with secondary antibody—Dylight 488-donkey anti-chicken (Jackson Immuno)—for 1 h at room temperature on a shaker. The samples are washed three times in the TBS, with the last wash containing DAPI (diluted 1:500 from 5 mg mL$^{-1}$ stock). The sample is then mounted with a glass coverslip using Prolong Gold Antifade reagent (Invitrogen).

## Results

### Rapid prototyping and material choice

Rapid prototyping is an inexpensive way to produce precision-manufactured 3D parts with a turn-around time of less than 1 week. Most rapid prototyping techniques form objects by additive fabrication. The typical paradigm is to print successive layers of the precursor material that are then fused through the inkjet deposition of binding agents (3D printing), the rastering of high power lasers (selective laser sintering) or electron beams (electron beam melting). Stereolithography works similarly by incrementally lowering a platform into a vat of UV-curable resin. Each layer of the final object is cured by drawing a UV laser across the top.

Additive manufacturing unfortunately often suffers from a stair-stepping effect that is a result of the layer-by-layer construction. By contrast, subtractive rapid prototyping, which removes a material by computer programmed machine tools, does not suffer from this same problem. In subtractive rapid prototyping, a 3D design file is automatically translated into toolpaths that can be programmed into a computer numerical control mill. The materials available for additive fabrication technologies are usually proprietary polymers with relatively low glass transition temperatures (<100 °C), high porosity and undetermined cytocompatibility. Our initial prototypes using a clear ABS-like polymer, DSM Somos Watershed XC 11122, showed poor cytocompatibility. Additionally, another ABS-like polymer absorbed the PDMS curing agent when PDMS was cast on the surface, thus inhibiting the curing process.

To produce our alignment jig, we use subtractive rapid prototyping services offered commercially. We chose to fabricate the jig with aluminum because it is heat resistant (which is useful for curing PDMS at high temperatures) and can be coated on a variety of metals and polymers.

Although pure aluminum oxide is highly corrosion resistant and supports the growth of cells [23], the machining processes in rapid prototyping use aluminum alloys. These alloys have a high content of metallic impurities—iron, copper, manganese, chrome and zinc—which corrode and form salt precipitates in the cell culture media, which have high salt concentrations.

To address this problem, we coat the entire jig with 10 $\mu$m of parylene by chemical vapor deposition. Parylene deposits as a highly conformal layer, and is chemically inert and safe for cell culture. After deposition of parylene, no salt precipitation was observed when the jigs were incubated in the cell culture

medium for up to 5 days. Although this solution suited our needs, a preferable, but more costly, approach would be to machine the jig from a material known to be cytocompatible, such as Teflon or stainless steel.

### Microwell traps achieve high efficiency in cell pairing

The adult hippocampal progenitor cells exhibit a tight distribution in their size (12.3 ± 1.6 $\mu$m) (figure 2(*a*)). This size monodispersity enables us to capture single cells and paired cells with high efficiency. The phase and DAPI images of the Hoescht-stained cells are shown in figure 2(*b*-i)–(*b*-iv). Over 90% of the 15 $\mu$m microwells capture single cells (figure 2(*d*)). Approximately 80% of the hourglass-shaped microwell traps captured paired cells (figure 2(*d*)).

The size of these microwells must be tightly tuned to the size of the cells of interest. Increases in the size of the round microwells results in increasingly broadening distributions in the number of cells captured (figure 2(*b*)). Additionally, adjusting the spacing between the centroids of the two circles comprising the halves of the centroids results in altered captured efficiencies. We find that a separation of 15 $\mu$m, equal to the diameter of the single trap, results in the best trapping efficiencies for pairs of cells. For all further experiments, we used the 15 $\mu$m diameter microwells for single cells and the hourglass traps with the 15 $\mu$m spacing for paired cells.

A scanning electron microscopy (SEM) scan is shown in the inset demonstrating cells trapped in proximity in the microwell making membrane contact. Some volumetric shrinking of the cells is observed due to the fixation and drying process used in the SEM preparation protocol.

### Outward migration in the cell differentiation medium

After the cells are trapped into the microwells, the medium is changed to one that results in mixed differentiation (1% FBS, 1 $\mu$M RA, 1% penicillin/streptomycin) down all three lineages: neurons, astrocytes and oligodendrocytes. In this medium, the cells begin to migrate outwards from the wells, and sometimes will crawl over and adhere to cells in neighboring wells (figure 3(*a*)). This disrupts the isolated or contact state of single cells or paired cells respectively. Tracking of the residence times of cells in the microwells shows that the initial trapping state is fully maintained for about 420 min (for wells with 90 $\mu$m spacings) (figure 3(*b*)).

The pitch of the microwells affects the maintenance of the initial state. The further apart that the microwells are spaced, the slower the cells migrate outward (figures 3(*c*), (*d*)). For a 35 $\mu$m spacing, the cells have a mean residence time of approximately 380 min, which increases to 1150 min for a spacing of 90 $\mu$m. However, when cells are in hourglass traps separated by 90 $\mu$m, they still migrate outward quickly, with a residence time of 470 min (figure 3(*d*)).

This outward migration demonstrates that microwells themselves are insufficient for constraining the cell contact for the lengths of time necessary to see early markers of fate commitment, which usually peak at 1–2 days after the initial stimulus to differentiate. Although it is possible to fabricate
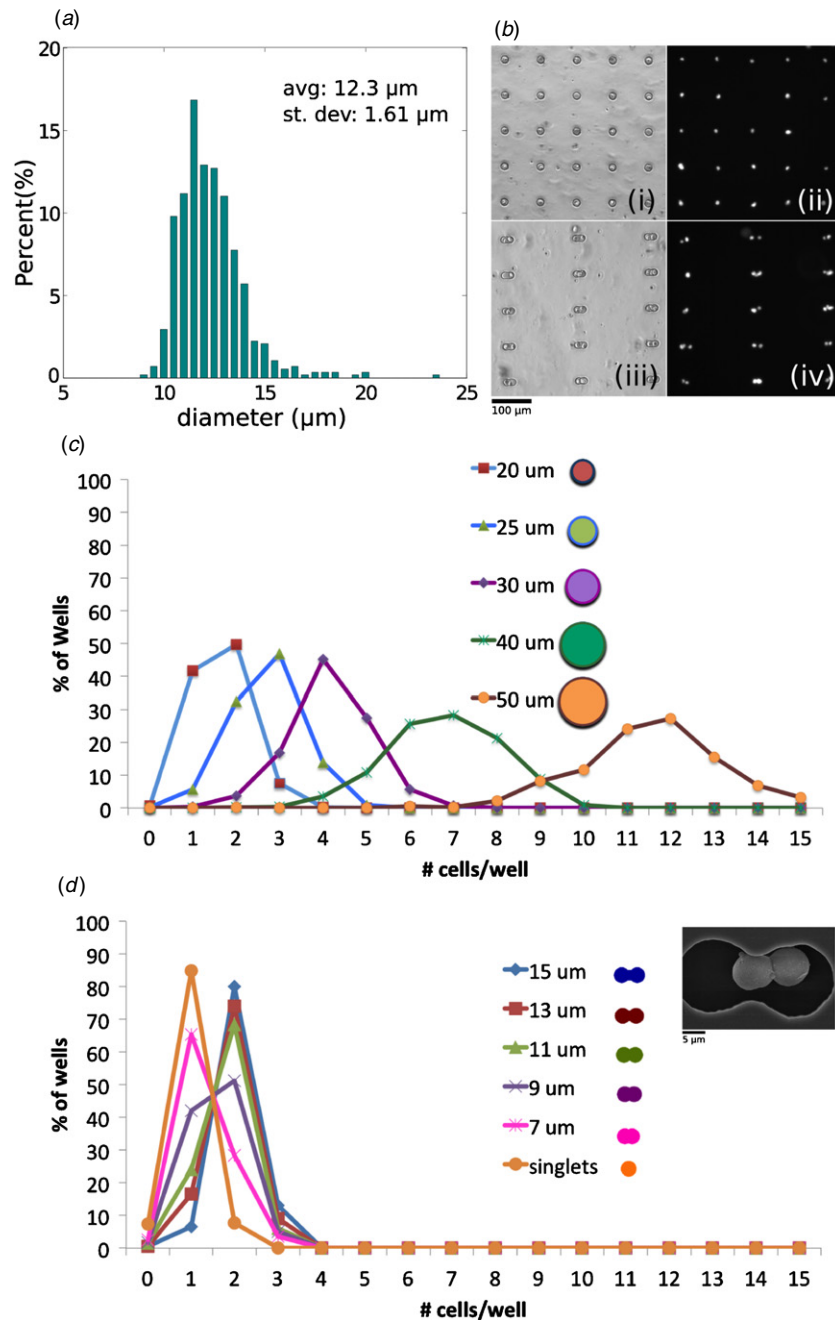
**Figure 2.** Microwells achieve high efficiencies in trapping single and paired cells. (*a*) The rat hippocampal progenitor cells are relatively uniform in size, averaging $12.3 \pm 1.6$ $\mu$m. (*b*) Rat hippocampal progenitor cells trapped in single-cell wells (i–ii) or double cell wells (iii–iv). Phase images are on the left (i, iii) and Hoescht-stained nuclei are shown on the right (ii, iv). (*c*) Varying the diameters of circular microwells tunes the average number of cells captured in each well but the distributions are broad. None of these well sizes are capable of capturing cell pairs with greater than 50% efficiency. (*d*) 15 $\mu$m diameter round microwells can capture single cells with greater than 90% efficiency. Hourglass-shaped wells, which are essentially the union of two adjacent single-cell traps, can capture two cells with over 80% efficiency. The optimal separation between the centroids of the two halves was found to be 15 $\mu$m.

deeper wells, cell viability is very low (data not shown). Thus, our approach is to align additional corrals on top of the traps after the cells have been seeded.

*PDMS sealing under liquids*

PDMS is a very hydrophobic material that forms a reversible conformal seal to a variety of materials by van der Waals forces. This conformal seal can resist up to 30 kPa of pressure

and can be used for low-pressure microfluidic applications [24]. However, when immersed in liquid, PDMS is subject to a buoyant force due to the lower density of PDMS (0.965 g mL$^{-1}$) versus the surrounding liquid medium (usually slightly higher than 1.0 g mL$^{-1}$, the density of water).

For thicker pieces, this buoyant force can be large enough to lift the piece up away from the surface to which it has been sealed. However, for thin films of PDMS (such as the mesh
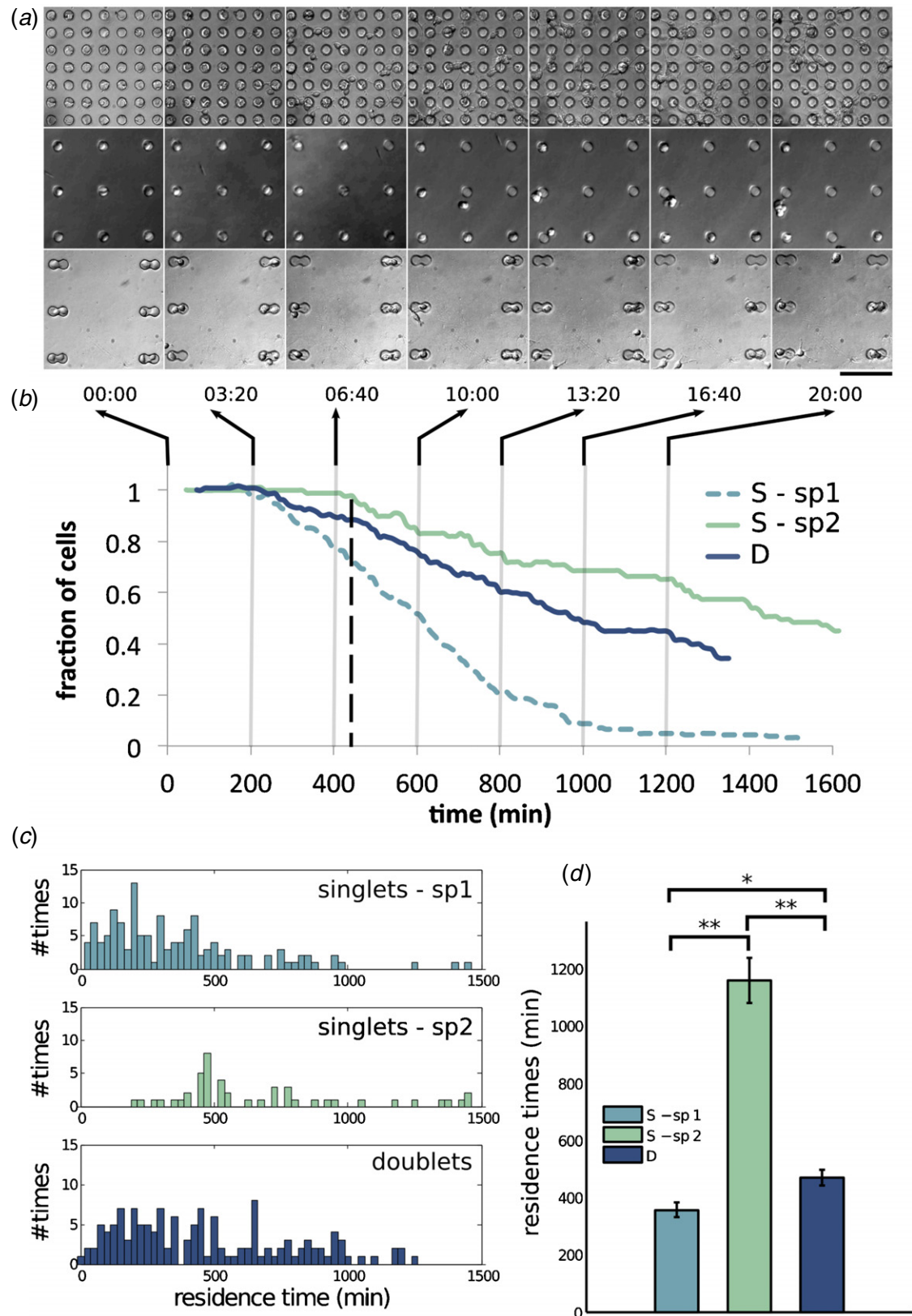
**Figure 3.** Outward migration from microwells in the mixed differentiation medium. (*a*) Timelapse data of cells trapped in single microwells with spacings of 35 $\mu$m (top) and 90 $\mu$m (middle) or hourglass microwells (bottom). (*b*) The maintenance of the initial separation or contact state decays over time. Single microwells with 90 $\mu$m retain the trapping state for the longest time, until approximately 420 min after the mixed differentiation medium is added. (*c*) Histogram of the residence times in each of the microwell conditions. (*d*) The mean residence times of cells in microwells is increased by over 800 min when the center-to-center spacing is increased from 35 to 90 $\mu$m. However, traps with more cells show more outward migration. The differences in distribution are significant by the Kolmogorov–Smirnov test ($^{**}$, $p < 10^{-9}$; $^*$, $p < 0.005$). Error bars show $\pm$ SEM. Scale bar: 100 $\mu$m.
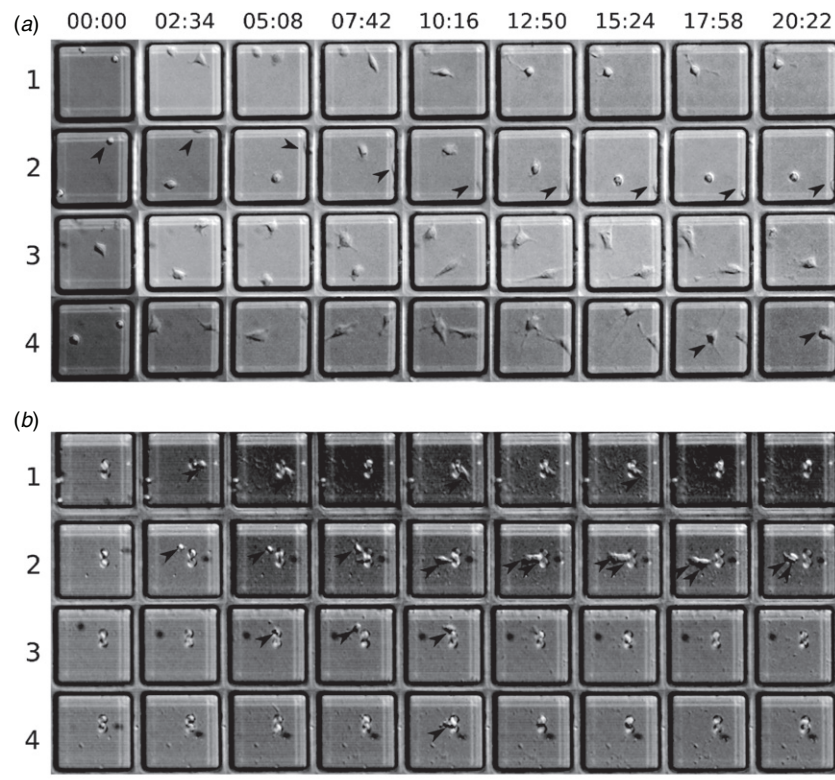
**Figure 4.** Migration of cell pairs when seeded into corrals (*a*) or trapped in microwells first and then corralled (*b*). Cells that are randomly seeded into corrals rarely make contact over a 24 h period but cells which have been pre-trapped maintain the contact state. In (*a*), the arrows show the position of cells that are difficult to decipher from still images. In (*b*) arrows are added to show the position of cells which have crawled out of the microwells. Though the cells often try to migrate away, they often bounce back (1, 3, 4) or both cells migrate out together (2).

in these experiments), the buoyant force is small enough to be insignificant. Thus, for the 85 $\mu$m thick PDMS meshes, the seal remains undisturbed in the cell culture medium for at least 7 days.

Due to the small dimension of the holes in the mesh and the hydrophobicity of PDMS, air bubbles get trapped in the meshes when they are immersed in aqueous solutions. To alleviate this problem, we make the PDMS surface hydrophilic by exposing it to oxygen plasma at 30 W of RF power for 2 min (Harrick Plasma, PDC-32G). However we have found that hydrophilic PDMS does not seal well with the PS surface under water. We suggest that the failure to form a seal is a result of water molecules forming a lubricating layer that prevents the PDMS from making contact with the PS surface. To protect the bottom surface of PDMS from being oxidized, we press the mesh to a clean glass slide during plasma oxidation, leaving that surface hydrophobic.

Meshes that have a hydrophilic top and sides are able to wet thoroughly and also seal to a PS surface in water. However, no seal can be achieved in the cell culture media due to undetermined interactions with the molecular constituents of the cell culture media. We found that phosphate buffered saline, an isotonic salt solution suitable for cells, does not present the same problem. Thus our protocol calls for replacing the media with PBS before aligning the mesh on top of the seeded cells.

*Trap and corral*

Timelapse imaging of the cells that have been trapped and corralled indicate that they maintain contact for up to a day after seeding (figure 4(*b*)). Low-density seeding of the PDMS corrals alone can result in two cells being trapped in each well, but in those conditions, the two cells often engage in very little contact or none at all (figure 4(*a*)). We show that the cells can be cultured for up to 6 days, enough time for cells to start expressing markers of differentiation (figure 5).

Occasionally, we observe that cells will migrate vertically up the walls of the corrals and migrate along the top surface of the mesh. Though many remain on the top surface of the PDMS mesh, some will descend into the same or a different corral. Since we use timelapse microscopy to monitor cell migration, we exclude any cases where this happens. We anticipate that this problem can be easily avoided by blocking the surface of the top and sides of the PDMS mesh using PLL-PEG, a cytophobic surface coating.

Since the PDMS meshes are only ∼100 $\mu$m thick, they can deform when brought down on top of the underlying substrate. Two features of the device design prevent them from deforming significantly. The first is that the entire mesh remains attached to the top part of the alignment jig as it is peeled away from the mold (figure 1(*d*)). Thus the top part of the jig maintains the mesh in a stretched, flat state so that it is easy to handle. The other feature is that the sides of the top part (arrows
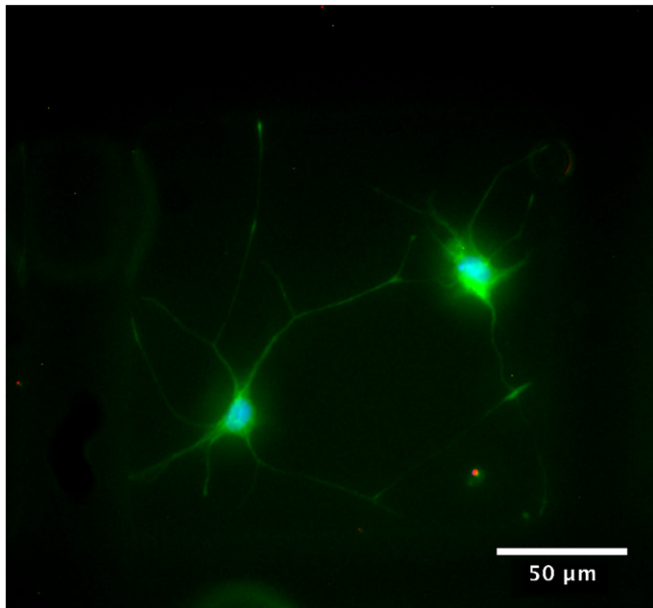
**Figure 5.** Cells can be cultured in the corrals for up to 6 days and then immunostained for differentiation markers. GFAP, an astrocytic marker, is in green and DAPI is in blue.

in figure 1(*d*)) fit snugly against the sidewalls in the recession of the bottom part. This restricts the motion of the top piece vertically so that there is no lateral deformation of the mesh as it is brought down. When the meshes are positioned by hand (such as when they are sealed to unembossed PS), deformation can be prevented by bringing the mesh down gently, allowing one side to seal against the substrate before slowly lowering the other side.

This corral alignment method is adaptable to many types of patterned substrates. Since the corrals are aligned on top of the substrate after the cells have been seeded, any substrate to which PDMS can conformally seal in aqueous solutions can be used. Conformal sealing is necessary to prevent cells and processes from burrowing through to adjacent chambers. In this paper, we track homotypic interactions between cells, but the technique can easily be extended to heterotypic cell interactions for the repertoire of signals that a stem cell may receive *in vivo*. For example, *in situ* addressable photopatterning [3] can be used to create heterotypic multicellular assemblies, which may then be cultured in isolation for extended periods of time using this architecture.

## Conclusions

The technology presented here possesses two important features. First of all, the cells can be trapped as single cells or cell pairs with high efficiency. This efficiency can be easily extended to trapping higher numbers of cells by modifying the spatial geometries of the traps. Secondly, the cells can continue to be cultured for many days after trapping, without significant change to the initial contact or separation state. Although sometimes paired cells will separate, the cells are generally observed to maintain their contact state for up to a day. Additionally, they will not migrate into neighboring wells because the PDMS mesh acts as a contact barrier. Although it is possible to seed one or two cells randomly into the corrals, the cells often never make contact over a 1 day period because they are likely to be seeded far apart.

The conjunction of these two features—high efficiency and long-term maintenance of the initial state—means that the technology can be used to dissect the downstream effects of contact-mediated signaling days after the cell–cell contact was initially specified. This is crucial because markers of these initial differentiation decisions often take from 1 to 6 days to become detectable. For these hippocampal progenitor cells, immunochemical staining cannot detect fate commitment until 4 days after fate induction by chemical inducers. The known markers, such as glial fibrillary acidic protein and beta-tubulin III, are cytoskeletal proteins that take many days to be expressed to adequate levels.

Alternatively, the detection of mRNA levels, either by mRNA microarray screens or qPCR, can be used earlier (at about 1–2 days) to detect changes in the expression levels of transcription factors that are markers of fate commitment. These mRNA detection methods generally require a substantial amount of starting material, the equivalent of approximately $10^3$–$10^6$ cells. Thus, the high efficiency of cell pairing in our method enables us to use mRNA detection techniques to probe the early transcriptional changes that occur as a result of cell-contact-mediated signaling processes.

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