A novel technique for positioning multiple cell types by liquid handling

Emma Luong-Van, Ryan Kok Chuan Kang, and William R. Birch[a)]
*Institute of Materials Research and Engineering, Agency for Science Technology and Research (A*STAR), 3 Research Link, Singapore 117602*

(Received 23 January 2009; accepted 30 March 2009; published 30 April 2009)

The spatial control of cells on a surface and the patterning of multiple cell types is an important tool for fundamental biological research and tissue engineering applications. A novel technique is described for the controlled seeding of multiple cell types at specific locations on a surface without requiring the use of specialized equipment or materials. Small-volume, quasi-hemispherical drops of cell solution are deposited onto a cell culture surface immersed under barrier oil, which serves to contain the drop and prevents evaporation of the cell culture medium during the time necessary for cells to attach to the cell culture surface. Subsequent flooding with an aqueous cell-compatible buffer displaces the barrier oil, allowing the cells to grow freely across the surface. This technique offers a simple and easily implemented solution for defining the initial position of cultured cells. The coculture of multiple cell types may be carried out by incorporating different cell types in each drop. A suitable drop volume was found to be 1 μl dispensed with a standard 0.5–10 μl pipette. The drop formed resulted in a footprint diameter of approximately 2 mm. Mineral oil and silicone oil do not compromise the viability of cultured cells when used in this technique. Moreover, a surface with heparin-immobilized FGF2 is shown to retain its bioactivity following drying of the substrate and contact with mineral oil. © *2009 American Vacuum Society.* [DOI: 10.1116/1.3122025]

I. INTRODUCTION

Techniques that facilitate the spatial control of cells on a substrate and the position of multiple cell types relative to each other provide a tool for fundamental biological studies. On patterned or gradient surfaces, control over the initial positioning of cells facilitates studies of their response to the functional surface. The positioning of multiple cell types also allows the interaction between different cell types to be controlled and measured.[1,2] Cell positioning techniques also find applications in tissue engineering, where multiple cell types are needed for complex tissue regeneration.[3]

A simple and commonly implemented technique for cell positioning relies on the patterning of attachment-permissive and nonpermissive regions on a substrate. This may be achieved by depositing cell adhesive or repellent molecules on the surface[4–7] or by using a removable cell-seeding stencil to prevent contact with the cell culture substrate.[8] The former generally restricts cell evolution to the attachment-permissive region, while the latter may allow cells to evolve freely over the cell culture surface. Physical barriers, such as cloning rings, are able to confine a cell to a specific location on a substrate. However, the sealing of the ring to the surface with grease generates a barrier to cell migration following the ring's removal. A cell culture substrate with specialized switching properties, such as a thermoresponsive polymer, has also been used to control cell attachment at specific locations.[9,10] When using these techniques, the seeding of more than one cell type may require multiple processing steps, where the attachment of one cell type is completed before the next can be introduced.[3,5,8]

More complex methods for accurate cell seeding make use of ink-jet printing and microfluidic devices to deposit cells at the desired positions.[11,12] These methods are suitable for implementing a high level of automation and they allow excellent spatial resolution. These advantages come with a high acquisition cost and an onerous experimental setup, rendering these techniques impractical for flexible laboratory-scale applications in exploratory research.

Glycerol and high serum concentrations have successfully been used to prevent evaporation from small volumes of cell culture media, deposited from an ink-jet printing apparatus.[13] However, the addition of high boiling point components into the cell solution may interfere with the function of the assay. Techniques for using sacrificial drops to reduce evaporation from small-volume drops have also been explored.[14]

The novel cell deposition technique described in this article provides confinement of the seeded cells via a wetting barrier. Droplets containing cells in culture medium are deposited on a cell culture substrate immersed in barrier oil. Their flexible positioning is not tied to a specific pattern and does not involve modification of the cell culture substrate. The barrier oil generates a boundary that is not crossed by the cells and prevents the evaporation of water during the time necessary for the cells to adsorb to the substrate. Its subsequent displacement by flooding with an aqueous buffer solution allows the cells to evolve and migrate across the surface. This use of a wetting barrier intrinsically provides a seal with the cell culture surface at the three-phase line. Inspiration for this wetting configuration was drawn from measurements of high surface energy solids, such as metals and silica-based glasses, where the energy balance of water drops deposited under octane yields finite and measurable contact angles.[15]

[a)]Author to whom correspondence should be addressed; electronic mail: w-birch@imre.a-star.edu.sg

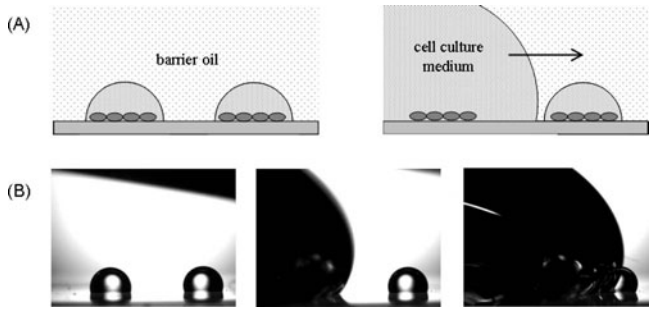

FIG. 1. (a) Schematic of the cell positioning technique. Drops of cell solution are deposited under barrier oil at the desired locations on the substrate. After cell attachment, the barrier oil is displaced by cell culture media, leaving the cells to grow freely from their seeding position. (b) Sequential photos of the displacement process.

The technique is implemented as shown in Fig. 1. Following immersion of the cell culture substrate in barrier liquid, small drops of solution containing cells are then deposited at desired locations on the surface under the barrier liquid. After allowing sufficient time for cell adhesion to the substrate, flooding with an aqueous medium that is compatible with the cells displaces the barrier oil from the cell culture substrate. Having thus defined their initial position, the cells may be cultured under normal conditions, allowing them to migrate across the surface.

While an essential property of the barrier oil is its immiscibility with aqueous (nonsurfactant) solutions, an additional desirable property is density lower than water. This is generally the case for hydrocarbon-based oils. A lower density barrier oil causes cell culture medium drops to sink onto the cell culture substrate and ensures its upward displacement when flooding the cell culture substrate with cell-compatible aqueous medium. The barrier liquid should not have specific interactions with the cell culture substrate. This avoids its adsorption onto the surface and into the underlying material. Importantly for biological applications, the barrier oil should neither affect the viability of the deposited cells nor compromise the bioactivity of the cell culture substrate. Biocompatible barrier oils are commonly used in small-volume embryonic cultures, utilizing mineral oil,[16] silicone oil,[17] and paraffin oil[18] to overlay the cell culture medium and prevent its evaporation.

This study tests the performance of three barrier oils: octane, mineral oil, and silicone oil, on a selection of cell culture surfaces, from standard cell culture plastics to surface-presented biomolecules. The accurate seeding of multiple cell types was demonstrated using a standard 0.5–10 μl pipette to deposit cell solution drops.

II. EXPERIMENT

A. Barrier liquids

Three barrier oil candidates were selected for testing: octane (Sigma-Aldrich 74821), mineral oil (Cell culture tested, Sigma M5310), and silicone oil (polydimethylsiloxane, Sigma-M6884). Their physical properties are listed in Table I. All three are immiscible with aqueous solutions and have densities lower than water.

TABLE I. Density, viscosity, and surface tension of the barrier liquids at 25 °C.

Barrier liquid	Density (g/ml)	Viscosity (Pa s)	Surface tension (mN/m)
Octane	0.703	0.0005	21.3 ± 0.2
Mineral oil	0.84	0.015	29.4 ± 0.2
Silicone oil	0.96	0.2	20.4 ± 0.1

B. Cell culture substrates

Cell culture surfaces tested were tissue culture plastic (TCP, Nunc), tissue culture grade glass slides (TCG, BD Falcon™ culture slides 354112), and surface-immobilized FGF2. TCP and TCG were used bare, coated with laminin (Sigma L2020), and coated with proteins from fetal calf serum (FCS, Invitrogen 26140-079). Laminin was used at 0.1 mg/ml in phosphate buffer saline solution (PBS). FCS was deposited from cell culture medium (CCM), consisting of 10% FCS in Dulbecco's modified eagles media (Invitrogen). The surfaces were coated by overnight incubation, followed by rinsing with PBS. A poly-(ethylene terephtalate) (PET, Goodfellow) substrate was functionalized with poly-(acrylic acid) (PAAc), as described by Li *et al.*[19] Biotin hydrazide (Sigma B7639) (1 mg/ml) was covalently attached to the PAAc via carbodi-imide cross-linking using 250 mM N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride for 1 h. The attached biotin was subsequently used to affinity bind streptavidin (0.1 mg/ml), which in turn immobilized biotinylated heparin (Sigma H3149) (0.1 mg/ml). Fibroblast growth factor 2 (FGF2, R&D Systems 133-FB-CF) was adsorbed to heparin by taking advantage of the specific interaction between the two biomolecules.[20] Unbound FGF2 was rinsed off with PBS and the presence of bound FGF2 confirmed by an enzyme-linked immunosorbent assay. This surface is referred to as heparin-immobilized FGF2.

C. Liquid handling

Liquid handling was evaluated by depositing sessile drops of CCM, mimicking the cell-seeding solution, on a range of model cell culture surfaces immersed in barrier oil. The substrates were immersed in barrier oil, contained in a transparent glass cell (Hellma 704.004), which allowed observation of the contact angle. Sessile drop contact angles were measured using a goniometer (Ramé-Hart Inc.), interfaced with DROPIMAGE advanced software (Ramé-Hart Inc.). This same instrument was used to image hanging drops, both suspended in air and under barrier oil, and calculate surface tension values.

D. Cell culture

NIH3T3 mouse fibroblast cells and S180 mouse sarcoma cells were maintained in CCM supplemented with 10% FCS, 2mM L-glutamine, and penicillin/streptomycin. This CCM formulation was used in all the cell culture experiments described below.

Cell viability was tested following seeding under the barrier oils on each of the substrates. The dry substrate was submerged in a 0.5 cm layer of barrier oil in a glass chamber. The cell solution consisted of NIH3T3 cells, dispersed at a concentration of 1×10^6 cells/ml in CCM. 1 μl drops of cell solution were deposited onto the substrate using a standard 0.5–10 μl range Eppendorf pipette. The chamber was then placed for 1 h in a 37 °C incubator to allow cell attachment. The barrier oil was subsequently displaced by tilting the chamber by approximately 10° and gently pouring in PBS. Floating oil was removed by aspiration and the cell culture substrate pulled out of the aqueous medium. To avoid adhesion of any remaining free-floating cells, the substrate was briefly rinsed in PBS and moved to a new culture vessel, containing CCM. The cells were then photographed and their expansion was monitored for several days' culture under normal conditions.

E. Coculture

1. Multiple cell types in a one-step seeding process

Two cell species, NIH3T3 and S180, were separately resuspended in CCM at a concentration of approximately 1×10^6 cells/ml. 1 μl drops of each cell solution were deposited in an alternating sequence onto the surface under mineral oil. The cells were allowed to attach for 1 h at 37 °C before displacing the barrier oil with PBS. After extracting the substrate and briefly rinsing in PBS, the cells were placed in a new culture vessel with fresh CCM. The cells were then photographed after 24 h to allow visual distinction between cell morphologies.

2. Multiple cell types in a two-step seeding process

NIH3T3 cells were seeded onto the surface under mineral oil using 1 μl drops. Cells were allowed to attach for 1 h at 37 °C and the barrier oil displaced with PBS. Following a brief rinse in PBS, the surface was then flooded with S180 cells dispersed in CCM for 1 h at 37 °C. The sample was then rinsed in PBS to remove unattached cells, placed in CCM and then photographed after 24 h.

F. Bioactivity of a functionalized surface after oil exposure

The impact of drying and immersion in mineral oil on the bioactivity of heparin-immobilized FGF2 was probed by measuring the proliferative response of NIH3T3 cells. The substrates with heparin-immobilized FGF2 were first air dried and then submerged in mineral oil for 15 min. Following displacement of the oil by PBS, the substrates were seeded with NIH3T3 cells at a density of 1.5×10^4 cells/cm^2. Cells were starved in serum-free CCM for 48 h prior to seeding onto the substrates. Cell proliferation on this surface was compared with an FGF2 substrate that received only air drying and an FGF2 substrate kept in contact with PBS before cell seeding. The control surface was surface-immobilized heparin, without FGF2. After 48 h, cell proliferation was measured using an XTT colorimetric assay (Roche), following the manufacturer's instructions. All assays were performed in triplicate and the significance was determined by students' t test and set to $p\leq0.05$.

TABLE II. Contact angles, in degrees, measured under mineral oil. (Mean is calculated from eight contact angle measurements. Standard deviation was ±1 for all surfaces.)

Substrate	Water	CCM
TCP bare	111	140
TCP+FCS	67	60
TCP+laminin	95	82
TCG bare	82	111
TCG+FCS	86	80
TCG+laminin	87	78
Heparin-immobilized FGF2	60	40

III. RESULTS AND DISCUSSION

The criterion of immiscibility with water allows several classes of barrier liquids to be considered. The low density and potential biocompatibility of hydrocarbon alkane oils and fatty acids render them suitable for implementing this technique. Silicone oil has a lower density differential with water and its viscosity is an order of magnitude higher than that of mineral oil. Despite these differences, both mineral oil and silicone oil had acceptable liquid handling properties. The latter refer to the successful displacement of the barrier oil when flooding with CCM and the subsequent aspiration of barrier oil. Early liquid handling experiments tested two vegetable-based oils (canola and sunflower oil), which contain triglycerides and have high viscosity (0.05–0.1 Pa s). These oils presented significant difficulties in liquid handling, with some areas of the substrate coated by a thin film of residual barrier oil after displacement by PBS. Cell viability with these vegetable oils was also poor (data not shown), possibly due to the amphiphilic nature of the fatty acid molecules causing them to adsorb to the cell culture surface.

A. Liquid handling

The wetting of aqueous CCM solution is measured from sessile drop contact angles on several model cell culture surfaces immersed in barrier liquid. These data are shown in Table II. Contact angles measured under octane and silicone oil are in a similar range (data not shown), providing the same liquid handling behavior on these substrates.

Differences in wettability between water and CCM are attributed to the amphiphilic biomolecules carried by CCM. These molecules segregate at interfaces, as evidenced by the surface tension of the air-water interface. The surface tension of CCM (62 mN/m for a freshly formed surface, decreasing

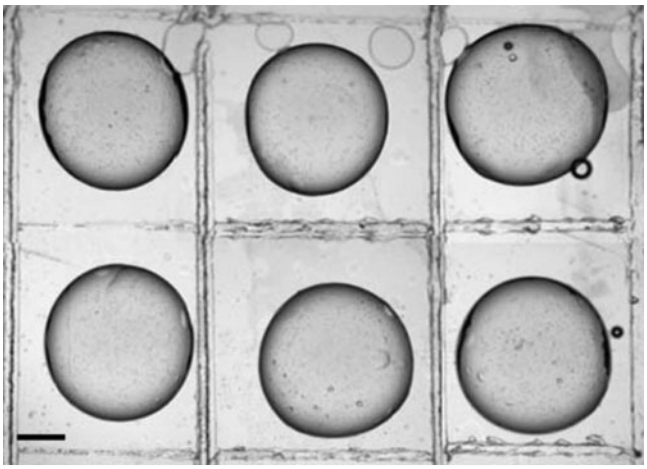

FIG. 2. Positioning 1 μl drops of cell suspension within a 2×2 mm^2 grid immersed in mineral oil. Scale bar=500 μm.

asymptotically toward 56 mN/m over approximately 3 min) is lower than that of water (72.7 mN/m). Biomolecules carried by CCM adsorb to bare TCP and TCG, enabling the cell culture function of these substrates. The increased contact angle of CCM versus water on TCP and TCG is attributed to these adsorbed biomolecules, which decrease the wettability of the bare TCP and TCG surfaces. On protein-coated surfaces, whose wettability is less altered by the adsorption of biomolecules, the lower surface tension of CCM decreases the contact angle.

Protein-coated substrates may be considered to represent biofunctionalized surfaces. The contact angles of CCM under barrier oil on these surfaces fall into a convenient range, from about 60° to 80°. Low contact angle values, below about 20°, lead to a high degree of cell solution spreading. Contact angles significantly higher than 90° can lead to a failure of PBS to displace the barrier oil following cell attachment. A heparin-immobilized FGF2 surface displays the lowest contact angle. This may be caused by the hydrophilic nature of the underlying PAAC scaffold and the immobilized heparin polysaccharide.

Drops placed on a square grid with spacing approximately one and a half times their diameter present no risk of coalescence when deposited on a surface with uniform wettability. In contrast, the positioning accuracy of spreading drops may be influenced by nonuniform surface wettability. The manual positioning of drops was demonstrated on a uniform surface within a 2×2 mm^2 grid (Fig. 2). This experiment used 1 μl drops, with a contact angle of approximately 90°, yielding a drop diameter of about 1.7 mm on the surface. While this spatial resolution is far lower than that achieved by ink-jet printing,[11] these drops were deposited by hand from a standard 0.5–10 μl range pipette. Although the use of a dispensing needle with smaller drop volume would increase the spatial resolution of this technique, care must be exercised to avoid excessive shear forces through a narrow needle or dispensing tip.

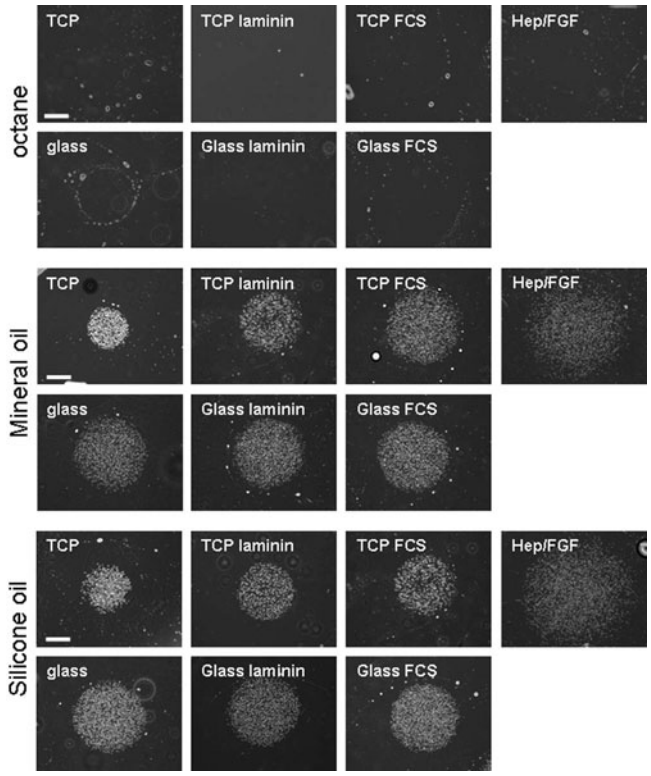

FIG. 3. Cells seeded under octane, mineral oil, or silicone oil on TCP or TCG. TCP and TCG are used bare, coated with laminin, and coated with FCS proteins. Heparin/FGF2 is immobilized on a polymer scaffold, grafted to a PET substrate. Scale bar=500 μm.

B. Cell attachment

Cell attachment under barrier oils was tested with NIH3T3 fibroblasts by depositing 1 μl drops, followed by an incubation time of 1 h. Seeding was successful under both silicone and mineral oil (Fig. 3). The incubation time was sufficient for cell seeding to form a confluent monolayer of cells within the footprint of the drop. These remained attached to the substrate after oil displacement. The seeded cells subsequently expanded to form a confluent layer over the entire substrate surface, indicating that the cell-seeding process did not impair cell viability.

In contrast to mineral oil and silicone oil, cells seeded under octane showed poor attachment to all substrates tested. No explanation was found for the lack of cell adhesion to the culture substrate immersed in octane. A toxicity study was performed to discount the possibility of toxic impurities leaching from octane into the aqueous phase. For this experiment, a layer of octane was spread over a culture of cells in cell culture media. Following 1 h contact of the cell culture medium with octane, no effect on cell viability was observed by live/dead staining (data not shown). This result implies that direct contact of cells with octane may be responsible for the lack of cell attachment. While octane may adsorb into polymeric substrates, it is fully displaced from glass surfaces by spreading water, as observed by wettability measurements on glass surfaces. To illustrate the wettability resulting from an ultrathin film of octane, the sessile contact angle of water

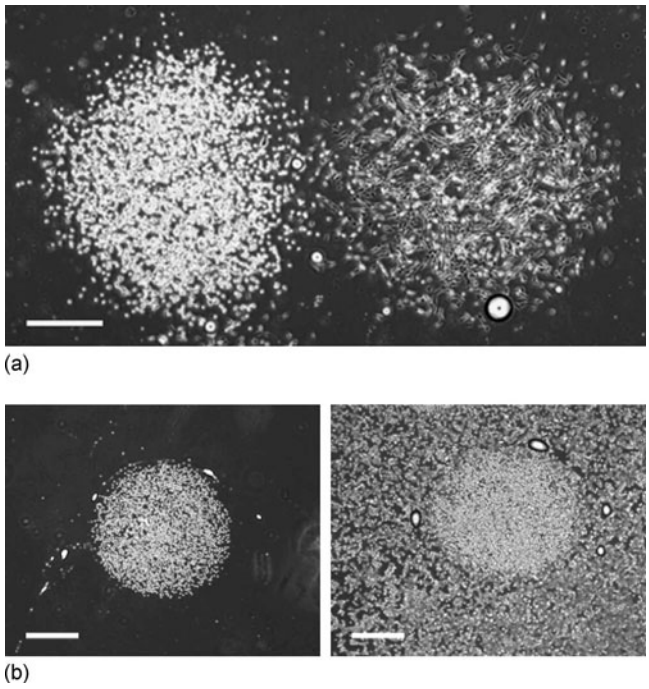

FIG. 4. (a) One-step seeding process under mineral oil: NIH3T3 cells adjacent to S180 cells. (b) Two-step seeding process: S180 cells seeded under mineral oil, followed by uniform seeding of NIH3T3 cells. Scale bar =500 μm.

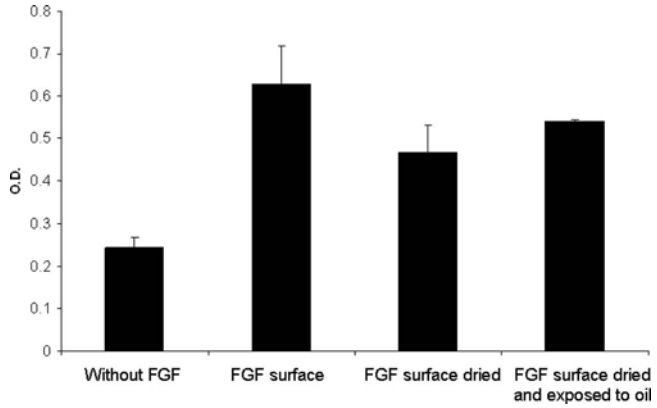

FIG. 5. Proliferation of NIH3T3 cells on heparin-immobilized FGF2 following drying and exposure to mineral oil. The surface without FGF2 is immobilized heparin.

was measured on a glass surface with a grafted monolayer of octadecyl silane. The contact angle of water on a monolayer of alkane immersed in octane was 165°, easily distinguishable from the 77° contact angle of water on bare TCG immersed octane. These measurements correspond to a wetting model where CCM fully displaces the barrier oil coming into direct contact with the substrate surface. This leads to the conclusion that seeded cells should come into contact with bare TCG.

No adverse effect of hypoxia on cell viability was observed during the time required for attachment. While cell types requiring longer attachment times may need a larger drop volume to prevent hypoxia, previous studies have shown that embryos cultured in a 5 μl drop volume under silicone oil remain viable after 120 h.[21]

Mineral oil was selected for all further cell-seeding experiments. Silicone oil was omitted due to its strong adsorption to exposed silanol groups on glass surfaces.

C. Cell coculture

An advantage of this technique is the lack of an upper limit on the number of cell types that may be placed on a common surface in one seeding step. In this sense, this technique lends itself to the creation a cell array for high throughput screening. Cells can be positioned at specific locations on a surface for a coculture study of cell interactions. Figure 4(a) shows an example of one-step cell seeding with two cell species: NIH3T3 and S180 cells. Figure 4(b) shows the result of a two-step version of the seeding method. In this variation, the first cell type is deposited at a desired location on the substrate using the barrier oil technique. Following attachment of the cells and displacement of the barrier oil, the surface is exposed to cell culture medium containing the second cell type, allowing these cells to attach to free areas on the surface. The ease of implementing this technique on a small laboratory scale facilitates the study of cell behavior on patterned or gradient surfaces, where initial cell position on the substrate may be critical to the experimental outcome.

D. Bioactivity of functionalized surfaces

When using barrier oil, a concern arises for its effect on the bioactivity of a substrate coated with biomolecules. Proteins immobilized on the cell culture substrate are susceptible to denaturation, which may compromise their function. Implementation of this cell-seeding technique requires drying of the surface before immersion in barrier oil. Both the drying process and the exposure of the surface to a hydrophobic environment are capable of denaturing proteins. FGF2, affinity bound to surface-immobilized heparin, was used as a model test of the impact of this cell-seeding technique on bioactivity. The proliferation of cultured NIH3T3 fibroblasts was measured on heparin-bound FGF2 after drying alone and after drying with subsequent exposure to oil. This was compared to the same cells cultured on a freshly coated heparin-bound FGF2 substrate. The proliferative effect of the immobilized FGF2 on the cells is shown in Fig. 5.

Freshly coated heparin-bound FGF2 generates a distinguishable enhancement in NIH3T3 proliferation. Despite a decrease in the bioactivity of heparin-bound FGF2 after drying the performance of the surface remains higher than the control and is not further degraded following exposure to mineral oil ($p \geq 0.5$). Denaturation of protein upon contact with a hydrophobic environment occurs at the aqueous/oil interface. However, denaturation is small when a solid protein is mixed with an organic phase.[22] The use of protective agents to mitigate the loss of bioactivity induced by drying surfaces functionalized with fragile biomolecules may enhance applications of this cell-seeding technique.

IV. CONCLUSION

The ability to seed cells at specific locations provides an enabling technology for probing cell response to gradient and patterned surfaces. It also enables the coculture of different cell types. This opens the possibility of creating cell arrays, suitable for high throughput screening or for probing interactions between cells. A novel cell-seeding technique is described and its implementation demonstrated with common laboratory supplies. This simple technique for positioning cells relies on the use of a wettability barrier, which confines the cells within cell culture drops. The overlay of cell culture medium drops with barrier oil prevents evaporation and allows the cells to survive for the time necessary for them to attach to the surface. This wettability barrier does not require the use of a stencil and its effective removal is accomplished by flooding with a cell-compatible aqueous solution. Mineral oil and silicone barrier oils used in this technique do not compromise the viability of the seeded cells, which expand freely across the cell culture substrate following displacement of the barrier oil. The bioactivity of heparin-immobilized FGF2 was not degraded from contact with barrier oil after drying the substrate. This technique is expected to allow the flexible positioning of cell populations on both standard cell culture substrates such as glass and plastic, as well as surfaces functionalized with different biomolecules.

ACKNOWLEDGMENTS

The authors would like to acknowledge the funding support of the Institute of Materials Research and Engineering and the Agency for Science, Technology and Research (A*STAR), Singapore.

[1]S. N. Bhatia, U. J. Balis, M. L. Yarmush, and M. Toner, J. Biomater. Sci., Polym. Ed. **9**, 1137 (1998).
[2]S. N. Bhatia, U. J. Balis, M. L. Yarmush, and M. Toner, FASEB J. **13**, 1883 (1999).
[3]Y. Tsuda, A. Kikuchi, M. Yamato, A. Nakao, Y. Sakurai, M. Umezu, and T. Okano, Biomaterials **26**, 1885 (2005).
[4]J. Fukuda, A. Khademhosseini, J. Yeh, G. Eng, J. Cheng, O. C. Farokhzad, and R. Langer, Biomaterials **27**, 1479 (2006).
[5]A. Khademhosseini, K. Y. Suh, J. M. Yang, G. Eng, J. Yeh, S. Levenberg, and R. Langer, Biomaterials **25**, 3583 (2004).
[6]V. A. Liu, W. E. Jastromb, and S. N. Bhatia, J. Biomed. Mater. Res. **60**, 126 (2002).
[7]T. Peterbauer, J. Heitz, M. Olbrich, and S. Hering, Lab Chip **6**, 857 (2006).
[8]D. Wright, B. Rajalingam, S. Selvarasah, M. R. Dokmeci, and A. Khademhosseini, Lab Chip **7**, 1272 (2007).
[9]X. Cheng, Y. Wang, Y. Hanein, K. F. Bohringer, and B. D. Ratner, J. Biomed. Mater. Res. Part A **70A**, 159 (2004).
[10]H. Hatakeyama, A. Kikuchi, M. Yamato, and T. Okano, Biomaterials **28**, 3632 (2007).
[11]J. A. Barron, D. V. Krizman, and B. R. Ringeisein, Ann. Biomed. Eng. **33**, 121 (2005).
[12]S. Takayama, J. C. McDonald, E. Ostuni, M. N. Liang, P. J. Kenis, R. F. Ismagilov, and G. M. Whitesides, Proc. Natl. Acad. Sci. U.S.A. **96**, 5545 (1999).
[13]E. Berthier, J. Warrick, H. Yu, and D. Beebe, Lab Chip **8**, 852 (2008).
[14]E. Litborn and J. Roeraade, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. **745**, 137 (2000).
[15]C. Vittoz, M. Mantel, and J. C. Joud, J. Adhes. **67**, 347 (1998).
[16]S. Lee, M. Cho, E. Kim, T. Kim, C. Lee, J. Han, and J. Lim, J. Vet. Med. Sci. **66**, 63 (2004).
[17]A. Vann Soon, A. R. Mahmoudzadeh, A. Christophe, M. T. Ysebaert, and A. de Kruif, Reprod. Domest. Anim. **36**, 169 (2001).
[18]J. C. Tae, E. Y. Kim, W. D. Lee, S. P. Park, and J. H. Lim, J. Assist. Reprod. Genet. **23**, 121 (2006).
[19]B. Li, S. Wang, and P. M. Moran, Biomaterials **26**, 1487 (2005).
[20]D. M. Ornitz and N. Itoh, Genome Biol. **2**, 3005 (2001).
[21]R. S. Canseco, A. E. T. Sparks, R. E. Pearson, and F. C. Gwazdauskas, J. Assist. Reprod. Genet. **9**, 454 (1992).
[22]H. Sah, J. Pharm. Sci. **88**, 1320 (1999).