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Micromolded Arrays for Separation of Adherent Cells

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Abstract

We present an efficient, yet inexpensive, approach for isolating viable single cells or colonies from a mixed population. This cell microarray platform possesses innovations in both the array manufacture and the manner of target cell release. Arrays of microwells with bases composed of detachable concave elements, termed microrrafts, were fabricated by a dip-coating process using a polydimethylsiloxane mold as the template and the array substrate. This manufacturing approach enabled the use of materials other than photoresists to create the array elements. Thus microrrafts possessing low autofluorescence could be fabricated for fluorescence-based identification of cells. Cells plated on the microarray settled and attached at the center of the wells due to the microrrafts' concavity. Individual microrrafts were readily dislodged by the action of a needle inserted through the compliant polymer substrate. The hard polymer material (polystyrene or epoxy resin) of which the microrrafts were composed protected the cells from damage by the needle. For cell analysis and isolation, cells of interest were identified using a standard inverted microscope and microrrafts carrying target cells were dislodged with the needle. The released cells/microrrafts could be efficiently collected, cultured and clonally expanded. During the separation and collection procedures, the cells remained adherent and provided a measure of protection during manipulation, thus providing an extremely high single-cell cloning rate (>95%). Generation of a transfected cell line based on expression of a fluorescent protein demonstrated an important application for performing on-chip cell separations.

INTRODUCTION

The selection and isolation of single cells from a mixed population is a common procedure performed throughout biomedical research. For example, during the development of clonal cell lines that are genetically engineered, derived from stem cells, or grown from patient samples, single cells must be isolated and then cloned to form a homogeneous population. A variety of strategies exist to selectively identify and collect individual non-adherent cells from a mixed population, including fluorescence activated cell sorting (FACS), limiting dilution, panning, column chromatography and magnetic sorting; furthermore, new techniques based on microfluidics and dielectrophoresis show promise in this area.^{1–6} To address the need to collect pure or enriched populations of cells that normally grow in an adherent fashion, investigators use these procedures combined with disaggregating or stripping the cells from their growth surface to create cell suspensions. Unfortunately, enzymatic or mechanical release imposes significant drawbacks including loss of cell morphology, removal of cell surface markers, damage to cell membranes, alterations in cellular physiology and loss of viability.^{7–14} New techniques for separation of adherent,

mammalian cells address some of these challenges, but remain limited for living cells. Laser microdissection (LM) has enabled single cells or small groups of selected cells to be obtained from tissue sections for genetic and proteomic studies; however the vast majority of applications utilize fixed or frozen specimens rather than living cells.^{15–18}

The ability to analyze adherent cells cultured on an array provides a unique cell analysis tool. Cells patterned in an array can be assessed based on morphology and time resolved characteristics that are not possible using conventional sorting methods.¹⁹ Sample sizes can also vary from tens of cells to over a million cells with an array format. Unfortunately, conventional cell arrays are not compatible with cell sorting. To address this issue, arrays of transparent, microfabricated polymer elements formed on glass slides were developed to sort adherent cells.^{19–24} Cells cultured on these elements can be isolated by release of the individual microstructures using the focused beam of a laser. Despite advantages of enhanced selection criteria and small sample sizes, multiple limitations remain. The most serious constraint is the requirement for a microscope with an integrated laser system to release the micron-scale structures. The optical system (including solid-state laser, beam splitter, mirror and lens) adds significant cost and must be carefully aligned and maintained. Furthermore, to effectively release individual structures, the laser pulse must be precisely focused within a distance of a few microns of the polymer:glass interface. Another drawback is that the microstructures must be fabricated from a photoresist with incorporated photoinitiator since the array is lithographically defined. The cured photoresists used to date possess autofluorescence in the range of 480 – 520 nm, which overlaps the wavelength range of many commonly used fluorescent dyes (*e.g.* fluorescein, Oregon green, Alexa Fluor 488, *etc.*). Thus, an inexpensive, easily maintained, cell-sorting platform capable of utilizing a wide variety of low-fluorescence-substrate materials for cell growth would be of high value in biomedical research.

In the current article, an improved microarray platform for cell sorting applications was fabricated and evaluated. An array of microwells possessing detachable concave elements, termed microrrafts, was manufactured using a polydimethylsiloxane (PDMS) mold combined with a dip-coating process. The ability to fabricate arrays of microrrafts from low fluorescence materials such as epoxy monomers or polystyrene was demonstrated. The compatibility of the arrays with standard brightfield and fluorescence microscopy methods as well as cell growth and colony segregation was evaluated. The efficiency of targeted microrraft release, as well as non-target microrraft release, by needle insertion through the PDMS substrate was quantified. Separation of viable cells from the array was established and measurement of the cell cloning rate was determined. Finally the feasibility of cloning molecularly engineered cells using the array was demonstrated.

MATERIALS AND METHODS

Materials

SU-8 photoresist was purchased from MicroChem Corp. (Newton, MA). 1002F photoresist was formulated according to a previous publication.²⁵ The Sylgard 184 silicone elastomer kit was purchased from Dow Corning (Midland MI). Gamma-butyrolactone (GBL), octyltrichlorosilane, propylene glycol monomethyl ether acetate, rhodamine B, glutaraldehyde, L-glutamine, acrylic acid, and azobisisobutyronitrile were obtained from Sigma-Aldrich (St. Louis, MO). EPON epoxy resin 1009F and 1002F (fusion solids) were purchased from Miller Stephenson Chemical Co. (Sylmar, CA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA). Polycarbonate plates (12 inch × 12 inch × 0.25 inch) were purchased from McMaster-Carr (Los Angeles, CA). Matrigel™ basement membrane matrix

and Falcon™ Petri dishes were obtained from BD Biosciences (San Jose, CA). All other reagents were from Fisher Scientific (Pittsburgh, PA).

Fabrication of PDMS mold imprinted with a microwell array

The PDMS mold was fabricated by casting PDMS on an SU-8 master. The PDMS mold was imprinted with microwell arrays with controlled depth and dimension over the range of 20 – 500 μm . The SU-8 master was fabricated by standard photolithography on a glass slide with 40 – 60 μm thick SU-8 as described previously.²¹ The surface of the master mold was treated to render it non-sticky to PDMS by spin coating 1 vol% octyltrichlorosilane in propylene glycol monomethyl ether acetate at 2000 rpm for 30 s, followed by baking at 120 °C on a hotplate for 10 min. PDMS prepolymer (10:1 mixture of base:curing-agent of Sylgard 184 kit) was spread on the master mold, and degassed under vacuum to remove trapped air bubbles. To control the thickness of PDMS, PDMS was spin-coated at 500 rpm for 30 s on the master to yield a 200 μm -thick PDMS layer. The PDMS was cured by baking the master at 100 °C on a hotplate for 30 min. The PDMS mold forming the multiwell array (Fig. 1A–i) was then obtained by peeling it from the master.

Replica molding of microrrafts by dip-coating

A liquid coating solution composed of polymer (polystyrene, poly[styrene-co-acrylic acid], or epoxy resin) in gamma-butyrolactone (GBL) was prepared at an appropriate concentration. Polystyrene solution was prepared by dissolving a Petri dish in GBL at 30 wt % concentration. 40 wt% poly(styrene-co-acrylic acid) (9:1 mol:mol) solution was prepared by copolymerization of styrene and acrylic acid in GBL as described in Supplementary Material. Epoxy resin solution was prepared by dissolving 30 - 50% EPON 1002F or 1009F epoxy resin in GBL. Fig. 1A–iii shows the schematics of the dip coating process which is also described in detail in the Supplementary Material (Fig. S1). The liquid molding solution was first spread on the microwell array (Fig. 1A–ii). The trapped air bubbles in the microwells were removed by degassing under vacuum. The PDMS mold was immersed in the solution and then withdrawn vertically at a controlled speed (Fig. 1A–iii). After the entire PDMS mold was removed, it was placed horizontally inside an oven and baked at 95 °C for 2 h to evaporate the bulk of the solvent leaving solid structures within the microwells. The array was then further baked in a vacuum oven (–30 inches Hg) at 120 °C for 1 h to completely evaporate any remaining solvent from the molded material.

To improve the flatness of the flexible PDMS-based array, the array was attached to a rigid plastic cassette made from polycarbonate with a 25.4 mm $\times$ 25.4 mm area. In-plane stretching of the PDMS mold was used to further reduce array sagging. PDMS was stretched along the axes parallel to the array surface to offset the out-of-plane bowing (z-axis) before it was glued to the support structure.

Cell culture on the microrraft array

A plastic support structure to hold the array and create a culture chamber similar to a Petri dish (25.4 mm $\times$ 25.4 mm $\times$ 6.35 mm) was machined from a polycarbonate plate using a computer numerical controlled (CNC) machine (Supplementary Material Fig. S2A). The array was attached to the structure by gluing it in place using PDMS and curing in an oven at 70 °C for 1 h. For arrays composed of epoxy and polystyrene microrrafts, the array was treated in an air plasma cleaner (Harrick Plasma, Ithaca, NY) for 1 min to produce a negative surface charge on the microrrafts. For arrays composed of poly(styrene-co-acrylic acid) microrrafts, the plasma treatment was omitted. The array was sprayed with 75% ethanol for sterilization, and then dried under sterile conditions in a tissue culture hood. Three milliliters of phosphate buffered saline (PBS) was added to the chamber. To remove the trapped air bubbles inside the microwells, the PBS-covered plate was placed under a

vacuum and degassed for 20 min at room temperature. The PBS buffer was aspirated, and a suspension of cells (15,000 cells) was added to the chamber. Three types of cells were used in this study: HeLa, the mouse embryonic stem cell line ES129, and primary cells isolated from a needle biopsy obtained from a resected pancreatic tumor (isolation protocol is described in Supplementary Material). Before loading the array with ES129 cells, the array was coated with Matrigel (1/20 dilution in DMEM) for 30 min in a 37 °C incubator. The cells were cultured on the array in DMEM supplemented with FBS (10%), and L-glutamine (584 mg/L) at 37 °C in a humidified, 5% CO₂ atmosphere. Penicillin (100 units/mL) and streptomycin (100 µg/mL) were added to the media to inhibit bacterial growth.

Release the targeted micrafts with a needle

A motorized system was built to release the targeted micrafts from the array with a needle (Fig. 4A and Supplementary Material Fig. S2C). A miniaturized DC motor (Pololu Robotics & Electronics, Las Vegas, NV) controlled by a simple switch-activated circuit was attached to the Z-micrometer of an XYZ micromanipulator mounted on an inverted microscope. An anodized steel microneedle (150 µm base diameter, 17.5 µm tip diameter, Fine Science Tools, Foster City, CA) was mounted the DC motor. The array was placed on the microscope stage in an inverted position with the needle above the base of the array. The cells remained immersed in media within the cassette created by mating the array and collection chambers (see below). To release the targeted structure, the needle was aligned with the micraft. The user then pressed a button to trigger the motor to lower the needle a specified distance to pierce the PDMS and extrude the micraft. The motor automatically reversed to bring the microneedle back to its original Z position in preparation for the next release action.

Cell collection after the release of micrafts

A collection chamber (40 mm × 40 mm × 6.35 mm) was machined from a polycarbonate plate by a CNC machine and a glass plate glued in place to form a clear base (Supplementary Material Fig. S2B). Prior to needle release, the micraft array was rinsed with fresh culture medium to remove non-adherent and dead cells. Then 5 mL of fresh culture medium was added to completely fill the cell culture chamber forming a convex fluid surface. The collection plate was mated directly on the cell culture chamber. Any excess liquid was aspirated from the assembled cassette at the conclusion of the experiment. The cassette assembled in this manner formed an enclosed compartment housing the array that was filled with culture medium and lacking air bubbles. The assembly was then inverted and placed on the microscope stage (Fig. 4A). Micrafts containing selected cells were released, whereupon the micrafts settled on the collection plate by gravity. The collection plate and array were then separated in a sterile environment and the collection plate containing the released cells/micrafts was transferred to a standard tissue culture incubator. The growth of the collected cells was monitored daily by brightfield imaging.

Transfection of HeLa cells with enhanced green fluorescent protein (eGFP)

HeLa cells (1×10^5 cells) were transfected with pmaxGFP® plasmid (Lonza Group, Switzerland) using DreamFect™ transfection reagent (OZ Biosciences, France) per supplier protocol. The transfected cells were allowed to recover for 2 days in DMEM and then were plated on the arrays.

Characterization of autofluorescence with standard microscopy filter sets

The 1002F photoresist (formulation 10) was formulated according to a previous publication.²⁵ SU-8 photoresist (formulation 10), 1002F photoresist, 1009F resin (30 wt% in GBL), or polystyrene (30 wt% in GBL) were used as dip coating solutions for fabrication of the

arrays. In these experiments, the microwells in the PDMS mold were 100 μm square, 40 μm deep, 20 μm inter-well spacing. The array was baked at 95 $^{\circ}\text{C}$ for 2 h. The SU-8 and 1002F micrafts were exposed to UV at a dose of 400 and 800 mJ respectively, and baked at 95 $^{\circ}\text{C}$ for 10 min to complete the photo-induced crosslinking reaction. Finally, all four types of micrafts were baked in 120 $^{\circ}\text{C}$ vacuum oven for 1 h. The native fluorescence of the micrafts was examined by a Nikon Eclipse TE300 inverted fluorescent microscope equipped with three fluorescent filter sets: a fluorescein filter set (B-2A; Nikon Instruments; excitation filter 450–490 nm, dichroic 500 nm long pass, emission 520 nm long pass); a CY3 filter set (G-2E; Nikon Instruments; excitation filter 528–553 nm dichroic 565 nm long pass, emission 590–650 nm); and a Cy5 filter set (41008; Chroma Technology, Rockingham, VT; excitation filter 590–650 nm, dichroic 660-nm long pass, emission 665–740 nm). Images were collected with a cooled CCD camera (Photometrix Cool Snap; Roper Scientific, Tuscon, AZ) using NIS-Elements software. A line profile of fluorescence intensities across four individual micrafts was obtained from the fluorescence images using NIS-Elements software (Nikon Instruments). The average intensity was used to quantitatively compare the autofluorescence of the molded structures.

Scanning electron microscopy (SEM) of cells

Cells cultured on the arrays were rinsed gently with PBS and then fixed with 2.5 wt% glutaraldehyde in PBS for 30 min. The sample was washed with PBS and dehydrated with a series of ethanol/water mixtures of increasing ethanol concentration (30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% ethanol, 10 min in each mixture). The fixed cells were observed by SEM (FEI Quanta 200 ESEM, FEI Company).

RESULTS AND DISCUSSION

Replica molding of micrafts by dip-coating a PDMS mold

The soft-lithographic fabrication via template-assisted, replica-molding process has been used for micro and nanoscale fabrication of polymeric structures.^{26–28} In the current work, the replica-molding process was modified to fabricate arrays of micrafts for cell separations. High quality arrays of micromolded structures were fabricated by a dip-coating process (Fig. 1A), in which a PDMS mold was first immersed into a liquid coating solution, and then was withdrawn from the solution at a controlled speed (see below). The mold was prepared by casting PDMS against a master, which was fabricated from SU-8 photoresist using standard photolithography processes. The mold was imprinted with microwell arrays with controlled depth and dimension in the range of 20 – 500 μm (Fig. 1A–i). The coating solution was added to the PDMS mold, and application of a vacuum was used to remove air bubbles trapped inside each well (Fig. 1A–ii). To generate an isolated micraft inside each microwell required that the coating solution be drained from the PDMS mold's surface by gravity without leaving any residue. Therefore, the coating solution must be dewetting on the PDMS. PDMS has a hydrophobic surface with a surface tension of 16 – 21 dyne/cm.²⁹ Common solvents (*e.g.* ethyl acetate, xylene) are known to significantly swell PDMS, and therefore were unsuitable for the molding process on PDMS.³⁰ GBL, a polar solvent, was tested to solubilize the material used to create the micrafts due to its relatively high surface tension of 40 dyne/cm.³¹ To test the compatibility of GBL with PDMS, PDMS swelling in GBL was assessed. A slab of PDMS (20 mm $\times$ 20 mm $\times$ 2 mm) was incubated in GBL for 15 min (the usual time needed for a dip coating process), and the swell ratio (defined as the gain of weight) was found to be only 1.0094 ± 0.0005 ($n = 3$) demonstrating that GBL does not swell PDMS. This property and its high surface tension made this solvent an appropriate choice for the application.

EPON 1009F epoxy resin, polystyrene and poly(styrene-co-acrylic acid) were chosen as test materials to create the micrafts since they could be dissolved in GBL and were expected to be biocompatible.^{25, 32} The PDMS mold was immersed then withdrawn from the solution created by dissolving one of the materials in GBL. In all cases, excess solution drained from the PDMS surface as a result of gravity and the dewetting phenomena caused by the discrepancy of the surface tensions (Fig. 1A–iii). The withdrawal speed was performed at a constant speed of 1 – 5 mm/min controlled by a slow DC rotary motor so that the solution was allowed enough time to drain from the PDMS surface. To maintain a constant concentration, the dip-coating was performed in a sealed glass jar to prevent solvent evaporation. After the entire PDMS mold was withdrawn from the solution, each microwell was observed to be filled with liquid possessing a convex surface (Fig. 1A–iv). The array was then baked in a horizontal position to evaporate the solvent. Evaporation resulted in shrinkage of the polymer and formation of a solid micraft with a concave surface (Fig. 1A–v, B, C). The thickness of this structure could be adjusted by altering the concentration of polymer in the solvent. For example, by using 30 wt% concentration the height of the micraft was approximately 1/3 of the depth of the well. The dip-coating strategy permits very large arrays to be created with ease. As an example, an extended array of 300 mm × 50 mm × 0.2 mm containing >10⁶ micrafts (100 μm square, 20 μm inter-raft spacing) could be readily processed by this dip-coating method (Fig. 1D). The approach provides the prospect for a simple mass production capability to create high quality microarrays inexpensively.

Micromolding adds versatility to the manufacture of these arrays. Identifiers such as numerical coding can be fabricated by placing the number on the PDMS mold. Four-digit numbers were fabricated every 5 × 5 micrafts on the array to indicate their X-Y location (Supplementary Material Fig. S3A). By virtue of the molding process, the numbers were imprinted onto the micrafts and allowed tracking the micrafts after they were detached (Supplementary Material Fig. S3B). The solution used to create the molded structures was composed of only a polymer and solvent as inclusion of photocatalyst and UV illumination were not required. Other molecules could be mixed into the solution for making the molded structures. As a simple example of the ability to create a composite molding material, an array of micrafts composed of polystyrene with 3% rhodamine B was fabricated (Supplementary Material Fig. S3C).

Molded micrafts have extremely low autofluorescence

Fluorescence-based assays are important tools for cell selection. Thus it was important to measure the native fluorescence of the molded structures. Micrafts of 1009F resin, and polystyrene were formed in PDMS molds (microwell dimensions L×W×D = 100 μm × 100 μm × 40 μm) and their fluorescence intensities were measured as described above. For comparison, micrafts were molded in an identical manner from the photoresists SU-8 and 1002F. The micraft thickness was around 15 μm for each material tested (Fig. 2). Using a fluorescein filter set (excitation: 450 – 490 nm, emission: >520 nm), the fluorescence of 1009F resin was only 4% of that of SU-8, and 24% of that of 1002F. Using CY3 (excitation: 528 – 553 nm, emission: 590 – 650 nm) and CY5 (excitation: 590 – 650 nm, emission: 665 – 740 nm) filter sets, the fluorescence of 1009F resin was 1% that of SU-8, and 30% that of 1002F. To determine whether the autofluorescence of the micrafts might be further reduced, polystyrene was used a raft material. The fluorescence of polystyrene micrafts was 48% of that of 1009F when using a fluorescein filter set, and 54% of that of 1009F using both the CY3 and CY5 filter sets. The fluorescence of these polystyrene structures was only somewhat higher (18 – 20%) than that of the PDMS mold, which is one of the least fluorescent polymers in the visible range.³³

Cells remain localized on the micrafts during culture

The array is made from polymers that are known to be biocompatible. To confirm that a cell-based array could be created with the micromolding procedure, arrays composed of 1009F or polystyrene were oxidized in a plasma cleaner to modify the micrafts for cell attachment by making their surfaces hydrophilic. While the PDMS walls between the micrafts were also oxidized, prior work indicates that PDMS rapidly reverts to its hydrophobic character after exposure to air leaving the PDMS regions unsuitable for cell attachment.^{34, 35} Nevertheless, in experiments, it was noted that cells growing on plasma oxidized arrays tended to migrate beyond the confines of the microwells after 2–3 days in culture even after exposing oxidized arrays to air for up to 3 weeks. For this reason, an alternate material for the micrafts, poly(styrene-co-acrylic acid) (PS-AA) was used when long-term cell culture was desired. This material possesses a negatively charged surface suitable for cell attachment and proliferation without the need for oxidation.³²

HeLa cells were cultured on the various arrays. Most cells settled into the wells by gravity. After 20 min, the array was gently rinsed with fresh medium to remove cells not captured in the wells. Arrays composed of 1009F micrafts were examined by microscopy after 6 h. In these experiments, $99.2 \pm 0.7\%$ of cells ($n = 3$ arrays of each type, 500 cells counted per array) were located inside the well and attached to the micraft surface (Fig. 3A). SEM images (Fig. 3B) corroborated these findings. The cell capture efficiency was similar on arrays composed of polystyrene and PS-AA micrafts. On arrays composed of PS-AA micrafts, cells were seen to remain sequestered in the microwells after 8 days in culture (Supplemental Materials, Fig. S4).

Extracellular matrices (ECMs) such as collagen can be coated onto the micraft surface to enhance cell attachment. As an example, a micraft array was coated with 100 $\mu\text{g/mL}$ collagen (type I from rat tail) for 1 h. Within 2 h after plating, HeLa cells attached to and spread out on this surface. As a control, HeLa cells cultured on uncoated rafts were also observed at 2 h. Cells on the uncoated surface were still rounded and weakly attached at 2 h. On the uncoated surfaces, the cells required >6 h to fully spread out on the surface. (Supplemental Materials, Fig. S5).

For isolation of single cells from mixed cell populations, the rafts should possess one or fewer cells. There are two strategies to maximize the placement of single cells on the micrafts in the array. These are: (1) Optimization of cell plating density. The distribution of cells on the microwell array follows a Poisson probability distribution. Based on theory, if the number of cells is $1/3$ of that of microwells, 72% of wells are empty, 24% wells capture single cells, and 4% of wells capture two or more cells. Based on our experimental observation, if 15,000 cells were plated on the array containing 45,000 rafts (100 μm size), we found that $71.8 \pm 9.1\%$ of the rafts possessed no cells, $25.0 \pm 7.6\%$ of the rafts possessed a single cell, and $3.0 \pm 2.0\%$ rafts contained 2 or more cells ($n = 3$ experiments with 140 rafts counted in each experiment). Therefore, the majority of cells ($>78\%$) were present as a single cell on a raft. If the number of cells is $1/10$ of that of microwells, $>96\%$ cells were present as a single cell on a raft. Our experimental observation corroborates the theoretical calculation. (2) Optimization of raft size. If the raft diameter is only slightly greater than that of a cell, the probability of capturing a single cell is greatly increased since two cells can not fit within the raft depression. As a demonstration, an array of circular rafts (30- μm diameter) was fabricated and HeLa cells were plated on the array. Both brightfield and scanning electron microscopy demonstrated that these rafts captured only single cells (Supplemental Materials, Fig. S6).

To determine whether other cell types could be grown on the arrays, ES129 embryonic stem cells were cultured in the presence of leukemia inhibitory factor (LIF) for 50 h on an array

composed of $200 \times 200 \mu\text{m}$ PS-AA micrafts (Fig. 3C). The array provided a suitable environment for stem cell renewal in an undifferentiated state as determined by the morphology of the cells and colonies.³⁶ The stem cells grew in a spherical colony as expected with the colony attached to the micraft surface. The same type of array ($n = 3$) was then used to culture primary cells obtained from a needle biopsy taken from a pancreatic tumor surgically removed from a patient. Fig. 3D shows the primary cells cultured on the array at 23 days. The cells appeared to be of a fibroblast phenotype.

Single micrafts can be effectively released from the array with a microneedle

Micrafts appeared to be poorly adhesive to the PDMS mold as there was no residue apparent in SEM images of the partially removed structures (Fig. 1C); nevertheless, the micrafts remained in place within the microwells during manipulation of the arrays. Since PDMS is an elastomeric material, it was reasoned that the thin PDMS layer ($\sim 200 \mu\text{m}$ thick) beneath the micraft could be easily pierced. To determine if the micrafts could be individually detached from the array by a mechanical force, a release system composed of a needle of micron dimensions actuated by a micromanipulator with a motor-driven z-axis was designed to puncture the PDMS layer (Fig. 4A and Supplementary Material, Fig. S2C). The arrays were inverted so that the needle pierced the PDMS layer from above and extruded the micraft, whereupon it settled into a collection dish mated with the array. To determine the effectiveness of the release using this process, micrafts were released from an array composed of PS-AA micrafts ($100 \mu\text{m}$, $20 \mu\text{m}$ inter-raft spacing). In these experiments 81% of the micrafts were released from the array with only one actuation of the needle ($n = 140$). In some cases multiple actuations of the needle were required to release an individual micraft (2 attempts in 14%, 3 attempts in 4%, and 4 attempts in 1%). In this experiment, 100% of the 140 micrafts were releasable and in no case was an adjacent micraft detached. In a second experiment, 8 micrafts were targeted for release in an alternating pattern. Again all 8 were released without detachment of adjacent micrafts (Fig. 4B–D). The released micrafts settled to the collection dish where they were examined with the microscope. All micrafts were intact with no damage from the needle seen on any of the released structures. The process was tested for micraft dimensions over the range of $30 - 500 \mu\text{m}$. All micrafts in this size range could be effectively released using microneedles with tip diameters of either $1 \mu\text{m}$ or $17.5 \mu\text{m}$.

Living cells on micrafts released from the array remain viable

To determine the feasibility of cell isolation by release of micrafts, HeLa cells were cultured on an array composed of PS-AA micrafts (Fig. 4E). Micrafts possessing a single cell were released with the microneedle and collected ($n = 3$ experiments with 20 cells released per experiment). After release, the cells remained adherent to their micraft and appeared intact by brightfield microscopy (Fig. 4F). A similar procedure was used to isolate colonies of cells (>30 cells) growing on $300 \mu\text{m}$ micrafts and single cells growing on $30 \mu\text{m}$ micrafts with the same results (data not shown). To assess the viability of cells isolated in this manner, the released micrafts were placed in culture. The cells were imaged by brightfield within 1 h of collection and at varying times thereafter. At 1 h after collection, $100 \pm 0\%$ of HeLa cells remained on the micraft surface. By 48 h, all of the cells had divided and begun to migrate from the micrafts onto the adjacent culture surface. At 144 h, $95 \pm 8.7\%$ of the single cells had formed a small colony, demonstrating the feasibility of sterile collection and single-cell cloning (Fig. 4G). The orientation of the micraft on the collection surface after release did not impact the cell growth rate which corroborated our previous results.²² The extremely high post-sorting viability as measured by the single-cell cloning efficiency indicated that the cells did not undergo undue stress during the release and collection procedures. Of note, after the release procedure the arrays were returned to an upright orientation and placed back in culture, yet fluid did not leak from the needle

puncture site due to the self-sealing properties of PDMS. In addition, bacterial or fungal contamination did not occur. Thus, the array could be used repeatedly to perform isolation of cells at multiple time points if desired, a feature not possible with sorting by magnetic beads or flow cytometry.

Cell separation based on eGFP expression

By examining the arrays using an inverted microscope, it should be possible to identify cells of interest based on a number of criteria including fluorescence, morphology, and time-dependent characteristics such as growth rate.¹⁹ Cells of interest can then be isolated by selectively releasing and collecting target micrafts. To demonstrate that the array could be used to sort based on expression of a fluorescent marker, a cloning experiment was performed using HeLa cells transfected with eGFP. The cells were transfected with a plasmid encoding eGFP under the control of a cytomegalovirus promoter using a lipid-based transfection reagent. At day 2 after transfection, the cells were plated onto 3 arrays in antibiotic-free media. A suspension containing 15,000 cells was added to each array which contained 44,800 PS-AA micrafts (100 μ m, 20 μ m inter-raft spacing). Under these conditions, each micraft on the array contained one or zero cells after 24 h in culture. Cells were maintained in culture in the absence of selective antibiotics for 5 days. At that time, the arrays were examined by fluorescence microscopy (Fig. 5A, B). Each colony contained >12 cells and were either non-fluorescent (the majority) or were composed of a mixture of fluorescent and non-fluorescent cells indicating loss of eGFP expression in the progeny. However, on average each array contained 1 – 3 colonies in which all daughter cells within a colony expressed eGFP. The micrafts containing these colonies were released and collected individually (n = 3 arrays) (Fig. 5C, D). At 72 hr after release, the cells were seen to be continually dividing and producing fluorescent progeny (Fig. 5E, F). At 6 days after release (*i.e.* 13 days after transfection), colonies of ~500 cells were present in which all cells remained fluorescent. Notably, the total time required to isolate eGFP expressing clones with this procedure was less than two weeks and selective antibiotics were not required.

CONCLUSIONS

An array of microwells with detachable bases termed micrafts has been manufactured by a simple dip-coating process using a PDMS mold as both the template and array substrate. The molding process used to create the array does not require a microfabrication clean room facility or photolithography, thus dramatically lowering the cost of manufacture. While a master is created photolithographically to produce the PDMS mold, this master is used repeatedly and can be purchased prefabricated. The manufacturing process can create arrays of any size and shape to tailor the array to a particular application. For instance, arrays with millimeter footprints may be of use with samples of only hundreds to a few thousand cells, while arrays of several centimeters and millions of culture sites would be valuable for automated, high-throughput screening of rare cell populations. A wide range of materials can be employed to create culture substrates tailored to a given application. In particular the molded micrafts can be composed of a variety of very low-fluorescence materials enhancing their versatility for microscopy-based cell selection. The mold itself provides the cell-localizing walls without need for additional surface modification to pattern the cells. Finally, the mechanical release system is robust and inexpensive in comparison to laser-based or dielectrophoretic methods. In combination, these innovations create a platform that provides biomedical scientists with access to diverse cell culture surfaces with integrated, easy-to-use cell separating capabilities at low cost.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Patel, D. Separating Cells. Springer-Verlag; New York: 2001.
2. Fu AY, Chou HP, Spence C, Arnold FH, Quake SR. Analytical Chemistry. 2002; 74:2451–2457. [PubMed: 12069222]
3. Wolff A, Perch-Nielsen IR, Larsen UD, Friis P, Goranovic G, Poulsen CR, Kutter JP, Telleman P. Lab on a Chip. 2003; 3:22–27. [PubMed: 15100801]
4. Young SM, Curry MS, Ransom JT, Ballesteros JA, Prossnitz ER, Sklar LA, Edwards BS. Journal of Biomolecular Screening. 2004; 9:103–111. [PubMed: 15006133]
5. Sohn LL, Saleh OA, Facer GR, Beavis AJ, Allan RS, Notterman DA. Proceedings of the National Academy of Sciences of the United States of America. 2000; 97:10687–10690. [PubMed: 10995481]
6. Wang XB, Yang J, Huang Y, Vykoukal J, Becker FF, Gascoyne PR. Analytical Chemistry. 2000; 72:832–839. [PubMed: 10701270]
7. Welm B, Behbod F, Goodell MA, Rosen JM. Cell Proliferation. 2003; 36(Suppl 1):17–32. [PubMed: 14521513]
8. Burridge K, Chrzanowska-Wodnicka M. Annual Review of Cell and Developmental Biology. 1996; 12:463–519.
9. Chiquet M, Matthisson M, Koch M, Tannheimer M, Chiquet-Ehrismann R. Biochemistry and Cell Biology. 1996; 74:737–744. [PubMed: 9164643]
10. Ingber DE. Annual Review of Physiology. 1997; 59:575–599.
11. Seidl J, Knuechel R, Kunz-Schughart LA. Cytometry. 1999; 36:102–111. [PubMed: 10554157]
12. Piercy KT, Donnell RL, Kirkpatrick SS, Mundy BL, Stevens SL, Freeman MB, Goldman MH. Journal of Surgical Research. 2001; 100:211–216. [PubMed: 11592795]
13. Mackie EJ, Pagel CN, Smith R, de Niese MR, Song SJ, Pike RN. IUBMB Life. 2002; 53:277–281. [PubMed: 12625364]
14. Miki M, Nakamura Y, Takahashi A, Nakaya Y, Eguchi H, Masegi T, Yoneda K, Yasouka S, Sone S. Journal of Medical Investigation. 2003; 50:95–107. [PubMed: 12630574]
15. Burgess DS. Biophotonics International. 2004; 11:46–49.
16. Todd R, Lingen MW, Kuo WP. Expert Review of Molecular Diagnostics. 2002; 2:497–507. [PubMed: 12271821]
17. Schutze K, Posl H, Lahr G. Cell and Molecular Biology. 1998; 44:735–746.
18. Burgemeister R. Journal of Histochemistry and Cytochemistry. 2005; 53:409–412. [PubMed: 15750030]
19. Wang Y, Young G, Aoto P, Pai JH, Bachman M, Li GP, Sims CE, Allbritton NL. Cytometry A. 2007; 71A:866–874. [PubMed: 17559133]
20. Wang Y, Sims CE, Marc P, Bachman M, Li GP, Allbritton NL. Langmuir. 2006; 22:8257–8262. [PubMed: 16952271]
21. Salazar GT, Wang Y, Young G, Bachman M, Sims CE, Li GP, Allbritton NL. Analytical Chemistry. 2007; 79:682–687. [PubMed: 17222037]
22. Wang Y, Young G, Bachman M, Sims CE, Li GP, Allbritton NL. Analytical Chemistry. 2007; 79:2359–2366. [PubMed: 17288466]
23. Wang Y, Salazar GT, Pai JH, Shadpour H, Sims CE, Allbritton NL. Lab on a Chip. 2008; 8:734–740. [PubMed: 18432343]
24. Xu W, Sims CE, Allbritton NL. Analytical Chemistry. 2010; 82:3161–3167. [PubMed: 20199088]
25. Pai JH, Wang Y, Salazar GT, Sims CE, Bachman M, Li GP, Allbritton NL. Analytical Chemistry. 2007; 79:8774–8780. [PubMed: 17949059]

26. Zhao XM, Xia YN, Whitesides GM. *Journal of Materials Chemistry*. 1997; 7:1069–1074.
27. Gates BD, Xu QB, Stewart M, Ryan D, Willson CG, Whitesides GM. *Chemical Reviews*. 2005; 105:1171–1196. [PubMed: 15826012]
28. Rolland JP, Maynor BW, Euliss LE, Exner AE, Denison GM, DeSimone JM. *Journal of the American Chemical Society*. 2005; 127:10096–10100. [PubMed: 16011375]
29. Jalbert C, Koberstein JT, Hariharan A, Kumar SK. *Macromolecules*. 1997; 30:4481–4490.
30. Lee JN, Park C, Whitesides GM. *Analytical Chemistry*. 2003; 75:6544–6554. [PubMed: 14640726]
31. Shaw, M.; Nawrocki, D.; Hurditch, R.; Johnson, D. Springer-Verlag; 2003. p. 1-6.
32. Jung H, Kwak B, Yang HS, Tae G, Kim JS, Shin K. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*. 2008; 313:562–566.
33. Duffy DC, McDonald JC, Schueller OJA, Whitesides GM. *Analytical Chemistry*. 1998; 70:4974–4984. [PubMed: 21644679]
34. Hillborg H, Gedde UW. *IEEE Transactions on Dielectrics and Electrical Insulation*. 1999; 6:703–717.
35. Kim J, Chaudhury MK, Owen MJ. *Journal of Colloid and Interface Science*. 2000; 226:231–236.
36. Heo J, Lee JS, Chu IS, Takahama Y, Thorgeirsson SS. *Biochemical and Biophysical Research Communications*. 2005; 332:1061–1069. [PubMed: 15922302]

Figure 1.

Fabrication of the molded micraft array. (A) Schematic of the fabrication process. i) A PDMS microwell array (shown in gray) is fabricated by a standard molding process. ii) A polymer solution (shown in purple) is added to the PDMS microwell array. iii) The wetted PDMS mold is immersed in the polymer solution and then slowly withdrawn. iv) The dewetting of polymer solution from PDMS results in isolated convex polymer solution in each well. v) Evaporation of solvent results in concave polymer micrafts inside each well. (B) SEM image of an array of 1009F micrafts ($175\ \mu\text{m}$ square, $40\ \mu\text{m}$ inter-raft gap). (C) A close-up of an SEM image of a ruptured section of an array showing that the micraft is loosely attached to the PDMS and is easily released. The micrafts in this image are $175\ \mu\text{m}$ -square, $25\ \mu\text{m}$ thick in the center and $31\ \mu\text{m}$ thick at the edges. (D) Large scale array of molded polystyrene micrafts numbering $>10^6$. The array size was $300\ \text{mm} \times 50\ \text{mm} \times 0.2\ \text{mm}$ and each micraft is $100\ \mu\text{m}$ square.

Figure 2. Autofluorescence of molded micrafts composed of SU-8 photoresist (PR), 1002F PR, 1009F resin, and polystyrene. (A) Fluorescence image of the arrays using a fluorescein filter set. (B) Fluorescence intensity of the arrays measured using three filter sets: fluorescein, CY3 and CY5.

Figure 3. Culture of cells on 1009F and PS-AA micraft arrays. (A) brightfield and (B) SEM images of HeLa cells cultured on a 70 μm 1009F micraft array (inter-raft spacing 30 μm ; micraft height 15 μm). (C) Mouse embryonic stem cell and (D) primary cells isolated from a pancreatic needle biopsy cultured on arrays composed of 200 μm PS-AA micrafts (inter-raft spacing 20 μm ; micraft height 25 μm). Scale bar is 100 μm .

Figure 4. Mechanical release system. (A) Schematic of needle-based release hardware composed of a XYZ micromanipulator and a DC motor. (B–D) Eight targeted PS-AA micrafts (indicated as *) were sequentially released from the array and successfully transported to the collection dish. The images show the array prior to micraft release (B), the array after release (C), and the released, collected micrafts (D). (E–G) Single HeLa cells were isolated from an array of 70 μm PS-AA micrafts (inter-raft spacing 30 μm ; micraft height 15 μm). (E) Brightfield image before release. (F) Image after release showing the targeted micraft/cell has been released and transported to the collection dish. (G) The single HeLa cell in “E” and “F” has formed a small colony 144 h after release.

Figure 5.

HeLa cell line expressing eGFP was generated by culture, analysis, and selection of clonal colonies on a PS-AA micraft array. (A) Brightfield and (B) fluorescence images of a region of an array (micraft size 100 μm , inter-raft spacing 20 μm , height 15 μm) containing clonal colonies of HeLa cells 7 days after gene transfection. A single micraft with a colony composed entirely of eGFP-expressing cells is present. (C) and (D) Images of the colony seen in “A” and “B” after release and collection. (E) and (F) Images of the same colony 72 h after selection. The fluorescent daughter cells are seen to be dividing and growing off the isolated micraft. (G) and (H) Images of the same colony 6 days after selection. A colony of ~500 cells in which all progeny are fluorescent has formed from the original cell.