Micropallet Arrays for the Separation of Single, Adherent Cells

Georgina To'a Salazar,[†,‡] Yuli Wang,[†,§] Grace Young,[§] Mark Bachman,[‡,§,∥] Christopher E. Sims,[†,§] G.P. Li,[*,‡,§,∥] and Nancy L. Allbritton[*,†,‡,§]

Department of Physiology and Biophysics, Integrated Nanosystems Research Facility, Department of Electrical Engineering and Computer Science, and Department of Biomedical Engineering, University of California, Irvine, California 92697

The selection and collection of single cells from within a heterogeneous population is required to produce genetically engineered cell lines, to develop new stem cell lines, and for single-cell studies. We describe a new platform for the positive selection of single live mammalian cells while the cells remain adherent to their growth surface. Cells were grown on arrays of microfabricated, releasable elements composed of SU-8 polymer termed "cell pallets". The presence of air between the elements restricted the cells to the top surfaces of the pallets. Single pallets situated within large arrays of pallets were released on demand using a single, focused, laser pulse. The laser pulses were low in energy (2−5 μJ) and did not detach nearby, nontargeted pallets. Since the SU-8 pallets and the underlying glass substrate were optically transparent, the cells on the pallets could be visualized by microscopy before and after release. Over 90% of cells remained attached to the pallet during laser-based release. The feasibility of growing the cells from the released pallets into clonal colonies was demonstrated. The pallet array system permits adherent cells to be inspected using conventional microscopy and selected cells released for further analysis. The ability to assess cells while they remain adherent to a surface will broaden the number of attributes that can be utilized for cell separation, for example, cell shape, cytoskeletal properties, and other attributes.

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 cell lines that are genetically engineered, derived from stem cells, or grown from patient cell lines, single cells must be isolated and then cloned to form a homogeneous population. A variety of strategies exist to selectively identify and collect nonadherent cells from a mixed population, including fluorescence-activated cell sorting, 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 adherent cells, investigators use these procedures by 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 adherent, mammalian cell selection address some of the challenges but remain limited for living cells. Laser capture microdissection (LCM) (Arcturus, Mountain View, CA) has enabled single cells or small groups of selected cells to be obtained from tissue sections for genetic and proteomic studies, although most applications utilize fixed or frozen specimens.[15] Protocols for use with live cells have been published, but are very low throughput and not suitable for isolating large numbers of single, living cells.[16] Most applications of LCM utilize fixed or frozen specimens.[15−18] Thus, these techniques have only partially

* Corresponding authors. E-mail: nlallbri@uci.edu. Phone: 949-824-6493. Fax: 949-824-9137. E-mail: gpli@uci.edu. Phone: 949-824-4194. Fax: 949-824-3732.

† Department of Physiology and Biophysics.

‡ Department of Biomedical Engineering.

§ Integrated Nanosystems Research Facility.

∥ Department of Electrical Engineering and Computer Science.

(1) Patel, D. *Separating Cells*; Springer-Verlag: New York, 2001.
(2) Fu, A. Y.; Chou, H. P.; Spence, C.; Arnold, F. H.; Quake, S. R. *Anal. Chem.* **2002**, *74*, 2451−2457.
(3) Wolff, A.; Perch-Nielsen, I. R.; Larsen, U. D.; Friis, P.; Goranovic, G.; Poulsen, C. R.; Kutter, J. P.; Telleman, P. *Lab Chip* **2003**, *3*, 22−27.
(4) Young, S. M.; Curry, M. S.; Ransom, J. T.; Ballesteros, J. A.; Prossnitz, E. R.; Sklar, L. A.; Edwards, B. S. *J. Biomol. Screening* **2004**, *9*, 103−111.
(5) Sohn, L. L.; Saleh, O. A.; Facer, G. R.; Beavis, A. J.; Allan, R. S.; Notterman, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 10687−10690.
(6) Wang, X. B.; Yang, J.; Huang, Y.; Vykoukal, J.; Becker, F. F.; Gascoyne, P. R. *Anal. Chem.* **2000**, *72*, 832−839.
(7) Welm, B.; Behbod, F.; Goodell, M. A.; Rosen, J. M. *Cell Proliferation* **2003**, *36* (Suppl 1), 17−32.
(8) Burridge, K.; Chrzanowska-Wodnicka, M. *Ann. Rev. Cell Dev. Biol.* **1996**, *12*, 463−519.
(9) Chiquet, M.; Matthisson, M.; Koch, M.; Tannheimer, M.; Chiquet-Ehrismann, R. *Biochem. Cell Biol.* **1996**, *74*, 737−744.
(10) Ingber, D. E. *Ann. Rev. Physiol.* **1997**, *59*, 575−599.
(11) Seidl, J.; Knuechel, R.; Kunz-Schughart, L. A. *Cytometry* **1999**, *36*, 102−111.
(12) Piercy, K. T.; Donnell, R. L.; Kirkpatrick, S. S.; Mundy, B. L.; Stevens, S. L.; Freeman, M. B.; Goldman, M. H. *J. Surg. Res.* **2001**, *100*, 211−216.
(13) Mackie, E. J.; Pagel, C. N.; Smith, R.; de Niese, M. R.; Song, S. J.; Pike, R. N. *IUBMB Life* **2002**, *53*, 277−281.
(14) Miki, M.; Nakamura, Y.; Takahashi, A.; Nakaya, Y.; Eguchi, H.; Masegi, T.; Yoneda, K.; Yasouka, S.; Sone, S. *J. Med. Invest.* **2003**, *50*, 95−107.
(15) Burgess, D. S. *Biophotonics Int.* **2004**, *11*, 46−49.
(16) Todd, R.; Lingen, M. W.; Kuo, W. P. *Expert Rev. Mol. Diagn.* **2002**, *2*, 497−507.
(17) Schutze, K.; Posl, H.; Lahr, G. *Cell. Mol. Biol. (Noisy-le-grand)* **1998**, *44*, 735−746.
(18) Burgemeister, R. *J. Histochem. Cytochem.* **2005**, *53*, 409−412.

10.1021/ac0615706 CCC: $37.00
Published on Web 12/09/2006

met the needs of investigators for the positive selection of adherent, mammalian cells. P.A.L.M. Microlaser Technologies (Bernried, Germany) markets an instrument that uses a laser to cut out a region of interest from a tissue section and then generate a shock wave that "catapults" the cells into an overlying collection device.[17] Again, most of the work with this technique has utilized fixed specimens, but collection of large colonies of living cells has been demonstrated.[18] Cells are subjected to stress due to the direct effects of the shock wave and desiccation from removal of fluid overlying the sample during collection. ClonePix (Genetix, Hampshire, UK) is an automated system that uses image recognition to guide a suction pipet that aspirates colonies of loosely adherent cells from plates. The system requires cells that grow in loosely adherent clusters or suspension-adapted versions of adherent cells growing in a semisolid methylcellulose media; thus it is not applicable to the vast majority of mammalian cells.

The current paper describes the development of a new platform for the positive selection of live, single mammalian cells while the cell remains adherent to its growth substrate. Arrays of microfabricated SU-8 pallets were fabricated on a glass substrate. Cells were localized to the top surface of the pallets so that the cells could be readily viewed with conventional microscopy. Single pallets were released with a single focused pulse from a laser without perturbation of adjacent pallets. Upon release of a pallet with an attached cell, the cell remained adherent to its underlying pallet. The feasibility of collecting and then cloning the cell on the released pallet was demonstrated. This platform has the potential to become a valuable and widely applicable tool for separation and cloning of adherent cells.

EXPERIMENTAL SECTION

Materials. SU-8 photoresist and SU-8 developer were purchased from MicroChem Corp. (Newton, MA). (Heptadecafluoro-1,1,2,2-tetrahydrodecyl)trichlorosilane was from Gelest Inc. (Morrisville, PA). Dulbecco's Modified Eagle Medium, fetal bovine serum, penicillin/streptomycin, calcein red-orange AM, and Oregon Green diacetate were obtained from Invitrogen (Carlsbad, CA). L-Glutamine and poly(D-lysine) hydrobromide (MW 70 000−150 000) were obtained from Sigma-Aldrich (St. Louis, MO). Collagen I from rat tail tendon was purchased from BD Biosciences (San Jose, CA). Fibronectin extracted and purified from human plasma was purchased from Chemicon International, Inc. (Temecula, CA). Silicone O-rings (24-mm outer diameter) were purchased from McMaster-Carr (Los Angeles, CA). All other reagents were from Fisher Scientific (Pittsburgh, PA).

Fabrication of SU-8 Pallets. Pallets composed of SU-8 were fabricated on a glass slide as described previously.[19] SU-8 films of 30-μm thickness were obtained by spin-coating the resist on the glass slides at 100 rpm for 10 s followed by 1000 rpm for 30 s using a WS-200-4NPP spin coater (Laurell Technologies Corp.). The coated slides were baked on a hot plate at 65 °C for 3 min followed by a second bake at 95 °C for 7 min to remove organic solvent. To prepare SU-8 pallets, the SU-8 film was exposed to UV light through a photomask with the designed features for 30 s using an Oriel collimated UV source (7.4 mW/cm^2). The postexposure baking was done on a hot plate at 65 °C for 1 min and 95 °C for 3 min. The SU-8 samples were then developed in SU-8 developer for 5 min, rinsed with 2-propanol, and dried by a nitrogen stream. Fabrication of SU-8 pallets of alternative thicknesses was performed using the same process, except that the appropriate time parameters for that thickness were substituted.[20,21]

Laser-Based Pallet Release. A frequency-doubled Q-switched Nd:YAG laser (ACL-1, New Wave Research, Fremont, CA) generated a single laser pulse (5-ns pulse width, TEM00, 532 nm), which was spatially expanded to a 4-mm diameter (Oriel Beam Expander). A polarizer (DP-100-VIS1, Thor Labs) was used to adjust the beam energy between 1 and 10 μJ, and the energy was verified using an energy meter (EPM 1000, Molectron). The beam was then directed into an inverted fluorescence microscope (TE300, Nikon). The pulse was focused at the pallet−glass interface by a microscope objective (20×, 0.5 NA). When the virtual walls were present on the pallet array, the droplets of water that condensed on the glass surface between the pallets and beneath the air bubbles were used to determine the focal plane of the glass-SU-8 interface.

Measurement of the Threshold Energy (E_p) for Pallet Release. E_p is the laser pulse energy at which 50% of the pallets are released by a single pulse. Formation of a plasma by a focused laser beam is stochastic. Consequently, the probability of plasma formation at a given energy (E) is described by a Gaussian error function.[22,23] Since the pallets are released by the mechanical energy generated by a plasma, the probability of pallet release ($P(E)$) was also fit to the Gaussian error function, $P(E) = 0.5(1 + \mathrm{erf}((E - E_p)/a))$, where a is a constant.

Surface Coatings for Virtual Air Walls. After fabrication of SU-8 pallets on a glass substrate, the pallet array was baked on a hot plate at 95 °C for 2 h to remove any solvent trapped on the surface. The formation of a hydrophobic perfluoroalkylsilane layer on the silicone oxide surface was carried out in a low-pressure reactor.[19] The array and a small plastic Petri dish containing 100 μL of (heptadecafluoro-1,1,2,2-tetrahydrodecyl)trichlorosilane were placed inside a 100-mm-i.d. Wheaton dry-seal desiccator. The desiccator was then attached to an oil-free diaphragm vacuum pump (Vacubrand, Fisher Scientific) for 1 min (7 Torr), and then the desiccator was closed under vacuum for 16 h at room temperature. Afterward, the array was placed under a high vacuum (2×10^{-3} Torr) for 2 h to remove any unreacted silane molecules using a standard oil vacuum pump (Fisherbrand, Fisher Scientific). The array was stored in the vacuum desiccator until use.

Surface Coating of Pallets for Cell Culture. After silanization, a chamber was constructed by using PDMS to attach a silicon "O"-ring (24-mm outer diameter) to the pallet array. The top surface of pallets was then modified to enhance cell adhesion. A two-step procedure was used to coat collagen on the pallet top surface. First the hydrophobic pallet top surface was converted to a hydrophilic surface by 16-h immersion in 100 μg/mL poly(D-lysine) in phosphate buffered saline (PBS: 138 mM NaCl, 27 mM KCl, 10 mM PO_4, pH 7.4). After chemical modification of

(19) Lorenz, H.; Despont, M.; Fahrni, N.; LaBianca, N.; Renaud, P.; Vettiger, M. *J Micromech. Microeng.* **1997**, *7*, 121−124.
(20) Wang, Y.; Sims, C. E.; Marc, P.; Bachman, M.; Li, G. P.; Allbritton, N. L. *Langmuir* **2006**, *22*, 8257−62.
(21) Shaw, J. M.; Gelorme, J. D.; LaBianca, N. C.; Conley, W. E.; Holmes, S. J. *IBM J. Res. Dev.* **1997**, *41*, 81−94.
(22) Rau, K. R.; Quinto-Su, P. A.; Hellman, A. N.; Venugopalan, V. *Biophys. J.* **2006**, *91*, 317−329.
(23) Vogel, A.; Venugopalan, V. *Chem. Rev.* **2003**, *103*, 577−644.

the SU-8 to form a hydrophilic surface, 400 μg/mL collagen solution (in 2 mM acetic acid) was added to the pallet array and then was removed by pipet suction within 1 min. The remaining acidic collagen solution formed a thin liquid layer on each hydrophilic pallet surface. After drying in air for 15 min, a conformal collagen film was deposited on the top surface of the pallets. The addition of PBS buffer to the array neutralized the acidic collagen film causing it to become insoluble. Alternatively, a single-step procedure was used to coat fibronectin on the pallet top surface. Fibronectin (25 μg/mL in PBS, 0.8 mL) was added to the chamber and incubated at room temperature for 16 h. When virtual walls were present on the pallet array, only the top surface of each pallet came in contact with the collagen or fibronectin.

Cell Culture. After the pallet array was silanized and coated with fibronectin or collagen, 3T3 RBL, or HeLa cells were grown on the array at 37 °C in a humidified 5% CO_2 atmosphere in Dulbecco's minimal essential media supplemented with fetal bovine serum (10%), and L-glutamine (584 mg/L). Penicillin (100 units/mL) and streptomycin (100 μg/mL) were added to the media to inhibit bacterial growth. Immediately prior to use, the growth medium was removed from the cell chamber and replaced with PBS.

Loading Cells with Oregon Green or Calcein Red-Orange. Cells were incubated with Oregon Green diacetate (8 μM) or calcein red-orange AM (200 nM) at 37 °C for 30 min. The cells were then washed with PBS. Fluorescence microscopy of Oregon Green was performed using a standard fluorescein filter set (excitation, 470 ± 20 nm; emission, ≥515 nm) and an inverted fluorescence microscope (TE300, Nikon). Fluorescence microscopy of calcein red-orange was similarly performed but with a different filter set (excitation, 540 ± 20 nm; emission, 625 ± 20 nm).

Pallet Collection. Pallets were collected into an overlying micropipet or small tube using an applied vacuum. Prior to use, the pipet or tube was cleaned by rinsing with ethanol and then sterile PBS. The pallet was then transferred into a tissue culture dish. Alternatively, the pallet array was inverted over a culture dish so that the fluid and released pallets were poured into the culture dish. The pallet/cells were then cultured as described above.

RESULTS AND DISCUSSION

Release of Individual Pallets from a Large Array. To form an array with a high density of pallets, microstructures composed of SU-8 were fabricated on a glass surface (Figure 1A). SU-8 photoresist is an epoxy-based material that becomes cross-linked upon exposure to near UV light.[20,21] This photoresist has become widespread through out the semiconductor industry since it can be used to fabricate microstructures with high aspect ratios and near vertical walls.[20,21,24] An advantage of SU-8 is that it is optically transparent at most visible wavelengths. Using conventional microfabrication methods, arrays of pallets with varying heights, shapes, and surface areas can be formed.[19] A critical feature is that large numbers of the pallets can be fabricated on a conventional biologic surface such as a microscope slide. For example, 20 000 square pallets with a 50-μm side and 20-μm spacing are present in 1 cm^2. Thus, a single array could possess hundreds of thousands of pallets in an area of practical dimensions.

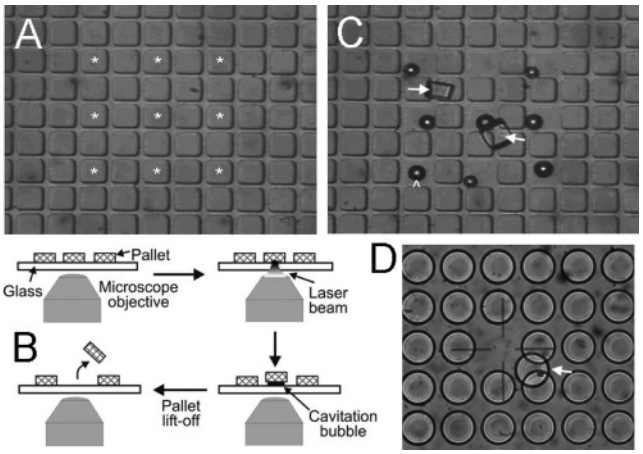

Figure 1. Release of SU-8 pallets by a single pulse of a focused laser beam. (A) Shown is an array of square pallets (50-μm side, 30-μm height, 15-μm spacing). The pallets marked with an asterisk were released as shown in (C). (B) Schematic of laser-based release of individual pallets. For details see text. (C) The six pallets marked in (A) were sequentially released with a single laser pulse (2 μJ). After release, two pallets settled back onto the array in the field of view. These pallets are marked by an arrow. One settled on its side and the other upright. A "^" marks an air bubble remaining after release of one of the pallets. Multiple air bubbles from the release process are present. (D) Shown is an array of circular pallets (100-μm diameter, 30-μm height, 30-μm spacing) with a single released pallet marked with an arrow.

For the pallet array to be suitable as a cell cloning method, individual pallets located in the midst of large numbers of nearby pallets must be releasable on demand. Typically, when using SU-8 in combination with glass, a metal layer is placed between the SU-8 and glass surface to enhance adhesion. Without the intervening metal layer, the SU-8 is weakly adherent to the underlying glass. Omission of the metal layer yielded arrays of pallets that could be detached with a mechanical force of the appropriate magnitude. The focused beam of a laser was used to generate a mechanical force localized to dimensions of micrometers. A single pulse (5-ns duration) of a Nd:YAG laser (532 nm) was focused at the interface between the glass and SU-8 pallet (Figure 1B). When a laser beam is focused to a sufficiently small diameter, a localized plasma is created, which in turn produces an outwardly propagating shock wave and an expanding cavitation bubble.[22,23,25] In an aqueous solution, up to 5% of the laser's energy can be transmitted to the cavitation bubble yielding a bubble tens of micrometers or more in diameter. To determine whether the shock wave and cavitation bubble generated by the laser-induced plasma could release a pallet, a single pulse of low energy (2−5 μJ) was focused at the SU-8 glass interface below a pallet. The pallet was released without disturbing neighboring pallets (Figure 1C,D). Under these conditions, 100% (n > 100) of targeted pallets were released and 0% of adjacent pallets were detached. The shock wave, cavitation bubble, or both yielded localized mechanical forces centered at the focal point of the laser beam and restricted to a single pallet. Multiple pallets in an array could be released by moving the

(24) O'Brien, J.; Hughes, P. J.; Brunet, M.; O'Neill, B.; Alderman, J.; Lane, B.; O'Riordan, A.; O'Driscoll, C. *J Micromech. Microeng.* **2001**, *11*, 353−358.

(25) Sims, C. E.; Meredith, G. D.; Krasieva, T. B.; Berns, M. W.; Tromberg, B. J.; Allbritton, N. L. *Anal. Chem.* **1998**, *70*, 4570−4577.

microscope stage to sequentially place pallets in the path of the focused beam (Figure 1A,C). For these small pallets (50-μm side), the mechanical energy was frequently sufficient both to detach the pallet and to propel the pallet from its array site (and often from the field of view of the microscope) (Figure 1C,D). When pallets were released, there was frequently a small defect on the face of the pallet that was in contact with the glass surface, suggesting that the plasma formed adjacent to this surface and at the interface between the SU-8 and glass surfaces. Movement of the focal point of the laser beam into the glass or SU-8 material resulted in damage within the pallet and even fracturing of the pallet.

Smaller and larger pallets could also be released using the focused laser pulse. Pallets with a 30-μm side were released at lower energies (<2 μJ) with 100% efficiency and 0% cross talk (release of adjacent pallets). Larger pallets (>100 μm) required higher energies to effect a 100% release rate. For example, square pallets with a 250-μm width required 6 μJ of energy. Even at these higher energies, no adjacent pallets were released. Multiple laser pulses could be used to release pallets at energies lower than a single pulse (data not shown). A variety of other pallet shapes (ovals and hexagons) and sizes (20−250 μm) were also successfully released with this laser-based method. Since the SU-8 pallets were individually addressable and releasable with the laser, the pallets were suitable candidates for the array-based scanning and cloning of adherent, mammalian cells.

Release of Individual Pallets with Cells. In previous studies, SU-8 was found to be biologically compatible.[26−29] However, cells do not adhere well to the surface of native SU-8. SU-8 slabs incubated with fibronectin or collagen did support attachment and growth of RBL. 3T3, and HeLa cells (data not shown). Pallet arrays were incubated with fibronectin or collagen followed by culture of 3T3, RBL, or HeLa cells on the array. While most cells did not attach to the top surface of the pallets, some pallets did possess cells on their top surfaces (Figure 2A,B). To determine the feasibility of releasing pallets with living cells, the pallets with cells on their surface were released using the focused beam of the laser (Figure 2C). Prior to release, the cells were loaded with a viability indicator, Oregon Green diacetate. Most cells on the top surface of the pallet retained the Oregon Green, suggesting that the plasma membrane was intact and that the cells were living (Figure 2D). In contrast, cells adherent to the sides of the pallets frequently did not retain the indicator, suggesting that they were often killed by the release process. When choosing and releasing pallets based on the properties of the cells on their top surface, the cells on the sides of the pallet may contaminate the cultures of the desired cells from the pallet top surface. This was especially problematic since many more cells grew on the sides of the pallets than on the top surface.

Laser-Based Release of Pallets Surrounded by Virtual Walls. To decrease the accessibility of cells to the pallet side walls, virtual walls of air were created between the SU-8 pallets. Wang and colleagues recently demonstrated that hydrophobic coatings placed on a glass surface between SU-8 structures could be used to trap air (Figure 3A).[19] The air trapped between the microstructures was stable for many weeks and excluded cells and molecules from the regions between the SU-8 structures. To determine whether SU-8 pallets surrounded by trapped air could be released by the focused laser, an array of pallets was coated with (heptadecafluoro-1,1,2,2-tetrahydrodecyl)trichlorosilane.[19] A pallet on an array with virtual walls was released by a single pulse. For pallets less than 50 μm in height with an interpallet spacing of greater than 30 μm, aqueous solution filled the gap vacated by the pallet (Figure 3C,D). By moving the microscope stage, pallets could be sequentially released while adjacent pallets remained attached to the glass surface (Figure 3E). Over 100 pallets were released without detachment of pallets adjacent to the targeted pallet. When pallets of greater than 75-μm height (50-μm side, 30-μm interpallet spacing) were detached, the trapped air rather than aqueous solution filled in the site of the released pallet (Figure 4E,F). Under these conditions, the virtual walls were stable despite the removal of the pallet from the array.

To compare the energy required to release pallets surrounded by air to that for pallets surrounded by aqueous buffer, the probability of pallet release was measured for arrays with and without virtual walls with respect to the laser pulse energy (Figure 3B). The curves of the probability of pallet release versus laser energy were fitted to a Gaussian error function to determine the threshold energy for pallet release (E_p). E_p for pallets with and without virtual walls was 1.9 and 1.5, respectively. Thus, the energy needed to release pallets surrounded by air or aqueous buffer was similar. No release of adjacent pallets was observed in these experiments (n > 100).

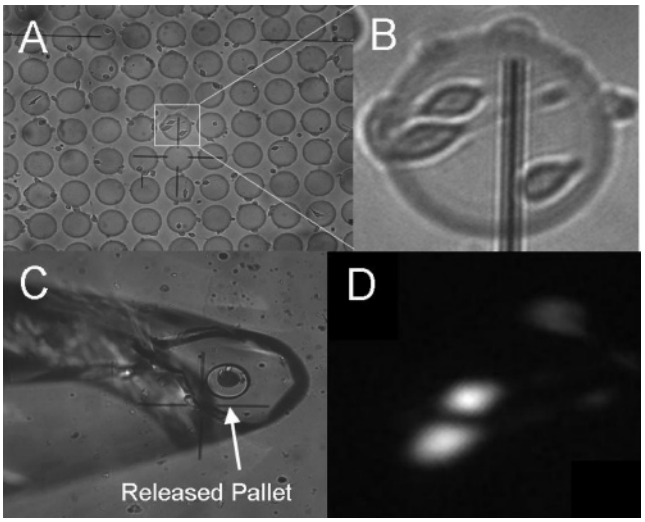

Figure 2. Release and collection of pallets with attached viable cells. (A) Shown is an array of circular pallets (75-μm side, 30-μm height, 20-μm spacing). 3T3 cells were cultured and then loaded with Oregon Green. (B) Shown is a closeup of a single pallet from (A). Six cells are attached to the pallet, three on the side wall and three on the top surface. (C) The pallet shown in (B) was released and then collected into a pipet. Shown is the tip of the pipet containing the released pallet. (D) The pallet collected in panel C was released into a culture dish and examined by fluorescence microscopy. Two of the cells on the top surface of the pallet retained the Oregon Green, indicating that they remain alive. In this particular instance, none of the cells on the side walls retained the viability indicator.

(26) Grayson, A. C. R.; Shawgo, R. S.; Johnson, A. M.; Flynn, N. T.; Li, Y.; Cima, M. J.; Langer, R. *Proc. IEEE* **2004**, *92*, 6−21.
(27) Kotzar, G.; Freas, M.; Abel, P.; Fleischman, A.; Roy, S.; Zorman, C.; Moran, J. M.; Melzak, J. *Biomaterials* **2002**, *23*, 2737−2750.
(28) Voskerician, G.; Shive, M. S.; Shawgo, R. S.; von Recum, H.; Anderson, J. M.; Cima, M. J.; Langer, R. *Biomaterials* **2003**, *24*, 1959−1967.
(29) Weisenberg, B. A.; Mooradian, D. L. *J. Biomed. Mater. Res.* **2002**, *60*, 283−291.

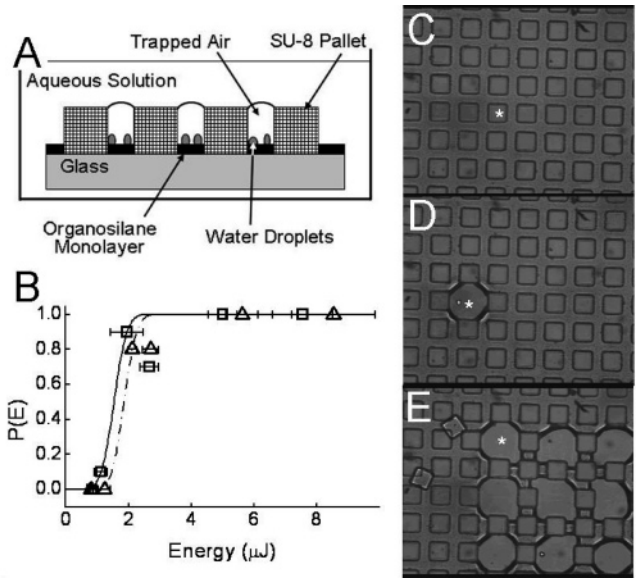

Figure 3. Virtual walls of air placed between pallets to shield the intervening glass and pallet sidewalls, leaving the upper surface exposed. (A) Schematic of the virtual air walls created by modification of the surface between the pallets. Air is trapped between the SU-8 pallets when an aqueous buffer is added to an array with a hydrophobic organosilane coating on the glass substrate. (B) The probability of pallet release is plotted against the laser pulse energy. The triangles and squares represent data from arrays with and without virtual walls, respectively. The lines are the best fits of the data to a Gaussian error function. The pallets were squares with a 50-μm side, 25-μm height, and 20-μm spacing between pallets. (C) Shown is an array of square pallets (50-μm side, 30-μm height, and 30-μm interpallet spacing) with virtual walls of air. The pallet to be released using a single laser pulse (2 μJ) is marked with an asterisk. (D) The site vacated by the pallet in panel C is marked with an asterisk. (E) Five more pallets were released sequentially. The asterisk marks the same array location as that in (D). Aqueous solution fills the regions vacated by the pallets. Most released pallets were propelled beyond the field of view, but two were visible in the left portion of the image.

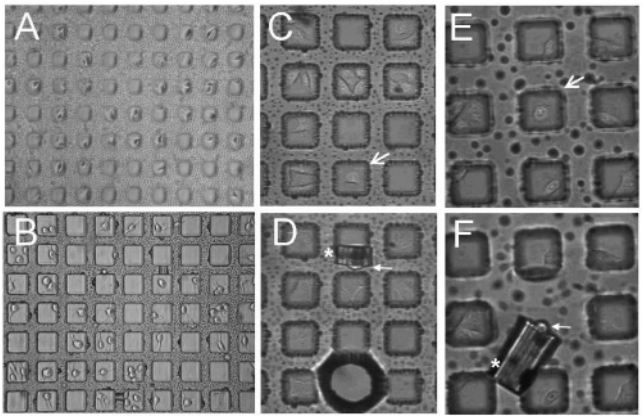

Figure 4. Culture and release of cells from arrays with virtual walls. (A) Shown is an array of square pallets (30-μm sides, 30-μm height, 30-μm interpallet spacing) plated with RBL cells in the presence of virtual walls. (B) Same as in panel A but with larger pallets (50-μm sides, 30-μm in height, 30-μm interpallet spacing). (C) HeLa cells were cultured on an array of pallets (50-μm sides, 30-μm height, 30-μm interpallet spacing). The pallet marked by the arrow was targeted for release. (D) The targeted pallet in panel C was released and permitted to settle back onto the array. The pallet marked with an asterisk settled on its side and the cell (marked by an arrow) is viewed in profile. The cell remains attached to the top surface of the pallet and aqueous buffer has filled the array site vacated by the released pallet. (E) HeLa cells were cultured on an array of pallets (50-μm sides, 100-μm height, 50-μm interpallet spacing). The pallet marked by the arrow was targeted for release. (F) The targeted pallet in panel E was released and permitted to settle back onto the array. The pallet marked with an asterisk settled on its side, and the cell (marked by an arrow) is viewed in profile. With these tall pallets, the virtual walls remain in place with air filling in the gap in array at the site of the released pallet.

Laser-Based Release of Cells/Pallets Surrounded by Virtual Walls. RBL and HeLa cells were cultured on pallet arrays with virtual walls. Square pallets with 30−40-μm sides provided adequate surface area for 1−2 RBL or HeLa cells per pallet since the size of these cells is ~25 μm (Figure 4A). Larger pallets (50−75 μm) could hold more cells due to the larger surface area (Figure 4B). The cells were localized to the pallet surfaces. Pallets with single cells were released by a focused laser pulse (2 μJ) (Figure 4C,D). SU-8 possesses a density slightly greater than that of water so the released pallets settled back down onto the array. The pallet frequently remained within the field of view after release. When the pallet settled on its side, the cell could be visualized in profile attached to the top surface of the pallet (Figure 4C−F). As for the arrays without cells, the fate of the entrapped air at the site of the released pallet depended on the array dimensions, pallet size, and interpallet spacing. The virtual wall at the site of the detached pallet was replaced by the aqueous buffer when the pallets were of limited height (Figure 4C,D). In contrast, the virtual wall of air was stable when the pallets were of sufficient height (Figure 4E,F). Following laser-based release, detached pallets were collected and examined to determine whether the cell remained on the pallet. For RBL cells, 94% of the collected pallets possessed cells (n = 17). For HeLa cells, 93% of collected pallets (n = 42) contained attached cells. The mechanical forces generated by the focused laser pulse at the glass−pallet interface were not sufficient to detach the majority of HeLa or RBL cells from the SU-8. In addition, the released cells appeared to have normal morphology by transmitted light microscopy, suggesting that the cells were viable. To further establish the viability of released cells, HeLa cells cultured on pallet arrays were loaded with a viability indicator (calcein red-orange AM) prior to release. Single cells on pallets were then released and immediately examined for retention of the dye. Over 90% of the HeLa cells (n = 21) retained the dye, demonstrating that their plasma membrane was intact and the cells were viable. These data demonstrate that each pallet with its cell was releasable on demand using the focused beam of the laser. Most importantly, the cells remained viable following release of the pallet to which they were attached.

Culture of Cells on Released Pallets. To determine the feasibility of collecting single cells for culture and expansion, pallets with single RBL or HeLa cells were released, collected, and placed into a culture dish. The cells were imaged by microscopy within 1 h of collection and then at varying times thereafter. At 1 h after collection, most HeLa and RBL cells remained on the pallet tops (Figure 5A,C). By 10 h after collection, many of the cells had migrated from the pallets onto the adjacent surface and some cells had also undergone cell division (Figure 5B,D). Within 2−4 days following culture, a small colony of cells was present with cells attached to both the pallet and surrounding

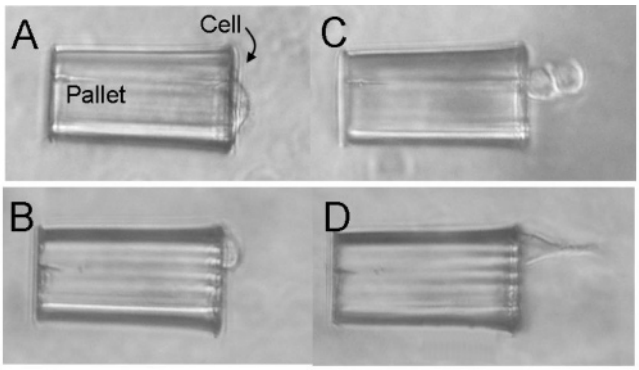

Figure 5. Culture of cells from released pallets. (A) and (B) RBL cells were cultured on an array of pallets (50-μm sides, 75-μm height, 50-μm interpallet spacing). Pallets with a single RBL cell were released and placed into separate culture dishes. Two pallets each with single RBL cell are shown immediately after collection in panels A and B. In both cases, the pallet lies on its side and the cell is seen in profile. The culture dishes were then placed in an incubator. (C) and (D) After 10 h of culture, the pallets were examined by transmitted light microscopy. The cell from panel A is shown in panel C and has migrated off of the pallet surface and divided into two cells. The cell of panel B now seen in panel D has sent a long pseudopodia out onto the surface of the culture dish as it migrated off of the pallet.

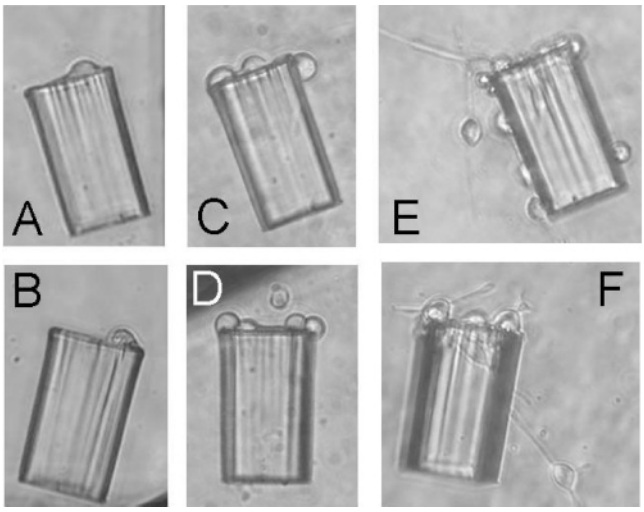

Figure 6. Growth of colonies from cells on released pallets. Pallets containing single RBL cells were released and transferred to a culture dish. Cells were examined under transmitted light over time after collection. Shown are time lapse images two different cells at 0 (A, B), 2 (C, D), and 4 (E, F) days after collection. Both pallets lie on their sides with the cell seen in profile at time 0. By 2 days, several cells were present on or around the top surface of each pallet. By 4 days, a small colony of cells surrounded each pallet. Multiple pseudopodia were extended from the cells.

culture dish (Figure 6A–F). All cells in the colony were likely clones of the original single cell. These data demonstrate the feasibility of collecting living RBL and HeLa cells from the pallet array and producing colonies of clonal cells. However, one weakness of the current system was the pallet collection strategy following release. With the current collection methods, released pallets were frequently trapped in regions of fluid dead volume in the tubing or culture dish or were lost due to adhesion to the tubing or vessel walls. For these experiments, the collection efficiency of the released pallets ranged from 10 to 50%. In addition, the maintenance of sterility during the collection process was a challenge due to difficulties in sterilizing the collection components. A future goal will be to simplify pallet collection to enhance the collection efficiency, preserve sterility, and maintain cell health.

CONCLUSIONS

The new array technology presented here incorporates a high density of elements with each element releasable on demand. The individual elements or pallets are composed of SU-8, a negative photoresist, that is easily patterned on micrometer-sized dimensions. While SU-8 is fully biocompatible, it does possess an autofluorescence with a peak emission wavelength of 470 nm. However, fluorescence microscopy with green-emitting fluorophores has been reported using thin pieces of SU-8.[19,30] The fluorescence of SU-8 is greatly diminished in the red wavelengths compared to the blue and green wavelengths (Wang, Y. unpublished data). Thus, it is expected that traditional fluorescence microscopy assays will be compatible with cells on the pallets especially when red fluorophores are employed.[30] Nevertheless, it will be important to develop low or nonfluorescent substrates for the pallets so that very low-level fluorescence measurements can be performed on cells grown on the arrays.

The pallet arrays possess attributes that significantly enhance current collection methods for live adherent cells. Thousands to millions of cells can be grown on micrometer-sized pallets in arrays with centimeter-sized dimensions. Each cell/colony remains adherent to its growth substrate throughout the analysis and collection process with concomitant reduction in manipulation. An important advantage is that individual elements of the array are indexed so that each cell has a unique address and can be followed over time prior to its selection. As discussed above, the microfabricated platform is expected to be compatible with standard imaging methods so that validated, commercially available reagents can be used for cell identification and analysis. This array technology provides a new approach for positive selection and cloning procedures that will confer significant benefits to biomedical investigations utilizing adherent cells.

ACKNOWLEDGMENT

We thank E. Stanbridge for his insightful comments and advice and Ruisheng Chang for pallet fabrication. This research was supported by grants from the NIH (EB004597 and CA104214).

Received for review August 22, 2006. Accepted November 2, 2006.

AC0615706

(30) Wu, Z. Z.; Zhao, Y.; Kisaalita, W. S. *Colloids Surf. B* **2006**, *52*, 14–21.