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Micropallet arrays for the rapid, nondisruptive isolation of adherent cells

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UNIVERSITY OF CALIFORNIA
IRVINE

Micropallet arrays for the rapid, nondisruptive isolation of adherent cells

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Engineering

by

Georgina To'a Salazar

Dissertation Committee:
Professors Nancy Allbritton & G.P. Li, Co-Chairs
Professor Eric Stanbridge

2008

The dissertation of Georgina To'a Salazar

is approved and is acceptable in quality

and form for publication on microfilm:

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Committee Co-Chair

_____
Committee Co-Chair

University of California, Irvine

2008

Die Kleinsten

Sag Atome, sage Stäubchen.
Sind sie auch unendlich klein,
Haben sie doch ihre Leibchen
Und die Neigung, dazusein.

Haben sie auch keine köpfchen,
Sind sie doch voll Eigensinn.
Trotzig spricht das Zwergeschöpfen:
"Ich will sein, so wie ich bin."

Suche nur, sie zu bezwingen,
Stark und findig, wie du bist.
Solch ein Ding hat seine Schwingen.
Seine Kraft und seine List.

Kannst du aus ihnen schmieden
Deine Rüstung als Despot,
Schieβlich wirst du doch ermüden,
Und dann heiβt es: "Er ist tot."

Wilhelm Busch (1904)

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Acknowledgements

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I would like to acknowledge members of the Allbritton Lab who have contributed in various ways to my work: Dr. Christopher Sims, for initiating me in skills I needed to work in the lab, Jim Palmer and Hsuan-hong Lai for patience in alignment issues, Yuli Wang, for overcoming key hurdles in the pallet project, Wei Xu, for considering me a fabrication person and always providing a good example working through frustration. Special thanks to those and other lab members, Neychelle Fernandes, Hamed Shadpour, Laura Borland, Scott Phillips, Allison Nelson, Lavanya Rau, Simon Mwongela, Shan Yang, Marlon Thomas, Jeng-hao Pai, Paul Marc, Andrew Kang, Sumith Kottegoda, Grace Young, Angie Proctor, Phil Gach, Jocelyn Wang, Mike Brenner, for sharing lab and life, for thoughtful discussions – scientific and non-scientific alike – and for their friendship.

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Finally, I would like to thank my parents, Iva and Edward Salazar, my sisters Nina and Vailala, my brother Victor, and our large and growing extended family for providing all the support that a family can give.

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- Assemble and maintain a system of optical components to direct a 532 nm ns pulsed Nd:YAG laser into a fluorescence microscope
- Employ microfabrication, cell culture, laser phenomena, optical and fluorescence microscopy to develop and characterize a new method for selection of adherent cells while cells remain adherent, broadening the number of attributes that can be utilized for cell separation
- Results of research were included in grant proposals and publications describing this method, that promises to be of use to many techniques in cell biology that require selection of a single or small groups of adherent cells

Research Technician. The Scripps Research Institute, La Jolla, CA. 11/01-6/03
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- Jimenez R, Salazar G, Baldridge KK, Romesberg FE. "Flexibility and molecular recognition in the immune system," *The Proceedings of the National Academy of Sciences*, vol. 100, no. 1, pp. 92-97 (2003)
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- Wang Y, Salazar GT, Pai JH, Shadpour H, Sims CE, Allbritton NA. "Micropallet arrays with poly(ethylene glycol) walls," Lab on a Chip, vol. 8, pp. 734–740 (2008)
- Quinto-Su PA, Salazar GT, Sims CE, Allbritton NL, Venugopalan V. "Examination of pulsed laser microbeam detachment of SU-8 polymer 'micropallets' for the collection and separation of adherent cells," Analytical Chemistry, vol. 80, pp. 4675–4679 (2008).

- Salazar GT, Wang Y, Sims CE, Bachman M, Li GP, Allbritton NL. "Characterization of the Laser-Based Release of Micropallets from Arrays," Journal of Biomedical Optics, vol. 13, no. 3, published online (2008)

Conference presentations:

<u>Poster</u>
Biomedical Engineering Society Conference. Chicago, Illinois. October 2006
UC Irvine Biomedical Engineering Department New Graduate Student Orientation. Irvine, California. September 2006
LifeChips Symposium. Irvine, California. May 2006.
<u>Oral</u>
Salazar G, *Rapid, nondisruptive isolation of single adherent cells from a heterogeneous population*, Biophysical Society Meeting, February 15, 2005

Abstract of the Dissertation

Micropallet arrays for the rapid, nondisruptive isolation of adherent cells

By

Georgina To'a Salazar

Doctor of Philosophy in Biomedical Engineering
University of California, Irvine, 2008
Professors Nancy Allbritton & G.P. Li, Co-Chairs

Techniques for rapid positive selection, the process of selecting and isolating an individual cell or a distinctive group of cells in the midst of a mixed cellular population, would enhance genetic engineering protocols, cell transformation studies, cell-based screening of random libraries, and many other studies. The goal of this work is to develop and characterize a system for analyzing, sorting, and collecting viable cells from a mixed population while they remain adherent. The attainment of this goal will be based on readily available microfabrication techniques and chemical surface modifications, an optical phenomenon already used in a wide range of cellular micromanipulation techniques, biological measures needed for isolation of single cells, and an understanding of how these elements can work together. This understanding will be built on findings of preliminary research. To make this research possible, dense arrays of cell-sized microstructures, or pallets, and a prototype laser-based release system were constructed. Single pallets within large arrays were selectively released with a single, focused, low energy laser pulse. Cells were cultured on pallet arrays. The system was able to isolate viable, proliferating cells. As a next

step, the laser-based release of pallets was characterized with respect to laser and pallet array parameters, including materials used for pallets and for walls between pallets. Progress was made toward characterizing cell health on laser-released pallets with respect to laser and pallet array parameters. Thus, this work demonstrates a technology for sorting rare cells from a heterogeneous population. The benefits of this separation strategy will be greater cell viability, reduction in time and manipulation of selected cells, and a broader set of cell attributes available for cell selection.

Introduction
The need for the development and optimization of micropallet arrays

Cell sorting is a procedure common throughout biomedical research. Cell transformation studies, cell-based screening of random libraries, the development of cell lines derived from primary patient cells and many other studies involve putting significant effort into selecting, separating, and collecting specific cells. 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 (Figure 1). 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]
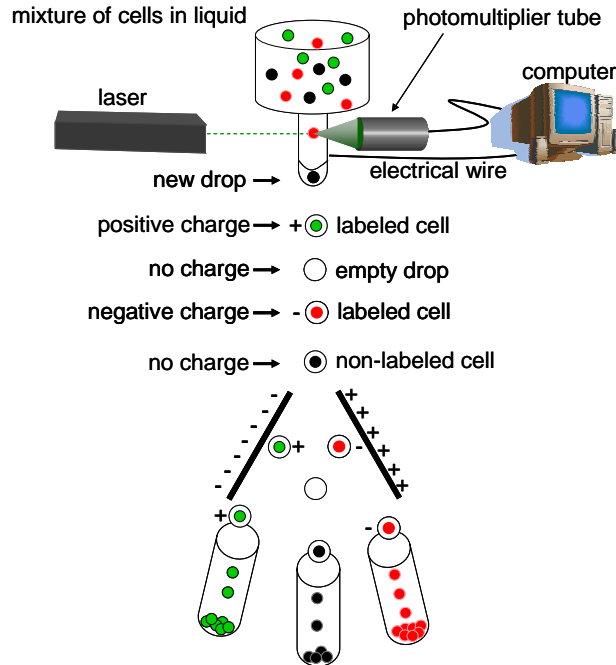
Figure 1. Schematic drawing of fluorescence-activated cell sorting (FACS). The requirement that the cells enter the system in a single-cell suspension allows sorting of extremely large numbers of cells, but imposes limitations including reducing the number of selection criteria available.

Most cells naturally grow in an adherent manner and can be analyzed for a broader range of attributes when in their native state. Alternative techniques to FACS are commonly used for collecting select adherent cells from a mixed population, but these are manpower and time intensive. In limiting dilution, adherent cells are disaggregated, extensively diluted, and then placed in culture wells so that on average one or fewer cells is plated per well (Figure 2). Since the 1990s, there has been an increase in research and development for the separation of adherent cells while they remain adherent. Pulsed lasers have been used for the direct transfer of cells from one surface or container to another. In laser-induced forward transfer (LIFT) of cells, cells are suspended in a thin fluid layer overlying a sacrificial silver layer on which a laser pulse is focused. The silver layer absorbs energy from a laser pulse, transforming

electronic excitation to kinetic energy to carry cells to soft accepting gelatin layer. While this technique is suitable for transfer of random suspended cells as for cell arraying, it is not readily compatible with cell sorting.[15, 16] Pulsed laser-mediated techniques for retrieving adherent cells are already commercially available. In laser capture microdissection (LCM) (Arcturus, Mountain View, CA) cellular or tissue samples are first placed on a film that is placed on a microscope cover glass. The area around the sample is manually selected. A pulsed laser beam activates the film underlying the sample, which is then lifted into a collection vial.[17] Laser microdissection techniques have been widely applied. For instance, Toner's and Revzin's groups have incorporated laser microdissection with microwell arrays to sort and collect lymphocytes and co-cultured hepatocytes and fibroblasts.[18-20] Most applications of laser microdissection use fixed or frozen samples(Figure 3).[17] Protocols for live cell sorting using laser microdissection have been described, but remain low throughput and not suitable for isolating large numbers of single, living cells.[21] Drying of the specimen, usually required for dissection and collection, limits live cell sorting by traditional laser microdissection approaches. 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(Figure 4).[22] Collection of live specimens has been demonstrated,[20, 23] but most of the work with this technique has utilized fixed specimens. The microdissection and catapulting process results in cellular injury emanating from direct UV photodamage associated with the dissection step or injury associated with catapulting. A foil used to protect cells from

UV-light-induced and thermal damage in laser microdissection and pressure catapulting (LMPC) fluoresces and scatters, interfering with fluorescence identification of cells of interest. Multiple layers are present in the current technique for live-cell catapulting, making catapulting dynamics and optimization complex.[20] 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. Thus, these techniques have only partially met the needs of investigators for the positive selection of adherent cells.
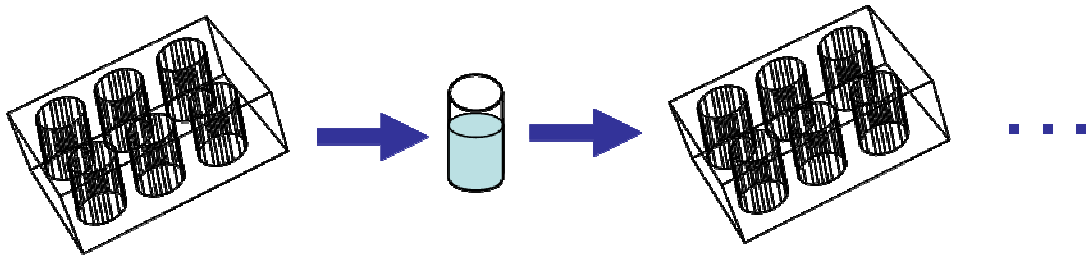
Figure 2. The weeks-long process of limiting dilution involves waiting for clones to become prominent, picking individual colonies, and subjecting cells to serial passaging.
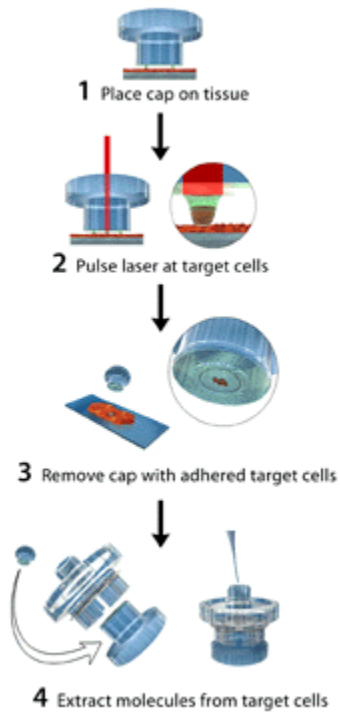
Figure 3. A laser capture microdissection (LCM) process. A cap is placed over sample of interest. Pulsing a lser through the cap activates a film to attach the sample to the cap. Source: http://www.actur.com/research_portal/products/pixcell_obtain_results.html

specimen cut out using focused laser beam

Glass slide

Microscope objective

Collection cap

isolated specimen ejected into the cap of a microfuge tube by defocused laser pulse
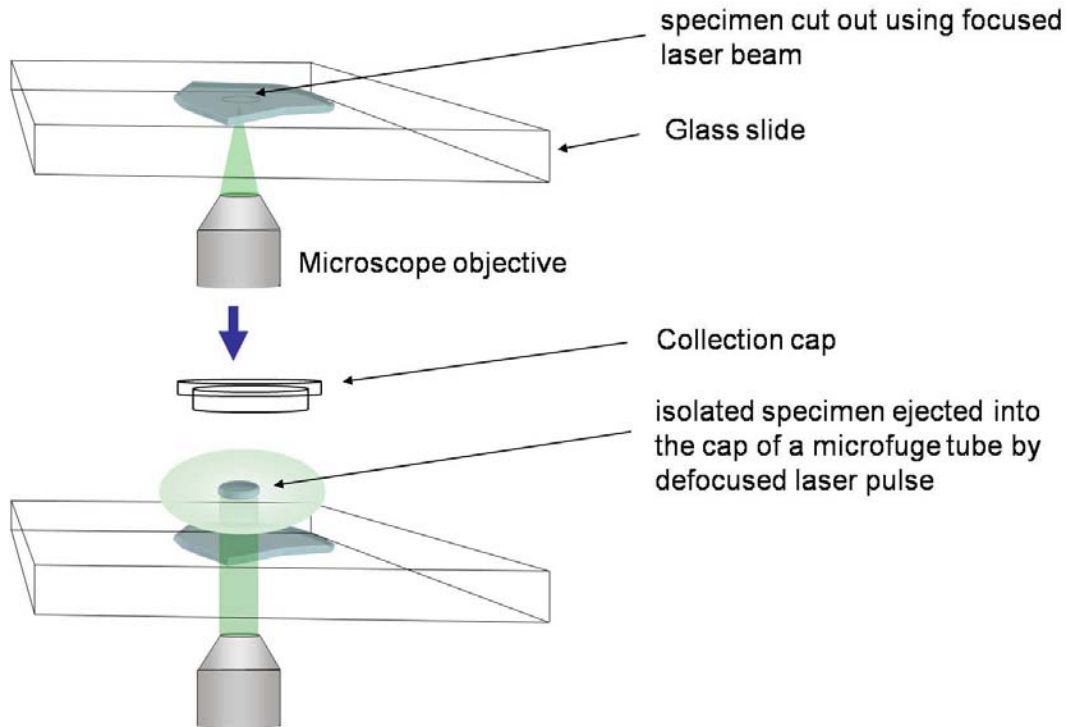
Figure 4. PALM's LMPC technology. A pulsed laser is focused through a microscope objective for cutting action. After, the isolated specimens are ejected out of the object plane and catapulted directly into a cap using a single defocused laser pulse.

In the following chapters, I will describe the development and characterization of a platform for the positive selection of live, single adherent cells while the cell remains adherent to its growth substrate. Initial efforts accomplished individual release of selected SU-8 pallets within large arrays of pallets using a single, focused, low-energy laser pulse without detachment of nearby, nontargeted pallets. The system was able to isolate viable, proliferating cells singly and in small groups.[24] Thus, the feasibility of using the pallet array technology for collecting and cloning adherent cells was demonstrated. As a next step, laser-based release of pallets was characterized with respect to pallet array and laser parameters. Pallet size, interpallet distance, and pallet height were varied. The laser parameters of pulse duration and

pulse number required for release at a given energy were also investigated.[25] Arrays were fabricated using alternate materials for pallets and for walls between pallets; the energy required for laser-based release of pallets from these arrays was measured.[26, 27] Examination of the pallet release process using fast-frame photography further provided mechanistic understanding of pallet release.[28] Understanding the pallet release process and its interplay with laser and array parameters informs strategic choice of parameters that reduce exposure of cells to stresses during sorting. Finally, progress toward examining the effect of pulse energy and pattern on the adherence of cells to laser-released pallets is discussed.

Chapter 1   Micropallet Arrays for the Separation of Single, Adherent Cells

1.1   Abstract

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. This work describes 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. 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. This work was published in the journal *Analytical Chemistry* (Salazar et al., 2007).

1.2 Introduction

1.2.1 *The need for a new approach to sorting live adherent cells*

This chapter 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 (Figure 5). 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.

1. Pallet
2. Microscope objective
3. 5 ns, 5 µJ laser pulse; formation of shock wave and cavitation bubble
4. Released pallet
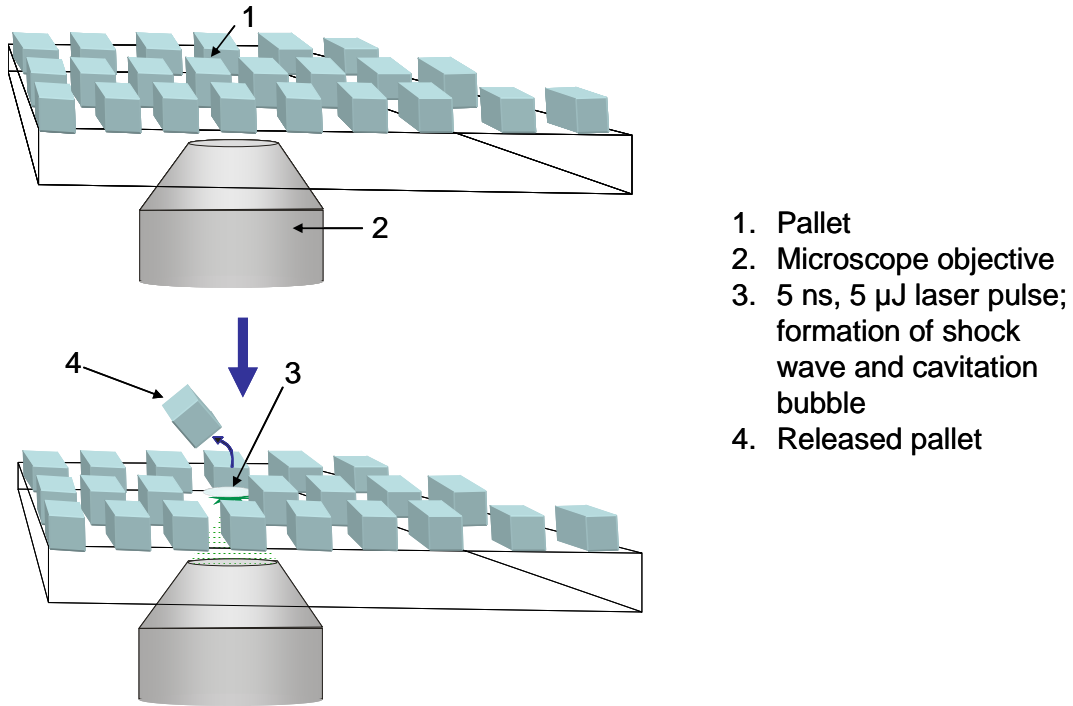
Figure 5. A laser pulse is aimed at the interface of the pallet and the glass slide. Optical breakdown drives formation of a bubble under the pallet, lifting it from the glass.

1.2.2   The experimental system

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 and silanization of SU-8 pallet arrays. Pallets composed of SU-8 were fabricated on a glass slide as described previously, varying time parameters as appropriate for the SU-8 thickness being prepared.[29-31] The coated slides were baked on a hot plate to remove solvent and exposed to UV light transmitted through an iron oxide photomask with the desired pallet features. The pallets were finally baked again then developed in SU-8 developer (Figure 6). A fuller description of the fabrication parameters is included in Chapter 5.
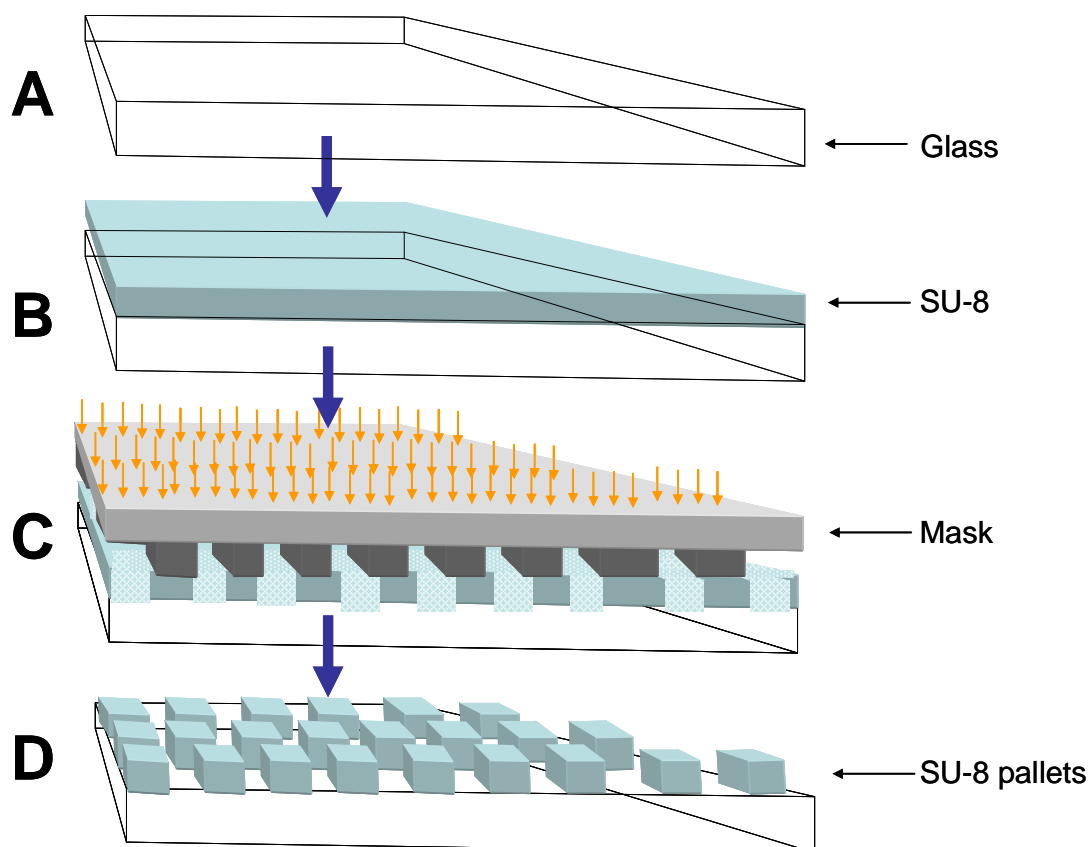
Figure 6. Cell pallets are formed by microfabrication techniques. A) A glass slide is cleaned. B) The polymer SU-8 is spun on the surface of the glass slide to a thickness between 25 μm and 100 μm. C) The SU-8 is exposed to UV light through a high resolution mask, then cured and heated to accomplish cross-liking in the areas that were exposed to

UV. D) Uncured material is rinsed away to reveal high resolution microstructures, pallets, in SU-8 polymer.

Laser-Based Pallet Release. Light from a pulsed Nd:YAG laser (New Wave Research ACL-1, Fremont, CA, 532 nm, 5 ns pulse width) was steered into the rear port of an inverted microscope (Nikon TE 300, Melville, NY). Beam intensity distribution on sample was TEM00 Gaussian. Beam size diameter at the focal point was approximately 1 µm (Figure 7). Laser-based pallet release, imaging on the pallet release system, and measurement of pulse energy is described in detail in Chapter 5.

Figure 7. Schematic of light path for laser-based pallet release. Reproduced with permission from The Society of Photo-Optical Instrumentation Engineers.

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.[32, 33] Since the pallets are released by the mechanical energy generated by a plasma, the

probability of pallet release was also fit to the Gaussian error function as described in Chapter 5.

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 as described previously(Figure 8).[34] Details of the silanization process for pallet arrays are described in Chapter 5.
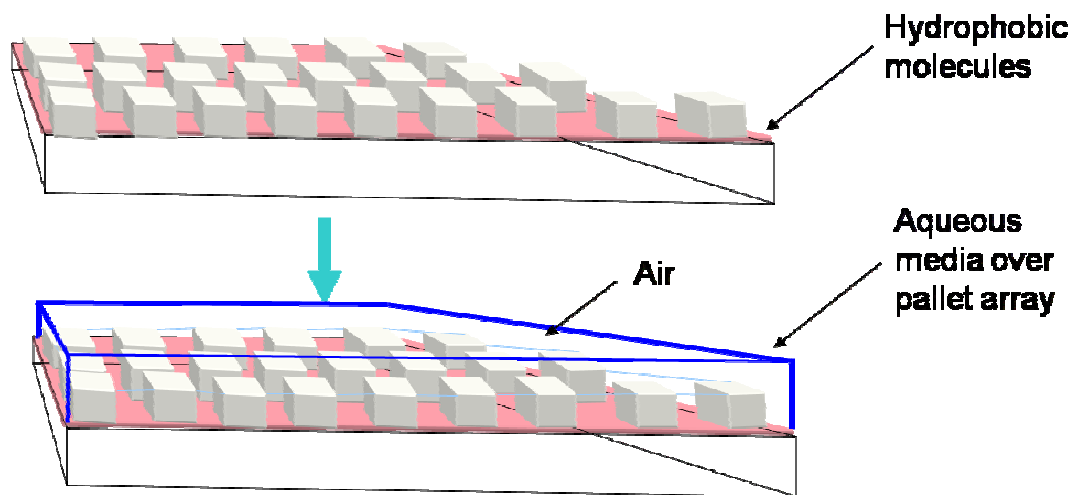
Figure 8. After fabrication of arrays of SU-8 pallets, silanization leaves a hydrophobic coating on the glass between the pallets. Immersion of the pallet array in aqueous solution leaves air trapped between the pallets.

Surface Coating of Pallets for Cell Culture. After silanization, the top surface of pallets was modified to enhance cell adhesion as described in Chapter 5. For the experiments described in the current chapter, the pallet top surface was coated with collagen using the two-step procedure or with fibronectin using the single-step procedure.

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.

1.3 Development of a selection system using a microfabricated array of individually releasable elements

1.3.1 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. SU-8 photoresist is an epoxy-based material that becomes cross-linked upon exposure to near UV light.[30, 31] This photoresist has become widespread throughout the semiconductor industry since it can be used to fabricate microstructures with high aspect ratios and near vertical walls.[30, 31, 35] 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.[29] 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. 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 5). 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.[32, 33, 36] 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. 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 microscope stage to sequentially place pallets in the path of the focused beam. 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). 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.

1.3.2 *Release of Individual Pallets with Cells*

In previous studies, SU-8 was found to be biologically compatible.[37-40] 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 9A, 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 9C). 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 9D). 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.
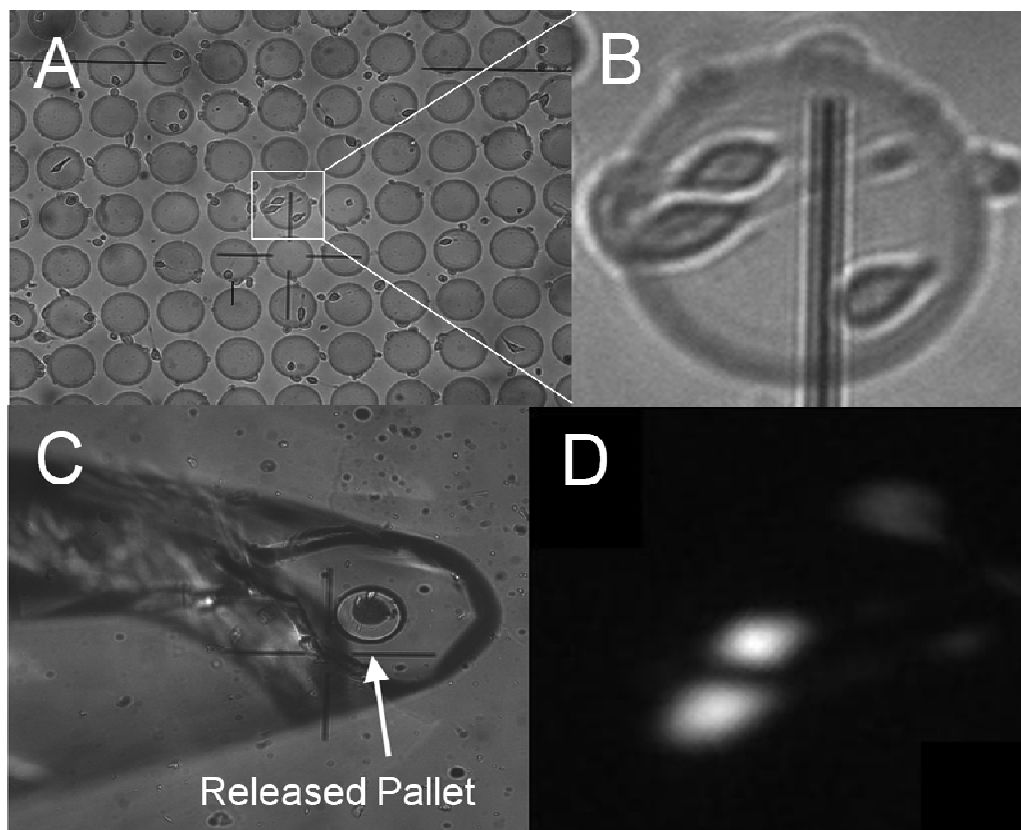
Figure 9. Release and collection of pallets with attached viable cells. (A) Shown is an array of circular pallets (75-µm side, 30-µm height, 20-µm spacing). 3T3 cells were cultured and then loaded with Oregon Green. (B) Shown is a closeup of a single pallet from (A). Six cells are attached to the pallets, three on the side wall and three on the top surface. (C) The pallet shown in (B) was relased 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. Figure reproduced with permission from the American Chemical Society.

1.3.3   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.

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 10). 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).
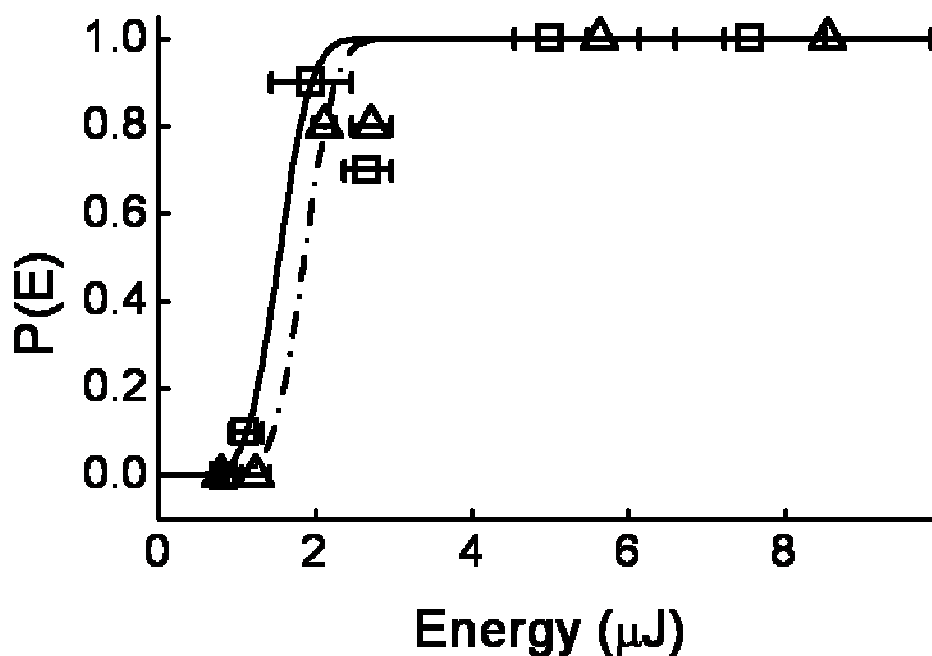
Figure 10. The probability of pallet release is plotted agains the laser pulse energy. The triangles and squares represent data from arrays with and without virtual walls, respectively. The lines are the best fits of the data to a Gaussian error function. The pallets were squares with a 50-μm side, 25-μm height, and 20-μm spacing between pallets. Figure reproduced with permission from the American Chemical Society.

1.3.4   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. Larger pallets (50-75 µm) could hold more cells due to the larger surface area. The cells were localized to the pallet surfaces. Pallets with single cells were released by a focused laser pulse (2 µJ). 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. 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. 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.

1.3.5   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 hour of collection and then at varying times thereafter. 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.

1.4   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.[29, 41] 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.[41] 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.

Chapter 2   Characterization of the laser-based release of micropallets from arrays

2.1   Abstract

The micropallet array system uses a pulsed laser to release pallets tens of microns to hundreds of micrometers in size from a larger array, enabling selective isolation of single cells adherent to the pallets. We characterize the laser-based release of pallets with respect to pallet array and laser parameters. The threshold laser energy required for pallet release increases linearly with the area of the pallet in contact with the underlying glass substrate. The spacing of the pallets within an array as well as the thickness or height of the pallet does not impact the energy required to release a pallet. Delivery of multiple laser pulses decreases the energy/pulse required for pallet release when the pallets were 100 μm or greater on a side. In addition to the square pallets, complex structures such as cantilevers and spirals could be released without damage using the pulsed laser. Identification of the pallet-array variables influencing the energy required for pallet release as well as strategies to minimize this energy will prove critical in optimizing the release of pallets with cells on the arrays. This work was published in the journal *Journal of Biomedical Optics* (Salazar et al. 2008)

2.2 Introduction

2.2.1 Relevance of quantitative release threshold information for the improvement of the pallet cell sorting system

Pulsed lasers have been used in other applications for the direct transfer of cells from one surface or container to another. Chief among these techniques are laser-induced forward transfer (LIFT) and laser microdissection with laser pressure catapulting (LMPC).[20, 42] LIFT was first described for the deposition of copper metal patterns inside a vacuum chamber.[43] In LIFT, a laser pulse heats a material past its boiling point so vapor-induced pressure ejects the material from a donor to an acceptor substrate. Modifications of the LIFT process allow the technique to be used to transfer material without subjecting that material to vaporization in order to transfer delicate substances or structures for deposition of electronic components, biological molecules, or cells.[15, 44-48] LIFT of biomolecules may prove useful in manufacturing DNA and protein microarrays.[44, 45] When applying LIFT for microarray spotting, the solvent acts as a transport vector and prevents decomposition of the soluble biomolecules.[49] Damage to biological materials during LIFT can also be mitigated by using a biocompatible sacrificial absorbing layer. It is this approach that has been used to transfer live cells for cell arraying.[15, 16] The cells are suspended in a thin fluid layer overlying the sacrificial layer upon which the laser pulse is focused. The focused pulse causes transfer of a droplet of overlying fluid containing cells in suspension onto an acceptor substrate. Measures of cell viability, stress, and proliferative ability after LIFT point toward its potential for applications in single-cell studies and tissue engineering.[15, 16] However, while the technique is

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suitable for transfer of random cells suspended in buffer, it is not readily compatible with identification or analysis of unique cells followed by their sorting.

Laser microdissection is used predominantly to obtain tissue sections for genetic and proteomic studies.[20] [50-55] The technique works well for fixed or frozen tissue as the laser-cutting systems utilized in these instruments are affected by moisture, and removal of fluid from the specimen is generally required for dissection and collection.[56] Drying of the specimen limits the use of this technique for live cell applications, although protocols for this purpose have been published.[21, 57] Zeiss (Göttingen, Germany) markets an instrument for laser microdissection that uses a pulsed UV laser to "catapult" the dissected tissue or cells into an overlying collection device.[58] LMPC has had greater success in live-cell applications than earlier laser microdissection technologies due to the fact that a thin layer of fluid can be present during cutting and laser transfer.[20] A 5-µm-thick UV-absorbing polymer foil is used to protect the specimen from UV-light-induced and thermal damage, but the foil scatters and fluoresces, interfering with histochemical and fluorescence identification techniques for cells of interest. A large number of layers are present in the current technique for live-cell catapulting, making catapulting dynamics and optimization complex.[20]

The aforementioned pallet-array system permits living cells or colonies of cells to be sorted while they remain on their growth surface, thus enabling analysis prior to the sort.[59] Studies to date using this sorting technique have documented a high rate of cell viability after laser-based release, and exceptional success in clonal expansion of sorted cells.[60] Stresses during sorting procedures, as is well known in flow

cytometry, can induce apoptosis (programmed cell death) particularly in non-cancer cells.[13, 61-64] Although high rates of cell viability have been demonstrated for cancer cell lines, optimization of laser-based pallet release remains a need for more fragile cells, e.g. primary cells, in order to maximize cell health and minimize cell stress. In addition, the various cell types and applications envisioned for pallet arrays will require a variety of pallet designs which will impact release parameters, most critically the pulse energy required for pallet removal. This work seeks to perform quantitative assessment of the effect of laser and array parameters on threshold energies for pallet release in order to understand and optimize the variables for laser-based release of living cells. A number of variables were examined to determine how they influence the energy required for laser-based pallet release. Pallet size, the distance between pallets, and pallet height were varied on a test pallet array. The laser parameters of pulse duration and the pulse number required for pallet release at a given energy were also investigated. Strategies to minimize the laser pulse energy for pallet release were described as well as alternative uses for the focused laser in the release of complex microstructures. The results of this study should provide a better understanding of the laser release process of pallets, and allow the choice of parameters that reduce the exposure of cells to physiologic stresses during the sort.

2.2.2   The experimental system

Microfabrication. We used an array of square pallets with different dimensions (25, 50, 100, and 200 µm squares 25, 50, or 75 µm tall) and regions containing pallets with different interpallet spacing (10, 25, 50, and 75 µm).   These pallets were

fabricated using SU-8 photoresist spin coated on glass slides. The coated slides were baked on a hot plate to remove solvent and exposed to UV light transmitted through an iron oxide photomask with the desired pallet features. The pallets were finally baked again then developed in SU-8 developer. The precise process of pallet fabrication is described in Chapter 5.

Optical geometry for plasma formation. Light from a pulsed Nd:YAG laser (New Wave Research ACL-1, Fremont, CA, 532 nm, 5 ns pulse width) was steered into the rear port of an inverted microscope (Nikon TE 300, Melville, NY). Beam intensity distribution on sample was TEM00 Gaussian. Beam size diameter at the focal point was approximately 1 μm. Laser-based pallet release, imaging on the pallet release system, and measurement of pulse energy is described in detail in Chapter 5.

Measurement of probability of SU-8 structure release by a single laser pulse. To release individual pallets, a pallet array was first placed on a microscope stage, and then the laser focus was set at the interface of the glass substrate and SU-8 pallet. A solution of polystyrene beads (approximately 1 μm diameter) in water was placed over the array, and beads were allowed to settle on the top surface of the glass to facilitate accurate and consistent focusing at the interface. Laser energies were chosen so at least two energies resulted in 0% release of targeted pallets, at least two energies resulted in release of 100% of targeted pallets, and at least two energies yielded between 0% and 100% release of targets. For a given pulse energy, 10 individual pallets were targeted with a single laser pulse. We counted the number of pallets that were released at a given energy and the average energy of ten pulses fired was determined.

Measurement of the probability of SU-8 structure release by multiple laser pulses. For experiments using multiple pulses for release of a single pallet, the pallet array was first placed on the microscope stage and laser focus was set at the interface between SU-8 and glass, as for release with a single pulse. Multiple pulses fired manually at a frequency of 1 Hz were then used to release the structures as described here.

Fit of the data to a Gaussian error function. To determine the threshold energy for pallet release, the pallet release frequency was plotted as a function of incident pulse energy calculated to reach the microscope stage. A Gaussian error function was fitted to this data. Fitting was performed using the nonlinear least-squares fitting capability of the software Origin 7.5 SR6 (OriginLab Corporation, Northhampton, Massachussets). The Gaussian error function was

$$p(E_p) = 0.5\{1 + erf[P_1(E_p - P_2)]\},$$

where p(Ep) is the probability of pallet release at a laser energy Ep. The values P_1 and P_2 were the fitted parameters, where P_1 determined the sharpness of the Gaussian error function and P_2 was the threshold energy, the pulse energy that resulted in a 50% probability of pallet release.

2.3 Studies of the dependence of pallet release energy on pallet size, interpallet spacing, pallet height, pulse duration and number

2.3.1 Pallet size

The size of the pallet used when sorting cells will depend on the cell type and the desired number of cells per pallet. Typically smaller pallet sizes ($\leq$50 µm) will be suitable for single cells while larger sizes (>50 µm) are more appropriate for cell colonies. The required laser energy for pallet release may depend on the size of the pallet. Thus, it is important to understand how the laser energy increases with the size of the pallet. To determine how the laser energy required for release scaled with the pallet size, pallets were released from an array of square pallets with a sides (s) of 25, 50, 100, or 200 µm (Figure 11A, B). For these pallets, the height (h) was 50 µm and the interpallet gap (g) was 50 µm. Six laser energies ranging from less than 1 µJ to greater than 10 µJ were chosen for pallet release. The pulses were aimed at the center of the targeted pallets, at the interface between the glass and SU-8. To minimize the effects of batch-to-batch variability in pallet release, the data for all sized pallets was obtained from a single array. The fraction of pallets released was recorded, along with the average energy of the ten pulses aimed at the pallets. The probability of pallet release as a function of pulse energy was fit to a Gaussian error function (Figure 12A). The Gaussian error function describes the stochastic nature of the plasma assumed to be the mechanism of laser-based pallet release. Comparison of thresholds for pallets of different sizes revealed a significant increase in threshold energy with increase in pallet size. The threshold energy required to release 25-µm squares, 50-µm squares, and 100-µm squares increased nonlinearly with size (Figure

12B, Table 1]. The 200 μm size pallets could not be released when tested with the highest laser energy available on the current system (14 μJ). To determine whether the threshold energy was linearly related to the surface area, the surface area of the pallet was plotted against the threshold energy (Figure 12C). The data points fell on a straight line with a y-intercept of 1.0 μJ. Since the release energies are proportional to the surface area, the 200-μm pallets would likely require about 28 μJ to be released. As the pallet surface area decreases to zero, a finite amount of energy is still required to release the pallet since the y-intercept is not zero. It is likely that this is the energy (1 μJ) required to form a plasma at the SU-8 – glass interface. This threshold energy for plasma formation acts as a necessary condition for pallet release to occur. For small pallet size (<25 μm), the magnitude of plasma formed at the threshold for plasma formation is sufficient to disrupt the adhesive forces between the glass and SU-8. As pallet size increases (>25 μm), the threshold energy for pallet release will be increasingly higher than the threshold for the necessary plasma formation, as larger plasmas will be required to disrupt the larger adhesive forces corresponding to larger SU-8 to glass contact area. Prior work studying the laser induced forward transfer of liquids demonstrated that droplet volume displayed a linear dependence on laser pulse energy.[49] Furthermore, in similarity to our work, a threshold energy density was a necessary condition for transfer. In the presented experiments, the laser is focused to a spot size smaller than the pallet area interface with the glass substrate. Similar to the mechanism described for LMPC, it is likely that the mechanism of pallet release relates to the generation of plasma with a

concomitant shock wave and cavitation bubble.[20] The mechanical forces created by

these phenomena are the probable source of energy used to dislodge the pallet.
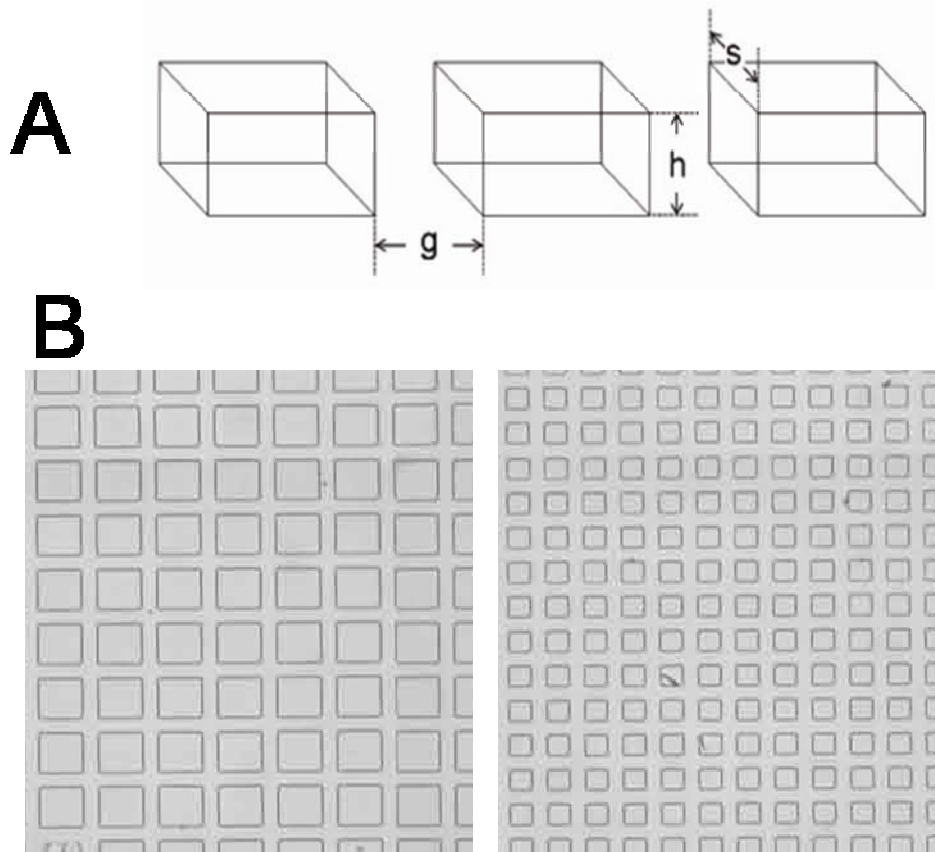
Figure 11. Experimental system: (A) Schematic of three pallets in an array with dimensions of height (h), size (s), and inter-pallet gap (g); (B) image of two sections of the pallet array. The right and left panels are a section of pallets with a side (s) of 200 and 100 μm, respectively. Both arrays possess other dimensions of 50 (h) and 30 (g) μm. Figure reproduced with permission from The Society of Photo-Optical Instrumentation Engineers.
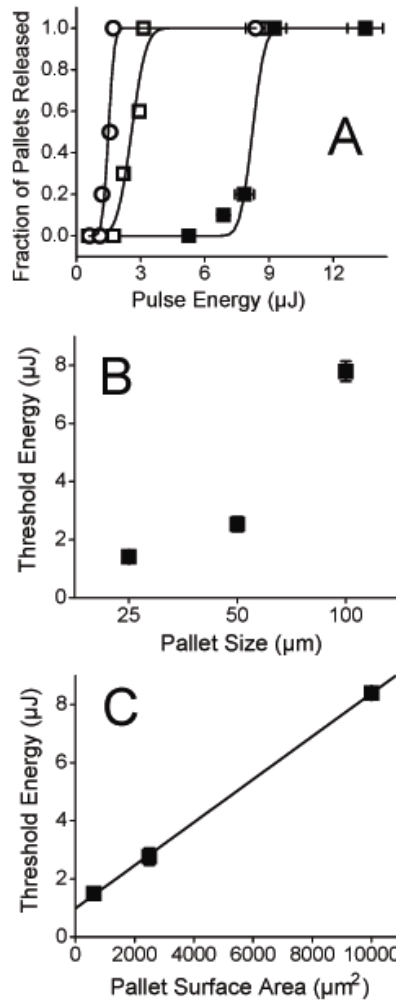
Figure 12 Dependence of release energy on pallet size. A) The probability of pallet release for different sized pallets was plotted against the average laser energy. The pallet side (s) was 100 (solid squares), 50 (open squares), or 25 (open circles) mm. The error bars represent the standard deviation of the laser energies. The solid lines represent fits of the data points to an error function. B) The threshold energy for pallet release was plotted as a function of pallet size. C) The threshold energy for plotted release was plotted as a function of the pallet surface area. The solid line is a straight line fit to the data points. For both B) and C) each data point is the average of 3 measurements and the error bars represent the standard deviation. Figure reproduced with permission from The Society of Photo-Optical Instrumentation Engineers.

2.3.2   Interpallet spacing

Different applications of the pallet array system may be best served by different interpallet spacings. For cell sorting, the distance between pallets on arrays will be optimized for cell isolation and the stability of air virtual walls between the pallets.

To determine whether interpallet spacing influenced thresholds for pallet release, experiments were performed on an array with regions containing square pallets spaced 10, 25, 50, or 75 μm from their neighbors. The height of the pallets was either 25 or 50 μm and the side of the pallet was 25, 50, or 100 μm. The probability of pallet release at different energies was measured and the threshold energy determined. For each pallet height, the data were determined from a single array to eliminate array-to-array variability. The pallets with a 50-μm height and a 10-μm inter-pallet gap could not be released due to residual SU-8 in the regions between the pallets. For all other pallets, comparison of the threshold energies for pallet release revealed no significant difference in threshold energy with respect to the inter-pallet spacing (Table 1). Since inter-pallet spacing does not affect the energy required for laser release, inter-pallet gap can be optimized to improve other array qualities. Air pockets (virtual walls) placed between the pallets are used to direct cells to the pallet tops. The stability of these air pockets is directly related to the size of the interpallet gap and pallet height. In future studies, interpallet gap can be optimized for virtual wall stability with no influence on the required laser energy for pallet release.[65]

2.3.3   Evaluation of interarray variability

In the preceding experiments, all pallets were fabricated on the same array to eliminate variability occurring at different fabrication times. However, it is not always possible to use pallets fabricated at identical times. Pallets with identical dimensions but fabricated at different times may have different threshold release energies since the adhesiveness of SU-8 to glass depends on multiple variables. These variables include the glass-cleaning procedure, the SU-8 baking parameters, the UV

exposure time, and the developing parameters of the SU-8 structures. Since these variables can be difficult to control precisely during manual fabrication of arrays, array-to-array variability in the SU-8:glass adhesion, and therefore, the threshold pallet release energies may occur. To identify the variation in release energies associated with arrays from different batches, threshold energies were measured for pallets [50 (*h*), 50 (*s*), and 75 (*g*) μm] on arrays from four different fabrication batches. The average release threshold and standard deviation were 4.0 ± 0.9 μJ, thus array-to-array variability can be substantial and must be taken into account in experiments that utilize arrays fabricated at different times. Further optimization of the pallet manufacturing variables as well as automation of the manufacturing process will likely decrease this variability.

2.3.4 Pallet height

Pallet height is an important design parameter of the pallet array system. SU-8 fluoresces in the green wavelengths so that an increased pallet height results in greater fluorescent background. This increased background may interfere with the detection of very low intensity fluorescence. For effective live cell sorting using laser-releasable pallets, the pallets must be thick enough to protect the cells during release, but thin enough to produce the least possible amount of background fluorescence. To determine how increasing pallet height affects the energy required for pallet release, arrays with pallets of differing heights (25, 50, and 75 μm) were fabricated. The probability of pallet release at different energies was determined and the threshold energy for pallet release was determined from the fit to the Gaussian error function as described above. For the 25-μm-size squares, no significant

difference in release threshold was observed between pallets 25 μm and 50 μm in height (Table 1). Pallets 25 μm in size and 75 μm in height were not manufactured due to the excessive aspect ratio required. For 50-μm squares, 25, 50, and 75 μm tall pallets had similar release thresholds. For pallets of 100 μm (s) and an interpallet gap of 50 μm, the variations in release thresholds for pallets of 50 μm (h) (7.8 μJ ± 0.3) and pallets of 75 μm (h) (12 ± 1) were within the range of the variability between batches of arrays (see above). Pallets of 100 μm (s) and 25 μm (h) did not release with a single laser pulse due to the flexibility of these very thin pallets. The interpallet gap did not introduce variation in the release thresholds (Table 1). These data suggest that pallet height and mass do not play a significant role in the threshold release energy. The energy required to disrupt the adhesion of the SU-8 to the glass is far greater than the energy required to lift the small mass. However, further decreases in the array-to-array variability might permit the detection of slight differences in the threshold energy of pallet release with respect to height. It is possible that differences in the curing of the SU-8 near the glass surface vary as the pallet height changes. Given the energy for laser release does not depend on the pallet height, this variable can be optimized to enhance other pallet array properties. For example, the viability of the cells on the pallets may depend on the height of the pallet since the pallet acts to shield the cells from the laser-generated phenomena at the glass:pallet interface. The stability of virtual walls between pallets is also directly related to the height of pallets.[66] In future studies, pallet height can be optimized for cell viability and virtual wall stability with minimal or no influence on the required laser energy for pallet release.

Table 1-Threshold energy (µJ) for pallet release. Figure reproduced with permission from The Society of Photo-Optical Instrumentation Engineers.

		10 µm gap	25 µm gap	50 µm gap	75 µm gap
25 µm squares	25 µm tall	1.8 ± 0.2	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.3
	50 µm tall	-	1.8 ± 0.4	1.4 ± 0.1	1.4 ± 0.1
50 µm squares	25 µm tall	4.3 ± 0.6	4.7 ± 0.2	5.2 ± 0.7	4.6 ± 0.5
	50 µm tall	-	2.4 ± 0.2	2.5 ± 0.3	2.9 ± 0.2
	75 µm tall	-	4.1 ± 0.1	4.4 ± 1	3.8 ± 0.1
100 µm squares	50 µm tall	-	8 ± 0.5	7.8 ± 0.3	8 ± 0.4
	75 µm tall	-	13.1 ± 1.2	12.1 ± 1.3	11.4 ± 1.1

Thresholds are calculated from data of triplicate experiments and shown as mean ± standard deviation.

2.3.5   *Multiple pulses*

Lower pulse energies for pallet release may lead to higher cell viability during cell sorting. One strategy for lowering the pulse energies is to deliver a train of pulses with each pulse disrupting a portion of the SU-8:glass bond. To determine whether a series of pulses could release a pallet at lower energies/pulse, a pulse was delivered to each corner of a pallet. No more than four pulses were delivered to a pallet. The average energy of the pulses delivered to the pallet was recorded as the release energy. The probability of releasing a pallet vs the average laser pulse energy was fitted to an error function to determine the release energy threshold. Release of

the 25 µm-sized pallets (h of 50 µm, gap of 50 µm) by multiple pulses manually fired at a frequency of 1 Hz required a threshold energy of 1.4 ± 0.3 µJ while pallet release by a single pulse required a threshold energy of 1.4 ± 0.1 µJ. Similarly multiple pulses released a 50 µm-pallet (h of 50 µm, gap of 50 µm) with a threshold of 1.7 ± 0.5 µJ, and a single pulse required a threshold of 2.5 ± 0.3 µJ. Thus, for small pallets, release thresholds achieved by aiming a pulse at each corner were similar to that obtained by aiming a single pulse at the center of the target pallet. For large pallets, 100 and 200 µm in dimension, the minimum energy needed to release a pallet was substantially lower for multiple pulses than for a single pulse. Release thresholds for 100 µm-sized pallets (h of 50 µm, gap of 50 µm) were reduced almost threefold (release threshold 2.9 ± 0.2 µJ) when four pulses were used to achieve release rather than a single pulse (release threshold 8.4 ± 0.2 µJ). Thus, the energy per pulse for pallet release was decreased although the total energy delivered was not decreased for the multiple pulse protocol. Pallets with dimensions of 100 µm (s) with 25 µm (h) or 200 µm (s) with 50 µm (h) could not be released with a single laser pulse aimed at their center due to the flexibility of these very thin pallets. However, pallets of 100 µm (s) with 25 µm (h) were easily released with a threshold energy of 5.4 ± 1.0 µJ when four pulses, one at each corner, were utilized. Pallets with dimensions 200 µm (s) and 50 µm (h) could be released with a threshold of 11.7 ± 1.6 µJ when a pulse was aimed at each corner of the target pallet. For large pallets, multiple pulses lower the required energy per pulse and may be required for the release of very thin, flexible pallets.

An alternative strategy to lower the energy/pulse for pallet release is to deliver a large number of subthreshold pulses, most of which will not impact the SU-8:glass bond. However a small portion may initiate a plasma leading to SU-8:glass separation. A train of pulses was fired at either the center or corners of a pallet [100 μm (*s*) and 50 μm (*h*)] until the pallet was released (Figure 13). The average number of pulses required to release a pallet was plotted against the energy/pulse of the laser. When directed at the pallet corners, as little as 2-μJ energy pulses could be used to release a 100 μm pallet. Although on average 50 pulses were required. When the laser pulses were targeted to the center of the pallet, twenty 5-μJ pulses were required to effect pallet release. It may be possible to further reduce the energy/pulse for pallet release by firing even larger numbers of pulses with a high frequency. While the energy/pulse was lowered by delivering a series of laser pulses, the total energy of all of the laser pulses exceeded that when a single laser pulse was used to initiate pallet release. A key future goal will be to determine whether cell health is most closely tied to the energy/pulse or the total energy of all pulses.
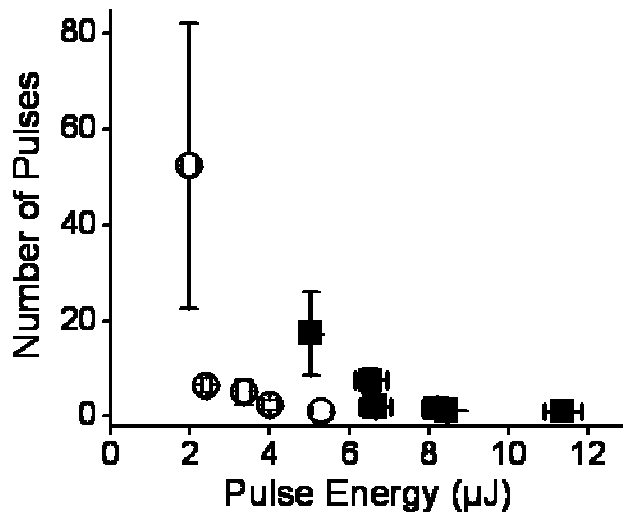
Figure 13. Release of pallets with a train of laser pulses. The average number of laser pulses required to release a pallet is plotted against the average energy/pulse. The y axis error bars represent the standard deviation in the number of pulses utilized to release ten different pallets. The x axis error bars represent the standard deviation of the laser pulse energy. The pulses were directed at the pallet corners (open circles) or at the center of the pallet (solid squares). Figure reproduced with permission from The Society of Photo-Optical Instrumentation Engineers.

2.3.6 Pulse duration

A likely mechanism for pallet release is the formation of a plasma by the focused laser beam at the interface of the SU-8 and glass substrate. The ensuing mechanical shock wave and cavitation bubble might also contribute to the disruption of the SU-8:glass adhesion. Since plasma formation depends more on the critical irradiance (power/unit area) rather than the critical radiant exposure (energy/unit area), the pulse energy needed to form a plasma decreases as the pulse duration decreases. Thus single laser pulses with a duration of 500 ps might mediate pallet release at lower energies than that of the 5-ns pulses. Pallets of 50-μm size were released with a single laser pulse of 5 ns or 500 ps and the energy threshold for energy release was measured. The release thresholds calculated for picosecond-laser-based release (1.4 ±

0.3 μJ) were not significantly lower than those for nanosecond-laser-based release (1.5 ± 0.1 μJ) for the tested pallet array. It is likely that the total energy needed to release a pallet is dominated by that energy needed to disrupt the SU-8:glass adhesion rather than that required to form a plasma.

2.4 Laser based release of complex structures

The pallet material SU-8 is used widely to microfabricate high aspect ratio structures. Frequently all or portion of a complex SU-8 structure must be released from the substrate on which it was fabricated. These SU-8 components are often synthesized on a sacrificial layer, which can be removed using a chemical etchant. However wet etching can chemically contaminate or degrade coatings on the SU-8 microstructures. Dry release processes use an anti-adhesion layer, such as Teflon or a self-assembled monolayer, between SU-8 and its substrate enabling SU-8 microstructures to be mechanically lifted from a substrate without immersion in a fluid. However all of these methods require bulk treatments, are relatively time-consuming, or cannot be spatially localized. To determine whether laser-based release of SU-8 could detach complex microstructures from an underlying surface, a variety of microcomponents (cantilever, anteater, and spiral) were fabricated in an array format (Figure 14A). The cantilever-shaped structures were 1.5 X 0.5 mm with 20-μm-wide arms while the anteaters were 650 X 250 μm and the spirals were 350 μm in diameter with 20-μm-wide arms. Each of the microstructures was released using a series (20 to 100) of focused pulses (3 to 5 μJ). No fragmentation of any of the structures occurred and only the targeted structure in the array was released

(Figure 14C). In contrast, mechanical scraping with a spatula led to both release and extensive fragmentation of the components (Figure 14B). Laser-based release of SU-8 from surfaces may be of utility in applications requiring localized, precise release of microstructures. Structures with dimensions of microns to millimeters can be released using the appropriate number and energy pulses.
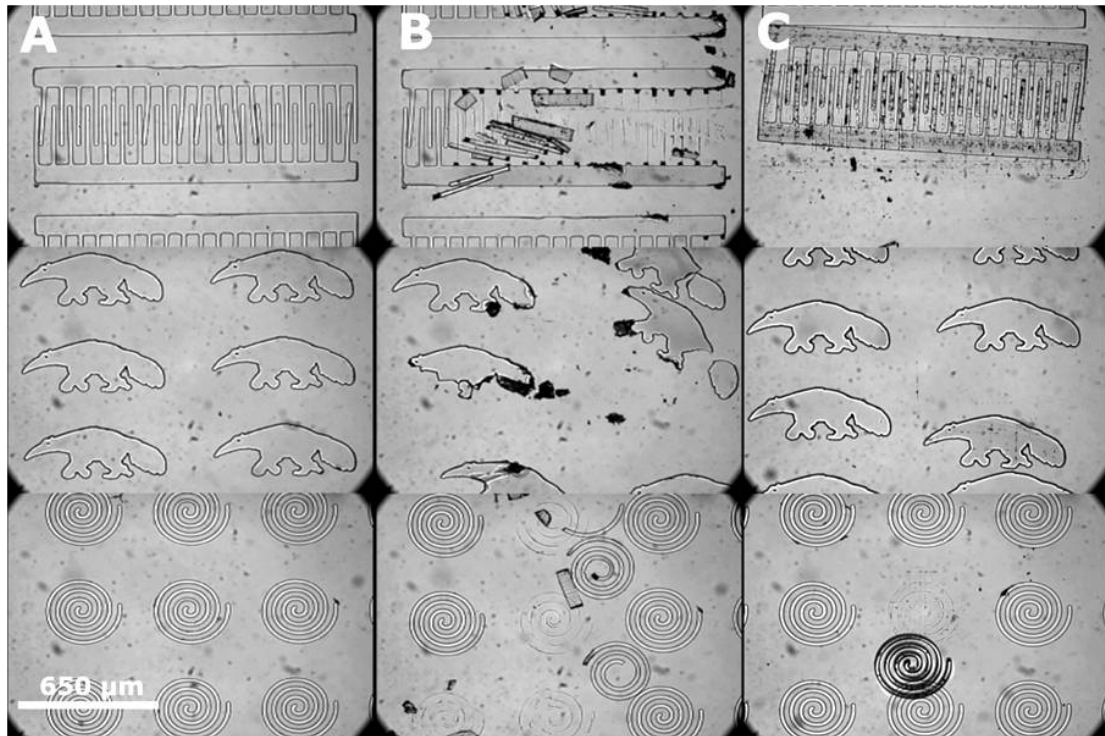
Figure 14. Laser-based release of complex structures: (A) arrays of cantilevers (top panel), anteaters (middle panel), and spirals (lower panel) were fabricated, a transmitted light image; (B) structures released by mechanical scraping; and (C) single structure released using a train of focused laser pulses. Figure reproduced with permission from The Society of Photo-Optical Instrumentation Engineers.

2.5 Conclusions

The threshold energy for pallet release was shown to be linearly related to pallet surface area. This analysis also showed that a finite amount of energy would be required to release a pallet whose surface area was zero. These data are consistent

with a threshold energy requirement that acts as a necessary, but not sufficient, condition for pallet release to occur. This energy is interpreted as the threshold for plasma formation at the focal point of the laser. This process creates a cavitation bubble and shock wave, which likely generate the mechanical forces to drive pallet release. The results further suggest that the optimal strategy for laser-based pallet release depends on the size of the pallet. Small pallets ($s \leq 50$ μm) are most efficiently released by a single, centered laser pulse of low energy. Larger pallets, especially when thin ($h < 50$ μm), may not be releasable with a single pulse, but can be released with multiple pulses aimed near the corners of the pallet. Each of these corner pulses likely detaches a quadrant of the pallet. A series of focused pulses of a few microjoules per pulse can be utilized to release not only pallets, but also complex, millimeter-sized structures with little to no damage. In addition to uses in the sorting of cells using pallet arrays, this method may find use when small regions of a larger structure must be detached, for example, the building of 3-D microstructures.

Chapter 3   Progress toward evaluating the effect of pallet array and laser parameters on cells adherent to released pallets

3.1   Abstract

To test the pallet system for sorting live cells, we aimed to identify pallet array and laser parameters most conducive to cell health after laser-based pallet release. To work towards examining the effect of pulse energy and pattern on the adherence of cells to laser-released pallets, I released pallets carrying either a more delicate cell line (3T3 cells) or a hardier cell line (HeLa cells). Targets were released either by aiming a single pulse at the pallet center or a single pulse at a pallet corner. Two laser settings were used: one generating a lower (about threshold) energy; the other generating a higher pulse energy (about 1.5x threshold). For both cell types, all patterns and energies tested left cells adherent to released pallets. The results of this study implicate collection of released pallets, rather than laser based pallet release, as the step that most challenges cell health in live cell sorting by laser releasable micropallets.

3.2   Introduction

3.2.1 *Relevance of quantitative assessment of sorting by pallet array effect on cells*

Arrays of laser-releasable micropallets have been employed to sort live, proliferating cells, addressing a prevalent need in biomedical research.[24, 27, 59, 60] To aid selection of optimal variables for laser-based release of living cells, an investigation was conducted examining the dependence of release threshold on array and laser parameters.[25] Further progress towards the aim of optimizing health of cells on laser-released pallets will quantitatively assess the effect of pallet dimensions and laser parameters on adhesion of cells to released pallets, viability of cells and cell's ability to proliferate after isolation by the pallet array system.

It is expected that optimum working conditions correspond to laser pulse energies only slightly higher than the pallet release threshold, where the kinetic energy of released pallets will be low enough to allow them to travel gently through the culture medium. However, releasing with higher energies may increase the possibility that released pallets can be easily separated from the array. It will be valuable to know how cells tolerate energies several times threshold.

Using a train of pulses to release a pallet, with each pulse disrupting a portion of the bond between the pallet and its glass substrate, is one strategy for lowering the pulse energies used to release pallets. The question of whether the cumulative energy or the energy per pulse has a greater effect on cell health has yet to be quantitatively addressed.

Laser pulses focused at the corner of a pallet induces rotational movement as the pallet travels through the culture medium. Vogel et al. found rotational movement reduces shear stress and increased recultivation rate of laser catapulted live-cell

specimens.[20] Aiming pulses at corners of pallets rather than at center allows maximum physical separation between cells and the location of laser-polymer interaction. This provides for better protection of cells from any peripheral laser effects associated with pallet release. However, rotational movement adds centrifugal forces that increase proportional to r the distance from the axis of rotation.

Larger, taller pallets are expected to provide better protection to cells from a given pulse energy. Cells may remain adherent more easily to larger released pallets if increased pallet size slows pallet travel after laser release. Or, cells may adhere better to smaller pallets after release if the lower pulse energy required for release of smaller pallets corresponds to less forceful lift-off.

Ultimately criteria for successful sorting of live cells are the fraction of cells on released pallets that can be recovered or collected, the percentage of cells remaining viable after release and collection, and the recultivation rate. Cell adhesion, viability and proliferative ability must be measured as a function of pallet size and height, pulse number, energy and pattern used to release pallets. This information will enable strategic choice of the laser and array conditions most favorable for cell viability and minimize the side effects experienced by cells on laser-released micropallets.

The following chapter describes a quantitative study of the effect of laser and array parameters on the health of cells isolated using micropallet arrays. Considering the fact that the study has not been completed in total, this requires in some part a theoretical discussion.

3.2.2 The experimental system

Microfabrication. I used arrays of square pallets with different dimensions (50, 100, and 200 μm squares 50 μm tall, and 100 μm squares 75 or 100 μm tall). These pallets were fabricated using SU-8 photoresist spin coated on glass slides. The coated slides were baked on a hot plate to remove solvent and exposed to UV light transmitted through an iron oxide photomask with the desired pallet features. The pallets were finally baked again then developed in SU-8 developer. The precise process of pallet fabrication is described in Chapter 5.

Optical geometry for plasma formation. Light from a pulsed Nd:YAG laser (New Wave Research ACL-1, Fremont, CA, 532 nm, 5 ns pulse width) was steered into the rear port of an inverted microscope (Nikon TE 300, Melville, NY). Beam intensity distribution on sample was TEM00 Gaussian. Beam size diameter at the focal point was approximately 1 μm. Laser-based pallet release, imaging on the pallet release system, and measurement of pulse energy is described in detail in Chapter 5.

Silanization of pallet arrays. After fabrication, the pallet array was treated to form a hydrophobic perfluoroalkylsilane layer on the silicone oxide surface in a low-pressure reactor.[67, 68] This step enables the array to retain a continuous air bubble (virtual wall) between the pallets, which prevents access of solutions or cells in the region between the pallets.[68] The array is stored in a vacuum desiccator until use.

Surface coating of pallets for cell culture. After silanization, a chamber is constructed using poly(dimethylsiloxane) (PDMS) to attach a PDMS ring to the pallet array. The top surfaces of pallets are then modified using fibronectin to enhance cell adhesion. For coating of the pallet top surface with fibronectin, a 1 mg/ml stock

solution of fibronectin was diluted 40 times with PBS to yield 25 µg/mL fibronectin in PBS. Fibronectin (0.8 ml, 25 µg/mL in PBS) was added to the chamber and incubated at room temperature for 16 h in the presence of the virtual walls. Arrays were washed with media prior to plating of cells.

Cell culture on pallet arrays. 3T3 and HeLa cells were grown on arrays at 37°C in a humidified 5% CO_2 atmosphere in DMEM supplemented with FBS (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. Cells (about one cell for each pallet on the array) were plated in a volume of 1 mL on each array.

3.3 Analysis of adhesion of cells on released pallets as a function of laser pulses and pulse energy

3.3.1 Cell adhesion as a function of pattern of laser pulses aimed at pallets

To determine the number of pallets with single cells that would retain their cell after release by a single pulse aimed at the pallet center, ten pallets carrying a single cell each were targeted for release by aiming a pulse at pallet center. Laser setting was chosen to generate threshold energy for this pallet size (about 2.6 µJ). The pulse energy used to release each pallet was recorded, and each released pallet was observed to verify whether it had retained its cell. Experiments were performed in triplicate.

To determine the number of pallets with single cells that would retain their cell after release by a single pulse aimed at a pallet corner, ten pallets with single cells were targeted for release by aiming a single pulse at one corner, with laser set to

generate pulses in the threshold energy range for that size pallet. The pulse energy used to release each pallet was recorded. Each released pallet was observed and note was made of whether its cell remained adherent. Experiments were performed in triplicate.

For both HeLa and 3T3 cell types, both conditions generated 100% cell adhesion to laser-released pallets.

3.3.2   Adhesion of cells to released pallets as a function of pulse energy

To assess the effect of pulse energy on the number of pallets with single cells that would retain their cell after release by a single pulse aimed at pallet center, ten pallets with single cells were targeted for release by aiming a single pulse at the pallet center, with laser set to generate pulses at about 1.5 x the threshold energy range for that size pallet. The pulse energy used to release each pallet was recorded. Each released pallet was observed and note was made of whether its cell remained adherent. Experiments were performed in triplicate.

To assess the effect of pulse energy on the number of pallets with single cells that would retain their cell after release by a single pulse aimed at a pallet corner, ten pallets with single cells were targeted for release by aiming a single pulse at one corner, with laser set to generate pulses at about 1.5 x the threshold energy range for that size pallet. The pulse energy used to release each pallet was recorded. Each released pallet was observed and note was made of whether its cell remained adherent. Experiments were performed in triplicate.

As in the case of pallet release with pulse energy set at about threshold, for both HeLa and 3T3 cell types, release using a single pulse of about 1.5 x threshold, aiming at either pallet center of a pallet corner, generated 100% cell adhesion to laser-released pallets.

3.3.3 Discussion

The completed work described in this chapter demonstrated 100% adhesion of cells to released pallets whether pulses were aimed at pallet center or a pallet corner, independent of pulse energy in a range between one and 1.5 x threshold, for both HeLa and 3T3 cell types. This result implies that the collection and recultivation step, rather than the release step, is the part of sorting live cells with micropallet arrays that presents the greatest challenge to health of sorted cells. This agrees with our group's observation in previous studies, where the processes of collection and culture after collection appeared to present the greatest health challenge to cells adherent to released and collected pallets.[60]

To complete checking and quantification of this observation, the health of cells on pallets after release but before collection can be further characterized using CellTrace calcein probes will be used as markers for viability assays. Other tests of cell health might also be used, for instance, in order to determine the amount of damaging heat or shear stress the cells experience during release, I could perform immunocytochemical studies utilizing antibodies specific to heat shock proteins 60 and 70 (anti-HSP60/HSP70). To examine the ability of the released cell to proliferate before it is subject to the challenge of collection, we could release pallets on a microscope with a stage incubator then monitor proliferation of the cell on the

released pallet on its array for several days. Pulse energies in the completed study remained in a limited range, since pallets released with energies much above threshold fly out of the field of view. A greater range of pulse energies could be tested if the pallets were labeled or we were otherwise able to track pallets that leave the field of view after release.

When completed, the results of this study should allow a better understanding of the process of laser-based release of cells on micropallets, informing strategic selection of parameters for isolation of cells with optimal viability and proliferative ability.

Chapter 4   A low-fluorescence alternative photoresist, poly(ethylene glycol) walls, and mechanistic examination of pallet release

4.1   Abstract

The negative photoresist SU-8 has a number of properties that make it attractive as a structural material in microfabrication of arrays of pallets for use in the pallet array system, but some features of SU-8 limit its application. Generating a continuous region of air between pallets, referred to as virtual air walls, successfully constrains cells to growing on the tops of pallets. However, the technique has its limitations. Release of individual micropallets from arrays using highly focused laser pulses was demonstrated for the efficient separation, collection, and expansion of single, adherent cells from heterogeneous cell populations. The mechanism producing pallet detachment was not initially examined. A negative photoresist, 1002F, was developed with decreased fluorescence and enhanced cell adhesive properties as compared with SU-8, broadening the use of pallet arrays. This work was published in the journal *Analytical Chemistry*, JH Pai et al., 2007. Relative to virtual air walls, walls of poly(ethylene glycol) between pallets offer the advantage of long-term stability in low-surface tension solutions and with any pallet size, height,

and spacing. This work was published in the journal *Lab on a Chip*, Y Wang et al., 2008. Visualizing the dynamics of pallet release using fast frame photography enables a mechanistic understanding of the pallet release process informs the strategic choice of parameters to refine laser release and minimize the exposure of cells to physiologic stresses during the sort. This work was published in the journal *Analytical Chemistry*, PA Quinto-Su et al., 2008.

4.2 Introduction

SU-8 photoresist has many assets, but significant weaknesses when used as a structural material for bioanalytical applications. Fluorescence detection in the green is possible, but at greatly reduced sensitivity due to SU-8's high background fluorescence at these wavelengths. A second weakness of the SU-8 pallets is that they must be coated with adsorbed layers or covalently attached molecules before most cells will attach and grow on the pallet surface[59]. Pai et al. developed a low-fluorescence alternative to SU-8 photoresist.[26] The fabrication process for the new 1002F photoresist was optimized. Since the ultimate goal for the new photoresist was as a structural material for pallet arrays for cell sorting, I measured the energy required to release 1002F pallets from an array using a focused, pulsed laser. Studies demonstrated the utility of the new 1002F photoresist for the fabrication of arrays of pallets for use in the pallet array system.

The use of pallet arrays would also be broadened by using alternative materials for the pallets. 1002F pallets were observed to require higher laser pulse energies for release. I found release thresholds for 50 micron squares composed of SU-8 where

they contact glass, so have the lower release energies of SU-8 pallets. But, these 'hybrid pallets' are mostly composed of 1002F, so they have the low fluorescence of 1002F and are better than bare SU-8 for cell adhesion.

To culture cells on pallet arrays, cells are initially placed in suspension, but are allowed to settle and grow on individual pallets prior to analysis. In past work, placement of cells only on the pallets has been accomplished by generating a continuous region of air between the pallets referred to as a virtual air wall.[34, 60] The ability to exclude cells from the region between the pallets is necessary to establish a cell-based array using pallets.[34] The virtual air wall works well in this regard, but has limitations. To overcome these limitations, a solid barrier that serves the same function as the air wall would be of great value. In the work Wang et al. published in *Lab on a Chip*, vol. 8, pp. 734–740 (2008), poly(ethylene glycol) (PEG) was selectively polymerized on the micropallet array within the inter-pallet space. I measured thresholds for releasing pallets from arrays with PEG walls using a single, focused laser pulses. Pallet arrays with PEG walls may have a wider range of applications than that of virtual walls.

Optical breakdown produced by a focused, pulsed laser has been studied in liquids where the cooling of the plasma results in cavitation bubble formation, expansion, and collapse.[69] The volumetric expansion of the plasma results in the emission of a shock wave and bubble formation that provide a potential mechanism for pallet release. However, the precise sequence of events that begins with pulsed laser microbeam irradiation of a SU-8 polymer micropallet and produces pallet detachment was not known and had not been examined mechanistically. The

objectives of the work published in *Analytical Chemistry*, Vol. 80, No. 12, 4675 – 4679 were to visualize the dynamics of this process and determine the underlying mechanisms of pallet detachment. I fabricated micropallet arrays and participated in initial laser-release experiments imaged by fast-frame photography. A mechanistic understanding of the pallet release and its interplay with laser parameters can inform strategies to refine and optimize the release process and minimize cellular damage.

4.3 Results and discussion

A key aspect of the SU-8 pallet arrays is that individual pallets can be released by a single focused pulse from a Nd:YAG laser (5 ns, 532 nm, 2 µJ). On-demand, single-pallet release permits the collection of pallets possessing a desired cell and, consequently, the separation of that cell from the mixture of cells on the array. Thus, for 1002F to be successful as a substrate for a pallet array, the 1002F pallets must be readily releasable using low pulse energies. I participated in performing comparison of energies required to release 1002F and SU-8 pallets, measuring threshold energies for pallet release of identically sized pallets composed of either SU-8 or 1002F (Figure 15). Although the energy for 1002F pallet release was greater than that for SU-8 pallets, successful release and collection of living cells on SU-8 pallets has been performed with laser release energies up to 10 µJ.[59] Thus, the 1002F pallets should be suitable for sorting many different types of cells. There are several possible reasons for higher energies required to detach the 1002F pallets from glass. For one, 1002F is a softer, more flexible material than SU-8, therefore may absorb more mechanical energy before the contact with the glass is disrupted. Additionally, the

lower absorbance of 1002F near 532 nm may result in the conversion of a smaller

fraction of the laser light into mechanical release energy.
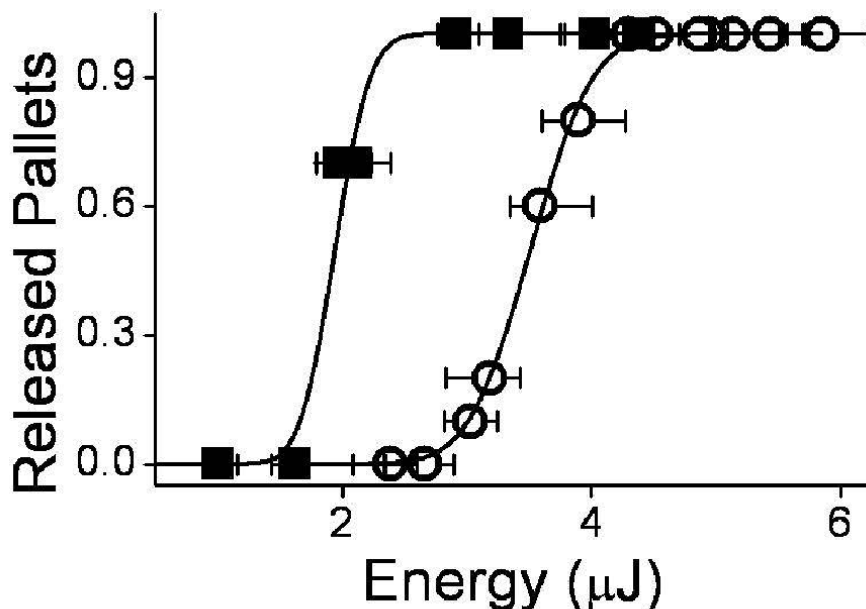
Figure 15. Measurement of the threshold energy for 1002F and SU-8 pallet release. Shown on the y axis is the fraction of pallets released at each energy tested. Ten pallets were released for each data point. The energy of each pulse sent from the laser was measured. The x coordinate represents the average energy at a given laser setting, and the error bars represent the standard deviation. The dimensions of the pallets were 50 μm (s) and 100 μm (h). The distance between each pallet was 50 μm. Figure reproduced with permission from the American Chemical Society.

Hybrid pallets are composed mostly of 1002F, but of SU-8 where they contact

glass were found to have the lower release energies of SU-8 pallets (Table 2). The

thresholds I found here are for 50 micron squares. Pallets fabricated at different times

may have different threshold release energies since adhesiveness of SU-8 or 1002F to

glass depends on many variables. Since these variables can be difficult to control

precisely during manual manufacture of arrays, array-to-array variability in the SU-

8:glass adhesion and therefore pallet release thresholds may occur. This variation has been identified as $1 - 2\ \mu J$ for this size pallet.

Table 2 – Threshold energy (μJ) for release of SU-8, 1002F and hybrid pallets

Array material	Threshold (μJ)
SU-8	2.3
1002F	5.1
2 μm hybrid	2.1
5 μm hybrid	1.6

The hydrated PEG walls surrounding each pallet might provide a frictional force opposing the release of the pallet. This force could be of sufficient magnitude to prevent pallet release or require higher laser energies that are detrimental to cell viability. To compare the energy required to release pallets in the presence or absence of PEG walls, I measured the probability of pallet release with respect to the laser pulse energy for arrays with and without PEG walls (Figure 16). The threshold release energy for pallets surrounded by water or PEG hydrogel was 2 and 6 μJ, respectively. Thus, the energy needed to release pallets surrounded by PEG was increased by 3-fold, likely as a result of frictional forces imparted by the PEG wall. The release threshold energy is dependent on the type of PEG monomer utilized to form the walls. I could not release pallets on arrays with walls formed from PEG dimethacrylate (MW 750) with energies as high as 20 μJ, the highest energy tested. Since SU-8 is hydrophobic, the additional methyl groups on the monomer may have enhanced the hydrophobic interactions between SU-8 and gel. In addition, the release threshold energy depended on the water content of the initial monomer solution. When the monomer solution possessed no added water, pallets on the arrays could not be released after immersion of the array in water. This was most likely due to the

swelling of the PEG wall after immersion in water which held the pallets tightly in place. It is possible that optimization of the chemical properties of the monomer as well as the water content of the PEG hydrogel could further lower the threshold energy for pallet release.
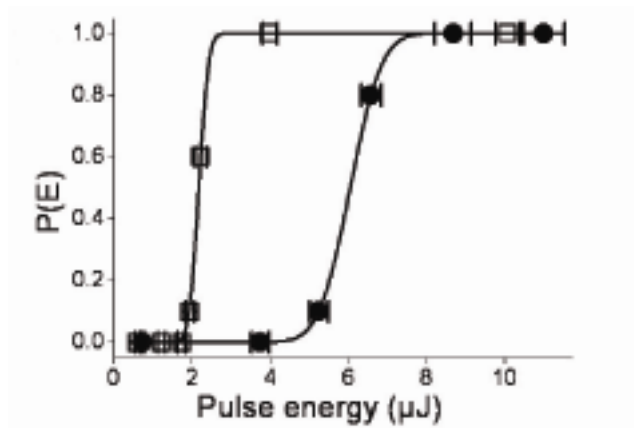
Figure 16. Laser-based release of SU-8 pallets from arrays with and without PEG walls. The probability of pallet release (P(E)) is plotted against the laser pulse energy for arrays with (solid circles) and without (open circles) PEG walls. P(E) is defined as the probability of the pallet becoming dislodged by a single focused pulse of energy E. Ten pallets were released at each pulse energy. Since the pulse to pulse energy was slightly variable, the energy of each pulse was measured and the average pulse energy with teh standard deviation (error bar) was plotted. The lines are the best fits of the data to a Gaussian error function. The threshold energy was defined as the pulse energy required to release 50% of the targeted pallets. The dimensions of the pallets were 70 μm (s) and 30 μm (h). The distance between pallets was 30 μm. Figure printed with permission from the Royal Chemical Society.

4.4 Conclusions

Pai et al. characterized the use of the new photoresist 1002F and demonstrated its applicability to the fabrication of arrays of pallets. Preliminary results, including low laser energies needed to detach 1002F pallets from a glass substrate suggest that 1002F is an excellent pallet substrate for the pallet array system.[26] PEG hydrogel walls formed microwells with releasable bases in studies performed by Wang et al.[27] The PEG walls offer a number of advantages compared to the virtual air walls,

though pallets with PEG walls do require higher laser energies for pallet release relative to that with air walls. The work of Quinto-Su et al. examined the release of optically transparent SU-8 polymer micropallets using a pulsed laser.[28] Time resolved photography of the pallet release process confirmed that laser microbeam-induced plasma formation is a prerequisite for pallet release. The mechanism and dynamics of pallet release do not significantly damage the sample; the thick SU-8 polymer pallets also provide substrate rigidity and attenuate any possible thermal effects produced by the laser-polymer interaction.

Chapter 5
Experimental Methods

5.1 Materials

The SU-8 10, SU-8 50 and SU-8 100 photoresists and SU-8 developer were purchased from MicroChem Corp. (Newton, MA). Pre-cleaned glass slides (75 x 25 x 1 mm^3) were purchased from Corning Glass Works (Corning, NY). (Heptadecafluoro-1,1,2,2-tetrahydrodecyl)trichlorosilane was from Gelest Inc. (Morrisville, PA). Dulbecco's Modified Eagle Medium, fetal bovine serum (FBS), penicillin/streptomycin and calcein red-orange AM were obtained from Invitrogen (Carlsbad, CA). L-Glutamine was obtained from Sigma-Aldrich (St. Louis, MO). Fibronectin extracted and purified from human plasma was purchased from Chemicon International, Inc. (Temecula, CA). Collagen I from rat tail tendon was purchased from BD Biosciences (San Jose, CA). Calcein Red-Orange AM and Oregon Green diacetate were obtained from Invitrogen (Carlsbad, CA). Silicone O-rings (24-mm outer diameter) were purchased from McMaster-Carr (Los Angeles, CA). Before use, the silicon O-rings were washed in distilled water for 24 hours, rinsed with ethanol, and then dried in a 50°C oven. The Sylgard 184 silicone elastomer kit was purchased from Dow Corning (Midland MI). RBL cells, 3T3 cells

and HeLa cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). All other reagents were from Fisher Scientific (Pittsburgh, PA).

5.2   Microfabrication of SU-8 pallet arrays

Substrate pretreatment

MicroChem datasheets recommend that substrates should be cleaned and dried prior to applying SU-8 resist for maximum process reliability. Previously, substrates were cleaned with a piranha wet etch. Glass slides were cleaned by immersing them in freshly prepared piranha solution (3:1 concentrated H_2SO_4/30% H_2O_2 by volume) for 30 minutes. The slides were then rinsed with deionized water and dried with a nitrogen stream. The slides were dehydrated on a 200 °C hot plate for at least 5 minutes before use.[27, 60] Alternatively, glass slides were cleaned by storing them in sulfuric acid (H_2SO_4) for a minimum of a month. The slides were then rinsed with deionized water and dried in a nitrogen stream. The slides were dehydrated in a 180°C oven for 5 minutes before use.[25] More recently, glass substrates were cleaned with an acetone wash, then rinsed with ethanol and dried in a nitrogen stream.

Coat

Programs for spin-coating SU-8 on a substrate depend on desired thickness and on the viscosity of the type of SU-8 resist being coated. For example, the viscosity of SU-8 photoresist formulation 50 (SU-8 50) is optimized to generate film thicknesses between 40 and 100 μm. Two steps are recommended for the spin coat. The first, the spread cycle, allows the resist covers the entire surface. The second step, the spin cycle, achieves the desired film thickness. Recommended spin speeds to produce selected SU-8 resists and film thicknesses are displayed in Table 3.

Table 3 Thickness vs. spin speed for select SU-8 resists. For all spin programs, the first step was a spread cycle using an acceleration of 100 rpm/s, holding a speed of 500 rpm for 10 seconds. The final spin speed given in the table is for the spin cycle that determines thickness of coated film. For each program, spin cycle used an acceleration of 300 rpm/s.

Resist name	Thickness (μm)	Final spin speed (rpm)
SU-8 10	25	1200
	30	1000
SU-8 50	50	2100
	75	1600

The Allbritton group used a WS-400B-6NPP/LITE spin coater (Laurell Technologies Corporation, North Wales, Pennsylvania). To prepare for spin coating, a chemically resistant oil-free pump was attached and turned on and compressed nitrogen was opened to 60 – 70 psi pressure.

Before the spin was started, the spin coater was set to desired program. The spread cycle was usually 500 rpm for 10 seconds with acceleration 100 rpm/second (Acl 1 on the WS-400B-6NPP), as recommended by the MicroChem datasheet.

The acceleration for the spin cycle, 300 rpm/second (Acl 4 on the WS-400B-6NPP), and duration of final spin speed (30 seconds) were chosen from the MicroChem datasheet. The final speed of the spin cycle depends on the desired film thickness and was chosen based on the manufacturer datasheet guidelines and experience. SU-8 films of 25 μm and 30 μm thicknesses were obtained by spin coating the SU-8 10 resist with final spin speed of 1200 rpm and 1000 rpm, respectively.[24, 25, 27] SU-8 films of 50-μm thickness and 75-μm thickness were obtained with SU-8 50 coated with a final spin speed of 2000 rpm and 1600 rpm, respectively.[25, 60]

When the spin coater was ready and the program set, the substrate was placed on stage in the middle of the spin-coater and held in place by vacuum. Approximately 2-

3 mL of resist was dispensed to the center of the substrate. Then the spin coater run was started to execute the selected program.

Softbake

The soft bake following spin coating evaporates solvent and increases the density of the SU-8 film. Better adhesion, reduced edge bead and better coating are obtained by more controlled rate of solvent evaporation made possible by a lower initial bake temperature.[70]

The Allbritton group has used a level hot plate with good thermal control and uniformity to bake SU-8, although convection ovens may be used. Bake times have been subject to adjustment as solvent evaporation rate is influenced by rate of heat transfer and ventilation, which may be different in different facilities and changing environmental conditions.

Recommended softbake times for SU-8 10 and SU-8 50 films of selected thicknesses are shown in Table 4. The coated slides were baked on a hotplate at 65°C for a first bake, followed by a second bake at 95°C to remove organic solvent. After baking, the slides were slowly cooled to room temperature.

Table 4 – **Recommended softbake parameters for SU-8 10 and SU-8 50 films of selected thicknesses.**

Resist name	Thickness (µm)	Prebake @ 65°C (min.)	Softbake @ 95°C (min.)
SU-8 10	25	3	5
	30	3	7
SU-8 50	50	6	20
	75	9	25

Expose

To prepare SU-8 pallets, the SU-8 film was exposed to UV light through iron oxide photomasks, fabricated according to traditional microfabrication processes,

with various micropatterns of designed features. The Allbritton group used a 500-W Oriel Flood Exposure Source (Newport Stratford, Inc., Stratford, Connecticut), a broad spectral output source used with filters to remove excessive energy. For exposure, the sample was placed resist-side up on a black cloth. The black cloth prevented reflection of light from below the sample. The mask was placed metal side down on top of the sample, and then pressed lightly to ensure full contact.

For a given setting, the exposure source generated light at a particular power. The Allbritton group used settings that generated powers in the range $6.8 - 14.9$ mW/ cm^2. The exposure power in mW/cm^2 is equal to the exposure energy in mJ/cm^2 divided by the exposure time in seconds. The MicroChem datasheets[70] give a chart of recommended exposure energy (in mJ/cm^2) vs. film thickness. Thicker films require higher dosage. Exposure required depends on the pattern too – more time (higher UV exposure dose) is needed for smaller features in pattern; less time (lower UV exposure dose) is needed for smaller spacing between features and for larger features in the pattern (to decrease chance of overexposure in smaller spaces or through larger windows in mask). Exposure time was adjusted to deliver total exposure energy appropriate to the film thickness and mask features.

Table 5 gives exposure energy dose for select film thicknesses. These values are examples that have worked in the past. But, the fabrication process is sensitive to environmental conditions and UV exposure dose is one of the most influential in the process. Therefore, it is important to examine the quality of pallet arrays each time arrays are fabricated. If a sample is much overexposed, an image of the mask will be visible in the resist immediately after exposure. A lower amount of overexposure will

cause pallets to appear 'T-topped,' or rounded and beginning to touch at the tops. Insufficient cross-linking may lead to catastrophic adhesion failure, or large portions of the array's pallets coming off their substrate during fabrication. The problem of insufficient cross-linking may be resolved by increasing exposure dose or increasing postexposure bake (PEB) time.[70]

Table 5 – Recommended exposure dose for select SU-8 film thicknesses. A figure in the MicroChem datasheet shows one curve representing the maximum recommended dose and another representing the minimum recommended dose for SU-8 films between 0 and 250 μm thick. The values in this table, taken from the middle of the exposure dose range recommended by MicroChem, represent a starting point for determining optimal UV exposure dose.

Resist name	Thickness (μm)	Exposure dose (mJ/cm^2)
SU-8 10	25	200
	30	230
SU-8 50	50	350
	75	400

Postexposure bake

After samples were exposed, they were subjected to a 2-step post exposure bake to selectively cross-link the exposed portions of the film. Gradual, two-step heating and slow cooling after minimize resist cracking and other problems resulting from stressing the film with excessively quick cross-linking.

Optimum cross-link density is obtained through careful adjustments of the exposure and PEB conditions, but amount of UV exposure is the more sensitive parameter. If the exposed samples are given too long, several hours, PEB, the photoacid will spread and parts of the film not exposed will crosslink. But change within a few minutes shouldn't make much of a difference.

The Allbritton group performed PEB using hot plates; a convection oven can also be used. The first PEB step was at 65°C, the second step was at 95°C. A faint image of the mask should be visible on the resist by the time the second PEB step begins. The absence of an image during PEB is a sign cross-linking is below optimal. In this case, exposure or PEB time or both should be increased [70] Recommended times for PEB are shown in Table 6. At the end of baking, cooling was allowed to happen slowly, minimizing generation of internal stresses that could crack microstructures. We avoided putting a hot sample on a cool surface.

Table 6 – Recommended post exposure bake (PEB) parameters.

Resist name	Thickness (μm)	PEB 1 @ 65°C (min.)	PEB 2 @ 95°C (min.)
SU-8 10	25	1	2.5
	30	1	3
SU-8 50	50	2	5
	75	2	9

Develop

After slowly cooling to room temperature, the SU-8 samples were developed in SU-8 developer. To improve effectiveness of development, Y. Wang implemented the strategy of using two Petri dishes with developer. The surface of the sample was scratched to make certain the sample is placed resist-side up in the developer. The sample sat in one Petri dish of developer on a rotator for several minutes then was moved to the other dish of developer for several more minutes. Table 7 shows recommended development times for selected SU-8 film thicknesses.

Table 7 – Recommended development processes.

Resist name	Thickness (μm)	Development (min.)	
		Dish 1	Dish 2
SU-8 10	25	1	2.5
	30	2	3

| SU-8 50 | 50 | 5 | 2.5 |
| | 75 | 6 | 3.5 |

Rinse and dry

At the end of developing, the sample was removed from developer using tweezers. Avoiding direct contact between liquid stream and microstructures, the array was rinsed with ethanol or isopropanol. The sample was dried using a stream from a compressed nitrogen tank.

After development, rinse and dry, pallet arrays checked for quality under a microscope. Height of fabricated pallets was verified by quick release (by scraping or laser) to image pallets lying sideways. In some cases,[25] after inspection, arrays were hardbaked (cured) at 150°C for 1 hour.

5.3 Optical Geometry for laser-based pallet release

Light from a pulsed Nd:YAG laser (New Wave Research ACL-1, Fremont, CA, 532 nm, 5 ns pulse width) was steered into a beam expander, then directed through an iris to yield a beam diameter of 6 mm (Figure 7). The light then passed through a lens (150 mm focal length) into the rear port of an inverted microscope (Nikon TE 300, Melville, NY). Arrays were imaged using a CCD camera (CCD Camera Model KP-M1AN, Hitachi, Brisbane, CA or CoolSNAP[TM] fx, Photometrics, Portland, OR). Images were captured using MetaFluor (Universal Imaging, Downingtown, PA). An objective with a magnification of 20X (N.A. 0.5, Nikon Plan Fluor) was used to focus the laser beam in order to release pallets. Beam intensity distribution on sample was TEM00 Gaussian. Beam size diameter at the focal point was approximately 1 micron. A coverslip was placed into the path of the laser beam prior to the back

entrance of the microscope. The light from the coverslip was directed to an energy meter (J4-09 probe, Molectron EPM 1000, Santa Clara, CA) and used to measure the energy of each laser pulse.

5.4   Measurement of the Probability of SU-8 Structure Release

To release individual pallets, a pallet array was first placed on a microscope stage, and then the laser focus was set at the interface of the glass substrate and SU-8 pallet. 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. When no virtual walls were present, a solution of polystyrene beads (approximately 1 μm diameter) in water was placed over the array, and beads were allowed to settle on the top surface of the glass to facilitate accurate and consistent focusing at the interface. Laser energies were chosen so at least two energies resulted in 0% release of targeted pallets, at least two energies resulted in release of 100% of targeted pallets, and at least two energies yielded between 0% and 100% release of targets. For each laser energy 10 pallets were targeted with each pallet receiving only a single pulse.   For each laser energy, the fraction of pallets released (out of ten targeted) and the average energy of the ten pulses fired were determined.

For experiments using multiple pulses for release of a single pallet, the pallet array was first placed on the microscope stage and laser focus was set at the interface between SU-8 and glass, as for release with a single pulse. Multiple pulses fired manually at a frequency of 1 Hz were then used to release the structures as described in the text.

5.5 Fit of the Data to a Gaussian Error Function

To determine the threshold energy for pallet release, the pallet release frequency was plotted as a function of incident pulse energy calculated to reach the microscope stage. A Gaussian error function was fitted to this data. Fitting was performed using the nonlinear least squares fitting capability of the software Origin 7.5 SR6 (OriginLab Corporation, Northhampton, MA). The Gaussian error function was:

$$p(E_p) = 0.5\{1 + erf[P_1(E_p - P_2)]\}$$

$p(E_p)$ is the probability of pallet release at a laser energy E_p. The values P_1 and P_2 were the fitted parameters where P_1 determined the sharpness of the Gaussian error function and P_2 was the threshold energy, the pulse energy that resulted in a 50% probability of pallet release.

5.6 Surface Coatings for Virtual Air Walls

After fabrication, the pallet array was treated to form a hydrophobic perfluoroalkylsilane layer on the silicone oxide surface in a low-pressure reactor.[67, 68] This step enables the array to retain a continuous air bubble (virtual wall) between the pallets, which prevents access of solutions or cells in the region between the pallets.[34]

The array and a small dish containing 100 µL of (heptadecafluoro-1,1,2,2-tetrahydrodecyl)trichlorosilane were placed inside a 100-mm-internal diameter Wheaton dry-seal desiccator. The desiccator was then attached to an oil-free diaphragm vacuum pump (Vacubrand, Fisher Scientific) for 1-2 minutes (7 Torr). The desiccator was detached from the pump and then maintained under vacuum for

16 hours at room temperature. Afterward, the array was placed under a high vacuum
(2 x 10^{-3} Torr) for 2 hours to remove any unreacted silane molecules using a standard
oil vacuum pump (Fisherbrand, Fisher Scientific). Silanized arrays were stored in a
vacuum desiccator until use.

5.7   Surface Coating of Pallets for Cell Culture

After silanization, a chamber was constructed using poly(dimethylsiloxane)
(PDMS) to attach a silicon "O"-ring (24-mm outer diameter) or a PDMS ring to the
pallet array. A pallet array was immersed in 75% ethanol for sterilization, and then
rinsed with PBS buffer five times to remove the ethanol. The top surface of pallets
was then modified to enhance cell adhesion. When virtual walls were present on the
pallet array, only the top surface of each pallet came in contact with the collagen or
fibronectin.

Initially, a two-step procedure was used to coat collagen on the pallet top
surface.[24] 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 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 minute. The remaining acidic collagen solution formed a thin liquid
layer on each hydrophilic pallet surface. After drying in air for 15 minutes, 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.

For coating of the pallet top surface with fibronectin instead of collagen, fibronectin (25 µg/mL in PBS, 0.8 mL) was added to the chamber and incubated at room temperature for 16 hours in the presence of the virtual walls. During rinse steps, the liquid was not completely removed from the chamber to prevent fibronectin fibrils from forming and bridging between the pallets. Arrays were washed with media prior to plating of cells.

5.8   Cell culture

3T3, RBL and HeLa cells were grown on the array at 37°C in a humidified 5% CO2 atmosphere in DMEM supplemented with FBS (10%), and L-glutamine (584 mg/L). Penicillin (100 units/mL) and streptomycin (100 g/mL) were added to the media to inhibit bacterial growth.

After the pallet array was silanized and coated with fibronectin or collagen, a suspension of cells was added to the chamber over pallet array, with cell density calculated to yield about one cell per pallet on the array. Cells were allowed to settle.

5.9   Loading Cells with Oregon Green or Calcein Red-Orange

Cells cultured on the array were washed twice with PBS. Cells were incubated PBS with glucose (10 mM) plus 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).

5.10 Fabrication of a Multiwell Collection Plate

A PDMS multiwell plate for collecting the released pallets was fabricated by a two-step molding process. First, a 150-µm-thick, SU-8 mold was fabricated on a glass surface using a process similar to that used for the SU-8 pallet fabrication. The SU-8 mold was the first mold of two molds and contained square (1-mm-long sides) or round (1 mm in diameter) wells with a 150-µm depth and a 250-µm gap between each well. The microwells were numerically labeled with numbers and alphabets adjacent to each well for identification. These numbers and alphabets were shallow notches of 25 µm in width created using a process similar to that used for pallet encoding.[59]. To make a nonstick surface, the mold was treated with vapor-phase (heptadecafluoro-1,1,2,2-tetrahydrodecyl)trichlorosilane for 16 hours as described above. Sylgard 184 elastomer mixture was then poured onto the SU-8 mold, degassed for 10 min, and cured on a 95 °C hot plate for 20 min. After cooling to room temperature, the PDMS was peeled off and used as the second mold. To make the PDMS surface nonsticky, the PDMS mold was oxidized in an oxygen plasma for 2 min (200 mTorr, 200 W) and baked in an 80 °C oven for 20 min. Sylgard 184 elastomer mixture was then poured on the PDMS mold and cured as described above. The final PDMS multiwell plate contained microwell structures identical to those of the original SU-8 mold.

5.11 Cell collection after pallet release

An early method of collecting released pallets involved using flow-based transfer of the pallets into an overlying micropipet and plastic tubing using 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.

A more recent strategy for collecting and tracking released pallets and their cells involved using labeled microwells. Using labeled microwells for collection allowed improved sterility during collection, more efficient pallet collection, better preservation of cells' viability and ability to grow into clonal colonies after collection. To collect released cell/pallets in microwells, a plate of labeled microwells 1-mm across, 150 μm deep and separated by walls 0.25 mm, was fabricated by casting PDMS against SU-8 molds as described above. The microwell plate was circular with a diameter of 17 mm and was designed to mate with the chamber containing the pallet array to form a water-tight seal. After fabrication, a chamber was constructed on the multiwall plate by attaching a silicon O-ring (24-mm outer diameter) to the surface of the plate using PDMS. Prior to usage the PDMS microwell plate was sterilized by autoclaving and then coated with fibronectin (25 μg/mL in PBS) for 6 hours at room temperature. Before pallet release the PDMS microwell plate was sealed to the pallet array using a sterile gasket. During pallet selection and release, the interior of the unit remained sterile. After pallet release, the microwell plate-pallet array unit was inverted so that the pallets and aqueous solution settled into the microwell plate by gravity.

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