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Fabrication and biological evaluation of uniform extracellular matrix coatings on discontinuous photolithography generated micropallet arrays

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ABSTRACT/SYNOPSIS

The recent identification of rare cell populations within tissues that are associated with specific biological behaviors, e.g., progenitor cells, has illuminated a limitation of current technologies to study such adherent cells directly from primary tissues. The micropallet array is a recently developed technology designed to address this limitation by virtue of its capacity to isolate and recover single adherent cells on individual micropallets. The capacity to apply this technology to primary tissues and cells with restricted growth characteristics, particularly adhesion requirements, is critically dependent upon the capacity to generate functional extracellular matrix (ECM) coatings. The discontinuous nature of the micropallet array surface provides specific constraints on the processes for generating the desired ECM coatings that are necessary to achieve the full functional capacity of the micropallet array. We have developed strategies, reported herein, to generate functional coatings with various ECM protein components: fibronectin, EHS tumor basement membrane extract, collagen, and laminin-5; confirmed by evaluation for rapid cellular adherence of four dissimilar cell types: fibroblast, breast epithelial, pancreatic epithelial, and myeloma. These findings are important for the dissemination and expanded use of micropallet arrays and similar microtechnologies requiring the integrated use of ECM protein coatings to promote cellular adherence.

Keywords

bioMEMS; micropallet array; extracellular matrix; discontinuous surface; adherent cells

1. INTRODUCTION

A driving force for the development of methodologies for the investigation of rare adherent cell populations is the increasing appreciation of cellular heterogeneity at the tissue level, including the recognition of mesenchymal stem cell and other progenitor cell compartments. The relative proportion of specific cellular subsets has been associated with specific biological behaviors¹⁻¹⁴ suggesting that the study of rare cellular subsets within tissues may provide additional capacity over genomic signatures to direct individualized therapeutic strategies, so called “individualized” or “personalized” medicine^{2-4,15}. Ideally, these studies would be conducted on primary tissue samples at the single cell level to potentially enable molecular and genomic analysis of individual cells. Primary tissues contain various broad cellular compartments including: stromal (e.g., fibroblasts), glandular or lining structures (e.g., epithelial and endothelial cells), along with cells of the hematologic and immune systems (e.g., macrophages and lymphocytes). Although technologies for the study of rare cell populations of hematopoietic and lymphoid cells has existed for some time¹⁶, the currently available technologies for selection and isolation of single adherent cells from large heterogeneous populations have significant drawbacks, including requirements for large tissue samples and poor viability of recovered cells.

Micropallet arrays are a recently developed technology designed to allow the identification, selection, and recovery of a single adherent cell within a given cell population, Fig. 1(a),¹⁷⁻²³. Micropallet arrays consist of tens of thousands of microscale pedestals, or “micropallets”, arrayed on a glass microscope slide and each designed to hold a single adherent cell. The micropallets are made from a transparent polymeric material that can be patterned using photolithographic microfabrication techniques. The cells are confined to the tops of the micropallets by chemical modification of the array such that their surfaces, i.e., the micropallet top surfaces, sidewalls, and underlying glass substrate, are highly hydrophobic. When an aqueous solution is applied to the arrays, air is trapped in the voids between micropallets creating an air network of “virtual walls” that sequesters cells to the top surfaces of individual micropallets, Fig. 1(b). The proof of concept for this technology was established by the use of particularly robust adherent cell lines with little or no specific requirements for optimal adherence, HeLa & RBL^{18,19,22}. Prior tissue culture experience and knowledge of fundamental biology suggests that the successful application of micropallet array technology to the examination of primary adherent and semi-adherent cells is likely to be critically dependent on the ability to effectively coat micropallet arrays with extracellular matrix (ECM) components. Extracellular matrix components that are commonly used for growth of adherent cells are generally proteins or glycoproteins capable of adhering to standard tissue culture surfaces and/or glass. The unique physical nature of micropallet arrays places additional constraints on the use of ECM components in order to maintain the full functional capacity of the micropallet array. Such considerations are not necessary when utilizing ECM proteins coatings for cell culture on traditional *ex vivo* culture technologies that employ flat, continuous growth surfaces.

The functionality of an ECM coating on micropallet arrays is determined by three critical parameters: the uniformity and stability of adherence of the ECM coatings on individual micropallets, restriction of the ECM to the top micropallet surfaces, and the affinity for the ECM proteins of the specific cell type being investigated. The growth surface of a micropallet array is in reality a vast number of uniformly spaced microscale growth surfaces, arrayed to present a pseudo-solid surface at the macroscale, but very non-uniform and discontinuous at the micro and cellular scale. This drives the need to develop specialized coating protocols to achieve proper and efficacious application of ECM components to the micropallet arrays. We report studies to define conditions and methodologies to generate

functional ECM coatings of various types (fibronectin, EHS basement membrane extract (BME), collagen, and laminin-5) on micropallet arrays and the effectiveness of these coatings in capturing cells from different cell lines representing the range of cellular compartments, noted above, that constitute specific tissues.

2. MATERIALS & METHODS

2.1 Cell Lines

The following cells were used for these studies: NIH/3T3, a murine fibroblast cell line; SK-BR-3, a human breast adenocarcinoma epithelial cell line; AsPC-1, a human pancreatic adenocarcinoma epithelial cell line (CRL-1658, HTB-30, CRL-1682, ATCC, Manassas, VA, respectively); and KMS-18, a human multiple myeloma cell line (established at Kawasaki Medical School, Kurashiki, Okayama, Japan)²⁴. These cells were selected to be representative of the three broad categories of cells residing in tissues: stromal (fibroblast), glandular or lining (epithelial), and hematologic or immunologic (multiple myeloma – a transformed B lymphocyte).

2.2 Extracellular Matrix Coating Reagents

Extracellular matrix components included: human plasma fibronectin and laminin-5 (Millipore, Billerica, MA), human type 1 collagen and EHS tumor basement membrane extract (BME) (Trevigen, Gaithersburg, MD), along with reagents to facilitate adherence to the micropallets: Poly-L-lysine (Trevigen, Gaithersburg, MD) for collagen and laminin-5, Sulfo-SANPAH (Thermo Scientific, Rockford, IL) for BME. Visualization of ECM coatings was performed using anti-fibronectin rabbit polyclonal antibody (Cat # F3648,); anti-collagen, type 1, mouse monoclonal antibody, (clone COL-1); and biotinylated concanavalin-A (all from Sigma-Aldrich, St. Louis, MO). Colorimetric detection was obtained using appropriate secondary reagents conjugated to horseradish peroxidase; goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch, West Grove, PA) or anti-mouse IgG (BD Biosciences Pharmingen, San Jose, CA) or Neutravidin (Thermo Scientific, Rockford, IL), and reacted with TrueBlue peroxidase substrate (KPL, Gaithersburg, Maryland) according to manufacturer's instructions.

2.3 Production of Micropallet Arrays

Micropallet arrays were fabricated from 1002F photoresist, which is similar to the high aspect ratio negative resist SU-8, but with less fluorescence and absorbance of visible light²³. The 1002F photoresist was made by dissolving EPON resin 1002F (phenol, 4,4'-(1-methylethylidene)bis-, polymer with 2,2'-((1-Methylethylidene)bis(cyclohexane-4,1-diylloxymethylene))bisoxirane) (Miller-Stephenson, Sylmar, CA) and UVI-6976 photoinitiator (triarylsulfonium hexafluoroantimonate salts in propylene carbonate) (Dow Chemical, Torrance, CA) in γ -butyrolactone (GBL) (Sigma-Aldrich, St. Louis, MO) at a ratio of 61% 1002F resin/6.1% photoinitiator/32.9% solvent (weight percentage), as previously described²³. Micropallet arrays were constructed in a manner similar to that previously described¹⁷⁻²³. Briefly, 50 μ m thick coatings of 1002F resist were obtained by spin-coating and baking the resist on clean 2 in $\times$ 2 in glass slides. The arrays were patterned by exposure to UV light through a photomask and developed with SU-8 developer. The arrays were rinsed with 2-propanol, dried by a nitrogen stream and hard-baked at 120 $^{\circ}$ C. The micropallets had dimensions of 40 $\times$ 40 μ m with 30 μ m gaps.

After microfabrication, the arrays were treated to deposit a hydrophobic (perfluoroalkyl) silane layer on the glass and micropallet surfaces. A micropallet array and a plastic weighing boat containing 100 μ L of (heptadecafluoro-1,1,2,2-tetrahydrodecyl)trichlorosilane (Gelest, Morrisville, PA) were placed inside a dry-seal desiccator and attached to an oil-free vacuum

pump for 1 min (~7 Torr) followed by a standing time of 12 h under vacuum. The desiccator was purged to remove any residual unreacted silane molecules and arrays were stored under vacuum until use.

Plastic 4-well chambers taken from LabTek chamber slides (Nunc, Inc., Naperville, IL) were reattached to the micropallet arrays using polydimethylsiloxane (PDMS). The chambers demarcated discrete 9 mm × 20 mm areas of the arrays, each containing roughly 40,000 micropallets, holding a maximal volume of 1.5 ml of fluid. Prior to use, arrays were sterilized by briefly submerging them in 70% ethanol, after which residual ethanol was allowed to evaporate completely in a sterile environment.

2.4 Fibronectin Coating Method

Human plasma fibronectin (FN) was diluted to 20 µg/ml in sterile-filtered double-distilled water (ddH₂O). One ml of solution was added to each LabTek chamber well and incubated for 1 h at room temperature (RT). After incubation, four serial half-volume exchanges were used to replace the FN solution with sterile ddH₂O to dilute and remove non-adhered FN. Subsequent serial half-volume exchanges were then used to replace the ddH₂O with 70% ethanol. 70% ethanol solution has lower surface tension than water, which compromises the integrity of the network of virtual air walls, causing them to collapse and the inter-micropallet spaces to fill with solution. After collapse of the virtual wall network, the ethanol solution was aspirated off of the array, and the array was allowed to dry completely in a sterile environment at RT.

2.5 Basement Membrane Extract Coating Method

Basement membrane extract was diluted to 50µg/ml in phosphate buffered saline (PBS), at 4°C and one ml was applied to the micropallet arrays. The arrays were incubated for approximately 16 h at 4°C, and washed three times with PBS prior to the plating of cells. All steps were carried out with reagents at 4°C and chilled serological pipettes to prevent gelling of the BME. The BME coating efficiency was improved with the functionalization of the micropallet top surfaces prior to BME application. Sulfo-SANPAH was diluted to a concentration of 0.5mM in 50mM HEPES buffer. Arrays were submerged in the Sulfo-SANPAH solution and exposed to top-down 365nm UV radiation for 15 min using a Loctite Zeta 7411 UV flood exposure system, then washed twice with 50mM HEPES. The Sulfo-SANPAH application/UV exposure was repeated. BME solution was then applied, incubated, and washed exactly as above.

2.6 Collagen Method

One ml of poly-L-lysine solution at stock concentration of 0.01% (0.1 mg/mL in PBS) was applied to the arrays and incubated for 1 h at 37 °C, after which the arrays were washed ×3 with PBS and allowed to dry at RT for 10 min. Human collagen was diluted to 75 µg/ml in PBS and one ml of solution per well was applied to the arrays and incubated for 3 h at 37 °C. The arrays were then washed ×3 with PBS.

2.7 Laminin Method

As above, one ml of poly-L-lysine solution at stock concentration of 0.01% was applied to the arrays and incubated for 1 h at 25 °C, after which the arrays were washed three times with PBS and the arrays were allowed to dry at RT for 10 min. Rat laminin-5 was diluted to 1 µg/ml in PBS and one ml per well was applied to the arrays and incubated for 3 h at 37 °C. The arrays were then washed ×3 with PBS.

2.8 ECM Protein Staining & Evaluation

Colorimetric histochemical staining techniques were used to visualize and evaluate the ECM protein coatings. Optimization of primary and secondary reagent concentrations was performed on ECM coatings applied to glass substrates. The ECM protein-coated micropallet arrays (or glass substrates) were incubated with 3% BSA in PBS for 30 min at RT to block nonspecific reagent binding then washed $\times 2$ with washing buffer (1% BSA in PBS) and incubated for 30 min at RT with the following concentrations of primary reagents: for FN, anti-fibronectin antibody at 0.1 $\mu\text{g/ml}$; for BME, concanavalin-A at 1 $\mu\text{g/ml}$; for collagen, anti-collagen, type 1 antibody at 1.9 $\mu\text{g/ml}$; for Laminin-5, concanavalin-A at 0.33 $\mu\text{g/ml}$. The arrays were washed $\times 3$ with washing buffer and incubated for 30 min at RT with the following concentrations of horseradish peroxidase-conjugated secondary reagents: goat anti-rabbit IgG (H+L) at 0.4 $\mu\text{g/ml}$; rat anti-mouse IgG1 at 0.25 $\mu\text{g/ml}$; or Neutravidin at 1 $\mu\text{g/ml}$. Finally, the arrays were washed $\times 3$ with washing buffer and developed with TrueBlue peroxidase substrate for 10 min at RT, washed $\times 3$ with water, and air-dried.

2.9 Cell Adherence Studies

Cell lines were maintained in culture according to recommendations from ATCC. For firmly adherent cell lines, 3T3 and AsPC-1, and semi-adherent SKBR-3, the cell monolayer was washed with PBS without Mg or Ca, then incubated with trypsin-EDTA solution (0.25% trypsin; 1mM EDTA) for 3-5 min at 37 °C to release the cells from the growth surface for subsequent application to the array. An equal volume of fresh media containing fetal calf serum was used to neutralize the trypsin. For the relatively non-adherent KMS-18 cells, the cells and media were collected directly from the tissue culture flask. The recovered cells were centrifuged at $200 \times g$ for 5 min and the cell pellet was re-suspended in fresh media, counted using a hemacytometer, and diluted to 10,000 cells/ml in fresh media, and 1.5 ml of cell suspension was applied to each LabTek well. The arrays were then incubated at 37 °C and 10% CO₂ to allow cellular adherence.

Initial investigations into cell-type-specific affinities for the various ECM coatings were conducted using solid 1002F resist substrates subjected to the silane treatment and with attached LabTek chambers. The adherence of the cells was evaluated after incubation on the ECM-coated 1002F substrate for 4 h. The presence of the ECM proteins was confirmed using duplicate wells that were stained by colorimetric histochemistry. Cell adherence was measured by using a micropipettor to induce a shearing force at the surface of the 1002F. The tip of the micropipettor was positioned at one end of the LabTek well, and 1 ml of culture media was repeatedly drawn up and expelled at a rate of 0.5 Hz to produce shearing fluid flow at the surface. The effect of the shear stress on the cells was monitored at the center of the LabTek well using an inverted microscope with attached digital camera. Images of the same field-of-view were acquired before and after agitation. Adherence was quantified as the percentage of cells adhered to the growth surface sufficiently that they remained in place after agitation. Each cell type was evaluated on each ECM coating.

2.10 Mixed Cell Population Studies

Micropallet arrays were coated with either FN or laminin-5 as described as above. SK-BR-3 cells were stained with CFSE according to the manufacturers' instructions to permit fluorescent identification and were used to prepare a mixed population of 10% SK-BR-3 and 90% KMS-18 cells at a combined density of 10,000 cells/ml. One ml of mixture was applied to each LabTek well. After incubation for 4 h at 37 °C to allow adherence, five half-volume media exchanges were performed to remove non-adhered cells. An LSM 510 Meta laser scanning confocal microscope was used to analyze the cells that remained on the arrays.

3. RESULTS AND DISCUSSION

A distinct advantage of the micropallet array is to be able to identify and recover viable single adherent cells from a heterogeneous mixture of cells. The unique geometry and characteristics of the micropallet array impose constraints on the use of ECM coatings. Such coatings are likely to be required for application of this technology to primary cells or cell lines with restricted growth conditions. Thus, specific issues surrounding the application of ECM components must be addressed, specifically the uniformity and stability of adhered ECM coatings, restriction of the ECM to the top micropallet surface, and cell-type-specific affinities for the various ECM components. Early on we identified that although single cells could be placed onto the micropallet array, we frequently saw cells bridging from one micropallet to another. We postulated that this was potentially due to bridging of the ECM coating. In order to test the hypothesis and to evaluate the various parameters involved in ECM coating of the micropallet array, we established imaging methods for the ECM coatings.

3.1 Detection Methods for ECM protein coatings

We established colorimetric staining methods for analysis of ECM coatings on the micropallet arrays, with both brightfield and confocal microscopy. Each coating type was applied to a glass substrate according to the manufacturers' instructions and/or by previously reported methods. For each ECM coating type, we employed two control conditions: 1) coatings incubated with the secondary HRP-conjugated reagent alone and developed using TrueBlue substrate solution, and 2) plain glass not coated with ECM proteins and subjected to the full staining protocol. All four ECM protein coatings, Hu FN, BME, Rat Laminin-5, and Hu Collagen, were visualized, Fig. 2(a), although each required different staining methods as described above (Materials & Methods). After establishing satisfactory methods to detect the coatings on a glass substrate, the experiment was repeated on an unpatterned 1002F photoresist substrate surface with concordant results. We noted that incorporation of the 3% BSA blocking step was important to reduce nonspecific background staining on the 1002F photoresist. Figure 2(b) depicts each ECM coating successfully applied to micropallets. The established staining protocols were used in the subsequent development of the various methodologies used to coat micropallet arrays with the different ECM coatings.

3.2 Fibronectin

Fibronectin adhered strongly to the 1002F photoresist and did not require application of a primer coating such as poly-L-lysine or modification of the surface to facilitate adhesion. However, while FN adheres well to the micropallets, the biochemistry of FN makes it particularly susceptible to self-polymerization resulting in the formation of fibrils and sheets during the coating of micropallet arrays. Fibronectin exists in two forms: in solution as a large (MW = 450-500 kDa) soluble glycoprotein or as a fibrillar ECM protein²⁵⁻²⁸. The soluble form, which consists of two covalently-linked subunits, remains in solution both *in vivo* (in normal plasma) and *in vitro*, and does not spontaneously undergo fibrillogenesis^{27,29,30}. However, when FN dimers are subjected to physical force, typically through cell-mediated cytoskeleton interactions^{27,31} or fluid shearing stresses *in vitro*³², their molecular conformations are altered, exposing specific FN-FN binding sites, resulting in self assembly into polymeric, insoluble fibrillar structures^{28,30,32-36}. This latter *in vitro* phenomenon has been studied and reported by others to occur at liquid-air interfaces in dilute solutions of FN due to accumulation of FN molecules at the interfaces and their subsequent susceptibility to sheering forces^{32,36}.

Upon investigation, we found that during application the FN was adsorbed and deposited evenly onto the top surface of the micropallets, but was also concentrated into a sheet-like

structure at the liquid-air interface of the virtual wall network, Fig. 3(a). It has been postulated that due to its self-interacting nature, FN remains immobilized at the liquid-air interface more so than other proteins such as BSA³². Under initial conditions for coating the arrays with FN, in which the FN solution was either removed directly or exchanged with ddH₂O prior to removal, heterogeneous patterns of FN deposition across the array were produced consisting of: 1) regions where the FN sheet was dismantled and FN remained exclusively on the tops of the micropallets, 2) droplets of liquid that remained on the surface of the array and drew the FN sheet into insoluble fibrils as they evaporated, bridging neighboring micropallets, Fig. 3(b), and 3) large remaining sheets of FN, Fig. 3(c). In practice, all three of these patterns were present across a single LabTek well. Cell adherence experiments upon arrays coated under these conditions showed cells that came to rest on the FN-covered virtual walls had the ability to adhere in place on the FN sheet, Fig. 3(d).

We developed a strategy to prevent the formation of FN bridging fibrillar and sheet-like structures between micropallets. After incubation to allow FN deposition, the solution was serially replaced using half-volume exchanges with ddH₂O, followed with serial replacement with 70% ethanol, which sufficiently decreased surface tension such that the virtual walls collapsed. Finally, the bulk liquid was removed and the residual ethanol evaporated from the inter-micropallet spaces. When the virtual wall network collapsed, the FN sheet was no longer supported and appeared to disintegrate as it collapsed. After the array was allowed to dry, the virtual wall network was again supported with the reapplication of aqueous solution. FN fibrils and sheets were not present, and the individual micropallets had uniform coatings of FN that remained well-adhered, Fig. 3(e). We recognize that the group of J.P. Spatz³² used alcohol break down of entrapped air bubbles in a micropillar array, which had hydrophobic properties imparted by the dimensions of the micropillars (5µm diameters, 10µm tall, 4µm gaps), to purposefully generate FN fibrils. There are several differences between our two systems to account for the discrepancy in results including differences in polymers used for the microstructures, differences in microstructure dimensions and geometry, use of ethanol vs. polyvinyl alcohol to decrease the surface tension, and our use of hydrophobic surface treatment. Nevertheless, in our hands with our micropallet array technology and this methodology, we can generate uniform FN coatings restricted to the top surface of the micropallets.

3.3 Basement Membrane Extract

EHS tumor BME is unique among the four ECM coatings that we evaluated, in that its physical properties include being liquid at 4 °C and a semisolid gel at 37 °C. It is typically applied as either a thick or thin film coating^{37,38}. We found that BME was ill-suited for use with micropallet arrays when applied using traditional application methods. Firstly, it was found that the gelled BME coating had poor adhesion to the 1002F photoresist that was further compromised by the geometry of the micropallets. When arrays coated with BME were evaluated histochemically, across large portions of the array we observed sections without adherent BME or only partial coatings of BME, suggesting poor adhesion of the BME to the micropallet surfaces, Fig. 4(a). Although in some areas of the array the BME appeared to have comparatively better adhesion, it also appeared that cell-generated tensional forces acting on the BME coating were sufficient to cause it to lose adhesion to the micropallets, as evidenced by the spherical and compact morphology that 3T3 fibroblastic cells acquired over time on BME-coated arrays, Fig. 4(b). An additional concern when using the BME was the tendency of the coating to form fibrils or swaths spanning across multiple micropallets, similar to those observed with FN, Fig. 4(c).

We developed a novel method of effectively coating the micropallet arrays with BME extract that resulted in a marked increase in coating adhesion and decrease in the formation of fibrillar bridging structures. We used a two-pronged approach that 1) prevented the

gelling of the BME during the coating process to eliminate fibril formation and 2) used a heterobifunctional crosslinker, Sulfo-SANPAH, to improve adhesion by crosslinking the proteins of the BME to the 1002F photoresist. Sulfo-SANPAH contains an amine-reactive N-hydroxysuccinimide (NHS) ester and a UV photoactivatable nitrophenyl azide element. The sulfo-SANPAH treatment covalently cross-linked the proteins in the BME directly to the 1002F photoresist micropallet material. Sulfo-SANPAH treatment increased the deposition and adherence of the BME proteins onto the surface of the individual micropallets as compared to no sulfo-SANPAH treatment, Fig. 4(d). In conjunction with the sulfo-SANPAH treatment, BME was applied at a much lower concentration than traditionally used to coat growth surfaces for cell adhesion and proliferation. BME is traditionally applied as either a thick or thin gelled coating, or as a non-gelled, thin-film coating, which utilize dilutions six or two orders of magnitude more concentrated, respectively, than we used in our application method. During initial experiments, we utilized concentrations of BME appropriate for applying a standard thin film coating (1 mg/ml) to coat micropallet arrays and observed significant micropallet-to-micropallet bridging. Thus, although the BME does not form thick gels at these concentrations (below 9 mg/ml, *source: manufacturer's datasheet*), the protein layer deposited on the surface of the arrays is dense enough and has sufficient self-interactivity to create a continuous fibrous mesh spanning multiple micropallets. Reducing the concentration of the BME solution two orders of magnitude from 1 mg/ml to 50 ug/ml greatly reduced the ability of the coating to support gap-spanning protein networks. Additionally, incubation of the BME solution during application to the arrays was performed at 4 °C to further reduce gelling behavior of the BME. Using this combinatorial approach of low protein concentration and sulfo-SANPAH crosslinking, we were able to coat the micropallet arrays with a uniform coating of BME with minimal fibril formation, Fig. 4(e), and obtain strong adhesion to the micropallets that was sufficiently dense to support the adherence of cells. The improved adhesion and ability to support cell adherence was confirmed by observing 3T3 cells on micropallets coated with BME using the developed protocol, which now had classical fibroblastic appearance, Fig. 4(f), in contrast to that seen under initial coating conditions.

3.4 Collagen and Laminin-5

The coating protocols for collagen and laminin-5 required less adaptation for effective use on the micropallet arrays than did those for FN and BME. For both of these coating types, micropallet-to-micropallet protein bridging was not observed. As expected from the literature, it was found that the use of the poly-L-lysine primer coating improved the adsorption of both collagen and laminin-5 onto the micropallets, which was detected by the previously discussed colorimetric staining methods, as well as by increased adherence of cells plated on the arrays as compared to uncoated arrays (data not shown). It was also found that the concentrations used to coat standard growth surfaces, e.g., glass and polystyrene, were appropriate and did not require adjustment for use with the micropallet arrays for both collagen and laminin-5. The collagen was diluted to 75 ug/ml and the laminin-5 was diluted to 1 ug/ml. Laminin-5 is a natural substrate for cells in contact with basement membranes, and these cells, compared to fibronectin or collagen, adhere faster to Laminin-5 via the $\alpha\beta1$ integrin. Laminin-5 can also be used at much lower concentrations (approximately 10-fold lower) than other ECM macromolecules to provide an effective coating for cellular adherence³⁹.

3.5 Cell-type-specific behavior on ECM coatings

Four discrete cell types were used to evaluate the biological property of cellular adherence to the ECM-coated micropallet arrays. The cell types consisted of 3T3, SK-BR-3, AsPC-1, and KMS-18, which are fibroblastic, breast and pancreatic epithelial, and hematopoietic (myeloma plasma cell), respectively. These cell lines were chosen as representative samples

of stromal, epithelial, and hematopoietic/inflammatory cell types that are likely to reside in any given tissue and which might be of interest in studies requiring the selection of a single cell from a heterogeneous population. We predicted that they would have distinctly different behaviors on the various ECM protein coatings.

Although we chose to evaluate rapid adherence, after 4 h of incubation, we acknowledge that some cells might require longer periods for firm adherence, but this must be weighed against the fact that with extended *in vitro* culture, there would be a greater risk of the well-documented phenotypic drift associated with *in vitro* cell culture, which may compromise some analyses. However, we evaluated adherence for up to 18 h and detected no significant differences between 4 and 18 h with any cell type or coating, with the exception of SK-BR-3 on fibronectin, which showed a marked increase in adherence over the 18 h period. The method of using shearing fluid forces to determine adherence was chosen because cells on micropallet arrays experience similar forces when the liquid in the LabTek chamber is removed or replaced, which is necessary for the analysis of the cells as well as media exchanges. An example of the method used to quantitate adherence is shown in Fig. 5(a). The percentage of cells of each type that were firmly adhered after a 4 h incubation was evaluated with replicate experiments for each coating type, Fig. 5(b). Poly-L-lysine, which was used as a primer coating for laminin-5 and collagen, was included as a stand alone coating in these experiments for control purposes and no cell type exhibited significant adherence to it. The fibroblastic 3T3 cells adhered well to laminin-5, FN, and BME, but not collagen. The breast cancer epithelial cells (SK-BR-3) adhered well to laminin-5 and collagen, and moderately to BME and FN, while the pancreatic cancer epithelial cells (AsPC-1) adhered well to all 4 coatings, which is not surprising given the strong desmoplastic response (dense stromal proliferation) associated with pancreatic adenocarcinoma⁴⁰. The plasma KMS-18 cells adhered very strongly to laminin-5 and strongly to collagen, but not at all to BME and FN. All four cell types adhered well to laminin-5. Adherence to laminin-5 occurs faster than to other ECM proteins, e.g., FN and collagen, for most cell types³⁹ and thus it may be possible to use even shorter incubation periods for some cell types.

3.6 Cell behavior on ECM-coated micropallet arrays

The value of micropallet arrays technology and its utility for analyzing adherent cell populations is critically dependent on the ability to sequester single cells to individual micropallets. We hypothesized that single cell isolation and capture depends on our ability to effectively coat micropallet arrays with ECM protein coatings, and also on the affinities of the cells for adherence to the ECM protein(s). We demonstrated our ability to properly coat micropallets with various ECM protein coatings and investigated cellular adherence to these coatings for three cell types; stromal, epithelial, and hematopoietic/inflammatory.

We investigated whether cellular behavior on coated micropallet arrays was analogous to typical ECM-coated culture surfaces and demonstrated our ability to effectively capture and isolate cells of each type. We found that the percentage of captured cells correlated well to the percentages found with the ECM-coated unpatterned 1002F surfaces as above, Fig. 6. This indicates that the biological integrities of the ECM coatings on the micropallet arrays were maintained and the various techniques developed to achieve proper ECM-coated micropallet arrays did not reduce the efficacy of the coatings at supporting cell adherence. Additionally, micropallet-to-micropallet cellular bridging was not observed for any coating type, supporting the hypothesis that cellular bridging is dependent on bridging structures of the ECM protein coatings.

3.7 Enrichment of mixed cell populations

Because different types of cell lines were shown to have differential adherence properties depending upon the ECM coating, Figure 5, one possible application would be to use the ECM coating to enrich for specific cell types. A mixed population of two cell types was employed to demonstrate this capacity, Figure 7. The relative frequency of a rare cell type among all adhered cells was dramatically increased by choice of ECM coating.

4. CONCLUSIONS

Our findings support our hypothesis that the success of various ECM protein coatings for use with micropallet arrays to capture single cells depends on 3 properties: the adherence of the ECM coating to the micropallet top surfaces, the prevention of ECM protein networks that bridge neighboring micropallets, and the cells' affinity for adherence to the ECM coating. We discovered that certain ECM coatings are problematic when applied in traditional manner to micropallet arrays due to poor adherence to the micropallets and/or formation of fibrillar bridging structures connecting multiple micropallets. This in turn, required the development of novel application strategies to overcome these issues and effectively coat micropallet arrays with each of 4 different ECM protein coatings: human FN, BME, collagen, and laminin-5. We investigated the ability of each coating type to capture cells representing stromal, epithelial, and hematopoietic/inflammatory cell types on individual micropallets and were able to capture all cell types with one or more of the coatings. Cell adherence to each ECM coating was not affected by the micropallet-patterned surface in comparison to adherence experiments performed on solid 1002F photoresist surfaces. Finally, the capacity for the relative enrichment of a given cell population dependent upon the strategic selection of ECM coatings reinforces the necessity to be able to effectively coat the micropallet array surface with various ECM components and provide the broadest range of applications for these types of technologies.

Micropallet arrays are a new technology with many versatile applications in the fields of adherent cellular analysis and separation, including single cell selection, colony selection, population characterization, and population purification. The successful application of this technology to the study of adherent cells is critically dependent on the ability to capture and isolate cells to single micropallets. The work presented in this paper will enable and guide the use of micropallet arrays for a multitude of future uses in the isolation and study of discrete adherent cell populations. In addition to micropallet arrays, these findings have particular relevance for other systems that utilize ECM coatings and microscopically nonuniform surfaces. Such devices are increasingly common as advances in microtechnology are tailored for biological applications.

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Figure 1.

Micropallet array and virtual walls. Micropallet arrays are vast arrays of microscale polymer pedestals uniformly arranged on a glass microscope slide. (a) Scanning electron microscope (SEM) micrographs of a micropallet array consisting of micropallets with 40 μm width, 50 μm height, and 30 μm spacing between adjacent micropallets. (b) The array is made to be very hydrophobic by adsorption of an organosilane monolayer onto the surfaces of the array and the hydrophobicity causes air to be trapped between the micropallets when an aqueous solution is applied to the array. The resulting network of air walls (virtual walls) prevents cells from falling between the micropallets and constrains them to the top surfaces of the micropallets.

Figure 2.

Colorimetric detection of ECM coatings. Colorimetric histochemical detection of ECM coatings on (a) clean glass with experimental zones demarcated using PAP pen hydrophobic circles (bar = 1 cm) and (b) micropallets coated using the optimized methodologies described within the text (micropallets are 40 μm $\times$ 40 μm). Individual columns represent the various coatings and the top rows in a and b represent the non-specific staining controls in which the primary antibody or detection reagent, for example, concanavalin-A, was omitted

Figure 3.

Micropallet array coating with fibronectin. (a) Micropallet arrays were coated with FN but stained after replacing the FN solution with pure ddH₂O without ever completely removing the ddH₂O or exposing the array's surface to air during the staining process. The arrays were imaged while still submerged under ddH₂O. The results revealed a continuous FN sheet covering micropallets and virtual walls (left). Also shown for comparison are properly coated micropallets with FN only on the micropallet top surfaces (middle) and a control case of no FN present (right). (b) Time lapse images over the course of 1 min showing the phenomenon of multiple fibrils being formed after the bulk of the FN solution has been removed and a remaining droplet evaporates from the surface of the array. Two fibrils are indicated by black arrowheads in the right most panel. (c) The use of standard coating methods to coat the arrays with FN results in a combination of FN sheet structures, FN fibrils, and properly coated micropallets. (d) Phase contrast micrograph of two 3T3 cells (white arrowheads) that have landed and adhered on a continuous FN sheet, the border of which is indicated by black arrowheads. (e) Using our developed coating method, all micropallets are evenly coated with FN with no sheets or fibrils observed (inset: staining control). Micropallets in all panels are 40 $\mu\text{m} \times 40 \mu\text{m}$

Figure 4.

Micropallet array coating with EHS tumor basement membrane extract. (a) Stained BME showing poor adhesion to untreated micropallets. (b) Phase contrast micrograph showing multiple 3T3 fibroblast cells (two indicated by white arrowheads) that have exerted sufficient tractional forces to pull the BME coating from the surfaces of the micropallets; a boarder of the BME coating is indicated by the black arrowhead. Note the spherical morphologies of the cells. (c) SEM micrograph of BME fibrils and cells bridging neighboring micropallets. (d) Stained BME on micropallets showing improved adhesion to the micropallet top surfaces when the Sulfo-SANPAH treatment is used. From top to bottom: Control (no BME); BME at 50 Bg/mL without Sulfo-SANPAH treatment; BME at 50 Bg/mL with Sulfo-SANPAH treatment. (e) Stained BME on micropallets showing improved adhesion and no fibril formation (inset: staining control). (f) 3T3 cells on a micropallet array coated with BME using the developed coating method. Individual cells are constrained and well adhered to single micropallets. Micropallets in all panels are $40\ \mu\text{m} \times 40\ \mu\text{m}$

Figure 5.

Cell adherence to ECM coated 1002F surface. (a) Example images of SK-BR-3 cells on a FN-coated 1002F substrate before (left) and after (right) being subjected to fluid shearing forces. Indicated are examples of cells that either remained adhered (black arrows) or were dislodged (white arrows). In this particular experiment, roughly 60% of the cells were characterized as being adhered. (bars = 50 μm) (b) Bar graph representation of multiple experiments ($n \geq 3$ for all cell types and all conditions) evaluating the mean percentage of cells strongly adhered to each ECM coating after 4 h incubation at 37°C, with error bars representing SD and outliers rejected using Grubbs' test for outliers, $p < 0.05$.

Figure 6.

Adherence of different cell types on various ECM coatings. Representative images of each cell type on micropallet arrays coated with each ECM coating using the developed methods. The percentages of cells for each condition that were appropriately captured correlated well with the percentages seen in Figure 5 for cells adhered to the ECM coatings on unpatterned growth surfaces. Micropallets in all cases are 40 μm μ 40 μm .

Figure 7.

Enrichment of rare cells by strategic choice of ECM coating. A mixed population of cells can be enriched for a rare cell type by use of an appropriate ECM coating. The percentage of rare SK-BR-3 cells in a background of KMS-18 cells was increased over sevenfold by using FN as compared with laminin-5 ($n = 2$ for each data point).