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Multicolor Immunofluorescent Imaging of Complex Cellular Mixtures on Micropallet Arrays Enables the Identification of Single Cells of Defined Phenotype

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The ability to simultaneously identify, recover, and study specific cellular elements of complex tissues has remained a challenge for all except the hematopoietic lineages, due in large part to the limited tools or platforms available for the analyses of adherent cells. The increasing recognition that distinct cellular subsets within a complex tissue or organ confer biological characteristics has dramatically increased the need for the development of new platforms to permit studies of defined cellular subsets contained within heterogeneous cell populations, with minimal perturbation.

The basic micropallet array platform is a micro- and nanotechnology that permits the isolation, selection, and recovery of single adherent cells.^[1] This platform consists of micro-scale pedestals, termed “micropallets,” that are fabricated using standard photolithography of high-aspect negative photoresist, 1002F,^[2] patterned on a glass surface (Figure 1). The micropallet array is treated post-fabrication to impart specific qualities, including the creation of a network of “virtual air walls”^[3] to limit cellular access to the channels between micropallets, coating with any of several extracellular matrix (ECM)

components to facilitate cell adherence to the top surfaces of the micropallets,^[4] and imparting magnetic properties to the micropallets by doping the 1002F photoresist with ferromagnetic nanoparticles for magnetic recovery.^[5] Individual micropallets carrying single adherent cells can be released using a low energy laser pulse^[6] and remain viable throughout the release and subsequent recovery process. These elements of the micropallet array technology and methodology, in conjunction with the demonstration of single cell analyses of collected cells,^[5] provide the foundation for this work.

The appreciation of the interactions between cellular subsets in tissues, organs, and neoplasms is the source of new hypotheses and provides a rationale for the increasing interest and activity in systems biology. Within neoplastic tissues, cancer stem cells are one of the cellular subsets of interest in part because it has been proposed that the proportion of putative cancer stem cells, or tumor-initiating cells, are associated with resistance to therapy and early metastasis.^[7] Additionally, the proportion of endothelial progenitor cells, another subset of interest, is associated with angiogenic phenotype and response to anti-angiogenic therapies.^[8] Differences in the cellular profile of tumors have been hypothesized to be a source of variability in clinical behavior and response to treatment strategies,^[9,10] and likely plays a role in other tissues undergoing normal (e.g., injury recovery) or pathologic processes (e.g., response to elevated blood sugar, diabetes). The complex relationships between cellular subsets residing in primary tissues will only be characterized when individual cellular subsets can be effectively identified, isolated, recovered, and studied with limited perturbation. The micropallet array is an attractive platform to meet this need.

Although the micropallet array provides an attractive platform for recovery and analysis of adherent cellular subsets, with previously described advantages over alternative technologies,^[1–3,5,6] the functional capacity to be able to identify specific cellular subsets within a heterogeneous adherent cell population has not been demonstrated. Characterization of adherent cell subsets typically requires interrogation of several molecules, in contrast to non-adherent cells that frequently have unique single identifying surface molecules. Thus, multichannel imaging is required to fulfill the functional capacity of this platform for studies of defined cellular subsets from primary tissues and tumors. As a model system, we employed three cell lines of disparate origins (human medulloblastoma, human breast adenocarcinoma, and human embryonic kidney). Using this model system we demonstrated the capacity to identify cell subsets of interest, expected to be contained within human breast adenocarcinomas, by integrating multicolor

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Figure 1. Overview of the micropallet array platform. A) Standard micropallet arrays are fabricated with $40 \times 40 \mu\text{m}$ square micropallets (blue arrows) that are $50 \mu\text{m}$ tall (purple arrow) and separated by $30 \mu\text{m}$ gaps (red arrow). B) Lab-tek chambers are attached to micropallet array slides and demarcate 40 000 micropallets per individual well. C) Virtual air walls are constructed on the micropallet array by creating a highly hydrophobic surface through silane vapor deposition to restrict cellular access to the top surfaces of the micropallets. Extracellular matrix is applied to the top surfaces of the micropallets to facilitate cell adherence.

immunofluorescent confocal imaging of up to five cell surface molecules selected to discriminate specific cellular subsets from heterogeneous mixtures of adherent cells, **Table 1**.

The capacity to identify different cell types and subsets within heterogeneous cell populations based on their cell surface molecule expression patterns provides a very flexible platform for a wide range of biological analyses, including those that require retention of cellular viability. To develop this capacity, we examined three cell lines, MCF7, D283 Med, and transiently transfected HEK 293T expressing CD309, selected for expression of the desired cell surface molecules (Table 1). Purified monoclonal antibodies (mAbs) specific for each cell surface molecule of interest were tested against each cell line to confirm their antigen reactivity using appropriate fluorophore labeled secondary IgG antibodies using flow cytometry (Figure S1, Supporting Information). Based on these findings, the following cell surface molecule expression patterns were determined for each cell line: MCF7 cells express ESA, CD24, and very low levels of CD44, and do not express CD133 and CD309; D283 Med cells express CD133 and CD44, and do not express CD309, CD24, and ESA; finally, HEK 293T cells express low levels of ESA, and when transiently transfected with pBLAST2-hFLK1 express CD309, but do not express CD24, CD133, and CD44 (Table 1). Similar to primary breast tumor cell subsets, there is no single molecule that uniquely identifies one cell type from another, reiterating the requirement for multichannel analyses. All cell lines adhered to micropallet arrays coated with either rat laminin-5 (rL5) or human fibronectin (huFN) and demonstrated the same surface molecule expression patterns as detected via flow cytometry (Figure S1, Supporting

Information). Thus, these cell lines had the necessary characteristics to be an appropriate model system to demonstrate proof of principle for the multicolor immunofluorescent confocal imaging strategy.

Successful multicolor imaging requires attention to both the emission and excitation spectra of selected fluorophores, methods of detection, and strategic construction of the imaging strategy. Although imaging software algorithms, such as linear unmixing and emission fingerprinting, can spectrally separate fluorophores whose emission spectra overlap, these techniques require multiple control samples to be prepared, imaged, and used to calibrate the system. Such techniques are not feasible for studies with limited sample size, such as would be obtained from a normal tissue or tumor biopsy.

We selected fluorophores Brilliant Violet (BV) 421, 605, and Alexa Fluor (AF) 488, 546, and 647 because their emission spectra can be spectrally separated using commercially available confocal scanning microscopes. Each fluorophore was paired with an mAb such that the brightest fluorophores were paired with mAbs directed against cell surface molecules expressed at lower levels and vice versa, **Table 2**. CD24-BV421 and CD44-BV605 mAbs were procured as fluorophore conjugated antibodies. CD309-AF488, ESA-AF546, and CD133-AF647 mAb-fluorophore conjugates were generated by established protocols using carboxylic acid, succinimidyl ester forms of Alexa Fluor dyes to directly conjugate the desired fluorophore to its respective antibody (Figure S2, Supporting Information). All in house generated antibody-fluorophore conjugates exhibited a degree of labeling (DOL) of 2–5 moles Alexa Fluor dye per mole of antibody. Appropriate isotype-matched antibodies were also procured either already fluorophore conjugated or purified and conjugated directly to the respective fluorophore

Table 1. Cell surface molecule panel for the identification of cell subsets within human breast tumors and on respective cell lines.

	ESA	CD44	CD24	CD309	CD133
Cancer stem cell	+	+	–	–	–
Epithelial tumor cell	+	+	+	–	–
Endothelial progenitor cell	–	–	+	+	+
MCF7	+	+/-	+	–	–
D283 Med	–	+	–	–	+
CD309+ 293T	+/-	–	–	+	–

Table 2. Antibody-fluorophore conjugates.

Fluorophore	Monoclonal antibody	Isotype antibody
Brilliant Violet 421	CD24	Mouse IgG2a
Brilliant Violet 605	CD44	Rat IgG2b
Alexa Fluor 488	CD309	Mouse IgG1
Alexa Fluor 546	ESA	Mouse IgG1
Alexa Fluor 647	CD133	Mouse IgG1

in the panel with a similar DOL and used at the same titers as the antigen recognizing mAb-fluorophore conjugate. To confirm maintenance of target antigen reactivity in the fluorophore conjugated primary antibody, fluorescence from cells stained with the primary conjugated antibody was compared to fluorescence of cells stained with the unconjugated primary antibody and detected by an appropriate commercial fluorophore labeled secondary antibody. Similar fluorescence intensity confirmed that antigen reactivity was not compromised by the conjugation procedure. When imaging each individual cell type on the micropallet array stained with the multicolor panel of isotype-fluorophores, none of the isotype-fluorophore conjugates could be detected, establishing that under these conditions non-specific background staining is very limited and essentially undetectable (Figure S3, Supporting Information). Individual cell lines were stained with single mAb-fluorophore conjugates and imaged to verify the specificity of the mAb-fluorophore conjugates for the cell lines, confirming the flow cytometry data above (Figure S3, Supporting Information).

A roughly equal part mixture of the three cell lines was applied to the micropallet array, simultaneously stained using the mAb multicolor panel, and imaged on an entire single micropallet array demarcating roughly 40 000 micropallets. The multicolor imaging strategy correctly identifies each individual cell population with no significant photobleaching (Figure 2). Individual cell types adhered to the micropallet array were identified by their surface molecule expression patterns. Magnified regions are provided for increased visualization (Figure 2), enabling discrimination of four cell types (CD309 transfected and untransfected HEK 293T cells, MCF7, and D283 Med).

We have demonstrated proof of principle of the micropallet array platform to identify cellular subsets of defined cellular phenotype within heterogeneous cell populations by the incorporation of multichannel (five channels) multicolor immunofluorescent laser scanning confocal imaging. The micropallet array is a well-developed platform capable of isolating and collecting single adherent cells with minimal perturbation that is suitable for a wide range of applications. The unique combination of fluorophores utilized in this particular multicolor imaging strategy allows the user to detect each individual fluorophore without the utility of spectral unmixing, thereby providing the opportunity to examine biospecimens with limited cell numbers. Although we have focused on cell surface molecule expression, intracellular molecules can also be detected, provided that an acceptable monoclonal antibody is available, but at the expense of cell fixation and permeabilization, potentially

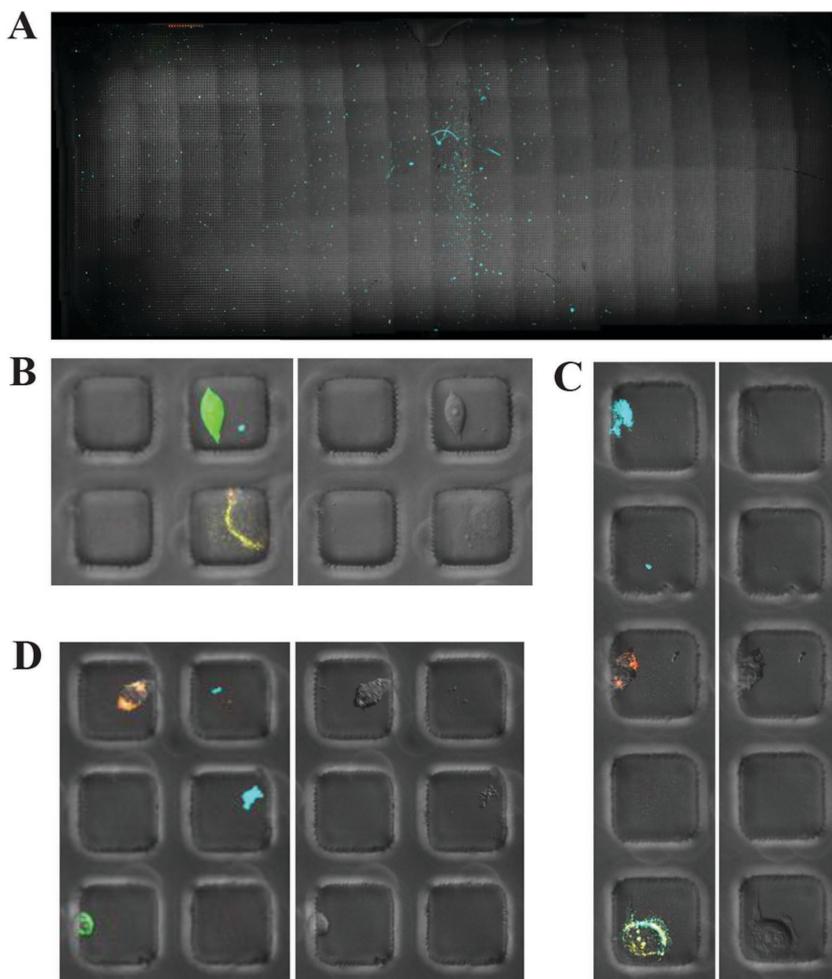


Figure 2. Multicolor imaging of complex heterogeneous cell mixtures for the identification of cellular subsets. A) Depicts representative multicolor immunofluorescent image of a heterogeneous mixture comprised of MCF7, D283 Med, and HEK 293T cells transiently transfected to express CD309, adhered to a rat laminin-5 coated micropallet array and stained with the panel of fluorophore conjugated mAbs directed against the five selected cell surface molecules. Simultaneous immunofluorescent imaging using a Leica Sp8 laser scanning confocal microscope to image an entire micropallet array containing $\approx 40\,000$ micropallets. B–D) Depicts representative areas of (A) containing cells identifiable by their surface molecule expression patterns, including the phase contrast channel for reference.

higher non-specific background staining, and inability to maintain viability. We expect that with continued development of imaging capacities, specifically focused on the micropallet platform, such as quantitative fluorescence, it will be possible to discriminate an even broader range of adherent cell subsets. The current data, along with the previously reported refinements to this platform,^[4,5] presents an innovative methodology that (1) permits the simultaneous enumeration and identification of various cellular elements present within a complex adherent cell sample, (2) provides the opportunity to assess the molecular profiles of single collected cells from various defined cellular subsets,^[5] (3) can accommodate high throughput analyses, and (4) overcomes sample size and throughput limitations to existing technologies, such as laser capture microdissection or fluorescence-activated cell sorting, among others. This new functional capacity for the micropallet array platform yields

a novel tool to allow investigators to address a multitude of fundamental biological questions involving complex, heterogeneous, normal, and pathological primary tissues dominated by adherent cells.

Experimental Section

Materials and Reagents: All chemicals, cell culture media, media supplements, disposables, and reagents were procured from Fisher Scientific (Pittsburgh, PA) unless otherwise noted. High precision 24 × 60 mm cover glass slides, No. 1.5 (Azer Scientific, Morgantown, PA) provided the base for the micropallet array. Micropallet fabrication materials included UV photoresist polymer: EPON resin 1002F (phenol, with polymer of 4,4'-(1-methylethylidene)bis-, polymer with 2,2'-(1-methylethylidene)bis(cyclohexane-4,1-dioxymethylene)) bisoxirane (Miller-Stephenson, Sylmar, CA), UVI-6976 photoinitiator (triarylsulfonium hexafluoroantimonate salts in propylene carbonate) (Dow Chemical, Torrance, CA), and γ -butyrolactone (GBL) (Sigma-Aldrich, St. Louis, MO). The following reagents were used for post-production modification of the micropallet arrays: Silane (heptadecafluoro-1,1,2,2-tetrahydrodecyl)trichlorosilane (Gelest, Morrisville, PA), four-well Lab-Tek chamber slides (Nunc Inc., Rochester, NY), human fibronectin (huFN, Millipore, Billerica, MA), poly-L-lysine (PLL, Trevigen, Gaithersburg, MD), and rat laminin-5 (rL5, Millipore, Billerica, MA).

mAbs recognizing specific human cell surface antigens, matching isotype controls, and fluorophore labeled secondary antibodies were procured; CD24 (clone ML5, pre-conjugated to Brilliant Violet 421), CD44 (clone IM7, pre-conjugated to Brilliant Violet 605), and isotype controls similarly pre-conjugated with the respective fluorophore (Biolegend, San Diego, CA); epithelial surface antigen (ESA) (clone 1B7) (eBioscience, San Diego, CA); CD133 (clone AC133) (Miltenyi Biotec, Auburn, CA); and CD309 (clone EIC) (ReliaTech, Wolfenbüttel, Germany).

Fluorophores for antibody-fluorophore conjugation included: Alexa Fluor 488, 546, and 647 carboxylic acid, succinimidyl esters (Molecular Probes, Eugene, OR). Reagents for antibody-fluorophore conjugation included: Anhydrous *N,N*-dimethylformamide (DMF) (Sigma, St. Louis, MO), and Amicon Ultra-0.5 NMWL 50 000 centrifugal filter units (Millipore, Billerica, MA).

Micropallet Array Fabrication: Micropallet arrays were fabricated from 1002F photoresist in a similar manner to that previously described.^[1] Briefly, 1002F photoresist was made by dissolving EPON resin 1002F and UVI-6976 photoinitiator in γ -butyrolactone at a ratio of 61% 1002F resin/6.1% photoinitiator/32.9% solvent (weight percentage).^[1] The 1002F photoresist was spin-coated and baked to achieve 50 μ m thick coatings on clean 24 × 60 mm high precision cover glass slides, patterned by exposure to UV light through a photomask, and developed with SU-8 developer. The arrays were rinsed with isopropanol, dried by a nitrogen stream and hard-baked at 120 °C. The fabricated micropallets have dimensions of 40 × 40 μ m cross section, 50 μ m height, with 30 μ m gaps. As previously described,^[2] virtual air walls were constructed around the micropallets by creating a highly hydrophobic surface through silane vapor deposition restricting access of the cells to the top surfaces of the micropallets. Plastic four-well Lab-Tek chamber slides were attached to the micropallet arrays using polydimethylsiloxane (PDMS) to demarcate areas of the array and to hold in cell culture media.^[3] The micropallet array was sterilized in ethanol (70%) and allowed to completely dry in a sterile environment before use.

To facilitate cellular adhesion the micropallets were coated with ECM proteins, human fibronectin or rat laminin-5, as previously reported.^[1,3] Briefly, huFN was diluted in sterile 1× PBS, pH 7.4 (20 μ g mL⁻¹) and applied to the LabTek chamber wells demarcating the micropallet arrays for 1 h at room temperature and then washed thrice with sterile ddH₂O to remove non-adhered huFN. The arrays were then subject to three half volume exchanges with ethanol (70%) to remove excess huFN and to re-establish sterility.^[3] The resulting huFN coated micropallet array was allowed to dry in a sterile environment at room temperature for several

hours before use. To coat micropallet arrays with rL5, arrays were first pre-treated with poly-L-lysine solution (0.01%) for 1 h at 37 °C before coating with rL5 diluted in sterile 1× PBS, pH 7.4 (1 μ g mL⁻¹) for 1 h at room temperature. The rL5 coated arrays were washed using three half volume exchanges with 1× PBS, pH 7.4 and maintained in solution until use.

Cell Culture and Transient Cell Transfection: MCF-7 breast adenocarcinoma (HTB-185) and D283 Med medulloblastoma (HTB-22) cell lines were procured from ATCC (Manassas, VA). Both cell lines were cultured per ATCC instructions, for the former in DMEM (Mediatech, Manassas, VA) supplemented with fetal bovine serum (10%), penicillin (100 U mL⁻¹), streptomycin (100 μ g mL⁻¹) (Mediatech, Manassas, VA), and Novolin R insulin (44 U L⁻¹) (Novo Nordisk, Plainsboro, NJ), and for the latter in MEM (Mediatech, Manassas, VA) supplemented with fetal bovine serum (10%), penicillin (100 U mL⁻¹), streptomycin (100 μ g mL⁻¹) (Mediatech, Manassas, VA), and β -mercaptoethanol (10 × 10⁻⁶ M).

HEK 293T cells (a gift from Dr. Craig Walsh, University of California, Irvine) were cultured per ATCC instructions in MEM supplemented with fetal bovine serum (10%), penicillin (100 U mL⁻¹), streptomycin (100 μ g mL⁻¹), sodium pyruvate (1 × 10⁻³ M), and non-essential amino acid mixture (1×). HEK 293Ts were transiently transfected with pBLAST2-hFLK1 (InvivoGen, San Diego, CA) using BioT transfection reagent (Bioland Scientific, Paramount, CA) using conditions established by the manufacturer. HEK293T cells transiently transfected with pBLAST2-hFLK1 were utilized for flow cytometry or micropallet array studies 48 h post transfection.

AF-Antibody Conjugation Reactions: Carboxylic acid, succinimidyl esters of Alexa Fluor 488, 546, and 647 were conjugated to anti-human CD309, ESA, and CD133 mAbs, respectively. Each mAb was concentrated and exchanged into 1× PBS, pH 7.4 using Amicon centrifugal filter units (NMWL 50 000), to an optimal concentration unique to each mAb clone. Sodium bicarbonate was then supplemented to the antibody solution at a final concentration of 0.1 M. The reactive Alexa Fluor dye was dissolved in anhydrous DMF and then immediately added to the mAb solution at molar excess concentrations unique to each mAb clone. The antibody-Alexa Fluor conjugation reaction was incubated at room temperature for 1 h and then overnight at 4 °C with continuous mixing (300 RPM), resulting in the formation of a stable amide bond between the fluorophore and primary amines of the mAb.^[1,4] Figure S2 (Supporting Information). The mAb-Alexa Fluor conjugate was desalted and exchanged into 1× PBS, pH 7.4 again using Amicon centrifugal filter units (NMWL 50 000) removing excess unconjugated Alexa Fluor dye. The degree of labeling for each AF-antibody conjugate was determined as instructed by protocols provided by Molecular Probes,^[1,5] and was optimized for each mAb used in the cell surface marker panel. Appropriate isotype mAbs were also conjugated to each of the Alexa Fluor dyes under conditions that led to a similar degree of labeling as their respective experimental mAb-Alexa Fluor dye conjugates.

Immunofluorescent Cell Staining and Confocal Microscopy: MCF-7, D283 Med, and transiently transfected HEK 293T cells transfected to express CD309 were released from their flasks and treated with RNase free DNase I (100 U mL⁻¹) (Agilent Technologies, Santa Clara, CA) for 10 min at room temperature, then washed with appropriate cell culture media (10 mL), and collected by centrifugation at 228×g for 5 min at room temperature. The cell samples were then resuspended in cell culture media, strained with either 40 × 10⁻⁶ M cell strainers for D283 Med and transiently transfected HEK 293T cells, and through 20 × 10⁻⁶ M cell strainers (pluriSelect, Leipzig, Germany) for MCF-7. Prepared cell suspensions were then seeded onto previously huFN coated micropallet arrays at 10 000 cells mL⁻¹, 1 mL per single four-well chamber and incubated at 37 °C/10% CO₂ for a minimum of 4 h to facilitate cellular adherence.

Micropallet arrays were washed twice with RPMI 1640 without phenol red and L-glutamine prewarmed to 37 °C, to remove any residual non-adhered cells from the micropallet array samples. Cells adhered on the micropallet arrays were then incubated with blocking buffer, HBSS (Mediatech, Manassas, VA) supplemented with casein solution (1%) (Roche Diagnostics, Indianapolis, IL) and NaN₃ (0.1%), for 30 min at room temperature. The arrays were then washed twice with wash buffer (HBSS supplemented with BSA (1%) and NaN₃ (0.1%)).

Predetermined appropriate concentrations of either fluorophore conjugated or unconjugated mAb, specific for the cell surface molecules of interest or isotype control antibodies were utilized to stain samples for 30 min. The arrays were then washed twice with wash buffer to remove any excess unbound mAb. In select cases, the arrays were then stained with FITC labeled secondary antibody for 30 min. Arrays were then washed twice with wash buffer to remove any excess unbound secondary antibody, and subsequently imaged. For samples interrogated using the established mAb-fluorophore conjugates, cells adhered to the micropallet array were stained with the mAb-AF, and mAb-BV421 conjugates all at $3 \mu\text{g mL}^{-1}$ concentration for 45 min. The arrays were then washed twice with wash buffer to remove any excess unbound conjugated mAb and subsequently imaged. An inverted Leica Sp8 laser scanning confocal microscope with LASX acquisition software was utilized for all the images obtained.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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