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Functionalization of Micropallet Arrays for Multichannel Immunofluorescent Imaging of Complex Cellular Mixtures

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Abstract **-**The micropallet array is a photoresist-based platform that provides a means to isolate and recover single adherent cells on individual pedestals, termed "micropallets". We present a strategy to functionalize the micropallet array by incorporating multichannel immunofluorescent confocal imaging to identify cellular subsets of defined surface phenotype within complex heterogeneous mixtures, such as would be obtained from a tissue biopsy. We present the capacity to interrogate the expression pattern of up to 5 cell surface molecules. As proof of principle, we have demonstrated the capacity to detect the panel of surface molecules (Epithelial Surface Antigen (ESA), CD24, CD44, CD133, and CD309) necessary to identify unique cellular subsets within human mammary tumors, e.g. putative cancer stem cells, endothelial progenitor cells, and bulk malignant epithelium. Appropriate selection of fluorophores is critical to this capacity; monoclonal antibodies were procured directly conjugated to Brilliant Violet (BV) fluorophores (CD24-BV421 and CD44-BV605), while Alexa Fluor (AF) conjugates (CD309-AF488, ESA-AF546, and CD133-AF647) were prepared using established conjugation chemistries for monoclonal antibodies not available directly conjugated to the desired fluorophores. Selected cell lines that collectively express the panel of cell surface molecules (MCF7, D283 Med, and transfected HEK 293T cells transiently expressing CD309), were applied to the micropallet array, stained with the antibody-fluorophore panel, and imaged using laser scanning confocal microscopy. The ability to interrogate the expression pattern of several cell surface molecules provides a critical addition to the existing micropallet array platform, in that it enables the identification, enumeration, and with the previously demonstrated capacity to release and retrieve micropallets carrying single adherent cells, the study of single cells of defined surface phenotype isolated from a complex heterogeneous population. This new capacity functionalizes the micropallet array platform technology to be broadly applied to the study of complex cellular systems or tissues.

Keywords**:** Micropallet Array, BioMEMs, multicolor immunofluorescent confocal microscopy, single cell analysis

1. Introduction

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 elements within a complex tissue or organ microenvironments confer biological characteristics has dramatically increased the need for the development of new platforms to permit studies of 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 (Salazar, G.T., et al., 2007, Gunn N.M., et al., 2010a, b, Sims C.E., et al., 2007). This platform consists of micro-scale pedestals, termed "micropallets", that are fabricated using standard photolithography of high-aspect negative photoresist, 1002F (Pai J.H. et al., 2007), 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" (Wang Y., et al., 2007) 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 (Gunn, N.M. et al, 2010a), and imparting magnetic properties to the micropallets by doping the 1002F photoresist with ferromagnetic nanoparticles for magnetic recovery (Gunn, N.M. et al, 2010b). Individual micropallets carrying single adherent cells can be released using a low energy laser pulse (Salazar, G.T., et al., 2008) 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 (Gunn, N.M. et al, 2010b) provide the foundation and basic platform for this work.

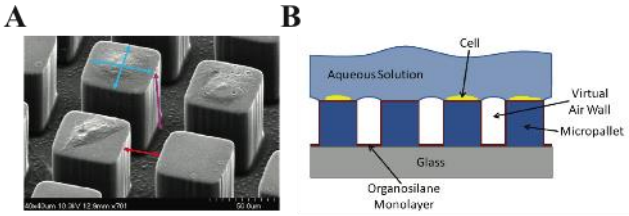

Fig. 1. Overview of the Micropallet Array Platform. (A) Standard micropallet arrays are fabricated with 40x40µm square micropallets (blue arrows) that are 50µm tall (purple arrow) and separated by 30µm gaps (red arrow). **(B)** Virtual air walls are constructed around the micropallets by creating a highly hydrophobic surface through silane vapor deposition to restrict cellular access to the top surfaces of the micropallets.

The appreciation of the interactions between cellular subsets in tissues, organs, and neoplasms is one of the factors driving interest and activity in systems biology. Within neoplastic tissues the proportion of putative cancer stem cells, or tumor-initiating cells, has been associated with resistance to therapy and early metastasis (Kraljevic P.S., et al., 2011, Ghadially, R., 2011, Frame, F.M. and N.J. Maitland, 2011). Similarly, the proportion of endothelial progenitor cells is associated with angiogenic phenotype and response to anti-angiogenic therapies (Mung, J.A., and J. Case, 2011, George, A.L. et al., 2011). Cancer patients, even those with histologically identical tumors, experience substantial variability in clinical behaviour and response to treatment strategies (Anderson, K.M., et al., 2007). Differences in the cellular profile of tumors may be a source of this variability and may contribute to the observed molecular heterogeneity between tumors of identical histology. Additionally, 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. The micropallet array is an attractive platform to meet this need.

The heretofore-undeveloped capacity is the ability to identify specific cellular subsets in a heterogeneous adherent cell population on the micropallet array platform. 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 analyses are required for the use of this platform for studies of defined cellular subsets from primary tissues and tumors. We report the ability to functionalize the micropallet array by integrating multicolor immunofluorescent confocal imaging of up to 5 cell surface molecules to discriminate specific cellular subsets from heterogeneous mixtures of adherent cells using breast tumors as a proof of principle tissue, Table 1.

Table 1: Cell Surface Panel for Identification of Cell Subsets of Interest within Human Breast Tumors

	CD44	ESA	CD24	CD133	CD309
Cancer Stem Cell	+	+	-	-	-
Epithelial Tumor Cell	+	+	+	-	-
Endothelial Progenitor Cell	-	-	+	+	+

2. Results and Discussion

2. 1. Detection of Cell Surface Molecules on Cell Lines

One application of the micropallet array technology is for the analysis of heterogeneous cell populations. To develop this capacity, it was important to establish the ability to identify different cell types based on their cell surface molecule expression patterns. A total of 3 cell types, MCF7, D283 Med, and transiently transfected HEK 293T expressing CD309 were selected for expression of the desired cell surface molecules. 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 2). 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; lastly, 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 2). 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 the human fibronectin (huFN) coated micropallets (Figure 2) and demonstrated the same surface molecule expression patterns as detected via flow cytometry. 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.

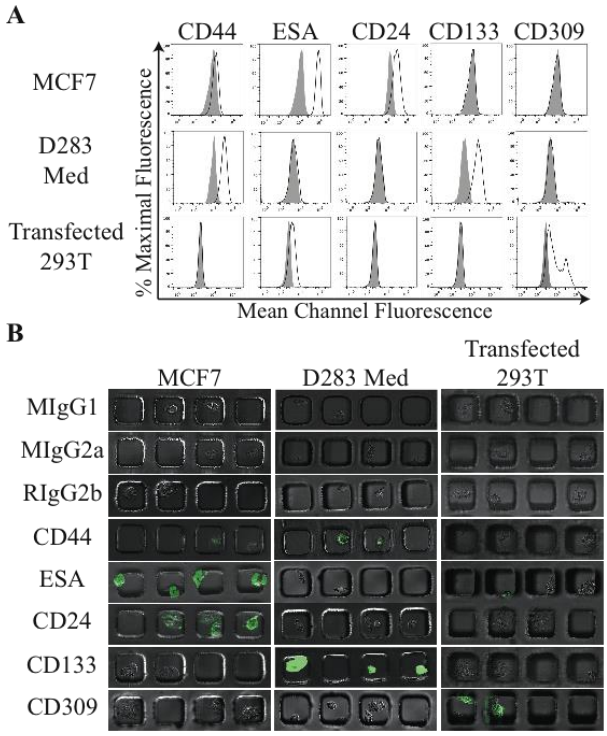

Fig. 2. Expression of Cell Surface Molecules on Cell Lines. (A) Representative flow cytometry histograms of cell surface molecule expression on MCF7, D283 Med, and HEK 293T cells transiently transfected for CD309 expression. Grey histograms depict isotype matched control antibody fluorescence. Black line open histograms depict fluorescence obtained from mAbs directed against the designated molecule noted above each column. Each plot depicts mean channel fluorescence by percent maximal fluorescence. Histograms were constructed from ≥ 50,000 events recorded as live cells using propidium iodide exclusion. (B) Representative confocal fluorescent microscopy images of cells from designated cell lines on huFN coated micropallet arrays, stained with the primary mAb and FITC-conjugated secondary antibody. Panels are an overlay of phase contrast and fluorescent images.

2. 2. Development of Multichannel, Multicolor Laser Scanning 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 a mAb such that the brightest fluorophores were paired with mAbs directed against cell surface molecules expressed at lower levels and vice versa, Table 3. CD24-BV421 and CD44-BV605 mAbs were procured already conjugated to the fluorophore. CD309-AF488, ESA-AF546, and CD133-AF647 mAb-fluorophore conjugates were generated using established protocols using carboxylic acid, succinimidyl ester forms of Alexa Fluor dyes to directly conjugate the desired fluorophore to its respective antibody. All 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 or conjugated directly to each fluorophore in the panel with a similar DOL and used at the same titers as the mAb-fluorophore conjugate panel. 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 3). Individual control 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 3).

Table 3: Antibody-Fluorophore Conjugates

Fluorophore	mAb	Isotype
BV605	CD44	RIgG2b
AF546	ESA	MIgG1
BV421	CD24	MIgG2b
AF647	CD133	MIgG1
AF488	CD309	MIgG1

A roughly equal mixture of all 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 4). Individual cell types adhered to the micropallet array were identified by their surface molecule expression patterns. Magnified regions are provided for increased visualization (Figure 4). Thus, we demonstrated proof of principle for integration of multicolor immunofluorescent cell identification strategy into the micropallet array platform.

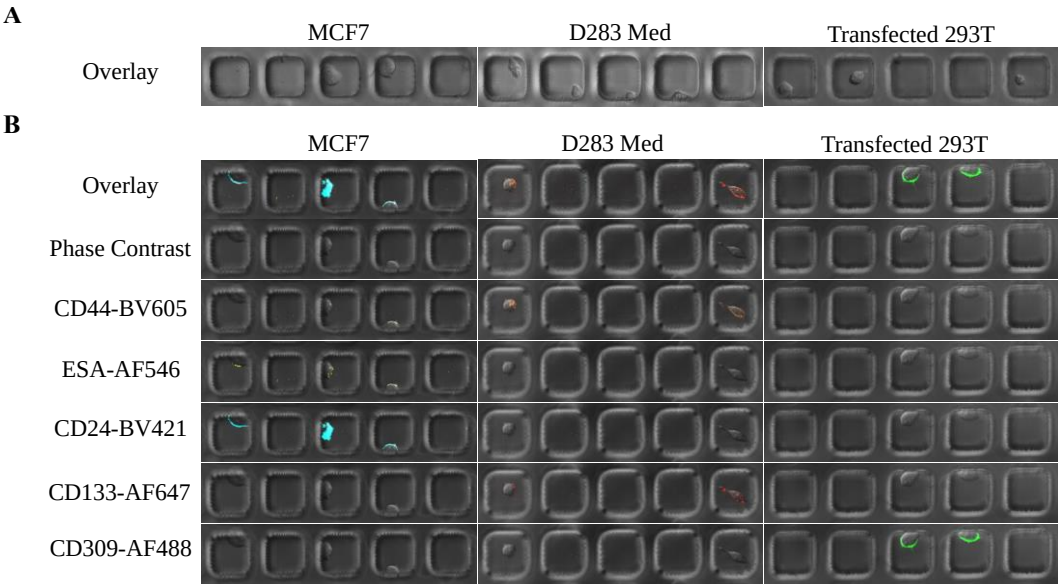

Fig. 3. Expression of Cell Surface Molecules on Cell Lines. (A) Representative multicolor immunofluorescent images of designated cell lines, adhered to huFN coated micropallet arrays and stained with the respective primary fluorophore conjugated isotype control antibody panel (imaged as negative controls). (B) Depicts representative multicolor immunofluorescent images using the mAb-fluorophore multicolor panel and imaged to reveal each cell type's surface molecule expression pattern.

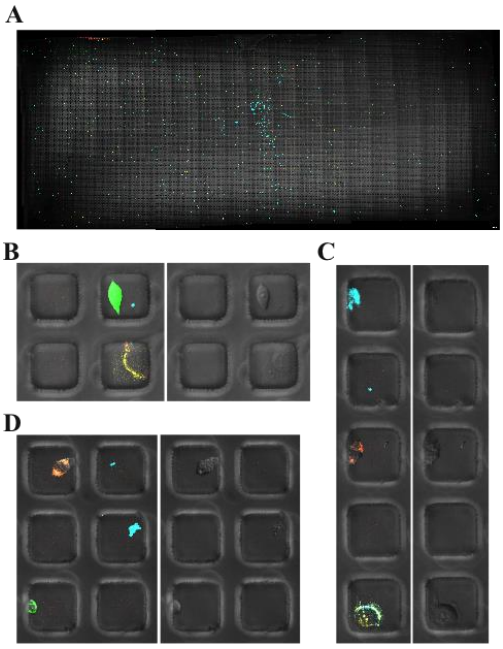

Fig. 4. Multicolor Imaging of Complex Heterogeneous Cell Mixtures for the Identification of Cellular Subsets. (A) Depicts representative multicolor immunofluorescent image of a heterogeneous mixture comprised of MCF7, D283 Med, and HEK 293T cells transiently transfected to express CD309, adhered to a huFN coated micropallet array and stained with the panel of fluorophore conjugated mAbs directed against the 5 selected cell surface molecules. Simultaneous immunofluorescent imaging using a Leica Sp8 laser scanning confocal microscope was utilized to image an entire micropallet array containing roughly 40,000 micropallets. (B-D) Depicts representative areas of 4A containing cells identifiable by their surface molecule expression patterns, including the phase contrast channel for reference.

3. Conclusion

We have demonstrated proof of principle for the functionalization of our micropallet array platform to identify cellular subsets of defined cellular phenotype within heterogeneous cell populations by the incorporation of multichannel (5 channels) multicolor immunofluorescent laser scanning confocal imaging. The micropallet array is a well-developed platform capable of isolation and collection of 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, and potentially higher non-specific background staining. These data, along with the previously reported refinements to this platform (Gunn, N.M., et al, 2010a, b), 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 (Gunn, N.M., et al., 2010b), 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. This functionalization of 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.

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