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Micropallet arrays for the capture, isolation and culture of circulating tumor cells from whole blood of mice engrafted with primary human pancreatic adenocarcinoma

Philip C. Gach [a], Peter J. Attayek [b,c], Rebecca L. Whittlesey [d], Jen Jen Yeh [d,e,f], Nancy L. Allbritton [a,b,c,d,f,*]

[a] Department of Chemistry, University of North Carolina, Chapel Hill, NC 27599, United States
[b] Joint Department of Biomedical Engineering, University of North Carolina, Chapel Hill, NC 27599, United States
[c] Joint Department of Biomedical Engineering, North Carolina State University, Raleigh, NC 27695, United States
[d] Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599, United States
[e] Department of Surgery, Division of surgical Oncology, University of North Carolina, Chapel Hill, NC 27599, United States
[f] Department of Pharmacology, University of North Carolina, Chapel Hill, NC 27599, United States

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ABSTRACT

Circulating tumor cells (CTCs) are important biomarkers of cancer progression and metastatic potential. The rarity of CTCs in peripheral blood has driven the development of technologies to isolate these tumor cells with high specificity; however, there are limited techniques available for isolating target CTCs following enumeration. A strategy is described to capture and isolate viable tumor cells from whole blood using an array of releasable microstructures termed micropallets. Specific capture of nucleated cells or cells expressing epithelial cell adhesion molecules (EpCAM) was achieved by functionalizing micropallet surfaces with either fibronectin, Matrigel or anti-EpCAM antibody. Surface grafting of poly(acrylic acid) followed by covalent binding of protein A/G enabled efficient capture of EpCAM antibody on the micropallet surface. MCF-7 cells, a human breast adenocarcinoma, were retained on the array surface with $90 \pm 8\%$ efficiency when using an anti-EpCAM-coated array. To demonstrate the efficiency of tumor cell retention on micropallet arrays in the presence of blood, MCF-7 cells were mixed into whole blood and added to small arrays (71 mm^2) coated with fibronectin, Matrigel or anti-EpCAM. These approaches achieved MCF-7 cell capture from $\leq 10\,\mu L$ of whole blood with efficiencies greater than 85%. Furthermore, MCF-7 cells intermixed with 1 mL blood and loaded onto large arrays (7171 mm^2) were captured with high efficiencies ($\geq 97\%$), could be isolated from the array by a laser-based approach and were demonstrated to yield a high rate of colony formation ($\geq 85\%$) after removal from the array. Clinical utility of this technology was shown through the capture, isolation and successful culture of CTCs from the blood of mice engrafted with primary human pancreatic tumors. Direct capture and isolation of living tumor cells from blood followed by analysis or culture will be a valuable tool for cancer cell characterization.

1. Introduction

Advances in clinical technologies have revealed the significance of low abundance biomolecules or cells for monitoring patient health. Many studies have demonstrated the utility of a non-invasive, 'liquid' biopsy towards tracking disease progression or patient health by quantifying low-concentration biomolecules in blood (Rissin et al., 2010). Recent reports have hypothesized that circulating tumor cells (CTCs) may provide valuable information regarding the phenotype and metastatic behavior of an individual's primary tumor (Cohen et al., 2008; Cristofanilli et al., 2004). Efficient strategies to isolate these low abundance cells from peripheral blood may enable novel clinical diagnostics as well as better understanding of cancer cell biology.

Many recent studies have sought to capture CTCs from the peripheral blood of patients or animal models with cancer. Magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS) are the oldest methods in use for isolating CTCs (Cristofanilli et al., 2004; Ameri et al., 2010). Unfortunately, MACS sorting is only capable of collecting CTCs based on their surface markers, rarely provides 100% collection efficiency and is plagued by contaminating

* Corresponding author at: Department of Chemistry, University of North Carolina, Chapel Hill, NC 27599, United States. Tel.: +1 919 966 2291; fax: +1 919 962 2388.
E-mail address: nlallbri@unc.edu (N.L. Allbritton).

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cells such as lymphocytes and other nonspecifically captured cells. FACS is a commonly used technology for retrieving pure cells from a heterogeneous population. The low abundance of CTCs present in blood necessitates removal of erythrocytes prior to cell sorting generally suffers from loss of CTCs as well as reduced cell viability (Takao and Takeda 2011). Many of the methods depending on surface expression of EpCAM may also suffer from poor CTC capture and identification as a result of down regulation of EpCAM as the cells undergo an epithelial to mesechymal evolution (Gorges et al., 2012).

The inefficiency of these technologies at isolating CTCs has spurred the development of a multitude of new capture technologies to sort and analyze CTCs. Microfluidic based devices for isolating CTCs enhance substrate-cell interactions by increasing the effective surface area (Nagrath et al., 2007) or by generating chaotic cell mixing to increase collisional contact with the surface (Wang et al., 2011). Some technologies have allowed collection of CTCs following capture by employing laser pressure catapulting (LPC) or integrating a releasable antibody–substrate linker (Helzer et al., 2009; Gunn et al., 2010; Gach et al., 2010). Microfluidic devices have also achieved isolation of CTCs from blood by employing enrichment within microwell arrays (Kang et al., 2012), fluidic switching (Schiro et al., 2012), dielectrophoresis (Gascoyne et al., 2009) and inertial focusing (Hur et al., 2011). Size-selective microfilters have been developed in a variety of geometries to effectively capture CTCs from whole blood (Zheng et al., 2011). These size-based collection strategies are able to capture and collect CTCs with excellent viability, however, they are unsuccessful at capturing small CTCs and also suffer from clogging by lymphocytes when high blood volumes are filtered. Though the tissue-cultured cells captured by the devices described above retain a high level of proliferation; few reports have successfully demonstrated culture of CTCs acquired from animal models and CTC culture from patient samples remains extremely challenging due the fragile nature of these cells (Ameri et al., 2010; Glinskii et al., 2003; Glinsky et al., 2006).

Our lab has previously demonstrated the utility of arrays of releasable elements microfabricated on glass substrates termed 'micropallets' for sorting single adherent cells (Wang et al., 2007). Micropallet technology has shown success at sorting single cells in a mixed cell population with low reagent requirements, high post-sorting yield and excellent viability. Additionally, this platform has exhibited utility for isolating small numbers of target cells from mixed populations especially when the target cells are present in low abundance (Wang et al., 2007). Functionalization of the micropallet surfaces with extracellular matrices (ECM) or capture antibodies has previously been reported to provide pre-enrichment of target cells prior to sorting (Gunn et al., 2010; Shadpour et al., 2009). In this report, the potential for using micropallet arrays to isolate tumor cells directly from whole blood is examined. The capture efficiency was tested for MCF-7 cells mixed into whole blood and loaded directly onto micropallet arrays functionalized with fibronectin, Matrigel, or anti-EpCAM. Isolation of MCF-7 cells from whole blood followed by laser-based release and culture was assessed along with the sorting of tumor cells directly from the whole blood of mice bearing patient-derived pancreatic ductal adenocarcinoma.

2. Materials and methods

2.1. Micropallet functionalization

Micropallets were initially fabricated, as described in the Supporting information, and functionalized with fibronectin or Matrigel. Micropallet surfaces were treated with various proteins to aid in cell capture and surface adhesion. Initially, micropallet arrays were sterilized by rinsing with 95% ethanol and dried in a sterile hood. Excess ethanol was removed with five rinses with phosphate buffered saline (PBS). For capture of adherent cells, the top surfaces of the micropallets on the array were coated with 1 mL of 25 μg/mL fibronectin in PBS for 1 h at room temperature. For attachment of primary cells, micropallets were incubated with 1% Matrigel (Matrix Growth Factor Reduced, BD Biosciences, Franklin Lakes, NJ) in complete media for at least 1 h at 37 °C. Following surface coating, the array was rinsed 5 × with PBS. Alternatively, micropallet tops were functionalized with anti-EpCAM antibody, as described in the Supporting information (Fig. S1).

2.2. MCF-7 cell culture immediately before and after array-based isolation

MCF-7 cells (a human breast adenocarcinoma cell line) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS) (10%), L-glutamine (584 mg L^{-1}), penicillin (100 units mL^{-1}) and streptomycin (100 μg ml^{-1}) in a 37 °C incubator with a 5% CO_2 atmosphere. To ascertain the capture efficiency of adherent cell lines from whole blood, MCF-7 cells were intermixed with sheep blood (BioChemed Services, Winchester, VA). The sample was then mixed with 2 mL (small arrays) or 10 mL (large arrays) of culture media and overlaid onto a fibronectin, Matrigel or anti-EpCAM functionalized micropallet array. The array was then transferred to an incubator for at least 2 h to permit the adherent cells to settle and attach to the substrate. Isolated MCF-7 cells were cultured in MCF-7-cell conditioned media.

2.3. Culture of primary human CTCs on arrays

Whole blood samples obtained from a PDX model of human pancreatic cancer were cultured in a manner similar to the MCF-7 cells with the following exceptions (Wang et al., 2012). Blood from the mice was collected into a BD Microtainer® tube (BD Biosciences, Franklin Lakes, NJ) and then diluted with 10 mL of pancreatic-cell conditioned media and incubated on the micropallet arrays for 48 h in a 37 °C incubator with a 5% CO_2 atmosphere. Isolated pancreatic CTCs were cultured in pancreatic-cell conditioned media, prepared as described in Supporting information. Prior to cell analysis and separation, the arrays were rinsed 5–10 times with PBS to selectively remove the blood components. All animal studies were approved by University of North Carolina at Chapel Hill Animal Care and Use Committee and comply with Nation Institute of Health guidelines.

2.4. Microscopy and micropallet isolation

Following immunostaining of CTCs, as described in Supporting information, screening for CTCs over the large micropallet array was achieved with an MVX10 MacroView microscope (Olympus, Center Valley, PA) and motorized stage (ProScan III motorized stage system, H138A/C ProScan upright microscope stage, ProScan III motorized focus control, Prior Scientific Inc., Rockland, MA) paired with an ORCA-Flash4.0 camera (Hamamatsu, Bridgewater, NJ). Custom MATLAB software was used to analyze the acquired images in addition to controlling the microscope, stage, and camera. Imaging small micropallet arrays and micropallet release was performed on an inverted microscope (Eclipse TE300, Nikon, Melville, NY) combined with a pulsed Nd:YAG laser (532 nm, 5 ns Polaris II laser, New Wave Research, Fremont, CA). Released micropallets were then magnetically collected onto a multiwell plate as described previously (Gach et al., 2010). Quantitative inaccuracies inherent to spiking low numbers of MCF-7 for cell capture experiments were reduced by collecting the waste generated during array