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Imaging multiple biomarkers in captured rare cells by sequential immunostaining and photobleaching

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ABSTRACT

Several technologies recently have been developed for separating and counting circulating tumor cells (CTCs) in the human blood. CTCs play an important role in the metastasis of cancer. Most of the current applications are focused on the enumeration of CTCs; however, analysis of the enumerated CTCs has been proven to be increasingly important. Ensemble-decision aliquot ranking (eDAR) is a high-throughput method that allows the isolation of the CTCs from the whole blood with high recovery and a zero false-positive rate. Coupling a CTC separation and capturing method, such as eDAR, with a downstream immunostaining test provides information about the cell's expression of certain protein biomarkers. In this article, we introduce a semi-automated system for sequential immunolabeling and photobleaching on the eDAR platform. With our new technique, we were able to evaluate the expression of eight different biomarkers on isolated CTCs.

1. Introduction

Circulating tumor cells (CTCs), defined as the cancer cells shed into the bloodstream from the primary tumor [1–3], are thought to be an important part of cancer metastasis in the "seed and soil" theory [4]. These cells have been detected in many different types of cancer, such as breast [5], lung [6], prostate [7] and pancreatic [8]. It has been shown that the number of CTCs directly correlates with the clinical outcome in metastatic patients, providing valuable prognostic information that can be helpful to manage the clinical care of these patients [2,9]. Other potential applications of the CTC studies include the prognosis of non-metastatic cancer patients [10] and the development of personalized medicine [11]. However, due to their very low concentration (1–10 CTCs per 1 mL of whole blood), the detection and enumeration of these rare cells in human blood have been very challenging [12]. Many new technologies—immunomagnetic separation [13], affinity chromatography like separation methods [14,15], negative selection [16], and various other microfluidic approaches [17–19]—only aim to count CTCs. Indeed, the only method currently approved by the U.S. Food and Drug Administration (FDA), the CellSearch system, is entirely based on the enumeration. It should be noted that CTC methods are often considered to be akin to a non-invasive and real-time biopsy.

But more studies are showing that the cellular and molecular analyses of CTCs may be more important than simple enumeration [20]. These analyses can potentially verify some biological and clinical hypotheses by studying the expression of many biomarkers on CTCs and comparing them to the biomarker expression profile of the primary tumor. For example, a recent study demonstrated by analyzing the expression of epithelial and mesenchymal markers on CTCs that the epithelial–mesenchymal transition (EMT) plays an important role in the blood-borne dissemination of breast cancer [21]. Downstream analysis post-enumeration can also improve our understanding of the mechanisms of metastasis. Some studies showed that cancer stem cells can be found in the population of CTCs [22,23], and may also correlate with the disease progression.

Among various potential genetic and molecular analyses, analyzing protein biomarkers may be an easy and important starting point to perform in the downstream analysis of CTCs for several reasons. First, some protein markers are direct targets of anti-tumor drugs, such as human epidermal growth factor receptor 2 (Her2) [24] or epidermal growth factor receptor (EGFR) [25]. By studying these biomarkers, more molecular details can be revealed, which may benefit the prognostic evaluation. Second, some protein markers can clearly define the sub-populations of CTCs, such as epithelial CTCs, mesenchymal CTCs or CTCs with stem-cell characteristics, which may provide more biological details about the metastatic tumor biomarker. Third, no matter what enrichment method is used, most of the current CTC-methods count and verify CTCs using immunostaining and fluorescence imaging techniques. These techniques are fully compatible with subsequent protein

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biomarker analysis after CTC enumeration. Therefore the immunostaining and fluorescence analyses of CTCs can be readily done once the cells are captured with minimum additional sample preparation or transfer processes.

Our group recently developed a high-throughput and sensitive method for detecting and isolating CTCs in human blood called eDAR [26]. The detection, separation and analysis were integrated into a single microfluidic chip, aiming for an "all-in-one" platform for the analysis of CTCs. eDAR took less than 20 min to analyze 1 mL of whole blood. The method had a 94% recovery rate and a zero false-positive value, demonstrating that eDAR has high throughput, sensitivity, and accuracy. We believe eDAR is an ideal platform for capturing CTCs and performing the subsequent analysis of the expression of protein markers for two reasons: first, cells are captured and enriched into a small area on the microfluidic chip so the imaging process can be done much faster than accomplished by other techniques [26]. The small area also minimizes the usage of secondary antibodies. Second, eDAR has an open-access design, which can facilitate the manipulation of single cells, such as picking up a cell of interest or delivering certain reagents to a cell.

In this paper, we developed and optimized a simple and semi-automatic method to perform the downstream analysis of the expression of protein markers on trapped CTCs. We designed an in-line immunostaining and photobleaching system which allowed us to perform labeling and fluorescence imaging tests on selected CTCs with a group of antibodies conjugated with different fluorophores followed by the photobleaching and re-labeling with different fluorescent antibodies against another group of biomarkers. This process can be repeated multiple times to study groups of protein biomarkers. In our experiment, two protein markers of interest, combined with a positive control marker (nuclear stain) and a negative control marker (CD45), are studied in each round. As proof of principle, we performed four rounds of the immunostaining and photobleaching process to look at the expression of eight protein markers of interest.

2. Materials and methods

2.1. Microfluidic components and line-confocal optics

The polydimethylsiloxane (PDMS) chips were fabricated using methods described previously [26]. Briefly, the features were designed using AutoCAD (AutoDesk, San Rafael, CA), and then written on a transparency mask by Fineline Imaging (Colorado Springs, CO). Micro-features were fabricated on a silicon wafer using SU-8-3050 (Micro-Chem Corp., Newton, MA) as a negative photoresist; the feature height was controlled to be 50 μm. Once the features were developed, uncured PDMS was poured onto the silicon master, incubated at 75 °C for 2 h, peeled off and then bonded to a glass coverslip using the plasma oxidation method.

Earlier, we had developed the line-confocal detection scheme [26–29] where two laser sources, 488 and 633 nm, were used to form the two detection windows using a series of dichroic mirrors, cylindrical lens and beam splitters. The first detection window, having the two laser beams overlapped at the same time, was used to detect the fluorescence signals from the labeled CTCs, and then controlled the sorting automatically. The second detection window was used to confirm the sorted aliquots and monitor the sorting efficiency.

2.2. Biological materials and eDAR process

Isoton (Beckman Coulter Inc., Chino, CA) was used as the buffer for all the experiments unless otherwise specified. The breast cancer cell lines MCF-7, SKBr-3 and MDA-MB-231 (American Type Culture Collection (ATCC), Manassas, VA) were used to characterize the system. Cell culture was performed under the conditions recommended by the vendor, and harvested once a week. MCF-7 was cultured in Eagle's Minimum Essential Medium (EMEM); SKBr-3 cells were cultured in McCoy's 5; and MDA-MB-231was cultured Dulbecco's Modified Eagle's Medium (DMEM) (ATCC, Manassas, VA). All media also contained 2 mM L-glutamine, 10% fetal bovine serum (FBS) (ATCC, Manassas, VA), and 50 μg/mL penicillin/streptomycin. Human whole blood drawn from healthy donors was purchased from Plasma Lab International (Everett, WA) and stored at 4 °C upon arrival. Each 20-mL draw came in four 5-mL Vacutainer tubes coated with EDTA as an anti-coagulant. We discarded the first tube of each draw to avoid potential contamination from skin cells.

Antibodies were centrifuged for 5 min at 14,000 rpm to remove possible aggregates before any labeling procedure. Each blood sample was labeled with anti-epithelial cell adhesion molecule (EpCAM) conjugated with phycoerythrin (PE) (Abnova, Taipei City, Taiwan) in darkness and incubated at room temperature for 30 min. The labeled blood sample was washed and centrifuged (2300 rpm for 10 min) to remove the free antibodies. The sample was immediately injected into the eDAR chip using a syringe pump. Typically, the flow rate was set to 50 μL/min for the operation of eDAR, although based on the previous optimization methods, it could be higher [26]. APD signal traces were collected by a PCI data acquisition card (PCI 6602, National Instruments, Austin, TX) and analyzed by a MATLAB (MathWorks, Natick, MA) script developed in-house. A home-built electronic box was programmed to give an automatic feedback control based on the detected APD signals, and apply a voltage on the solenoid (S-10-38-H-40, Magnetic sensor systems, Van Nuys, CA) connected to the microfluidic chip. More details about eDAR were described previously [26].

2.3. Sequential immunostaining and photobleaching process

After washing the cells isolated by eDAR, main, side and waste channels were closed by turning off the inline valves. A 400-μL aliquot of cell fixation buffer (BioLegend, San Diego, CA) was introduced into the microchip by a peristaltic pump (Fisher Scientific, Pittsburgh, PA) at a flow rate of 15 μL/min. After washing with the buffer for 5 min at the same flow rate, the cells are permeablized by flowing through 250 μL of 2.5% surfynol 465 surfactant (Air Products and Chemicals Inc., Allentown, PA) for 15 min. After this step, four rounds of immunostaining and photobleaching of the cells are performed. For each round of staining, 220 μL of a staining solution with four biomarkers conjugated to four different fluorescent dyes were prepared. Nine primary antibodies conjugated with different fluorophores were used in the four rounds of labeling and photobleaching, including anti-EpCAM-PE, anti-Cytokeratin-Alexa 647, anti-MUC1-PE, anti-Her2-Alexa 647, anti-CD24-PE, anti-CD44-Alexa 647, anti-CD166-PE and anti-EGFR-APC. The sources and experimental conditions about the antibodies and nuclear stain used in each round are summarized in Table 1S. After a centrifugation step (14,000 rpm for 5 min) to remove the aggregates, 200 μL of the supernatant was collected as the staining buffer. We injected it into the microchip at a flow rate of 20 μL/min. When the antibody solution filled the whole filtration area, the flow was stopped. Incubation took place for 20 min in dark to ensure all the trapped cells came into contact with the antibodies efficiently. After this step, the cells were washed for 10 min to remove any free antibodies and minimize the fluorescence background. Photobleaching was performed using a xenon arc lamp as the light source (Sutter instrument, Novato, CA). Each bleaching step took 15 min. A 20X objective was used for epi-fluorescence imaging and photobleaching. The imaging condition was