CONCURRENT MULTI-SAMPLE ANALYSIS OF LOW EXPRESSED BIOMARKERS ON SINGLE HUMAN CELLS BY ENZYMATICALLY AMPLIFIED IMMUNODETECTION IN DROPLETS

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ABSTRACT

We have developed a novel microfluidic droplet based assay for analysis of low expressed cell surface proteins on individual cells at rates of hundreds of cells/s by antibody coupled enzymatic amplification in monodisperse droplets [1]. Here we expand the method to include concurrent analysis of multiple populations of single cells. We report the validation of the method by analyzing the human monocytic cell line U937 for two low expressed markers, CCR5 and CD19. Comparing our method to standard flow cytometry, we demonstrate increased peak separation, which should allow sorting by these low expressed biomarkers unavailable to flow cytometry.

KEYWORDS: Droplets, Enzymes, Antibodies, Cell Surface Biomarkers

INTRODUCTION

The presence and quantity of a certain cell surface protein indicate the state of a cell. The expression of a protein ranges between very few copies and 10⁹ copies per cell [2]. The standard method for measuring protein expression levels on single cells is flow cytometry using fluorophore-coupled antibody labels. Low expressed proteins (<1000 copies/cell) are important for many cell functions such as signaling [2] and thus may be important biomarkers, like HER2 in breast cancer. Flow cytometry is limited to detecting proteins expressed at moderate to high levels. Using droplets that encapsulate the fluorescent product of an amplifying enzymatic reaction together with the single cell and a color code we analyze multiple cell samples concurrently. Since the droplets are monodisperse the amplified signal is quantitative.

Figure 1: Schematic description of the enzymatic amplification assay with color-coding. Cells are labeled, encapsulated on-chip, incubated and re-injected for analysis.
EXPERIMENTAL

Microfluidic devices were manufactured in Polydimethylsiloxane using soft lithography techniques [3]. The device has three components; a droplet generation nozzle, a temperature controlled droplet incubation vial and a module for fluorescence read-out from individual droplets [4]. Figure 1 shows a schematic of the assay. Three samples of U937 cells were labeled with biotinylated antibodies for the cell surface biomarkers CCR5 and CD19 as well as a biotinylated isotype negative control antibody (eBiosciences) respectively. Antibodies were coupled to Beta-Galactosidase enzyme (Rockland) via a biotin-streptavidin linkage. Lastly cells were labeled with 655nm quantum dot conjugated wheat germ agglutinin (Invitrogen). Each labeled cell population was encapsulated together with 100µM of the Beta-Galactosidase specific fluorogenic substrate fluorescein beta-digalactopyranoside (FDG, Invitrogen) and a specific concentration of the Alexa405 (Invitrogen) fluorophore as a color code, forming 40µm aqueous droplets in a fluorocarbon carrier phase (fig. 2).

Figure 2: Generation of 40µm diameter droplets containing single cells. The number of cells per droplet follows a Poisson distribution. Scale bar is 50µm.

All samples were collected in the same incubation vial, cooled to 4°C to inhibit enzymatic hydrolysis of FDG, then incubated at 37°C for 2 hrs to allow for the enzymatic reaction to take place and subsequently re-injected into the microfluidic chip and dispersed in an oil stream. The droplets individually passed through a site where the fluorophores were excited by 405nm and 488nm lasers. The emitted fluorescence was collected in three photomultiplier tubes associated with optical filters.

Figure 3: Fluorescent signal development over time for a number of different Beta-Galactosidase concentrations encapsulated with FDG substrate. Solid lines show linear models. For 40µm droplets 1.1pM signifies a mean occupancy of 22 enzyme molecules/droplet. For very low average enzyme content the fluorescent signal exhibits a discrete behavior consistent with Poisson statistics and can therefore not be averaged.
RESULTS AND DISCUSSION

To investigate the kinetics and limit of detection of the system, the fluorescein signal produced by Beta-Galactosidase FDG containing droplets is analyzed. The enzyme reaction shows a linear behavior over 5 hours for enzyme concentrations ranging from 110pM to 1.1pM (fig. 3). 1.1pM corresponds to on average 22 Beta-Galactosidase molecules per droplet. Single U937 cells are assayed for the low expressed cell surface proteins CCR5 and CD19 along with a negative control. Figure 4A shows a scatter plot for ~12000 cells analyzed concurrently using droplet color-coding to distinguish the differently labeled cell populations. Comparing the droplet based enzymatically amplified assay (fig. 4B) to flow cytometry (fig. 4C) peak separation from background is improved. Enzymatic amplification allows a greater number of cells to be distinguished from the negative control sample. Thus the enzymatically amplified assay should enable cell sorting by low expressed biomarkers with increased sensitivity compared to conventional flow cytometry.

Figure 4: (A) Scatter plot of the three differently labeled cell populations distinguished using blue droplet color codes. Comparison between the sensitivity of (B) enzymatically amplified immunodetection in droplets with 2hrs incubation and (C) flow cytometry for analyzing cells labeled with antibodies for CCR5, CD19 and a negative control antibody specific to mouse (NC). In flow cytometry analysis, Alexa488 coupled antibodies were used. The vertical line shows a threshold set to allow <0.7% false positives. Using this gate the enzyme amplified assay can distinguish 95% of cells labeled for CCR5 and 56% of cells labeled for CD19 from those labeled with NC compared to 22% and 6.6% respectively using flow cytometry.

CONCLUSIONS

We have demonstrated a method for concurrent multi-sample analysis of low expressed biomarkers using color-coded droplets and enzymatic amplification. Using this method we have analyzed three cell samples labeled with different antibodies concurrently. Enzymatic amplification in droplets increased signal-to-background separation compared to standard methods. The increased sensitivity afforded by enzymatic amplification used together with droplet sorting devices [5] should enable previously unattainable cell sorting by low expressed biomarkers.

REFERENCES