Enzymatic Amplification

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# Detection and Analysis of Low-Abundance Cell-Surface Biomarkers Using Enzymatic Amplification in Microfluidic Droplets**

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We describe a method for detection and analysis of cell-surface protein biomarkers on individual human cells using enzymatic amplification inside microscopic droplets in a microfluidic device. The method provides high sensitivity with high throughput and permits concurrent analysis of several cell samples by incorporation of droplet optical labels. We demonstrate detection of the low-abundance biomarkers CCR5 and CD19 on single human monocytic cells with a significantly higher signal resolution above background than can be achieved using a standard fluorescence-activated cell sorter (FACS).

Microfluidics hold great promise for miniaturizing and improving many biological methods. One appealing approach creates monodisperse[1] aqueous droplets compartmentalized in a continuous oil phase[2,3] using devices derived from soft lithography.[4] Schemes for manipulating droplets have been developed, including splitting,[5] fusing,[6,7] sorting,[8] and reintegrating droplets to a continuous aqueous phase.[9] To date, microfluidic droplet-based systems have been used for cell-free protein expression,[10] protein crystallization,[11] fluorescent reporter protein expression on single bacteria,[12] nucleic acid based assays,[13,14] enzyme kinetics studies,[6,15,16] encapsulation of antibody-producing hybridoma cells,[17] and reporter enzyme expression in mammalian cells.[18] Human cells have been shown to survive for several days in droplets.[19]

Cell-surface proteins constitute an important class of biomarkers, which have already proven to be useful diagnostic indicators of disease state and clinical outcome, for example Her2 in breast cancer.[20]

FACS analysis of cells labeled with fluorescent-dye-coupled antibodies is one of the most commonly used methods for analyzing cell-surface protein biomarkers. However, this technique can be used only for studying highly and moderately expressed biomarkers; a minimum of several hundreds to thousands of proteins per cell is required to achieve a measurable signal.[21] Cellular protein expression levels range from a few copies to $10^7$ copies per cell,[22] and many important proteins, such as cytokine receptors, are expressed at levels too low to detect easily.[21]

Enzymatic amplification methods have been used extensively for standard antibody-based detection of antigens in microliter-scale ELISA assays.[23] Enzyme-based amplification techniques have been developed for FACS[24] but have not been widely used. Microfluidic assays using the enzyme β-galactosidase have been developed for measuring reporter gene expression in single-cell assays[25] and have shown accordance with Michaelis–Menten kinetics even at concentrations as low as a single enzyme molecule per microfluidic chamber.[26] We present a sensitive, high-throughput analytical method for detecting a very small number of surface biomarkers on single cells in droplets formed using a microfluidic device. The method, shown schematically in Figure 1, uses enzymatic amplification, with the fluorescent product remaining confined inside each droplet and increasing in concentration over time. Encapsulation permits amplified detection of extremely low levels of molecules.

Cells were labeled for cell-surface biomarkers using specific biotinylated antibodies and standard protocols (see the Supporting Information). After washing, streptavidin-coupled β-galactosidase was bound to the antibody-labeled cells by a biotin–streptavidin linkage. The enzyme-labeled

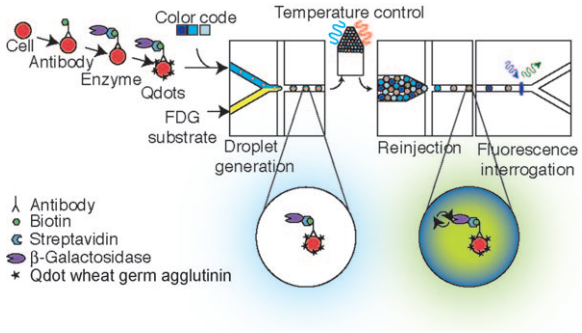


***Figure 1.*** Schematic depiction of the complete assay. Cells were labeled for a specific cell-surface protein with an antibody-coupled enzyme and a quantum dot wheat germ agglutinin (WGA) stain for visualization. Cells were then encapsulated in droplets on the microfluidic chip with a sample-specific color code and a fluorogenic enzyme substrate. After incubation to let the signal develop, each droplet's fluorescence was analyzed. FDG = fluorescein-di-β-D-galactopyranoside.

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cells were injected into the microfluidic device, where the cell stream was combined with a fluorogenic substrate (fluorescein-di-β-D-galactopyranoside, FDG). Monodisperse aqueous droplets form and snap off at the microfluidic nozzle, owing to the force of two opposing immiscible oil streams. The nozzle dimensions and the flow rates determine the droplet size. The device generates 40 μm diameter droplets at rates of up to 2500 droplets per second. Droplets were collected in an oil-filled vial cooled to 4 °C to minimize enzymatic turnover during collection, after which the vial temperature was raised to 37 °C to begin incubation for enzymatic amplification. After a defined incubation time, the packed droplets were reinjected into a microfluidic readout circuit and spaced out into single-droplet trains by infusion of oil. The droplets pass through a laser focused in the channel, and the induced fluorescence from each droplet is quantified using photomultiplier tubes. We analyzed 500–1500 droplets per second.

To characterize the limits of detection for the method, we generated droplets containing decreasing concentrations of β-galactosidase and a constant amount of the substrate FDG (100 μM). After incubation, the droplets were reinjected at intervals and analyzed for the fluorescent reaction product (fluorescein). This signal developed linearly for 5 h using concentrations of β-galactosidase as low as 1.1 pM (Figure 2A), which corresponds to an average of 22 molecules per 40 μm diameter droplet. Below this concentration the number of enzyme molecules in each droplet will begin to follow a Poisson distribution. Figure 2B shows two separate experiments in this concentration regime fitted with a theoretical Poisson model. The model curve indicates an occupancy of 2.65 enzyme molecules per droplet, equivalent to a concentration of 132 fM, corresponding well with the 110 fM concentration used.

When cells are encapsulated using limiting dilution, the number of cells per droplet also follows Poisson statistics.[12,18] To assure single-cell occupancy in the cell-containing droplets, only a small percentage of the droplets contain cells. Calculations using a Poisson distribution model, disregarding cell aggregation, show that diluting the cell suspension such that 9.3 % of the droplets contain a single cell should result in fewer than 0.5 % of the droplets containing more than one cell (90.2 % of the droplets have no cells). We typically generated droplets in this concentration regime at a rate of 200 cell-containing droplets per second.

To characterize our method, we analyzed a human monocytic cell line (U937) for expression of the low-abundance cell-surface biomarker CCR5,[27] a co-receptor in HIV-1 infection.[28] We separately injected two cell samples labeled with CCR5 antibody and an isotypic negative control antibody and analyzed fluorescent signals after 2 h incubation (Figure 3A). The signal developed linearly over time, similar to the experiments using solution-phase β-galactosidase. Increasing the incubation time resulted in an increased peak separation between the CCR5 and negative control samples (Figure 3B).

We also devised an optical coding scheme enabling concurrent analysis of multiple labeled cell populations. For this scheme, droplets are color-coded when they are generated (using different concentrations of a fluorescent dye), and all of the coded droplets are collected into the same vial for incubation and enzymatic amplification. Upon reinjection,

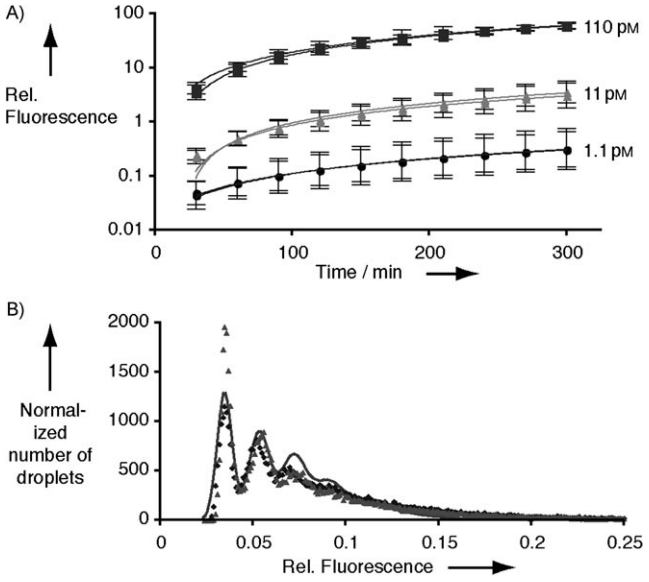


***Figure 2.*** A) Enzyme-amplified signal development in replicate experiments. Geometric mean of signals using three concentrations (110, 11, and 1.1 pM) of streptavidin β-galactosidase incubated with 100 μM FDG at 37 °C for 30–300 min. Solid lines show linear approximations. Error bars show two standard deviations. 48000–74000 droplets were analyzed at each time point. B) Dots show two replicate histograms of the droplet fluorescence signals for 110 fM streptavidin β-galactosidase incubated for 7 h. Solid line shows the theoretical Poisson distribution model.

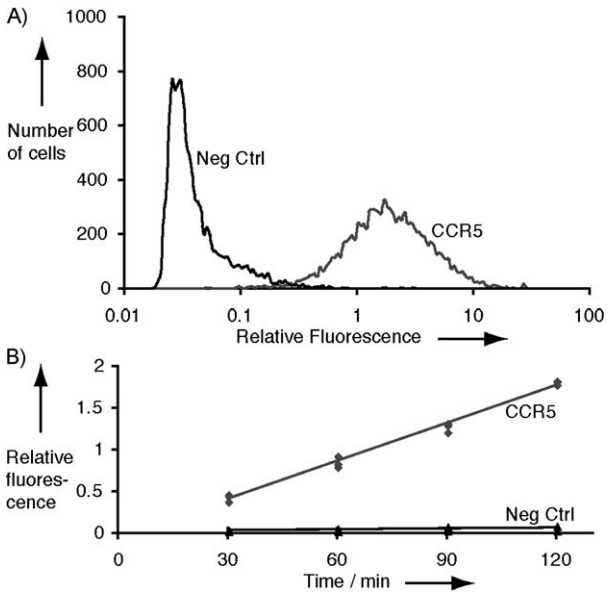


***Figure 3.*** Enzymatic amplification of CCR5 or negative control antibody-labeled U937 cells. A) Histograms of fluorescence from approximately 10000 cells each of the CCR5 and negative control samples. B) Signal development over time described by the geometric mean signal from approximately 10000 cells per point in replicate, fitted to a linear regression model. The average coefficient of variation of the geometric mean of the CCR5 signal for three replicates is 6%.

  

each different droplet population can be distinguished based on its dye label. We examined the enzyme-amplified signal from three populations of the same cells labeled using three different antibodies; antibodies directed against CCR5, CD19 (a B-cell lineage marker), or a negative (isotype) control antibody (Figure 4A). Each population was color-coded with

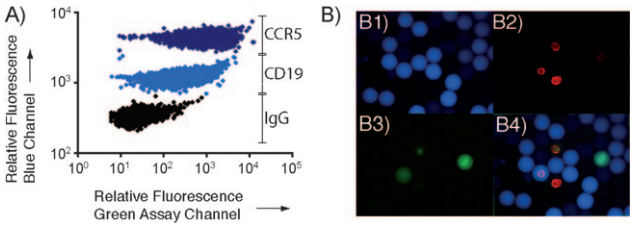


***Figure 4.*** A) Scatter plot showing concurrently analyzed CCR5, CD19, and negative control samples (12750 cells analyzed in total) and corresponding colored microscope images acquired with three filter sets: B1) blue filter set showing the three Alexa405 color-coding concentrations, where the high concentration appears dark blue, medium concentration appears light blue, and very low concentration appears black; B2) red filter set showing the WGA-655 Qdot cell label; B3) green filter set showing the fluorescein assay signal; B4) images of (B1–3) overlaid to demonstrate that only the specifically labeled cells yield a fluorescein signal.

a different concentration of the blue dye Alexa405. Furthermore, cells were stained with a red quantum-dot surface stain (WGA) to discern cell-containing droplets. Figure 4A shows the three clearly distinguishable labeled samples, enabling analysis of the expression levels of the different biomarkers concurrently. Microscope images of the three color assay (Figure 4B) show two droplets with CCR5-labeled cells and two containing cells labeled with the negative control antibody. Only the droplets with the enzyme-labeled cells show the fluorescein assay signal.

Sorting a specific cell population from contaminating background cells, for enrichment or for further analysis, places even more stringent demands on resolving discrete antibody-labeled cell populations. To compare the droplet-based enzyme-amplified method to FACS, we labeled the U937 monocytes for FACS analysis using the exact same protocol used for enzymatic amplification, only exchanging the streptavidin-conjugated β-galactosidase for streptavidin-conjugated Alexa488 in the second binding step. Figure 5 shows histograms from concurrent color-coded enzymatic amplification analysis in the microfluidic droplet assay (Figure 5A) and standard FACS analysis (Figure 5B). The enzyme-amplified droplet assay resolves both the CCR5- and CD19-positive cells away from isotype control stained cells with greatly improved separation than standard FACS. Using a gate allowing 0.7% false positives (vertical line), 95% of cells labeled for CCR5 and 56% of cells labeled for CD19 can individually be discriminated from the negative controls by enzymatic amplification in droplets, compared to 22 and 8.1% using the FACS machine. Further improvements in resolving separation would be possible using longer incubation times.

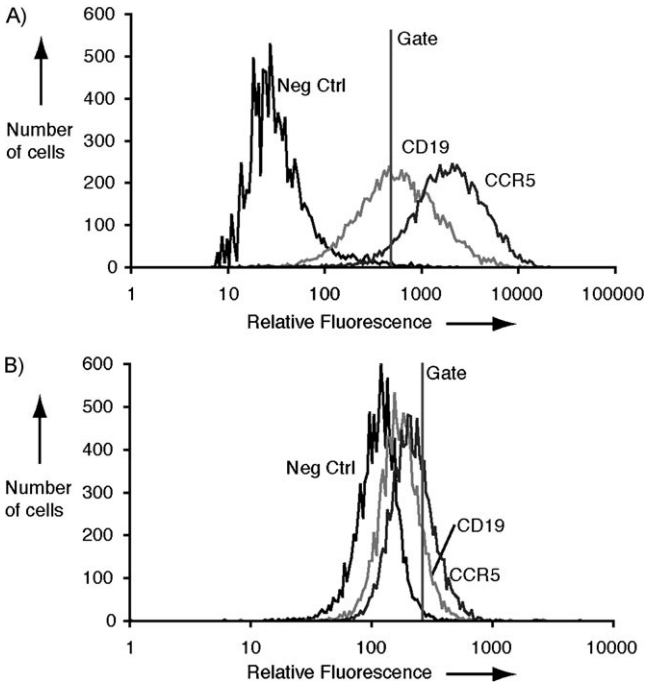


***Figure 5.*** Comparison of the resolution and potential sorting capabilities between A) concurrent multisample analysis by enzymatic amplification in droplets and B) standard FACS analysis. In (A), the histogram shows the fluorescence signal from 8331, 8781, and 9998 cells labeled with biotinylated CCR5, CD19, or a negative control antibody, respectively, with subsequent enzymatic amplification. Each histogram in (B) contains approximately 10000 cells labeled with the same antibodies and a streptavidin-conjugated fluorescent dye. The vertical line shows a gate allowing 0.7% false positives.

In summary, we have presented a method for studying low-abundance cell-surface biomarkers on individual human cells using enzymatic amplification in microfluidic droplets. We also demonstrate the use of droplet color codes, allowing for concurrent analysis of multiple samples. In this proof-of-principle study, we have analyzed single human U937 cells for the low-abundance cell-surface receptors CD19 and CCR5. Increased signal discrimination compared to standard FACS methods will enable sorting of cells on the basis of these low-abundance markers.

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