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Ultrasensitive Detection of Low-Abundance Surface-Marker Protein using Isothermal Rolling Circle Amplification in Microfluidic Nano-Liter Platform

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Abstract

With advances in immunology and cancer biology, there is an unmet need for increasingly sensitive systems to monitor the expression of specific cell markers for the development of new diagnostic and therapeutic tools. To address this challenge, we have applied a highly sensitive labeling method that translates antigen-antibody recognition processes into DNA detection event that can be greatly amplified via isothermal Rolling Circle Amplification (RCA). By merging the single-molecule detection power of RCA reaction with microfluidic technology we were able to demonstrate that identification of specific protein markers can be achieved on tumor cell surface in miniaturized nano-liter reaction droplets. Furthermore, this combined approach of signal amplification in a microfluidic format could extend the utility of existing methods by reducing sample and reagent consumption and enhancing the sensitivities and specificities for various applications, including early diagnosis of cancer.

Keywords

Microfluidic droplets; RCA; single-molecule detection; antigen-antibody; tumor markers

1. Introduction

Both single-molecules detection (SMD) methods and microfluidic techniques have been increasingly applied to biological systems over the last ten years. SMD and microfluidic techniques, which belong to the expanding field of nano-science, open up new horizons in analytical chemistry, biology and medicine. Observing a single molecule removes the usual ensemble average, allowing the exploration of hidden heterogeneity where the individual characteristics can be measured. The use of nano-liter reaction volumes and parallel sample processing represent potential advantages of microfluidic devices, making them ideally suited to total chemical and bioassay analysis, ultra-high-throughput screening applications, and other cases where reagents are available in limited quantities. By merging both SMD

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and microfluidic techniques, it may be possible to achieve the analysis and manipulation of samples on a scale comparable to the size of biological cell markers and macromolecules.

Detection and subsequent analysis of cell specific surface markers are important for the development of diagnostic and therapeutic modalities for the management of cancer [1]. Low expression of many of these markers prevents their practical use as suitable targets for manipulation of the associated tumors [1,2]. Current strategies for detection of these low-expression markers use flow cytometric analysis with amplification either by enzymatic staining or by increasing the number of fluorophore molecules, utilizing quantum dots (Qdots), fluorescent microspheres, or extra “layers” of reagents [2]. Some of the important limitations of the above methods include the time needed to perform multiple steps and the necessity for relatively large volumes of reagents and samples as well as false-positive events. Thus, development of comparable target amplification methods with single-molecule resolution for protein markers using small volumes of reagents and samples could substantially improve development of diagnostic and therapeutic modalities.

Although it is not yet possible to chemically duplicate protein markers for identification purposes, it is possible to tag them with oligonucleotide markers. These oligonucleotide tags can then be amplified with nucleic acid amplification methods and detected with DNA detection methods [3]. A labeling protocol with single-molecule sensitivity and ultrahigh detection specificity of antigen-antibody recognition would further enhance identification.

To address this need, we have developed a robust, highly specific and sensitive screening protocol for detection of protein markers in a microfluidic nano-liter reaction droplet system. This method employs highly specific antigen-antibody recognition combined with DNA-amplification using a nano-liter Rolling Circle Amplification (RCA) platform. RCA is a simple amplification method featuring single-molecule sensitivity with specificity owing to the DNA stringent strand matching requirement and its high signal amplification efficiency [3]. Also, since the amplification is isothermal, the RCA process preserves the integrity of the antibody-antigen complexes and therefore can be used for surface protein targeting. In our approach, cell surface proteins are labeled with specific biotinylated-antibodies (Figure 1 A(1)) that are later conjugated to DNA tags via biotin-avidin bridge (Figure 1 A (2, 3)). These conjugated constructs [4] serve as scaffolds for the directed assembly of RCA on cell specific markers. The RCA assembly produces a long one-dimensional DNA substrate with a designed DNA sequence of hundreds to millions of linear replicas of DNA mini-circles (Figure 1 A(4)). These amplified repeat units of DNA (i.e., RCA amplicon) are directly labeled during the synthesis by introducing fluorescently labeled nucleotides at high density ensuing 10^4 -fold amplification efficiency for easy visualization (Figure 1 B(2)).

2.Results and discussion

2.1 Detection of individual RCA amplicons

To demonstrate the sensitivity of our design we first performed experiments focusing on detection of individual RCA amplicons using streptavidin-coated glass slides (10^5 streptavidin molecules per mm^2). A measured amount (i.e., 0, 1, 10, 100, 1,000 or 10,000 molecules per mm^2) of a pre-hybridized equimolar mixture of DNA-tagged primer and mini-circles was spread onto slides. The slides with the resultant streptavidin-biotin rolling circle complex (i.e., constructs) [4] (Figure 2 A) were then immersed into RCA reaction buffer with all RCA necessary components in the presence of CY-3 labeled dCTP. The samples were incubated at 37 °C for 4 hours and then extensively washed and dried in air (see supplementary data for details), before being subjected to high-resolution imaging scanner analysis. A GenePix fluorescence-imaging scanner provided the measurement of total fluorescence intensity of individual RCA amplicons per specified area. An area of 10

mm × 10 mm (i.e., 10⁶ pixels ~20–25% of slide surface) was analyzed using SigmaPlot software and images were processed using ImageJ software. Three different detection areas for each concentration were analyzed.

Using a microarray scanner with multiple pixel resolutions of 10 microns² (1 pixel = 10 micron × 10 micron) we demonstrated a linear dynamic response in total fluorescent intensity within the 1 to 10000 molecules/mm² surface-immobilized DNA rolling circle complex concentration range (Figure 1 B, C). This allowed differentiation at a level between 1 and 10 amplicons and corresponds to the detection limit of approximately a single DNA tag molecule per cell. Thus, a single target molecule can be observed clearly compared with the background noise.

2.2 Protein amplification protocol for cancer cell surface protein markers detection

Next this protein amplification protocol was employed to cancer cell surface protein markers detection. Circulating tumor cells (CTCs) offer an excellent source of surface markers to monitor progress of treatment of cancer patients. An epithelial cell adhesion molecule (EpCAM) is frequently expressed on these carcinomas and is typically used as a convenient surface marker [5]. However expression of EpCAM in some tumors is below the threshold of direct detection using flow cytometry systems. Hence multiple tumor cell markers must be applied to provide reliable identification of those cells using conventional detection methods [6]. We therefore decided to use the expression of an EpCAM on the PC3 human prostate cancer cell line as a model target for amplification. Our proposed single target amplification method was then compared to a traditional direct immunoassay detection technique using anti-EpCAM antibodies labeled with fluorescents.

The interrogated PC3 cells were fixed on glass slides. To create the rolling circle complex in our design, the cells were first conjugated with biotinylated antibodies specific for the protein epitope of interest, i.e., EpCAM, (Figure 1 A(1)) and then with specific DNA tag primer via an avidin-biotin bridge (Figure 1 A(2)). Next, the DNA mini-circle was coupled to the previously immobilized primer (Figure 1 A(3)). These constructs were then isothermally amplified by phi29 DNA polymerase in the presence of CY-3 labeled dCTP (Figure 1 A(4)). In the direct approach, the cells were labeled with biotinylated anti-EpCAM antibodies and then detected with Cy3-fluorescence labeled streptavidin. All cells were counterstained by DAPI (4, 6-diamidino-2-phenylindole) (see methods in the supplemental material).

Figure 1 B(2, 3) shows that our constructed assembly combined with RCA technique provides clearly distinguishable fluorescently labeled PC3 cells enabling reliable analysis of the expression of EpCAM biomarkers. In contrast, minimal staining was observed in the direct detection method (Figure 1 B(1)). Since the DNA amplification product in linear RCA method is in a coiled shape, in our design this gives rise to local clusters of fluorescent molecules tethered to cell surface that are unambiguously visible with a standard fluorescent microscope as a bright “dot” (Figure 1 B(3)). Thus, a single target molecule can be observed clearly compared with the background noise as have been proved by our data presented above (Figure 2 B, C).

To confirm the specificity of detection and to exclude the possibility of nonspecific probe binding to cell structures, a set of reference experiments were carried out. Table 1S (supplemental material) shows the conditions used for the set of control experiments. Negative control cells, i.e., lymphocytes, which do not express EpCAM (supplemental material Figure 1S) were also included in the test. Based on the results obtained from this set of experiments, no false positives were produced implying that the system is highly specific.

2.2 Microfluidic Nano-Liter Platform for identifying low abundance proteins on cancer cell surfaces

To take full advantage of RCA for identifying low abundance proteins on cell surfaces, we integrated the RCA reaction inside monodisperse aqueous emulsion nano-liter droplets containing single cell and linear RCA reaction mix (detailed in supplemental material). Recently, this type of microfluidic droplet system has attracted significant interest as they allow pico- or nano-liter volumes of samples and reagents to form extremely high-density microreactors [7]. Such well-defined miniaturized systems prevent sample loss, cross contamination, achieve fast mixing, enhance thermal transfer, and chemical reaction rates [7]. This microfluidic system for RCA offers the advantage of reduced reagent and sample consumption, which is critical when limited sample material is available.

In order to evaluate our method for screening with single-cell resolution, a drop-based microfluidic device was used to encapsulate PC3 cells in distinct nL-sized drops of RCA reagents (Figure 3 A). The Polydimethylsiloxane (PDMS) microfluidic system which generates monodisperse droplets in a microchannel through shearing flow at a T-junction or a flow-focusing zone has been previously described ^[17] (Figure 3 B(1, 2)). Figure 3 A schematically illustrates the microfluidic platform for droplets generation. Three perpendicular inlet channels form a nozzle shown in Figure 3 B(1). The center stream is the suspension that contains cells (10^5 cells mL^{-1}) and RCA mix, while the two opposing side streams contain the oil phase. Individual syringe pumps control the flow rate of oil and cell suspension. To form droplets, the flow rate ratio of water to oil is adjusted to the $Q_w/Q_o = 0.5$ ($Q_w = 1 \mu\text{L}/\text{min}$ and $Q_o = 2 \mu\text{L}/\text{min}$). The generated droplet volume is ~ 1.8 nL corresponding to spherical drop diameter of $150 \mu\text{m}$. For single cell studies, all drops should contain at most, one cell, so that the majority of drops contain no cell at all since the encapsulation process follows Poisson statistics [7]. While the reported frequency of single-cell-bearing drops is rather low, the high production and screening rate that can be achieved with microfluidic devices can result in the isolation of a large number of defined secreting cells.

Individual droplets were focused in the incubation channel for amplification reactions of cell surface markers and optical interrogation (Figure 3 B (3–7)). Since RCA is isothermal, there is no need for thermal cycling that requires sophisticated and expensive instrumentation such as seen in microfluidic droplets using polymerase chain reactions (PCR) [7b] or enzymatically catalyzed amplifications with beta- galactosidase or horseradish peroxidase [1c]. Assuming a replication rate of phi29 DNA polymerase of 1400–1500 bases/min [8] and a circle size of 80 nucleotides, each rolling circle product (RCA amplicon) results in approximately 80,000 nucleotides per hour, with total circle amplification as high as 1000 fold per hour amplification resulting in accumulating fluorescent product directly on the cell surface marker. Figure 3 B(6) shows the fluorescent image of encapsulated cells in RCA reaction at the beginning of the incubation, and Figure 3 B (3,4, 7) after 1.5 hours. Figure 3 B(4) shows the interactive 3D surface plot of fluorescence intensity distribution (axis (z)) of image 3 within each droplet at the end of the amplification process. The distinguish signal can be recorded and quantified for each cell in the droplet when compared to the empty droplets which represent a background signal. Therefore, our results clearly demonstrate that the RCA was successfully performed on a single PC3 cancer cell enclosed in the droplets (Figure 3 B(3, 7)). The control experiments were conducted to establish the unspecific binding in the droplet format (supplementary), based on the results obtained from this set of experiments, no false positives were produced implying that the system is highly specific.

Despite the advantages and potentials mentioned above, this prototype nano-liter RCA platform remains to be improved for high throughput screening analysis. As the future direction, we intend to expand the current assay by incorporating sorting systems similar to

the one schematically illustrated in Figure 3 A. In this setting, real-time RCA in nL-drops can provide with quantitative measurements, if fluorescence is recorded using a custom optics system and software similar to one described in the literature 4,¹⁴. In addition, a high-speed droplet sorter based on fluorescence intensity recently has been reported [9]. This droplet sorting method combined with our nano-conjugated RCA approach may allow isolation of positive EpCAM cells for further studies.

3. Conclusions

In summary, we have developed a RCA strategy for ultrasensitive detection of low-abundance protein markers. This constructed RCA assembly allows visual detection of 1–10 surface protein molecules per cell, with at least 10^3 fold amplification of fluorescent intensity over a range of conventional direct labeling procedure. Furthermore, by merging the single-molecule detection power of RCA strategy with microfluidic technology we were able to demonstrate that identification of specific tumor marker, EpCAM, can be achieved on tumor cell surface in miniaturized nano-liter reaction droplets. Various therapeutic strategies such as antibody- or gene-directed therapy, exploiting the selective expression of markers on the surface of tumor-associated cell, could benefit from this sensitive and rapid protein surface detection method. The correlation between surface protein expression data obtained from tumor cells before and after treatment and after the response of patients to various therapeutic regimens can be obtained on a single cell level. In addition, individual tumor cell analysis, if applied for detection of CTCs cells, could provide important information, once cells are recovered from the drops for future testing. Moreover, the application of this approach to microfluidic droplets could extend the utility of existing methods by enhancing the sensitivities and specificities for various applications, we expect that it might become a promising tool for early-stage diagnosis of cancer and proteomic studies.

4. Experimental Section

Microfluidic device fabrication

Microfluidic flow chambers are fabricated by soft lithography. Negative photo resist SU-8 2025 or SU-8 2100 (MicroChem, Newton, MA) is deposited onto clean silicon wafers to a thickness of 50 μm , and patterned by exposure to UV light through a transparency photomask (CAD/Art Services, Bandon, OR). The Sylgard 184 poly(dimethylsiloxane) (PDMS) (Dow Corning, Midland, MI) is mixed with crosslinker (ratio 10 : 1), poured onto the photoresist patterns, degassed thoroughly and cured for at least 1 h at 65° C. The PDMS devices are peeled off the wafer and bonded to glass slides after oxygen-plasma activation of both surfaces. To improve the wetting of the channels with mineral oil in the presence (1% w/w) of the surfactant (span80), the microfluidic channels are pretreated with Aquapel (PPG Industries, Pittsburgh, PA) by filling the channels with the solution as received and then flushing with air. Polyethylene tubing with an inner diameter of 0.38 mm and an outer diameter of 1.09 mm (Becton Dickinson, Franklin Lakes, NJ) connects the channels to the syringes. Syringes are used to load the fluids into the devices, and flow rates are controlled by syringe pumps.

PC3 Cells

The ATCC adherent cells line PC-3 (V/N CRL-1435) were sub-cultured between 1:3 and 1:6 rations in serum filled cell specific media F12K (ATCC, C/N CRL-1435). The cells were fixed on the slide in methanol/glacial acid (3:1) for 15 min at room temperature. Slides were washed three times for 2 min in 50 ml of PBS. Next, the fixed cells were incubated with biotinylated anti-EpCAM antibodies (1 $\mu\text{g}/\text{ml}$, R&D systems (BAF960)) diluted in

binding buffer (PBS 1%, BSA 0.2% Tween-20) for 45 min at room temperature (RT). Slides were washed in PBS, as above, and incubated with strep-avidin (1 µg/ml) for 45 min at RT and then washed with PBS, as above. Next the biotinylated primer (1 µg/ml) was applied. Slides were washed in PBS, as above, and the circle padlock (108 circles) was then added. For the droplet studies, the same steps for labeling the cells with the circle padlock were preformed in suspension.

Counting of individual RCA amplicons

A 5'-phosphorylated 88-nt circularized probe was circularized by ligation with T4 DNA ligase in the presence of biotinylated primer (splint), also employed as a primer in RCA. A specified amount (10 ml) of the DNA minicircle complex with the primer was spread onto streptavidin-covered glass slides (105 streptavidin molecules per mm²; 22 × 60 of total area). After a few minutes, to the slides with the resultant streptavidin-biotin rolling circle complex 40 ml of solution with all RCA necessary reaction components was added. Specifically, the samples were incubated at 37 °C for 4 hours with 1 U of Phi29 DNA polymerase and 20 mM dNTPs in the buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 25 mM NaCl, and 5 mM DTT. 10% of dCTP were substituted by their Cy3-fluorescent analogs. The spot was covered with the thin cover glass and sealed with glue. Then the samples were washed by immersing the slides in (i) 2xSSC with 2% sodium dodecyl sulfate, (ii) 2xSSC with 50% formamide, (iii) 2xSSC, and (iv) 0.2xSSC; dried in air; and subjected to GenePix fluorescence-imaging scanner analysis. The images were processed by ImageJ software.

RCA

Previously immobilized biotinylated primer on the cell was employed as a primer in RCA. The RCA reaction mix consisted of 1x phi29DNA polymerase buffer, 10 units phi29 DNA polymerase (New England Biolabs), 200 µM dNTPs and a 40 µM of Cy3-dCTP fluorescently labeled dCTP (GE Healthcare). For RCA in droplets, suspension of PC3 cells (105 cells /ml) previously labeled with circle padlock in RCA reaction mix were encapsulated. After encapsulation the RCA reaction was performed in droplets in the incubation channel at 37°C for 1.5 hours.

For the slide studies, the RCA reaction mix (50 µl) was dropped on the slide, the slide was then covered with a cover slip and sealed with rubber cement. The RCA reaction was performed at 37°C, for 1.5 hours in a humidifying chamber. Then the slides were washed twice in buffer A (100 mM TrisHCl, 150 mM NaCl and 0.05% Tween20) and once in 4XT buffer (4X SSC, 0.05% Tween 20). After washing, a drop of mounting medium counterstaining DAPI (4, 6-diamidino-2-phenylindole) at 50 mg/ml in PBS was added. The slides were then recovered with a cover slip, and after standing at RT for 5 min, the cover slip was secured by using nail polish.

Image analysis

Fluorescence images of droplets were captured on a Zeiss 200 Axiovert microscope using an AxioCAM MRm digital camera. For the cell studies fluorescence signal were captured separately using appropriate filter sets for DAPI and Cy3. Image process and analysis was conducted by ImageJ software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic illustration of constructed assembly for the detection of a target surface protein. (A) (1) binding of biotin-labeled antibodies; (2) coupling of biotinylated DNA-tag primer via an avidin bridge; (3) hybridization of the DNA mini-circle; (4) Rolling Circle Amplification (RCA). (B) Microscope images of PC3 cell fixed on the glass slide and counterstained with DAPI (Blue). Expression of an EpCAM marker (Red) detected by (1) direct immunoassay labeling with anti- EpCAM antibodies and (2, 3) by conjugated RCA strategy.

Figure 2.

Visualization of individual RCA amplicons tethered to the surface-immobilized proteins. (A) Schematic drawing of rolling circle construct assembly. (B, C) Detection of surface-immobilized RCA amplicons with GenePix scanner. A specified amount ("no circle" control, 10⁰, 10¹, 10², 10³ and 10⁴) of the DNA minicircle complex with the DNA tag primer was spread onto approximately 1 cm² area of streptavidin-covered glass slides (10⁵ streptavidin molecules/mm²). Images were processed at 10 micron resolution by ImageJ software. Left to right: Areas 1mm × 1mm are shown for approximately 1000, 100 and 10 molecules/mm², correspondently. Total fluorescence measured as a function of rolling circle complex load. Six different areas of 10 mm × 10 mm (10⁶ pixels) for each concentration were analyzed using SigmaPlot software. Pixels with fluorescence less than 200 FU were considered as background and were not counted. Data point with standard deviation is mean values of triplicate data from two arrays.

Figure 3. Schematic illustration of the RCA strategy. (A) The microfluidic cell sorting system for nano-RCA droplets. (B) (1) Drop-based microfluidic device which was applied to encapsulate PC3 cells in distinct nL-sized drops of RCA reagents; (2) The RCA reaction droplets are conveyed by the oil focused in the channel for amplification of cell surface marker and optical interrogation, (3–7) Incubation channel, (4) Shows the interactive 3D surface plot of fluorescence intensity distribution in image 3 (6) The fluorescent image of encapsulated cell with constructed RCA assembly in RCA reaction mix at the beginning of the incubation and (3,7) after 1.5 hours of incubation.