DIGITAL READOUT PLATFORM FOR WATER-IN-OIL DROPLET IMMUNOASSAYS RUNNING ON A CELL-PHONE FOR POINT OF CARE VIRAL LOAD SENSING

Patrick A. Sandoz[1], Ahmet F. Coskun[2], Aram J. Chung[1], Westbrook M. Weaver[1], Oladunni Adeyiga[1], Delaram Khodadadi[2], Aydogan Ozcan[2], Dino Di Carlo[1]

[1]Department of Bioengineering, [2]Department of Electrical Engineering
University of California, Los Angeles

ABSTRACT

We report a new technique to improve signal fidelity for implementing on-chip digital immunoassays running on a cell-phone. The assay targets the human immunodeficiency virus (HIV) p24 capsid protein to quantify viral load as part of a compact device for point of care (POC) testing. The sample is digitized as water-in-oil droplets and integrates advanced techniques utilizing Poisson distributions of target molecules, robust emulsion generation, and increasing sensitivity up to previously unachievable levels.

KEYWORDS

Digital ELISA, Water in oil emulsion, Fluorophore stabilization, Wide field of view fluorescence imaging

INTRODUCTION

Recent progress in multi-phase digital microfluidics has opened new horizons for viral load determination coupling highly sensitive single-molecule assays to quantified readouts. Sensing by digital PCR which targets and amplifies the two viral nucleic acid sequences present per viral particle is currently the most widespread method [1,2,3]. Nevertheless improvements in assay sensitivity, dynamic range, and reduced platform size are needed to achieve automated portable cost effective devices.

Alternatives to digital PCR were demonstrated over the last decade targeting more abundant proteins in a sandwich enzyme-linked immunosorbent assay (ELISA) [4]. From this perspective and according to the current clinical assay [5], we have developed an optimized on-chip digital readout platform towards HIV p24 capsid protein sensing which integrates a reproducible water-in-oil emulsion generation, a rapid and stable signal amplification and a cell-phone based cost effective portable fluorescent imaging system.

EXPERIMENTAL

As presented, our device targets more abundant HIV p24 capsid protein in a sandwich ELISA (Fig.1a). At low protein concentration, one target protein is associated to one 10µm magnetic microparticle (MµP). Using MµPs allows simple magnetic catch-and-release of the particles and improves on-chip conjugation efficiency. Target protein capture is then reported from each droplet with p24-bound MµPs by the horseradish peroxidase (HRP) enzyme conjugated to a secondary antibody. In the presence of Amplex UltraRed fluorogenic substrate (a resorufin derivative), HRP produces a strong fluorescent signal. Conjugated antibodies against p24 were purchased from ViroStat Inc. (USA), synthetic p24 core protein was purchased from ProSpec-Tany TechnoGene Ltd (IL), streptavidin-coated MµPs were purchased from Corpuscular Inc. (USA), Amplex Ultra Red substrate was purchased from Life Technologies Corp. (USA). Digitization is achieved by encapsulating the MµPs in water-in-oil droplets (Fig. 1b).

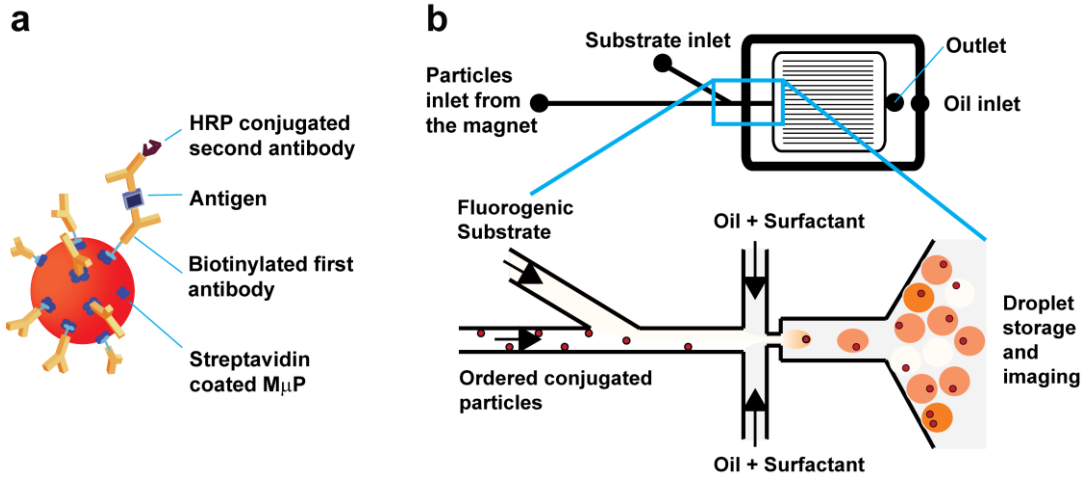

Figure 1. (a) On-MµP sandwich ELISA of the p24 antigen using a HRP enzyme conjugated to the second antibody for reporting a successful capture. (b) The conjugated MµPs are washed and then injected and mixed with the enzyme fluorogenic substrate prior to being encapsulated as a water-in-oil emulsion. The droplets are then stored and the signal is amplified in the droplet population according to the initial antigen concentration.

The microfluidic chip was fabricated by using standard photolithography and polydimethylsiloxane (PDMS) replica molding protocols. SU-8 was spun at 3000 rpm to produce a 51µm thick layer on a 4'' silicon wafer. The three phases were injected at constant flow rate using standard syringe pumps (Harvard apparatus, USA).

The droplets are collected downstream in a massively parallel two dimensional array of traps to be imaged. Due to the dense storage of the droplets in the 2-dimensional parallel array, the emulsion was stabilized using a 10% (w/w) surfactant mixture of span80 and tween80 dissolved in mineral oil.

Inertial ordering of ELISA particles prior to droplet generation ensures each droplet contains one particle, allowing Poisson statistics to be employed to analyze sample readout (adapted from [6]). This greatly simplifies detection using a POC-type device, and negates the need for high sensitivity and resolution imaging systems.

Readout fluorophores are generally amphipathic, resulting in poor signal stability with water-in-oil digital systems. We have overcome this previously fundamental limitation with the addition of sucrose in the aqueous phase, significantly conserving the signal stability. The fluorophore leakage was quantified on-chip from the droplet signal intensities in chambers with different sucrose dilutions to identify a relevant concentration. This technique significantly improves signal fidelity for generating and imaging droplets.

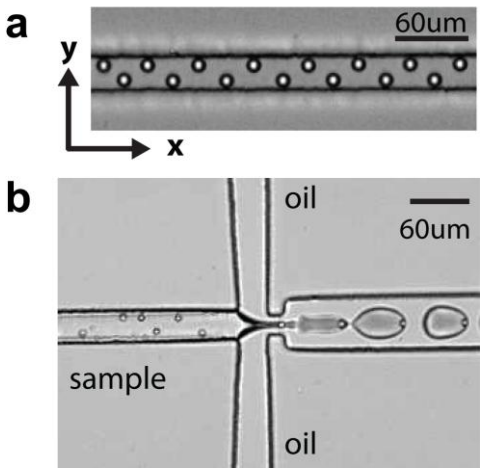

Figure 2. (a) The conjugated MµPs are ordered by inertial focusing in high aspect ratio channel. (b) the conjugated MµPs are single encapsulated at a flow focusing junction.

Finally, we increased the dynamic range of concentrations that can be detected by imaging over a large field of view using a variant of cell-phone based fluorescence imaging adapted from previous work [7]. This cost effective and portable imaging device has a low cost lens and using plastic color filters enables future multiplexing possibilities. The imager is directly mounted on a cell-phone camera or on a webcam and is well adapted for POC testing or telemedicine applications.

RESULTS AND DISCUSSION

In order to avoid any nonspecific binding observed from the conjugated antibodies, the platform was first calibrated by conjugating biotinylated HRP directly on the streptavidin coated MµPs using a 5-orders of magnitude serial dilution of the enzyme. Ordering of the conjugated particles was obtained at a flow rate of 12µl/min (Fig. 2a). Reproducible, stable, monodisperse droplets of 40µm were generated at a production rate of 1 kHz (Fig. 2b). Around 15'000 droplets were stored in the massively parallel 2-dimensional array and efficiently stabilized by the surfactant presence.

As a primary observation, it appeared that the readout fluorophore produced by the HRP turnover had a higher stability in the oil phase and immediately leaked out of the droplets inducing a bright surrounding environment and an intensity decrease of more than 80% in the positive droplets. This problem was overcome by incorporating 25% (w/v) of sucrose in the aqueous phase. Indeed, this leakage phenomenon is observed with our system in the presence of surfactant (Fig. 3a), and seems to result from an interaction of the fluorophore with the surfactant at the droplet interface. The addition of sucrose allowed for conservation of more than 90% of the signal (Fig. 3b).

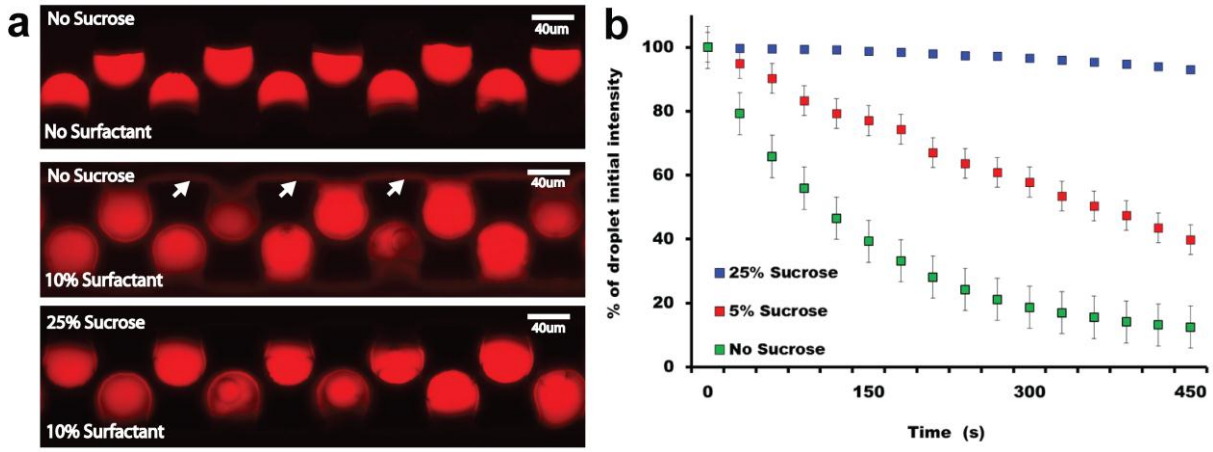

Figure 3. (a) On-chip intensity-based quantitative assay of the resorufin fluorophore stability in mineral oil. Aqueous droplets containing the fluorophore were compartmentalized by oil in lateral chambers. In the presence of surfactant the fluorophore leaked out of the droplets into the oil phase (white arrows). With the addition of sucrose, the fluorophore leakage was highly reduced. (b) The droplet intensity measurements showed that in the presence of surfactant, the addition of sucrose preserves 90% of the signal whereas more than 80% of the signal was lost in the standard case.

Thereby, the signal was stably conserved inside a positive droplet during the substrate turnover period of time, enabling discrimination and counting of the number of encapsulated single particles and the corresponding number of positive droplets (Fig. 4a).

Finally, all the droplets stored in the massively parallel array were imaged using a variant of cell-phone based fluorescent imaging. This cost effective and portable imaging device showed a resolution of ~10µm (Fig. 4b) and had a wide field-of-view of 1cm^2. The sensitivity and the dynamic range can be improved as the complete 2-dimentional array is imaged in one picture and all chambers in the array are successively reloaded with new droplets. Currently, the digital level is observed at a theoretical conjugated concentration of 1000 enzyme per MµP. Obviously, the conjugation efficiency and the enzyme activity have to be assumed lower than 100% and future developments of the platform will be focused in improving the sensitivity of the sandwich ELISA.

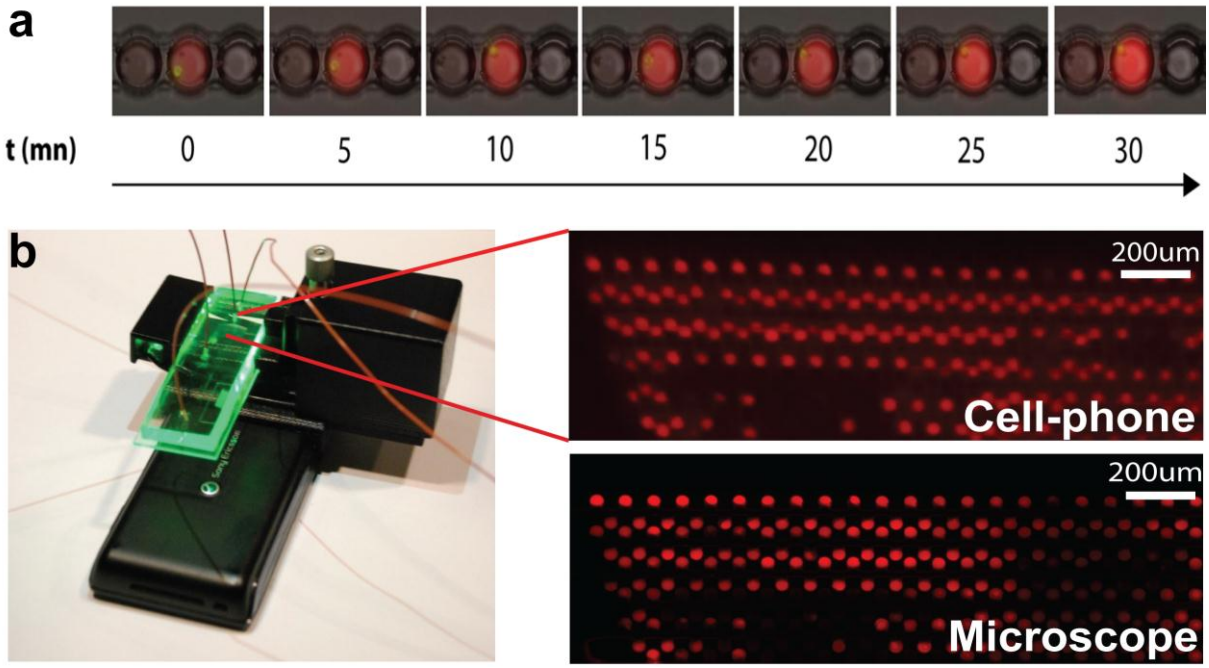

Figure 4. (a) Superposed bright-field, red and green fluorescence pictures in a time lapse imaging sequence. With the addition of sucrose, positive droplets showed stable signal amplification. A FITC coating of the MµPs improved the particle tracking in the droplet population. (b) After a digital zoom in, the cell-phone based fluorescence imaging resolution macthed with a standard microscope 10x objective.

CONCLUSION

Here we have demonstrated the assembling of a digital readout portable and cost effective platform for viral load sensing through a sandwich enzyme-linked immunosorbent assay running on a cell-phone. Previously, one of the most promising techniques to stabilize digitization used fluorinated oil, requiring complex surfactant synthesis as well as high flow rates in droplet generation and screening. The presented resorufin fluorophore stabilization by the addition of sucrose provides signal fidelity improvement as our approach enables now a variety of emulsion handling, on-chip storage and screening possibilities at lower costs and with better availability.

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CONTACT

Dino Di Carlo +1-310-983-3235 or dicarlo@seas.ucla.edu
Patrick Sandoz +41-79-327-9548 or psandoz@ucla.edu