A SIMPLE SYSTEM FOR IN-DROPLET INCUBATION AND QUANTIFICATION OF AGGLUTINATION ASSAYS

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ABSTRACT

This work reports on a simple system for quantitative sensing of a target analyte based on agglutination in microchannels. Functionalized microbeads and analyte with no prior incubation are flowed in droplets (~2μL) through a thin silicone tube filled with mineral oil at a flow rate of 150 μL/min. Hydrodynamic forces alone produce a highly efficient mixing of the beads within the droplet, without the need of complex mixing structures or magnetic actuation. The setup allows rapid observation of agglutination (<2 min), which is quantified using image analysis, and has potential application to high-throughput analysis.

KEYWORDS: Microfluidic, Agglutination Assays, Image Processing, Droplets.

INTRODUCTION

Agglutination assays are typically performed manually on cards and are qualitative or semi-quantitative. Performing them in a microfluidic device has advantages such as increasing their sensitivity and speed while reducing the required sample volume [1]. Antibody and protein agglutination assays in plugs have been demonstrated previously using microfluidic devices [1-3]; however, these studies have been done at low flow rates and use either winding channels to enhance the mixing of the agglutination components (5.5μl/min) [2] or a concentrated magnetic field (up to 1μL/min) [3], and require previous incubation. Simple droplet velocity control can be used to affect the circulation and aggregation of non-functionalized beads, as demonstrated by Kurup et al. [4].

In card agglutination assays, the user is required to manually stir the agglutination mixture and then rock the card for several minutes. Mixing is an important step when performing these assays, as the beads need enough circulation to come into close proximity with each other and agglutinate, therefore a more efficient mixing is desirable.

This work further expands and refines a preliminary system previously presented by our group [5], which takes advantage of the efficient mixing generated within a droplet at high velocities to perform agglutination in a simple and rapid way, without the need for magnetic fields and at flow rates that allow for much higher throughput in the future. Internal vortices inside a droplet are created by the parabolic profile present in laminar flow, which within the frame of reference of a plug results in a forward flow at the center and a reverse flow towards the edges in contact with the channel walls [6].

EXPERIMENTAL

The system used to perform the agglutination assays is illustrated in Figure 1. It consists of a 150 cm length of 0.51 mm internal diameter silicone tube, driven on one end by a syringe pump. For each test a droplet of agglutination mixture (~2μL), formed by functionalized microbeads and analyte, was pipetted into a container with mineral oil, and then flowed through the tube at 150 μL/min using a syringe pump. A polydimethylsiloxane (PDMS) block was cast around the tube at mid-length for index matching to facilitate optical observation.

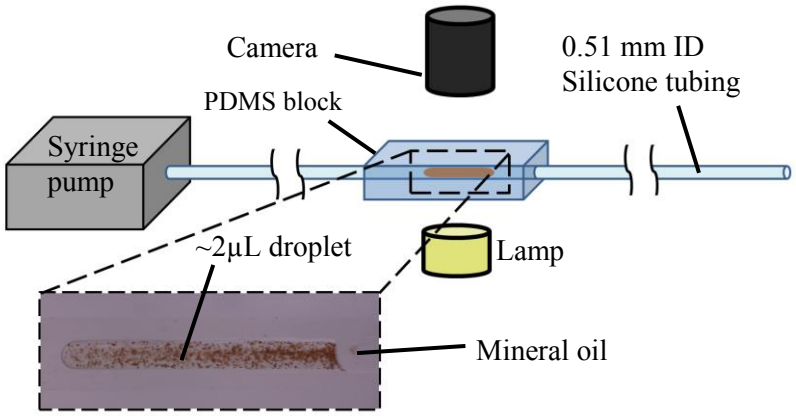

Figure 1: Schematic representation of the experimental setup. A 150 cm silicone tube is filled with mineral oil and driven by a syringe pump. Droplets containing a bead and analyte mixture are inserted into the tube by the open end. Images are taken with a camera placed over a PDMS block (used for refraction index matching with the tube) at mid-length of the tube.

A Canon EOS 5D Mark II camera with a 1-5x macro lens was placed above the PDMS block and a white LED lamp with a diffuser underneath. Videos at 30 frames per second with a resolution of 1920 x 1080 pixels were taken of the passing droplets. Images were extracted and processed with a script in Matlab. A relative measure of agglutination was quantified by the distribution of the image's grayscale values, Figure 2. Regardless of bead concentration, negative tests have a narrow color distribution, whereas positive tests have a wider one (dark agglutinates and lighter background), which can be measured by the standard deviation of the pixel values.

Silicone oil AR20 (Sigma) was selected as the carrier fluid for index matching. We found early on during experimentation that the aqueous droplets would split and leave a trail of smaller droplets that had been pinched off during the flow. Nonionic surfactant Span 80 was added at 1% (w/w) to the mineral oil, allowing the droplets to maintain their integrity at the flow rates used.

The agglutination mixture was composed of Dynabeads M-270 streptavidin beads in 1x PBS/1% BSA buffer, at concentrations ranging from 25k beads/μL to 500k beads/μL. For protein testing, the target analyte used was biotinylated BSA (Sigma) at a wide range of concentrations, from 1 x 10^{-3} nM up to 6 x 10^{3} nM. For DNA testing, beads were functionalized with oligonucleotides to match a target 47 base pair section of the SRY gene.

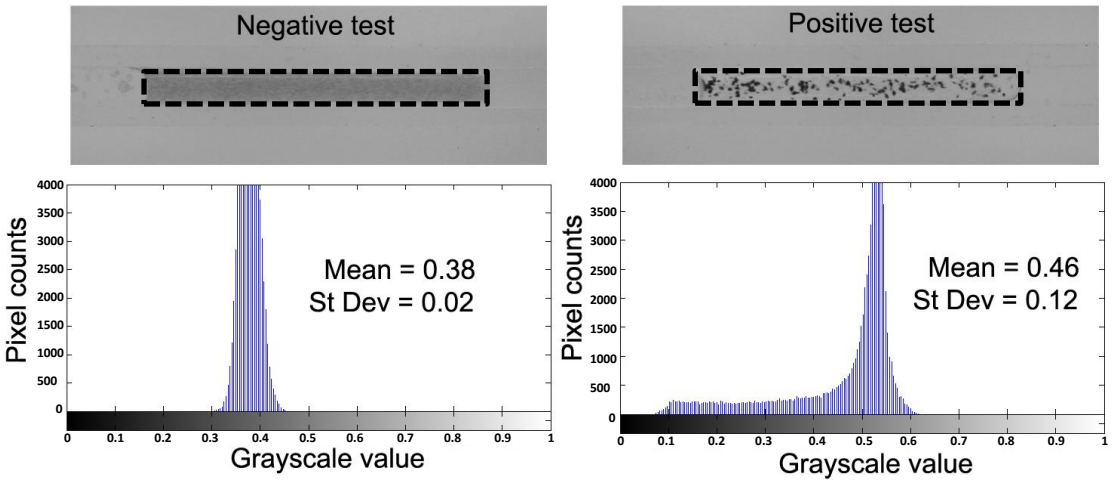

Figure 2: Representation of the quantification of agglutination. The histogram of the image at the cropped region will have a narrow distribution (low standard deviation, σ) in negative tests (left), and a wide distribution (higher standard deviation, σ) in positive tests (right).

RESULTS AND DISCUSSION

For each test, 5 μL of agglutination mixture were prepared, out of which 2 μL were immediately introduced into the system without additional incubation time. In each experiment, the droplet was allowed to flow forward for the entire length of the tube, after which the flow was reversed and the droplet was discarded as it exited the tube. Measurements were taken on the return pass of the droplet.

We studied the effect of varying both the microbead and analyte concentrations, the results are shown in Figure 3. Each point of the graph represents the average measurement of five frames of the video, to minimize error caused by the random motion of the clumps of beads. Error bars show their standard deviation, showing low variation between each measurement.

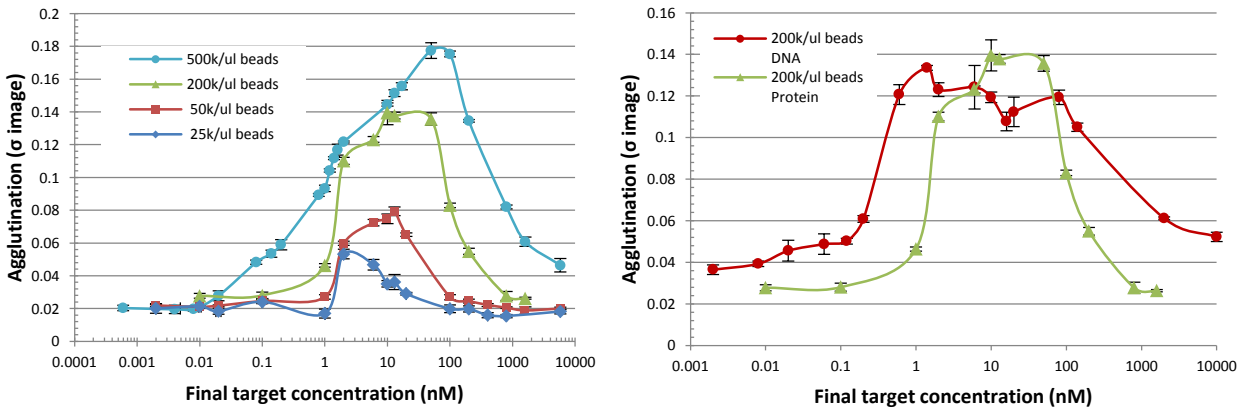

Figure 3: Curves showing agglutination strength, each point is the average measurement of 5 photographs of the droplet, error bars are their standard deviation. Left: Effect of varying the concentration of the target analyte and beads in a biotin-streptavidin assay. Right: Agglutination at 200k beads/μL for biotin-streptavidin and DNA tests.

When experiments were run continuously using the same silicone tube, a loading effect of analyte on the system occurred after performing assays at very high concentrations (> 1 x 10^3 nM), which produced a false positive when running low concentration tests. However this effect was eliminated if the system was flushed with negative tests consisting of 2 µL of agglutination mixture of only the bead solution. The false positive effect diminished with consecutive flushes, and was eliminated after the fifth repetition.

With increasing analyte concentrations, agglutination degree increases, but afterwards decreases after reaching a peak value. This false negative or false low is the so-called hook or prozone effect, which occurs when all the linkage sites of the bead surfaces are saturated by an excess of analyte, impeding agglutination. We found that both the maximum signal value of the detection peak (Prozone threshold) and the analyte concentration at which it occurs, increases linearly as bead concentration increases. Both protein and DNA were successfully measured at a wide range of concentrations, its width increasing linearly with bead concentration. Ranges as high as six orders of magnitude were obtained for the highest bead concentrations. These parameters are summarized in Table 1.

Table 1. Summary of measurement parameters

Assay	Bead concentration (Beads / µL)	Prozone Threshold (nM)	Detection Threshold (nM)
Biotin-Streptavidin	25,000	2.5	2
Biotin-Streptavidin	50,000	13	1.5
Biotin-Streptavidin	200,000	30	1
Biotin-Streptavidin	500,000	70	0.02
DNA	200,000	100	0.02

CONCLUSION

In this work we were able to reliably quantify the agglutination of both protein and DNA based agglutination assays. The detection peak and lower detection limit increase linearly as the bead concentration increases. At high analyte concentrations the hook or prozone effect is observable, which is characteristic of agglutination assays.

Increasing the concentration of microbeads tends to increase the range and degree of agglutination, with DNA tests showing an even wider range. It should be noted that a remarkable degree of agglutination was observed during the DNA testing, where in the range of 6 – 80 nM the beads in the droplet agglutinated consistently into a single clump, which could not be achieved in traditional card agglutination assays.

We were able to perform these measurements rapidly and at high flow rates, without the need for incubation, complex mixing structures or magnetic actuation, which has potential for high throughput applications.

Future work includes further characterization of the system parameters and more extensive repeatability studies. Different tubing material, such as teflon could reduce the loading effect seen after measurements of very high concentrations of analyte, which would help minimize cross-contamination between tests.

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