Rapid Microfluidic Assay for Immunocapture and Enumeration of *Escherichia coli* O157:H7 cells in Aqueous Samples

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The objective this paper was to demonstrate a simple protocol for rapid screening of *Escherichia coli* (*E. coli*) O157:H7 cells from aqueous samples using a series of three antibody arrays in a microfluidic chip (µ-CHIP). The protocol involved fabricating antibody arrays on glass cover slides using adsorptive coatings the µ-chip with streptavidin and then immobilizing biotin-antibodies against *E. coli* O157:H7. The arrays were patterned using a straight poly dimethyl siloxane (PDMS) channel to create rectangular shaped arrays with the protein streptavidin deposited on the bottom glass surface. Streptavidin served as the anchor for biotin-labeled antibodies to attach, since biotin and streptavidin spontaneously bind to each other due to their receptor ligand interaction. Employing an array of three rectangular surfaces with immunoassays immobilized in the µ-chip, we were able to capture fluorescently labeled *E. coli* O157:H7 cells from known samples. After immunocapture and enumeration of the *E. coli* O157:H7 cells on the antibody-coated arrays, the fluorescent signal from each cell was used to estimate the number of colony forming units. After an initial cell enrichment process, of capturing fluorescently labeled *E. coli* O157:H7 cells on the arrays, imaging and counting them, we were able to observed a linear correlation between the cell densities and the fluorescent intensities for cell densities in the range from $2x10^4$ - $2x10^9$ CFUs/ml. We were also able to evaluate immune-capture of biotin-labeled polystyrene beads as they interacted with the streptavidin arrays under flow conditions, into the micro-channel of a microfluidic chip. The flow rate in the chip was experimentally optimized and set at 20 µl/min. A single wash step was performed for both the array and the microfluidic chip enclosed array by flowing 0.5 ml of tris buffer (pH 7.4) solution over the area. The number of cells attached to a 100 x 100 μm^2 area was counted using the freeware ImageJ. The assay was able to enumerate *E. coli* cells over the *Bacillus sphaericus* (*B. sphaericus*) negative control at greater than 99% efficiency. The total time for performing the assay is less than two hours (hr), which includes one hr for fabricating the microfluidic chip. These results demonstrate the feasibility of using our µ-chip method for screening for *E. coli* O157:H7 cell in aqueous samples.

Keywords: *E. coli* O157:H7; array; rapidly screening; immunoassay; microfluidic chip.

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Introduction

While bacterial species are ubiquitous in the environment, several bacterial species are pathogenic to human. For this reason, it is important that their presence be closely monitored in our food and water supplies [1]. In the United States, each year there are millions of cases of food borne illness with thousands of hospitalizations and death, resulting from

bacterial contamination [2, 3]. The recent introduction of new legislation by the United States Food and Drug Administration (FDA), called the food modernization act, mandates monitoring of irrigation water quality by farmers and food producers to prevent contamination of food supplies [4, 5]. The food modernization act proposes an improvement in the monitoring standards for irrigation water and food samples to allow more expedient tracing of the source of outbreaks from foodborne pathogens [6, 7]. In recent years, there has been an increase in the volume of research has been done as it relates to the development of technology for rapidly screening and detection of bacterial pathogens, particularly in resource-limited regions where access to modern laboratories are normally limited [8].

There are several bio-analytical methods that are currently applied to the detection of bacterial pathogens. The gold standard among them involves using culturing method, which is often performed using a polymer or glass vessel that is filled with a nutrient rich media (i.e. growth on semi-solid or liquid media in an incubator) which has a colorimetric assay in the media that is specie specific [9]. There are several other popular methods for positively identifying bacterial pathogens including the use of Polymerase chain reaction (PCR)-based assays [10, 11], enzyme-linked immunosorbent assays (ELISA) [12, 13], detection by mass spectroscopy [14, 15], and using immunoassays [16], among others [17-19]. All of these methods, however, have limited capability for multiplexing the screening of pathogens. It is believed, however, that microfluidic biosensors have a huge potential for multiplexing which would allow the rapid detection of multiple pathogens in a single test [20]. Our hypothesis is that simple and rapid biosensors can be easily designed and fabricated using a poly dimethyl siloxane (PDMS) channel to create antibody arrays in a μ-chip. These antibodies can be immobilized by exploiting the binding of the small molecule biotin to the protein streptavidin.

The enterohemorrhagic strain of *Escherichia coli* (*E. coli*), *E. coli* O157:H7, is a well known pathogenic specie which often cause gastroenteritis in humans [21]. Infections resulting from this pathogen are typically associated with eating undercooked meats [22-24]. Infections resulting from *E. coli* O157:H7, have also been reported from eating contaminated fresh fruits and vegetables [25, 26], raw milk [27], and from contaminated water [28-30]. Unfortunately, infections resulting from *E. coli* O157:H7, frequently result in hospitalizations and event death [31, 32]. The use of biosensors could potentially improve rapid diagnostics and advance early treatment options for dealing with bacterial infections.

One simple yet rapid approach for producing protein-coated biosensors is to pattern the surface of the microfluidic device with antibodies for different bacterial species [21, 33]. Here we describe using a PDMS channel to pattern a glass cover split with three arrays aligned in series. To fabricate the arrays, we immobilize biotin-labeled antibodies against *E. coli* O157:H7 to surface adsorbed streptavidin molecules as illustrated in a rectangular array as illustrated in figure 1(a). To fabricate the microfluidic chips, a PDMS channel was sealed over an antibody array on a glass cover slide. Recently, Gehring *et al.* reported using a micro-spot array to rapidly detect *E. coli* O157:H7 [34]. This previous attempt to use micrometer-sized spots was inconsistent and suffered from low detection limits. These limitations can be overcome by using a larger, continuous sensory area with surface-bound antibody against *E. coli* O157:H7.

To try to address the lack for rapid monitoring protocols for *E. coli* O157:H7 in food and water samples, we are proposing a protocol that could lead to the development of a cost-effective monitoring strategy. Currently, the major approaches for being utilized for screening *E.*

coli includes either immunoassays [35-40], gene amplification methods using PCR approaches [41-50], and antibody tagged magnetic beads [51]. All three methods offer high sensitivity with great precision but they also require pre-culture to enrich the sample before analysis. The pre-culture step requires significant time to allow the cells to grow. Our hypothesis is that if were used an antibody coated biosensor, we could potentially removed the pre-culture step and rapidly detect *E. coli* O157:H7 in aqueous solutions.

In this paper, we investigated the potential of using µ-chip to capture and enumerate cells on-chip. The patterning of the arrays were performed by creating a device using the elastomeric polymer PDMS, sealing the PDMS stamp to the glass cover slide after plasma treatment and then depositing streptavidin. This step is followed by the deposition of biotin-labeled antibodies against *E. coli* O157:H7 directly above the layer of streptavidin. The spontaneous interaction between biotin and streptavidin resulted in a strong non-covalent bond forming between the antibody and the streptavidin. *E. coli* O157:H7 cells were successfully captured onto the patterned surfaces at flow rates between 2 – 200 µl/min. In addition to imaging live *E. coli* O157:H7 cells that were immobilized on the antibody array, we also used polystyrene beads to simulate bacterial cells flowing at 20 µl/ml in a microfluidic chip with antibody arrays. The polystyrene beads that were enumerated on the arrays and imaged did not show a strong correlation to the live cells immobilized on the antibody arrays.

Materials and method

Materials

The materials used in this study included the following: unlabeled goat anti-*E. Coli* O157:H7 antibody at a working solution of 0.5 mg/ml in tris buffer, streptavidin solution at 13 units/ml or 1 mg/ml from Kirkegaard & Perry Laboratories (Gaithersburg, MD), 3,3'-diethylthiacyanine iodide (THIA) at a working concentration of 6 µM in tris buffer from Sigma-Aldrich (St. Louis, MO), tris buffer solution from Sigma-Aldrich (St. Louis, MO), sylgard 184 pre-polymer and curing agent (Dow-Corning, NY), Su-8 epoxy-based photo-resist (MicroCem, USA), 200 proof ethanol from Thermo Fisher (Carlsbad, CA) .

Preparation of antibody array on glass cover slip

Glass coverslips from Fisher Scientific (Carlsbad, CA) was cleaned using 70% ethanol in water and air-dried at room temperature. The glass slide was covered in a 1 mm layer of poly dimethyl siloxane in a 10:1 ratio of pre-polymer to curing agent and allowed to cure at 70^{o}C for 2 hours. Two 0.9 x 0.9 mm^2 square wells were cut from the PDMS and filled with 50 µl of a 1 mg/ml streptavidin solution with 13 units/mg, as seen in figure 1. After 2 hours, 50 µl of a 0.5 mg/ml of biotin-label Goat anti-*E. Coli* O157:H7. The antibody-coated glass cover slips were then stored at 4^{o}C until they were used.

Fabrication of the microfluidic chip

The microfluidic device consists of two parts: the top which is made of PDMS and has the micro-channel and the bottom which is the glass cover slip which has arrays or bacterial antibodies arranged in series. The micro-channel (height: 100 µm, width: 360 µm) was formed by soft lithography. The shape of the microfluidic chip was designed using a computer aided drawing software package (Adobe Illustrator, San Jose, CA), which was then printed at ultra-high resolution on a Mylar sheet, forming a photo-mask. A 5-inch (5") silica wafer was then spin-coated with the photo-resist Su-8 to a thickness of 100 µm. The silica wafer was baked for 1 minute (min) at 65^{o}C and then 10 min at 95^{o}C. The photo-mask was used to hide some areas while allowing others to be exposed at high intensity ultraviolet light. The photo-resist was exposed to high intensity ultraviolet light from an OAI mask-aligner, and then baked at 65^{o}C for 5 min and 95^{o}C for 30 hr.

(a) (b)

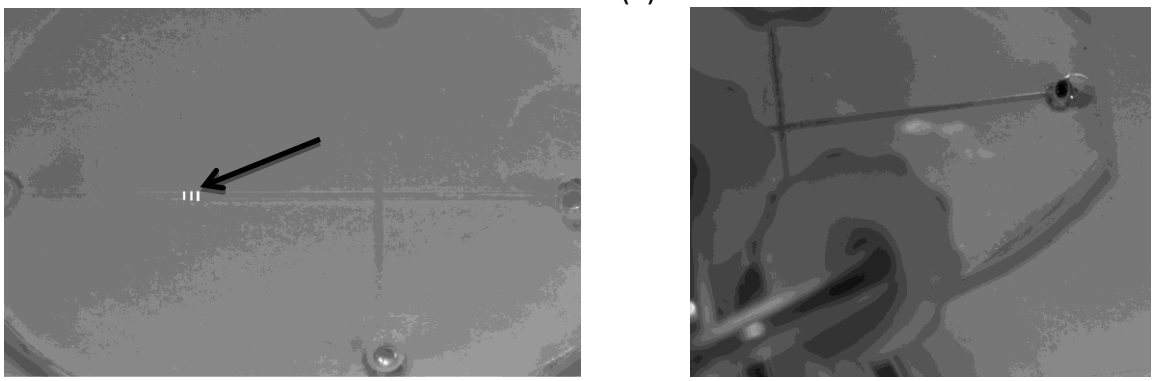

Figure 1. Images of the PDMS μ-chip on a microscope stage (a) top view image of a PDMS microfluidic device used for capturing *E. coli* O157:H7 cell in water samples using positive selection; (b) Image of a PDMS μ-chip with cell solution flowing in the channel. The arrow in figure 1 (a) outlines the approximate area of the protein array.

The Su-8 regions where the UV light was allowed to radiate allowed cross-polymerization of the Su-8 epoxy-based photo-resist. The unexposed areas were dissolved using a Su-8 developer (MicroChem, USA) the top section of the microfluidic chip was formed by casting PDMS against a master mold that formed the shape of the micro-channel. Punching them with a biopsy punch created the inlets and outlets for the microfluidic chip. The PDMS section was treated for 90 seconds (sec) with air plasma and sealed to the glass cover slip. Care was taken to ensure that the micro-channel was sealed over an area that contained the antibody arrays. For precise positioning, a stereo-microscope was used to direct the placement of the PDMS channel. The completed device was stored at 4^{o}C. All devices were used within 48 hr after fabrication.

Growth and enumeration of *E. coli* O157:H7

One colony of *E. coli* O157:H7 was removed from petri dish stored at 4^{o}C and added to 8 ml of LB Broth. The cell solution was incubated at 37^{o}C for 18 hr with shaking at 200 RPMs. Cultures were counted using a Petroff-Hausser counting chamber. A 1 ml aliquot of cells was pelleted by centrifugation at 4,200 RPMS for 5 min, the media was decanted and the pellet re-suspended in a 6 µM solution of THIA and kept in the dark for 15 min [52]. The solution was then centrifuged; dye solution decanted and the pellet washed three times with phosphate buffered saline (PBS). The pellet was re-suspended in PBS, and serially diluted to the desired concentrations. The arrays were then imaged via bright field and fluorescence microscopy. The cell solution was used within 1 hr after preparation.

Antibody microarray screening of *E. coli* O157:H7

The microfluidic chips were removed from the refrigerator (4^{o}C) and warmed to room temperature (23^{o}C). One hundred microliters of bacterial solution was added, to the inlet of the microfluidic chip. The solution was allowed to flow for 15 min and then bright field and fluorescence microscopy imaged the chip.

Microscopy of arrays in the microfluidic device

Phase-contrast and epi-fluorescence images were taken using an inverted microscope, Leica DMI 6000B equipped with the Leica AF6000 Modular System (Wetzlar, Germany). The images were taken using a Leica 345 FX. The selected array spots were visualized by fluorescence microscopy. The arrays in the channel were imaged using the appropriate fluorescent filters with 488 nm excitation and 515 nm emission using procedures similar to those reported elsewhere. Fluorescent microspheres used were 1 µm diameter fluorospheres that were obtained from Life

Technologies (Carlsbad, CA). The fluorospheres were used to simulate the flowing of cells in the channel.

Data analysis

Experiments performed in the μ-chips were done in quadruplets to ensure that the results from experiments would be statistically relevant. Analysis of cell immobilization on the three-line antibody arrays, were evaluated by manual counting and automated cell counts using the freeware ImageJ. Each cell represents an experimental unit. The background fluorescent measurements were taken from pre-selected locations that were proximal to but outside the antibody coated areas. The negative control samples, consisiting of *B. sphaericus* cells, were introduced to identical μ-chips to the ones used for the immobilization experiements for *E. coli* O157:H7. Images were captured using the Leica AF6000 software and exportd as Bitmap files (tiff files). The bacterial cell counts was obtained by taking an image of region of interest that is approximately a 100 micron square region in the channel using with the Leica DMI 6000 microscope and using the Leica AF6000 software package, and then counting the number of cells in the region of interest using ImageJ. An identical procedure was carried out for all images. Statistical analysis was used to relate the experimental data by reporting the mean ± standard deviation.

Results and discussion

***E. coli* O157:H7 sample processing on antibody array**

The processing steps for *E. coli* O157:H7 screening in water samples involved: (i) coating streptavidin arrays on a glass cover slip aided by a PDMS mold; (ii) layering of the biotin-labeled anti-bodies on the streptavidin surface; (iii) staining cells of interest with the thiacyanine dye, 3,3'-diethylthiacyanine iodide for 5 min and washing three times to remove excess dye; (iv) introduce cells to the arrays and allow to incubate for 15 min or to the (v) microfluidic chip and allow to incubate for 15 min. The negative controls used for this study was to introduce the gram-positive bacteria species *Bacillus sphaericus* by using differential staining of cell solutions with equal cell density of *B. sphaericus* and *E. coli* O157:H7. The *B. sphaericus* cells were retained at 1% relative to *E. coli* O157:H7 or 1:99 ratio.

The relative intensity of the auto-fluorescent for this study for each of the micro-channels was significantly lower than the fluorescent signal generated by the fluorescently labeled cells (figure 2). The signals generated were equal to the localized background fluorescence. The parameters for the camera were adjusted to a single sample of fluorospheres; the gain, intensity and exposure times were all recorded and used for subsequent experiments. At the start of each experiment, the values were then manually inputted before each experiment to ensure consistency of the data collected.

Limit of detection

To establish the limits of *E. coli* O157:H7 detection with an antibody on a microfluidic array surface, we performed a static-experiment using live cells on antibody-coated array on a glass slides, which were exposed to *E. coli* O157:H7 cells over a range from 2 $x10^3$ – $2x10^9$ cell/ml. The fluorescent intensity of the cells was strong, which enabled us to detect cells even at the lower limits of the experiment. This procedure was accurate and reproducible down to cell densities at $2x10^4$ cells/ml. Below this level, the data was inconsistent with high variability in the standard deviations. For this reason, we claim that our device is sensitive down to $2x10^4$ cells/ml. The higher fluorescent emission from our high cell density samples saturated the surface, which made differentiating individual cells difficult. The high fluorescent intensity also saturated our CCD camera, which increased the error in our measurements. Based on our limited set of experiments, we found that using a starting cell density of 2 x 10^4 – 2 x 10^9 cell/ml was optimum

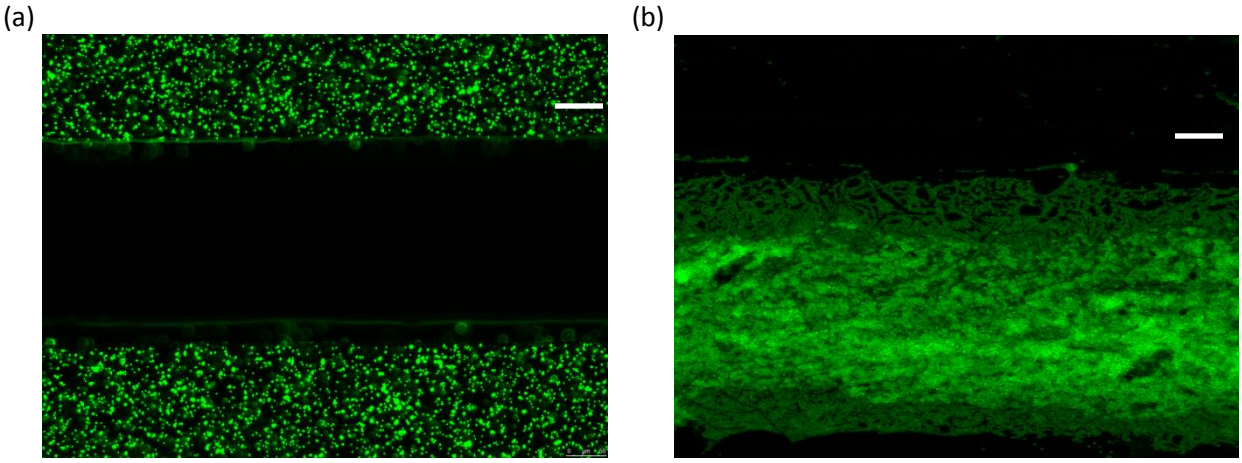

Figure 2. Fluorescent micrographs of (a) poly styrene beads in a flow system as 2 µl/min on a glass cover slip and (b) *E. coli* O157:H7 cells captured on an antibody-coated micro-array both flowing at 20 µl/min. A correlation was established between the starting bacterial cell density and the emitted fluorescence from the surface. The fluorescent particles were counted using the freely available imaging and image analysis software Image J. Scale bar represents 50 µm.

when using a THIA concentration of 6 µM. The bacterial cell counts was established for *E. coli* solution with a starting cell density of $2x10^3$ cell/ml, as seen in figure 3. The highest cell count was established at $2x10^9$ cell/ml.

Cells attachments appeared to be best at the 2 µl/min and worst for the 200 µl/min, as expected because of the absence of force acting to prevent the binding of the cells to the surface. Cells require some finite amount of time to interact with the surface to form a bond. In addition, if the magnitude of the flow rate is such that it induced shear stress on the cell with sufficient force that it can break the bonds formed between *E. coli* O157:H7 cells and the array surface, then the cells will simply wash away.

In this study, we confirmed that *E. coli* O157:H7 cells could be selectively immobilized from an aqueous solution on by an antibody coated glass surface of a microfluidic device. The number of cells immobilized followed a linear correlation with respect to the number of fluorescent that were originally in the starting cell solutions of each tested sample. This strongly suggested that the arrays are able to capture cells from solution at a given rate. This rate seems to be insensitive to cell density. We were able to demonstrate reproducible data over the range of cell densities from $2x10^4$ – $2x10^9$ CFUs/ml. In this study, all samples were analyzed n quadruplets (n=4), while the error was reported as the standard deviation from the mean, as seen in figure 3.

In this study, we were able to demonstrate that our simple protein-coated µ-chips could be used to immobilize and enumerate *E. coli* O157:H7 cells from an aqueous solution. The design of the device simply allowed our sample (i.e. bacterial solution) to flow past the rectangular sensory area at a range of velocities between 2-200 µl/min. It is important to note that none of the conditions were optimized beyond what was stated in the experiments therefore, enhanced performance could potentially be realized in a fully optimized system. Using fluorescently labeled polystyrene beads that were biotin-labeled, we investigated the immune-capture ability of the arrays. It was experimentally determined that a flow rate of 2 µl/min was ideally suited because the 2 µl/min was too slow and fluid flow rates of 20 µl/min or faster had too great of a force and delaminated cells for the surface of the microfluidic chip. The polystyrene beads appear to give a linear relationship between the fluorescent cell counts and the starting bead

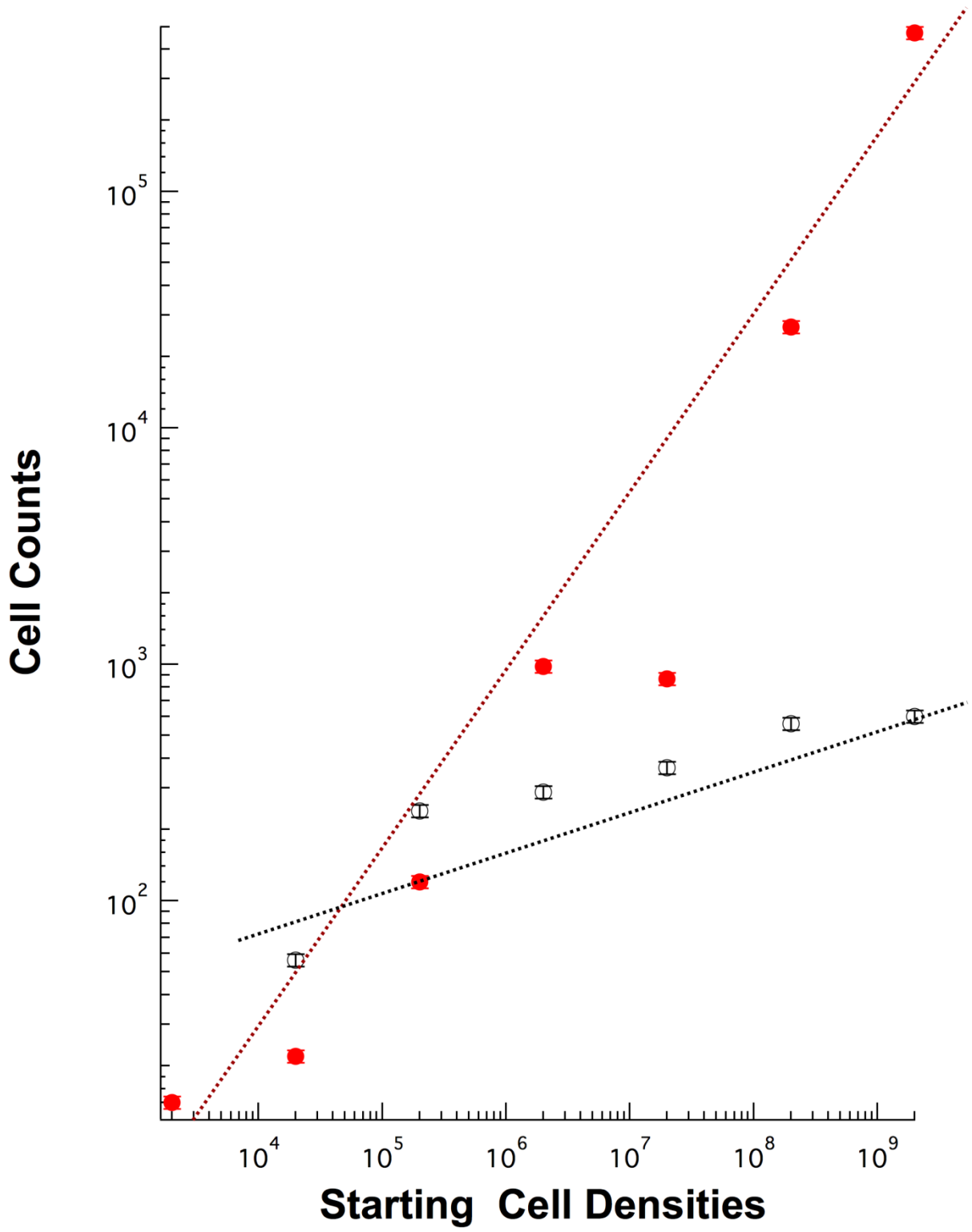

Figure 3. Cell counts from fluorescent *E. coli* O157:H7 bacterial cells immobilized on the region of the microarray as a function of starting cell density. The red solid circles represents data collected from magnetic beads that flow in at a rate of 2 µl/min while the open circles represents cells captured at 20 µl/min. Fluorescent emission resulted from staining with THIA concentration of 6 µM. Error bars represent the standard deviation from.

densities in solution. At bead densities lower than $2x10^5$ beads/ml, the fluorescent counts for the fluorescent signal is greater for the beads than for the live cells. At concentrations greater than $2x10^5$ CFU/ml, the capture rate of lives gave a higher fluorescent count than that of the fluorescent beads at similar cell densities. This may suggest that there is cooperative cell binding to the array surfaces, which is more significant at high cell densities. This suggests that a flow system would need to be highly optimized. While we did not do an extensive optimization procedure, we did find that a flow rate of 2 µl/min was ideally suited for our experiment. This technology could easily be operated and compliment many existing technologies. The microfluidic chip tested in this report has the potential of being quantitative and displaces a linear correlation on the optimized range of detection, which spans $2x10^4$ – $2x10^9$ for the fluorescent bead in the flow system. This correlation was similar to the linear correlation we saw between the starting cell densities and fluorescent cell counts established for live *E. coli* O157:H7 cells. Further work will seek to test a fully integrated microfluidic chip for testing *E. coli* O157:H7 strains over our optimized range. We believe that the rate of flow has a stronger influence over cell adhesion than the starting cell density. There is likely some optimum time need for the beads, or cells, to interact with the surface in order to achieve binding to the antibody array. Our study has outlined the basis for the development of simple, rapid and low-cost protocols for fabricating microfluidic-based biosensors with antibody arrays for rapid bacterial detection form aqueous samples.

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