Accepted Manuscript

Title: Immunocapture of *Escherichia coli* in a fluoropolymer microcapillary array

Authors: Isabel P. Alves, Nuno M. Reis

PII: S0021-9673(18)31492-4
DOI: https://doi.org/10.1016/j.chroma.2018.11.067
Reference: CHROMA 359846

To appear in: *Journal of Chromatography A*

Received date: 31 May 2018
Revised date: 21 November 2018
Accepted date: 24 November 2018

Please cite this article as: Alves IP, Reis NM, Immunocapture of *Escherichia coli* in a fluoropolymer microcapillary array, *Journal of Chromatography A* (2018), https://doi.org/10.1016/j.chroma.2018.11.067

Immunocapture of *Escherichia coli* in a fluoropolymer microcapillary array

Isabel P. Alves[1], Nuno M. Reis[1,2,*]

[1]*Department of Chemical Engineering, Loughborough University, Leicestershire LE11 3TU, UK*
[2]*Department of Chemical Engineering, Claverton Down, University of Bath, Bath BA2 7AY, UK*

**Corresponding author. Phone: +44(0)1225 383 369. Email address: n.m.reis@bath.ac.uk (N.M. Reis)*

Highlights:

- Immunocapture of *E. coli* in FEP microcapillaries reported for first time
- Identified the key role of interrogation volume and gravity settling
- Up to 100% of *E. coli* cells were captured with a limit of detection of just 1 colony forming unit (CFU)
- Immunospecificity was shown by performing ELISA with a different bacterial specie

Abstract

This study presents novel experimental insights into the direct quantitation and immunocapture of bacteria cells in a fluoropolymer microcapillary array, using *Escherichia coli* as work model, a pathogen responsible for around 80% of urinary tract infections (UTIs). In spite of the current clinical demand for sensitive tests for rapid identification and quantitation of pathogens in human samples, portable diagnostic tests developed to date lack the specificity, limit of detection and speed for effective implementation in bacteria detection at point-of-care. The 'open microfluidic' approach presented in this work directly addresses those challenges. We report for the first time evidence of immunocapture of bacteria using polyclonal antibodies immobilized on the inner surface of an inexpensive 10-bore, 200 μm internal diameter FEP-Teflon® MicroCapillary Film, with a limit of detection (LoD) of at just 1 colony forming unit (CFU). In capillaries coated with less than a full monolayer of capture antibody, we observed a first order equilibrium, with bacteria captured (in CFUs/ml) linearly proportional to the CFU/ml in the incubated sample. We captured up to 100% of *E.coli* cells, with clear evidence of immunospecificity as demonstrated by testing with a different bacteria specie (in this case *Bacillus subtillis*). We noticed gravity settling of bacteria within the capillaries created a gradient of concentration which on the overall enhanced the capturing of cells up to 6 orders of magnitude beyond the theoretic full monolayer (~4.5×10^4 CFUs/ml), which washings having an unnoticeable effect. Our data particularly highlights quantitatively the relevance of interrogation volume in respect to the miniaturisation of bacteria quantitation, which cannot be solved with the most sophisticated imaging equipment. A further set of continuous flow experiments at a flow rate of just ~1 μl/min (corresponding to a wall shear rate of ~101 s^{-1} and superficial flow velocity ~53 μm/s) showed a degree of flow focusing, yet the mobility, antibody affinity capturing and gravity settling of bacteria cells enabled successful capturing in the microcapillaries. These results will inform the future development of effective microfluidic approaches for rapid point-of-care quantitation of bacterial pathogens and in particular rule-in or *E. coli* in UTIs.

***Keywords**: immunocapture, bacteria detection, E.coli, miniaturisation, MicroCapillary Film*

1. Introduction

Bacterial infections represent a significant burden to global health and economy. It is estimated that multidrug resistance 'superbugs' is responsible for around 25.000 deaths per year in Europe resulting annually in healthcare costs of €1.5 billion and significant productivity losses [1]. Although treatable, most diseases caused by bacterial infections including *Escherichia coli* are the cause of high annual mortality rate in both developing and developed countries. Difficulty in early identification of pathogens and inaccurate treatment remain the two major clinical challenges to be solved [2,3]. Bacterial identification at point-of-care (POC) has proved particularly difficult, with no rapid and cost-effective tool yet available to identify and quantify pathogens with the high sensitivity and specificity required for clinical use. Identification of pathogens is currently done in centralised microbiology laboratories, involving complex logistics and long waiting times, typically between 20 and 72 hr [4,5]. New diagnostics tools for rapid bacteria identification and quantitation are regarded essential for managing the over prescription of antibiotics and tackling antimicrobial resistance [6,7,8]. Consequently, microfluidic detection of pathogen is emerging as a new solution to tackle bacterial infections, offering high throughput combined with small fluid volumes and short assay time in a portable format compatible with POC requirements [2,9,10]. However, development of those devices remains mostly empirical, driven from an analytical chemistry perspective that is disconnected to the engineering challenges represented by e.g. the high-shear and focused flow of bacteria in microchannels or microcapillaries.

Methods currently available for bacterial detection are based on a variety of laboratory-based tests including microscopy [11], plate culture [4], antimicrobial peptides [9], immunoassays [4,7], nucleic-acid amplification [7,11], electrochemical impedance microscopy [7] and magnetic beads [11]. Though well established, some of those techniques present relevant drawbacks for application at POC such as complex sample preparation, expensive reagents, lack of specificity and sensitivity and/or demanding expensive readout systems only available in sophisticated centralized labs [3,10-12]. Microbiological identification of bacteria intrinsically relies on the doubling time and growth time of bacteria in agar-rich plates, which is set by the nature of the microorganism and growth conditions. Immunoassays are highly sensitive bioanalytical tools that rely on the high antibody specificity and can be used in several applications including clinical diagnostics [2,5,10,13,14], environmental monitoring [10,15], bioterrorism [3] and drug kinetics [3,10] for pharmacology industry. They utilise enzymatic amplification for detection of very low concentrations of proteins or bacteria, so several authors have attempted detection of bacteria with immunoassays [2,5,10,13-17]. Miniaturization of bacteria detection tests presents the problem of reduced signal strength which demands very sophisticated detection and quantitation equipment. This is one of the biggest challenges for POC microfluidic devices [10,18,19]. Limitations reported for immunoassay detection of

bacteria are the poor sensitivity, cross-reactivity, reproducibility and reduced limit of detection (LoD) [20,21]. So far there has been no systematic study showing how the miniaturisation and immunoassay format affects the performance of an immunoassay aiming rapid POC bacteria quantitation.

In this study we used optical imaging techniques such as Laser Scanning Confocal Microscopy (LSCM) and Scanning Electron Microscopy (SEM) to understand immunocapture of bacteria in plastic microcapillaries coated with a polyclonal antibody. We used *E.coli K12* fluorescently labelled to gather quantitative information about avidity of antibody-bacteria binding [9,22]. Plastic microcapillaries offer large surface-area-to-volume ratio (SVR), are inexpensive and enable larger volumes of sample to be used typical of 'open' microfluidics. We employed a new cheap miniaturised immunoassay platform based on a 10-bore Microcapillary film (MCF) melt-extruded from Fluorinated ethylene propylene (FEP-Teflon®). MCF detection present optical characteristics that favour high signal-to-noise-ratio and simple optical detection with low-cost readout systems, essential for high performance detection in an affordable, portable format. The hydrophobic surface of FEP microcapillaries allows simple antibody immobilization by passive adsorption and avoids complex fluid handling [23]. The large SVR ratio of ~200μm microcapillaries enabled a performance comparable to microtiter-based immunoassays with at >10-fold reduction in total assay time [24]. These features were key to our recent MCF developments: PSA biomarker detection using smartphone based system and screening from whole blood [24,25] multiplexed femtomolar quantitation of human cytokines (IL-1β, IL-6, IL-12 and TNF-α) [26]; Lab on a stick for multi-analyte cellular assays as antibiotic susceptibility and microbiological screening [27] and one-step quantitation of PSA using nanoparticles labels [28].

2. Experimental

2.1. Reagents and materials

Unconjugated Rabbit Polyclonal Antibody Immunogen: O and K antigenic serotypes (all types) of *Escherichia coli* (# PA1-7213); LB Agar, Miller (# BP1425-2) and LB Broth, Miller (Granulated) (# BPE9723-2) were supplied by Fisher Scientific UK Ltd, Loughborough, UK). Phosphate Buffered Solution, 0.01M at pH 7.4 (PBS, # P5368-10PAK), washing buffer PBS with 0.05% v/v of Tween-20, Nunc maxisorp ELISA 96-well MTPs and SIGMAFAST™ OPD (o-Phenylenediamine dihydrochloride) tablets (# P9187) were sourced from Sigma Aldrich (Dorset, UK). Pierce™ Protein-Free Tris-Buffered Saline (TBS) Blocking Buffer (# 37584), LIVE/DEAD® BacLight™ Bacterial Viability Kit, for microscopy (# E2069) and High Sensitivity Streptavidin–HRP (# 21130) were supplied by Thermo Scientific (Northumberland, UK). Female Luer ¼ (# P-624) were obtained from C M Scientific (Silsden, UK). A 10-bore MCF material and push-fittings seals were provided by Lamina Dielectrics Ltd (Billingshurst, UK).

MCF fittings were designed by Lamina Dielectrics Ltd and manufactured in-house. *E.coli K12* 'wild-type' (NCIMB 11290) and *Bacillus subtillis* (NCIMB 8054) were supplied by Loughborough University, obtained from NCIMB Ltd, (Aberdeen, UK).

2.2. Fluoropolymer MCF

A 10-bore MCF was used (see Figure 1) which presents a parallel array of microcapillaries with a mean hydraulic diameter of 206 ± 12.6 µm and external dimensions 4.5 ± 0.10 mm width and 0.6 ± 0.05 mm thickness [23]. This material was manufactured by a novel melt-extrusion process from Teflon® FEP (Dow, USA) [23,25]. The cost for pelleted FEP material is in range of £20/Kg, with a unit weight of 5 grams per meter of material, meaning the material cost for a 10-plex, 50 mm long MCF test strip is less than 1 pence. This excludes the cost of reagents, manufacturing and fittings required for carrying out immunoassays in the MCF. FEP was chosen for its excellent transparency resulting from refractive index matching with water, allowing good signal-to-noise detection with low cost readout systems [23] but also with sophisticated fluorescence imaging equipment such as confocal microscope.

2.3. *E.coli* sample preparation

A colony of *Escherichia coli K12* "wild type'' was inoculated in a 100 mL LB broth and incubated at 37 ºC overnight under sterile conditions. Afterwards the culture media was washed 3 times with PBS buffer and cells recovered after centrifugal separation (4,500 rpm, 20 min) and re-suspended in the original volume. *E.coli* sample aliquots were prepared in 2 ml Eppendorf tubes with an OD_{600} of ~0.7 and stored at 20 ºC. Serial dilutions were made from a volume of 0.1ml *E.coli* samples in 0.01 M PBS and spread onto LB agar plates at 37 ºC overnight for estimating the viable cells density as colony forming units (CFUs) present in fresh media. Control plates with PBS and agar without *E.coli* were performed to assess any contamination at this stage.

2.4. Microcapillary *E.coli* capture and flow experiments using LSCM

Confocal microscopy experiments were carried with LSCM (Nikon inverted Microscope ECLIPSE TE300 with Bio-Rad RAD200 (scan head 60X-1.20NA objective lenses, excitation peak wavelength of 488 nm and emission peak wavelength of 530 nm) operating Laser Sharp 2000 software. Post-acquisition and particle tracking analysis were carried out using Image J [29]. For affinity capture of *E.coli* in the microcapillaries, *E.coli* was stained fluorescently with LIVE/DEAD® BacLight™ Bacterial Viability Kit according to supplier specifications for live cells.

For each experiment, a 80 cm long MCF strip was coated with 40 µg/ml of polyclonal anti-*E.coli* antibody (capAb) in PBS 0.01 M during 2 hours and Protein Free (TBS) blocking buffer for another 2 hours. We used pushed-fit seals to connect MCF strips to a Terumo Syringe 2.5

ml luer Lock (Figure 1D) to promote a uniform filling of the microcapillaries. Flow experiments were performed using a PHD-ultra Harvard Pump syringe (Instech Laboratories, Inc., USA) and all MCF strips positioned flat in LSCM window with a built-for-purpose MCF holder. Because of the small dimensions of the capillaries, flow was assumed laminar and therefore characterized by low Reynolds number described by:

$$\boldsymbol{R_e = \frac{\rho . d_h . u}{\mu}} \quad (1)$$

where ρ (Kg/m^3) is the fluid density, u (m/s) the superficial flow velocity of the fluid, μ (Ns/m^2) the fluid viscosity and d_h the mean hydraulic diameter of capillary. The volumetric flow rate,Q (m^3/s) affects the superficial flow velocity, u of bacterial cells in the capillaries, according to:

$$\boldsymbol{Q = u . A} \quad (2)$$

where A (m^2) is the cross-sectional area. It can be shown the (maximum) wall shear stress, τ_w (Pa) and shear rate, $\dot{\gamma}$ (s^{-1}) are given, respectively by:

$$\boldsymbol{\tau_w = \mu \times \dot{\gamma}} \quad (3)$$

$$\boldsymbol{\dot{\gamma} = \frac{\partial u}{\partial x}} \quad (4)$$

where $\frac{\partial u}{\partial x}$ is the velocity gradient across the microcapillary.

2.5. Study of *E. coli* binding into MCF capillaries with SEM

We used a JSM-7800F Field Emission SEM to observe surface bound *E.coli* incubated in MCF strips with and without antibody coating. Polyclonal anti-*E.coli* antibody (40 μg/ml capAb) in PBS 0.01M was immobilized in a 50 cm long MCF strip and incubated for 2 hours and then further 2 hours with Protein Free TBS blocking buffer. Fluid aspiration was done with 1ml syringes connected to the MCF via a 2 cm long silicone tube. As controls we incubated a 20 cm long MCF strip with the same concentration of capAb in PBS but without blocking buffer and a 20 cm long MCF strip with just Protein Free TBS blocking buffer during 2 hours without any capAb. Thereafter all strips were washed with PBS 0.05% v/v Tween-20 and trimmed into shorter, 10 cm long strips. Each strip was then incubated with 200 μl of a synthetic sample consisting of 10^9 CFU/ml *E.coli* in PBS for 20 minutes and then gently washed with 1ml PBS 0.05% v/v Tween-20 buffer and dried at room temperature during 24 hours. One of the strips was incubated with 200 μl of 10^9 CFU/ml of *E.coli* in 2.5% w/v glutaraldehyde in 0.01M PBS and washed consequently. Before SEM imaging cell MCF strips were sliced through the middle of the capillaries aiming to expose the *E.coli* on the microcapillary walls. Samples were fixed to a pin stub with double-side tape and coated with 5 nm layer of gold using Au-Sputter Coater Quorum Q150T ES for 90 seconds.

2.6. Avidity of antibody-coated microcapillaries

A 80 cm long MCF strip was first coated with 40 µg/ml of capture for 2 hours, followed by blocking buffer solution for another 2 hours and finally flushed with washing buffer and trimmed to produce individual 40 cm long strips. Each 40 cm long strip (having a total internal volume of 133 µl) was then gently flushed with 1.2 ml *E.coli* sample and incubated for 20 min with a 10-fold serial dilution from 2 ml aliquots of *E.coli* samples in PBS. The solution from each strip was then fully recovered into a microwell by pushing the fluid out of the capillaries with a plastic syringe full or air attached to a 2 mm i.d. silicone tube and platted for CFU counting. The ratio between the CFU/ml in the recovered solution and the CFU/ml in the incubated solution enabled plotting the equilibrium curve and computing the percentage of CFU captured in the MCF strips coated with capAb with a simple mass balance. Assuming bacteria capture only occurred during batch incubation of the strips, it can be shown that in equilibrium conditions the CFUs/ml captured is given by the difference between CFU/ml in the aliquot and the CFUs/ml in the solution withdrew from the microcapillaries. In order to understand the effect of washing on bacterial binding, similar experiments were repeated at same conditions but adding a final step of washing with 200 µL PBS with 0.05% v/v Tween-20.

As CFUs/ml tested covered several orders of magnitude, results were plotted as $\log_{10}$ CFU/ml and $\log_{10}$ CFU/cm^2, the last based on an inner surface area of 25.84 cm^2 for 40 cm long strip. As a guideline, we estimated a full monolayer capacity of ~4.5×10^4 CFUs/ml assuming each *E. coli* cell has a footprint of 0.5 µm^2 when lying in a flat position. As antibody-cell interaction is likely to involve more than one antibody molecule, the equilibrium can be regarded as a representation of the binding avidity to capAb immobilized onto FEP-Teflon microcapillaries.

2.7. Immunoassay detection of *E.coli K12* and *Bacillus subtillis*

To demonstrate immunospecificity of the immobilized capAb in respect to *E. coli* capturing, a colorimetric sandwich immunoassay was devised in the MCF strips inspired on immunoassay protocol previously reported by e.g. Castanheira *et al.* [26] for protein biomarkers. We used the following work assay conditions: 40 µg/ml unconjugated polyclonal capAb in 0.01M PBS incubated for 2 hours; 1ml Protein Free TBS blocking buffer incubated for another 2 hours; 150 µl of *E.coli K12* or *Bacillus subtillis* diluted in 3% of BSA incubated for 30 min; 40 µg/ml biotinylated detection antibody incubated for 3 min; 4 µg/ml of HSS-HRP incubated for further 4 minutes; chromogenic substrate consisting of 4 mg/ml OPD in 1mg/ml H_2O_2. The antibody manufacturer reported the possibility of some cross reactivity with other bacteria in particular related enterobacteriaceae, in this case we selected *Bacillus subtilis* for being a common, gram-positive bacteria.

3. Results and discussion

3.1. LoD of bacteria is set by level of miniaturization

We noticed the LoD of bacteria is intrinsically linked to the level of miniaturisation. The advantages of microfluidic bioassays are well understood and explained in literature, however detection and quantitation of bacteria is intrinsically more challenging in miniaturised systems because of the small volumes being dealt with. The volumes herein mentioned could refer to the sample volumes and/or interrogation volume. The most sensitive theoretical technique will allow detecting just 1 CFU, however this represents a minimum volume of 1 μL for a LoD of 1×10^3 CFUs/ml, typically the clinical threshold for Urinary Tract Infections (UTIs) [30]; note counting viable cells as CFUs is a norm in microbiology as it reflects the uncertainty connected to visual detection of a colony resulting from a single cell or group of cells. To illustrate this, we have imaged plastic microcapillaries strips loaded with a serial dilution of fluorescently labelled *E.coli* in PBS into a confocal microscope. For optimum sensitivity and resolution, we have set the interrogation window to be 200×200 μm^2, representing an estimated interrogation volume of just 62.5 nL for a 200 μm perfectly cylindrical capillary which is a fair approximation for the MCF.

Figure 2A shows a rapid drop of fluorescence signal as cells are diluted, suggesting it is not possible to directly quantify *E.coli* cells below 10^4 CFUs/ml even with a sophisticated microscope. We used ImageJ to count the number of cells that could be detected in the interrogation window and established a relation to the theoretical number of bacteria expected to be found in that volume (Figure 2B). We estimated a minimum $\log_{10}$ CFUs/ml of 5.20 required to have at least one CFU present in that small interrogation volume and that becomes experimentally visible. This finding shows that even the most advanced optical imaging equipment is unable to detect anything below 4-5 $\log_{10}$ CFUs/ml in a synthetic sample. We hypothesised the use of an 'open microfluidic' system as exemplified by Figure 1E like the MCF, enabling parallel replicas and cells capturing via immobilized high affinity antibodies, would offer the opportunity to combine within a single device capturing and quantification of bacteria cells with a level of amplification, enabling the use of large sample volume for yielding detection at reduced CFU/ml values.

3.2. SEM evidence of immune affinity of *E.coli* cells in microcapillaries.

We studied the capture of *E.coli* cells in microcapillaries coated with polyclonal antibodies by SEM and main results are summarized in Figure 3. All MCF strips were flushed with 1 ml of washing solution to remove any unbound cells before imaging. We confirmed *E.coli* is unable to adhere non-specifically to raw, hydrophobic microcapillaries uncoated with capAb or

in absence of blocking buffer (Figures 3A and 3B). Figures 3C-F revealed *E.coli* binding only occurred in microcapillaries coated with capAb, with Figures 3C and 3D showing detailed morphology between cells commensurate with the typical size for *E.coli*. This agreed with e.g. Chang *et al.* [9] who reported binding of *E.coli* DH5α and O157:H7 to the surface of the AMP-labelled beads with similar morphology and shape [9]. The SEM study highlighted an important role of gravity in the capture of *E.coli* cells, with Figures 3E and 3F showing very distinct degrees of bacteria capture between the top and bottom parts for a given capillary cut along its length. These optical observations of uneven *E.coli* capture in microcapillaries are original and to our knowledge not previously reported in the literature, yet they can have high relevance to the design of fluidic devices suited to efficient capture of bacteria cells. Conventional microfluidic devices are usually only coated with antibody in one surface, in contrast to the cylindrical microcapillaries. In spite of all cross section of capillaries being evenly coated with the capture antibody [24], settling created a gradient of concentration reducing density of bacteria in the top part of the capillary and consequently increasing efficiency of cells captured at bottom part of the capillary.

3.3. Study of *E.coli* capture with fluorescence techniques

LSCM experiments enabled studying the spatial and time-lapse immunocapture of fluorescently labelled *E.coli* (10^8 CFU/ml in 0.01M PBS) in the plastic microcapillaries. We initially used MCF strips in the absence of flow (to avoid blurring microphotographs) and samples incubated between 5 and 20 min without any subsequent washing. Figure 4A shows a *z*-stack series of fluorescently labelled *E.coli* cells in a random microcapillary, starting from bottom to the centre of microcapillary. We noticed a gradient of cells captured, with higher density of bacteria measured at the bottom part of the capillary in line with SEM data reported in section 3.2, as a result of bacteria settling during the incubation of *E.coli* sample (Figure 4C). We hypothesized gravity settling enhanced the overall capturing of cells within the microcapillaries. To validate this we carried out a series of in-flow capture experiments of *E.coli* at very low flow rate or 1 μl/ml. Note this corresponds to the total flow rate split between the 10-bore MCF strip, and we assumed an even flow distribution between the 10 parallel microcapillaries. Figure 4B (i) shows a time sequence at four different *z* heights; film files are provided as Supplementary Information and further explained in Appendix A. We then used image analysis to determine the fraction of *E.coli* cells that remained mobile between consecutive frames (i.e. cells not captured by capAb immobilized) and the fraction *E.coli* cells that remained static (therefore captured) by the coated microcapillaries, these are summarised in Figures 4B (ii) and 4B (iii), respectively. In general, we observed a decrease on the surface density of cells captured with the increasing *z* position in the capillary, confirming the relevant role of gravity and suggesting cell capture is more effective at higher cell densities.

Park *et al.* [33] demonstrated a strip-based biosensor using ELISA and monoclonal antibodies as immunocapture probes to quantify *E.coli* O157:H7 diluted in PBS buffer after 30 min from a range CFU/ml of $1.8x10^3$-$1.8\ x10^8$. Jayamohan *et al.* [34] reported immunomagnetic separation (IMS) of *E.coli* O157:H7 in a range of 3-300 CFU per 100 ml of PBS buffer in 2 hr, with 95% extraction efficiency. Naja *et al*. [35] proposed capture and detection of *E.coli* suspended in PBS, using magnetic immune-nanoparticles. The immunoseparation was assured by attaching specific anti-*E.coli* polyclonal antibodies to the nanoparticles achieving an overall 82% yield. Wang *et al.* [36] validated a multiplex quantum dot-based immunoassay to separate foodborne pathogens (*E.coli* O157:H7, *Salmonella Typhiurium* and *L. monocytogens*) resuspended in PBS buffer with 82-90% capture efficiencies, however the immunoseparation process took 2hr overall for a bacterial level range of 10 to 10^3 CFU/ml. Furthermore Tu *et al.* [37] emphasized the importance of sedimentation and motion of immunobeads in respect to improving the efficiency of bacterial capture, which is in line with the role of gravity settling for cells retained within capillaries in our approach. Although those authors have attempted the immunocapture/separation, they did not address the miniaturization challenges and presented complex and lengthy preparation steps not suitable for POC applications in contrast to our proposed approach.

Note that immunocapturing of cells as presented in this work is unable to distinct between viable and dead cells in contrast to e.g. microbiological cells culturing, however clinical samples of active infections tend to have a very high fraction of viable cells (and in case of *E. coli* only live cells remain mobile). This disadvantage of our methodology based on capturing bacterial cells in plastic microcapillaries is surpassed by several advantages compared to other methods. For example, the gold standard method for diagnosis of UTI is entirely based on microbiological plate culture, requiring a trained microbiologist therefore totally limited to laboratory setting [3,4,7]. Urine samples are presented with unknown cell density, requiring the need to plate a whole dilution series. The procedure can be cost-efficient but is very slow, requiring 2-3 days for counting CFUs [2,4,5]. More modern procedures including enzyme amplification and polymerase chain reaction (PCR) are also complex in terms of fluid handling, requiring enriched samples and pre-selective steps [2, 4, 7, 21], therefore financially un-attractive and restricted to analytical labs. Fluorescence cell sorting or flow cytometry is an effective method for counting cells yet it requires fluorescent labelling and separation of cells (which can be done with e.g. magnetic-activated cell sorting), therefore the method is complex and again limited to lab setting. Immunoassay methods based on plastic surfaces coated with specific capturing antibodies are widely accepted including at point-of-care and sensitive for bacterial detection.

We imaged all capillaries on a given MCF strip (results not shown) and in general observed a small variation (d_h = 206 ± 12.6 µm) between the capillaries in the middle of the strips and

those at the edges [23,31]. This is due to a 6% of variation on d_h for each capillary across the strip inherent from the melt extrusion fabrication process. According to Haggen-Poiseuille's equation pressure drop is proportional to $(d_h)^4$ a significant variation in flow distribution and mean residence time of the sample across all MCF strips [31]. We tracked individual cells in the in-flow experiments of confocal imaging in-flow to determine the velocity profile of *E. coli* cells flowing along the straight microcapillaries. Note the magnification used in the confocal imaging aimed maximum resolution, therefore a small field of view of just 200×200 μm^2 was used. All experimental data was collected assuming as reference capillary number 5 (at centre of MCF). We noticed higher bacteria velocities at the centre of the capillary, and negligible as it approached the walls, agreeing with parabolic velocity profile characteristic of laminar flow in microcapillaries (Figure 5) and described by:

$$\boldsymbol{u(r) = u_{max} \times \left[1 - \left(\frac{r}{R}\right)^2\right]} \quad (6)$$

where $u(r)$ (µm/s) is the superficial flow velocity at radial position r (µm) estimated, u_{max} (m/s) the maximum flow velocity achieved in centre of capillary, and R (µm) the radius of the capillary (we assumed $R = d_h/2$). We estimated a maximum fluid velocity in the centre of the wall capillary of 53 µm s^{-1} and wall shear rate of 101 s^{-1}. We observed a degree of flow focusing, with cells preferring to flow through the centre of the capillary, corresponding to streamlines with reduced shear rate therefore reduced resistance. This focusing of cells in microcapillaries has a large implication in antibody capture of cells by reducing the probability of cells interacting with the wall and being captured by antibody immobilized at the wall, this observation is very relevant to any method replying on wall immobilization of antibody for quantitation and capture of bacteria. The maximum velocity of bacteria was always observed to be lower (on average 40-50%) than the estimated core fluid velocity. This was consistent across multiple replicas and is presumably indicative of mobility of *E.coli* cells. According to Wioland *et al*. [32], *E.coli* trends to walls, however in presence of continuous flow they follow the orientation direction of fluid flow. Those are consistent with our LSCM observations.

3.4. Capturing equilibrium and immunospecificity of antibody-bacteria binding

We studied the equilibrium and efficiency of *E.coli* capture in MCF strips coated with immobilized capAb and incubated with *E. coli* samples for 20 min (Figure 6). All experimental runs were duplicated and values quantified with a standard deviation within 5%. Note that a capillary entirely coated with capAb presents a SAV ratio 4 times larger than the one obtained by coating just one surface in a microchannel with the same dimensions, so the cylindrical shape of the microcapillaries offers larger surface for capturing cells but also helps pushing the equilibrium towards bacteria-capAb complex formation.

All independent experimental runs 1 to 3 (without washings) and run 4 (with washing) showed a great consistency with a cross-correlation coefficient of $R^2 > 0.9973$, with both CFU/ml and CFU/cm^2 increasing linearly with enhancing bacteria loading, up to 6 orders of magnitude beyond the CFU/ml estimated from a full monolayer (~4.5 $\log_{10}$ CFU/ml). The equilibrium plot showed bacteria capturing as a first order process with no clear trend in respect to saturation of the surface in the whole window of concentration tested (up to ~10^{12} CFUs/ml). The efficiency for *E.coli* capturing was 100% for small values of CFU/ml (up to 2-3 $\log_{10}$) and followed an exponential decay with increasing CFU/ml in the incubated sample, however surprisingly it remained meaningful for the whole range of CFU/ml tested. For the range relevant to UTIs (up to 10^8 CFUs/ml), the capturing remained between 100 and 20% with just 20 min incubation, so in alignment with the capturing yields reported by e.g. Naja et al. [35] and Wang et al. [36].

The bacteria capturing resulted from a combination of immunoaffinity capturing by the immobilized capAb and gravity settling of cells within the microcapillaries. The last was especially noticeable at high cell densities and presumably linked to aggregation of *E. coli* cells which is widely reported in literature. Confocal imaging experiments summarised discussed in section 3.3 showed settling of *E. coli* cells occurred within few minutes, and definitely within the 20 min incubation time used for these set of experiments. Although the lowest CFUs/ml tested was 148 (equivalent to 2.15 $\log_{10}$), the plots in Figure 6 suggest our approach for immunocapturing of *E. coli* is 100% efficient at very low CFUs/ml, representing a limit of detection of at just 1 CFU (equivalent to 7.5 CFU/ml for a 40 cm long MCF strip). Based on the correlation presented for the CFU/ml vs CFU/cm^2 plot, with a cross-correlation coefficient, $R^2 = 0.9973$, the capturing of 1 CFU (equivalent to 0.04 CFU/cm^2 on the *y* axis) occurs at 10 CFU/ml in solution (*x* axis), which means ~1 CFU for the volume of MCF used (133 μl), confirming the best possible limit of detection.

Run 4 aimed testing the effect of adding a washing step with 0.05% w/w Tween 20 in PBS for separating bound from unbound cells following incubation of sample with bacteria, and showed negligible effect of washing at higher cell densities. In spite of a 62-fold difference in diameter for an IgG antibody molecule (160 Angstroms) [38] and *E. coli* cell (between 0.5 and 5 μm) [37], cells capturing revealed resistant to fluid shear given by equations (3)-(4).

Although a methodology for bacteria capturing relying on gravity as the one proposed in this work can sound unpredictable and inaccurate, the data from independent experimental runs demonstrate this method is very reproducible. It is however not possible at this stage to comment on whether this methodology shows any level of selectivity to enable its use for separation of cells or capturing of cells from mixed cultures. This will be subject of future studies.

We tested the immunospecificity of coated microcapillaries for *E.coli* capture by carrying out a sandwich immunoassay amplification following a step of immunocapture of bacteria sample. Figure 7 shows the polyclonal antibody (specific to K and O serotypes of *E .coli*) demonstrated specificity for capturing *E.coli* (gram-negative) when tested bacterial samples of *Bacillus subtillis* (gram-positive) suspended in PBS 0.1M buffer. Statistically, no significant difference was observed in the absorbance signal obtained for the sandwich immunoassays incubated with *Bacillus subtillis* and PBS buffer (negative control), however it should be noted that the immunoassays were carried at very high cell densities and the signal shown by *Bacillus sp.* will drop as cell density decreases. Also, *Bacillus sp.* are not present in UTI infections. 'Sanvicens et al. [39] have used the same set of polyclonal antibodies for a quantum dot-based array for sensitive detection of E.coli O157:H7 and reported a negligible interference with pathogenic *S. aureus* (gram- positive) and *P. aeruginosa* (gram-negative) at low cell densities. The cross-reactivity of the antibodies should ideally have been tested against all the most common bacterial strains present in urine samples, which according to the European guidelines for urinalysis [40] are *Enterobacter spp*., *Enterococcus spp*., *Klebsiella spp*., *Proteus mirabilis* and *Pseudomonas aeruginosa* (majority enterobacteriacea), however any limitations with cross-reactivity of antibodies can be solved by using more specific, monoclonal antibodies for capturing and/or detection stages. Cross-reactivity of capture antibody with other species can actually be an advantageous feature for offering identification of multiple bacteria strains with a single capture antibody. A comprehensive cross-reactivity study will be the focus of future studies. It is common for some polyclonal antibodies to show cross-reactivity with other species, this will need to be fully examined using pure and mixed cultures before implementation of this method into an actual diagnostic test. Nevertheless, this new data related to immunocapturing is believed very useful for future developments of rapid test for UTIs, from clinical samples likely to contain pathogenic and non-pathogenic bacteria.

4. Conclusions

This experimental study has demonstrated efficient capturing of *E.coli* in plastic microcapillaries enabled by a combination of immunocapturing via an immobilized polyclonal antibody against *E.coli* and gravity settling, as evidenced by a range of optical imaging techniques. MCF platform showed immunospecificity for capturing *E.coli K12* cells and high 'affinity' of capturing, with 100% capturing efficiency at ~10^2 CFUs/ml. This study also highlighted the importance of interrogation volume in respect to the minimum CFUs/ml that can be detectable which intrinsically sets the limit of detection and the development of rapid miniaturised immunoassays based on affinity capturing of bacteria. Washings are an important feature in high-performance heterogeneous immunoassays (utilising immobilized antibodies), therefore the washing resistant cell capturing herein reported open up the opportunity to

integrate cells capturing with antibody and enzymatic labelling for quantitation of cells in future works. These new insights into microfluidic capture of *E.coli* are believed to be a major step towards the development of rapid immunoassay-based POC tests for quantitation of *E.coli* and other microbial pathogens. This method for capturing bacterial cells can certainly be applied to capture and eventually separation of other bacteria strains, to be subject of future publications.

Acknowledgements

The authors are grateful to Patrick Hester from Lamina Dielectrics Ltd for providing the MCF material, to Dr Ana Barbosa for assistance with antibody coating of microcapillaries, Abdulkadir Hussein Sheik for assistance with LSCM and Dr Karen Coopman for encouragement and assistance during the project. This work was funded by Loughborough University.

Appendix A. Supplementary data

Description of films provided as supplementary data associated with Figure 4 in this article can be found in the electronic file in the online version.

References

[1]. European Commission, Antimicrobial Resistance. 2016, acessed on 16th June, 2017; https://ec.europa.eu/health/amr/antimicrobial-resistance_en.

[2]. Kokkinis, G., Plochberger, B., Cardoso, S., Keplinger, F., & Giouroudi, I., A microfluidic, dual-purpose sensor for in vitro detection of Enterobacteriaceae and biotinylated antibodies, Lab Chip 16, (2016), 1261-71, https://doi.org/10.1039/c6lc00008h.

[3]. Foudeh, A., Didar, T., Veres, T., & Tabrizian, M., Microfluidc designs and techniques using lab-on-a-chip devices for pathogen detection for point-of-care diagnostics, Lab Chip 12, (2012), 3249-3266, https://doi.org/10.1039/c2lc40630f.

[4]. Mairhofer, J., Roppert, K., & Ertl, P., Microfluidic Systems for pathogen sensing: A review, Sensors 9, (2009), 4804-4823, https://doi.org/10.3390/s90604804.

[5]. Olanrewaju, A., Ng, A., DeCorwin-Martin, P., Robillard, A., & Juncker, D., Microfluidic Capillaric Circuit for Rapid and Facile Bacteria Detection, Anal. Chem. 89, (2017), 6846-6853, https://doi.org/10.1021/acs.analchem.7b01315.

[6]. O'Neil, Jim. RAPID DIAGNOSTICS: stop the unnecessary use of antibiotics, The review on antimicrobial resistance, October 2015.

[7]. Su, W., Xinghua, G., Lei, J., & Qin, J. , Microfluidic platform towards point-of-care diagnostics in infectious diseases, J. of Chromatog. A 1377, (2015), 13-26, https://doi.org/10.1016/j.chroma.2014.12.041.

[8]. Cho, S., Park, T., Nahapetian, T., & Yoon, J. , Smartphone-based, sensitive uPAD detection of urinary tract infection and gonorrhea, Biosens. Bioeletron. 74, (2015), 601-611, https://doi.org/10.1016/j.bios.2015.07.014.

[9]. Chang, M.-s., Yoo, J., Woo, D., & Chun, M.-S., Efficient detection of Escherichia coli O157:H7 using a reusable microfluidic chip embedded with antimicrobial peptide-labeled beads, Analyst 140, (2015), 7997-8006, https://doi.org/10.1039/c5an01307k.

[10]. Stratz, S., Eyer, K., Kurth, F., & Dittrich, P., On-Chip Enzyme Quantification of Single Esherichia Coli Bacteria by Immunoassay-based analysis, Analyt. Chem. 86, (2014), 1237-12381, https://doi.org/ 10.1021/ac503766d.

[11]. Sin, M., Mach, K., Wong, P., & Liao, J., Advances and challenges in biosensor-based diagnosis of infectious diseases, Expert Review of Molecular Diagnostics 14, (2014), 255-244, https://doi.org/10.1586/14737159.2014.888313.

[12]. David, S., Polonshii, C., Gheorghiu, M., Bratu, D., Dobreand, A., & Gheorghiu, E., Assessment of pathogenic bacteria using periodic actuation, Lab Chip 13,(2013), 3192-3198, https://doi.org/10.1039/C3LC50411E.

[13]. Eltzov, E., & Marks, R., Miniaturized Flow Stacked Immunoassay for Detecting Escherichia coli in a Single Step, Analyt. Chem. 88, (2016), 6441-6449, https://doi.org/10.1021/acs.analchem.6b01034.

[14]. Clotilde, L., Bernard IV, C., Salvador, A., Lin, A., Lauzon, C., Muldoon, M., Carter, J., A 7-plex microbead-based immunoassay for serotyping Shiga toxin-producing Esherichia coli, J. of Microbiol. methods 92, (2013), 226-230, https://doi.org/ 10.1016/j.mimet.2012.11.023.

[15]. Wang, N., He, M., & Shi, H.-C., Novel indirect enzyme-linked immunosorbent assay (ELISA), Anal. Chim. Acta 590, (2007), 224-231, https://doi.org/ 10.1016/j.aca.2007.03.041.

[16]. Kong W., Xiong J., Yue, H., Fu, Z., Sandwich Fluorimetric Method for Specific Detection of Staphylococcus aureus Based on Antibiotic-Affinity Strategy, Analyt. Chem. 87, (2015), 9864−9868, https://doi.org/10.1021/acs.analchem.5b02301.

[17]. Shih, C., Chang, C., Hsu, M., Lin, J., Kuan, C., Wang, H., Cheng, C., Paper-based ELISA to rapidly detect Escherichia coli, Talanta 145, (2015), 2-5, https://doi.org/10.1016/j.talanta.2015.07.051.

[18]. Ikami, M., Kawakami, A., Kakuta, M., Okamoto, Y., Kaji, N., Tokeshi, M., & Baba, Y., Immuno-pillar chip: a new plataform for rapid and easy-to-use-immunoassay, Lab Chip 10, (2010), 3335-3340, https://doi.org/10.1039/c0lc00241k.

[19]. Gervais , L., & Delamarche, E., Towards one-step point-of-care immunodiagnostics using capillary-driven microfluidics and PDMS substrates, Lab Chip 10, (2009), 3330-3337, https://doi.org/10.1039/b906523g.

[20]. Chin, C., Linder, V., & Sia, S., Lab-on-a-chip devices for global health: Past studies and future opportunities, Lab Chip 7, (2007), 41-57, https://doi.org/10.1039/b611455e.

[21]. Andreotti, P. E., Ludwig, G., Peruski, A., Tuite, J., Morse, S. S., & Peruski, L., Immunoassay of infectious agents review, BioTechniques Euro Edition 35, (2003), 850-859.

[22]. Kunst, B., Schots, A., & Visser, A., Detection of flowing fluorescent particles in a Microcappillary using fluorescence correlation spectroscopy, Analyt. Chem. 72, (2002), 5350–5357, https://doi.org/10.1021/ac0256742.

[23]. Edwards, A., Reis, N., Slater, N., & Mackley, M., A simple device for multiplex ELISA made from melt-extruded plastic microcapillary film, Lab Chip 11, (2011), 4267-4273, https://doi.org/10.1039/C0LC00357C.

[24]. Barbosa, A., Castanheira, A., Edwards, A., & Reis, N., A lab-in-briefcase for rapid prostate specific antigen (PSA) screening from whole blood, Lab Chip 14, (2014), 2918-2928, https://doi.org/10.1039/c4lc00464g.

[25]. Barbosa, A., Gehlot, P., Sidapra, K., & Edwards, A., Portable smartphone quantitation of prostate specific antigen (PSA) in a fluoropolymer microfluidic device, Biosens. Bioelectron. 7, (2015), 5-14, https://doi.org/10.1016/j.bios.2015.03.006.

[26]. Castanheira, A., Barbosa, A., Edwards, A., & Reis, N., Multiplexed femtomolar quantitation of cytokines in a fluoropolymer microcapillary film, Analyst 140, (2015), 5609-5618, https://doi.org/10.1039/C5AN00238A.

[27]. Reis, N., Pivetal, J., Loo-Zazueta, A., Barros, J., & Edwards, A., Lab on a stick: multi-analyte cellular assays in a microfluidic dipstick, Lab Chip 16, (2016), 2891-2899, https://doi.org/10.1039/C6LC00332J.

[28]. Barbosa, A., Wichers, J., Amerongen, A., Reis, N., Towards One-Step Quantitation of Prostate-Specific Antigen (PSA) in Microfluidic Devices: Feasibility of Optical Detection with Nanoparticle Labels, BioNanoScience (2017), https://doi.org/10.1007/s12668-016-0390-y.

[29]. Schneider, C.A., Rasband, W.S and Eliceiri, W.S., NIH Image to ImageJ: 25 years of image analysis, Nature Methods 9, (2012), 671-675, https://doi.org/10.1038/nmeth.2089.

[30]. M. Grabe , R. Bartoletti, T.E. Bjerklund Johansen, T. Cai , M. Çek, B. Köves, .B. Wullt., 2015, Guidelines on Urological Infections, acessed September 2017; http://uroweb.org/wp-content/uploads/19-Urological-infections_LR2.pdf.

[31]. Reis, N., & Puma, G., A novel microfluidic approach for extremely fast and efficient photochemical transformations in fluoropolymer microcapillary films, Chem. Comunications 51, (2015), 8389-8560, https://doi.org/ 10.1039/C5CC01559F.

[32].Wioland H., Lushi E. and Goldstein E., Directed collective motion of bacteria under channel confinement, New J. Phys. 18, (2016), 075002, https://doi.org/10.1088/1367-2630/18/7/075002.

[33]. Park S., Kim H., Paek S-H., Hong J., Kim Y., Enzyme-linked immuno-strip biosensor to detect Escherichia coli O157:H7, Ultramicroscopy (2008), 1348-1351, https://doi.org/ 10.1016/j.ultramic.2008.04.063.

[34]. Jayamohan H., Gale B., Minson B., Lambert C., Gordon N., Sant H., highly Sensitive Bacteria Quantification Using Immunomagnetic Separation and Electrochemical Detection of Guanine-Labeled Secondary Beads, Sensors 15, (2015), 12034-12052, https://doi.org/10.3390/s150512034.

[35]. Naja G., Bouvrette P., Hrapovich S., Liu Y., Luong J., Detection of bacteria aided by immuno-nanoparticles, J. Raman Spectrosc. 38, (2007), 1383-1389, https://doi.org/ 10.1002/jrs.1785.

[36]. Wang H., Li Y., Wang A., Slavik M., Rapid, sensitive and simultaneous detection of three foodborne pathogens using Nanobead-based Immunoseparation and Quantum Dot-based Immunoseparation and Quantum Dot-based multiplex Immunoassay, J. Food Prot. 74, (2011), 2039-2047, https://doi.org/10.4315/0362-028X.JFP-11-144.

[37]. Tu S.I., J. Uknalis, M. Gore, P. Irwin, I. Feder, Factors affecting the bacterial capture efficiency of immuno beads: A comparison between beads with different size and density, J Rapid Meth. Aut. Mic 11, (2003), 35-46, https://doi.org/10.1111/j.1745-4581.2003.tb00406.x.

[38]. Buijs, J.; Lichtenbelt, J. W. T.; Norde, W.; Lyklema, J. Adsorption of Monoclonal IgGs and Their F(ab′)2 Fragments onto Polymeric Surfaces, Colloids Surfaces B Biointerfaces, 5 *(*1995), 11–23, https://doi.org/10.1016/0927-7765(95)98205-2.

[39]. N. Sanvicens, N. Pascual, M.T. Fernández-Argüelles, J. Adrián, J.M. Costa-Fernández, F. Sánchez-Baeza, A. Sanz-Medel, M.P. Marco, Quantum dot-based array for sensitive detection of Escherichia coli, Anal. Bioanal. Chem. 399 (2011) 2755–2762. https://doi.org/10.1007/s00216-010-4624-5

[40]. O. Aspevall, H. Hallander, V. Gant, T. Kouri, European guidelines for urinalysis: A collaborative document produced by European clinical microbiologists and clinical chemists under ECLM in collaboration with ESCMID, Clin. Microbiol. Infect. 7 (2001) 173–178. https://doi.org/10.1046/j.1198-743x.2001.00237.x

Figure captions

Figure 1 Fluoropolymer MCF platform and accessories used to carry out in-flow LSCM experiments. **A** Top view of MCF strips. **B** Cross section photograph of a 10 bore MCF. **C** MCF accessories used in LSCM: **1** - MCF fitting connected to the luer with lock syringe, **2** - MCF holder capable of holding several MCF parallel MCF strips. **D** MCF accessories to connect MCF strips to Harvard Pump: **1** - MCF fitting with female luer, **2** - MCF male fitting, **3** - Push-fit seal, **4** - Terumo Syringe 2.5 ml luer lock. **E** 'Open fluidics' design of microcapillaries that enable passing a large volume of sample through the microcapillaries with immobilized capAb.

Figure 2 LSCM imaging of fluorescently labelled *E.coli* in a plastic microcapillary. **A** Serial dilution of *E.coli* in PBS imaged in a 200×200 μm^2 section of a 200μm i.d microcapillary. **B** Relationship between theoretical and experimental CFUs in interrogation volume (estimated as 62.5 nL).

Figure 3 SEM microphotographs of inner wall of fluoropolymer MCF microcapillaries. **A** MCF strip coated with blocking buffer solution and then incubated with synthetic sample **B** Uncoated MCF strip incubated only with synthetic sample. **C** and **D** MCF strips coated with capture antibody, blocking buffer solution and synthetic sample, showing size and morphology of *E.coli* cells. **E** and **F** Detail of top half and bottom half of the same microcapillary coated with capAb, blocking buffer solution and incubated with synthetic sample, revealing bacteria binding is not spatially uniform in horizontal capillaries.

Figure 4 LSMC imaging of *E.coli* cells in plastic microcapillaries coated with polyclonal anti-*E.coli* antibody. **A** *z*-stack series of MCF strip coated with 40 μg/ml capAb and blocking solution, and incubated for 5 min (in continuous flow) with a synthetic sample of *E.coli* stained fluorescently in 0.01M PBS. Sequence shows confocal images from bottom of capillary, *z* position = 35 μm (left) towards the middle of capillary, *z* position = 100 μm (right). **B** In-flow capture of *E.coli* cells, total flow rate of 1 μl/min (for 10-bore MCF), (i) snapshot of time sequenced films imaged at 4 fixed *z* positions as provided in Supplementary Information in Appendix A. Snapshot shows last frame in time sequence; for each stack two consecutive time frames were subtracted yielding in (ii) the cells that remained mobile (i.e. not captured by capAb) and in (iii) bacteria cells successfully captured. **C** $\log_{10}$ total cells and cells captured in interrogation window with *z* position in capillaries.

Figure 5 Speed velocity of bacteria flow in microcapillary number 5 versus normalized radial position, for rate of 1μl/min in the 10-bore MCF. Individual *E.coli* cells were tracked in two separate runs (1 & 2) and the mean velocity plotted.

Figure 6 Equilibrium of *E.coli* capturing in the MCF coated with polyclonal antibody based on 3 independent, experimental replicates without (run 1-3) and with (run 4) sample washing. Full monolayer capacity was based on footprint of individual *E.coli* cells. Results are plots as CFU/ml and CFU/cm and efficiency of capturing showing coated capillaries are particularly efficient capturing *E.coli* at low cell densities and washings had no detectable effect in reducing cells capture efficiency, validating immunocapture of polyclonal capture antibodies

Figure 7 Colorimetric sandwich immunoassay showing immunospecificity of cells captured in microcapillaries using 10^8 CFU/ml of *E.coli K12, Bacillus subtillis* and buffer diluent (0.01M PBS).

Figures

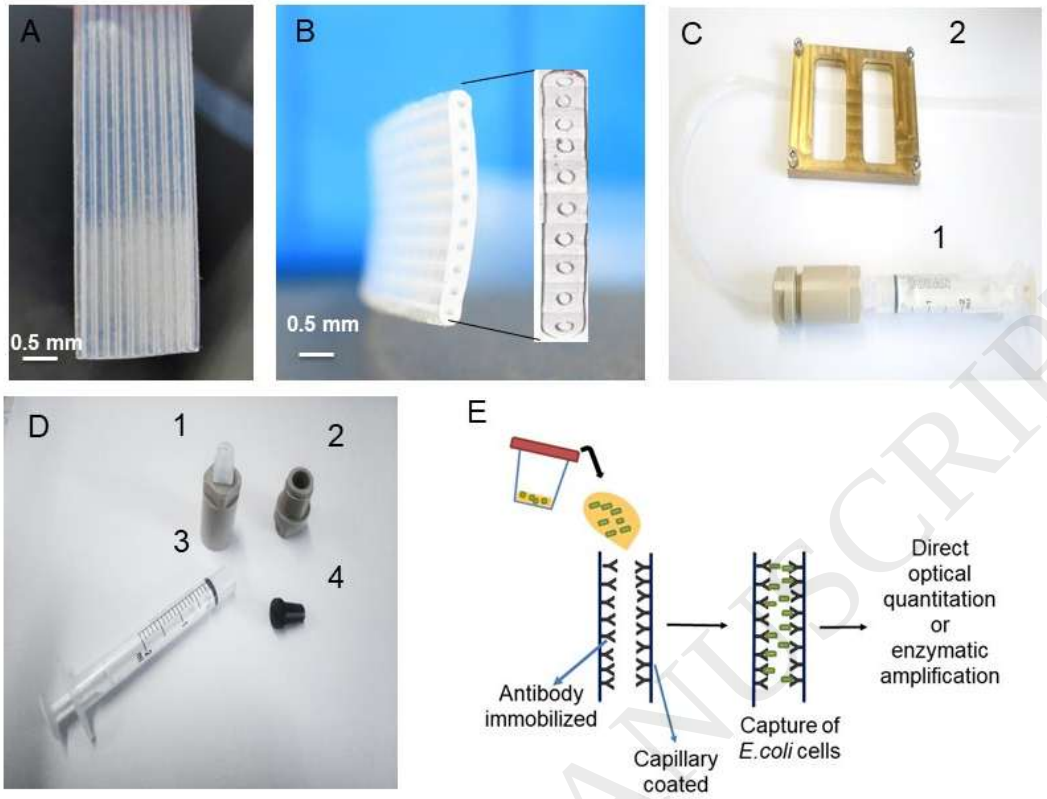

Figure 1

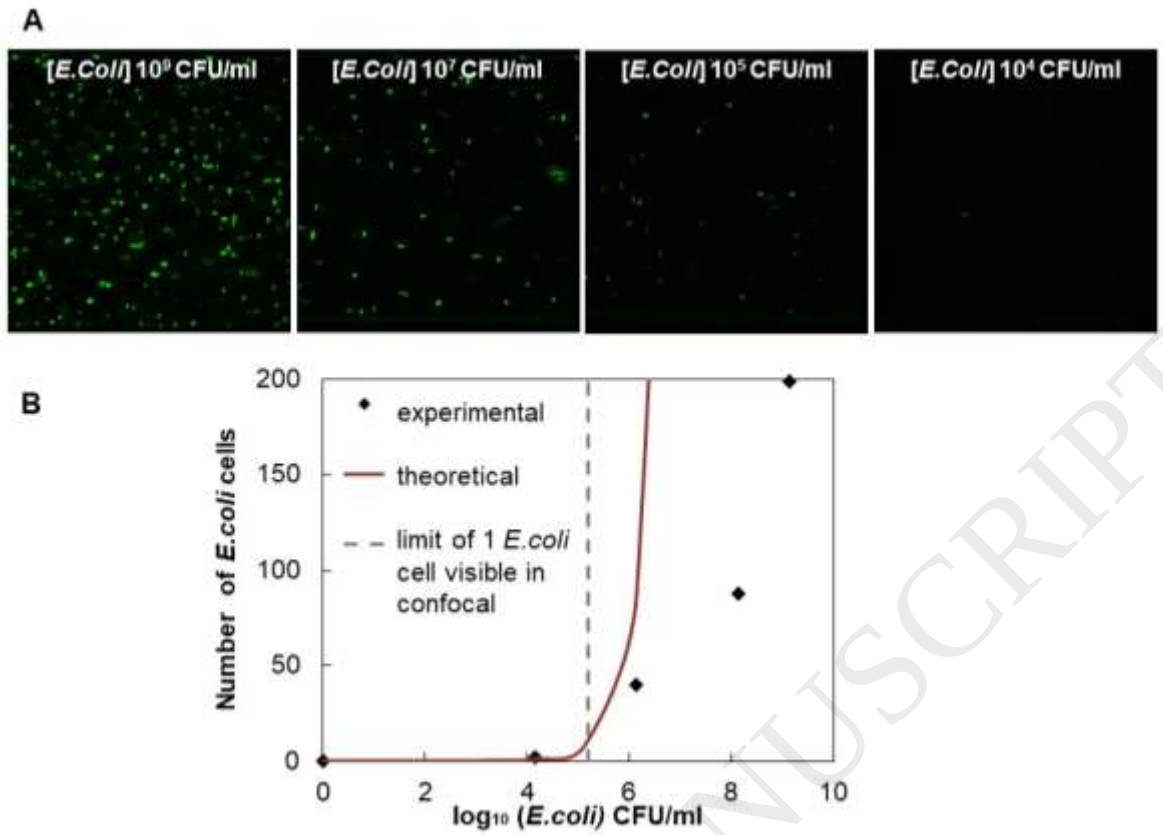

Figure 2

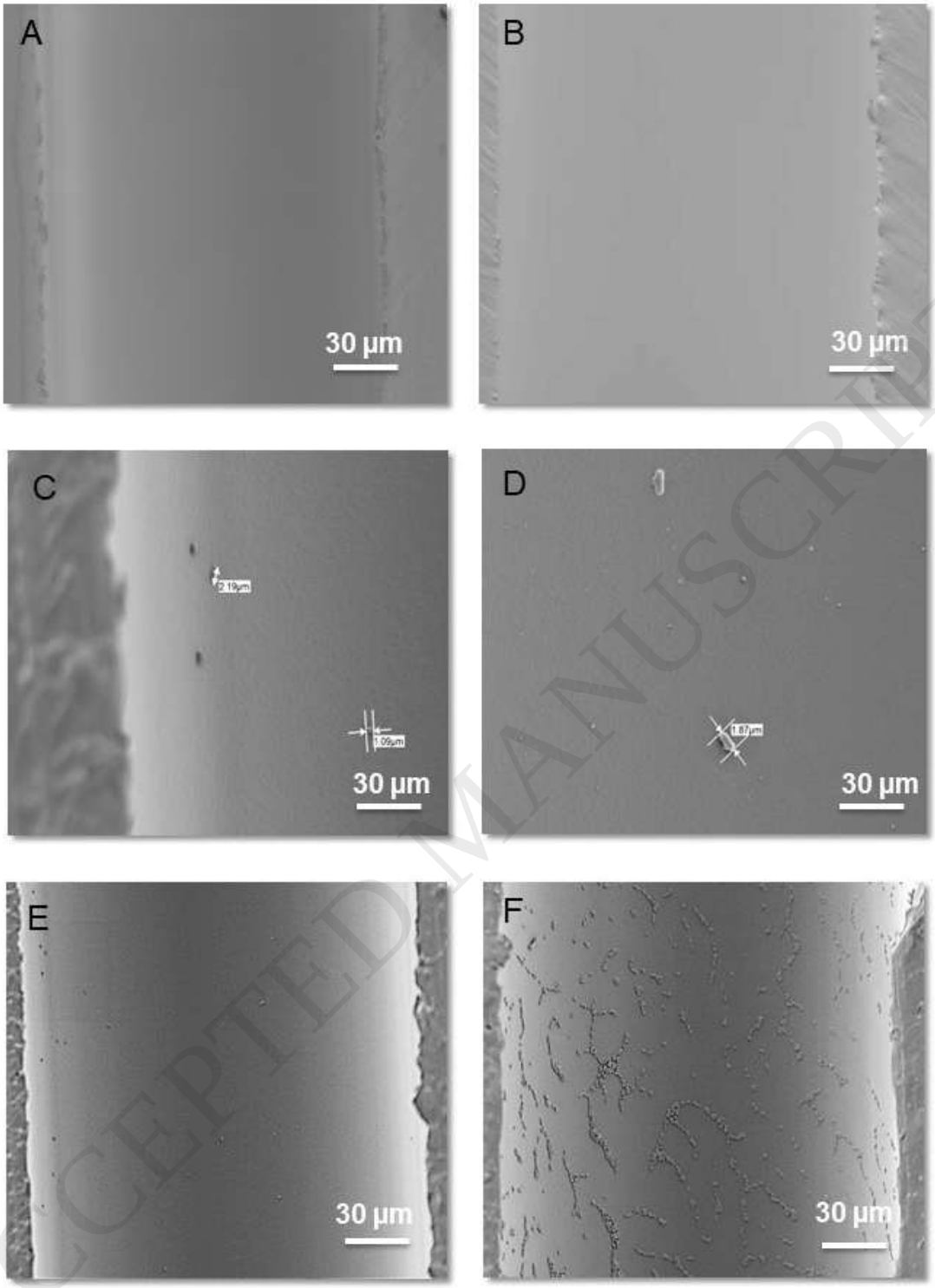

Figure 3

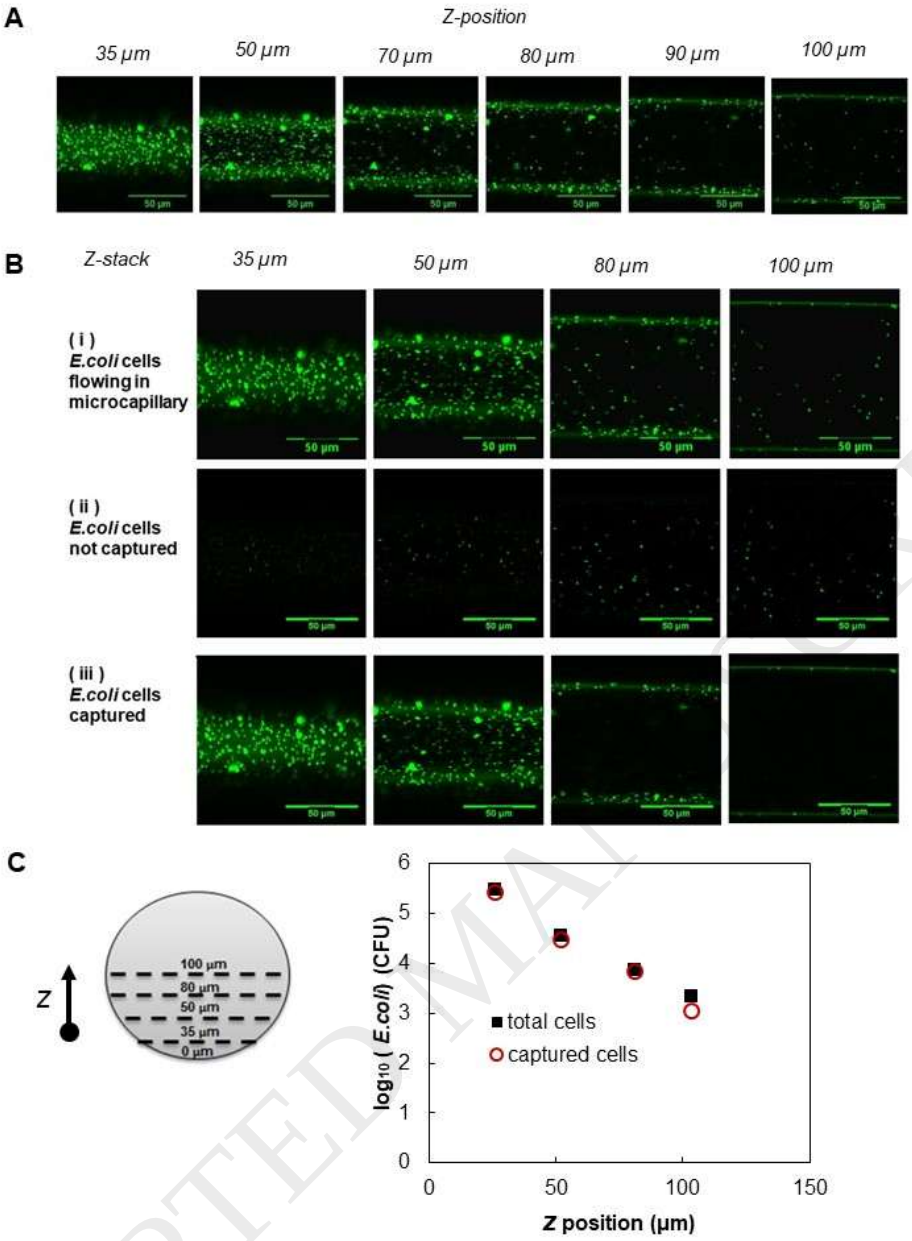

Figure 4

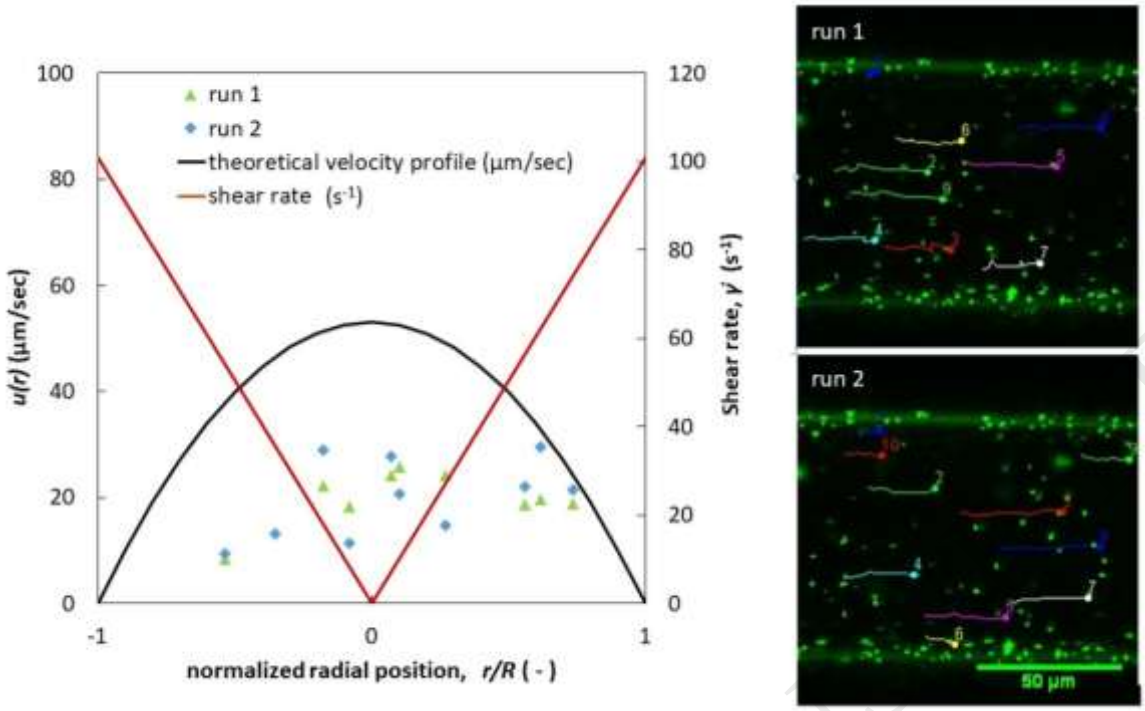

Figure 5

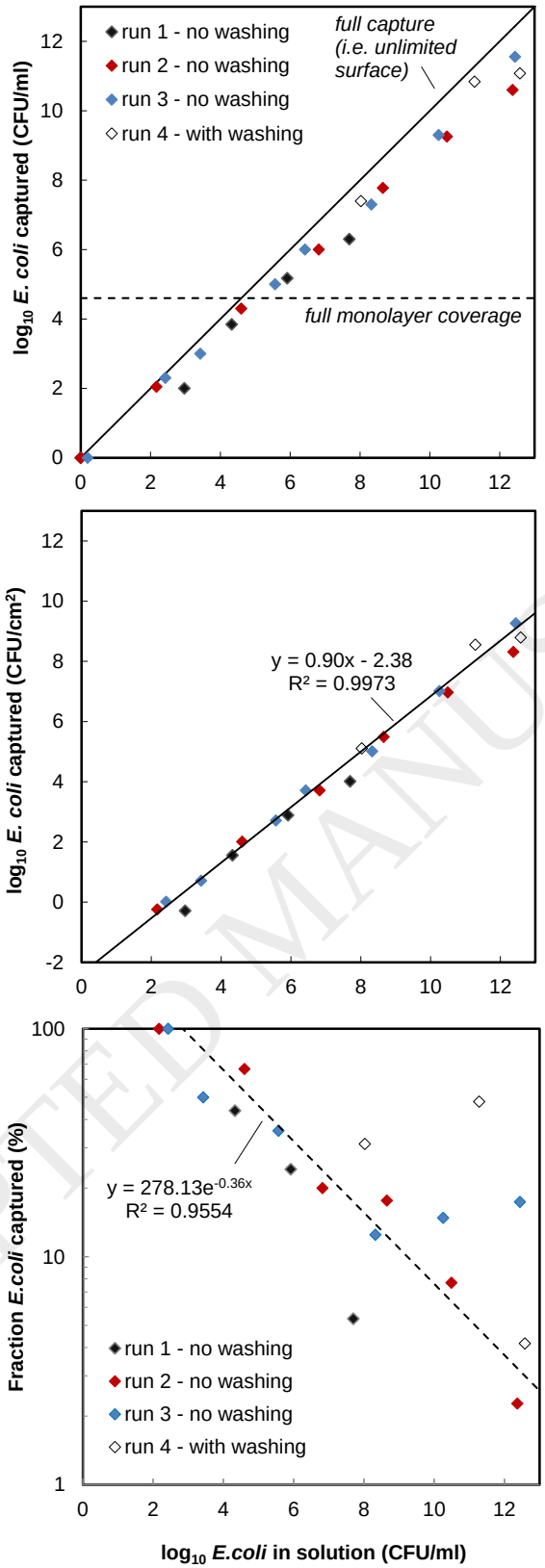

Figure 6

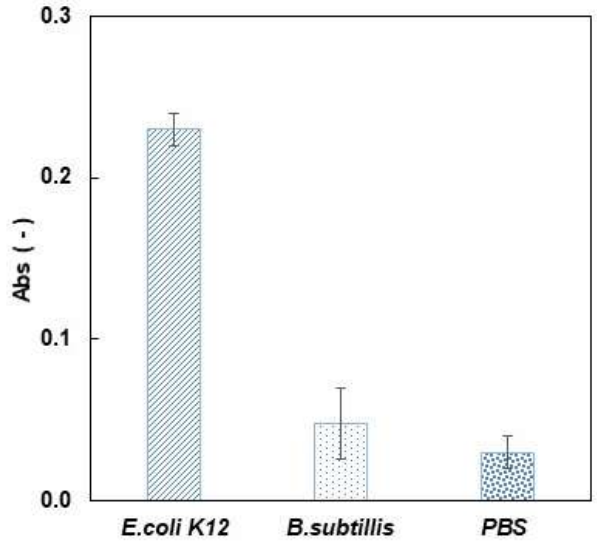

Figure 7