2020 IEEE 17th International Symposium on Biomedical Imaging (ISBI)
April 3-7, 2020, Iowa City, Iowa, USA

ENUMERATION OF AMPICILLIN-RESISTANT *E. COLI* IN BLOOD USING DROPLET MICROFLUIDICS AND HIGH-SPEED IMAGE PROCESSING

[1] Yiyan Li, [3-8] Hemanth Cherukury, [3-8] Jan Zimak, [2] Jacob Harrison, [9] Ellena Peterson, and [3-8] Weian Zhao

[1] Department of Physics and Engineering, Fort Lewis College, Durango, CO, USA
[2] Department of Chemistry & Biochemistry, Fort Lewis College, Durango, CO, USA
[3] Sue and Bill Gross Stem Cell Research Center, University of California, Irvine, Irvine, CA, USA
[4] Department of Pharmaceutical Sciences, University of California, Irvine, Irvine, CA, USA
[5] Chao Family Comprehensive Cancer Center, University of California, Irvine, Irvine, CA, USA
[6] Edwards Life Sciences Center for Advanced Cardiovascular Technology, University of California, Irvine, Irvine, CA, USA
[7] Department of Biomedical Engineering, University of California, Irvine, Irvine, CA, USA
[8] Department of Biological Chemistry, University of California, Irvine, Irvine, CA, USA
[9] Department of Pathology and Laboratory, University of California Irvine, Irvine, CA, USA.

ABSTRACT

Bacteria entering the bloodstream causes bloodstream infection (BSI). Without proper treatment, BSI can lead to sepsis which is a life-threatening condition. Detection of bacteria in blood at the early stages of BSI can effectively prevent the development of sepsis. Using microfluidic droplets for single bacterium encapsulation provides single-digit bacterial detection sensitivity. In this study, samples of ampicillin-resistant *E. coli* in human blood were partitioned into millions of 30 μm diameter microfluidic droplets and followed by 8-hour culturing. Thousands of fluorescent bacteria from a single colony filled up the positive droplets after the culturing process. A circle detection software based on Hough Transform was developed to count the number of positive droplets from fluorescence images. The period to process one image can be as short as 0.5 ms when the original image is pre-processed and binarized by the developed software.

Keywords *– Ampicillin-resistant bacteria, microfluidics, image processing, single-cell analysis*

1. INTRODUCTION

Single-cell screening and sorting have benefited from the unique liquid handling capabilities of microfluidic droplets. Microfluidic droplets in the forms of fine aerosol or an emulsion can be produced by flow focusing technology [1-3]. Producing massive droplets in the form of an emulsion is achievable; however, enumerating the positive droplets in the emulsion within an acceptable turnaround time is challenging. A traditional flow cytometer uses a single photodetector to receive and process one-dimensional (1D) fluorescence emission spikes from the sample [4]. The 1D fluorescence spikes read by the photodetector may come from other fluorescent particles and debris in the channel instead of positive droplets, which causes false-positives [5]. Multiple detectors can be used at the same time in an integrated setup, but the addition of more signal lines limits the bandwidth of the communication ports from the flow cytometer and the computer, which in turn slows down the screening process. Therefore, there is a high demand for rapid and accurate screening techniques to enumerate positive droplets from the emulsion [6-8].

In this study, we use a 2D high-speed image processing technique to count positive droplets from an emulsion to avoid false-positive reports in traditional flow cytometers. We tested the image processing software using static fluorescence microscope images which can be applied to the batch processing of thousands of video frames from a high-speed camera. Single bacterium encapsulation was completed by a high-throughput PDMS microfluidic device and followed by 8-hour culturing at 37 °C [9]. The fluorescence dye penetrated the membrane of the bacteria in droplets, bound to the DNA inside bacteria, and lit up the bacteria. After 8 hours, positive droplets emitted strong fluorescence with thousands of fluorescent bacteria being confined within the positive droplets. A high-speed image processing software batch processed all the 2D images and reported the count of the positive droplets which indicated the count of bacteria in the original sample.

2. MATERIALS AND METHODS

2.1. Microfluidic chip fabrication

The microfluidic chips were fabricated from a silicon wafer mold. Undoped 100 mm silicon wafers (University Wafer Inc.) were used as the substrate for

the SU-8 (negative photoresist, Micro Chem Corp.) patterns. SU-8 2050 was spin-coated onto the silicon wafer substrate at 2000 rpm for 30 seconds, followed by 10 minutes of soft-bake at 65 °C and a 1-hour hard-bake at 95 °C. The photomask was printed by Photronics Inc at the 25,000 dpi resolution. After UV exposure, the wafer was baked for 10 minutes at 65 °C followed by a 1-hour hard bake at 95 °C. Wafers were then developed in the SU-8 developer (Micro Chem) for 1-3 minutes before being cleaned by both SU-8 developer spray and by the IPA (isopropanol). The cured SU-8 and its substrate were hard-baked at 180 °C to finalize the patterning. The microfluidic chip was made using the SYLGARD silicone elastomer kit with a 10:1 base to catalyst ratio. The PDMS mixture was degassed in a desiccator for 20 min and cured on a hot plate for 10 min at 85 °C. The cured PDMS chip was peeled off from the wafer mold and bound to plasma-treated glass slides. The plasma treatment on both the PDMS side and the glass side effectively removed the hydrocarbon groups and allowed strong Si – O – Si covalent bonds to form between the two materials via the process.

2.2. Single bacterium encapsulation and droplet bacteria culture

Ampicillin-resistant *E. coli* (clinical isolates from Dr. Ellena Peterson's group at the Department of Pathology and Laboratory at UC-Irvine) in LB liquid culture were mixed with human blood samples from healthy donors at the Department of Pharmaceutical Science of the University of California, Irvine (with approved IRB (UCI HS# 2012-9023)). The bacteria culture, fluorescence dye (SYBR Green I), human blood, and buffer were mixed at a ratio to form a 10 ml sample with 280,000 bacteria and 10% blood. The sample preparation was followed by droplet generation carried out by microfluidic devices. SYBR Green I is a traditional fluorescence dye that binds double-stranded DNA molecules. It is used for quantitative PCR because the fluorescence intensity is correlated with the amount of DNA after every amplification cycle. In this study, the DNA inside bacteria was stained by allowing the SYBR Green I molecules to migrate through the membrane while the bacteria were still alive. The experimental procedure for microfluidic single bacterium culture and analysis can be found in Fig. 1.

Droplets were imaged before the culturing step to ensure the absence of any fluorescence signals. Droplets were cultured at 37 °C for 8 hours before being imaged under a fluorescence microscope. Static images were taken for further image processing and counting.

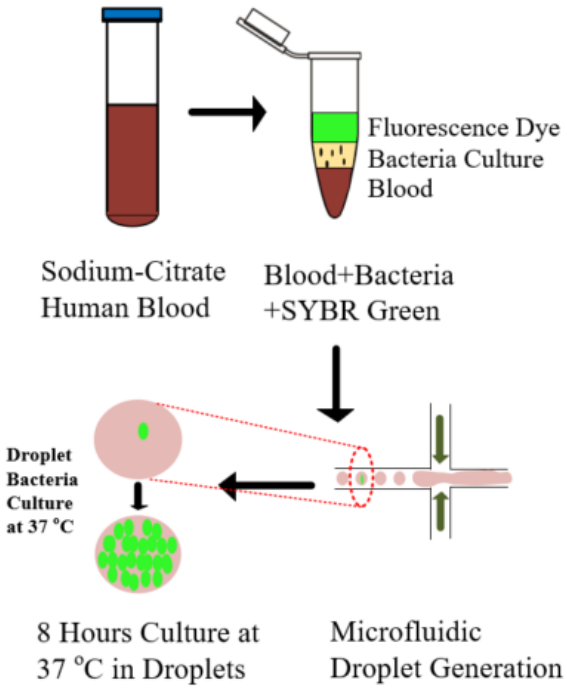

Fig. 1 The experimental procedure for microfluidic single bacterium culture and analysis.

2.3. Image processing and counting (Hough Circle Transform)

This pilot study uses static fluorescence images to simulate the image frames from a high-speed camera which will be used for future study. The image processing software developed in this study is based on the Hough Circle Transform library in OpenCV2 (Python 3.8).

The image was imported and immediately converted into grayscale and then smoothed by a median filter using the 'cv2.mideianBlur()' function in Python. The Hough Circle Transform was executed to detect the positive droplets in an image. The 'Image' class in the PIL library in Python was used to resize the image proportionally. The 'cv2.threshold()' function was used to convert the RGB image into a binary image. The Python script was executed in Spyder, Anaconda.

3. RESULTS

3.1. Droplet culture and time-course imaging

Ampicillin-resistant *E. coli* were cultured in droplets with 10% sodium-citrate human blood, 1 x SYBR Green I, 10 mM Tris (pH 8.0), 90% Terrific Broth, and Ampicillin. Time-course imaging of the droplet bacteria culture was conducted (Fig. 2). The small fluorescence

dots in all the images are white blood cells with their DNA stained by SYBR Green I (Fig. 2 and Fig. 4).

Differences between negative and positive droplets can be observed at the 5th hour but the signal to background ratio was low. The fluorescence signal of positive droplets saturated at the 8th hour. No significant differences were observed after the 8th hour.

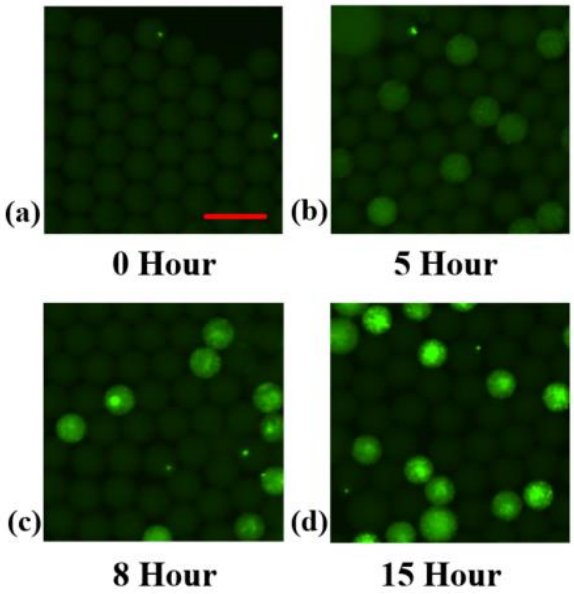

Fig. 2 Time-course imaging of the droplet bacteria culture. Images from Time 0 to Time 15 hours were presented. The fluorescence signal was saturated in the 8th hour. Scale bar: 60 μm.

Coagulation of the red blood cells (RBCs) in droplets is the main reason for the uneven fluorescence signal inside each positive droplet. We use 0.1% Saponin mixed with the bacteria culture before the droplet encapsulation. RBCs were lysed instantly once they are exposed to Saponin (Fig. 3).

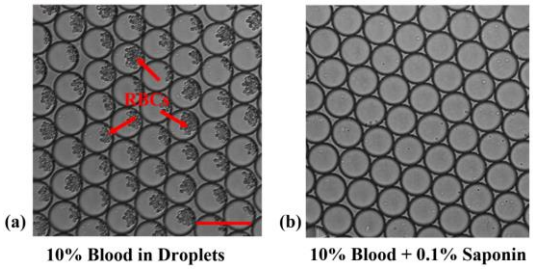

Fig. 3. Red blood cells (RBCs) in droplets in (a) were lysed instantly once they are exposed to Saponin in droplets (b). Scale bar: 60 μm.

3.2 Pre-process the microscope images

The fluorescence images were saved as '.jpg' images and then compressed into a smaller size (339 x 326 pixels in this experiment) for further processing. Negative droplets have background fluorescence which may be reported as positive droplets by the software (Fig. 4a).

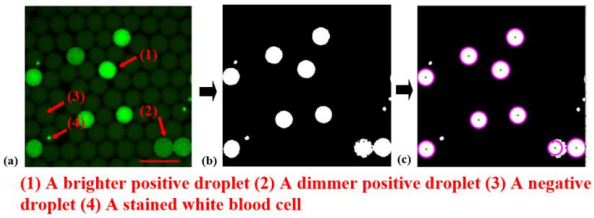

Fig. 4 (a) A 339 pixels x 326 pixels image of positive and negative droplets under a fluorescence microscope. (b) A 339 pixels x 326 pixels binary image converted from the RGB image in (a). (c) The circle detection software developed in this study detected the positive droplets and labeled them using purple circles. Scale bar: 60 μm.

The RGB images were converted into low-resolution binary images for the circle detection and the computation time was significantly reduced. The fluorescent images showed the positive droplets which encapsulated a single bacterium at time 0 were filled with bacteria stained by Sybr Green I after culturing. Not all the droplets showed the same fluorescence intensity, but all the positive droplets are distinguishable from the negative (background) droplets.

Images with different formats and resolutions were tested in the same software (Fig. 5). Images smaller than 50 x 48 pixels showed missing circles. No significant differences between the 100 x 96 and the 50 x 48 images which are probably due to the bottle-neck of the time spent on Hough Transform. The pre-processing and compression can reduce the computation time spent on each image to less than 500 μs (Fig. 5e).

The static fluorescence images used in this study are taken from a microscope. A high-speed camera (Photron MiniUX 32 GB high-speed camera (>5000 fps)) will be used to take video streams of the microfluidic droplet flow while sending on-chip data from the camera to the computer in future studies. The strategy is to schedule half of the on-chip memory in the camera for recording and the other half of the memory for data transmitting to achieve the high-throughput and long-term video stream recording and transmitting. The Photron MiniUX 32

high-speed camera provides the programmable ports for this future work. The software developed in this study will be used for massive image processing with frames from a high-speed camera.

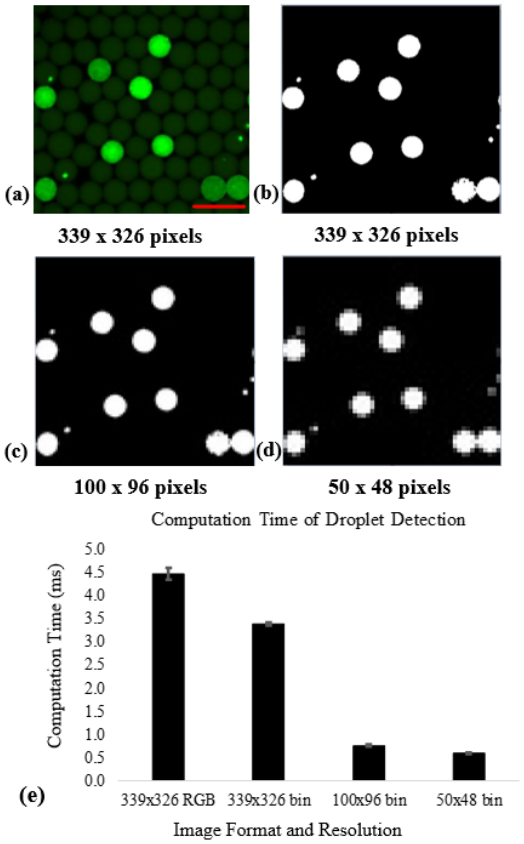

Fig. 5 (a-d) Images were pre-processed and compressed before being executed by the circle detection software. (e) Comparison of the computation time among images with different formats (RBG/binary) and resolutions.

4. DISCUSSION

This is a proof-of-concept study on image processing software aiming for rapid bacteria detection from a large volume of droplet emulsion. This software can be transplanted to a batch processing workstation to process massive frames from a high-speed camera to enumerate bacteria in patient blood samples.

5. ACKNOWLEDGMENTS

This study is supported by NIH/NIAID (R01 AI117061). J. Z. is supported by NIH TL1 training grant (TR001415). J. H. is supported by the NIH Maximizing Access to Research Careers (MARC) Award at FLC. Yiyan Li is supported by EPA P3 (SU83988001). This publication (the image processing part) was developed under Assistance Agreement No. SU83988001 awarded by the U.S. Environmental Protection Agency to Yiyan Li. It has not been formally reviewed by EPA. The views expressed in this document are solely those of Yiyan Li and do not necessarily reflect those of the Agency. EPA does not endorse any products or commercial services mentioned in this publication.

6. REFERENCES

[1] D. K. Kang, K. Zhang, M. M. Ali, L. Liu, L. Labanieh, M. Lu, *et al.*, "Digital quantification of miRNA in blood using integrated comprehensive droplet digital detection (IC3D) system," in *19th International Conference on Miniaturized Systems for Chemistry and Life Sciences, MicroTAS 2015*, 2015, pp. 200-202.

[2] L. Miotke, B. T. Lau, R. T. Rumma, and H. P. Ji, "High sensitivity detection and quantitation of DNA copy number and single nucleotide variants with single color droplet digital PCR," *Analytical chemistry,* vol. 86, pp. 2618-2624, 2014.

[3] A. R. Wheeler, W. R. Throndset, R. J. Whelan, A. M. Leach, R. N. Zare, Y. H. Liao, *et al.*, "Microfluidic device for single-cell analysis," *Analytical chemistry,* vol. 75, pp. 3581-3586, 2003.

[4] H. M. Shapiro, *Practical flow cytometry*: John Wiley & Sons, 2005.

[5] F. Li, M. Yang, L. Wang, I. Williamson, F. Tian, M. Qin, *et al.*, "Autofluorescence contributes to false-positive intracellular Foxp3 staining in macrophages: a lesson learned from flow cytometry," *Journal of immunological methods,* vol. 386, pp. 101-107, 2012.

[6] M. T. Guo, A. Rotem, J. A. Heyman, and D. A. Weitz, "Droplet microfluidics for high-throughput biological assays," *Lab on a Chip,* vol. 12, pp. 2146-2155, 2012.

[7] K. Churski, P. Korczyk, and P. Garstecki, "High-throughput automated droplet microfluidic system for screening of reaction conditions," *Lab on a Chip,* vol. 10, pp. 816-818, 2010.

[8] G. Du, Q. Fang, and J. M. den Toonder, "Microfluidics for cell-based high throughput screening platforms—A review," *Analytica chimica acta,* vol. 903, pp. 36-50, 2016.

[9] J. Friend and L. Yeo, "Fabrication of microfluidic devices using polydimethylsiloxane," *Biomicrofluidics,* vol. 4, p. 026502, 2010.