Development and Implementation of Analysis Program for Peritrichous Bacteria-based Nanorobot (Bacteriobot)*

Sunghoon Cho, Sung Jun Park, Young Jin Choi, Han-earl Jung, Shaohui Zheng, Seong Young Ko, Jong-Oh Park* and Sukho Park*, *Member, IEEE*

Abstract— This paper proposed an analysis program which can filter the moving phase of Peritrichous bacteria and analyze the rotational motion of a bacteria-based nanorobot (bacteriobot) with a spherical body. Using this program, the chemotactic steering of Salmonella typhimurium was quantitatively analyzed. The program used the bacterial running phase only to obtain an exact direction of the bacteria. As another implementation of this program, the motility of a bacteriobot which consists of an alginate microbead and flagellated bacteria has been analyzed. It showed a slow translational velocity and a relatively high angular velocity of a bacteriobot with a single attached bacterium. These results mean the propulsive force of a single bacterium gives some torque to the microbead. Therefore, the bacteriobot needs an additional external sources for an efficient translational motility such as chemical gradients, light intensity and magnetic fields.

I. INTRODUCTION

There are many research groups which are challenging medical nanorobotics. DNA based robot, bacteria based robot (bacteriobot) and magnetically driven nanocomposite show remarkable results for medical usage [1-3]. In case of the bacteriobot, it has a big difference with others that it has a biological organism component. So it is relatively difficult to expect when and where the bacteriobot goes and stops. Generally speaking, bacteria never stop for their lifetime so do bacteria based nanorobots. In this view, the analysis for bacterial behavior is important to model the bacteriobot.

Recently, our group succeeded to treat a Samonella based bacteriobot to mouse [4]. This approach proposes the use of biological microorganisms, such as the peritrichous bacteria. The peritrichous bacteria have several flagella in their body projecting in all directions. When the bacteria "run" toward one direction, they form a bundle and they "tumble" to switch their direction with flagella unwinding. Repeating these steps, they reach desire location [5]. In other way, "circle motion" is a variation of running motion when the bacteria swim near solid surfaces [6-8]. The *S. typhimurium* has good properties for cancer therapy especially; it is already proved that the species are the most effective bacteria for tumor killing [9, 10]. Although the wild type *salmonella* can bring an infection for human, it can be genetically modified and reduced its toxicity. Our main scenario is that, the bacteria are attracted by cancer chemically, and then the drug loaded microbeads which are attached with bacteria release drugs. Our group already developed many kinds of bacteria based nanorobot (Bacteriobot) and now we are considering the behavior of bacteria for more effective kinetic performance.

So far, many researchers analyzed bacterial running and tumbling motion by singular cell approach [5, 11, 12]. So they could expand our knowledge of individual bacterial motion. With regard to applying their result to the bacteriobot analysis, we face the necessity of development of software which can distinguish, filter and extract the motion of bacteria. Although there are several 2D and 3D tracking algorithms for bacteria and bacteriobots, the algorithms only focused on the trajectories of it [13-18]. So we've developed an algorithm which can classify the bacterial behaviors into three types on the tracking software base. Also, we expanded this program to analyze behavior of bacteriobots for revealing rotational motion caused by propelling bacterial torque. Using this program, we will be able to understand what bacterial motion makes what bacteriobot motion.

In this study, a program which can distinguish and classify the motion of peritrichous bacteriobot was developed and implemented to bacterial chemotaxis experiment. For the chemotaxis experiment, directionality of the *S. typhimurium* in a microfluidic channel was evaluated. Although they move through many narrow sections when used in human body, most researchers studied motilities of the bacteria on a boundary-free surface only for the *S. typhimurium*. So we evaluated the motility in a microfluidic channel. And for the bacteriobot experiment, translational and rotational motion was analyzed.

II. DEVELOPMENT OF THE ANALYSIS PROGRAM

A. Tracking and noise reduction

Darnton and Jaffe's particle tracking code was customized for tracking and visualizing of bacterial traces [19]. Briefly, it searches the edge of the object to recognize bacteria and changes the original images to the binary images. Then it eliminates the objects which have a size below threshold. After that, the centroid coordinates of each bacterium and frame information, i.e. timing data could be obtained from these images. This data can reconstruct each bacterium's trace to visualizing. Since bacterial speed is so fast, frame rate per minute of recorded video usually should be 15fps at least. To

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prevent some noise that masks bacterial mobility data, the program discarded objects which appeared less than 5 frames. Some trace that shows very short movement, e.g. less than 10 micrometers, is also removed from the data. The threshold values depend on the condition of the original data.

B. Bacterial motion sorting

Three-point method was used to estimate of curvature of the bacterial trace. The method determines a circle passing through three points by using following determinant equation.

$$\begin{vmatrix} C_{x}^{2} + C_{y}^{2} & C_{x} & C_{y} & 1 \\ P_{x}^{2} + P_{y}^{2} & P_{x} & P_{y} & 1 \\ Q_{x}^{2} + Q_{y}^{2} & Q_{x} & Q_{y} & 1 \\ R_{x}^{2} + R_{y}^{2} & R_{x} & R_{y} & 1 \end{vmatrix} = 0$$
(1)

Where $P(P_x, P_y)$, $Q(Q_x, Q_y)$, $R(R_x, R_y)$ are the first, middle and final point of the trace and $C(C_x, C_y)$ is the center of circle. Our program judges bacterial motion by using the method. If the radius of the circle is relatively large, i.e. over thousands of micrometers, the trace is classified as a running phase. Next, distances from the center of the circle to every point of bacterial trace are calculated to classify tumbling phases. Since normal circle motion shows relatively same curvature for running, the distance between the center of the circle and bacterium is constant but tumbling motion makes some of the distance cannot be constant. Fig.1 describes these steps.



Figure 1. Flow chart of bacterial motion sorting. To distinguish various motion of bacteria, curvature calculations of each bacterial traces are essential.

A general result of this program is shown Fig.2. Plenty of bacteria were captured for 10 seconds and tracked with the appropriate threshold at first. Even though most of bacteria show circle motion, many bacteria run straight for 10 seconds and a few bacteria showed tumbling motion. One can say most of these bacteria move near the surface and some of them are far from the surface so that they run and rarely change their direction. In addition, tumbling angle also can be estimated from these results.



Figure 2. Example of bacterial motion sorting. Recorded video is tracked at first (a). Then the radii of curvatures of each traces are estimated so that program extract 'running only' bacteria which are relatively large radius of curvature (c). If some bacterial traces are not on the expected circle, they are 'tumbling' bacteria (d). Remaining traces are considered as 'circle motion' bacteria (b). Unit of each axis are micrometers, circles were drawn with the corresponding radii of curvatures.

C. Recognition of bacteriobot's rotational motion

To evaluate bacteriobot's rotational motion, the attached bacterium should be distinguished. The rotational angle can be estimated by a vector from the center of the microbead to the position of the attached bacterium. First, region of interest (ROI) was defined around the microbead, i.e. the circle 10 micrometer larger than original detected microbead in radius was selected to detect attached bacterium. Next, extra bacteria which are not attached to microbead but in the ROI were rejected. Then the trace of attached bacteria was reconstructed. Fig. 3. shows these processes.



Figure 3. Recognition of attached bacterium. Region of interest is selected around the spherical microbead at each frame of recorded video. Then the program detects bacteria in the region of interest from binary image (a). Several bacteria are detected within tens of video frames (b). Bacterium attached onto microbead is recognized with developed algorithm and given threshold (c).

The rejection of non-attached bacteria is critical and difficult since the bacteria are too small so that tracking program lost them in some frames with given constant threshold. Hence, some data handling is needed to compensate broken traces of the microbead-attached bacterium. For this reason, the time derivations of angles were used. Non-attached bacteria shows different time derivation of angles in contrast with attached-bacterium. The program designed to find and link a trace that can minimize variation of time derivation among traces. And also a threshold which is determined by velocity of bacterial traces is used for the program. In addition, the distance between the microbead and bacterium also are used since attached-bacterium have almost constant distance. And finally the longest trace were chosen by program among the traces which satisfy given condition. For experiment, 200x magnification, 80ms exposure time and 4.0x gain was used for lightening condition to get appropriate image captures.

III. IMPLEMENTATION OF THE ANALYSIS PROGRAM

A. Directionality of bacteria within chemo-attractant

For the fabrication of the microchannel, we used photo-lithography and soft-lithography. SU-8 2050 photoresist and SU-8 developer solution were obtained from Microlithography Chemical Corp. (Microchem, Newton, MA) for photo-lithography and Agarose FR was obtained from CoreBio (Seoul, Korea) for soft-lithography. In the case of photo-lithography, we have taken conventional steps. Briefly speaking, the SU-8 photoresist was coated onto wafer with spin-coater and exposed to UV rays covered with a microfluidic channel patterned-mask. And then, unexposed area was eliminated by developing procedure to make a microfluidic channel mold. Using soft-lithography, the agarose channel was made with this mold since the glass channel can makes bacterial attachment [20]. The channel has three big chambers and two thin connections as described in our previous study [4]. The width of connection channel is 100µm to see at one view. Diameters of the chambers are 5-10 milliliters for easy loading. Central chamber is for loading bacteria/bacteriobot, left chamber is for loading chemo-attractant or chemo-repellent and right chamber is for loading Phosphate buffered saline (PBS) as a control. More dimensional parameters are in Fig. 4.



Figure 4. Geometric of used microchannel. Chemo-attractant, PBS and bacteria/bacteriobot are loaded left, right and center chamber respectively. The height is 0.5mm and other geometric values are represented in millimeter.

S. typhimurium which is genetically modified by the defection of guanosine provided by Professor J. Min (Chonnam National University Medical School, Korea) was used in this experiment [21]. We used Luria-Bertani (LB) agar plate (1% Bacto-Tryptone, 0.5% Bacto-Yeast extract, 1% NaCl and 1.5% Agar) and Kanamycin (Duchefa Biochemie) for bacterial growth. The bacteria were grown in 37°C incubator for 12hrs and then in shaking incubator for 5hrs.

For the chemotaxis test, whole channel was filled with PBS to minimize the effect of flow before bacteria and chemo-attractant are loaded. Then 10µl of 0.1mM aspartic

acid is poured to left chamber as a chemo-attractant and 10µl of PBS also poured right chamber as a control.

Directionalities of bacteria before after and chemo-attractant were evaluated with developed program. Since the bacteria movement is generally random walk, the moving direction of bacteria is uniform for all the directions without attractant. However in our case, direction of bacterial motility showed most bacteria headed for left and right side chambers respectively before chemo-attractant loading. This is because the bacterial concentration of center chamber was higher than side chamber so that concentration difference made diffusion phenomenon. Bacteria in the left and right sides of the central channel were recorded using microscope 10 minutes before and after loading the bacteria with 15 frames per second. The results are in Fig. 5.



Figure 5. These bacterial direction distribution shows the directional changing of bacteria before and after chemo-attractant loading. Results show bacterial moving direction was headed to left chamber after chemo-attractant. Horisontal axis is bacterial direction in degree and zero represents bacteria head for right chamber straightly, vertical axis is number of bacteria.

After chemo-attractant loading, the directionality of bacterial motility showed bias toward attractant chamber. In



Figure 6. Process of microbead-attached-bacterium (a-c) and the result with trace of microbead (d). Traces of bacteria within region of interest have several noise and broken link caused by passing bacteria, bacterium-bacterium crossing, defocusing with z-axis movement and so on (a). Developed program connects each traces or dots with given threshold and alogorism (b). Longest trace, i.e. the trace which is connected every broken link with given conditions are chosen. Black line indicates it(c). Unit of every axis are micrometeres in this figure.

the case of the left region of central chamber, 65% of bacteria headed for left direction before attractant but 63% of bacteria headed for right direction after attractant dropping. In the case of the right region of central chamber, 61% of bacteria headed for right direction before attractant and 78% of bacteria headed for right direction after attractant dropping. This result corresponds with common expectation.

B. Bacteriobot's rotational and translational motion

Alginate microbeads the body of the bacteriobot was made by modified spray method [22]. The nebulizer has two channels. One channel is for alginate through-put and has needle shape with 100 μ m inner diameter and 200 μ m outer diameter. Another channel is for air through-put with 2000 µm inner diameter. 1% alginate from Sigma-Aldrich (St. Louis, MO) was used for making microbeads and 2% calcium chloride (CaCl₂, Sigma-Aldrich Chemical Co.) was for chemical cross-linker. The alginate was extruded constantly by syringe pump with 5ml/hr. Air flow was controlled by mass flow controller (KRO-4001, Korea Instruments T&S, Seoul, Korea) with 200ml/min. CaCl₂ is located in front of nebulizer with 30cm distance for polymerization.

To make bacteriobots, alginate microbeads were transferred to 0.5% chitosan (Sigma-Aldrich Chemical Co., St. Louis, MO) solution. The chitosan coating makes bacteria can be attached to microbeads with charge interaction. After that, chitosan-coated microbeads were mixed with bacteria and let stand it for 30 min in 37°C incubator. During this time, bacteria attached onto the microbeads.

Using bacteriobot, translational and rotational motion was analyzed with the analysis program. To follow the trace of microbead-attached-bacterium, it is needed to pre-filter with the time, distance and angular criteria for pre-selection of dominant traces. Time and distance is from the data of bacterial traces and angle is from the direction vector between a point of bacterial traces and center of microbead at that time.

In this study, the threshold was 1 second, 4μ m and -45 to 20 degrees for time, distance, angle threshold respectively.



Figure 7. The result of trace and the evaluation of bacterial translational, rotational motion. Developed program can distinguish attached bacteria on the microbead well (a). Time variation plot for bacteriobot's moving angle, x and y coordinate (b).

From these pre-selection, given algorithm linked several traces to satisfy. In this satisfied traces, longest trace is the exact trace for attached bacterium since the bacterium can be detected almost every frames but not every frames. (Note that, the algorithm only can be used single bacterium attached bacteriobot. The bacteriobot on/to which two or more bacteria are attached cannot be analyzed by this algorithm.) And finally, rotational angle of the bacteriobot could be analyzed from the result. This process are shown in Fig.6 and the results are in Fig.7.

After tracing of microbead and attached bacterium, the angular result were estimated. Quantitatively speaking, the rotational speed was about 0.53rad/s and orbital circular speed was 0.42rad/s. Rotational angle showed constant increase in contrast to x and y coordinate of the center of bacteriobot vibration. This means single bacterium mostly makes to rotate bacteriobot. It explains the propulsion direction of bacterium is far from the line of center of mass of microbead. As the theory of torque reveals, propulsion direction should be in the line of center of mass of microbead, i.e. bacterium should be attached on microbead perpendicularly to minimize rotational motion and maximize translational motion. But it is difficult to develop with today's technology and it will be a next challenge of the bacteriobot.

IV. CONCLUSION

A software program can classify bacterial behavior to three main motions and evaluate rotational motion of bacteriobot has been developed. It has been used for chemo-taxis experiment and represented effective result. The program also has been used for analyze translational and rotational motion of bacteriobot. As a result, bacterial propulsion force made constant angular velocity. These two experimental results are already expected and fit with other studies [23-26].

The program can be considered as an advance version of tracking program which has more functions for bacterial motion analysis and bacteriobot's intrinsic rotational motion. It can be used for micro scale object for more applications. For example, partial modification on input/output part can make the program suitable for real-time servoing study. Since the algorithm can be refreshed during data processing, it is available for real-time process. And a relation between defocusing and z axial movement also can be added for 3D tracking and analysis. In further studies for modeling of bacteriobot, our program will be used with fitted function which can describe running and stopping behavior of bacteriobot.

REFERENCES

- Z. Zhang, Z. Li, W. Yu, K. Li, Z. Xie, and Z. Shi, "Propulsion of liposomes using bacterial motors," *Nanotechnology*, vol. 24, p. 185103, 2013.
- [2] S. N. Tabatabaei, S. Duchemin, H. Girouard, and S. Martel, "Towards MR-navigable nanorobotic carriers for drug delivery into the brain," in *Robotics and Automation (ICRA), 2012 IEEE International Conference* on, 2012, pp. 727-732.
- [3] J. Elbaz and I. Willner, "DNA origami: nanorobots grab cellular control," *Nature materials*, vol. 11, pp. 276-277, 2012.

- [4] S. J. Park, S.-H. Park, S. Cho, D.-M. Kim, Y. Lee, S. Y. Ko, et al., "New paradigm for tumor theranostic methodology using bacteria-based microrobot," Sci. Rep., vol. 3, 12/02/online 2013.
- [5] N. C. Darnton, L. Turner, S. Rojevsky, and H. C. Berg, "On torque and tumbling in swimming Escherichia coli," *Journal of Bacteriology*, vol. 189, pp. 1756-1764, 2007.
- [6] E. Lauga, W. R. DiLuzio, G. M. Whitesides, and H. A. Stone, "Swimming in circles: Motion of bacteria near solid boundaries," *Biophys J*, vol. 90, pp. 400-412, Jan 2006.
- [7] B. Misselwitz, N. Barrett, S. Kreibich, P. Vonaesch, D. Andritschke, S. Rout, et al., "Near Surface Swimming of Salmonella Typhimurium Explains Target-Site Selection and Cooperative Invasion," PLoS Pathog, vol. 8, p. e1002810, 2012.
- [8] J. Dunstan, G. Mino, E. Clement, and R. Soto, "A two-sphere model for bacteria swimming near solid surfaces," *Physics of Fluids*, vol. 24, Jan 2012.
- [9] N. S. Forbes, "Engineering the perfect (bacterial) cancer therapy," Nat Rev Cancer, vol. 10, pp. 785-794, 2010.
- [10] P. Chorobik and J. Marcinkiewicz, "Therapeutic vaccines based on genetically modified Salmonella: a novel strategy in cancer immunotherapy," *Polskie Archiwum Medycyny Wewnetrznej*, vol. 121, pp. 461-6, Dec 2011.
- [11]S. Chattopadhyay, R. Moldovan, C. Yeung, and X. Wu, "Swimming efficiency of bacterium Escherichiacoli," *Proceedings of the National Academy of Sciences*, vol. 103, pp. 13712-13717, 2006.
- [12] H. Jeon, Y. Lee, S. Jin, S. Koo, C.-S. Lee, and J. Y. Yoo, "Quantitative analysis of single bacterial chemotaxis using a linear concentration gradient microchannel," *Biomedical microdevices*, vol. 11, pp. 1135-1143, 2009.
- [13] B. Behkam and M. Sitti, "Bacterial flagella-based propulsion and on/off motion control of microscale objects," *Applied Physics Letters*, vol. 90, pp. 023902-023902-3, 2007.
- [14] D. Kim, A. Liu, E. Diller, and M. Sitti, "Chemotactic steering of bacteria propelled microbeads," *Biomedical microdevices*, vol. 14, pp. 1009-1017, 2012.
- [15]M. A. Traoré, A. Sahari, and B. Behkam, "Computational and experimental study of chemotaxis of an ensemble of bacteria attached to a microbead," *Physical Review E*, vol. 84, p. 061908, 2011.
- [16] A. Sahari, D. Headen, and B. Behkam, "Effect of body shape on the motile behavior of bacteria-powered swimming microrobots (BacteriaBots)," *Biomedical Microdevices*, vol. 14, pp. 999-1007, Dec 2012.
- [17]B. Behkam and M. Sitti, "Effect of quantity and configuration of attached bacteria on bacterial propulsion of microbeads," *Applied Physics Letters*, vol. 93, 2008.
- [18] M. R. Edwards, R. W. Carlsen, and M. Sitti, "Near and far-wall effects on the three-dimensional motion of bacteria-driven microbeads," *Applied Physics Letters*, vol. 102, p. 143701, 2013.
- [19] N. Darnton and J. D. Jaffe, "Particle/bacterial tracking software," ed. Rowland Institute at Havard University. Cambridge. MA., 2003.
- [20] P. S. Meadows, "The attachment of bacteria to solid surfaces," Arch Mikrobiol, vol. 75, pp. 374-81, 1971.
- [21] J.-J. Min, V. H. Nguyen, H.-J. Kim, Y. Hong, and H. E. Choy, "Quantitative bioluminescence imaging of tumor-targeting bacteria in living animals," *Nature Protocols*, vol. 3, pp. 629-636, 2008.
- [22]S. Fusco, M. S. Sakar, S. Kennedy, C. Peters, R. Bottani, F. Starsich, et al., "An Integrated Microrobotic Platform for On-Demand, Targeted Therapeutic Interventions," Advanced Materials, 2013.
- [23] J. Diao, L. Young, S. Kim, E. Fogarty, S. Heilman, P. Zhou, et al., "A three-channel microfluidic device for generating static linear gradients and its application to the quantitative analysis of bacterial chemotaxis," *Lab on a chip*, vol. 6, pp. 381-388, 2006.
- [24]S.-Y. Cheng, S. Heilman, M. Wasserman, S. Archer, M. Shuler, and M. Wu, "A hydrogel-based microfluidic device for the studies of directed cell migration," *Lab on a chip*, vol. 7, pp. 763-769, 2007.
- [25] D. Park, S. J. Park, S. Cho, Y. Lee, Y. K. Lee, J. J. Min, et al., "Motility analysis of bacteria-based microrobot (bacteriobot) using chemical gradient microchamber," *Biotechnology and Bioengineering*, 2013.
- [26] A. A. Julius, M. S. Sakar, E. Steager, U. K. Cheang, M. Kim, V. Kumar, et al., "Harnessing bacterial power in microscale actuation," in *Robotics* and Automation, 2009. ICRA'09. IEEE International Conference on, 2009, pp. 1004-1009.