J. Microbiol. Biotechnol. (2015), 25(3), 381–385
http://dx.doi.org/10.4014/jmb.1401.01012

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Viable Bacterial Cell Patterning Using a Pulsed Jet Electrospray System

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Received: January 7, 2014
Revised: April 2, 2014
Accepted: April 3, 2014

First published online
April 7, 2014

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pISSN 1017-7825, eISSN 1738-8872

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In the present study, drop-on-demand two-dimensional patterning of unstained and stained bacterial cells on untreated clean wafers was newly conducted using an electrospray pulsed jet. We produced various spotted patterns of the cells on a silicon wafer by varying the experimental conditions, such as the frequency, flow rate, and translational speed of the electrospray system in a two-dimensional manner. Specifically, the electrospray's pulsed jet of cell solutions produced alphabetical patterns consisting of spots with a diameter of approximately 10 μm, each of which contained a single or a small number of viable bacteria. We tested the viability of the patterned cells using two visualization methods. This pattering technique is newly tested here and it has the potential to be applied in a variety of cell biology experiments.

Keywords: Electrospray, two-dimensional pattern, bacteria treatment methods, pulsed jet, microorganism arrays, bioaerosols

Introduction

A patterning method for the immobilization of biological materials such as bacterial cells and human cells onto solid surfaces has become important for the development of biosensors, drug discovery, diagnostics, and fermentation microbiology [5, 8, 9]. Microcontact printing of adhesives, photolithography, and dip-pen nanolithography are the techniques generally used as steps and methods for patterning biomaterials on substrates [6, 9, 10]. However, most of these methods involve modifying the surface chemically to facilitate adherence of specific binding material [9]. Therefore, these methods need more than two steps to bind biological materials on solid surfaces. The best optimal method of patterning involves picking up biomaterials from a liquid suspension and depositing them at specific sites on a surface without any chemical treatment. We have recently proposed picking up cells in a liquid suspension and depositing them at specific sites on untreated surfaces *via* an electrospray pulsed jet, which has been considered to approach the optimal patterning of bacterial cells [4].

An electrospray system can generate highly charged tiny droplets of a liquid substance in the gaseous phase by using a highly intense electric field between the nozzle and the deposition plate [2]. An electrospray jet based on the electrospray system was found to be able to produce patterns of ink droplets with a resolution of a few micrometers [4, 7]. As mentioned above, for the first time, we have applied the electrospray method of generating highly charged droplets for the production of variously sized one-dimensional and two-dimensional patterns of bacterial cells on raw (untreated) silicon wafers at a cellular resolution [3, 4]. However, the viability of patterned bacterial cells had been questioned. In the previous study [4], the bacterial cells in the 190-μm-sized pattern were incubated and the viability test with cultivation was conducted. However, the viability of bacterial particles within dozens of micrometer-scale spots could not be tested. In addition to this limitation, the patterning was allowed only for simplified shapes [4]. Therefore, the applicability of the findings has been limited.

In the present study, we have newly produced alphabetical patterns consisting of unstained and stained bacterial cells using the electrospray drop-on-demand patterning method of bacterial cells (Chong ES. 2014. Master thesis, Konkuk University). We also newly succeeded in checking the viability of the patterned bacterial cells by using genetically modified cells and staining methods. The cellular resolution of various types of two-dimensional patterns of cells shows the potential of this new electrospray pattering method as a new tool for cellular immobilization and microbial experiments.

Materials and Methods

Patterning System

Fig. 1 shows a schematic diagram of the experimental setup for the pattering system. The experimental setup from our previous work [4] was modified to render it capable of pattering cells in two dimensions. In the present study, various bacterial cells and several staining methods were also used. The experimental system consisted of a silica capillary, a ground plate, a translation system of the ground plate, a liquid feeding system, a high-voltage power supply system (HV), and a visualization system [4]. The silica capillaries (PicoTip emitter, New Objective, USA) had diameters of 30 and 100 μm with a sharp tip. The ground plate was a stainless steel plate with a diameter of 12 cm, to which a silicon wafer was attached. The wafer on the ground plate was installed 100 μm below the capillary tip. The motorized stage translation apparatus translated the ground plate at an adjustable constant speed in two dimensions. For the liquid feeding part, 25 μl syringes (1702TLL; Hamilton, USA) and a syringe pump (Model 220; KD Scientific, USA) were used. The syringe and the capillary were connected through a microflow fitting set (P-662, 1572, F-242, U-322; Upchurch, USA). The sinusoidal high-voltage electricity of several kilovolts was applied to the capillary by means of an AC high-voltage power supply (AC +15 kV; Korea Switching, Korea) and a function generator (FG-7002C; EZ Digital, Korea). High-voltage electricity was applied to the liquid jet spray by means of electric charge on a stainless steel ZDV union (U-322; Upchurch, USA) [4].

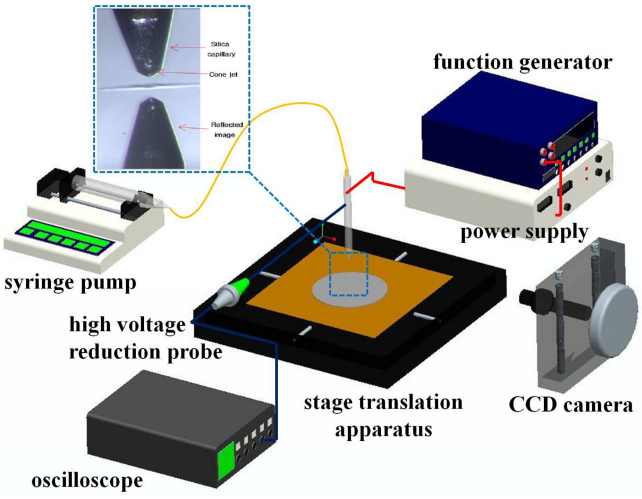

Fig. 1. A schematic diagram of the experimental setup.

To accurately observe the characteristics of the applied electric voltage, a 1/1000 AC high-voltage reduction probe (P6015A; Tektronix, USA) and an oscilloscope (TDS2014; Tektronix, USA) were used. The capillary tip and the pulsed jet were visualized by means of a CCD camera (Marlin F-145C2; Allied Vision Tech., Germany), a zoom lens (70XL; OPTEM, Korea), and a light source (LS-100W; Light Solution, Korea). We used an optical microscope (ECULIPSE ME600; Nikon, Japan) connected to a CCD camera (INFINITY1; Lumenera Co., Canada), a fluorescence microscope (Olympus BX51; PA, USA) equipped with a halogen light 470-490 excitation filter, and a 520 nm barrier filter for observing the produced patterns [4].

Bacterial Suspensions, Staining of Cells, and Observation of Patterns of Cells

Bacillus subtilis (KCTC 1021), *Staphylococcus aureus* (KCTC 1621), or *E. coli* strain (DH5α) (ATCC 53868; Rockville, MD, USA) were used in the present experiments. The bacteria were suspended in a mixture of 8 g of nutrient broth medium (beef extract 0.3%, peptone 0.5%; Difco) with 1 liter of deionized filtered water with an electrical conductivity of 1.39×10^{-3} S/cm at a concentration between 3.0×10^{7} and 9.0×10^{7} CFU/ml.

After electrospraying the bacterial cells onto the wafer, parts of the patterned cells were stained by crystal violet for visualizing the stained bacterial cells in the patterns. The wafers were dried for 20 min, and 100 μl of a 0.1 % (v/v) crystal violet solution (0.1 % of crystal violet in 2% ethyl alcohol in 0.1 M borate buffer, pH 9.0) was added for 40 min. After the staining, the wafers were washed gently with sterile distilled water to remove the excess stains.

Direct visualization of viable bacterial cells was obtained with the BacLight viability kit. The two BacLight stain solutions, SYTO 9 (300 μl) and propidium iodine (300 μl), were mixed together and diluted 1:10 in a NaCl solution (0.085%), providing 6 ml of a BacLight stock solution. For the staining, an appropriate volume (generally 50 μl) of the stock solution was added onto the wafer, and the wafer was incubated for 15 min in the dark at room temperature. The incubated sample was then observed with the fluorescence microscope. The *E. coli* strain utilized in this experiment, DH5α, contains a deletion mutation of the β-galactosidase gene in its genome, and such a bacterium expresses a non-functional β-galactosidase protein. If this type of bacteria is transformed with an extrachromosomal element called a plasmid that expresses a functional β-galactosidase, it can hydrolyze a galactoside, 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal), and develop a dark blue color. In our experiment, we utilized the pUC 19 plasmid, and treated the plasmid-transformed cells with X-gal for detecting gene expression. *E. coli* cells were grown overnight in LB broth in the presence of ampicillin (100 μg/ml) at 37°C, and diluted 1:10

with LB broth containing IPTG (1 mM) and X-gal (50 μg/ml) just before electrospraying.

Overall Experimental Procedures

The experimental procedures for two-dimensional patterning and the viability checking of bacterial cells on the wafer by using expressing gene were as follows. A syringe pump supplied the liquid bacterial suspension, either *B. subtilis* (KCTC 1021) or *S. aureus* (KCTC 1621) or *E. coli* strain (DH5α) (ATCC 53868; Rockville, MD, USA)), *via* a syringe to the capillary of the electrospray at flow rates ranging from 0.1 to 0.7 μl/h (where 1 μl = 10^{-9} m^3). A high-voltage power supply system coupled with a function generator supplied electricity to the electrospray system. The supplied electricity, which had a maximum voltage range of 0.5 to 0.8 kV and a central point voltage range of 0.25 to 0.4 kV, had a sinusoidal waveform with a frequency ranging from 10 to 20 Hz. The liquid flow and the high-voltage AC sinusoidal electric field together formed the pulsed jet of the electrospray. When the jet was formed, the ground plate of the electrospray was translated horizontally at a fixed speed ranging from 0.1 to 0.7 mm/s. The pulsed jet from the tip of the capillary then made regular contact with the clean silicon wafer on the ground plate.

In the experiments for checking the viability of bacterial cells using staining with fluorescence microscopy, we mixed a bacterial suspension (*Staphylococcus aureus* KCTC 1621) together with a dye called BacLight live/dead 7007 with a concentration of 3 μl/ml, and this mixture was loaded in the syringe. The syringe pump supplied this solution in a syringe to the capillary of the electrospray at a flow rate of 0.7 μl/h. A high-voltage power supply system with a function generator supplied electricity to the electrospray system. The supplied electricity, which had a maximum voltage of 1.23 kV and a central point voltage of 0.6 kV, had a sinusoidal waveform with a frequency of 20 Hz. When the jet was formed, the ground plate of the electrospray was translated horizontally at a fixed speed of 0.1 mm/s. A pulsed jet from the tip of the capillary then made regular contact with the clean thin glass plate on the ground plate. These procedures for electrospraying cells onto a thin glass plate could be completed within 5 min; it took approximately 5 min to dye the bacteria. Once the electrospray jet was formed for each pulse of the supplied electric voltage, this spray jet formed regular patterns of spots of bacterial suspension when it touched the silicon wafer and the thin glass plate.

Results and Discussion

At a voltage of 0.75 kV, 20 Hz (AC), and a bacterial suspension (5.0×10^6 CFU/ml) injection flow rate of 0.3 μl/h through a nozzle of 30 μm diameter when the translational speed of the silicon wafer was 0.4 mm/s, the electrospray pulsed jet could produce a two-dimensional alphabetical "KU" pattern of unstained bacterial cells (Fig. 2A). The pattern consisted of spots with a regular diameter of 11 μm, and the average interval between spots was 82 μm.

Fig. 2B shows a "KU" pattern consisting of bacterial cells that were stained by crystal violet. The pattern comprised spots with a regular diameter of 15 μm, and the average interval between spots was 27 μm. In this case, the electrospray jet was maintained at 0.6 kV with a frequency of 20 Hz (AC). The flow rate of the supplied suspension (bacteria at a concentration of approximately 5.0×10^7 CFU/ml) was 0.5 μl/h. The translational speed of the silicon wafer was 0.4 mm/s, and a nozzle of 30 μm diameter was used. The results presented in Figs. 2A and 2B demonstrate that electrospray pulsed jet patterning could produce complicated bacterial cell patterns on demand.

To examine whether bacterial cells spotted on the surface of a disk of a wafer maintain their metabolically active status even after the electrospray process, we utilized *E. coli* DH5α (ATCC 53868; Rockville, MD, USA) harboring a plasmid that produces the enzyme beta-galactosidase. In the presence of X-gal, an inert chromogenic substrate, all

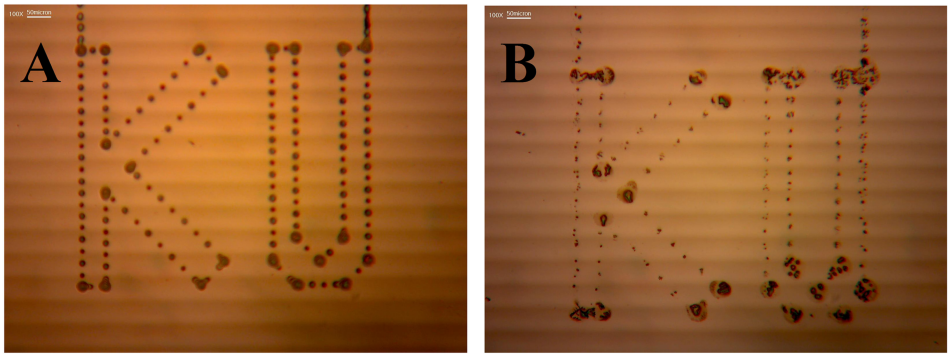

Fig. 2. Two-dimensional "KU" patterns of stained and unstained cells.
(**A**) "KU" – a symbol of the affiliation of the authors – pattern made of suspension drops containing unstained bacterial cells (*Bacillus subtilis* KCTC 1021) on a silicon wafer *via* electrospray jet patterning; (**B**) "KU" pattern made of stained (crystal violet) bacterial cells (*Bacillus subtilis* KCTC 1021) on a silicon wafer *via* electrospray jet patterning.

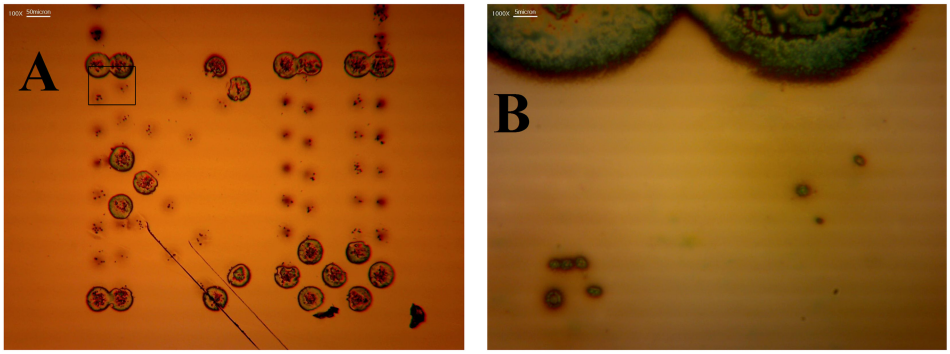

Fig. 3. Two-dimensional "KU" pattern of β-galactosidase-expressing *E. coli* strain DH5α.
(**A**) "KU" – a symbol of the affiliation of the authors – pattern made of bacterial cells (*E. coli* strain DH5α (ATCC 53868; Rockville, MD, USA)) on silicon wafer *via* electrospray jet patterning. (**B**) Magnified picture of a part of Fig. 3A (upper-left part of K) showing blue bacterial cells expressing β-galactosidase.

the cells expressing β-galactosidase hydrolyzed X-gal into colorless galactose and 4-chloro-3-brom-indigo, forming an intense blue precipitate. In this system, for the induction of the β-galactosidase gene, we treated cells with IPTG, a lactose metabolite that triggered transcription of the gene. Fig. 3A also shows a "KU" pattern consisting of the above special bacterial cells. The pattern comprised spots having a regular diameter of 20 μm. The average interval between spots was 25 μm. The applied electricity was a 0.58 kV sinusoidal waveform with a frequency of 10 Hz. The flow rate of the supplied suspension was 0.4 μl/h. The translational speed of the silicon wafer was 0.7 mm/s. A nozzle of 30 μm diameter was used. The concentration of bacterial cells was approximately 5.0×10^6 CFU/ml. Fig. 3B is a 1,000-fold enlarged picture of Fig. 3A (upper left part of K). The bacterial cells shown in Fig. 3 are blue, indicating that they expressed β-galactosidase. To the best of our knowledge, this is the first verification that the bacterial cells patterned on the wafer were metabolically active.

A different bacterial suspension (5.0×10^7 CFU/ml of *S. aureus*) stained by a bacterial viability kit called BacLight live/dead 7007 was patterned on a thin glass plate using an electrospray jet maintained at 1.23 kV, 20 Hz (AC) using a flow rate of 0.7 μl/h through a nozzle of diameter 100 μm at a translational speed of 0.1 mm/s. Fig. 4A shows the resultant line pattern. Direct visualization of live bacteria was achieved with the LIVE/DEAD BacLight Viability kit. This staining kit evaluates the structural integrity of the bacterial membrane by employing the fluorescence dyes SYTO 9 and propidium iodide. Post staining, all bacterial surfaces would be red under light of wavelength ranging from 510 to 550 nm, whereas living and hence metabolizing cells would appear green under fluorescence microscopy

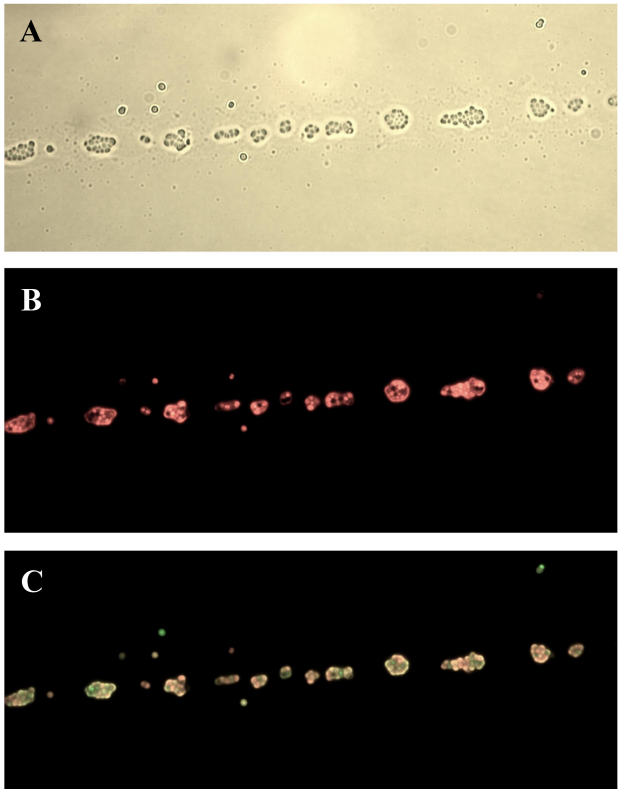

Fig. 4. BacLight live/dead 7007 staining pattern of *Staphylococcus aureus* cells.
(**A**) A linear pattern consisting of bacteria (*S. aureus* KCTC 1621) stained by a dye called BacLight live/dead 7007 on a thin glass plate. This picture was taken by an optical microscope. (**B**) The same pattern as that in Fig. 4A imaged using a fluorescence microscope with incident light of wavelengths ranging from 510 to 550 nm. All the bacterial cell membranes patterned on the thin glass plate were stained in red. (**C**) The same pattern as that in Fig. 4A imaged using a fluorescence microscope with a light of wavelength ranging from 460 to 490 nm. The green bacterial cells were metabolizing.

with light of wavelength ranging from 460 to 490 nm.

Fig. 4B is another picture of Fig. 4A taken by fluorescence microscopy when illuminated by light of wavelength ranging from 510 to 550 nm. It shows that all bacterial cell membranes patterned on the thin glass plate were stained in red. When the same cells (Fig. 4A) were illuminated with light of a different wavelength (*viz*., ranging from 460 to 490 nm), the bacterial cells appeared green, as shown in Fig. 4C. This result indicates that the bacterial cells on the thin glass plate were actively metabolizing. Compared with bacterial cells in the control groups, few changes of metabolic activity were observed. The above experimental results in Fig. 4 also demonstrate that patterned bacterial cells maintain their metabolic activity.

In conclusion, overall, our results in Figs. 1 to 4 illustrate that two-dimensional bacterial patterns can be generated on a thin glass plate as well as on a clean silicon wafer by using the developed electrospray system for several seconds without the need for any chemical treatment, pressure exposure, or heat treatment, and that the size and shape of the patterns could be controlled by adjusting the experimental conditions. The number of bacteria in the patterns could also be controlled by varying the experimental conditions, such as the concentration of the bacteria in suspension, the flow rate, the diameter of the nozzle, and the applied electrical conditions. In this experiment, we succeeded in producing various patterns of spots of bacteria on demand; furthermore, for the first time, the bacterial cells in the patterns were found to be metabolically active. However, we tested two kinds of plates and three types of microorganisms; therefore, the tested experimental conditions of the study can be a current limitation of this finding for applications.

Our new technique enables viable bacterial cells to be manipulated in solid and gaseous phases at cellular resolution. This technique has the potential to be used in several industrial applications. For example, this technique can be applied for biochemical analyses of viable individual cells in a gaseous environment. This new patterning technique could also be used for analyses of liquor production during fermentation or hormone synthesis to screen for more efficient microorganisms, and thereby increase the yield and efficiency of manufacturing. The electrostatic painting of bacterial cells for coating the surfaces of ocean plants could be yet another application of this new patterning technique, because coating with special bacteria endospores can protect ocean plants from microbially influenced corrosion in seawater [1]. The applicability of this new technique to other cells, such as human cells, on various plates has to be investigated in future studies.

Acknowledgments

This work was supported by a National Research Foundation of Korea (NRF) grant ('Measurement of airborne microorganisms in public facilities and development of control methods against airborne pathogenic microorganisms', No. 2010-0007615) funded by the Ministry of Science, ICT, and future planning.

The participation of Prof. Hyo Il Jung in this study was partly funded by the Public Welfare & Safety research program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (NRF-2013M3A2A1073991).

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