

DEVELOPMENT OF A BACTERIAL CULTURE SYSTEM USING A PAPER PLATFORM TO ACCOMMODATE MEDIA AND AN INK-JET PRINTING TO DISPENSE BACTERIA

¹Tithimanan Srimongkon, ²Takuya Ishida,
²Kiyohiko Igarashi and ¹Toshiharu Enomae

¹Graduate School of Life and Environmental Sciences, University of Tsukuba, Japan

²Graduate School of Agricultural and Life Sciences, University of Tokyo, Japan

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ABSTRACT

Generally, bacterial culture is performed manually and is subject to error. Here, we created a novel, well-ordered and reliable system for dispensing bacteria microscopically by using paper and an ink-jet printer for controlled patterning. For paper to accommodate a culture medium, hydrophobic/hydrophilic patterns were incorporated onto the paper by immersing paper in a toluene solution of polystyrene and drying for complete hydrophobization, followed by etching discrete, small areas of hydrophilicity by ink-jet printing with toluene. Agar was hydrolyzed with sulfuric acid for appropriate viscosity and dispensed with an ink-jet printer. In a separate experiment, bacterial cells were sequentially printed on a medium and colonies were observed microscopically. The results of this experiment ensured the successful dispensing of bacteria using ink-jet printing. An almost constant number of particles per droplet were ejected using a polystyrene latex as a model of bacterial dispersion. Consequently, we expect this technology to be adapted for the development of a paper-based bioassay system.

Keywords: Paper Platform, Bacterial Cells Printing, Hydrophobic/Hydrophilic Patterning

1. INTRODUCTION

Bacteria are present everywhere: In soils, deep in rocks, in all water bodies and in the atmosphere, including on and inside other living organisms (Kaláb, 2008). Several kinds of bacteria are commercially important in industries such as pharmaceutical and food industries (Hossain *et al.*, 2012). However, some bacteria are harmful. To extensively research bacteria species, microbiologists isolate pure cultures and further analyze them. To culture bacteria, a nutrient medium is required in a container, such as a Petri dish or test tube. If growth under different conditions is needed, several sets of containers are required. To culture bacteria, a nutrient medium is required as well as a Petri dish or test tube as a container. In preparation for nutrient media, an autoclave is always required for sterilization at so high a

temperature and pressure that high electric power is consumed. Then, bacterial cells are transferred to a medium plate in a clean bench. In the same way, an autoclave was used again for disposing bacteria. In case of observation under different conditions, several sets of containers are additionally required. Therefore, we came with an idea to use paper that is easy to handle as a container instead of a Petri dish. The study container would use ethylene oxide gas for sterilization and combustion for disposal.

Recently, various novel paper-based devices have been presented. Paper is ubiquitous, inexpensive and recyclable; it is easy to store, transport, manipulate and dispose. Attempts have been made to fabricate paper-based medical sensors to analyze clinical body fluids and metabolic substances, such as blood, urea (Martinez *et al.*, 2008; Abe *et al.*, 2008) and glucose

Corresponding Author: Tithimanan Srimongkon, Graduate School of Life and Environmental Sciences, University of Tsukuba, Japan

(Ornatska *et al.*, 2001). Paper sensor aiming at the detection of mesothelin as a cancer biomarkers was presented (Lei *et al.*, 2013). A multiplex detection system of *Escherichia coli* (referred-to *E. coli* hereafter) was developed using an enzyme-based lab-on-paper test strip (Martinez *et al.*, 2008). Furthermore, fabrication methods and design techniques of paper-based microfluidic channels using wax for portable bioassay were discussed (Lu *et al.*, 2009). Paper can be divided into multi-channels for liquid-transporting sensor tests by using a patterning technique to separate hydrophobic/hydrophilic areas. We considered that we would apply this useful technique to bacterial cultures under various conditions. Hydrophilic areas were tried to be built on a small sheet of hydrophobized filter paper in order to accommodate dispensed agar and then bacteria. In addition, paper is a combustible substrate, therefore grown bacteria could be simply disposed with the paper substrate after combustion. Thus, no autoclave is required in the disposal process.

There is another factor for inconvenience caused by manual operation. Bacterial cells are commonly transferred by manually in culture tests. The transferred volume cannot be constant or well-controlled. To solve the instability problem due to manual procedure, we proposed an ink-jet dispensing method. However, (John *et al.*, 1983) observed, under microscope, that rod-shaped cells like *E. coli* grew so simply that freshly-divided cells elongated with little or no increase in girth. Eventually a transverse wall was laid down near the center of the cell, When the cell reached approximately twice its original size, it separated into two cells nearly equal in size. Therefore, *E. coli* cells transfer was tested as a model of bacterial cells by using ink-jet technology. Because ink-jet technology is no longer only an office printing technology (Abe *et al.*, 2008); it has gradually become a versatile tool in various fields for accurately dispensing very small quantities of fluids on substrates, such as human organs or cells (Mironov *et al.*, 2009; Guillemot *et al.*, 2010; Xu *et al.*, 2005; Cui and Boland, 2009). Furthermore, numerical and experimental investigation of a fluid flow through porous media is important for a wide range of applications varying from environmental analyses to inkjet printing technology (Mohd Irwan *et al.*, 2010). Therefore, ink-jet technology is an essential technique for transferring bacterial cells in a small, stable and controlled volume. In addition, ink-jet ejection is easily controlled using software that can print any designed patterns and a charge-coupled device camera, which is usually equipped with high-performance ink-jet printers to

examine the pattern dispensed on a substrate. Processing, such as hydrophilic/hydrophobic patterning, with an ink-jet printer is a time-saving, low-cost and controllable technique that is an alternative to manual operation. Through this technology, researchers can avoid harmful or pathogenic bacteria from directly touching them as a concomitant effect.

In this study, we present a new method to create a well-ordered and reproducible system for dispensing bacteria on a microscopic scale by using advantages of paper and ink-jet technology. We used paper as a media platform for bacterial culture and ink-jet printing technology to dispense small amounts and for manageable patterning of bacterial suspension. Bacterial growth on a nutrient medium contained in a Petri dish is sometimes evaluated viaually by positive/negative judgement followed by a statistical analysis (Overdeest *et al.*, 2010), counting the number of colonies (Tortoli *et al.*, 2002), color of colonies (Glupczynski *et al.*, 2007) and more semi-quantitatively, scores based on the number of quadrants in which growth was observed (Huang *et al.*, 2010). The system we are creating aims to quantify bacterial growth with a microscope and image analysis as the future work, which will be useful to routine laboratories using agar plates.

2. MATERIALS AND METHODS

2.1. Preparation of the Hydrophilic/Hydrophobic Pattern

We selected paper as a potential new substrate to facilitate efficient observation of bacterial growth under various conditions. Previous research (Abe *et al.*, 2008) presented a method to modify paper to form hydrophobic and hydrophilic areas in the same substrate by using Poly Styrene (PS) and toluene. In the present study, we used PS to serve as a hydrophobic barrier to block spreading of the aqueous medium that was printed on hydrophilic areas. In addition, filter paper was chosen as a substrate to settle the medium because of its high absorbency. First, the filter paper was soaked in a 3.0 wt% toluene solution of PS for 1 h and then allowed to dry at room temperature for 15 min, to allow the entire filter paper to become hydrophobic. Squares (10×10 mm) were etched to make hydrophilic areas by printing toluene with Solvent Blue 35 at 0.015 wt% (blue toluene), for visualization, with 3 head nozzles and overprinting the area twice for the best organized patterns with distinctive borders. Finally, hydrophilicity was tested by dropping 2 μ L of water on the etched areas and the degree of penetration was observed and compared.

2.2. Modification of Agar

A common culture medium for bacteria is an agar medium that contains necessary nutrients. We attempted to print a nutrient agar medium by using the ink-jet printer for dispensing small quantities of the medium on hydrophilic areas of the filter paper. However, the medium quickly solidified at room temperature, which is not conducive to printing. Therefore, we modified the molecular structure of agar by hydrolysis. Agar contains agarose as a major component, which is a linear polymer made up of the repeating unit of agarobiose-a disaccharide made up of D-galactose and 3, 6-anhydro-L-galactopyranose. Glycosidic bonds between each agarobiose are easily hydrolyzed by acids, such as Sulfuric Acid (H₂SO₄), that shorten polysaccharide chains. The optimum hydrolysis of agar was sought to generate agar polymers conducive to printing. First, 0.5 g of agar powder was dissolved in 20 mL of 0.05, 0.1 and 0.02% H₂SO₄ aqueous solutions and then the solutions were heated at 100°C for 10, 15, 20, 25, 30, 40, 45 and 50 min. To stop the acid hydrolysis, the agar solutions were cooled on ice water for 5 min. Sodium Hydroxide (NaOH) was added to neutralize the solutions for bacterial culture. Sodium Sulfate (Na₂SO₄) produced during neutralization was removed by electro dialysis (Microacilyzer S1 with a membrane cartridge AC110-10, ASTOM, Japan). Viscosity of each hydrolyzed agar solution was measured by using a Cannon-Fenske Routine Viscometer. Finally, the hydrolyzed agar was mixed with yeast extract, Bacto tryptone and Sodium Chloride (NaCl). The agar mixture was loaded into an ink cartridge wrapped with a film-type heater and was printed on rectangular hydrophilic areas (5×5 mm). The drying speed of printed hydrolyzed agar was recorded to evaluate water retention.

2.3. Bacterial Culture on Paper

Bacterial growth was examined by transferring a suspension of bacteria and comparing the growth on a normal agar plate and on hydrolyzed agar medium on paper. First, bacteria floating in the air were taken randomly from stationary phase and isolated for pure culture by the streak technique. At least 3 repetitions of this technique were performed to confirm the purity of the bacteria and classify the morphology of the bacterial colonies. Serial dilution was applied to reduce the concentration of the bacterial suspension. Finally, a bacterial suspension of 2.7×10^7 CFU/mL was transferred onto hydrolyzed agar to observe the bacterial growth.

2.4. Ejection of *Escherichia coli* on Medium

E. coli cells were printed with a testing ink-jet printer (Dimatix DMP-2831, Fujifilm, Japan), with a piezoelectric actuator, on a standard culture medium to confirm bacterial cell applicability and viability. First, a 5-mm-thick square sheet of culture medium was prepared and placed on the stage of the ink-jet printer. Next, *E. coli* cells were loaded into a cartridge and printed following patterns designed using a specialized application. The pattern was considered to be a rectangle of 5×10 dots arranged on a sheet at intervals of 5 mm. The printed *E. coli* cells were incubated at 37°C for 24 h and the resultant colonies were examined.

2.5. Preparation of Emulsified Particulates

Polystyrene acrylate hollow latex particles with a diameter of about 1 μm (latex particles; PAT8125; ZEON Corporation, Japan) were selected as a bacterial cell model to ensure the ability to print a constant number of particles. The stock latex emulsion, consisting of 26.5% solids, was diluted to 0.001% and then mixed with a small amount of glycerol to obtain an appropriate viscosity for ink-jet printing. Latex particles were subsequently printed on a sheet of photo-grade ink-jet printing paper (Super photo grade; Kassai, Fujifilm, Japan) using an application to manage printing conditions such as printing frequency and number of droplets in 1 dot on the paper. A square pattern of 11×11 dots was printed only once or overprinted 3 or 5 times on the paper and photographs of the printed dots were taken by a scanning electron microscope (S-4200, Hitachi, Japan) to count the number of latex particles.

2.6. Confocal Laser Scanning Microscopy

Automatic and selective monitoring of bacterial growth by a Confocal Laser Scanning Microscope (CLSM 700; AxioObserver, Carl Zeiss Microscopy) was performed to develop this bioassay system. A dried piece of filter paper with a bacterial colony on the agar medium was subjected to fluorescence microscopic observation with an excitation wavelength (ex) of 555 nm (green) and an emission wavelength (em) ranging from 500-630 nm (blue to red) for the agar and ex of 639 nm (red) and em>640 nm (red) for the hydrophilic area. CLSM was used to obtain a regular light transmission image showing the fiber network and the composite image was created from the 3 images.

3. RESULTS AND DISCUSSION

3.1. Hydrophilic/Hydrophobic Patterning

After hydrophobic/hydrophilic pattern was checked by dropping water, the results show that 40 min printing method including 3 head nozzles and overprinting twice was chosen for optimum hydrophobic/hydrophilic patterning.

3.2. Optimized Modification of Agar for Ink-jet Printing

As shown in **Fig. 1**, hydrolyzed agars were classified into 3 groups, depending on the state of the material: Gel, paste and aqueous states. Agar powder dissolved in 0.05% H₂SO₄ aqueous solution and heated for 30 min attained an optimum hydrolysis level, which was a viscosity conducive to ink-jet printing. Additionally, a film-type wrapping heater was used to change the hydrolyzed agar from the paste to aqueous state. As a result, heating guaranteed smooth ejection of the hydrolyzed agar medium by maintaining the aqueous state and avoided clogging the nozzle heads. However, 50 μ L aliquots of the hydrolyzed agar medium dried so quickly that the moisture content of the agar was maintained only for 16 h. Because we developed a culture system for bioassays that will be processed within a few hours, the drying time of 16 h is sufficient to maintain a suitably moist environment.

To confirm the survival of bacterial cells on paper, the isolate originally from air-floating bacteria were transferred on the hydrolyzed agar medium manually. Colonies were not observed clearly on the hydrolyzed agar medium on paper as shown in **Fig. 2**.

3.3. Ejection of *E. coli* on Agar Medium

After a suspension of *E. coli* cells was printed, the normal agar medium sheet was immediately observed microscopically. Individual *E. coli* cells were not visible; however, mature *E. coli* colonies appeared as white spots after culture for approximately 30 h and were photographed (**Fig. 3**).

These results suggest that *E. coli* cells that were ejected through narrow nozzles were still viable, although they were subject to high shear stress in the nozzles. Previous reports discuss problems of low survival rates in such kinds of ink-jet bio-dispensing systems (Harrigan, 1998; Hossain *et al.*, 2012). However, the survival rate obtained using this system was satisfactory, considering that bacterial growth was

observed for all printed dots. These results also suggest that this ink-jet printing method is capable of dispensing fairly regular arrangements of *E. coli* cells, although some satellite colonies are visible next to the primary colonies in the photograph (**Fig. 3**). This deviation in the regular arrangement was due to droplet split, which occurred immediately as the droplets were released from the nozzle; deviation was solved by increasing viscosity and decrease surface tension by adding a small amount of glycerol to the bacterial solution.

3.4. Stability in Number of Ejected Particles

After we confirmed the ability to dispense viable bacterial cells by ink-jet, we verified that the ink-jet printer could dispense regular arrangements of non-ink liquid dots and eject consistent numbers per droplet of latex particles, a model for bacterial cells. The ink-jet printer is designed to eject droplets with volumes of 1-10 μ L. Although, the average diameter of the latex particles is approximately 1 μ m and latex particles were expected to print smoothly, we sought to avoid clogging of the head nozzles or aggregation between particles. A small amount of glycerol was added to latex particles to increase the viscosity and decrease the surface tension (Segur, 1953). **Figure 4** shows printed dots of latex particles at certain intervals on photo-grade ink-jet paper, which resulted in the formation of well-dispersed particles for easy counting and a potentially desirable inoculation level of bacterial cells. For the measurements, the number of primary particles with a diameter of approximately 1 μ m was counted and coexisting particles much smaller in size were excluded from counting. We randomly selected 10 out of 121 dots for counting. The stability of number of latex particles per droplet was tested by using the Analysis Of Variance (ANOVA) method. The statistical data are listed in **Table 1**. When F-values critical at $\alpha = 0.95$ was compared with the F-values calculated from ANOVA, the result showed that number of latex particles per droplet was not significantly different among different overprinting times. In addition, the average number of latex particles per droplet was consistently approximately 15. This finding suggests that liquids containing bacterial cells can be dispensed evenly and regularly onto a culture medium. In addition, the circular shape of 1 dot evenly formed from 5 droplets (**Fig. 4**), indicating that overprinting several times can be applied to adjust the number of cells per dot.

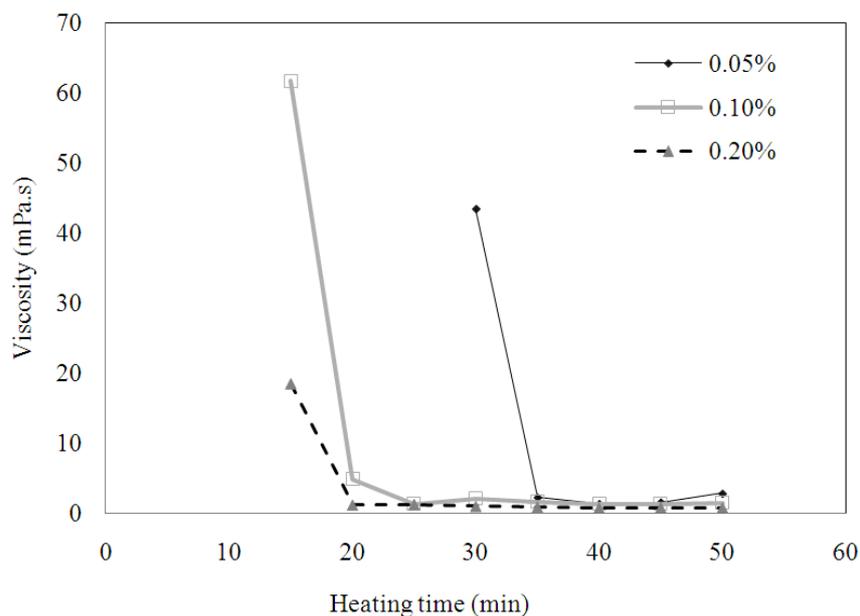


Fig. 1. Viscosity of agar hydrolyzed at three different concentrations of H₂SO₄

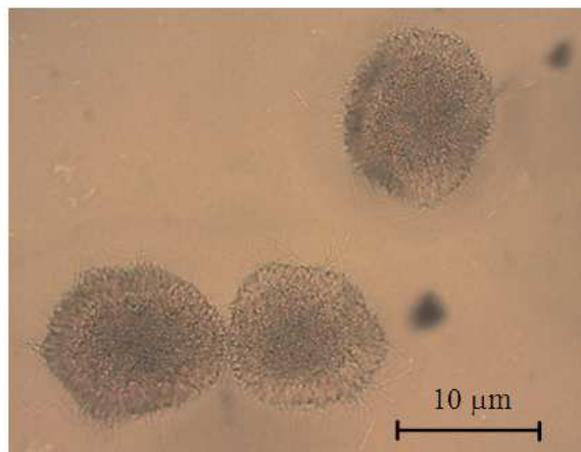


Fig. 2. Colonies of bacteria growing on modified agar medium



Fig. 3. Arrangement of cultured *E. coli* colonies after printing on a sheet of agar medium

3.5. Fluorescence Monitoring of *E. coli* Growth and Agar Location

Light transmission and fluorescence optical micrographs were captured by CLSM. **Figure 5** shows a combined image of an overall paper structure consisting of fibers of the filter paper with a dark area, agar medium with a yellow and toluene blue adsorbed by fibers in red, respectively. It should be noted that the false-colors appearing in the images are not the same as those visible to the human eye.

The combined image (**Fig. 5**) clearly indicates that the agar medium spread and settled within the hydrophilic area and the dark area. Unfortunately, the bacterial colonies could not be distinguished by CLSM, because both of bacteria and medium contains protein so the auto-fluorescence was same. However, CLSM is a promising technique to distinguish mixed components in the paper. This technique would be incorporated in the bioassay system as an automatic evaluation of bacterial growth.

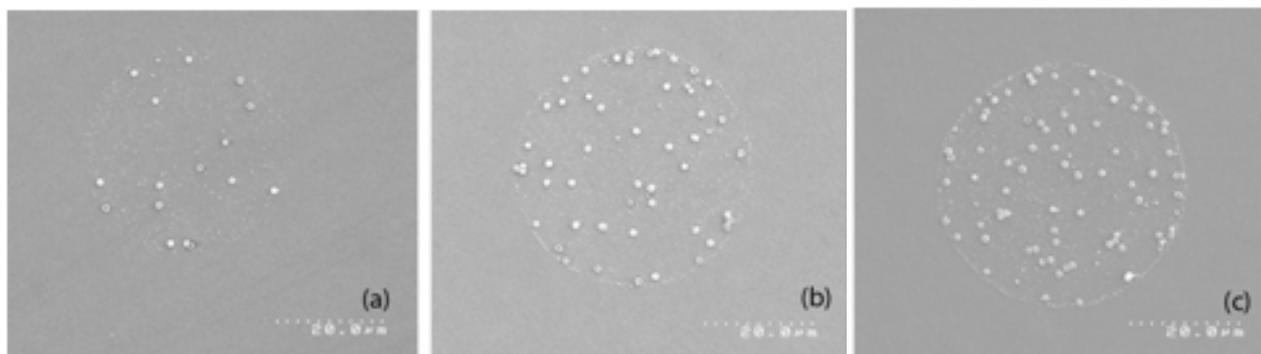


Fig. 4. Latex particles in 1 dot observed by scanning electron microscope; (a) 1 time overprinting, (b) 3 times overprinting and (c) 5 times overprinting

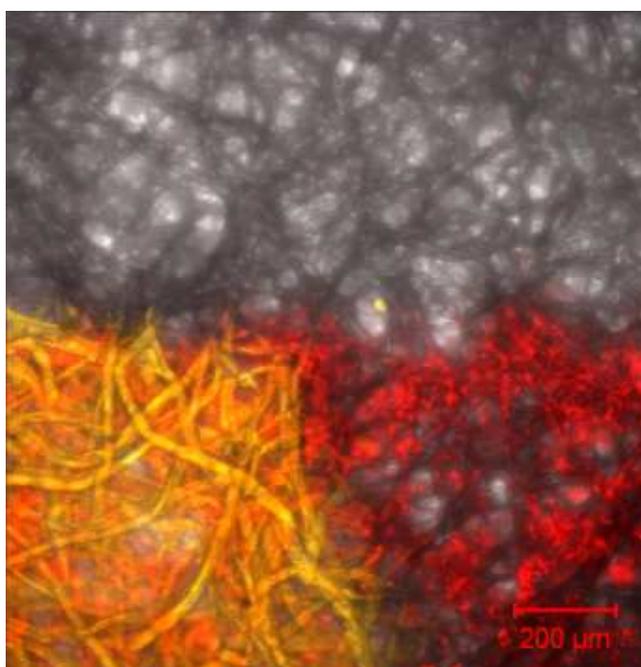


Fig. 5. Combined image of agar medium on hydrophilic areas of filter paper as observed by CLSM

Table 1. ANOVA result of latex particles per droplet at $\alpha = 0.95$

Source of variation	df	SS	MS	F
Treatments	9	1.73	0.19	0.0256
Error	20	149.75	7.49	
Total variation	29	151.48		

4. CONCLUSION

The ink-jet printer-assisted bioassay system we are currently developing in combination with a paper substrate is a promising technique to more efficiently

evaluate bacterial growth rates. The hydrophilic/hydrophobic patterning provided a paper substrate suitable for accommodating an ink-jet printed culture medium. Agar was hydrolyzed to adjust the viscosity, making it conducive to printing on paper and bacterial growth. CLSM was performed to detect the locations of the agar, blue dye and fibers. The discrete locations on the paper substrate were discernible in the fluorescence images. In a separate experiment, *E. coli* cells were printed on a sheet of agar medium and the growth of colonies with high survival rates were

confirmed. We confirmed the regularity of the number of ejected cells in 1 droplet with latex particles as a cell suspension model. Finally, we developed ink-jet assisted paper-based substrate as a part of a bioassay system.

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