Construction of High-Density Bacterial Colony Arrays and Patterns by the Ink-Jet Method

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Abstract: We have developed a method for fabricating bacterial colony arrays and complex patterns using commercially available ink-jet printers. Bacterial colony arrays with a density of 100 colonies/cm^2 were obtained by directly ejecting *Escherichia coli* (*E. coli*) onto agar-coated substrates at a rapid arraying speed of 880 spots per second. Adjusting the concentration of bacterial suspensions allowed single colonies of viable bacteria to be obtained. In addition, complex patterns of viable bacteria as well as bacteria density gradients were constructed using desktop printers controlled by a simple software program. © 2004 Wiley Periodicals, Inc.

Keywords: ink-jet printer; bacterial colony array; complex pattern; cell density gradient; biosensor

INTRODUCTION

Ink-jet printing is a noncontact reprographic technique that takes digital data from a computer representing an image or character, and reproduces it onto a substrate using ink drops (Mohebi and Evans, 2002). In recent years, the applications of the technology have been successfully extended from its traditional areas in electronics (Le, 1998) and microengineering (Mott et al., 1999) into bioengineering fields such as genomics, biosensors, and drug screening (Blanchard et al., 1996; Hart et al., 1996; Lemmo et al., 1998). Although biological molecules and structures are often viewed as fragile, molecules such as DNA have been directed onto glass by a Canon bubble jet printer to fabricate high-density DNA microarrays without molecular degradation (Okamoto et al., 2000). Moreover, proteins such as horseradish peroxidase have been deposited onto cellulose paper to create active enzyme arrays for bioanalytical assays (Roda et al., 2000). We have also shown recently that active biosensors based on biotin-streptavidin linkages can be deposited onto glass (Pardo et al., 2003). Although ink-jet technology has proven feasible for a broad spectrum of biological samples, until recently, most of its use has been limited to nonviable materials (Wilson and Boland, 2003). Here we describe the use of modified ink-jet printers to deliver viable *E. coli* DH5α cells onto a nutrient-covered glass substrate to create high-density colony arrays and organizationally complex patterns.

High-density colony arrays are routinely used for the construction of genomic and expression libraries. These require high-throughput screening of thousands of bacteria to identify specific DNA sequences, to investigate gene expression, and/or to search for differentially expressed genes. Currently, fabrication of bacterial multi-colony arrays is performed mainly by commercially established robotic spotting systems, such as the Genentix QBot (http://www.genetix.com) and the ORCA robot system (Copeland and Lennon, 1994). These are expensive and complex systems that employ a multi-pin contact-delivery mechanism to transfer bacteria onto a membrane filter. Moreover, there is an increasing need for suitable methods to create bacterial patterns to build bacterial biosensors for environmental monitoring, detection of toxicological contamination (Ptitsyn et al., 1997; Rettberg et al., 1999), and applications in the area of public defense (Pancrazio et al., 1999; Ringeisen et al., 2002). The method of printing described here presents an easy and reliable way to create complex patterns of viable bacterial cells.

MATERIALS AND METHODS

Thermal Ink-Jet Printer and Print Substrate

Two different commercial ink-jet printers (Hewlett-Packard DeskJet 550C and Canon Bubble Jet 2100) were modified to accommodate delivery of bacteria to a precise location on a certain substrate. Details of the modifications are published elsewhere (Pardo et al., 2003). Print substrates were made from soy agar. 1.5 mL and 25 mL of prewarm sterilized Trypticase® Soy Agar solution (Becton

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Dickinson & Co, Cockeysville, MD) was poured into 35 mm and 100 mm Petri dishes, respectively, containing sterilized coverslips or a microscope slide. The solution was allowed to cool to room temperature, at that point a thin gel layer was formed on the substrates. A modified Canon bubble jet printer was used to deliver the bacteria onto the coated coverslips because of its narrow distance between cartridge head and substrate-loading base. For delivery of the bacteria onto the microscope slides, a modified HP DeskJet printer was used, due to its wide space between cartridge head and substrate-loading base.

Bacterial Strain and Suspension

Escherichia coli DH5α cells (Gibco-BRL, Life Technologies, Rockville, MD) were grown overnight at 37°C on a Trypticase® Soy Agar plate. Two loopfuls of organisms (representing approximately two large colonies) were transferred into a centrifuge tube containing 5 mL sterilized water. This formed the original print suspension of bacteria. The cell concentration in the *E. coli* solution was determined to be 3×10^7 cells/mL by the standard plate count method (Benson, 1998). This solution was diluted to different concentrations of bacterial suspensions for subsequent printing. The tubes containing bacterial suspensions were forcefully shaken before printing, to break up clumps and ensure good distribution of the bacteria. The movement of the cartridge during printing allowed the cells to be maintained in suspension.

Fabrication of Colony Array

Microsoft PowerPoint software was used to edit a colony array pattern with a 2 dots/mm density and a 0.13 pt weight. A black ink-jet cartridge was emptied of its contents, thoroughly washed, rinsed with a 100% ethanol solution, and autoclaved water and dried in a sterilized hood before being filled with 1 mL of a bacterial printing suspension. This procedure proved to be an effective method for the cleaning and sterilization of the cartridges. This was determined by imprinting coverslips with sterilized water-filled cartridges and subsequent absence of colonies on agar-coated coverslips 2 days after incubation at 37°C.

Using the modified HP Desktop 550C printer, droplets of the *E. coli* suspension were ejected onto the agar-coated glass slides according to a predesigned colony array pattern. Different dilutions of print suspensions (1:10, 1:100, and 1:1000 from the original print suspension) were also used. All printed slides were incubated at 37°C overnight. The *E. coli* colony arrays were then scanned using an Epson Perfection 1640SU scanner, and the images were recorded for further analysis.

Complex Pattern Printing

We also proceeded to print *E. coli* directly using some irregular patterns and complex shapes, including the picture of a cartoon tiger paw and black color gradient. Using the modified Canon ink-jet printer, the 3×10^7 cells/mL suspension of bacteria was deposited onto an agar-coated coverslip according to these specially designed patterns. After printing, cells were incubated overnight. A digital camera optically recorded these complex patterns of viable *E. coli*.

RESULTS

Escherichia coli Colony Array Fabrication

The *E. coli* imprinted agar-coated slides, after incubation for 20 h at 37°C, were placed onto a high-resolution scanner, which provided an even light source for the optimal recording of the imprinted colony arrays. Individual colonies had a circular shape with an approximate diameter of 500 μm. In the following ''designed dots'' refers to the actuation of the nozzle according to software instructions. The designed dots could grow into single colonies, or overlapping colonies, which we refer to as ''imprinted dots'' hereafter. Designed dots not resulting in imprinted dots were also possible at low concentration of the bacterial print suspension.

Figure 1 shows the optical recording of the colony arrays from different bacterial concentrations that were printed on identically designed patterns. Few imprinted colonies are observed on slides A and B, where the bacterial print concentrations were 3×10^4 and 3×10^5 cells/mL, respectively. On slides C and D, where the bacterial print concentrations were 3×10^6 and 3×10^7 cells/mL, respectively, the many bacterial colonies present matched the designed array patterns. Overlapping colonies were observed on the slides with the highest bacterial concentrations. Furthermore, it appears that occasionally, during the nozzle firing, satellite droplets formed, and resulted
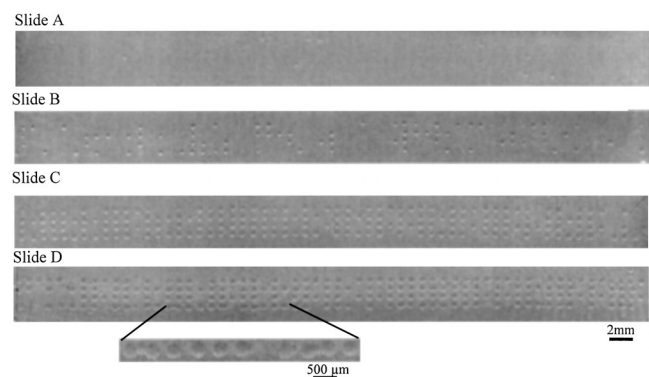

Figure 1. Optical recordings by scanner of *E. coli* colony arrays created using print suspensions of varying *E. coli* concentrations. Slide A: 3×10^4 cells/mL; slide B: 3×10^5 cells/mL; slide C: 3×10^6 cells/mL; slide D: 3×10^7 cells/mL. The insert shows a higher magnification of several colonies on slide D, revealing an overlap and a satellite defect.

in bacterial colonies growing outside the designed dots (slide D).

To analyze the ability of our ink-jet printing method to obtain optimal single-colony arrays we counted the relative number of imprinted dots and isolated colonies on each slide as a function of the concentration of the bacterial suspension (Fig. 2). Four rows (each containing 60 designed dots) of bacterial colony arrays were analyzed. A bacterial concentration of 3×10^6 cells/mL resulted in single colony arrays with the highest fidelity to the pattern.

Complex Patterns Printing

The cartoon tiger paw was designed as an example of printing bacteria in an irregular shape. No obvious visual pattern could be seen by the naked eye immediately after printing the *E. coli* suspension onto the agar substrate. However, after overnight incubation at 37°C, the pattern became visible. The bacteria thus created the complex image of a tiger paw (the unofficial Clemson University logo). An optical micrograph of the tiger paw pattern obtained by direct printing of the *E. coli* suspension is depicted in Figure 3.

The black color gradient was designed to represent a continuously changing pattern, where the darkness of color should represent the higher density of imprinted bacteria. The deposition of cells onto the soy agar surface with the Canon printer following the grayscale pattern, showed a bacterial density-gradient that changed from complete coverage on the left side, to virtually no colonies on the extreme right, hence closely matching the designed pattern. The designed gradient pattern and the imprinted bacterial density gradient (24 h after incubation at 37°C) are shown in Figure 4.
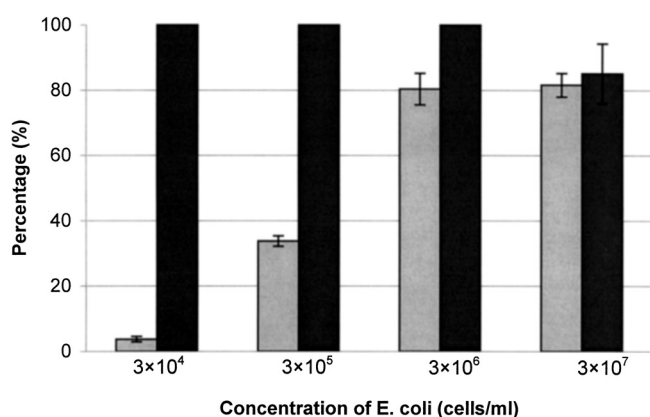

Figure 3. Photograph of cartoon tiger paw generated by printing viable *E. coli* cells, according to a predesigned pattern, onto an agar-coated coverslip.

DISCUSSION

Bacteria Printing and Fabrication of Colony Arrays

We have investigated the direct printing of bacteria on agar-coated substrates using different ink-jet printers. Our experiments provide evidence that viable *E. coli* cells can be reliably delivered to target substrates thus making it possible to produce high-density arrays and complex patterns with a method that is fully automated and high throughput. No nozzle blockage was observed and the cartridges could be re-used many times, provided a cleaning protocol was employed.

A probable reason for absence of any reports of printing bacteria by ink-jet printers is the prevailing thought that the high temperate of the heating system and the high shear stresses (up to 10 ms^{-1}) (Okamoto et al., 2000) for ejected samples in nozzles of ink-jet printers would damage or even kill viable cells during their passage through the nozzle. In Hewlett-Packard and Canon ink-jet printers a similar print system is used, in which a heated plate in the nozzle causes a vapor bubble to form and eject a droplet of ink. The current pulse lasts a few microseconds and raises the plate temperature as high as 300°C (Calvert, 2001). The *E. coli* strain (DH5α) used was a competent cell strain traditionally used for transformation and cloning. These
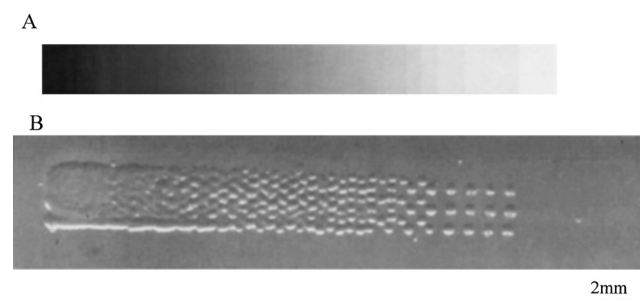

Figure 2. Graphic representation of the relative number of imprinted dots and isolated colonies, with respect to the density of the bacterial suspension. (▨) Average percentage and Standard Deviation ($n = 4$) of imprinted dots with respect to the total designed dots. (■) Average percentage and Standard Deviation ($n = 4$) of isolated bacterial colonies with respect to the total imprinted dots on the agar-coated slide.

Figure 4. Cell density gradient of *E. coli* cells imprinted on an agar-coated coverslip, following a grayscale pattern. (A) Black color gradient edited in Microsoft PowerPoint to provide patterns for cell density gradient. (B) Scanner recording of *E. coli* density gradient created by direct ink-jet printing of *E. coli*.

cells are more fragile and heat-sensitive than most other *E. coli* strains. However, our results showed that although the temperature of the heated plate can reach 200–300°C, the system still reliably delivered viable cells onto the agar-coated surfaces. One possible explanation for their survival is that the limited amount of heat transferred to the ejected droplets in the timeframe of the ejection causes only a small temperature increase in the liquid and therefore little, if any, damage occurs to the bacteria inside the droplets.

The optimal concentration of bacterial print suspension was determined by both experimental and theoretical means. Ink-jet printers can deliver as little as 8 pL/droplet, or as much as 95 pL/droplet. The droplet sizes vary according to the temperature gradient applied, the frequency of the current pulse, and the sample viscosity (Allain et al., 2000). The diameter of a single *E. coli* is approximately 2 µm (Harley et al., 2002) and the volume of the bacterium is about 4×10^{-3} pL, which is about 1/2000 or 1/25,000 of the volume of a droplet. Therefore, the concentration of the printing suspension was carefully adjusted to achieve printing of a single bacterium per drop. Based on the specifications of each printer it was decided to use the value of 95 pL/droplet to estimate the maximum concentration of bacteria in the print suspension to ensure single colony imprinting. Under these conditions the calculated maximum concentration of bacterial suspension needed was estimated to be approximately 1.1×10^7 cells/mL, but this value was then fine-tuned by experimental observations.

In our experiments the optimum concentration was found to be approximately 3×10^6 cells/mL in the print suspension to achieve approximately one bacterium per droplet. When the concentration of the bacterial suspension was 3×10^7 cells/mL the probability that two or more bacteria would be imprinted onto the same dot was higher (Fig. 1, slide D). On the other hand, when lower concentrations were used, there was a much lower probability for two or more bacteria to occupy the same site (Fig. 1, slides A, B, C). Similarly, the results detailed in Figure 2 show that using 3×10^7 cells/mL concentrations resulted in 90% isolated colonies. When a higher concentration of cells was used, such as 1×10^8 cells/mL, the percentage of isolated colonies dropped to about 80% (data was not shown). Moreover, no colony overlap was observed at concentrations below 3×10^7 cells/mL. In addition, some printed droplets did not contain any bacteria, as shown by spots devoid of bacterial colonies. The probability of the absence of bacterial colonies increases as the concentration of the bacterial suspension decreases. Therefore, there is a fine balance between colony overlap and missing bacterial colonies. This optimum concentration can also depend on the temperature gradient applied, the frequency of the current pulse, the sample viscosity, and the cell size. In summary, based on these results the optimum concentration was determined to be 3×10^6 cells/mL, in good agreement with the calculated volume of 1.1×10^7 cells/mL.

The density of colony arrays created by the ink-jet print method was about 100 dots/cm² resulting in 48,400 colony dots onto a 22 mm × 22 mm positively charged nylon membrane target filter. To construct colony arrays, the conventional methods, such as the Genentix QBot or the ORCA robot system, are based on a time-consuming multi-pin contact process that transfers bacteria onto a membrane filter in ordered arrays. A gridding head with 96 or 384 pins is used to dip into 96 or 384 well source plate to pick target bacteria and then direct them onto the filter. Washing and sterilization of the multi-pins must be performed before the gridding head can be moved to a new 96/384-well source plate. Conventional systems grid 3456 clones onto 8×12 cm filters, far less than can be done using the ink-jet approach. The gridding speed of conventional systems is about 100,000 spots/h (http://www.genetix.com), while the ink-jet based systems, such as the one presented here, can operate at 50,000 spots/min. Furthermore, the noncontact mode of ink-jet printers eliminates the need of washing and sterilization between depositions of bacteria. Additionally, loading different bacteria strains into different color cartridges provides a potential for creating mixed colony arrays from different bacteria types.

Construction of Complex Patterns and Bacteria Density Gradients

The capacity of desktop printers to orchestrate the printing of bacteria into complex patterns is illustrated in Figures 3 and 4. In fact, the method is as simple as it is elegant. The tiger paw pattern has an irregular shape, which is not conveniently fabricated by conventional patterning methods. Competing methods, such as microcontact printing, for example, would have difficulty creating complex arrangements of cells. Most methods use a two-step approach of orchestrating cell patterns by employing masks to pattern cell-adhesive substrates using self-assembled monolayers or certain proteins (Bernard et al., 2000) to which the cells are then exposed. The ink-jet approach is not only faster, but it is also easy to automate. In addition, cell density gradients are difficult, if not impossible to fabricate by the two-step approach. Finally, with the ink-jet method the creation of new patterns and modification of existing patterns is simply accomplished by editing conventional word-processing or graphics software.

CONCLUSION

We developed a noncontact ink-jet print method in which viable bacteria can be delivered onto precisely targeted positions that reproducibly generate isolated colony arrays, as well as complex patterns. Our system provides a cost- and time-effective alternative to the traditional method for creating arrays for bacterial colony screening and the construction of genomic and expression libraries. In addition, the ink-jet printer patterning technique, with its simplicity and high throughput, may hold considerable potential for the fabrication of bacterial biosensors. Moreover, the ink-jet printing could also be used to generate cell

density gradients that may be of use in pharmacological screening, where drug effectiveness and toxicity measurements require testing on varying densities of cells.

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