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Microcontact Printing of Living Bacteria Arrays with Cellular Resolution

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ABSTRACT

Arrays of living bacteria were printed on agarose substrate with cellular resolution using elastomeric stamps with a high aspect ratio generated by reverse in situ lithography (RISL). The printed bacteria reproduced the original stamp patterns with high fidelity and continued growing as in bulk culture. This methodology provides a simple route to any desired bacterial spatial 2D distribution and may be applied to screening as well as to studies of bacteria phenotypic variability, population dynamics, and ecosystem evolution.

The development of systems biology requires experimental procedures to control the spatial organization and interactions between individuals within a large population. In recent years, significant progress has been made in producing arrays of mammalian cells, which have great potential in fields such as gene expression profiling, drug screening, cell−cell communication study, and population dynamics.[1,2] However, a convenient and well-controlled method to make high-resolution arrays of widely studied bacteria (e. g., *Escherichia coli*) is still lacking due to their small size (∼1−5 μm) and motility. Up until now, very few methods have been proposed to pattern bacteria with micrometer precision, albeit with distinct limitations.[3−7] The holographic optical trapping technique by Akselrod et al. is precise yet technically demanding.[3] The heterogeneous surface functionalization method by Rowan et al. is hard to combine with routine biological investigations, and patterned bacteria do not grow under their physiological conditions.[4] The stamping technique proposed by Weibel et al. is biocompatible, but accessible pattern sizes range from 250 μm to 2 mm, which are far from single-bacteria dimensions.[6] Therefore, it is necessary to find a straightforward method capable of producing high-resolution arrays of living bacteria and suitable with eventual biological investigations. In this paper, we describe a novel yet simple methodology to print living bacteria on an agarose substrate with high aspect ratio stamps made of polydimethylsiloxane (PDMS). With this method, arrays of *E. coli* with a micrometer-resolution, down to single bacteria, can be printed directly on agarose in several seconds over a large area (cm^2). After printing, patterned bacteria keep growing and dividing as in bulk culture conditions.

The principle of the methodology depicted in Figure 1 is based on conventional microcontact printing techniques for pattern generation of biomolecules or particles.[8−11] First, 50 μL of LB medium containing *E. coli* (exponential phase) chromosomally engineered to express yellow fluorescence protein (YFP) were deposited on an agarose gel block (1 cm × 1 cm × 5 mm, Figure 1a). The absorption of the liquid by agarose led to the formation of an "inkpad" covered by a monolayer of *E. coli* (Figure 1b). Then, a PDMS stamp with a desired pattern was used to transfer bacteria from the "inkpad" to another agarose substrate (Figure 1c−f). This methodology resulted in an array of living bacteria reproducing the original stamp pattern.

The success of this method required reproducible stamps with high aspect ratio motifs and resolution. These were obtained by PDMS casting over high aspect ratio moulds fabricated by introducing a new improved UV lithography protocol, reverse in situ lithography (RISL), to minimize diffraction blurring. The stamp fabrication procedure and the comparison between RISL and conventional photolithography are described in the Supporting Information section. The only limit of RISL technology is the pillar diameter, which can hardly be smaller than 1 μm due to diffraction limit in the first lithography procedure (step "a" in Figure 1 of Supporting Information section). Above 1 μm in diameter, any desired pillar size and shape with high aspect ratio can

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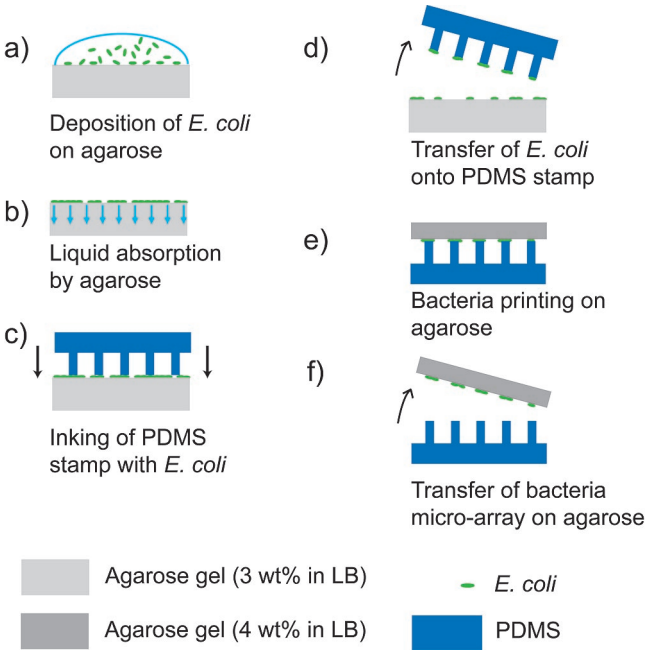

Figure 1. Schematic representation of the methodology to produce arrays of bacteria. (a) A droplet of *E. coli* in LB culture medium is deposited on an agarose gel (3 wt % in LB). (b) The liquid is absorbed by the agarose gel. (c) A PDMS stamp is inked by contact with bacteria covering the agarose gel. (d) When the stamp is removed from the "inkpad", a fraction of bacteria are transferred onto the stamp. (e−f) The bacteria are transferred by contact with a 200 μm thick slide of agarose (4 wt % in LB) into a regular microarray.

be produced, which is sufficient for precise patterning of bacteria; any period larger than 1 μm can also be obtained. Before bacteria printing, PDMS stamps were activated with oxygen plasma for 1 min and then treated with 1 wt % poly-(ethylenimine) for 1 h to increase the efficiency of bacteria transfer.[12] The bacteria transfer (steps c−e in Figure 1) was done as fast as possible (typically in less than 1 s) to avoid possible damage to bacteria when they are exposed to air. Because bacteria should be kept alive during and after the printing procedure, we chose agarose gel in LB, a widely used material for bacteria culture,[13] as the material of both "inkpad" and substrate for bacteria arrays. The adjustment of agarose concentration is crucial for a good-performance printing. A too-small concentration led to distortion of the printed pattern, while a too-high concentration is not suitable for bacteria culture. We found that appropriate concentrations were 3 and 4 wt % for the inkpad and the substrate, respectively. Under the above-mentioned conditions, we managed to generate living bacteria arrays with high resolution and good reproducibility.

We characterized the efficiency of our method to produce regular arrays of bacteria. For this purpose, we used stamps with hexagonal patterns of different characteristic periods p and motif diameters d and a constant height of 12 μm (Figure 2A). We analyzed the resulting bacteria arrays (Figure 2B) by means of phase-contrast and fluorescence microscopy. Parts C and D of Figure 2 show that the obtained bacteria array reproduces well the original pattern of the stamp (d = 12 μm, p = 60 μm). Moreover, the high-magnification

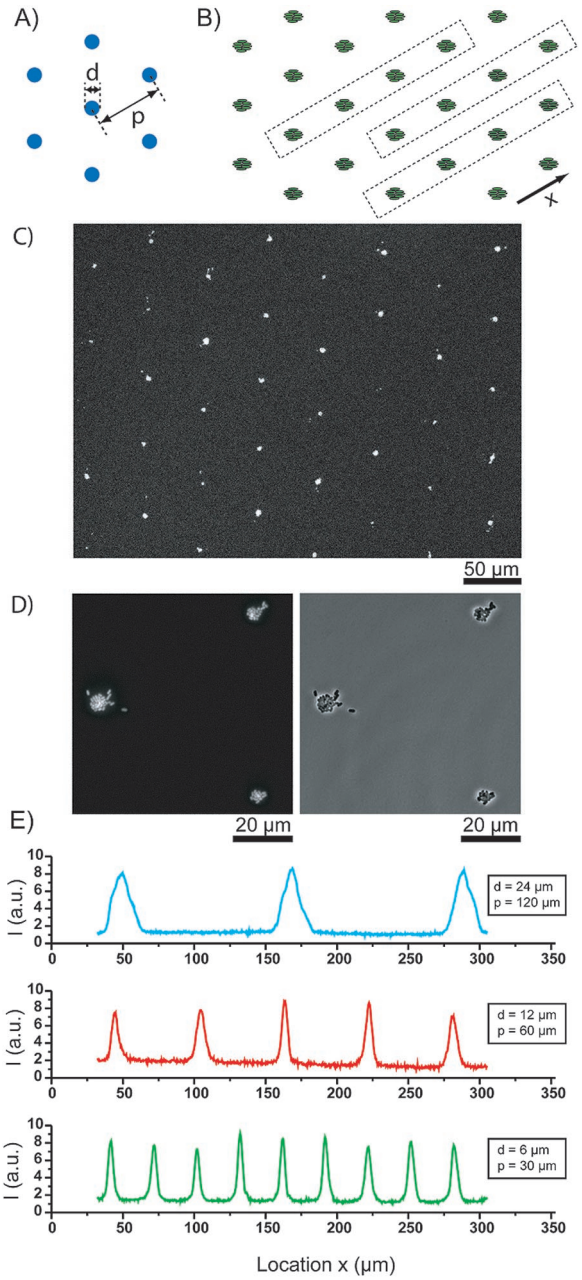

Figure 2. (A) PDMS stamp design: we used hexagonal arrays (period p) of cylindrical micropillars of diameter d and a constant height of 12 μm. (B) Schematic representation of the printed array of bacteria. The dashed boxes represent the sampling windows used to characterize bacteria distributions (see E). (C) Typical low-magnification fluorescence microscopy image of a bacteria microarray obtained with a PDMS stamp (d = 12 μm and p = 60 μm) on an agarose gel. (D) High-magnification fluorescence (left) and phase-contrast (right) microscopy images of a detail of the array shown in C. (E) Fluorescence intensity profiles (arbitrary units) of printed bacteria arrays obtained with stamps of various diameter d and period p. Each graph provides the intensity profile averaged on 50 sampling windows (275 μm × 25 μm) as defined in B. The x-axis is along the direction indicated by the arrow in B.

fluorescence image of Figure 2D indicates that the arrays are suitable for fluorescence studies and that bacteria have a normal state (shape, fluorescence emission, etc.). To quantitatively characterize the produced bacteria arrays, we measured systematically the fluorescence profile along one

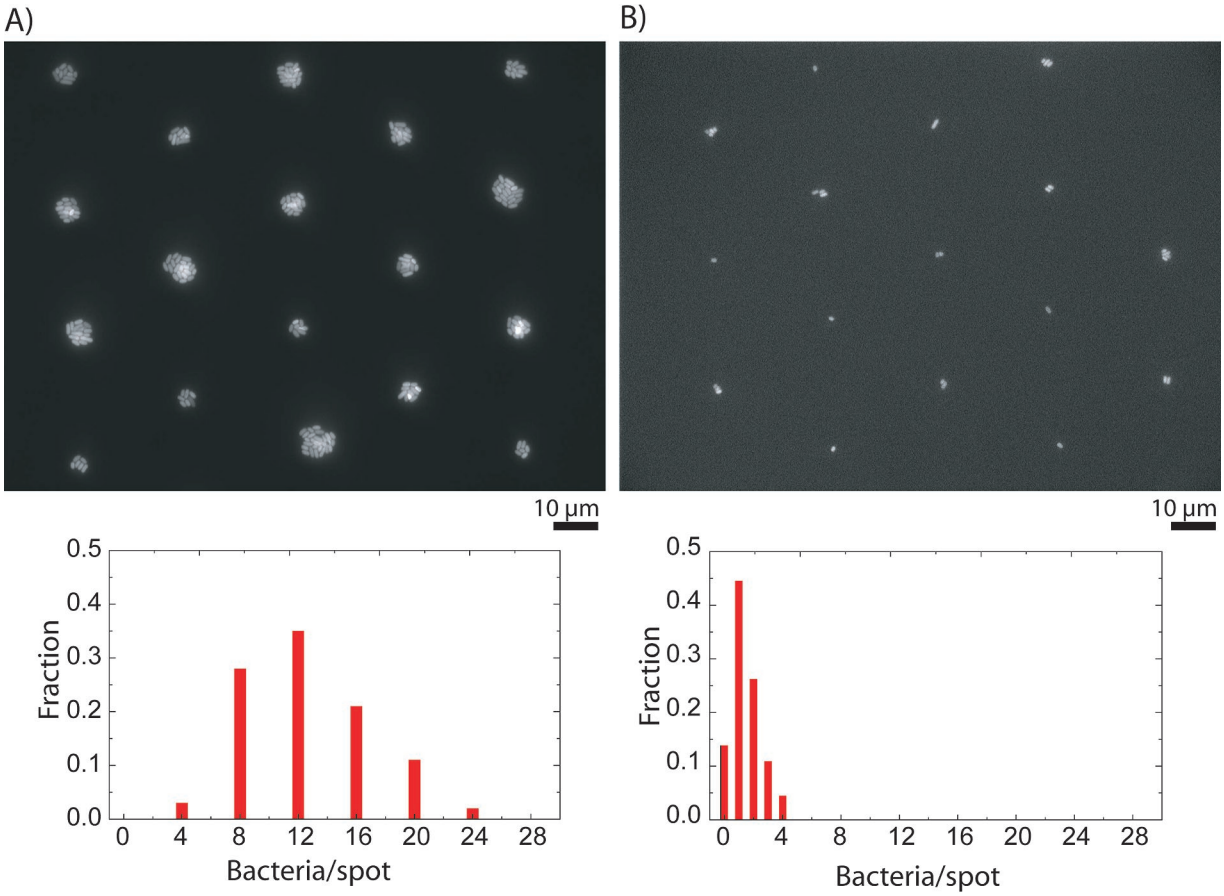

Figure 3. Fluorescence microscopy images (top) and distribution of number of bacteria per printed spot (bottom) of bacteria arrays obtained with a PDMS stamp ($d = 6$ μm and $p = 30$ μm) on an agarose gel. The initial concentration of bacteria (step "a" in Figure 1) was 10^9 cells/mL (A) and 10^8 cells/mL (B), respectively. Distributions have been established on 200 spots.

array axis by averaging the fluorescence intensity over 50 sampling windows (275 μm $\times$ 25 μm) (Figure 2B). Figure 2E shows the fluorescence intensity profiles of bacteria arrays produced with stamps of various periods and motif diameters. Regardless of geometrical parameters, all profiles have a similar general shape, which consists of periodically spaced intense peaks. The low-intensity value of the baseline corresponds to the average background and demonstrates that almost no bacteria are present between patterned spots. In contrast, the high intensity and symmetry of peaks indicate that bacteria are concentrated on the spots with no preferential direction. The similarity (intensity and shapes) between peaks of a given profile indicates a homogeneous distribution of bacteria over a large scale. Moreover, the distance between peaks corresponds exactly to the stamp period p and the half width of the peaks is directly correlated to the stamp motif diameter d. All these features show that the bacteria arrays reproduced the initial stamp pattern with a high fidelity over a large scale.

With the objective to obtain arrays of single bacteria, we studied the effect of a decrease in initial concentration of bacteria in the droplet spread on the agarose used as "inkpad" (step "a" in Figure 1). We observed that the average number of bacteria per spot decreased with a decrease in bacteria concentration. Figure 3 shows the effect of bacteria concentration when we used a stamp of diameter $d = 6$ μm and period $p = 30$ μm. In this figure, two bacteria concentrations, 10^9 cells/mL and 10^8 cells/mL, were used, respectively. A concentration of 10^9 cells/mL led to the situation where bacteria form a dense monolayer (approximately 5×10^5 bacteria/mm^2) on the "inkpad", as for the experiments of Figure 2. At a concentration of 10^8 cells/mL, the bacteria density on the "inkpad" decreased to approximately 5×10^4 bacteria/mm^2. Fluorescence microscopy pictures of resulting bacteria arrays (Figure 3, top) show that the typical spot size of the printed bacteria decreases strongly with the decrease in bacteria concentration while the hexagonal arrangement and the period of the stamp pattern are perfectly preserved. Moreover, at the low bacteria concentration, we observe that each spot is composed by a very small number of individuals. The bottom part of Figure 3 shows the distribution of number of bacteria per spot (established on 200 spots) for the two concentrations. For initial concentrations of 10^9 and 10^8 cells/mL, the average number of bacteria per spot was measured to be 12.1 and 1.4, respectively. For the high concentration, Figure 3A shows that the distribution is relatively wide, in agreement with Albrecht et al.[14] In contrast, at the low concentration, the distribution is very narrow, with 44.6% of spots having exactly a single *E. coli* cell and the other 40.1% having 0 or 2. These results demonstrate that our methodology can produce regular arrays of bacteria with a single-cell resolution.

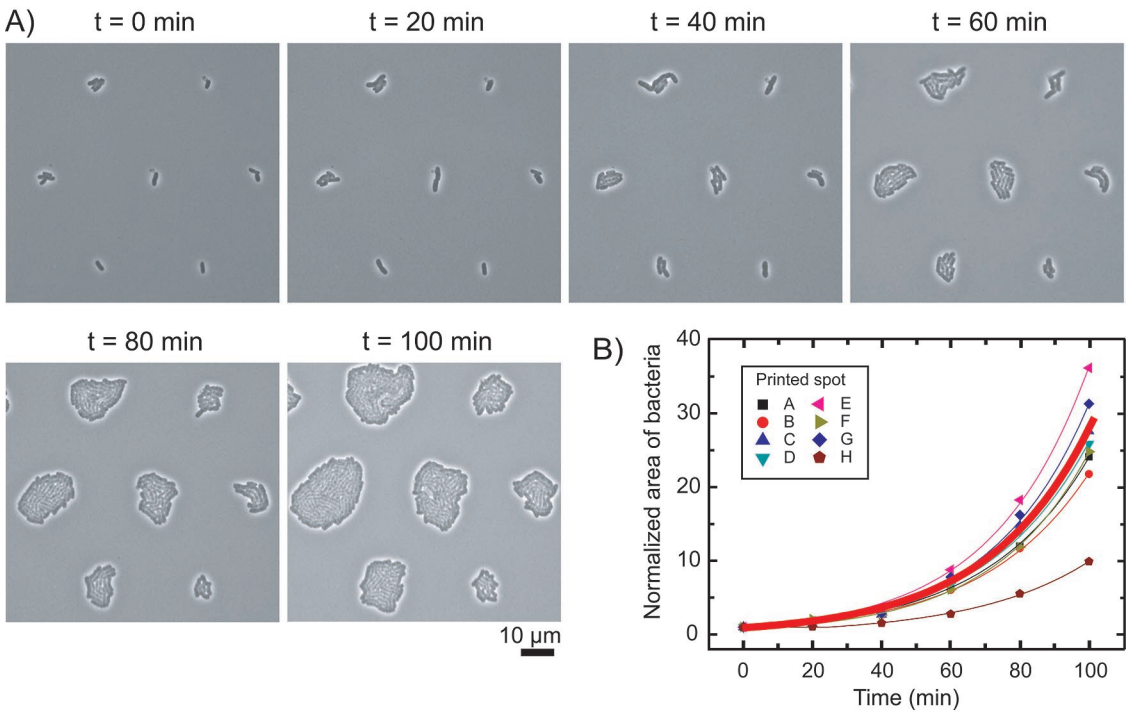

Figure 4. (A) Sequence of phase-contrast time-lapse microscopy images on a selected portion of a bacteria array obtained with a PDMS stamp ($d = 6\ \mu$m and $p = 30\ \mu$m) on an agarose gel. (B) Area of bacteria per spot normalized by area at $t = 0$ as a function of time. Each symbol corresponds to one printed spot. Thin lines are single-exponential fittings for each printed spot. The thick red line is a single exponential averaged fitting curve providing an average division time of 20.6 ± 1.3 min.

Finally, to further combine this technique with biological investigations, it is essential to address the influence of our patterning method on the physiological state of cells. To this end, we quantified the growth rate of patterned wild type *E. coli* (MG1655 strain) bacteria in the following way. The agarose gel substrate with bacteria array was mounted on a microscopy cover slide and covered by a 2 mm thick agarose gel (1.5 wt % in LB) for nutrient supply. The system was kept at 37 °C on a microscope stage, and images of bacteria were taken every 20 min. (Figure 4A) (see Supporting Information for details). Each spot contained initially a few bacteria that were followed through division. The overall hexagonal arrangement ($p = 30\ \mu$m) was perfectly preserved while the microcolonies were growing. (Figure 4A) We found that the overall growth rate of bacteria in the printed array, averaged over large number of microcolonies (0.034 ± 0.002 min^{-1}, corresponding to an averaged division time of 20.6 ± 1.3 min, Figure 4B *thick line*), reproduced well the bacterial growth rate measured in bulk liquid culture (21.4 ± 0.9 min). This demonstrates that bacteria keep their normal physiological behavior after printing. Moreover, our approach allows the analysis of the growth rate of individual lineages. Single-exponential fittings are shown for individual microcolonies in Figure 4B (*thin lines*). A model where exponential growth is preceded by a lag phase[13] gave better fittings to the individual microcolony growth data (Supporting Information). Interestingly, a dispersion of both the lag time (0.5−25 min) and division time (19−23 min) was recorded (Table 1, Supporting Information), revealing underlying phenotypic variability among lineages sharing the same genetics and environment.[13,15] This aspect is now under investigation.

In conclusion, we successfully produced high-resolution arrays of living bacteria by a new, fast, and simple procedure without any requirement of sophisticated microprocessing technology. Our methodology can serve as a ubiquitous strategy to generate and replicate any kinds of 2D patterns of various types of living microbes and may be easily adapted to high-throughput protocols. Arrays with different periods and spot sizes might find applications in many areas, such as phenotypic variation screening,[2,13,15] population evolution analysis,[1,16] signal transduction-based pattern formation,[17] cellular differentiation,[18] and metapopulation dynamics.[19] A future challenge is to extend our methodology to the generation of multistrain bacteria arrays with which complex ecosystems can be constructed with a microscale resolution. Dynamic interactions (competition, cooperation, game process, communication, etc.) between individuals/populations of various species could be then studied in real-time and with a single-cell resolution. Furthermore, combined with surface gradients or isolated culture nanochambers, this methodology will provide a versatile platform for high-throughput screening (e.g., death rate measurement, drug screening, directed evolution). The wide range of novel experiments and procedures made possible by this methodology is expected to bring practical and fundamental applications in nanobiotechnology, ecology, biosafety, microbiology, and systems biology. More generally, it should enlarge our knowledge on the ecological and evolutionary properties of living systems.

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Supporting Information Available: Materials, bacteria culture, stamp fabrication, comparison between standard UV photolithography and RISL, experimental setup for observation of bacteria growth after printing, distribution of growth rates of individual microcolonies. This material is available free of charge via the Internet at http://pubs.acs.org.

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