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Methods for Fabricating Microarrays of Motile Bacteria

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Abstract

Motile bacterial cell microarrays were fabricated by attaching *Escherichia coli* K-12 cells onto predesigned 16-mercaptohexadecanoic acid patterned microarrays, which were covalently functionalized with *E. coli* antibodies or poly-L-lysine. By utilizing 11-mercaptoundecyl-penta(ethylene glycol) or 11-mercapto-1-undecanol as passivating molecules, nonspecific binding of *E. coli* was significantly reduced. Microcontact printing and dip-pen nanolithography were used to prepare microarrays for bacterial adhesion, which was studied by optical fluorescence and atomic force microscopy. These data indicate that single motile *E. coli* can be attached to predesigned line or dot features and binding can occur via the cell body or the flagella of bacteria. Adherent bacteria are viable (remain alive and motile after adhesion to patterned surface features) for more than four hours. Individual motile bacterial cells can be placed onto predesigned surface features that are at least 1.3 μm in diameter or larger. The importance of controlling the adhesion of single bacterial cell to a surface is discussed with regard to biomotor design.

1. Introduction

Biomolecular nanotechnology is an emerging area of scientific research that holds great promise for the development of new technologies with broad applications in the area of optics, electronics, catalysis, and biodiagnostics.[1] An interesting and potentially important application of biomolecular nanotechnology is the construction of biomolecular motors.[2–4] Biomotors can convert chemical energy produced by biological systems into mechanical and electrical energy. The ability to efficiently convert chemical energy into mechanical output using simple fuels such as glucose or ATP at ambient temperatures will open the door to hybrid nanodevices that can be assembled, maintained, and repaired using basic physiological and biochemical methods. Bacterial cells may provide the ideal “power generators” for microscale biomotors[5] because their motility in liquid media may be exploited to either push or pull microfabricated features in the form of propellers or turbines.[6] Depending on the device design and the food source, microorganisms could power nano- and micromachinery for extended periods of time in a potentially fuel-efficient manner.[7]

Motile bacteria typically adhere to surfaces through extracellular components (lipopolysaccharides, phospholipids, proteins) via both specific and nonspecific interactions involving hydrophobic, hydrophilic, electrostatic, and/or van der Waals interactions.[8–10] Bacterial attachment is also mediated by flagella and pili.[5, 11, 12] A great deal of effort has been devoted to the development of modified surfaces that resist cell adhesion[9, 13–16] and such surfaces have been used in sensor development, cell assays,[13, 17] and biomaterials.[13, 18–20] Even though the attachment of bacterial cells to surfaces is now quite common, the fabrication of predesigned microarrays of living bacteria on surfaces has largely been unexplored. Recently it was shown that motile *Escherichia coli* could be randomly adhered to a surface through interactions with an antibody,[21, 22] and similar results have been obtained for motile bacteria such as *Salmonella typhimurium* and *Helicobacter pylori.*[10, 23–25] In addition, whole bacteria have been shown to randomly adhere to surfaces coated with polysaccharides, polystyrene, poly-L-lysine, and even hyperbranched polymer film templates.[8, 9, 15, 17, 26–28] An elegant study of bacterial-cell attachment involved building bacterial “corrals” that ranged from 50 to 120 square micrometers;[26] however, the limitation for biomotor applications is that large surface features hold multiple bacterial cells that are randomly arranged within the corral structure.

In order to understand how to control the attachment of single, motile bacteria to a surface in a predesigned microarray, we examined the adhesion of motile bacteria to surfaces using a variety of linking strategies capable of controlling attachment. Herein, we report the attachment of motile *E. coli* bacteria to gold surfaces in specific, predesigned microarrays via poly-L-lysine (PLL) or antibodies directed against *E. coli*.

2. Results

The attachment of motile bacteria to predesigned self-assembled-monolayer (SAM) modified surfaces was achieved via both nonspecific and specific interactions involving electrostatic interactions and antigen–antibody interactions. PLL, goat and rabbit anti-*E. coli* polyclonal antibodies, as well as rabbit *E. coli* anti-lipopolysaccharide (LPS) antibody were investigated as biological linkers in order to prepare motile bacterial microarrays. Early studies have shown that 11-mercaptoundecyl-penta(ethylene glycol) (PEG-SH) effectively resists cell adhesion to solid surfaces.[9, 13] In this study we examined PEG-SH and a few different commercially available compounds that were reported to resist bacterial and mammalian cells binding. Our analysis revealed that 11-mercapto-1-undecanol (MOU) was capable of significantly reducing *E. coli* adhesion as compared with unmodified gold.

Attachment of bacteria to gold surfaces via PLL: To follow the adsorption of 16-mercaptohexadecanoic acid (MHA), MOU, or PEG-SH, and PLL molecules, surface topography changes were measured by atomic force microscopy (AFM; Figure 1). MHA, which is more hydrophilic than gold, is observed as the light contrast areas in the lateral force microscopy (LFM) images (Figure 1 A**1**, top). The height profile of the patterned MHA structures revealed a 1.8–1.9 nm increase in height upon the attachment of MHA to bare gold (Figure 1 A**1**, bottom) that is consistent with the height of a MHA SAM.[29] AFM images do not exhibit sufficient contrast between MHA and MOU (or PEG-SH) resist layers (Figure 1 B**1**, bottom) due to the similar heights of their SAMs.[30] The typical height of a MOU SAM is 1.3 nm and a PEG-SH SAM is ≈2.0 nm.[30] The MHA–MOU (or PEG-SH) patterned areas can be differentiated by LFM (Figure 1 B**1**, top) due to the higher frictional force between the AFM tip and the MHA SAM.[31] The substrate with adsorbed MHA–MOU SAMs was soaked in a 10 mM aqueous solution of PLL. Based on literature precedent,[32] the PLL is presumably bound to a partially deprotonated MHA layer at pH 7.0, forming an ammonium–carboxylate (NH_4^+/COO^-) ion pair. The functionalization of the MHA layers with PLL results in a slight decrease in LFM image contrast (Figure 1 C**1**, top) and an increase in AFM image contrast (Figure 1 C**1**, bottom) of MHA–PLL and MOU patterned areas. This is in good agreement with the frictional behavior of PLL, which is less hydrophilic than MHA, and it is consistent with the adsorption of PLL.

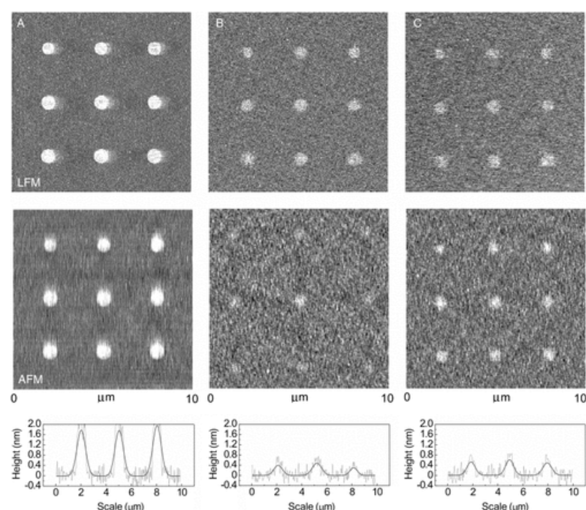


Figure 1 LFM and AFM images of gold substrates monitoring frictional force and topography changes after surface patterning: A) After 16-mercaptohexadecanoic acid has been patterned via dip-pen nanolithography on bare gold; B) after unpatterned areas have been passivated with 11-mercapto-1-undecanol; C) after immobilization of poly-L-lysine on the MHA patterns.

The addition of motile *E. coli* bacterial cells to PLL-modified surfaces resulted in *E. coli* attachment to prefabricated MHA–PLL surface structures. The motility of adsorbed bacterial cells onto prefabricated surface

structures was monitored at room temperature with an optical microscope with the substrate immersed in a liquid cell containing fresh M9 media. Based on direct optical monitoring, surface-adhered *E. coli* cells remained alive and motile for a minimum of 4 h under these conditions (see the movie in the Supporting Information).

To further characterize bacterial-cell binding to prefabricated surface structures, samples containing *E. coli* cells were dried in air and AFM studies were then conducted. Based on AFM/LFM images, *E. coli* cells only adhere to the patterned areas but not to passivated portions of the substrate (Figure 2 A–C**2**). Therefore, PLL-modified surfaces, as prepared herein, appear to be excellent for the attachment of motile *E. coli* bacterial cells, and MOU significantly inhibits cell adhesion.

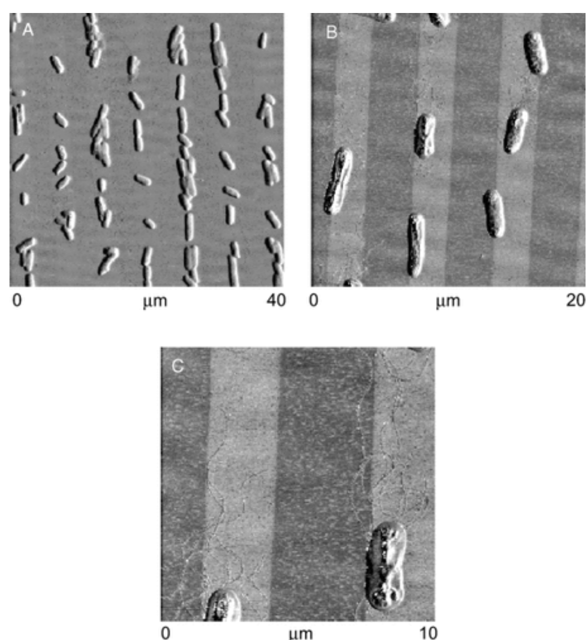


Figure 2 Low- and high-resolution LFM images of bacterial arrays formed on a PLL-modified gold surface: A, B) LFM images demonstrating *E. coli* cell attachment to gold surfaces in accordance with PLL–MHA pattern size and shape; C) a high-resolution image of *E. coli* flagella spreading along a MHA–PLL line pattern. 11-mercapto-1-undecanol was used as a bacteria adhesion resist layer and surrounded all pattern features.

Attachment of bacteria to gold surfaces via antibodies: Cross-reaction studies utilizing three different antibodies were performed to determine which corresponding IgG molecule effectively immobilizes *E. coli*. Figure 3 shows bacteria attached onto predesigned microarrays containing goat anti-*E. coli* immobilized on a MHA pattern with an *N*-hydroxysuccinimide–1-ethyl-3-(dimethylamino)propyl carbodiimide hydrochloride (NHS–EDAC) coupling reaction. The AFM image clearly indicates selective binding of *E. coli* K-12 to the antibody. Similar experiments also show that rabbit anti-*E. coli* antibody as well as rabbit anti-LPS antibody immobilized on MHA-modified gold surfaces maintained selective binding activity to K-12.

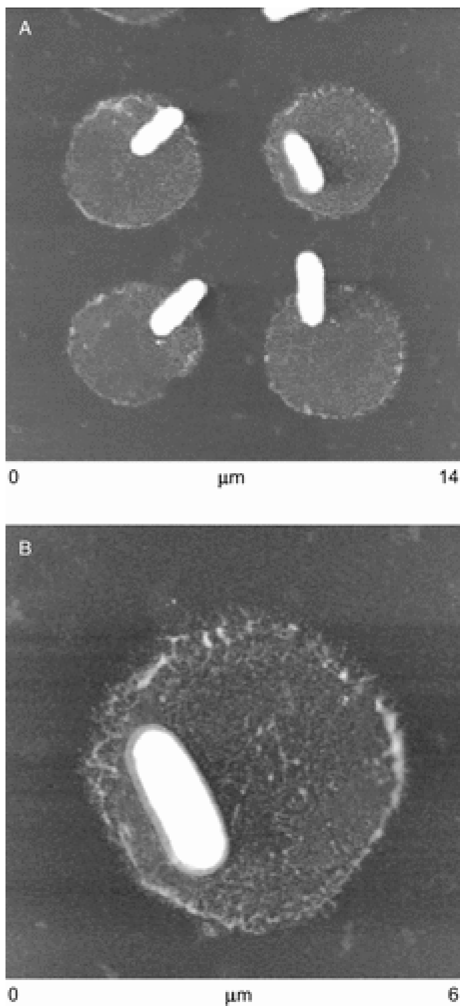


Figure 3 A, B) AFM images of single bacteria attached to polyclonal goat anti-*E. coli* immobilized on a gold substrate.

To distinguish live from dead bacteria in a population of cells transferred to the functionalized Au-coated substrates, a two-color fluorescence assay was used. Patterned bacterial cells were treated with two nucleic acid stains, the green-fluorescent SYTO 9 and the red-fluorescent propidium iodide. SYTO 9 labels both live and dead bacteria when used alone. Propidium iodide, however, penetrates only bacteria with damaged membranes, thereby reducing SYTO 9 fluorescence in the presence of both dyes. Thus, healthy living bacteria with intact membranes fluoresce green, while dead bacteria with damaged membranes fluoresce red. As shown in Figure 4, both live and dead bacteria can be viewed, which indicates that the majority (>70 %) of the cells are viable when captured on protein microarrays. These observations suggest that cross reactions of the targeted *E. coli* K-12 strain with its complementary antibody do not negatively affect the biological activity of these cells. Moreover, these results also indicate that dead bacteria also appear to exhibit selective binding to the *E. coli* antibody. A two-color fluorescence viability control was also performed for cells attached to PLL patterns, which revealed that the percentage of live bacteria was greater than 70 %.

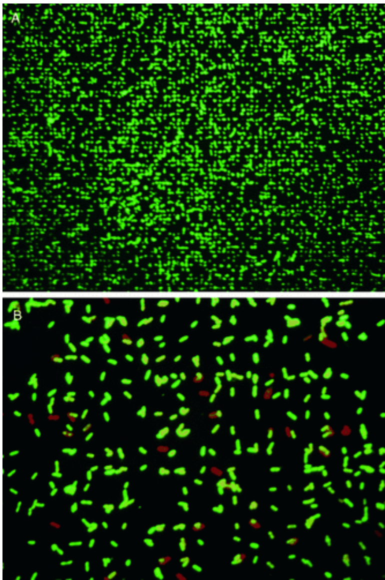


Figure 4 Fluorescence micrographs of both live (green) and dead (red) *E. coli* cells bound by rabbit anti-*E. coli* antibody: A) Green fluorescence image shown at 100× magnification; B) both red and green fluorescence images shown at 500× magnification.

3. Discussion

The adhesion of motile *E. coli* bacterial cells to PLL-modified MHA patterns likely occurs via an electrostatic interaction between negatively charged lipopolysaccharide (LPS) groups on the surface of bacterial cells and the positively charged PLL microarray. LPS groups, which reside in the external layer of the outer membrane, typically protrude from the outer shell of bacterial cell walls and appear to contribute significantly to the adhesion affinity of gram-negative bacterial cells to non-biological surfaces.[23, 33, 34] Based on control experiments, motile *E. coli* bacterial cells could also be directly attached to MHA patterns, although weakly, since the MHA surfaces are hydrophilic due to the carboxylate end groups. A plausible explanation for this interaction is that phosphates or acidic sugar groups of LPS, which impart a negative charge on the cell surface, are damaged.[33, 34] As a result, attractive electrostatic and hydrogen-bonding or van der Waals interactions dominate bacterial cell adhesion, however, hydrophobic interactions can not be entirely ruled out.[34]

The observed attachment of bacteria to surfaces does not occur exclusively via the cell wall. Optical monitoring studies have indicated that the flagella of motile bacterial cells can bind to surfaces.[35] Studies on the dynamics of bacterial-cell attachment show that flagella anchor to the surface while the body stays free and rotates around the flagella saddle point[35, 36] (see the movie in the Supporting Information). In addition, AFM images obtained on air-dried samples clearly show that the attachment of motile *E. coli* bacterial cells to PLL surfaces occurs via both their cell walls and flagella (Figure 2 C**2**).

AFM images of PLL–MHA-patterned areas bound by *E. coli* indicate that bacteria adopt a conformation that can be related to the pattern feature size and shape. By preparing dots with similar dimensions to motile bacterial cells (3 µm), single bacterial cells can be placed on a surface in predesigned patterns (Figure 5 A, B and C**5**).

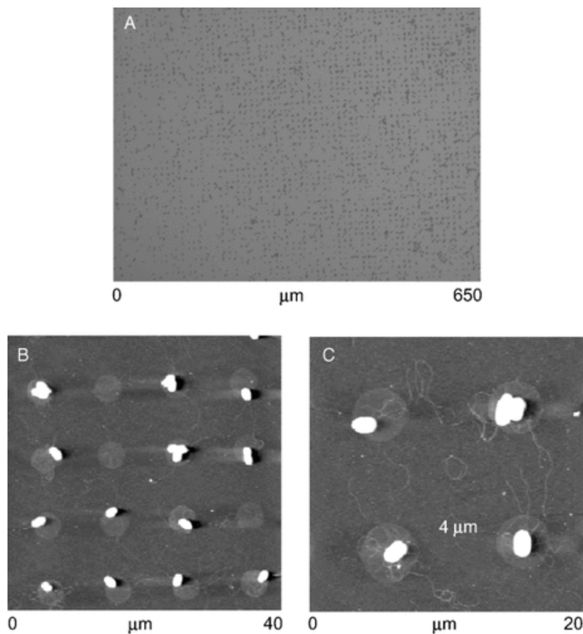


Figure 5 Optical (A) and AFM images (B, C) of single-cell bacterial attachment to MHA–PLL dot patterns.

In order to determine the minimum surface feature size able to bind an individual *E. coli* K-12 cell, a size-variable (1.0 to 3.0 μm via 0.1 μm steps) PLL–MHA dot array was prepared (Figure 6 A**6**). The PLL–MHA dot array was immersed in M9 media containing *E. coli* cells for ≈20 min. This substrate was washed in fresh M9 media followed by Milli-Q (18 MΩ) water. The motility of adsorbed bacterial cells on the size-variable PLL–MHA dot array was monitored with an optical microscope with the substrate immersed in a liquid cell containing fresh M9 media. The surface-adhered *E. coli* cells remained alive and motile for more than 4 h and bound only to PLL-modified dots that were at least 1.3 μm in diameter. These data indicate that the minimum surface feature size that effectively binds *E. coli* must be at least 1.3 μm (Figure 6 B and C**6**). Other bacterial cell types may prefer different surface feature sizes and this aspect is currently under investigation.

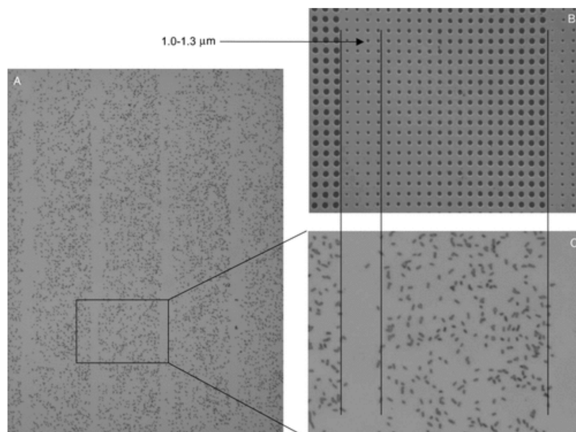


Figure 6 Bacterial attachment to a MHA–PLL dot array where the size of the features has been intentionally varied from 1 to 3 μm: (A) An optical microscopic image of bacterial adhesion to MHA–PLL dots patterned onto a gold substrate via microcontact printing; B) the silicon master used to fabricate the PDMS stamp; C) a high-resolution image of bacterial attachment to the dot array. Images B) and C) are shown at the same scale to define the lowest pattern size to which bacteria can adhere.

4. Conclusions

In conclusion, protocols for the fabrication of microarrays of motile *E. coli* on gold surfaces have been developed. Motile *E. coli* can easily be attached to predesigned functionalized surfaces, and bacteria binding can be controlled, so that a single bacterium can be attached to a single surface feature. Binding *E. coli* with its complementary antibody does not negatively affect the biological activity of cells, and more than 70 % of cells remain alive and motile for a minimum of 4 h after attachment to the surface. The best adhesion results for motile *E. coli* bacterial cells were obtained from PPL-functionalized patterns with a minimum spot size of 1.3 µm where the bare gold was passivated with PEG or MOU. However, a disadvantage is that PLL is not selective for a particular stain of *E. coli*.

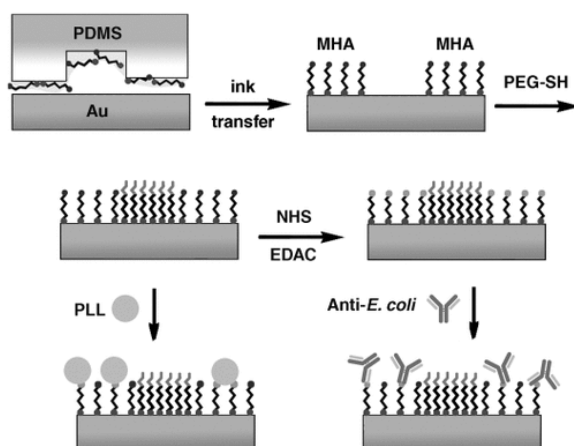
The ability to control surface adhesion of motile bacteria allows for the specific placement of microorganisms onto a surface in specific orientations that provides us with the unique opportunity to specifically design and prepare highly ordered nanoarrays of microorganisms on a surface that can potentially function as the power source for a biomotor.

5. Experimetal Section

Materials: All commercially acquired compounds and reagents were used as received. 16-Mercaptohexadecanoic acid (MHA), 11-mercapto-1-undecanol (MOU), and 1-octadecanethiol (ODT) were purchased from Aldrich (Milwaukee, WI). Poly-L-lysine hydrobromide (M_w=70,000–150,000) and protein A were purchased from Sigma (Milwaukee, WI). Affinity-purified polyclonal anti-*E. coli* antibodies (goat and rabbit) were purchased from Biodesign International (Saco, ME). The polyclonal antibody to *E. coli* lipopolysaccharide (LPS) type O128:b12 (rabbit) was purchased from Calbiochem (San Diego, CA). K-12 *E. coli* cultures were purchased from Carolina Biological Supply Company (Burlington, NC). 11-Mercaptoundecyl-penta(ethylene glycol) (PEG-SH) was provided by Professor Milan Mrksich at University of Chicago and was also synthesized in house.

Substrate preparation: Gold substrates were fabricated by thermal evaporation of a layer of gold (60 nm) onto a titanium (10 nm) coated silicon oxide wafer (8×12 mm^2). Si wafers (4 in, 475–575 µm thickness with a 500-nm thermal oxide layer, Wafernet, Inc. (San Jose, CA)) were cleaned with acetonitrile, ethanol, and Milli-Q water prior to use.

Microarray construction: MHA was patterned on surfaces via microcontact printing or through dip-pen nanolithography (DPN).[37] Poly(dimethylsiloxane) (PDMS) stamps were fabricated by casting an elastomeric polymer against photolithography-prepared silicon masters.[38]The silicon masters used in this experiment consisted of 2 µm lines spaced 2 µm apart and 3.0 µm holes with a separation 3.0 µm. The PDMS stamp surface was coated with a thin layer of the linker molecule by dropping a small amount of ink solution using a microliter pipette (Rainin (Oakland, CA)). After stamping, the substrate surface was allowed to dry in air for ≈5 min. Substrates patterned with MHA were soaked for ≈30 min in 10 mM ethanol solutions of PEG-SH or MOU to passivate unpatterned areas against bacterial adhesion. After passivation, the substrates were treated for 1 h in a 1:1 solution of *N*-hydroxysuccinimide (NHS; 0.2 M, Sigma) and 1-ethyl-3-(dimethylamino)propyl carbodiimide hydrochloride (EDAC; 0.1 M, Sigma) to crosslink the MHA-patterned area with incoming NH_2 groups of proteins. The surface of the activated stamp was then incubated with the anti-*E. coli* antibody (0.2 mg mL^{-1}) or with the anti-LPS antibody (1:100 dilution) for 2 h at room temperature. The wafer was then rinsed thoroughly with PBS followed by sterilized Milli-Q water. Poly-L-lysine was directly incubated with an MHA-patterned array for 30 min at room temperature. The schematic of surface modification is presented in Scheme 1.



Scheme 1 The surface modification protocol for attaching bacteria to a patterned gold substrate.

Bacteria cell preparation: *E. coli* K-12 cells were grown from a single colony in Luria–Bertani (LB) broth in a rotary shaker incubator at 37 °C and 225 rpm for 7–8 h. When the bacterial biomass reached an optical density (OD_{600}) of ≈0.8–1.0, bacteria were centrifuged at 4000 rpm for 20 min and resuspended in M9 media prepared from commercially available M9 minimal salts. The final bacterial concentration was approximately $1–2\times10^7$ cells mL^{-1}, which was determined by measuring the absorbance of the culture at 600 nm in a UV/Vis spectrophotometer (Agilent 8453; Palo Alto, CA) and by cellular counting methods using a Bright-Line hemacytometer (Fisher Scientific). Modified substrates were seeded with bacteria in M9 media for 20 min at 37 °C, rinsed with fresh M9 solution, Milli-Q water, and dried in air.

Cell viability assay: The LIVE/DEAD BacLight Bacterial Viability Kit (L-13152, Molecular Probes, Eugene, OR) was used to examine the viability of *E. coli* bacteria attached onto patterned microarrays. In this two-color assay, live bacteria fluoresce green and dead bacteria fluoresce red. The staining procedure was followed according to the protocol provided by Molecular Probes (MP07007). Bacteria on the patterned Au-coated substrates were incubated in a (2:2:1) solution of nucleic acid stains, SYTO 9 and propidium iodide, and PBS buffer (pH 7.4), respectively, for 15 min in the dark. The wafer was then rinsed with PBS followed by sterilized Milli-Q water twice and then allowed to air dry. The relative percentage of live *E. coli* on patterns was further analyzed by estimating the number of green and red cells under a fluorescence microscope (Zeiss Axiovert 100A, Thornwood, NY) equipped with a Zeiss bandpass filter set. The excitation/emission maxima for used dyes were about 480 nm/500 nm for the SYTO 9 stain and 490 nm/635 nm for propidium iodide.

Imaging: Fabricated microarrays were characterized by atomic force microscopy (AFM). Either a Veeco CP-Research or Nanoscope IV (Nanoman) microscope was employed to acquire topography, frictional force, and phase images. Optical images were obtained with a Zeiss Axiovert 100A inverted optical/fluorescence microscope (Thornwood, NY) equipped with a Penguin 600CL digital camera and StreamPix software.

Supporting Information

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38 The silicon masters used to make the PDMS stamp molds were manufactured at the University of Illinois at Urbana-Champaign in the Micro and Nanotech laboratory of Prof. Chang Liu.