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TITLE:

Patterning of Microorganisms and Microparticles Through Sequential Capillarity-assisted Assembly

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SUMMARY:

We present a technology that uses capillarity-assisted assembly in a microfluidic platform to pattern micro-sized objects suspended in a liquid, such as bacteria and colloids, into prescribed arrays on a polydimethylsiloxane substrate.

ABSTRACT:

Controlled patterning of microorganisms into defined spatial arrangements offers unique possibilities for a broad range of biological applications, including studies of microbial physiology and interactions. At the simplest level, accurate spatial patterning of microorganisms would enable reliable, long-term imaging of large numbers of individual cells and transform the ability to quantitatively study distance-dependent microbe-microbe interactions. More uniquely, coupling accurate spatial patterning and full control over environmental conditions, as offered by microfluidic technology, would provide a powerful and versatile platform for single-cell studies in microbial ecology.

This paper presents a microfluidic platform to produce versatile and user-defined patterns of microorganisms within a microfluidic channel, allowing complete optical access for long-term, high-throughput monitoring. This new microfluidic technology is based on capillarity-assisted particle assembly and exploits the capillary forces arising from the controlled motion of an evaporating suspension inside a microfluidic channel to deposit individual microsized objects in an array of traps microfabricated onto a polydimethylsiloxane (PDMS) substrate. Sequential depositions generate the desired spatial layout of single or multiple types of micro-sized objects, dictated solely by the geometry of the traps and the filling sequence.

The platform has been calibrated using colloidal particles of different dimensions and materials:

it has proven to be a powerful tool to generate diverse colloidal patterns and perform surface functionalization of trapped particles. Furthermore, the platform was tested on microbial cells, using *Escherichia coli* cells as a model bacterium. Thousands of individual cells were patterned on the surface, and their growth was monitored over time. In this platform, the coupling of single-cell deposition and microfluidic technology allows both geometric patterning of microorganisms and precise control of environmental conditions. It thus opens a window into the physiology of single microbes and the ecology of microbe-microbe interactions, as shown by preliminary experiments.

INTRODUCTION:

Spatial patterning of single microorganisms—particularly within experimental arenas that enable full control over environmental conditions, such as microfluidic devices—is highly desirable in a broad range of contexts. For example, arranging microorganisms into regular arrays would permit the accurate imaging of large numbers of individual cells and the study of their growth, physiology, gene expression in response to environmental stimuli, and drug susceptibility. It would also allow studying cell-cell interactions of particular interest in research into cellular communication (e.g., quorum sensing), cross-feeding (e.g., algal-bacterial symbiosis), or antagonism (e.g., allelopathy), with full control over the spatial localization of cells relative to each other. Cell physiology and evolution studies[1], cell-cell interaction studies[2], phenotypic differentiation screening[3], environmental monitoring[4], and drug screening[5] are among the fields that can greatly benefit from a technology able to achieve such quantitative single-cell analysis.

Several strategies to isolate and handle single cells have been proposed in recent years, from holographic optical traps[6] and heterogeneous surface functionalization methods[7-10] to single-cell chemostats[11] and droplet microfluidics[12]. These methods are either technically very demanding or affect cell physiology and fail to provide a high-throughput platform to pattern microbes that can be studied over long periods, ensuring single-cell resolution, full optical access, and control over environmental conditions. The goal of this paper is to describe a platform to pattern bacteria with micrometric precision into prescribed spatial arrangements on a PDMS surface through capillarity-assisted assembly. This platform allows precise and flexible spatial patterning of microbes and enables full optical access and control over environmental conditions, thanks to its microfluidic nature.

The technology behind this platform is an assembly technology developed in recent years, named sCAPA[13-15] (sequential capillarity-assisted particle assembly) that was integrated into a microfluidic platform[16]. The meniscus of an evaporating liquid droplet, while receding over a patterned polydimethylsiloxane (PDMS) substrate inside a microfluidic channel, exerts capillary forces that trap the individual colloidal particles suspended in the liquid into micrometric wells microfabricated on the substrate (**Figure 1A**). Suspended particles are first transported to the air-liquid interface by convective currents and then placed into the traps by capillarity. Capillary forces exerted by the moving meniscus act on a larger scale compared to forces involved in particle interactions.

Thus, the assembly mechanism is not influenced by the material, dimensions, and surface properties of the particles. Parameters such as particle concentration, the speed of the meniscus, temperature, and surface tension of the suspension are the only parameters that influence the yield of the patterning process. The reader can find a detailed description of the influence of the aforementioned parameters on the patterning process in [13-15]. In the original sCAPA technology[13-15], the colloidal patterning process was carried out in an open system and required a high-precision piezoelectric stage to drive the suspension across the template. This platform exploits a different strategy and allows the patterning to be carried out with standard equipment generally used in microfluidics in a controlled environment, thus minimizing the risks of contaminating the samples.

This microfluidic platform was first optimized on colloidal particles to create regular arrays of inert particles and then successfully applied to bacteria. Both microfluidic platforms are described in this paper (**Figure 1B,C**). Most of the preparatory steps and the experimental equipment described in the protocol are common for the two applications (**Figure 2**). We report colloidal patterning to demonstrate that the technique can be used to perform multiple sequential depositions on the same surface to create complex, multimaterial patterns. In particular, one single particle was deposited per trap for each step to form colloidal arrays with a specific geometry and composition, solely dictated by the traps' geometry and filling sequence. As for bacterial patterning, single depositions are described, resulting in one bacterium being deposited per trap. Once cells are patterned on the surface, the microfluidic channel is flushed with medium to promote bacterial growth, the preliminary step of any single-cell study.

PROTOCOL:

1. Silicon master preparation

NOTE: The PDMS templates bearing the microfabricated traps that form the template for colloidal and microbial patterning were fabricated according to the method introduced by Geissler et al.[17]. The silicon master was prepared by conventional lithography in a cleanroom. See the following steps for the procedure and the **Table of Materials** for the equipment.

1.1. Design the features using computer-aided design (CAD) software.

1.2. Prepare the chrome-glass mask with a layer of positive photoresist by exposing the designed features with a UV direct laser writer.

1.2.1. Develop the chrome-glass mask with a spin developer using a developer at 1:4 photoresist to water ratio for 15 s.

1.2.2. Immerse the mask in chromium etchant for 50 s.

1.2.3. Plasma-treat a 10 cm silicon wafer for 3 min at 600 W.

1.2.4. Vapor-deposit a layer of hexamethyldisilizane and bake at 110 °C to improve the adhesion toward the photoresist.

1.2.5. Deposit a layer of photoresist at 4,500 × *g* for 2 min.

1.2.6. Expose the feature to UV through the chrome-glass mask with a mask aligner for 2 s and develop with a developer as for the mask.

1.3. To complete the fabrication of the silicon master, etch the wafer via deep reactive ion exchange, adjusting the etching time (<2 min) to achieve the desired depth (measured with a profilometer).

2. Microchannel mold preparation

2.1. Design the channel with CAD software and slice it with a slicer software to convert the designed model into instructions for the 3D printer, setting the slicing distance at 0.05 mm.

2.2. Print the channel with a 3D printer for ~1 h.

2.3. Wash and postcure the mold in a dedicated curing and washing machine.

2.3.1. Put the mold in a container filled with pure isopropyl alcohol (IPA) and vortex the liquid (wash for 20 min). Take the container filled with IPA out of the machine.

2.3.2. Once the washed mold is removed from the IPA container, put it back into the washing and curing machine and postcure it for 15 min at 35 °C.

NOTE: The **Table of Materials** reports the 3D printer and the curing and washing machine used in the mold preparation protocol. The 3D model was sliced with the 3D printer's proprietary slicer software.

2.4. Place the print in a UV oven for 12 h and place it in an oven at 80 °C for 12 h.

NOTE: This step ensures that all the polymer is cured and all uncured polymer is removed from the print as it would prevent PDMS from curing.

2.5. Silanize the mold through vapor deposition of Trichloro (1H, 1H, 2H, 2H-perfluorooctyl) silane.

2.5.1. Place the 3D mold in a vacuum desiccator along with 20 µL of 100% concentrated Trichloro (1H, 1H, 2H, 2H-perfluorooctyl) silane pipetted on an aluminum foil placed close to the mold.

2.5.2. Create a vacuum in the desiccator to generate vapor and leave for 40 min.

2.5.3. Remove the mold from the desiccator and rinse it with pure ethanol before pouring PDMS on it.

3. Fabrication of the microfluidic chip

3.1. Prepare a PDMS mixture by mixing the elastomer with its crosslinking agent (**Table of Materials**). Stir the mixture vigorously to blend the two components uniformly until air bubbles are formed, and the PDMS mixture looks opaque.

NOTE: The amount of cross-linker should be 10% by weight of the amount of elastomer.

3.2. Degas the mixture of elastomer and crosslinking agent in a vacuum desiccator to remove the trapped air bubbles. Transfer the mixture in the desiccator to the container used for mixing (step 3.1). Continue the degassing process until all the bubbles have been removed and the mixture looks transparent again.

NOTE: The time typically required for desiccation is 30 min.

3.3. Pour 3 g of the mixture on the silicon master to produce the template, which will serve as the “floor” of the microfluidic chip.

NOTE: The thickness of the PDMS layer can be tuned by changing the amount of mixture poured on the silicon master.

3.3.1. To obtain a 400 µm-thick template, pour 3 g of PDMS on the silicon master, place the silicon master on a spin coater, and spin coat at 21 × *g* for 5 s and 54 × *g* for 10 s, and then degas again as described in step 3.2 to remove trapped air bubbles.

3.4. Pour 20 g of the PDMS mixture on the 3D-printed mold to make the microchannel, which will serve as the “roof” of the microfluidic chip, and degas it as described in step 3.2 for 30 min.

3.5. Bake both the silicon wafer and the 3D printed mold at 70 °C for at least 2 h.

3.6. Peel the PDMS layer off the 3D printed mold, cut the PDMS with a blade around the microchannels, and punch the holes that will serve as inlet and outlet of the microfluidic channel.

3.7. Peel the PDMS off the silicon master and cut the PDMS layer into smaller pieces with the same dimensions of the microfluidic channels that will be bonded on top of the templates.

3.8. Gently rub the templates and microchannels using a 1% detergent solution (see the **Table of Materials**) for 5 min and then rinse with deionized water. Next, rinse the templates and microchannels with isopropanol and rinse them with deionized water. Dry the templates and microchannels at room temperature for 1 min with compressed air at 1 bar.

3.9. Place the templates and the microchannels in a plasma cleaner with the surfaces to be bonded facing up. Turn on the plasma cleaner, and plasma treat the templates and the microchannels for 40 s. Take them out from the plasma cleaner and immediately bond the microchannels on top of the templates by putting them in contact with one another.

3.10. Store the microfluidic chips in an oven at 70 °C for five days to ensure PDMS hydrophobic recovery and have a receding contact angle within the optimal range, between 30 and 60°[10,11].

4. Bacterial patterning

4.1. One day prior to the experiment, grow a population of *Escherichia coli* (strain MG1655 prpsM-GFP). Inoculate the culture directly from the frozen stock and grow overnight for 20 h in lysogeny broth (LB) medium in a shaker incubator at 37 °C. Add 50 µg/mL of kanamycin for the cells to retain the prpsM-GFP plasmid.

4.2. On the day of the experiment, set the box incubator (**Figure 2A**) at 37 °C several hours before the experiment to have a uniform and stable temperature before starting the experiment. Set up the syringe pump and the heated glass plate on the microscope stage (**Figure 2B,C**), setting the temperature at the same temperature as the box incubator.

NOTE: The box incubator in which the entire system, including the microscope (**Figure 2E**), is enclosed ensures that a uniform and constant temperature is maintained throughout the entire experiment when the channel is flushed with medium.

4.3. Ninety minutes before the experiment, put the microfluidic chip in a vessel filled with 100% ethanol (EtOH) and flush the channel with 100% EtOH for at least 10 min. Place the microfluidic chip in a vacuum desiccator and degas for at least 30 min. Exchange the EtOH with distilled water and vacuum-treat the chip for at least 30 min. Put the microfluidic chip in the oven at 70 °C for 10 min to remove any traces of liquid left in the channel.

NOTE: This step is necessary to prevent bubble formation in the channel when flushed with culture medium. This bubble prevention protocol was adapted from Wang et al.[18].

4.4. Pipette 1 mL of 3-(*N*-morpholino)propanesulfonic acid (MOPS) medium (1x) into a centrifuge vial and add 10 µL of 0.132 M potassium phosphate (K_2HPO_4). Take the overnight culture out of the 37 °C incubator and aliquot 100 µL into the centrifuge vial and centrifuge the culture at 2,300 × *g* for 2 min. Gently pipette the supernatant out of the centrifuge vials, and resuspend the pellet in 1 mL of fresh MOPS medium with 0.015% v/v of Tween 20 and 0.01% v/v of potassium phosphate.

NOTE: The typical concentration of the bacterial suspension is in the range of 0.015–0.15 vol%, which ensures the formation of an extended and mobile accumulation zone and prevents the accumulation of particles on the substrate. Replacing the overnight medium with fresh medium

minimizes the risks of releasing films of bacteria on the template. The lack of carbon source in the fresh medium prevents cells from growing in the suspension during template patterning. A concentration of 0.015% v/v of Tween 20 in the suspension is necessary for the contact angle of the receding liquid on the PDMS template to fall within the optimal range, between 30 and 60°.

4.5. Load the bacterial suspension in a 1 mL syringe and connect the syringe to the chip through microfluidic tubing.

4.5.1. To secure the connection between the syringe and the tubing, directly insert a needle with an outer diameter of 0.6 mm into the tubing. To avoid scattering any suspension remaining in the inlet vicinity across the channel, flush fresh medium through the hole used as an outlet during the patterning process (**Figure 3A**–III), rather than the hole used as the inlet.

4.5.2. Mount the syringe on the syringe pump and inject the suspension into the microfluidic chip through the inlet located at the upstream part of the channel until the suspension covers the template region with traps.

NOTE: During the liquid injection process, air can escape through an outlet located at the downstream end of the channel.

4.5.3. Set the syringe pump to withdraw the bacterial suspension (**Figure 3A**–I) at a flow rate of 0.07–0.2 µL/min, which in the described geometry corresponds to a meniscus receding speed of 80–100 µm/min.

4.5.4. Monitor the patterning process via microscope software.

NOTE: Here, a 10]x magnification was used to monitor the receding meniscus on the template, and a 20x magnification was used to monitor the deposition of individual bacteria into the microfabricated traps.

4.6. Once the template has been patterned with cells (**Figure 3A**–II), increase the withdrawal flow rate to quickly empty the microfluidic channel and flush it with fresh LB that was previously degassed for at least 30 min and prewarmed at 30 °C.

4.7. Set the syringe pump at a flow rate of 2 µL/min to gently flush the channel. Once the channel has been filled, increase the flow rate (15 µL/min) according to the specific experimental needs.

4.8. Acquire images of growing bacteria at the desired magnification and time interval.

5. Colloidal patterning

5.1. Pipette 900 µL of a 0.015% v/v Tween 20 aqueous solution into a centrifuge vial and pipette 100 µL of the original colloidal suspension into it. Centrifuge the suspension at 13,500 ×

g for 1 min. Gently remove the supernatant and replace it with the aqueous Tween 20 (0.015% v/v) solution. Repeat this process three times to ensure complete replacement of the supplier's solvent from the stock suspension.

5.2. Load the colloidal suspension in a 1 mL syringe and connect the syringe to the chip through microfluidic tubing. Inject the suspension into the microfluidic chip through the inlet located within the central section, at the upstream part of the channel, and gradually push the suspension until the template is covered.

5.3. Withdraw the colloidal suspension at a flow rate of 0.07–0.2 µL/min, which corresponds to a meniscus receding speed of 1–2 µm/min, and image the patterning process via microscope software. Use 10x magnification to monitor the receding meniscus on the template and 20x magnification to observe the deposition of individual particles into the microfabricated traps. Increase the flow rate once the meniscus reaches the end of the template to empty the channel quickly.

NOTE: Colloidal arrays composed of several particles can be produced by running the patterning process from steps 5.1 to 5.3 in series. The channel is loaded with a new colloidal suspension at each iteration according to the desired colloidal arrays' composition. Each patterning step adds one particle to the colloidal array and can be run sequentially until the traps have been filled with the desired particles' sequence. The composition of the resulting colloidal arrays is solely given by the sequence of colloidal suspensions used to fill the traps (**Figure 3B**).

REPRESENTATIVE RESULTS:

A microfluidic platform that exploits capillarity-assisted assembly to pattern colloidal particles and bacteria into traps microfabricated on a PDMS template was developed. Two different channel geometries have been designed to optimize the patterning of colloids and bacteria through the capillarity-assisted assembly. The first channel geometry (**Figure 1B**) consists of three 23 mm long parallel sections with no physical barrier between them. The two sections on the sides are 5 mm wide and 1 mm high, while the central section is 7 mm wide and 500 µm high. This design helps maintain a well-defined moving droplet with a receding convex-shaped meniscus. The working principle of this platform is described in detail by Pioli et al.[11]. If the experiment requires filling the lateral channels, as in the case of bacteria culturing, air pockets are usually formed. For this reason, we designed and tested a second geometry that simplifies the filling process when the channel is flushed with medium. In this case, the platform (**Figure 1C**) consists of a single 20 mm long, 3 mm wide, and 500 µm high straight channel.

The temperature in the microfluidic channel is an important parameter to ensure an efficient deposition process. It must be maintained 15 °C above the dew point of water to avoid condensation on the template. The heated glass plate underneath the channel (**Figure 2D**) ensures a uniform temperature across the template to prevent condensation during the patterning process in the region close to the liquid-air interface, characterized by a high vapor concentration in the air. The temperature of the heated glass plate must be the same as that of the box incubator to avoid condensation on the template.

The straight channel geometry was exploited to pattern stationary-phased cells (**Figure 4A**) of a fluorescent *E. coli* strain (MG1655 prpsM-GFP). Bacteria were deposited in 83% of the 5,000 analyzed traps. The cells were first placed in the traps according to the presented protocol and subsequently grown for 4 h at 37 °C in LB flushed at 10 mm/min. Patterned bacteria resumed growth at different times within 1.5 h from when the channel was filled with fresh LB (**Figure 4B–**I), with the median at 44 min. Once growth is resumed, single bacterial cells start forming individual colonies, which expand (**Figure 4B–**II) until a surface layer is formed (3.5 h) and single-cell resolution is lost (**Figure 4B–**III).

This proof-of-concept experiment shows that capillarity-assisted assembly in a microfluidic channel can be used to pattern a surface with thousands of viable single-bacteria cells. Eleven replicates show that patterned cells grew in 45.5% of cases within a 7 h window. Four tests conducted adding propidium iodide (PI), a live-dead stain[19], to the fresh LB flushed into the channel after the deposition proved that non-growing, patterned bacteria did not get stained. As PI binds to DNA but cannot penetrate cells with an intact membrane, the PI staining experiment shows that neither the patterning process nor the desiccation and rehydration processes damaged the cells' membrane.

The three-section channel geometry was used to produce linear colloidal arrays with different compositions by performing sequential patterning of colloidal particles. **Figure 5** shows the different colloidal arrays formed through sequential patterning, including dimers (**Figure 5A,B**) and trimers (**Figure 5C**), containing two and three sequentially trapped particles, respectively. Green and red fluorescent polystyrene particles were used to assemble both dimers and trimers. Analysis conducted over 55,000 traps show that green-red (G-R) dimers (**Figure 5A,B**) made by particles with 2 µm and 1 µm in diameter were formed in 93% and 89% of analyzed traps, respectively. Green-red-green (G-R-G) trimers (**Figure 5C**) were formed in 52% of the 55,000 analyzed traps[11]. Dimers and trimers were assembled by running sequential depositions, with the colloidal suspensions moving in the same direction across all sequential depositions (**Figure 3B**). As a result, particles trapped in each deposition step are in direct contact with those trapped in the previous one.

Precise positioning of particles does not require direct contact between deposited particles. The distance between patterned particles can be precisely controlled by performing two depositions in opposite directions, thus trapping such particles at the opposite ends of each trap (**Figure 5D**). The distance between the trapped particles can be tuned by designing traps with the desired length. An additional possibility offered by the platform is chemical patterning of the surface with micrometric precision. This result can be achieved by patterning the template with chemically functionalized particles[11].

FIGURE AND TABLE LEGENDS:

Figure 1: Microfluidic channels' geometries and patterning process. (**A**) Schematic of a side-view section of the microfluidic channel during patterning of colloidal particles. The liquid suspension

evaporates inside the microfluidic channel, and convective currents transport suspended particles toward the air-liquid interface. Particles are thus accumulated and form the accumulation zone. The syringe pump pulls the liquid suspension, prompting it to recede, and individual particles get trapped during the liquid recession on the template. The arrow represents the direction in which the suspension is moving. (**B**) Schematic of the channel geometry used to pattern colloidal particles on the PDMS template. The channel is 23 mm long and 17 mm wide and consists of three sections: a central one that is 7 mm wide and two lateral ones that are 5 mm wide. The template is on the channel's floor, within the central section. The cross-section shows that the central section of the microfluidic channel, where the liquid suspension is confined throughout the patterning process, is the shallowest part of the channel and is 500 µm high, while the two lateral sections are both 1 mm high. (**C**) Schematic of the straight channel geometry used to pattern bacteria. The microfluidic device consists of a 20 mm-long, 3 mm-wide, and 500 µm-high straight channel. The rectangular section of this microfluidic device, shown in the cross-section, simplifies the filling process of the channel with medium. The SEM image shows a small portion of the PDMS template with 2 µm-long, 1 µm-wide, and 500 nm-deep traps microfabricated on it. Scale bar = 2 µm. Abbreviations: PDMS = polydimethylsiloxane; SEM = scanning electron microscopy.

Figure 2: Schematic of the platform for sequential capillarity-assisted assembly in a microfluidic channel. (**A**) Box incubator. The box incubator maintains a uniform and constant temperature (here, 30 °C) inside the microfluidic channel. (**B**) Syringe loaded with liquid suspension. The syringe is controlled by a syringe pump (not shown) and is used to control the motion of the liquid during the patterning process. (**C**) Heated glass plate. The heated glass plate is placed underneath the microfluidic channel and ensures a uniform temperature (here, 30 °C) across the template, which is critical to avoid condensation in the vicinity of the air-liquid interface. (**D**) Microfluidic chip loaded with a liquid suspension. The microfluidic chip's floor bears the traps in which bacteria and colloids get patterned during the patterning process. (**E**) Microscope. The heated glass plate and the microfluidic chip are placed on a microscope stage, granting full optical access during the patterning process and all the following steps.

Figure 3: Schematic of the steps involved in bacterial and colloidal patterning. Each panel shows an inner view of the microfluidic channel and only includes a small portion of the PDMS template. (**A**) Patterning of bacteria through capillarity-assisted assembly. (I) The bacterial suspension evaporates inside the microfluidic channel, causing convective currents to transport suspended bacteria to the air-liquid interface, thus forming the accumulation zone. Meanwhile, the suspension is pulled by the syringe pump and recedes on the template. The arrow represents the direction in which the bacterial suspension is moving. The receding suspension sweeps across the template, and individual cells get deposited into the microfabricated traps. (II) Deposited bacteria are exposed to air until the channel is flushed with fresh medium. The deposition process is over when the liquid suspension reaches the end of the template. (III) The microfluidic channel is flushed with fresh medium (i.e., LB). The arrow represents the direction in which the fresh medium is flushed. (**B**) Patterning of colloidal particles through sequential capillarity-assisted assembly. (I) The colloidal suspension recedes on the template while evaporating, and particles accumulate at the air-liquid interface, forming the accumulation zone. Individual particles are

deposited into the traps microfabricated on the template. The arrow represents the direction in which the colloidal suspension is moving. (II) The deposition process is complete when the liquid suspension reaches the end of the template. (III) A second deposition is run in the same direction as the first deposition to place a second particle into each trap on the template. (IV) Once the second deposition is over, the template is patterned with dimers of one green and one red particle. Abbreviations: PDMS = polydimethylsiloxane; LB = Lysogen broth.

Figure 4: PDMS template patterned with *E. coli* (strain MG1655 prpsM-GFP) cells. (**A**) SEM image of individual *E. coli* cells trapped in 2 µm-long, 1 µm-wide, and 500 nm-deep traps. Cells were trapped after one single deposition. Scale bar = 2 µm. (**B**) Epifluorescence images of a small portion of the PDMS template (approximately 80 µm x 80 µm) with trapped cells of *E. coli* after the microfluidic channel is filled with culture medium (Lysogen broth) at an initial speed of 1.3 mm/min, which is then increased to 10 mm/min. Trapped cells grow and divide multiple times for 4 h, eventually merging with cells from neighboring traps and covering the surface. Scale bars = 10 µm. Abbreviations: PDMS = polydimethylsiloxane; GFP = green fluorescent protein.

Figure 5: Colloidal clusters assembled through sequential capillarity-assisted particle assembly in the microfluidic platform (first channel geometry). Epifluorescence microscopy image of (**A**) 15 dimers assembled from polystyrene particles with 2 µm in diameter with two sequential depositions. (**B**) Dimers (n = 15) assembled from polystyrene particles with 1 µm in diameter with two sequential depositions. (**C**) Trimers (n = 15) assembled from polystyrene particles with 1 µm in diameter with three sequential depositions. (**D**) Traps (n = 15) with particles patterned at the extremities of each trap by running two sequential depositions in opposite directions. The distance between particles in a trap is 2 µm. Scale bars = 4 µm.

DISCUSSION:

The microfluidic platform described here allows the patterning of micro-sized objects, such as colloids and bacteria, into prescribed spatial arrangements on a PDMS substrate. The full control over environmental conditions offered by microfluidics and the ability to pattern cells with micrometric precision granted by sCAPA technology makes it a very promising platform for future physiology and ecology studies.

In the experiments presented in this work, the silicon master was realized using the photoresist reported in the **Table of Materials**. However, any photoresist suited to produce features in the size range described and usable with PDMS molding could be used. The same applies to the 3D printing resin used to prepare the microchannel mold. Any resin capable of producing features of the described dimension and compatible with PDMS molding could be used.

In this protocol, storing the microfluidic chips at 70 °C for 5 days optimizes the surface hydrophilicity (contact angle falling between 30° and 60°) when Tween 20 is added to the bacterial or colloidal suspension. This procedure ensures the best reproducibility for the receding contact angle under these experimental conditions; however, other storing times and temperatures may work as well.

Two applications of this microfluidic technique have been presented: 1) bacterial patterning

involving individual depositions of bacterial cells into the traps on the PDMS substrate, and 2) colloidal patterning involving sequential depositions of colloids, obtaining colloidal arrays of different compositions. This platform has been fully optimized to ensure high yields of bacterial deposition, with thousands of cells patterned on the PDMS template. The combination of capillarity-assisted assembly and microfluidics ensures single-cell resolution and stable flow of nutrients for several hours, with full optical access throughout the process. The patterned cells are capable of resuming growth once the microfluidic channel is flushed with fresh medium, although the viability of the patterned cells still needs to be optimized.

Currently, the major limitation of this technique is the lack of growth of patterned cells in 54.5% of experiments within a 7 h window. This aspect may be related to the specific bacterial species and needs to be further investigated to determine the root cause of the stress that prevents cells from regrowing. Air exposure and the force exerted by the meniscus during the patterning process may be among the major contributors to bacterial stress. Several possible solutions can be implemented to reduce such sources of stress, including the use of deeper traps to reduce the force applied on cells by the meniscus during the patterning process. An additional option to reduce the stress associated with air exposure would be to decrease the temperature of the heated glass plate. A temperature of 30 °C ensures a sufficient evaporation rate of the liquid suspension during the patterning process and would reduce the heat bacteria are exposed to after they are patterned on the template before medium is flushed.

Once fully optimized, we envision that this platform will be used in a variety of biological studies involving quantitative single-cell analysis. The high-throughput nature of the technique allows patterning of thousands of cells, providing large statistics within the same experimental arena. Full optical access enables tracking the behavior, growth, and molecular activities of large numbers of single microorganisms over time, thus providing major advantages in the study of phenotypic heterogeneity under homogeneous environmental conditions. The microfluidic nature of this platform ensures full controllability over environmental conditions such as media composition, flow rate, and temperature, just to name a few.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

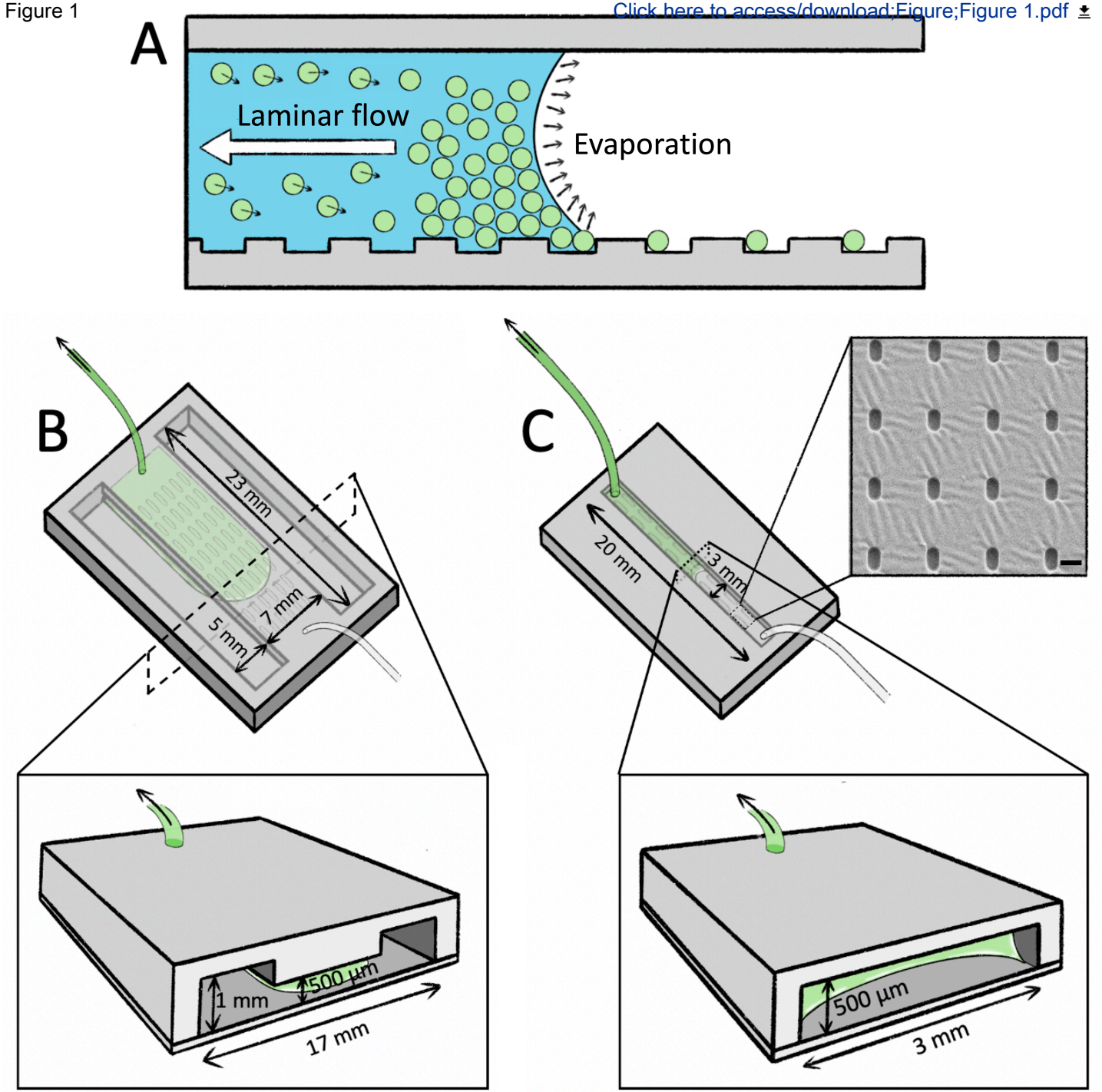


Figure 2

Click here to access/download;Figure;Figure 2.pdf

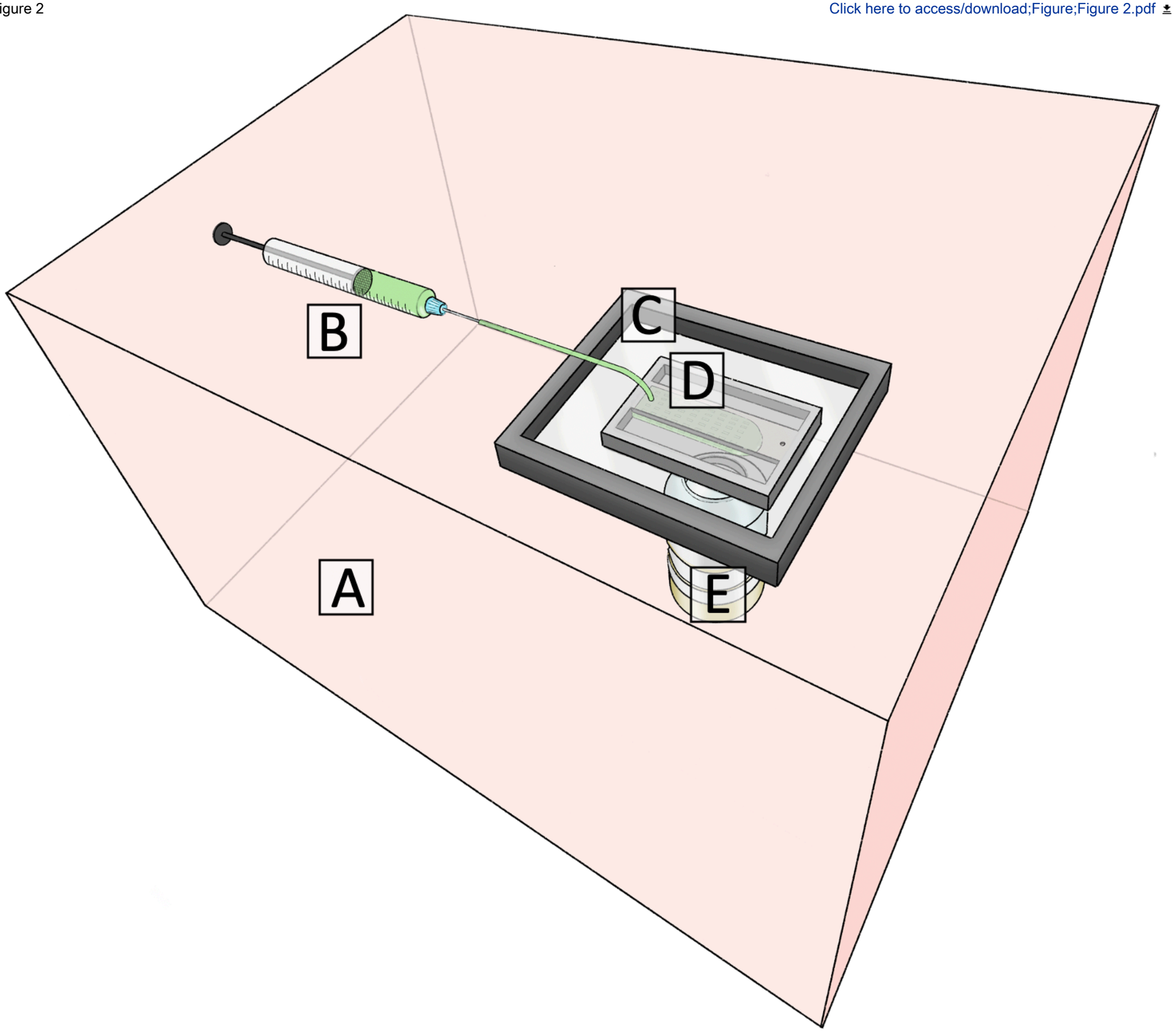


Figure 3

Click here to access/download;Figure;Figure 3.pdf

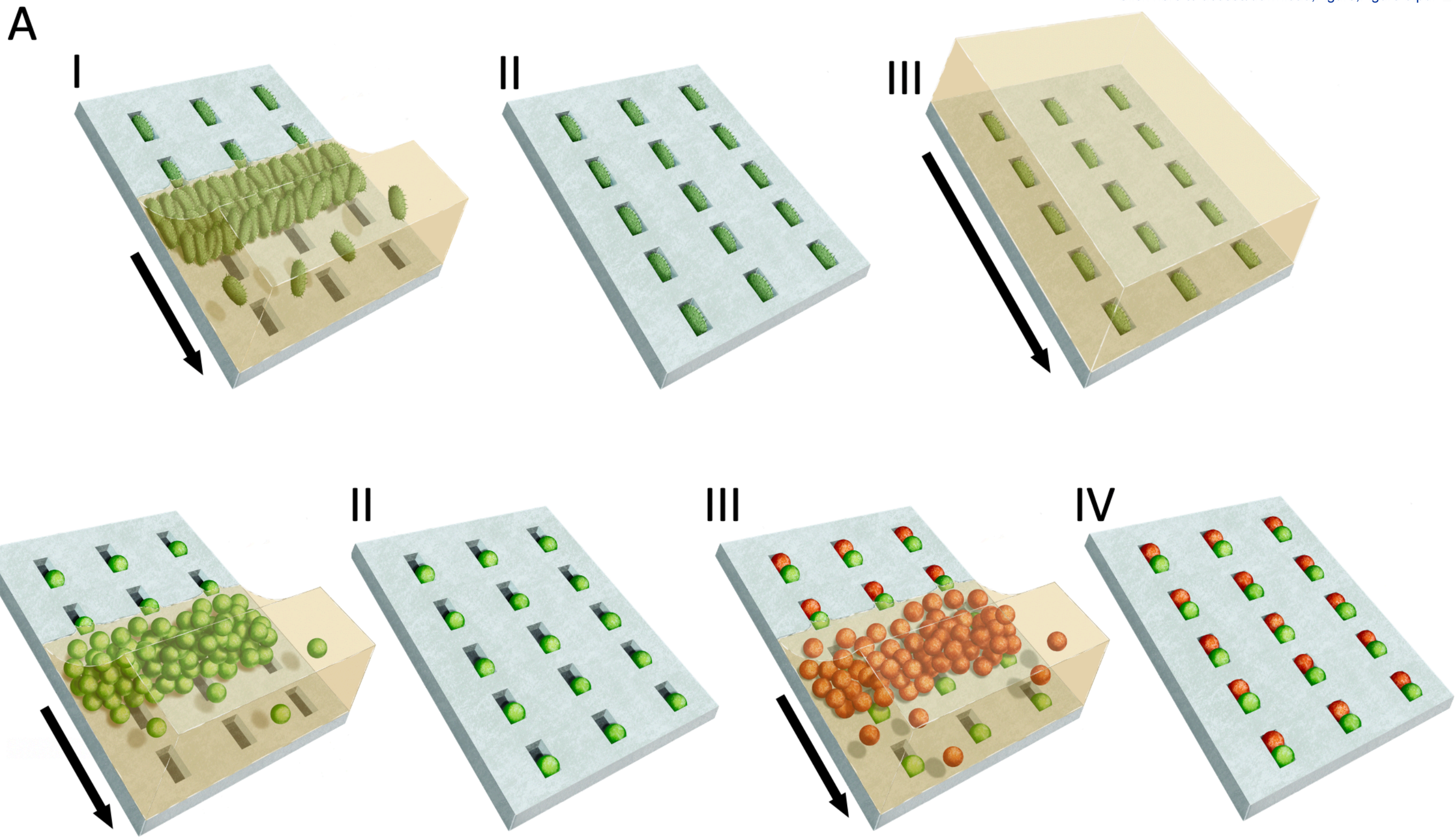


Figure 4

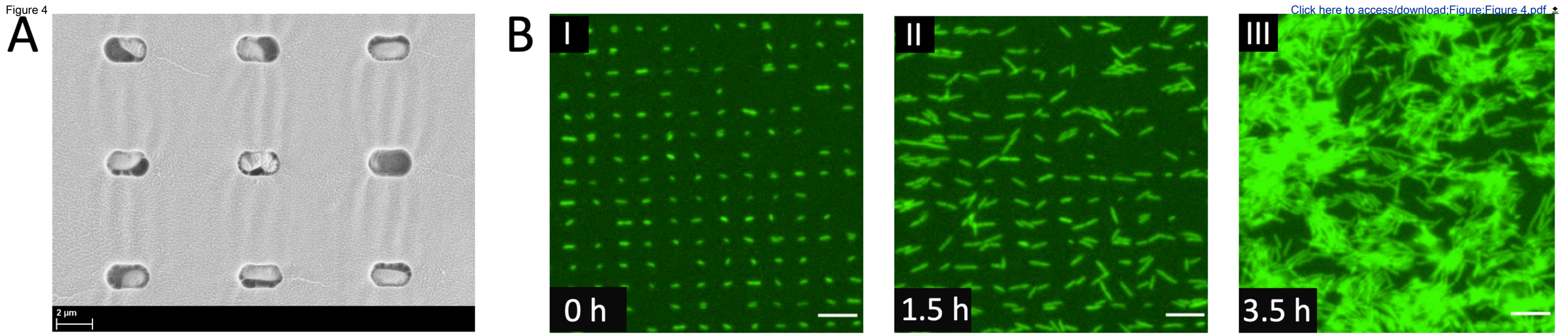
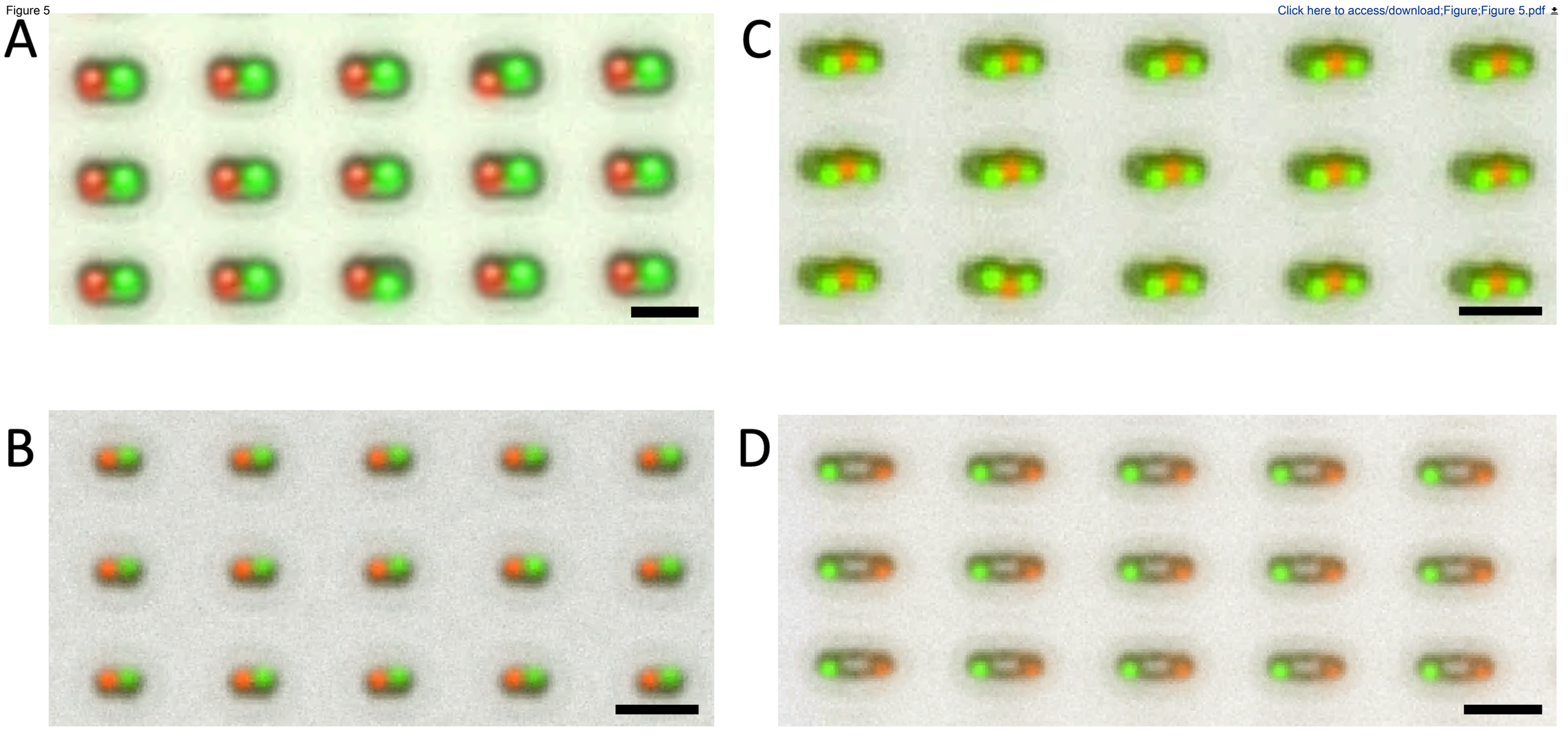


Figure 5



Reply to editorial comments.

[Reviewer and editorial comments are included in italics. Changes in the Manuscript and in the Supplementary information are reported in red.]

Editorial changes

We thank the editor for the numerous comments, which we address in detail below. We would like to stress that the sections we would like to film are:
Step 3: Fabrication of the Microfluidic Chip
Step 4: Bacterial Patterning
Step 5: Colloidal Patterning.

Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript was thoroughly proofread and no spelling or grammar issue was found.

2. Please revise the following lines to avoid previously published work: 38-42, 83-84, 106-108,120-124, 141-144, 161-163, 189-190, 217-220, 242-243. Please refer to the iThenticate report attached.

We have carefully checked the data reported in the iThenticate report. It is shown that 504 words are in common with our group's website (stockerlab.ethz.ch) where only a brief description (less than 50 words) of our Lab on a Chip paper (ref 16, *Pioli et al.*) is reported. Since we could not understand where the repetition came from, we did not edit this part. If edits are needed, we will ask for further guidance. Regarding the 102 words that are in common with the Lab on a Chip paper, they include grant numbers, affiliations and nouns, which we did not change. We revised, whenever possible, the related scientific part.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

The text was revised and any personal pronoun and adjective were removed from the protocol. Personal pronouns and adjectives were left in the discussion section in compliance with other papers published in JoVe.(i.e. Carrara, F., Brumley, D.R., Hein, A.M., Yawata, Y., Salek, M.M., Lee, K.S., Sliwerska, E., Levin, S.A., Stocker, R. Generating Controlled, Dynamic Chemical Landscapes to Study Microbial Behavior. *J. Vis. Exp.* (155), e60589, doi:10.3791/60589 (2020)).

4. Please define all abbreviations upon first use. For example, PDMS, MOPS, etc.

The manuscript has been thoroughly proofread and all abbreviations have now been defined upon first used.

5. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial

products should be sufficiently referenced in the Table of Materials. For example: AZ400K developer, etc.

Commercial names such as the AZ400K developer were removed from the manuscript.

6. Please provide an appropriate citation(s), wherever applicable, for the following lines: 54-64, 86-87

Citations have now been added to both sections.

7. Use SI units as much as possible and abbreviate all units: L, mL, µL, cm, kg, etc. Use h, min, s, for hour, minute, second. Please use centrifugal force (x g) for centrifugation speed.

We replaced all centrifugation speeds with centrifugation forces.

8. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Readers of all levels of experience and expertise should be able to follow your protocol.

9. Step 1.2, 1.3, 1.5, 1.6,1.7,1.9: How were these steps done? Please remember that our scripts are directly derived from the protocol text. Please include all actions associated with each step.

The silicon master preparation step will not be included in the filming script as it is a well-established method and is not unique to this manuscript, nor this is the only method that can be used to print microscopic features. Our protocol is resumed from a paper (*Geissler et al*), which was cited in the introductory section to the silicon master preparation step to provide details and further information to the readers. We could also directly refer to *Geissler et al* and avoid including details on the protocol to increase the consistency between scripts and protocol text.

10. Step 1.4: Does chrome etchant refer to chromium etchant. If yes, please use chromium etchant instead of chrome etchant. Also, please provide all the steps associated which describe how this was done.

Yes, chrome etchant refers to chromium etchant and has now been changed.

11. Step 2.1, 2.2: How was the channel designed, sliced, and printed? If this step needs to be filmed, please make sure to provide all the details such as "click this", "select that", "observe this", etc. Please mention all the steps that are necessary to execute the action item. If using long scripts, please include them as a supplementary file.

The microchannel mold preparation will not be included in the filming script as it is a standard 3D printing protocol. We have provided additional details to increase the reproducibility, but we did not go into the level of details needed for the filmed protocol.

12. Step 2.3: How was the washing of the printed channel done? What was used for washing? How long was the washing step? Please provide all steps associated.

We provided additional information on the washing step in the step 2.3 of the Microchannel Mold Preparation protocol.

13. Step 2.5: What was the concentration of silane used? How was the vapor deposition done? What was the concentration of ethanol used for rinsing? Is vapor deposition done using any established method? If so, consider adding a citation.

We have added details for the process at step 2.5 in the manuscript.

14. Step 3.2, 3.3,3.4: How was degassing and spin coating of PDMS done? Please provide all associated steps.

We have added details for the process at steps 3.2, 3.3 and 3.4 in the manuscript.

15. Step 3.8: What was the concentration of soap and isopropanol used? For how long were the samples dried and at what temperature? What was the pressure of the compressed air used?

We have added details in step 3.8.

16. Step 3.9: How was the plasma treatment and bonding done?

We have added details in step 3.9.

17. Step 4.4: What is the concentration of potassium phosphate? What was the volume of MOPS added for re-suspending the pellet?

We have added both information to step 4.4.

18. Step 4.5: How was a secure connection ensured between the syringe and the tubing?

We have added this information in step 4.5.

19. Step 4.7: What was the flow rate used in this study, how was it determined? How were the images acquired? Please elaborate on the steps for acquiring images. If this step needs to be filmed, please make sure to provide all the details such as "click this", "select that", "observe this", etc.

The flow rate used in the reported experiment (shown in Figure 4) was 15 µl/min. We have added this information to step 4.7. We note that this is the flowrate used in our study but it is by no means necessary to specifically use this flow rate. The flowrate was set via the pump controlling software. Images were taken via microscope software (NIS Elements). This step does not need to be filmed since it is not relevant to the patterning process itself or to the growth.

20. Step 5.3, NOTE: Do the author mean steps 5.1 to 5.3? If yes, please correct this.

Yes, thanks for raising the issue. We corrected the steps numbers.

21. Please provide all the actions steps associated, so that a reader may replicate your

analysis including buttons clicked, inputs, screenshots, command lines, etc. Please keep in mind that software steps without a graphical user interface (GUI) cannot be filmed.

There is no software that needs to be filmed for the analysis, so we did not add further details.

22. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

We have revised the use of notes according to the instructions and moved some to the discussion.

23. Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have highlighted the essential steps of the protocol for the video in yellow in the manuscript.

24. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

We have removed the figures from the Manuscript and left the figure title and the caption.

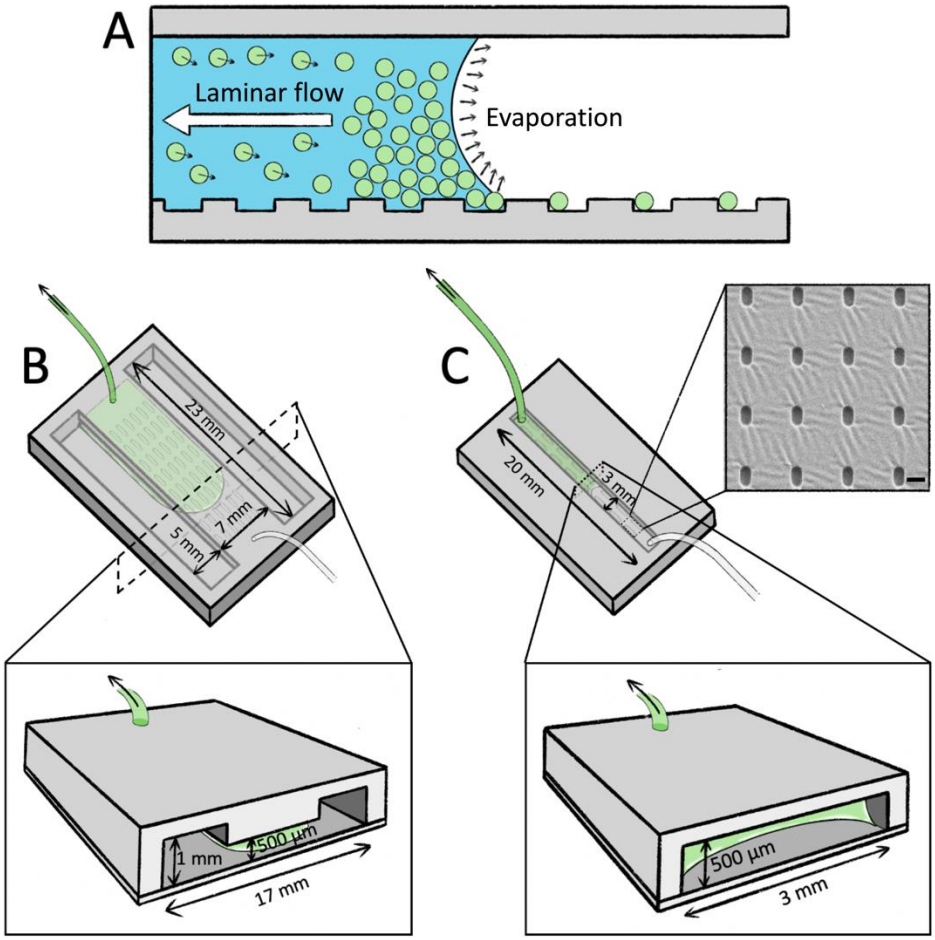
25. As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

26. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

We removed trademark and registered symbols from the Table of Equipment and Materials.

27. Figure 1: Please label the figure to make it more informative.

We added labels indicating evaporation and laminar flow in Figure 1. The figure is reported below.



Reply to Reviewer #1

The paper by Pioli et al describes a protocol for micropatterning of colloids or micro-organisms through capillary assembly.

The protocol is based on a previously developed and tested method (termed sCAPA) but has been improved and made more robust. The methodology uses a micropatternd PDMS surface integrated in a microfluidic channel. When a meniscus of a particle loaden liquid is receding over the pattern the microparticles are deposited into the surface wells. Sequential assembly permits to deposit for example two different particles into each well. The proposed protocol is very versatile as the microparticle properties play a negligible role in the deposition process. This methodology is very appealing as it enables not only full control of the geometric pattern, but subsequently also control over the environment and can as such be used for the investigation of the physiology of single microbes or microbe-microbe interactions.

The protocol is well described, and first experiments demonstrate its feasibility and robustness for passive colloids as well as micro-organisms. The prepared figures are clear and visually appealing.

I am convinced this methodology will be very useful for other groups working on colloidal

science, microbiology or biomedical applications. I thus think that this submission is a very good fit for JoVE and I recommend its acceptance.

We thank the reviewer for the appreciation of our work.

Reply to Reviewer #2

In this work, Pioli et al describe a microfluidic system to pattern micron-scale objects (bacteria or colloidal particles) using capillary-assisted deposition of single objects onto traps. The approach results in very interesting patterns of different configurations that could be used for a variety of studies of individual behavior or cell-cell or object interaction. This clever idea has been previously used by the authors, and sharing details on the approach is important, as this seems like a platform with wide applicability. The manuscript is well described and easy to follow, and the level of detail is enough to enable others to reproduce it, for the most part. I recommend this manuscript for publishing, although clarification of a few points is recommended.

Major Concerns:
1) Details regarding the scaling of the trap relative to the cell or particle of interest are not provided. Is there an optimal size matching? For example: would a 5 um trap work with a 1 um particle, or would the opposite be true?

We thank the Reviewer for the positive feedback on our work. The patterning mechanism is described in detail in the cited papers (Refs 13 – 16 in the manuscript). We have added a sentence to inform the reader on where to find a detailed discussion on the patterning parameters. The patterning process is based on a purely geometric effect, where if the particle's radius is in the order of magnitude of the trap's depth, due to an interplay of forces between the particles and the receding meniscus, one particle is trapped per trap. In the scenario described by the reviewer, i.e. 5 µm trap with a 1 µm particle, the outcome would depend on the depth of the trap in question. In case of a 500 nm deep trap, one single particle would be trapped. The results presented in the paper focus on this scenario, where the depth of the trap is equal to the radius of the particle.

2) Is the use of a positive photoresist necessary? I imagine this could be performed with other photoresists as long as it can be used for PDMS molding?

The Reviewer is right. Any photoresist suited to produce features in the size range described and usable with PDMS molding could be used. The replication with PDMS requires a negative of the template in the form of a photoresist mold (or of any other material).

3) Overnight UV and 80C treatment are necessary for the 3D mold. I imagine this is specific to the material the authors used for 3D printing, but there is a wide variety of available printing materials. Can the authors provide information on which material(s) work? And which one needs the UV/80C treatment?

The resin we used to print the mold is Prusa resin, Tough for 3D Printing, Prusa, Azure Blue. We have added this information and a detailed description of the curing process to the manuscript. The resin was chosen because is the one routinely used in our lab, but any resin

capable of producing features of the described dimension and compatible with PDMS molding could be used.

4) Can the authors clarify why two different devices are needed? Should both be able to accomplish the same purpose?

Both devices can be used to successfully pattern particles and bacteria on the PDMS template. The second geometry consists of a straight channel, which is easier to fill when flushed with culture media without forming bubbles and air pockets. Since bacterial patterning requires flushing the channel after bacteria have been successfully patterned, the second device is preferred to the first since it is easier to fill with media. We now discuss this aspect at the beginning of the Results section.

5) What bacterial/particle concentration is needed? Does this affect patterning?

Thanks for raising the point. We have now commented on this point in the manuscript (step 4.4). The typical concentration range used in the literature is between 0.1 and 1 vol% for sub-micron particles and from 0.0002 to 0.05 vol% for sub-100 nm particles (see ref 13: Ni, S., Isa, L., Wolf, H. Capillary assembly as a tool for the heterogeneous integration of micro- and nanoscale objects. *Soft Matter*. **14** (16), 2978-2995 (2018)). This concentration range ensures that an extended accumulation zone can be created without the aggregation of particles onto the substrate and away from the meniscus, which may hinder the controlled motion of the latter.
The same is true for bacteria. We have now added a comment in step 4.4.

6) Regarding lack of growth, did the authors test cell viability after patterning? I know this might be out of the scope of this work, but if it is known, it would be useful to state it.

We have tested cell viability by injecting LB with the addition of propidium iodide (PI), a dead-life staining agent. PI binds to DNA, but cannot penetrate into cells with an intact membrane. Consequently, cells that uptake PI and become fluorescent are dead, while alive cells with an intact membrane will not be stained. Non-growing patterned cells did not get stained by PI, showing that neither the patterning process nor the desiccation and rehydration processes damaged the cells' membranes. We discuss this aspect in the Results section.

Minor Concerns:
1) It is unclear why the PDMS chips need to be stored at 70C for 5 days (3.10), if the system is treated afterwards with etOH (for bubble avoidance) and then with TWEEN20. Is the 5-day treatment really necessary?

We found that storing the device at 70°C for 5 days helps ensuring the optimal surface hydrophilicity (contact angle falling between 30° and 60°) when TWEEN 20 is added to the bacterial or colloidal suspension. We found this ensures the best reproducibility for the receding contact angle under our experimental conditions, but we cannot exclude that different procedures may work as well.

2) "patterning process from 6.1 to 6.3 in series". Do the authors refer to 5.1 to 5.3?

Thanks for raising the issue. We meant 5.1 to 5.3 and changed the nomenclature in the manuscript.

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Author(s):	Roberto Pioli, Roman Stocker, Lucio Isa, Eleonora Secchi

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