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.

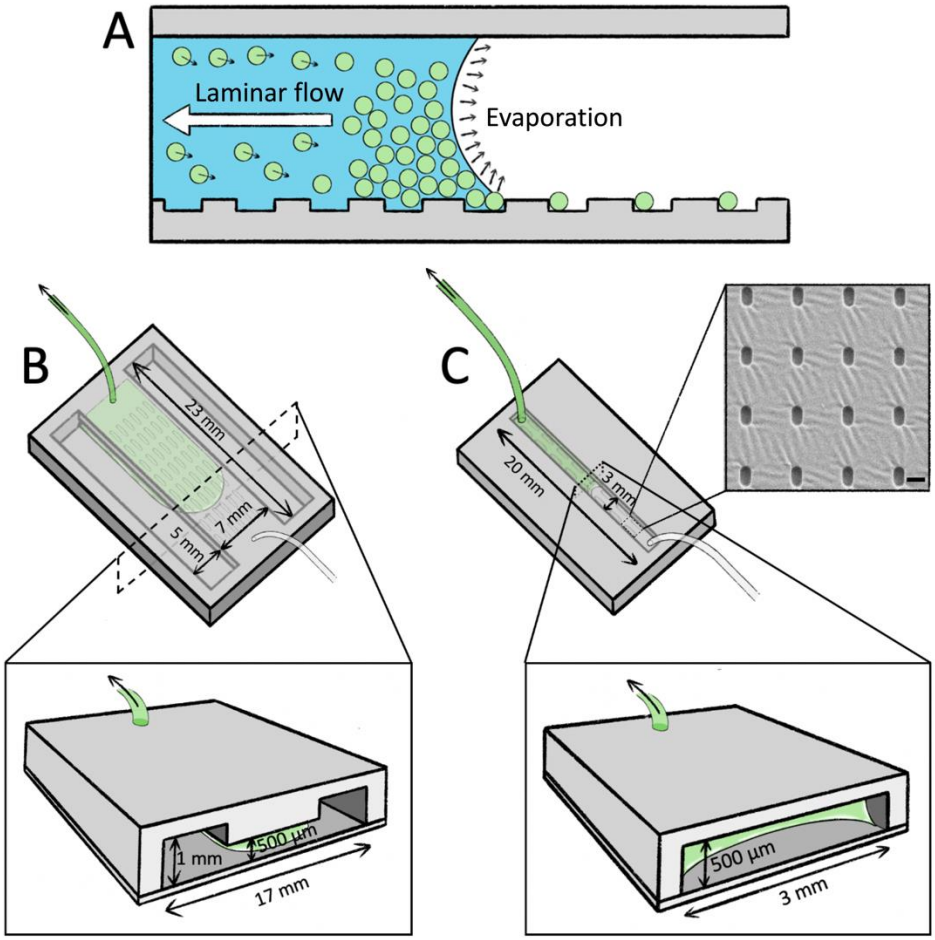
25. As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect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