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# Three-Dimensional Patterning of Engineered Biofilms with a Do-It-Yourself Bioprinter

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**TITLE:**

# Three-Dimensional Patterning of Engineered Biofilms with a Do-It-Yourself Bioprinter

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**KEYWORDS:**

bacteria 3D printing, biofilms, synthetic biology, 3D bioprinter, bacterial applications, spatially structured materials, 3D printing, additive manufacturing, bio-ink

**SUMMARY:**

This article describes a method of transforming a low-cost commercial 3D printer into a bacterial 3D printer that can facilitate printing of patterned biofilms. All necessary aspects of preparing the bioprinter and bio-ink are described, as well as verification methods to assess the formation of biofilms.

**ABSTRACT:**

Biofilms are aggregates of bacteria embedded in a self-produced spatially-patterned extracellular matrix. Bacteria within a biofilm develop enhanced antibiotic resistance, which poses potential health dangers, but can also be beneficial for environmental applications such as purification of drinking water. The further development of anti-bacterial therapeutics and biofilm-inspired applications will require the development of reproducible, engineerable methods for biofilm creation. Recently, a novel method of biofilm preparation using a modified three-dimensional (3D) printer with a bacterial ink has been developed. This article describes the steps necessary to build this efficient, low-cost 3D bioprinter that offers multiple applications in bacterially-induced materials processing. The protocol begins with an adapted commercial 3D printer in which the extruder has been replaced with a bio-ink dispenser

connected to a syringe pump system enabling a controllable, continuous flow of bio-ink. To develop a bio-ink suitable for biofilm printing, engineered *Escherichia coli* bacteria were suspended in a solution of alginate, so that they solidify in contact with a surface containing calcium. The inclusion of an inducer chemical within the printing substrate drives expression of biofilm proteins within the printed bio-ink. This method enables 3D printing of various spatial patterns composed of discrete layers of printed biofilms. Such spatially-controlled biofilms can serve as model systems and can find applications in multiple fields that have a wide-ranging impact on society, including antibiotic resistance prevention or drinking water purification, among others.

**INTRODUCTION:**

There is currently an increasing need to develop environmentally-friendly, sustainable solutions for the production of spatially-patterned materials, due to the expanding number of markets for such materials[1]. This article presents a simple, economical method for the production of such materials and therefore offers a large spectrum of future applications. The method presented here allows three-dimensional (3D) printing of spatially-patterned structures using a bio-ink containing living bacteria. Bacteria remain viable within the printed structures for over one week, enabling the bacteria to perform natural or engineered metabolic activities. Printed bacteria can thereby produce and deposit desired components within the printed structure, for example creating a functional cross-linked biofilm[2].

Traditional methods for the production of advanced materials involve high energy expenditures (e.g., high temperatures and/or pressures) and can produce large quantities of chemical waste, often toxic substances that require cost-extensive utilization[3,4]. In contrast, multiple bacterial species are able to produce materials that can be readily applicable in various industries. These materials include polymers such as polyhydroxyalkanoates (PHA)[5] or poly(glycolide-co-lactide) (PGLA)[6], bacterial cellulose[7], bacterial concrete materials[8], biomimetic composites[9], amyloid-based adhesives[10], or bio-based electrical switches[11], among others. Moreover, bacterial production of valuable materials typically takes place at near-ambient temperatures and pressures and in aqueous environments, without requiring or producing toxic compounds. While producing materials with bacteria has been demonstrated in the literature and some industrial applications have already emerged[12,13], a reliable method for spatial patterning of such materials remains a challenge.

This article demonstrates a straight-forward method of converting a low-cost commercial 3D printer into a 3D bacterial printer. The protocol shows how to prepare a bio-ink containing and sustaining the living bacteria, as well as how to prepare substrates onto which the 3D printing can be performed. This method is appropriate to use with a variety of natural and engineered bacterial strains able to produce materials. These bacteria can be spatially distributed within a 3D printed structure and still continue their metabolic activity, which will result in a spatial distribution of the desired materials produced by the bacteria.

This printing method enables additive manufacturing of biofilms, aggregates of bacteria surrounded by a self-produced extracellular matrix. Biofilms are heterogeneous 3D networks in

which proteins, polymers, bacterial cells, oxygen, and nutrients are all spatially structured[14]. While in the form of a biofilm, bacteria exhibit an increased antibiotic resistance and structural robustness, making them difficult to eradicate from surfaces including medical catheters and implants. The key to biofilm properties, and also the largest challenge to biofilm research, seems to be the heterogeneity of biofilms[15-17]. Production of spatially-controlled model biofilms is of special interest as it would allow for either reproducing or tuning the spatial patterns of biofilm components, aiding the understanding of the stable deposition of biofilms on virtually any surface in nature.

This article presents a method for the production of biofilms using 3D-printed hydrogels containing engineered *E. coli* bacteria that produce biofilm proteins in the presence of an inducer, as well as methods of verification of biofilm formation[2]. The major extracellular matrix components of these biofilms are curli amyloid fibers[18] that contain self-assembled CsgA proteins. When engineered *E. coli* bacteria are induced to express CsgA proteins, they form a stable model biofilm that protects the cells against being washed off of the printing surface. Such a 3D printed biofilm can be spatially controlled and can serve as a useful research tool for the investigation of multiscale biofilm structure-function mechanics or materiomics[19]. These bespoke biofilms will aid the understanding of the principles of biofilm formation and their mechanical properties, enabling further research into the mechanisms of antibiotic resistance among other applications.

**PROTOCOL:**

## 1. Conversion of a commercial 3D printer into a 3D bioprinter

1.1. Remove the extruder and the heater of a commercial 3D printer (**Table of Materials**) from the printer frame, and unplug the wiring controlling these elements from the main circuit board (**Figure 1A**). Since the sensor that controls the operational temperature of the printer needs to be functional to communicate with the printer software, remove from the printing software the algorithm that delays printing until operational temperature is reached.

1.2. Connect a pipette tip (200 µL tip) via silicon tubing (inner diameter of 1 mm) to a 5 mL syringe loaded into a syringe pump. Mount the pipette tip onto the 3D printer extruder head as a replacement for the original extruder (**Figure 1B**).

1.3. If more than one type of bio-ink will be used, mount additional tubing system(s) and pipette tip(s) to the printer.

## 2. Substrate preparation for 3D printing

2.1. Add 4 mL of 5 M $CaCl_2$ solution to 400 mL of 1% w/v agar dissolved in Luria-Bertani broth (LB) medium, supplemented with appropriate antibiotics and inducers (here 34 µg/mL chloramphenicol and 0.5% rhamnose).

2.2. Dispense 20 mL of the LB-agar solution into each 150 mm x 15 mm Petri dish. Dry 30 min at room temperature with the lid half-open.

NOTE: The protocol can be paused here by storing these printing substrates at 4 °C for up to several days.

## 3. Bio-ink preparation

3.1. Prepare a sodium alginate solution (3% w/v), and heat to the boiling point three times to sterilize the solution. Store at 4 °C until used.

3.2. Grow *E. coli MG1655 PRO ΔcsgA ompR234* (*E. coli ΔcsgA*) bacteria carrying plasmids pSB1C3-green fluorescent protein (GFP) (constitutive GFP expression)[2] or pSB1C3-GFP-CsgA (constitutive GFP expression, rhamnose-inducible CsgA expression) overnight at 37 °C with shaking at 250 rpm in 50 mL of LB medium containing 34 µg/mL chloramphenicol and 0.5% rhamnose.

3.3. Centrifuge the cell culture for 5 min at 3220 x *g* to pellet the bacteria. Remove the supernatant.

3.4. Re-suspend the bacteria pellet in 10 mL of LB medium and add 10 mL of sodium alginate (3% w/v).

## 4. 3D printing process

4.1. Install and open the 3D printing software (**Table of Materials**) on a computer. Connect the 3D printer to the computer. Move the printhead to its home position (specifically, X = -20 mm, Y = -15 mm, Z = 0 mm) by clicking the home button for the X, Y, and Z axes.

4.2. For each print, place a prepared printing substrate onto a particular location on the printing bed.

4.3. Calibrate the height of the printhead in the Z axis.

4.3.1. Raise the printhead to a height of 20 mm under manual control, so that it will not collide with the edge of the petri dish when moving to the desired position. Position the printhead overtop of the plate, and move it down until the pipette tip contacts the printing surface. Assign this Z-axis position as Z1 (the height of the printing surface).

4.3.2. Raise the printhead, and move it outside of the plate area by manual control in the X, Y, and Z axes. If the working distance between the printhead and the plate surface is defined as Z2, enter Z1 + Z2 into the printing program as the Z-value during printing.

4.4. Program the printing shape by a self-developed point-by-point coordinate-determined

method according to the desired trajectory.

4.4.1. If the desired trajectory is a straight line, define only the start and end points. Including additional points on curved lines will result in smoother curves. Move the printhead manually to every point sequentially, and record the coordinates of these points in order. Enter all of these coordinates as well as the printhead moving speed for each printed segment into the G-code editor.

4.5. Both before and after printing, lift the printhead to a distance higher than the plate edge (20 mm), and move directly out of the plate region. Save this program as a G-code file and load directly for use in subsequent prints, while re-measuring the Z axis height for each new printing substrate.

NOTE: See **Table 1** for an example G-code for printing a square.

4.6. Load the pre-programmed G-code file. Open the G-code editor in the software, and program in the commands for printing the desired shape. At each command line, the position of the printhead may be changed in the X, Y, and/or Z axis. Input the Z value during all printing steps as Z1 + Z2 (height of printing surface + working distance).

NOTE: The moving speed is also adjustable; 9000 mm/min is a suitable value for typical printing rates.

4.7. Load the liquid bio-ink into syringe(s), and mount them in the syringe pump(s) of the 3D bioprinter.

4.8. Print the bio-ink onto the printing substrate by clicking the **Print** button.

4.9. During printing, control the printhead movement entirely by the software. Manually start the syringe pump before the printhead comes into contact with the printing surface.

NOTE: The coordination of the syringe pump and the printer is empirically determined depending on the extrusion speed, the speed at which the printhead moves to the first print point, and the initial position of the printhead. If the initial printhead position is 20 mm, with a printhead speed of 9000 mm/min and an extrusion speed of 0.1 mL/h, start the syringe pump immediately after the printing is started. If the extrusion speed is changed from 0.1 mL/h to 0.3 mL/h, then wait 2−3 s to start the syringe pump after the printing is started.

4.10. Stop the syringe pump as soon as the printhead arrives at the last point of printing. Halt the syringe pump before the printhead lifts up at the end of the printing process, otherwise excess bio-ink will drop onto the printing substrate and reduce the printing resolution.

4.11. For the construction of 3D structures, wait 10 min after the first layer is printed. Increase the printing distance by 0.2 mm by changing the Z value in the G-code editor from Z1 to Z1 +

0.2, and repeat the printing process again. Do not move the plate during the printing process.

4.12. To measure the width and height of the printed hydrogel, use a steel ruler placed underneath or alongside the sample.

**5. Growing and testing the effectiveness of biofilm production by *E. coli***

5.1. Incubate the printed samples at room temperature for 3–6 days to allow the production of biofilm components (curli fibers). Image the plates using a camera or fluorescent scanner.

5.2. To dissolve the alginate matrix, add 20 mL of 0.5 M sodium citrate solution (pH = 7 adjusted with NaOH) to the printing substrates, and incubate for 2 h with 30 rpm shaking at room temperature. Discard the liquid and image the plates again to compare with the images of the plates before citrate treatment.

**REPRESENTATIVE RESULTS:**

The first step for successful 3D printing of biofilms is converting a commercial 3D printer into a bioprinter. This conversion is achieved by removing the extruder and heater of the printer, designed for printing with a polymeric ink, and replacing these with components appropriate for printing bio-ink containing living bacteria (**Figure 1A**). The extruder is replaced by a pipette tip (or tips, if multiple bio-inks will be used in the printing process) attached to a tubing system connected to a syringe pump (**Figure 1B**). The successful conversion of the commercial printer into a bioprinter can be assessed based on the ability to transfer desired bio-ink(s) from the syringe pump through the tubing system and pipette tip(s) onto a printing surface without leaking or heating the bio-ink. If the tubing bulges due to the flow of bio-ink during printing, it may be replaced by tubing with thicker walls. It should be noted that this printing technique should be able to work with any type of commercial 3D printer for which tubing can be attached to the printhead.

The 3D bioprinter can create bacteria-encapsulating hydrogels in a variety of two-dimensional (2D) and 3D shapes (**Figure 2**). Calcium ions in the printing substrate induce solidification (chelation of calcium ions with alginate carboxyl groups) of the bio-ink upon printing, converting the liquid bio-ink into a solid hydrogel. The resolution of bioprinting will depend on the extrusion speed, the size of the pipette tip, the speed of the printhead, the volume and concentration of $CaCl_2$ solution applied onto the printing surface, the flatness of the printing surface, and the viscosity of the bio-ink used. The concentration of $CaCl_2$ solution has a great influence on hydrogel sharpness. Four different concentrations of $CaCl_2$ (0.1 M, 0.2 M, 1 M, and 5 M) were sampled, and only 5 M $CaCl_2$ solution resulted in hydrogel that did not become blurred after printing. Therefore, 5 M was chosen as the optimal concentration of $CaCl_2$ solution.

The volume of $CaCl_2$ applied to printing substrates can also have a critical influence over printing quality and resolution. When using a 150 x 15 mm Petri dish, applying a volume of calcium chloride solution of more than 200 µL results in too much liquid remaining on the

printing surface. This liquid may spread unevenly when the plate is moved, which can change the working distance and cause blockage of the pipette tip. Too much volume of $CaCl_2$ can also cause printed hydrogels to float and slide across the solution, changing the shape and position of the printed hydrogel. If the volume of calcium chloride solution spread onto the printing surface is less than 50 µL, the liquid amount is too small to allow the liquid to be spread across the entire printing surface. Some regions of the plate may then not receive $CaCl_2$ solution, affecting hydrogel solidification. The optimal volume of the $CaCl_2$ solution applied on the plate surface is 100 µL for 150 x 15 mm Petri dishes, and 30 µL for Petri dishes with a diameter of 90 mm.

The extrusion speed and printhead movement are interdependent and can be tuned in a coordinated manner to alter the printing resolution. For example, if the printer is operated with extrusion speed between 0.1 mL/h and 0.5 mL/h with a constant printhead movement speed of 300 mm/min, the diameter of the printed hydrogel increases with the increase of extrusion speed[2,20]. At extrusion speeds over 0.5 mL/h, the outer edges of the printed lines of hydrogel change from straight, parallel lines to wavy lines, and the line width also increases. The velocity of the printhead also has an influence on the printing resolution. With a constant extrusion speed of 0.3 mL/h, increasing the speed of the printhead from 300 mm/min to 500 mm/min results in the width of the printed hydrogel becoming narrower, decreasing from 1.8 mm to 0.9 mm. If the printhead moving speed is over 500 mm/min, the gel line will easily become discontinuous. For a 200 µL pipette tip and the bio-ink used in the current study, several combinations of the printing resolution are considered optimal (**Table 2**). At pumping speed 0.3 mL/h, printhead movement speed 500 mm/min, and working distance 0.2 mm, printed hydrogel is produced with a width of approximately 0.9 mm.

One crucial achievement of the bacterial 3D printing method is its ability to create engineered biofilms. To create an engineered and spatially-controlled biofilm, the bacteria should not only survive the 3D printing process but should also produce biofilm components while remaining within the printed pattern. The engineered *E. coli* bacteria used in this protocol, *E. coli ΔcsgA* bacteria carrying the plasmid pSB1C3-GFP-CsgA, enable controllable expression of curli proteins. The use of a *csgA*-knockout strain ensures that CsgA protein is only expressed when it is induced from a plasmid with rhamnose. The bacteria export the induced CsgA protein subunits, which then self-assemble[21] onto CsgB proteins on the bacterial outer membrane[22] to form curli fibers. These amyloid-like fibers are the major proteinaceous components of biofilm extracellular matrix: a connected network of proteins and polymers in which the bacteria are embedded. The printed alginate matrix of the 3D-printing bio-ink lends physical support and structure to the bacteria during the curli production process. The use of constitutive GFP expression allows for visualization and quantification of printed cells via fluorescence imaging.

In order to assess whether the formation of biofilm was successful, the alginate matrix was dissolved using a sodium citrate solution, and the shape of the printed bio-ink was assessed after the citrate treatment (**Figure 3**). In the case of bio-ink without the inducible curli production plasmid, the printed pattern was completely dissolved after the sodium citrate treatment, signifying that no biofilm curli network had formed (**Figure 3A,B**). In the case of

bacteria containing the inducible curli production plasmid, the gel was not dissolved after sodium citrate treatment (**Figure 3C,D**). This result indicates that the printed bacteria were able to form a curli network extensive enough to stabilize the printed pattern of bacteria[2].

To construct multi-layered structures, additional layers were printed 10 min after the previous layer was printed (**Figure 4**). Increasing the number of printed layers in a sample caused the width and the height of the printed structures to increase incrementally (**Figure 5**)[2,20], but even 5-layer printed structures could be created with a resolution of millimeters to sub-millimeters. When *E. coli* engineered to inducibly produce curli proteins were printed into multi-layered structures, sodium citrate treatment did not dissolve the samples, whereas multi-layer structures containing non-curli-producing *E. coli* were dissolved in sodium citrate solution (**Figure 6**). This experiment demonstrates that engineered biofilms can be created in multi-layered, three-dimensional printed structures, as well as in single-layer printed structures.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Photos showing the conversion of a commercial 3D printer into a 3D bioprinter.** (**A**) The components of the 3D bioprinter after conversion from a commercial 3D printer. (**B**) The bio-ink extruder formed by a tubing system attached to a pipette tip. Additional printing tips can be added in the second printhead hole or by adding additional holes to the printhead, for use in printing multiple types of bio-ink.

**Figure 2: Examples of 3D bioprinted patterns containing *E. coli* pSB1C3-GFP-CsgA.** These images were taken two days after printing. This printing resolution was obtained with pumping speed 0.3 mL/h, printhead movement speed 300 mm/min, and working distance 0.2 mm. The G-codes for printing these shapes may be found in the **Supplemental Files**.

**Figure 3: A method of verifying whether biofilm components have been produced by *E. coli* bacteria within a printed pattern.** When printed *E. coli* contained a plasmid that did not encode for curli induction, the printed pattern was completely dissolved by sodium citrate treatment (**A** and **B**). When *E. coli* containing a plasmid encoding inducible curli proteins was used, the printed biofilm was resistant to sodium citrate treatment (**C** and **D**). The programming process and explanations of the G-code for printing this square pattern are provided in **Table 1**.

**Figure 4: Top view (A) and side view (B) of multi-layered printed structures containing *E. coli* pSB1C3-GFP-CsgA.** This sample was printed with pumping speed 0.3 mL/h, printhead movement speed 200 mm/min, and working distance 0.2 mm.

**Figure 5: The line width and height of printed hydrogels containing different numbers of printed layers.** The measurements were performed on samples printed with pumping speed 0.3 mL/h, printhead movement speed 500 mm/min, and working distance 0.2 mm.

**Figure 6: A method of verifying whether biofilm components have been produced by *E. coli* bacteria within multi-layer printed structures.** Engineered *E. coli* was printed into 1-, 3-, or 5-

layer hydrogels and incubated for 6 days. When the printed *E. coli* contained a plasmid that did not encode for curli induction, the printed pattern was completely dissolved by sodium citrate treatment (**A** and **B**). When the printed *E. coli* contained a plasmid encoding inducible curli proteins, the printed biofilm was resistant to sodium citrate treatment (**C** and **D**).

**Table 1: Programming process and explanations of G-code for printing a square.**

**Table 2: The optimal printing parameters for hydrogels with high resolution.** Five points were measured for each condition. The average value and standard deviation are shown in the table.

**DISCUSSION:**

The protocol presented here for 3D printing of engineered biofilms has two critical steps. First is the preparation of the agar printing surface, which is the most critical factor to producing a specific printing resolution. It is important to ensure that the printing surface is flat and that the pipette tip on the printhead is positioned at the correct height from the surface. If the surface is not flat, the working distance will change during the printing process. If the working distance is less than 0.1 mm, the $CaCl_2$ solution could enter inside the pipette tip and cause hydrogel formation, causing the pipette tip to become clogged. If the working distance is more than 0.3 mm, the gel cannot be printed continuously. The optimal working distance in this study is 0.2 mm. Good approaches for preparing flat agar printing surfaces are to use larger-diameter Petri dishes (150-mm-diameter Petri dish rather than a 90-mm-diameter plate), place the plates on a flat table, pour the agar solution with fast and even speed, and avoid moving the agar plate during its solidification.

The second critical step is the selection of desired printing parameters including pumping speed, viscosity of the bio-ink used, and printhead speed, which determine the resulting printing resolution. To select these parameters in an efficient manner, the user can sample several extreme values for printhead speed with a constant extrusion rate, noting the width of the printed hydrogel for each set of conditions. Then, repeat this experiment with 4 other extrusion rates. Next, take the five combinations that produced the best printing resolution for the application, and vary both printing parameters (pumping and printhead speeds) in smaller and smaller steps until the desired resolution is obtained.

The thickness of the printed lines has an impact on the ability of the printed engineered bacteria to form stable biofilms. Under optimal printing conditions (pumping speed 0.3 mL/h, printhead speed 300 mm/min, and working distance 0.2 mm), printed lines of bio-ink will produce stable biofilms after 3 days of incubation at room temperature. If the lines become thicker, such as by increasing the pumping speed, the middle regions of each line may not be induced sufficiently to produce citrate-stable biofilms.

When printing a multi-layer bio-ink hydrogel, each printed layer is solidified upon contacting the calcium ions that have diffused into the previous printed layer. Each layer should be printed with a 10-minute waiting interval between printing different layers to allow time for sufficient calcium ions to diffuse up through the lower layers to allow solidification of the upper layers.

Additionally, the printing distance of the upper layer should be restricted to only 0.2−0.3 mm higher than the printing distance of the previous layer. If the added printing distance is less than 0.2 mm, the tip will drag across the first layer and reduce the resolution of the printed hydrogel. If the added printing distance is larger than 0.3 mm, the bio-ink will form drops of liquid during extrusion, causing the printed hydrogel to become discontinuous.

The current bioprinting approach enables the production of reproducible, spatially-controlled engineered biofilms, suitable for use in the study of biofilm mechanical properties or biological resistance of biofilm bacteria to various factors including antibiotics, surfactants, etc. This capability ensures a direct usability of the proposed method. The development of higher-precision do-it-yourself (DIY) bioprinters will likely be possible by maintaining the printing working distance but lowering the pumping speed and the moving speed of the printhead, or by sampling different extruder geometries and bio-ink chemistries. With future improvements to the printing resolution, additional applications can be enabled such as tissue engineering or drug delivery. The 3D bioprinting approach described here should also be able to be expanded to printing additional types of bacteria species that are biocompatible with our alginate-based bio-ink. The current protocol provides sufficient sterility by repeatedly boiling the bio-ink during preparation, using sterile syringes and printing tips, and utilizing antibiotics in both the bio-ink and printing plate. Future experiments using wild-type bacteria may require additional sterilization measures such as replacing or disinfecting the tubing system between prints.

To the authors' best knowledge, the presented method (originally developed in Lehner et al.[20]) is the first published example of an additive manufacturing style for 3D printing of bacteria. In the first part of this protocol, this general method is described in detail for the 3D printing of bacteria, which is applied to the production of engineered biofilms[2]. Multiple future applications of 3D-printed biofilms are possible using this method. In nature, multiple bacterial systems have evolved that create various types of biofilms, of which in this article a single system was explored. Multiple other systems can be easily examined by creating 3D-printed biofilms with other bacterial systems, such as *Bacillus subtilis* or *Acetobacter xylinum*. Alternative methods[23,24] have also been developed for spatial patterning of bacteria at high resolution using optical signals. These approaches require more expensive, complicated equipment to achieve them in comparison to this printer, and are only suitable for patterning of genetically engineered bacteria.

The ability to spatially pattern 3D-printed biofilms with this method can allow for the creation of engineered biofilms that reproduce the spatial heterogeneity of natural biofilms[25]. Because of the highly detailed arrangement of protein and polymeric fibers within a biofilm, bacteria in a biofilm state achieve a much higher resistance to chemical and physical stimuli, such as an increased resistance to antibiotics as compared to the same bacteria in a planktonic state. Moreover, bacteria within a biofilm show an increased resistance to fluid flow, making the maintenance and sterility of implantable medical devices much more difficult[26]. Printed engineered biofilms that attempt to reproduce the specific spatial distributions of biofilm components are powerful tools for studying the mechanisms by which bacteria within a biofilm achieve resistance phenotypes.

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**DISCLOSURES:**

The authors have nothing to disclose.

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Figure 1



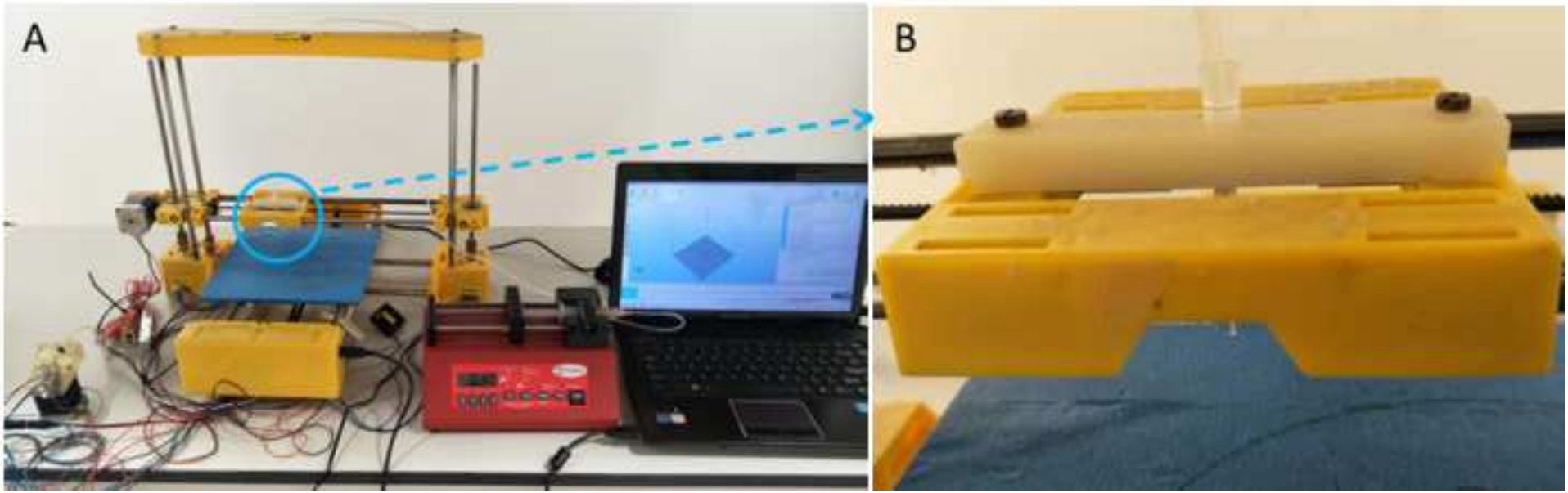

Figure 2



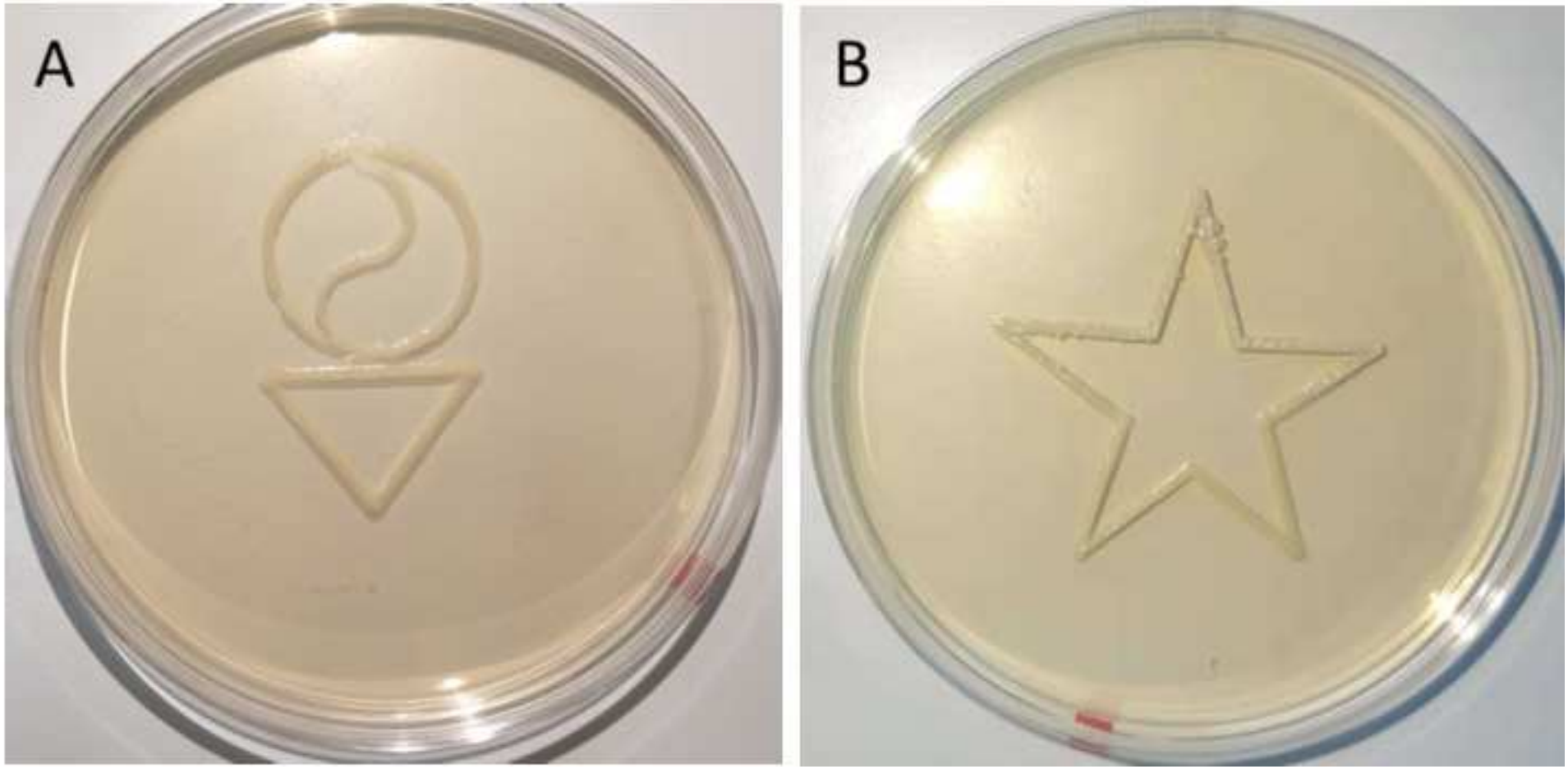

Figure 3



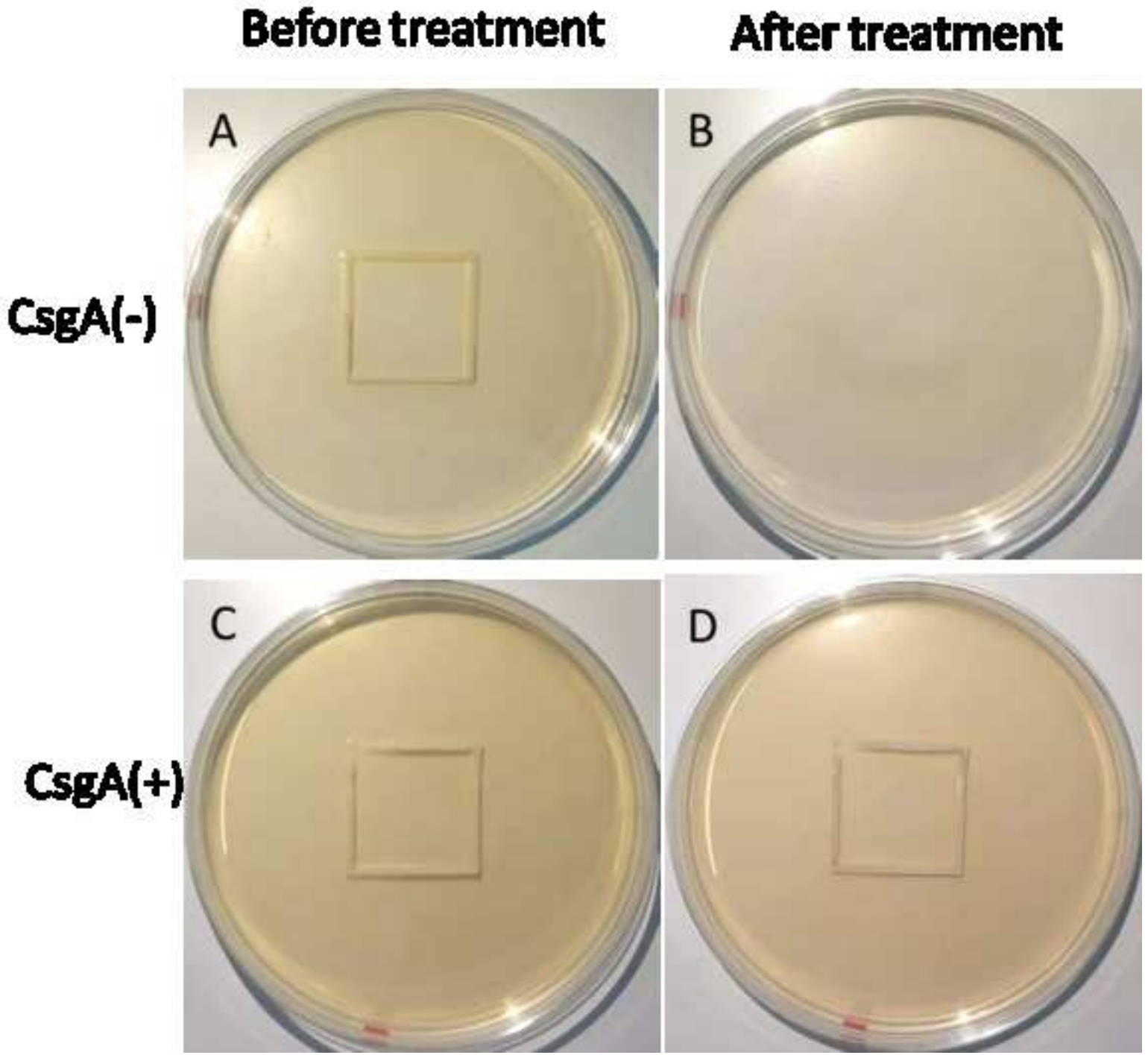

Figure 4



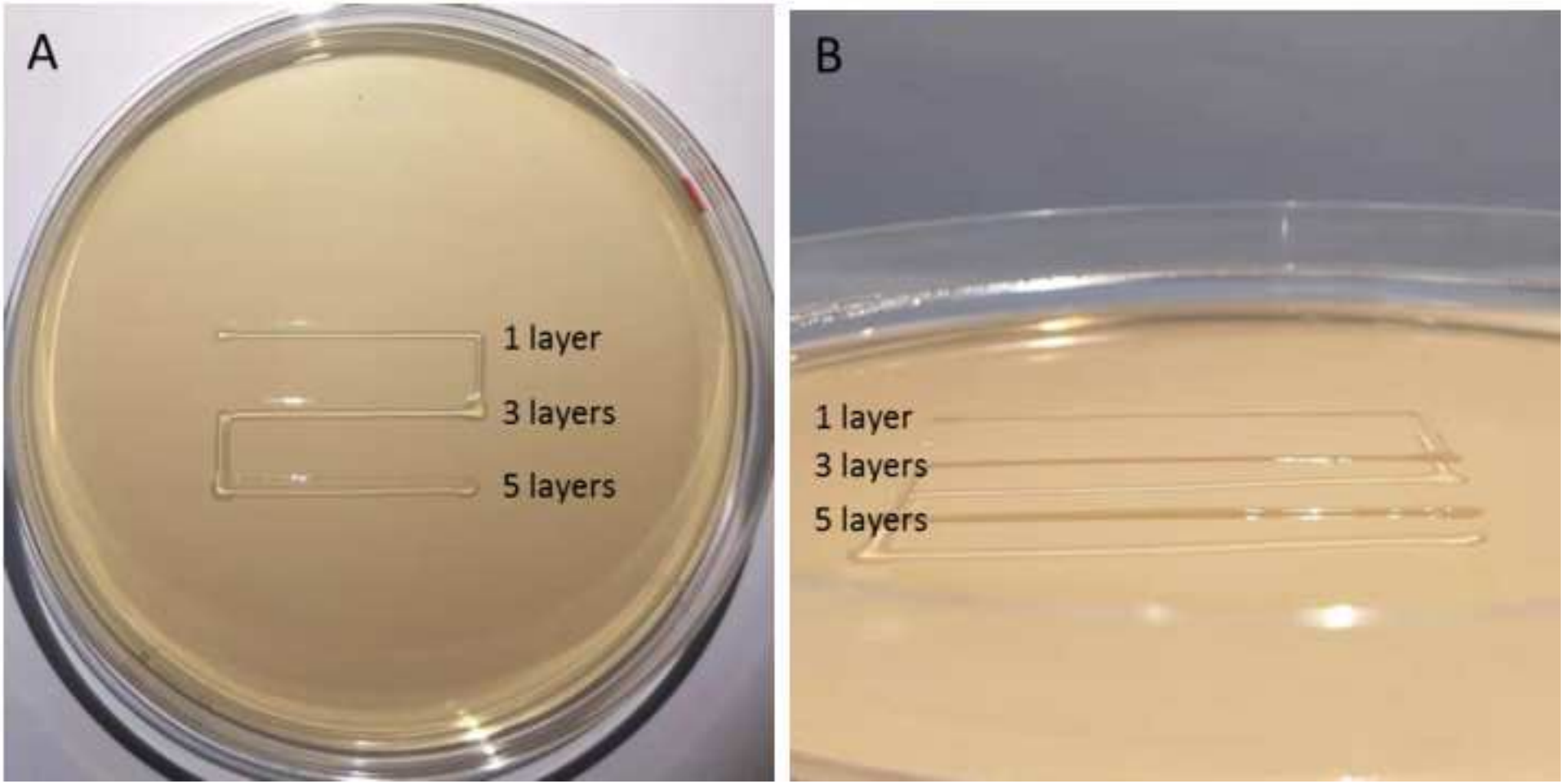

Figure 5



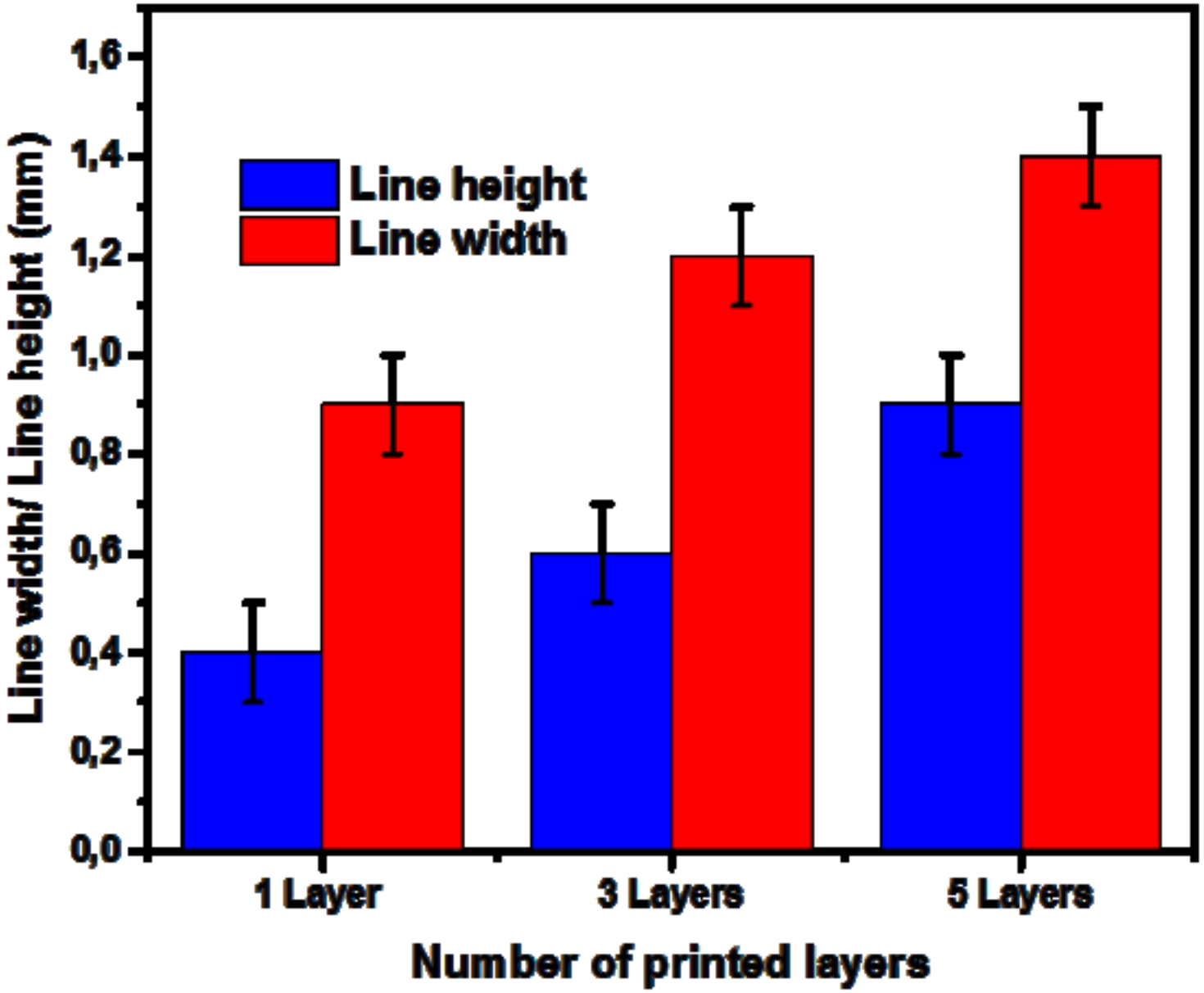

Figure 6



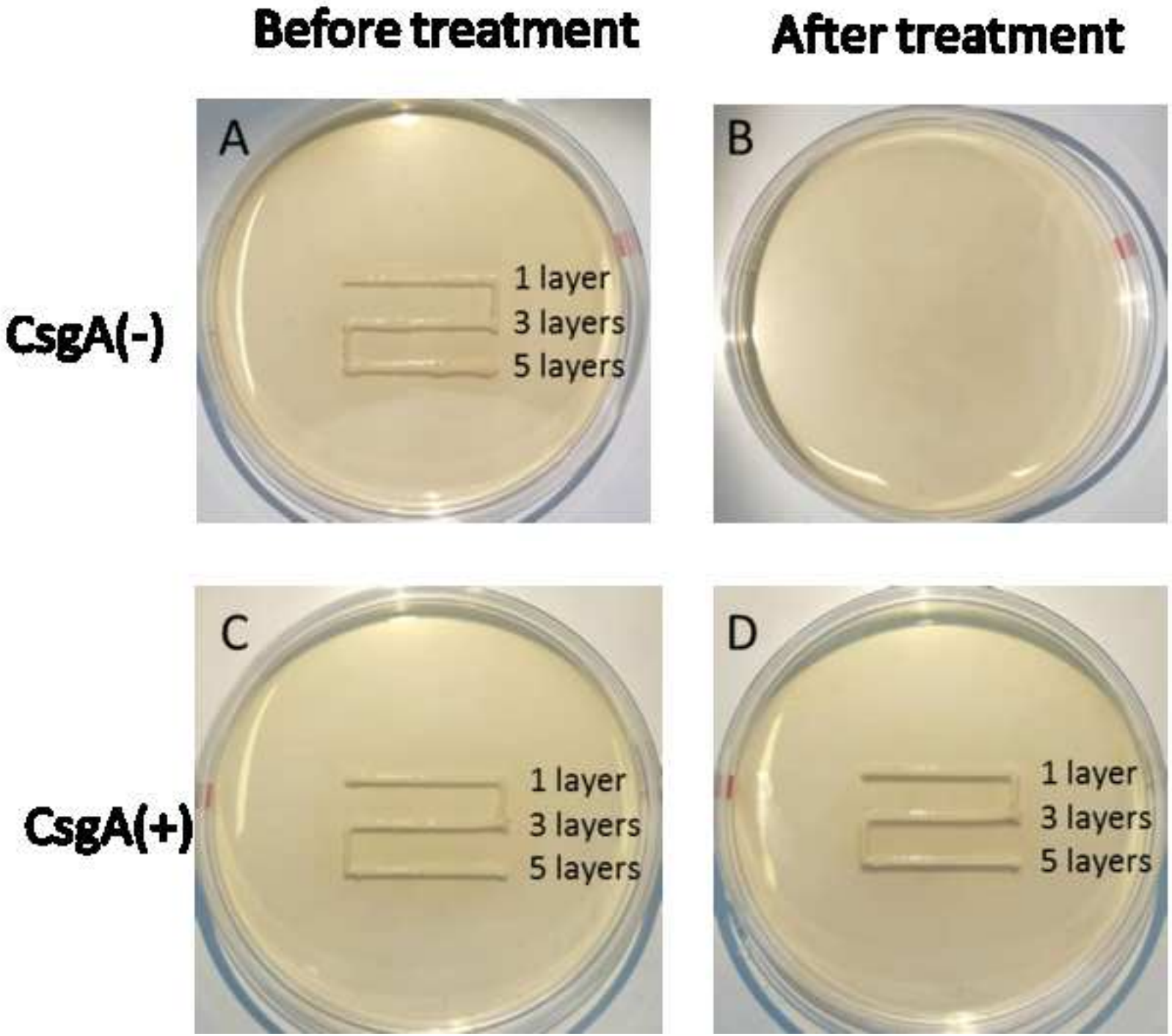

Table 1



| G-code commands |
| --- |
| G1 Z20 F9000 |
| G1 X95 Y65 F9000 |
| G1 Z6 F9000 |
| G1 X95 Y105 F300 |
| G1 X135 Y105 |
| G1 X135 Y65 |
| G1 X95 Y65 |
| G1 Z20 F9000 |
| G1 X55 Y40 F9000 |

| Tasks |
| --- |
| Lift the Z-axis to a height of 20 mm with a 9000 mm/min moving speed. |
| Move to the starting point of the first line with a 9000 mm/min moving speed. |
| Move downwards in the Z-direction to a proper (here Z = 6 mm) printing distance. |
| End point of the first line and starting point of the second line. |
| End point of the second line and starting point of the third line. |
| End point of the third line and starting point of the fourth line. |
| End point of the fourth line and starting point of the first line; a square is formed. |
| Lift the Z-axis to a height of 20 mm at 9000 mm/min. |
| Move to a coordinate (55, 40) outside of the Petri dish range. |

Table 2



| Extrusion speed (mL/h) | Printhead moving speed (mm/min) |
|---|---|
| 0.1 | 100 |
| 0.1 | 200 |
| 0.1 | 300 |
| 0.3 | 300 |
| 0.3 | 400 |
| 0.3 | 500 |
| 0.5 | 200 |
| 0.5 | 1200 |
| 0.7 | 200 |
| 0.7 | 1200 |

| Gel width (mm) |
| --- |
| 1.6 ± 0.1 |
| 1.1 ± 0.1 |
| 1.0 ± 0.1 |
| 1.8 ± 0.1 |
| 1.2 ± 0.1 |
| 0.9 ± 0.1 |
| 2.2 ± 0.2 |
| 1.2 ± 0.2 |
| 2.8 ± 0.1 |
| 1.3 ± 0.1 |

| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| 3D printer | CoLiDo | 3D-P Kit | |
| 3D printing software | CoLiDo | Print-Rite ColiDo Repetier-Host v2.0.1 | |
| Agar | Sigma-Aldrich | 05040 | |
| $CaCl_2$ dihydrate | Sigma-Aldrich | C7902 | |
| Centrifuge | Eppendorf | 5810 R | |
| Chloramphenicol | Sigma-Aldrich | 3886.1 | |
| LB broth powder | Sigma-Aldrich | L3022 | |
| Orbital shaker | VWR | 89032-092 | Model 3500 |
| Petri dish | VWR | 25384-326 | 150 x 15 mm |
| Rhamnose | Sigma-Aldrich | 83650 | |
| Silicon tubing | VWR | DENE 3100103/25 | |
| Syringe pump | ProSense B.V. | NE-300 | |
| Sodium alginate | Sigma-Aldrich | W201502 | |
| Sodium citrate monobasic | Sigma-Aldrich | 71498 | |
| Sodium hydrooxide | VWR | 28244.295 | |

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Title of Article: 3D patterning of engineered biofilms with a DIY bioprinter

Author(s): Ewa M. Spiesz, Kui Yu, Benjamin A.E. Lehner, Dominik T Schmieden, Marie-Eve Aubin-Tam, Anne S. Meyer

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| Department: | Dept. Biology |
| Institution: | University of Rochester |
| Article Title: | 3D patterning of engineered biofilms with a DIY bioprinter |
| Signature: | Anne S. Meyer |
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March 4, 2019

Dear Xiaoyan Cao
*JoVE* Review Editor,

Thank you for sending us the referee comments for our manuscript, "3D patterning of engineered biofilms with a DIY bioprinter" by Ewa M Spiesz *et al.*, manuscript ID JoVE59477.

In response to the referees' comments, we have improved our manuscript. All changes are indicated with tracked changes in the marked-up version of the manuscript. They are also described in a point-by-point fashion on the subsequent pages.

We are confident that we have addressed the referees' concerns appropriately, and we are hopeful that you will consider our manuscript suitable for publication in *JoVE*.

Please do not hesitate to contact me with any further questions. We look forward to hearing from you soon.

Yours sincerely,

Anne S. Meyer

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We thank both the reviewers as well as the editorial office for their comments on our manuscript. Our responses to the reviewers' comments are indicated in blue.

# Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Reply 1: We have thoroughly proofread the manuscript again to remedy as many spelling and grammar issues as possible.

2. Are any of the authors of this article affiliated with an institution in the United Kingdom?

Reply 2: No authors of this manuscript are affiliated with an institution in the United Kingdom.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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 and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: CoLiDo, VWR DENE, ProSense B.V., Print-Rite ColiDo Repetier-Host, etc.

Reply 3: All the commercial language in this manuscript has been replaced by generic terms.

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

Reply 4: All the personal pronouns in this manuscript have been replaced by suitable statements.

5. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Reply 5: The protocol has been revised to contain only action items in the imperative tense. Phrases such as "could be," "should be," and "would be" have been removed or replaced. Discussion about the protocol has been moved or added as a "Note."

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6. 3.3, etc.: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Reply 6: We have converted the centrifuge speeds to centrifugal force (x g) in the revised manuscript.

7. 4.4: Can the pre-programmed G-code file be provided as a supplemental file? Please provide an example of programing a desired shape.

Reply 7: The pre-programmed G-code files (for all the shapes designed in this manuscript) have been provided as supplemental files.

8. 4.5: Please split this into two steps so that individual steps contain only 2-3 actions per step.

Reply 8: This part has been split into two steps: 4.4 and 4.5.

9. 4.8: Please write the text in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "NOTE".

Reply 9: The text has been rewritten in the imperative tense throughout.

10. Section 5: Please consider incorporating these steps into the previous steps so that the protocol shows specific examples that can be followed in chronological order.

Reply 10: We have incorporated several steps into previous sections of the protocol. Section 5 now includes only steps related to growing and testing the printed biofilm.

11. Please include single-line spaces between all paragraphs, headings, steps, etc.

Reply 11: Single-line spaces have been inserted between all paragraphs, headings, and steps.

12. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less 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.

Reply 12: The headings and crucial parts of the protocol have been highlighted.

13. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting.

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Reply 13: The highlighted parts are complete sentences, and each part includes at least one action that is written in imperative tense. Notes are excluded from the highlighting.

14. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Reply 14: The details in the sub-steps are also highlighted.

15. Please remove the embedded figures and tables from the manuscript. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

Reply 15: The embedded figures and tables have been removed from the manuscript. All the Figure Legends have been included at the end of the Representative Results in the manuscript text.

16. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

Reply 16: All the figures have been uploaded individually in the proper format to the Editorial Manager account.

17. Please upload each Table individually to your Editorial Manager account as an .xls or .xlsx file.

Reply 17: All the tables have been uploaded individually in the proper format to the Editorial Manager account.

18. References: Please do not abbreviate journal titles.

Reply 18: All the abbreviated journal titles have been replaced by their full names.

19. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Reply 19: We have sorted the items in alphabetical order according to the name of the material or equipment.

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# Reviewers' comments:

## Reviewer #1:

In the manuscript, the authors provided a well-written protocol based on their previously published paper ("Printing of Patterned, Engineered E. coli Biofilms with a Los-Cost 3D Printer." ACS Synth. Biol. 7(5): 1328-1337), which allows readers to follow their method easily and reproduce the patterned biofilms formed by E. coli. In this manner, I suggested that the manuscript should be published. Before that, there are some technical issues that have been to be addressed.

1) Currently printed examples here (were shown in Fig.2, more like to a 2D patterned biofilms) are not sufficient to show that the 3D-printer can construct a living biofilm with well-defined 3D architecture. Therefore, a successful 3D demonstration must be included.

Reply 1: We thank the reviewer for this comment. With our current technology, it is possible to create printed biofilms with 3D architecture. When printing a multi-layer bio-ink hydrogel, each printed layer is solidified upon contacting the calcium ions that have diffused into the previous printed layer. Each layer is printed with a 10-minute waiting interval between printing different layers to allow time for sufficient calcium ions to diffuse up through the lower layers to allow solidification of the upper layers. This mechanism is described in newly added text on lines 406-414.

In our revised manuscript, we have added three new figures, showing images of the top view and side view of printed hydrogels containing gels 1 layer, 3 layers, or 5 layers (Figure 4). We report on the height and width of printed hydrogels containing different numbers of layers (Figure 5), which fall in the millimeter-to-sub-millimeter range and increase incrementally with the deposition of additional layers. Finally, we printed engineered *E. coli* containing a plasmid encoding for the inducible production of curli biofilm proteins into multi-layered structures of 1, 3, or 5 layers. We demonstrate that sodium citrate treatment does not dissolve these multi-layer printed structures, while it is able to dissolve equivalent structures containing *E. coli* that do not produce curli proteins (Figure 6). These new figures provide a full demonstration that our 3D printer can print engineered bacteria that form three-dimensional biofilms.

2) Some key references should be cited and discussed **to further expand the section of Discussion** (Huang, Y. J., et al. (2018). "Bioprinting Living Biofilms through Optogenetic Manipulation." ACS Synthetic Biology 7(5): 1195-1200; Jin, X. F. et.al. (2018). "Biofilm Lithography enables high-resolution cell patterning via optogenetic adhesin expression." PNAS 115(14): 3698-3703.), where the authors provided a bioink-free strategy to construct patterned living biofilms.

Reply 2: We thank the reviewer for this suggestion. We have added the two suggested key references into the manuscript and cited them in the Discussion to further expand this section. Our new text is at lines 439-442.

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# Reviewer #2:

Manuscript Summary:

This manuscript describes a method to pattern biofilms using a commercially available 3D printer. This report uses an alginate-based bio-ink to and genetically engineered E. coli. In this demonstration, the alginate-based ink that contains the bacteria solidifies upon contact with the calcium-containing printing substrate. A biofilm phenotype was induced in E. coli strain used in these experiments using a plasmid for curli, and the appropriate control of a strain without the plasmid showed no curli amyloid fibers. When the 3D-printed alginate matrix is chemically dissolved using a sodium citrate treatment, the printed biofilm of the strain containing the inducible plasmid remains intact, whereas the control without the plasmid is dispersed.

Overall, I think that this manuscript provides a nice demonstration of a new technique for spatially patterning biofilms that is interesting to scientists in this field. As the authors note, the ability to spatially control biofilms is useful for generating better model systems to study a variety of applications, including the development of antibiotic resistance. The method described in this report allows scientists to 3D print live bacteria at room temperature/ambient conditions using low cost, commercially available equipment. This inexpensive technique will help drive the field forward as it can be done in nearly any lab. This paper details how to convert the 3D printer to accept bio-ink, preparing the bio-ink, the commands to input to the printer to pattern biofilms, characterization of optimal printing parameters, demonstration of the method and confirmation of the biofilm phenotype. There is also a meaningful discussion about the technique, why it is useful, and the impact of biofilms/biofilm research in the field.

I do have some questions that I think the authors should address in the text, but I feel that this paper should be accepted for publication. I have outlined these questions in the "concerns" section of this peer review.

Major Concerns:

1. The only strain used in this paper is the engineered strain of E. coli, where curli amyloid fibers are produced upon induction, and indicate that the cells are expression a biofilm phenotype. **Have you tried the bioprinting with any other strains?** Another engineered strain with an inducible biofilm phenotype? Or even a wild type strain known to be a good biofilm former? I really like the experiment showing that the sodium citrate dissolves the printed matrix and the strain without the plasmid disperses. However, I'd like to address whether biofilms form naturally within these patterned matrices as a result of cells being confined within close proximity to one another, similar to how a biofilm would form in nature. Could you comment on what would happen (or if you have tried) using a wild type strain of bacteria that is known to be a good biofilm former (e.g., P.

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aeruginosa). I suspect in a wild type experiment your biofilm would remain intact when the alginate gel is dissolved. I think it would be important to comment about whether this printing technique can be used with more than one species - either by saying an experiment has been done.

Reply 1: We thank the reviewer for the nice comments on our manuscript and the useful suggestions. The reviewer's suggestion provides broader insights into the biofilm development process. In this manuscript, we just use one strain of bacteria, *Escherichia coli* MG1655 *ΔcsgA*. We plan to test other strains in future work.

In our current work, we have not tried using a wild-type strain of bacteria that is known to be a good biofilm former. Our bioink is composed of sterilized alginate, which is a natural polymer. Our 3D printing application should be able to be expanded to those strains that have good biocompatibility with alginate. We have added text describing this potential at lines 424-425.

2. Although this is a 3D printing technique (it uses a 3D printer and a bio-ink), it seems like everything that can be printed is really 2D. **Can you build in the z dimension via an additive process?** It seems like, because of the requirement to contact calcium to solidify the ink, that you can really only build a single layer. **Is it possible to coat the printed material with calcium so that you can build in an additive manner?** Has this been explored? If not, or if it is not possible to build truly 3D objects, **I think this needs to be addressed as a limitation in the text.** Perhaps it is possible, but that is not clear to me the way the manuscript is written. This essentially seems like a biofilm layer rather than patterning in three dimensions, which is still a useful way to develop better model systems. I feel like the way it is written, it is implied that there is more 3D control than there actually is due to the requirement of calcium contact.

Reply 2: We thank the reviewer for this crucial comment and suggestion. We agree that it is important to address that we have this ability to creating 3D structures. With our current technology, it is possible to create printed biofilms with 3D architecture. When printing a multi-layer bio-ink hydrogel, each printed layer is solidified upon contacting the calcium ions that have diffused into the previous printed layer. Each layer is printed with a 10-minute waiting interval between printing different layers to allow time for sufficient calcium ions to diffuse up through the lower layers to allow solidification of the upper layers. This mechanism is described in newly added text on lines 406-414.

In our revised manuscript, we have added three new figures, showing images of the top view and side view of printed hydrogels containing gels 1 layer, 3 layers, or 5 layers (Figure 4). We report on the height and width of printed hydrogels containing different numbers of layers (Figure 5), which fall in the millimeter-to-sub-millimeter range and increase incrementally with the deposition of additional layers. Finally, we printed engineered *E. coli* containing a plasmid encoding for the inducible production of curli biofilm proteins into multi-layered structures of 1, 3, or 5 layers. We demonstrate that sodium citrate treatment does not dissolve these multi-layered printed structures, while it is able to dissolve equivalent structures containing *E. coli* that do not produce curli proteins (Figure 6). These new figures provide a full demonstration that our 3D printer can print engineered bacteria that form three-dimensional biofilms.

3. Is contamination an issue? The authors don't describe sterilizing the ink and then inoculating the

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cells. Also, do you need to sterilize the tubing and any printer components? Since the only strains used required antibiotics for selection, it is possible that you are reducing contaminants by adding antibiotics to the ink and/or media. If you move to any wild type experiments, contamination and sterilization/disinfection of the printing equipment may be a bigger issue.

Reply 3: We thank the reviewer for this important comment. We have described in the protocol at step 3.1 (lines 151-152) that the 3% alginate solution should be heated up to the boiling point three times before use. This process is to sterilize the bioink; we have added new text explaining this feature at line 152.

Contamination can be avoided during the experimental process for many bacterial strains. We apply an antibiotic both in the bioink and the printing plate. The printing tip, tubing system, and syringe have already been sterilized before we open the commercial package, and the tip and syringe are replaced for each print. We appreciate the reviewer's comment that experiments utilizing wild-type bacterial strains may require additional sterilization measures, such as replacing or disinfecting the tubing system between prints. We have added new text discussing this at lines 425-429.

Minor Concerns:

1. The authors say that you can attach multiple pipette tips in the print head for multiple bio-inks. Has this been attempted? It should be feasible. Could you potentially use this to pattern multispecies biofilms? I think this could be a useful extension of this technique and worth mentioning in the manuscript.

Reply 1: It is possible to attach multiple pipette tips in the printhead of our printer for printing multiple bio-inks. The printhead contains two holes of different sizes (see below). Both are large enough to accommodate a printing tip, and additional holes could be created in the future using a metalworking lathe. While we did not print multiple species of bacteria in our experiments, it is a good idea to try in future work. Text discussing this has been added to the text at lines 334-336.

2. You show that curli production (a proxy for biofilm formation here) is inducible. My question is whether a biofilm phenotype can develop naturally based on the cells being patterned in close proximity and being fixed in space? It is implied that cells are metabolically active since curli are produced, but how do growth and metabolism correlate to a control biofilm grown in a different setting. For example, if you grow a control biofilm on a membrane outside of the alginate ink, is the growth rate and overall metabolism similar to inside the ink? Does the ink influence growth rate or metabolic pathways at all??

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Reply 2: We thank the reviewer for this question. We have studied this question previously with *E. coli* MG1655 in various growth states, where we saw that growth in solidified bio-ink vs. liquid bio-ink has minimal effect on growth rate and survival over the course of several days [Lehner et al., ACSSynBio 2017, 6:1124-1130; Schmieden et al., ACSSynBio 2018, 7:1328-1337]. The question of overall metabolism of printed bacteria is quite an interesting and promising topic, which could be studied intensively in future work. For the current manuscript, the development of the bioink and printing method is what we have highlighted.

3. Have you tried changing or optimizing the ink at all? Does changing the modulus/stiffness of the material change the biofilm? Could additives be used to mimic conditions found in nature? For example, biofilms in water cooling tanks may have very different mechanical properties from biofilms found in wounds. Can you "customize" your environment, or have you only tried the one ink described here? You show a table with the gel width at varying extrusion and print head speeds. Do these printing conditions change the rigidity of the material? Changing the ink or printing conditions may allow you to tune properties of the biofilm (such as diffusion) to match what is found in nature and create a better model.

Reply 3: We thank the reviewer for the specific questions. Our bio-ink has been optimized to produce high-resolution printed hydrogels that solidify quickly and support robust bacterial production of engineered proteins. We have not tested the material properties of our ink or tried to tune them by changing the printing conditions or the bio-ink composition. We plan to investigate this more intensively in our future work; we thank the reviewer for this nice suggestion.

4. You demonstrate this technique with one 3D printer (CoLiDo 3D-P). Will this only work with that one printer or could you modify other commercially available printers as well? If it is specific to this printer, I think that should be noted. It looks like it could potentially work with other extrusion-based printers.

Reply 4: Thanks for this comment. This printing technique should be able to work with any type of commercial 3D printer for which tubing can be attached to the printhead. We have added new text discussing this at lines 255-257.

Square shape G-code

Star shape G-code

Yin-yang and triangle shapes G-code