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High-resolution patterned biofilm deposition using pDawn-Ag43

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INGMAR RIEDEL-KRUSE, Ph.D.
Assistant Professor of Bioengineering

June 12th, 2018

RE: Article submission Jin & Riedel-Kruse "Optically patterning biofilms using pDawn-Ag43"

Dear Editor,

We are pleased to submit our manuscript, "Optically patterning biofilms using pDawn-Ag43" for consideration for publication in *Journal of Visual Experiments*. All authors have approved this manuscript.

This manuscript is closely related to and intended to accompany our earlier publication, "Biofilm Lithography enables high-resolution cell patterning via optogenetic adhesin expression" published in *PNAS* in March 2018, where we described a new and flexible method for depositing bacteria onto surfaces with high spatial resolution by using light to optogenetically drive the expression of adhesins. This manuscript documents in greater detail the established protocol for patterning bacterial biofilms, discussing the nuances involved in the technique, critical steps, alternatives and modifications of the protocol, its advantages and disadvantages, as well as offering tips and tricks regarding troubleshooting.

This manuscript will be of interest to the interdisciplinary audience of *Journal of Visual Experiments*, as it provides a detailed insight into our biofilm patterning technique, for readers interested repeating and extending on our published results, and in using it to investigate structure of natural biofilm colonization, bacterial community growth, and microbial ecology, as well as applications such as metabolic engineering and patterning/functionalization of biomaterials. Moreover, we hope this protocol may be of interest to a wider community of bio-artists and biology educators, given its convenience and reasonable material requirements.

Sincerely,

Xiaofan Jin

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TITLE:

High-resolution Patterned Biofilm Deposition Using pDawn-Ag43

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KEYWORDS:

Biofilm, patterning, optogenetics, synthetic biology, *E. coli*, lithography

SUMMARY:

We demonstrate a method for depositing *Escherichia coli* bacterial biofilms in arbitrary spatial patterns with a high resolution using optical stimulation of a genetically encoded surface-adhesion construct.

ABSTRACT:

Spatial structure and patterning play an important role in bacterial biofilms. Here we demonstrate an accessible method for culturing *E. coli* biofilms into arbitrary spatial patterns at high spatial resolution. The technique uses a genetically encoded optogenetic construct—pDawn-Ag43—that couples biofilm formation in *E. coli* to optical stimulation by blue light. We detail the process for transforming *E. coli* with the pDawn-Ag43, preparing the required optical set-up, and the protocol for culturing patterned biofilms using pDawn-Ag43 bacteria. Using this protocol, biofilms with a spatial resolution below 25 µm can be patterned on various surfaces and environments, including enclosed chambers, without requiring microfabrication, clean-room facilities, or surface pretreatment. The technique is convenient and appropriate for use in applications that investigate the effect of biofilm structure, providing tunable control over biofilm patterning. More broadly, it also has potential applications in biomaterials, education, and bio-art.

INTRODUCTION:

Biofilms are surface-attached communities of microbes, and are well-known for their strong structure-function coupling—namely, spatial geometry and patterning of biofilms play an important role in biofilms' overall community function (and *vice versa*)[1]. The small length scales involved in biofilm structure—on the order of tens of microns[2]—make tunable and convenient control of biofilm patterning a challenging problem. Here we demonstrate a protocol that allows for biofilms to be precisely patterned in arbitrary geometries, based on optical illumination.

The protocol presented here uses pDawn-Ag43[3], an optogenetic construct that couples biofilm formation in *E. coli* bacteria to optical illumination by driving the expression of Ag43 (an adhesin gene responsible for surface adhesion and biofilm formation) under the control of pDawn[4] (a transcriptional regulator controlled by optical illumination). The method is convenient to use and can pattern biofilms on various surface environments, including enclosed (transparent) culture chambers. Compared to existing cell deposition methods, such as droplet-based deposition[5] or surface prepatterning/treatment[6], pDawn-Ag43 does not require microfabrication or clean-room facilities and does not require materials beyond those available to a typical microbiology laboratory. It is able to pattern with a spatial resolution below 25 µm, approaching the spatial dimensions of microcolonies in naturally existing biofilms[2]. Overall, this technique provides the ability to manipulate biofilm structure, which then opens many avenues to study microecology in bacterial communities[7]. Additionally, patterned biofilms may provide a convenient platform upon which to engineer useful biomaterials[8,9]. In this paper, we discuss the basic protocol required for patterning biofilms using pDawn-Ag43 and, furthermore, address potential modifications and troubleshooting related to the method.

PROTOCOL:

1. Preparation of pDawn-Ag43 Bacterial Strains

1.1. Transform pDawn-Ag43 into an *E. coli* strain of interest (**Figure 1**).

1.1.1. Grow a cloning strain hosting pDawn-Ag43 plasmid (obtainable from a plasmid repository) by inoculating the strain in LB broth supplemented with 50 µg/mL spectinomycin (LB+spec) in a culture tube (overnight in a shaking incubator at ~250 rpm, 37 °C). Then, use a miniprep kit to harvest the purified pDawn-Ag43 plasmid[10].

1.1.2. Choose an *E. coli* strain of interest to be patterned. Thus far, pDawn-Ag43 has been verified to work in MG1655[3] and BW25113.

1.1.3. Use an established protocol to generate competent (*e.g.*, chemically competent[11] or electrocompetent[12]) stocks of the chosen *E. coli* strain (*e.g.*, MG1655).

1.1.4. Transform pDawn-Ag43 plasmid into the competent bacteria[11,12], followed by 1 h recovery, and plate it on LB+spec agar plates. Allow colonies to grow overnight at 37 °C.

1.2. Store the pDawn-Ag43 transformed strains.

1.2.1. Inoculate a single colony of pDawn-Ag43 from an LB+spec agar plate into LB+spec broth in a culture tube and grow it in a shaking incubator (at ~250 rpm, 37 °C) to exponential phase (OD600 ~0.4 - 0.8).

1.2.2. Prepare stock to store the pDawn-Ag43 transformed strain at -80 °C long-term by mixing 1 mL of culture with 1 mL of 50% sterile glycerol in a cryo-tube to obtain a 25% glycerol freezer stock. Store this in a -80 °C freezer.

1.2.3. If additional plasmids (*e.g.*, fluorescent reporter plasmids) need to be transformed, create competent cells[11,12] from the pDawn-Ag43 transformed strain and repeat the transformation process[11,12] for additional plasmids as needed.

Note: If using electroporation[8], it is possible to cotransform multiple plasmids (including pDawn-Ag43) simultaneously; however, a simultaneous transformation is not recommended using chemical transformation methods, as the large size (> 10 kB) of pDawn-Ag43 means transformation efficiencies are reduced.

2. Preparation of the Projector Optical Set-up for Illuminating Bacteria

2.1. Obtain a bacterial incubator with non-transparent walls and a hole for passing cables, a portable projector capable of fitting and functioning inside the bacterial incubator, and a laptop equipped with software for presentation-projection (see **Table of Materials**). When choosing the projector and incubator, ensure that the minimal focus distance of the projector is less than the interior height of the incubator.

2.2. Place the projector inside the incubator at the bottom, with the aperture pointing directly upward, at the ceiling, where the biofilm culture chamber is attached (**Figure 2**).

2.3. Fix the projector in place by constructing a set-up with an optical breadboard base connected to a vertical post, in turn connected to a horizontal post which screws into the projector (**Figure 2**). Note that this set-up can be modified depending on the projector/available parts. Ultimately, the key requirement is that the projector is solidly fixed near the bottom of the incubator, with the aperture pointing upward.

2.4. Connect the laptop *via* the display cable (*e.g.*, HDMI) to the projector inside the incubator.

2.5. If the surfaces—especially the ceiling—of the interior of the incubator are reflective (*e.g.*, metal polished surface), cover them with dark matte surfaces to minimize reflections.

2.6. Use tape to attach an empty 'dummy' culture dish to the ceiling of the incubator. Note that, as with the projector, there are multiple acceptable ways to attach a culture dish; ensure that the transparent bottom surface of the culture chamber, where illumination occurs, is not covered.

2.7. Adjust the focus on the projector by turning the focusing knob so that the focusing plane coincides with the bottom surface of the biofilm culture dish (*e.g.*, a well plate) attached to the ceiling of the incubator (**Figure 2**). The projector should illuminate a sharp, non-blurry image onto

the bottom of the culture dish. Remove the 'dummy' culture dish once the projection has been optimized.

2.8. Using laptop software, direct the projector to illuminate the full field of view with maximum blue illumination (*e.g.*, RGB = [0, 0, 255]) by presenting a full blue slide.

2.9. Measure the illumination intensity of the projector using an optical power meter by placing the photodetector head at the incubator ceiling and reading the intensity on the corresponding power meter calibrated to 460 nm of wavelength light. Follow the instructions on connecting and calibrating the photodetector for the specific power meter used.

2.9.1. Reduce ambient light (*e.g.*, turn off room lights, or place the incubator away from light sources) as much as possible prior to making illumination intensity measurements.

2.10. Adjust the illumination intensity using an adjustable neutral density filter placed at the projector aperture. Rotate the filter to adjust the illumination intensity measured by the power meter, until the illumination intensity in the center of the blue-light projected region reads 50 $\mu W/cm^2$.

Note: While it is possible to adjust the illumination intensity by using lower blue RGB values on the software end, using a filter while maximizing the blue RGB value has the advantage of maximizing the optical contrast of the system between illuminated *vs.* dark regions.

2.11. Draw arbitrary patterns using the presentation/projector software on the laptop and display these patterns on the ceiling of the incubator, using the projector.

Note: Biofilm-forming regions should be drawn with maximum blue illumination (*e.g.*, RGB = [0, 0, 255]), non-biofilm-forming regions with no illumination (*e.g.*, RGB = [0, 0, 0]).

3. Culturing Patterned Biofilms

3.1. Prior to illumination, prepare the pDawn-Ag43 bacteria, starting from the glycerol freezer stock, so that they are reliably induced at the late exponential growth phase for illumination (**Figure 3A**).

3.1.1. Streak a pDawn-Ag43 strain from glycerol stock onto LB+spec agar plates. Allow colonies to grow overnight (37 °C).

Note: From here until the illumination culture step, ensure the cells stay in the dark as much as possible—brief periods at ambient illumination (*e.g.*, for subdilution) are no problem.

3.1.2. Inoculate a colony of pDawn-Ag43 bacteria from an agar plate in LB+spec broth and grow the culture overnight to the stationary phase (in a shaking incubator at ~250 rpm, 37 °C, for ~16 h).

3.1.3. Subdilute the culture at a ratio of 1:1,000 with LB+spec broth (*e.g.*, add 1 µL of overnight culture to 1 mL of fresh LB+spec).

3.1.4. Allow the subdiluted culture to grow until the late exponential/early stationary phase (OD ~1.0, ~6 h in a shaking incubator at ~250 rpm, 37 °C).

3.1.5. While waiting for the culture to grow, prepare M63 media with 1x M63 salts, 1 mM $MgSO_4$, 0.2% glucose, 0.1% casamino acids, and 50 µg/mL spectinomycin in water (ensure the constituent parts are sterile).

3.1.6. Subdilute the late-exponential-phase culture at a ratio of 1:100 with M63 media supplemented with 50 µg/mL spectinomycin. Then, introduce the dilution into a biofilm culture dish (*e.g.*, pipette the diluted sample into a well plate).

Note: The volumes required for this 1:100 subdilution depend on the culture dish being used (*e.g.*, if using a 6-well polystyrene well plate [non-tissue-culture treated], a single sample would require adding 20 µL of culture to 2 mL of M63+spec, as the standard well volume of a 6-well plate is ~2 mL).

3.2. As the samples are now ready for illumination, tape the culture dish to the ceiling of the incubator, ensuring that the surface at the bottom of the dish is transparent for illumination from below by the projector.

Note: It is important to ensure that the ceiling of the incubator is not reflective, to minimize stray illumination. Stray illumination can also be reduced by using black-walled plates as culture dishes, although this is not strictly necessary—if using such plates, ensure the bottom surface is transparent.

3.3. Allow the biofilms to culture in the incubator overnight (16 h with no shaking, at 37 °C). Note that some projectors become less reliable at higher temperatures. If that is the case, culture at lower temperatures (*e.g.*, 30 °C), and do not forget that the incubation time may need to be increased, depending on the *E. coli* strain.

4. Imaging Patterned Biofilms

4.1. After the overnight growth of the biofilm samples, remove the culture dish from the incubator. The dish will have biofilm bacteria attached to its bottom surface where it has been illuminated, as well as planktonic bacteria dispersed in the liquid media above.

4.2. Discard the planktonic cells by removing the liquid media from the culture dish (*e.g.*, by gently aspirating with a pipette).

4.3. Rinse the sample 2x with a phosphate-buffered saline (PBS) solution to remove the remaining planktonic cells (**Figure 3B**) by gently pipetting in PBS, followed by aspiration.

4.4. If the cells are fluorescently tagged, directly image the samples using fluorescence microscopy[13] (*e.g.*, wide-field[13], 3-D confocal[14], *etc.*).

Note: Fluorescent biofilms can also be preserved using a self-hardening mounting medium. Apply one drop of mounting media to a biofilm sample, cover it with a glass coverslip, taking care not to capture any bubbles underneath, and allow it to harden overnight before imaging.

4.5. If the bacterial cells used are not fluorescently tagged, apply the crystal violet stain technique[15] (**Figure 3B**) to enhance biofilm contrast prior to imaging.

5. Protocol Modifications/Alternatives

5.1. Grow pDawn-Ag43 bacteria on different surfaces.

5.1.1. Put glass or poly-dimethyl-siloxane (PDMS) coupons (*e.g.*, coverslips or thin strips of PDMS) into well plates prior to the addition of a bacterial sample/illumination, and follow the same protocol as before to pattern pDawn-ag43 biofilms on glass and PDMS.

5.2. Grow pDawn-Ag43 bacteria inside a transparent, enclosed culture chamber.

5.2.1. Generate a culture chamber mold for PDMS.

5.2.1.1. For a basic culture chamber, generate mold by attaching a hard, rectangular prism to a flat surface (the prism will become a cavity in the PDMS that serves as the culture chamber once the mold is cast). Note that more intricate culture chamber molds can be fabricated using soft lithography[16].

5.2.2. Cast a PDMS cavity by pouring PDMS into the mold and allowing it to cure (for a detailed soft lithography protocol, see JoVE's Science Education Database[16]).

5.2.3. After curing, trim any excess PDMS, punch inlet/outlet channels into the cavity/culture chamber and bond the cavity to a flat surface (*e.g.*, glass/polystyrene) by firmly pressing the PDMS onto the flat surface, leaving the cavity between the surface and the PDMS ceiling as the biofilm culture chamber.

Note: More permanent bonding based on plasma treatment[16] can also be used, but the chips will then not be reusable.

5.2.4. Follow the culture protocol as before, using a syringe with blunt tip needles (instead of a pipette) to introduce the bacterial sample into the culture chamber/rinse with PBS buffer (**Figure 3C**).

5.2.4.1. If using a temporarily bonded cavity, use only negative pressure to pull liquid into/out of the chamber, to ensure that the cavity does not become unbonded from the underlying glass/polystyrene surface.

5.3. Grow pDawn-Ag43 bacteria using a film photomask for structured illumination.

5.3.1. Design a biofilm pattern using CAD software compatible with a film photomask printer/print service. The photomask film design should be clear in regions where the biofilm is meant to be printed, and black/opaque elsewhere. When complete, send the photomask file to the printer/printing service and await the return of the physical photomask.

5.3.2. Instruct the projector to illuminate a full field of view with maximum blue illumination (*e.g.*, RGB = [0, 0, 255]) using laptop software.

5.3.3. Cut out a region of interest from the larger film photomask, and tape it directly to the bottom of the biofilm culture dish prior to introducing the bacterial sample for overnight illumination (**Figure 3D**). Culture the biofilms as before and remove the photomask after culturing, prior to imaging.

REPRESENTATIVE RESULTS:

As seen in **Figure 4A**, pDawn-Ag43 bacteria were used to generate biofilms patterned in polystyrene well plates with projector illumination (the projector was set to illuminate a polka-dot pattern), imaged through brightfield microscopy with crystal violet stain as a contrast agent, and fluorescence microscopy using red-fluorescent-protein-expressing bacteria. Fluorescent biofilm samples can also be imaged using confocal microscopy[14] to obtain images of the biofilm in 3-D (**Figure 4B**). In **Figure 4C**, we illustrate the high-resolution patterning possible by using a film photomask to provide patterned illumination to the biofilm sample. Finally, in **Figures 4D** and **4E**, we demonstrate examples of patterning on glass and PDMS surfaces, as well as enclosed PDMS culture chambers—these illustrate the different types of environments where pDawn-Ag43 patterning can be applied.

FIGURE AND TABLE LEGENDS:

Figure 1: Preparation of pDawn-Ag43 bacteria (protocol section 1). Preparing pDawn-Ag43 bacteria capable of light-regulated biofilm formation involves purifying pDawn-Ag43 plasmid from a host cloning strain, transforming it into an *E. coli* strain of interest, and creating bacterial freezer stock for long-term storage.

Figure 2: Preparation of an optical set-up for biofilm sample illumination (protocol section 2). The optical set-up is housed inside a bacterial incubator and consists of a computer-connected projector illuminating a biofilm sample.

Figure 3: Culture protocol for patterning biofilms (protocol section 3). (**A**) Prior to illumination, pDawn-Ag43 bacteria are prepared prior to patterning such that they are reliably induced at the proper growth phase. (**B**) After overnight illuminated growth, a patterned biofilm will be present at the bottom of the culture dish, along with planktonic cells in the liquid media, and after some further processing, the biofilm is ready for imaging. (**C**) As an alternative to well plates, biofilms can be cultured in enclosed culture chambers such as a molded PDMS cavity. In this case, syringes attached to blunt tip needles can be used to introduce the sample and flush liquids out of the chamber. (**D**) As an alternative to projector-based illumination patterns, patterns can also be generated by taping film photomasks directly to the bottom of biofilm culture chambers. In this case, the projector should be set up to illuminate blue light across the full field.

Figure 4: Representative results of biofilms patterned using pDawn-Ag43. All results were obtained using an MG1655 *E. coli* host strain. (**A**) pDawn-Ag43 bacteria were used to generate biofilms patterned in polystyrene well plates with projector illumination (the projector was set to illuminate a polka-dot pattern), imaged through brightfield microscopy with crystal violet stain as a contrast agent, and fluorescence microscopy using red-fluorescent-protein-expressing bacteria. (**B**) Fluorescent biofilm samples are imaged with confocal microscopy to obtain 3-D images of the biofilm. (**C**) High-resolution biofilms can be patterned with a film photomask to provide patterned illumination to the biofilm sample. (**D**) Biofilms can be patterned on glass and PDMS surfaces. (**E**) Biofilms can be patterned in enclosed culture chambers. This figure has been adapted from previous work[3].

Table 1: Common troubleshooting issues.

DISCUSSION:

In light of the need for research tools that allow for biofilm structure control, we have presented an easy-to-use protocol for patterning bacterial biofilms using the pDawn-Ag43 optogenetic construct. With this technique, *E. coli* biofilms can be optically patterned on various surface environments, including enclosed chambers, with a spatial resolution below 25 µm.

Overall, this protocol can be broken down into four main sections: (1) the preparation of the pDawn-Ag43 bacteria, (2) the preparation of the optical and culture set-up hardware, (3) the pre-illumination bacterial growth steps, and (4) the post-illumination rinses and imaging.

The critical part of section 1 is the successful transformation of pDawn-Ag43 plasmid into the *E. coli* strain of interest. This is facilitated by isolating high-quality purified plasmid and generating high-quality competent cells for transformation (**Table 1**, troubleshooting).

The critical part of section 2 is the optimization of the projector set-up so that the illumination intensity is adjusted to 50 µW/cm^2 at the 460-nm wavelength, and the projector is properly focused at the biofilm sample height. Note that in this protocol, we describe an inverted illumination set-up where the projector shines light from below, upward toward the biofilm

sample. The advantage of this set-up is that the light only needs to travel through the bottom of the culture dish before reaching the biofilm formation surface. Illumination from above means that the light would have to travel through the liquid media above the biofilm surface, which, during the course of the growth, gets cloudy with planktonic cells. In addition to these concerns, it is also important to minimize stray light in the optical set-up as much as possible, for example, by covering up reflective surfaces on the interior of the incubator—this helps to obtain sharper patterned biofilms. On a related note, sharper biofilm patterns can also be obtained by using a photomask to control illumination patterning (**Figure 4C**). Common issues requiring troubleshooting include projector reliability issues at higher temperatures (*e.g.*, 37 °C), which can be minimized by incubating the biofilm growth at lower temperatures (*e.g.*, 30 °C), as well as computer software that causes operating system updates or blue light filtering during overnight growth (**Table 1**). It is also important to note that, depending on the projector and incubator model used, it is also possible that heat generated from the projector will result in a higher interior temperature than the incubator set temperature, which may need to be corrected.

The critical part of section 3 is obtaining reliable and repeatable bacterial samples before they are induced by illumination. For this reason, it is recommended to obtain clonal colonies of pDawn-Ag43 bacteria by streaking them out on an agar plate and then using the liquid culture steps to ensure that the bacteria are illuminated/induced at the late exponential growth phase in a repeatable manner.

Finally, the critical part of section 4 is to thoroughly, but also gently, wash away the planktonic cells remaining after the biofilm patterning protocol; thus, it is recommended to perform multiple gentle rinse steps with PBS.

Compared to existing techniques for cell patterning[5,6], optical biofilm patterning based on pDawn-Ag43 has a reasonably low barrier of entry to use, in that it does not require microfabrication, clean-room facilities, complex chemistry, or surface pretreatment, yet is still able to pattern with the high resolution (25 µm) typically associated with microfabrication techniques. The method extends previous work on bacterial photolithography for controlling gene expression[17]. Currently, pDawn-Ag43 plasmid is limited to *E. coli*, as it uses a pUC-based origin of replication, but pDawn and Ag43 are both compatible in other (Gram-negative) bacterial species. Genetic techniques are available for potentially introducing light-regulated biofilm formation to different bacterial species and represents a potential direction for future research. Another potential limitation of the technique is that it works by increasing biofilm formation in strains with weak native biofilm formation (*e.g.*, MG1655 *E. coli*). However, strains with strong native biofilm formation have biofilms form regardless of illumination conditions, precluding patterned biofilm formation using pDawn-Ag43 as described here; yet optogenetic techniques may still prove applicable in regulating biofilm formation. We note that in other contexts, alternative methods of biofilm patterning may be available, such as *via* optical c-di-GMP modulation[18].

Overall, pDawn-Ag43 based patterning will be appropriate for use in applications that investigate the effect of biofilm structure on function[1] and, therefore, could benefit from tunable control

over biofilm patterning—a particularly relevant example to highlight is the study of microbial ecology in biofilms[2]. Future directions include making patterned biomaterials[8,9] and/or structured bacterial communities. Alternative applications of this accessible protocol also include bio-art[19], given the clear aesthetic potential, as well as formal and informal life science education[20-22]. From an educational perspective, the protocol described here combines many relevant techniques (bacterial culture, transformation, optics/optogenetics) and is also modularly extendable (*e.g.*, include microfluidics).

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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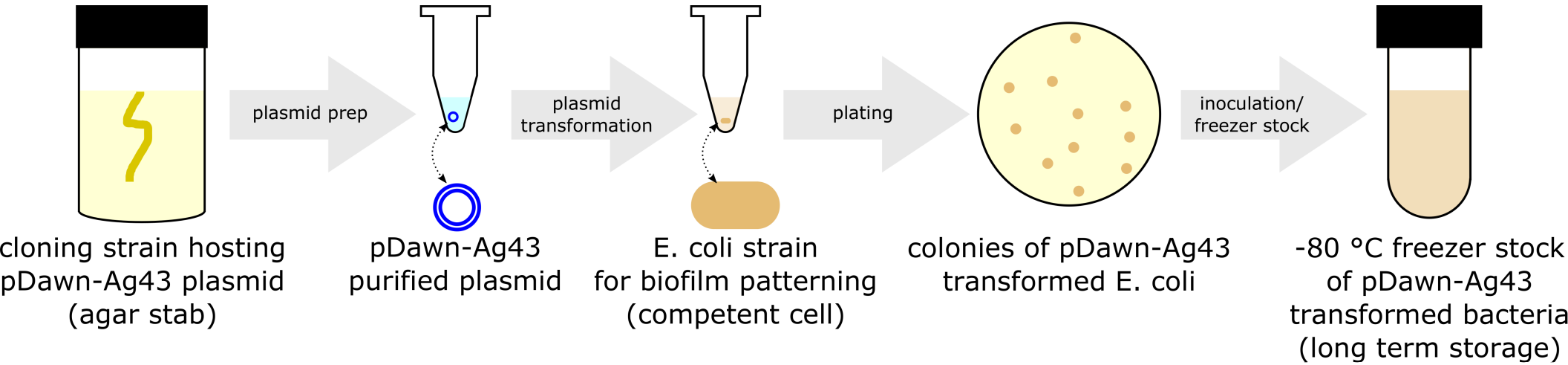

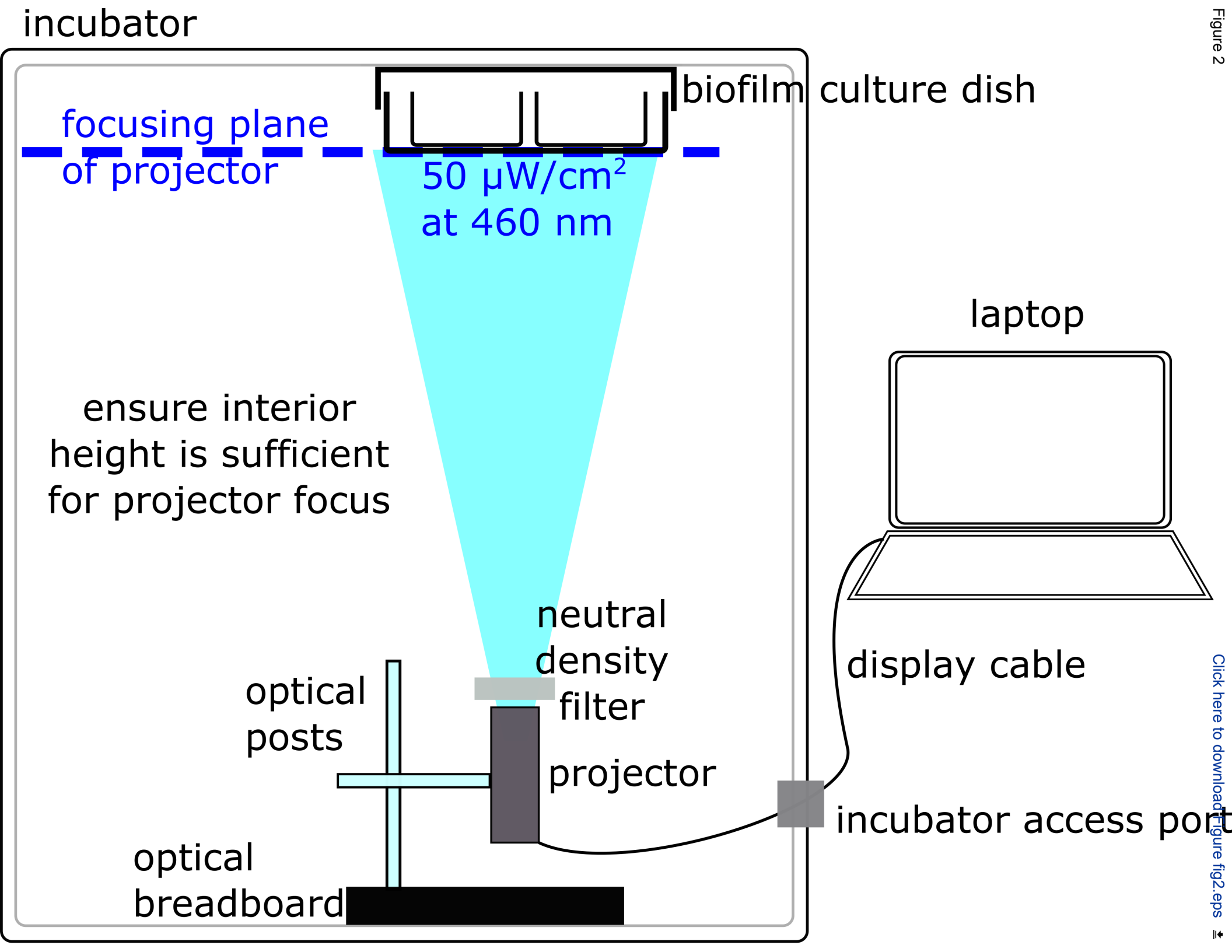

Figure 2

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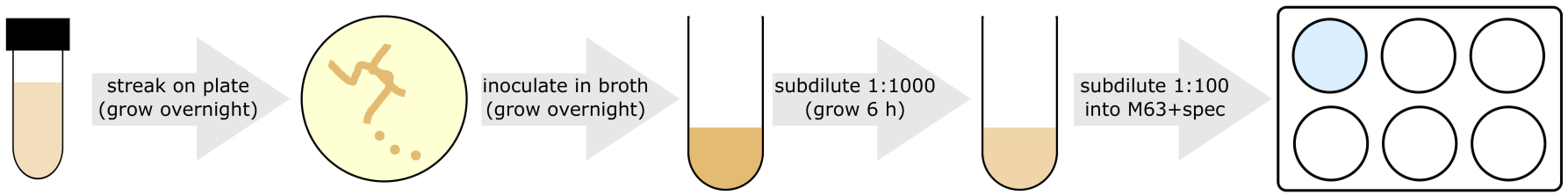
Figure 3

A pre-illumination



pDawn-Ag43 bacteria freezer stock

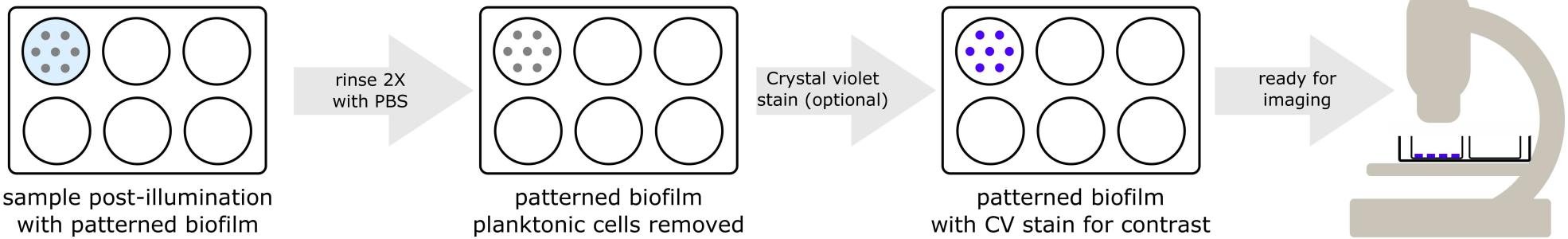
Colonies on LB agar+spec

Stationary phase culture in LB+spec

Late exponential phase culture in LB+spec

sample in culture dish ready for illumination

B post-illumination

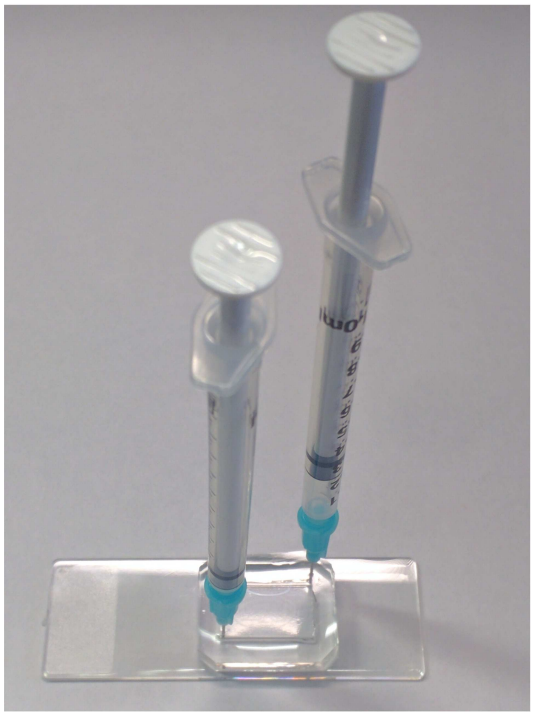


sample post-illumination with patterned biofilm

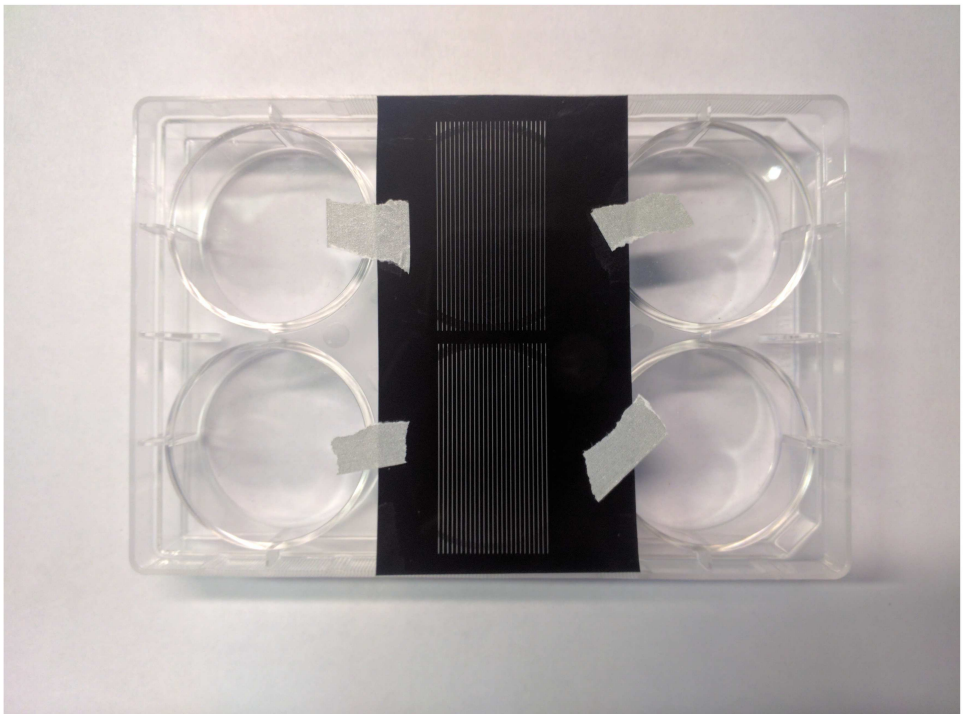
patterned biofilm planktonic cells removed

patterned biofilm with CV stain for contrast

C



D



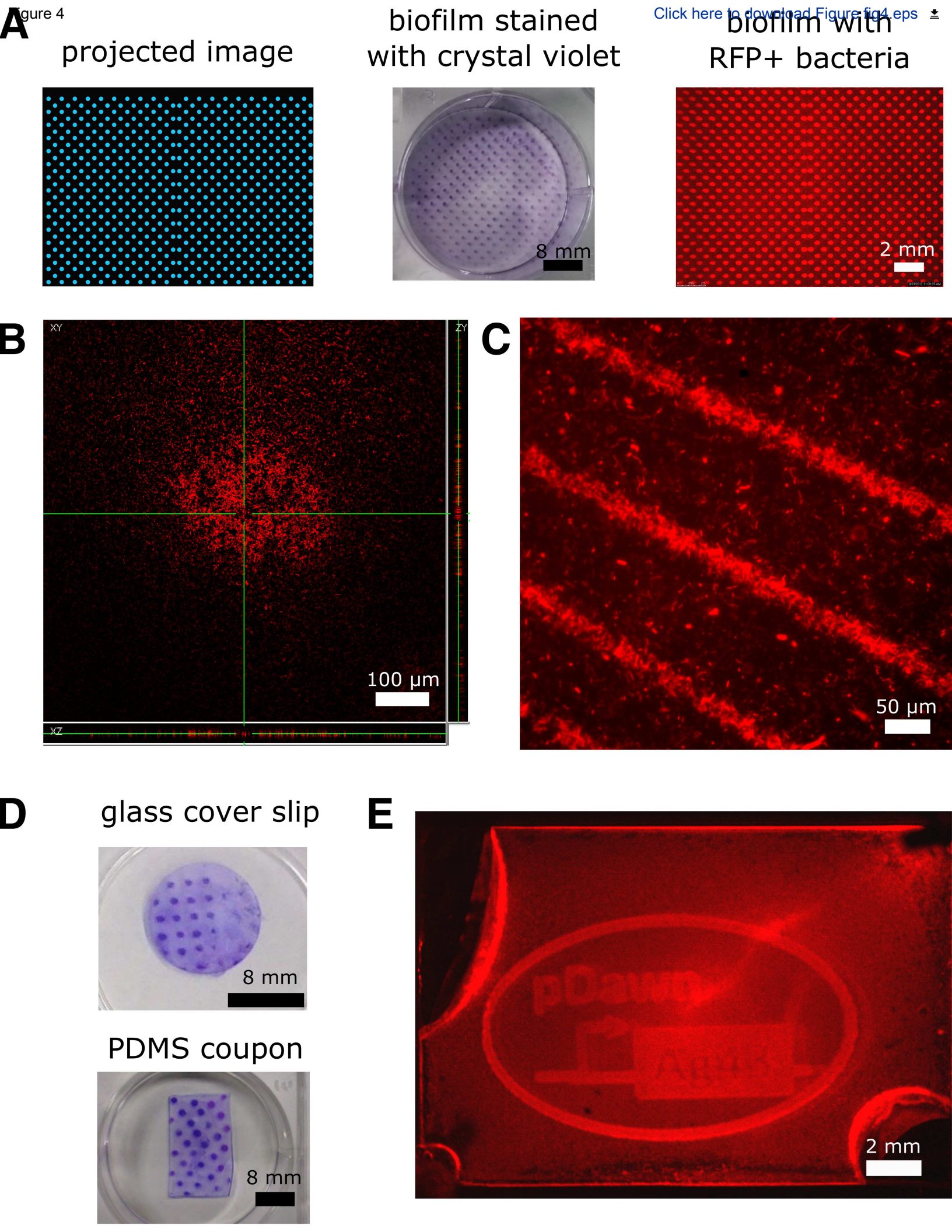


Table 1

Click here to download Table Table 1 - Common troubleshooting 080218.xlsx

Problem
Tranforming pDawn-Ag43 into host strain - no colonies
Projector illumination turns off / inconsistent overnight
No/low levels of biofilm formed after overnight illumination, no planktonic cells growth either (i.e. liquid is clear)
No/low levels of biofilm formed after overnight illumination, only planktonic cells (i.e. liquid is cloudy)
Fuzzy biofilm patterns, high levels of background noise

Potential causes/solutions
Low plasmid concentration - check plasmid concentration on spectrometer. A typical miniprep of pDawn-Ag43 sho
Check/remake competent cells: competent cells should have transformation efficiency at least 10^6 cfu/µg verifie
Wrong (level of) antibiotic on LB agar plate - make sure to use 50 µg/mL spectinomycin for selection
Disable problematic software such as: automatic overnight software/OS updating, night-time blue-light filter
Projector may be overheating - set incubator to lower temperature while projector is turned on (e.g. 30 °C instead
Remove unnecessary sources of humidity from incubator, as these may affect projector electronics
Wrong (level of) antibiotic - make sure to use 50 µg/mL spectinomycin
Check everything is added to M63 recipe properly
Check light level, projector should be illuminating blue light at 50 µW/cm^2 at 460 nm wavelength
Try letting bacteria grow for shorter/longer time after 1:1000 LB subdilution step prior to adding to M63
Restreak bacteria on LB plate, start from fresh colony to generate overnight stationary phase culture
Ensure projector is working consistently overnight - see point above
Reduce stray light from optical illumination system, cover reflective surfaces on interior of incubator
Consider using photomask-based (as opposed to projector-based) structured illumination
Check projector is properly focused at the bottom surface of the biofilm culture chamber

ɔuld yield at least 100 ng/µL; use up to 10-100 ng for transformation
ɐd using a standard plasmid such as pUC19 - if not, remake competent cells

ɹ of 37 °C) - note projector as heat source can overheat incubator beyond set point

Table of Materials

Click here to download Table of Materials JoVE_Materials_080218.xlsx

Name of Material/ Equipment	Company	Catalog Number
DH5alpha/pDawn-Ag43	Addgene	107742
MG1655 E. coli	Coli Genetic Stock Center - Yale University	CGSC #6300
RFP expression plasmid	iGEM biobricks	J04450-pSB4K5bb
Plasmid miniprep kit	Qiagen	27104
LB broth powder	Affymetrix	75852
LB agar powder	Affymetrix	75851
Petri dishes	Fisherbrand	431760
Spectinomycin hydrochloride pentahydrate	abcam	ab141968
Glycerol	Sigma-Aldrich	G5516
M63 media salts 5X solution	Bio-world	705729
Casamino acids	Amresco	J851
D-glucose	Sigma-Aldrich	G8270
Magnesium sulfate	Sigma-Aldrich	M7506
Crystal violet	Acros organics	212120250
Self-hardening mounting media (Shandon immumount)	Thermo Scientific	9990402
Phosphate buffered saline (PBS) solution	Gibco	10010023
6 well plate	Fisherbrand	351146
PDMS kit	Dow	SYLGARD 184
1 mL syringe	BD syringe	309659
Blunt tip needle	CML supply	901-23-050
Lab tape	Fisherbrand	15-901
Bacterial incubator	Sheldon Manufacturing	SMI6
Portable projector	Ivation	IV-PJ-PRO-4-1
Optical breadboard base	ThorLabs	MSB6
Optical post	ThorLabs	TR8
Optical post right-angle clamp	ThorLabs	RA90
Mounting base	ThorLabs	BA1S

1/4"-20 screw	ThorLabs	SH25S050	
1/4"-20 set-screw	ThorLabs	SS25E63D	
Optical power meter	Newport		840
Optical power meter detector	Newport	818-UV	
Adjustable ND filter	K&F Concept	SKU0689	
Presentation-projector software	Microsoft	Powerpoint	
CAD software	Autodesk	AutoCAD	
Film photomask	Fineline Imaging	n/a	

Comments/Description

DH5alpha cloning strain hosting pDawn-Ag43 plasmid - plasmid needs to be moved to E. coli strain of interest prior to use
MG1655 was used as E. coli strain of interest in this paper's representative results

Many options exist to obtain fluorescent bacteria - if using plasmid, ensure backbone does not conflict with colE1 ori of pDawn-Ag43

Add 20 g/L to water, autoclave, add 50 µg/mL spectinomycin to get sterile LB+spec
Add 35 g/L to water, autoclave, add 50 µg/mL spectinomycin, pour into petri dishes to get sterile LB+spec plates

Make 1000x stock 50mg/mL in water, filter sterilize and dilute into media as needed
Mix at 1:1 ratio with water, sterilize by autoclave or filter to obtain 50% glycerol
Add cas-amino acids, glucose and $MgSO_4$, bring to 1X salts concentration by adding sterile water
Make 20% stock in water, filter sterilize and add to M63 as supplement (final concentration 0.1%)
Make 20% stock in water, filter sterilize and add to M63 as supplement (final concentration 0.2%)
Make 1 M stock in water, autoclave and add to M63 as supplement (final concentration 1 mM)
Dilute to 0.1% in water prior to use

Use to preserve samples over long term for fluorescence imaging

Can also use PBS prepared from powder / tablets
Used as biofilm culture dish for representative results
Can be used to make enclosed microchamber cavities using soft lithography
For use with liquid handling with enclosed microchambers
Attaches to 1 mL syringe
Use to attach culture chamber to incubator ceiling
Ensure interior height of incubator is tall enough to focus projector at the ceiling
Many portable projector models exist, pDawn-Ag43 has been tested with multiple models including LED/laser based, with blue light channel
Base for optical setup to hold projector - many other setups possible, just need to hold projector firmly at bottom of incubator, pointing upw
2 posts needed - one to be set up vertically extending out of breadboard base, one horizontally attached via right-angle clamp
Connects vertical and horizontal posts
Connects optical breadboard base and vertical post

Attaches vertical post to mounting base, mounting base to breadboard base
Connects horizontal post to projector via tripod screw-hole
Use with power meter detector to measure projector illumination intensity - many power meter models exist, using one that has extendable
Connects to power meter (above) - UV detector not strictly necessary as blue light is within visible range
Adjustable (by rotating) neutral density filter - place above projector aperture

Any software that allows drawing / presentation will suffice
Used for designing photomasks, many mask printing services are compatible with AutoCAD files
Many photomask printer services exist for high resolution (>30000DPI) film photomask printing

ranging from 450-460nm central wavelength
ards

detector will facilitate measurement

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STANFORD
UNIVERSITY

INGMAR RIEDEL-KRUSE, Ph.D.
Assistant Professor of Bioengineering

July 27th, 2018

RE: Revised article submission Jin & Riedel-Kruse "High-resolution patterned biofilm deposition using pDawn-Ag43"

Dear Editor,

Thank you for your time in assessing our original submission and coordinating with reviewers. We are pleased to submit our revised manuscript, "High-resolution patterned biofilm deposition using pDawn-Ag43" for consideration for publication in *JOVE*. All authors have approved this manuscript.

We would like to thank the reviewers and editors for their time and thoughtful comments. Please find below a copy of the original comments, alongside our responses (italicized) detailing the corresponding changes in the manuscript. We carried out the suggested changes, and we believe we were able to address all the comments in full.

Editorial comments:
Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
    a. *We have gone through the manuscript again multiple times to ensure there are no spelling or grammar issues.*
2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
    a. *Please find the permission information in the 'reprintPermission.docx' file. We have cited where appropriate in the figure legend.*
3. Figures: Please include a space between all numbers and their corresponding units, i.e., -80 °C (Figure 1), 460 nm (Figure 2), 50 µW/cm2 (Figure 2), 8 mm (Figure 4), 100 µm (Figure 4), etc.
    a. *Thank you for pointing this out, we have added spaces where appropriate*
4. Please shorten the figure legends. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.
    a. *Thank you for this suggestion, we have shortened the figure legends as requested and moved the bulk of the text to the Protocol section.*
5. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.
    a. *Thank you for pointing this out, we have corrected where appropriate*
6. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.
    a. *Thank you for pointing this out, we have corrected where appropriate*
7. 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. For example: Addgene, Microsoft Powerpoint, etc.
    a. *We have removed commercial language as appropriate and moved them to the Table of Materials and Reagents*

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8. 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. Please move the discussion about the protocol to the Discussion.
    a. *We have made changes throughout the protocol section to ensure imperative tense whenever possible, and added "Note" as appropriate, while also moving several points to the discussion section.*
9. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. 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.
    a. *We have increased the level of detail in the protocols throughout, as well as specifically in addressing the following three points*
10. 1.1.4: Please specify the temperature for growing the bacteria.
    a. *Thank you for pointing this out, temperature at 37 deg C is now explicitly stated*
11. 1.2.2: Please specify at what wavelength the OD is recorded.
    a. *Thank you for pointing this out, OD600 is now explicitly stated*
12. 4.6: Please describe how this is done.
    a. *Thank you for pointing this out, given the multitude of variations on confocal microscopy for biofilms, we have added a reference on confocal microscopy for reference.*
13. 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.

14. 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.

15. 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.
    a. *Video section has been highlighted*

16. Please reference all data and figures in the manuscript. Currently Figure 3 and Table 1 are not described in the manuscript.
    a. *Thank you for pointing this out, we have modified the text to more closely refer to the figures, and Table 1 + Figure 3 are now explicitly referred to.*

17. As we are a methods journal, please revise the Discussion to explicitly cover 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

- a. *We have added a large section on critical steps of protocol, and reference modifications and troubleshooting in Table 1. We have also expanded section on technique limitations and existing methods to address comments by Reviewers 1 and 2.*

Reviewers' comments:

Reviewer #1:
In the manuscript, the authors provided a well-written protocol based on their previously published paper ("Biofilm Lithography enables high-resolution cell patterning via optogenetic adhesin expression." PNAS 115(14): 3698-3703), 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) Tuning the bacteria-surface interaction is the key to control the formation of patterned biofilms. Here, the authors regulated/controlled the expression of an adhesion protein on the E. coli via optogenetics tools. But this strategy may not work well in the case that bacteria-surface interaction is sufficient strong, for example, i) E. coil with a surface modified by polylysine; ii) bacterial species, like Pseudomonas aeruginosa or Bacillus subtilis, can spontaneously attach to the surface. This point should be explicitly mentioned in the section of discussion, which can help the readers to understand the limitations for applying this method in different scenarios, including different surfaces and/or different bacterial species.

Thank you for pointing this out, we have updated our discussion section to mention this potential limitation

2) A key reference published in the ACS synthetic biology should be cited (Huang, Y. J., et al. (2018). "Bioprinting Living Biofilms through Optogenetic Manipulation." Acs Synthetic Biology 7(5): 1195-1200) , where Huang et. al. provided a different strategy to show that patterned biofilms of Pseudomonas aeruginosa can be printed on surfaces.

Thank you for pointing this out, we included this in our updated discussion section

3) L. 62 e coli -> E. coli

Thank you for pointing this out, we have now corrected this typo.

Reviewer #2:
Manuscript Summary:
The paper of Jin & Riedel-Kruse describes a protocol for patterning of E.coli biofilm using blue light activated expression of the adhesion protein Ag43. It is complementary to their PNAS 2018 paper describing this system. There are a couple of minor issues that need to be addressed.

Minor Concerns:
(i) Two essential references are missing: one on the paper describing the pDawn plasmid (Moeglich's lab), another on of the first paper describing bacterial photolithograthy (Voigt's lab).

Thank you for pointing this out, we have now added these references as well as expanded the discussion on previous work.

(ii) p75: Describing E. coli at OD 0.4-0.8 as a "late exponential phase" is inaccurate. Delete 'late'.

Thank you for pointing this out, we have now corrected this mistake.

(iii) It would be helpful to have some guidance about the choice of a projector. How does one reproduce the protocol if the projector model listed in the manuscript is no longer available? Same guidance would be useful for the choice of an optical power meter.

Thank you for pointing this out, we have updated the JOVE Table of Materials to provide guidance on projector and power meter choice, pointing out that the exact models listed are not the only ones that will work.

(iv) Is there a reason to place the projector at the bottom and tape a plate at the top? It seems counterintuitive. Explain.

Thank you for pointing this out, we have updated the discussion section to mention this point: "Note in this protocol we describe an inverted illumination setup where the projector shines light from below upwards towards the biofilm sample. The advantage of this setup is that the light only needs to travel through the bottom of the culture dish before reaching the biofilm formation surface. Illuminating from above means that the light would have to travel through the liquid media above the biofilm surface, which during the course of growth gets cloudy with planktonic cells."

We look forward to hearing back from you regarding this revised submission, and are happy to respond to any further questions and comments.

Sincerely,

Xiaofan Jin

Ingmar Riedel-Kruse

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