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TITLE:

Quantifying Bacterial Surface Swarming Motility on Inducer Gradient Plates

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SUMMARY:

Here, we describe the use of inducer gradient plates to evaluate bacterial swarming motility while simultaneously obtaining multiple concentration responses.

ABSTRACT:

Bacterial swarming motility is a common microbiological phenotype that bacterial communities use to migrate over semisolid surfaces. In investigations of induced swarming motility, specific concentrations of an inducer may not be able to report events occurring within the optimal concentration range to elicit the desired responses from a species. Semisolid plates containing multiple concentrations are commonly used to investigate the response within an inducer concentration range. However, separate semisolid plates increase variations in medium viscosity and moisture content within each plate due to nonuniform solidification time.

This paper describes a one-step method to simultaneously test surface swarming motility on a single gradient plate, where the isometrically arranged test wells allow the simultaneous acquisition of multiconcentration responses. In the present work, the surface swarming of *Escherichia coli* K12 and *Pseudomonas aeruginosa* PAO1 were evaluated in response to a concentration gradient of inducers such as resveratrol and arabinose. Periodically, the swarm morphologies were imaged using an imaging system to capture the entire surface swarming process.

The quantitative measurement of the swarm morphologies was acquired using ImageJ software, providing analyzable information of the swarm area. This paper presents a simple gradient swarm plate method that provides qualitative and quantitative information about the inducers' effects on surface swarming, which can be extended to study the effects of other inducers on a broader range of motile bacterial species.

INTRODUCTION:

Bacterial swarming motility refers to the collective migration of bacterial cells across the surface of a substance. In addition to semisolid agar plates specially prepared in the laboratory[1], this phenotype is also observed on some soft substrates such as animal tissues[2], hydrated surfaces[3], and plant roots[4]. While a semisolid surface is considered one of the fundamental conditions for bacterial swarming, some species also require an energy-rich medium to support their swarming motility[5]. Flagella rotation powers both swimming and swarming motility—swimming describes the unicellular motility within a liquid environment, whereas swarming is the synchronous movement of a microbial population across semisolid surfaces.

Substrate viscosity influences bacterial motility; studies of pathogenic microbes, such as *Helicobacter pylori*, have shown that the pathogen's motility changes depending on the mucin layer viscosity, which is influenced by environmental acidification in the human host[6]. To replicate these environments, earlier studies using agar concentration above 0.3% (w/v) restrict bacterial swimming motility to effect a gradual shift into surface swarming. The use of agar concentration above 1% (w/v) prevents the swarming motility of many species[7]. The colony patterns formed on the surface are diverse, including featureless mat[8], bull's eye[9], dendrites[10], and vortex[11].

Although the relevance of such patterns remains unclear, those patterns seem to be dependent on environmental and chemical cues[12]. Environmental cues cover different aspects, including temperature, salinity, light, and pH, whereas chemical cues include the presence of microbial quorum sensing molecules, biochemical byproducts, and nutrients. Autoinducer quorum sensing signaling molecules such as AHL (N-hexanoyl-L homoserine lactone) can impact surface swarming by regulating the production of surfactant[13,14]. Resveratrol, a phytoalexin compound, could restrict bacterial swarming motility[15].

In the present work, we investigate the effect of gradient concentrations of resveratrol on wild-type *Escherichia coli* K12 strain and investigate arabinose-inducible swarming motility of engineered *E. coli* K12-YdeH and *Pseudomonas aeruginosa* PAO1-YdeH species. The production of the YdeH enzyme is induced by arabinose via the *araBAD* promoter, resulting in cellular c-di-GMP perturbation and affecting bacterial swarming motility[16,17]. This inducible swarming behavior is studied using arabinose gradient swarm plates with *E. coli* K12-YdeH and *P. aeruginosa* PAO1-YdeH strains.

The gradient swarm plates are prepared by successively solidifying double-layer medium (**Figure 1B**). The bottom layer comprises the medium added with the inducer, poured on one side of a propped-up Petri dish. Upon the solidification of the bottom layer, the Petri dish is returned to a flat surface, where the upper layer containing the medium without the inducer is added from the other side of the plate. After the swarm plates are completely solidified, isometrically arranged holding-wells are produced by boring holes on the swarm plates following a fixed layout (**Figure 1C**) or by imprinting the wells using a 3D printed model of the plate cover containing pegs during the medium curing process (**Supplemental Figure S1**). A gel imaging system is used to capture

the swarming morphologies at different time points (**Figure 2**). Analysis of surface swarming using ImageJ software (**Supplemental Figure S2**) provides quantitative results of the surface swarming process (**Figure 3**).

Thus, we propose a simple method to test surface swarming motility within a concentration range of inducers. This method can be used to test multiple concentration responses of other inducers by mixing the inducer into the bottom-layer medium and can be applied to other motile species (e.g., *Bacillus subtilis*, *Salmonella enterica*, *Proteus mirabilis*, *Yersinia enterocolitica*). This approach could provide reliable qualitative and quantitative results for screening a single chemical inducer, and separate plates may be employed to evaluate more chemical inducers.

PROTOCOL:

1. Preparation of gradient swarm plates

1.1. Preparation of swarm medium

NOTE: See the discussion section for a brief comparison of different medium viscosities; 0.7% (w/v) agar concentration of swarm medium was used in this protocol.

1.1.1. Prepare Lysogeny broth (LB) powder with agar in two conical flasks; each flask contains 2 g of tryptone, 2 g of sodium chloride, 1 g of yeast extract, and 1.4 g of agar. Add double-distilled water (ddH_2O) and stir the suspension using a magnetic stir bar. Adjust the final volume to 200 mL by adding additional ddH_2O.

1.1.2. Autoclave the solution at 121 °C for 20 min. Use an air-permeable cap or bottle sealing film with an air vent.

NOTE: Agar will dissolve when heated in the autoclave.

1.1.3. When the temperature drops to 65 °C, mix the solution to ensure homogeneity, and transfer the medium to a 65 °C incubator or water bath for short-term usage.

1.2. Preparation of bottom-layer swarm medium

NOTE: The bottom-layer medium is the mixture of swarm medium and inducer stock solutions. The formulation of inducer gradient swarm plates is shown in **Table 1**.

1.2.1. Prepare a 100 mM resveratrol stock solution by dissolving 114.12 mg of lyophilized resveratrol powder into 5 mL of dimethyl sulfoxide (DMSO), and store the solution at -20 °C.

1.2.2. Prepare a 20% (w/v) arabinose stock solution by dissolving 6 g of arabinose powder in 30 mL of ddH_2O; wait for 10–15 min to allow the arabinose to dissolve; and store the solution at room temperature.

1.2.3. Take out the medium from the 65 °C incubator and place it at room temperature; allow the swarm medium to cool until the Erlenmeyer flask is cool enough to hold (~50 °C). Do not place the swarm medium at room temperature for long periods, as this will cause the solidification of agar.

1.2.4. Add the required volume of the inducer stock solution to the swarm medium at 50 °C (**Table 1**). Use a pipette to dispense the inducer solution instead of pouring it. Gently swirl to mix the inducer with the swarm medium.

NOTE: This step is for inducers that cannot be autoclaved. Be careful not to introduce bubbles into the medium.

1.3. Preparation of double-layer gradient swarm plates

NOTE: The upper-layer medium is LB medium containing 0.7% (w/v) agar.

1.3.1. Label 13 x 13 cm square Petri dishes with inducer name and strains, and prop the dishes up over the edge of the lids (**Figure 1B**).

1.3.2. Add 40 mL warm bottom-layer medium (50 °C) using a 50 mL pipette or a 50 mL centrifuge tube.

NOTE: Alternatively, for 13 x 13 cm square Petri dishes, 40 mL bottom-layer and upper-layer medium is suitable; for 10 x 10 cm square Petri dishes, 25 mL bottom layer and upper-layer medium is suitable.

1.3.3. Allow the bottom-layer medium to cure uncovered for 1 h inside a laminar flow hood. Do not disturb square Petri dishes while the medium solidifies.

NOTE: While curing the bottom layer, swarm medium not containing inducers should be maintained in a 65 °C incubator or water bath.

1.3.4. Once the bottom layer is completely solidified, remove the lids and lay the square Petri dishes inside a laminar flow hood.

1.3.5. Add 40 mL of warm upper-layer medium (50 °C) using a 50 mL pipette or 50 mL centrifuge tube.

NOTE: The upper-layer medium does not contain inducers.

1.3.6. Cure the double-layer plates on the benchtop covered and undisturbed for 1 h. Store the prepared plates at 4 °C for up to 24 h.

NOTE: Longer curing times would reduce the moisture content and restrict swarming motility.

2. Growth of *E. coli* K12 and *P. aeruginosa* PAO1

2.1. Prepare 500 mL of LB medium by adding 5 g of tryptone, 5 g of NaCl, and 2.5 g of yeast extract into ddH_2O, and top up the solution to 500 mL. Autoclave the solution on liquid cycle for 20 min at 121 °C, and store it at 4 °C.

2.2. Prepare 100 mL of 1.5% (w/v) LB-agar medium by adding 1 g of tryptone, 1 g of NaCl, 0.5 g of yeast extract, and 1.5 g of agar into ddH_2O and top up the solution to 100 mL. Autoclave the solution on liquid cycle for 20 min at 121 °C. Transfer the medium to a 50 °C water bath to prevent the agar from solidifying.

2.3. When the LB-agar medium flask is comfortable to hold, add 20 mL of LB-agar medium into a Petri dish (10 cm in diameter) using a 25 mL pipette. Leave the plate at room temperature overnight, and store the LB-agar plate at 4 °C.

2.4. Take stock cultures stored at -80 °C, streak *E. coli* K12, *E. coli* K12-YdeH, *P. aeruginosa* PAO1, and *P. aeruginosa* PAO1-YdeH strains on LB-agar Petri dishes using disposable inoculation loops. Incubate the Petri dishes inverted overnight at 37 °C.

2.5. Pick single colonies for different strains from the Petri dishes, inoculate each colony into 5 mL of LB medium, and incubate the culture at 37 °C in a laboratory orbital shaker set at 220 rpm.

2.6. When the culture density reaches OD_{600nm} ~1.0, remove the culture from the shaker and place it at room temperature. Adjust the culture density to OD_{600nm} = 1.0, as described in step 3.2.1.

3. Inoculation and incubation of gradient swarm plates

3.1. Preparation of inoculation wells

NOTE: 3D printing cover models capable of generating wells separated by a standard distance can be used instead of the method described below (**Supplemental Figure S1**).

3.1.1. Mark the well positions on A4 paper shown in **Figure 1C**. Set three test concentrations in one square Petri dish with two or three replicates.

3.1.2. Place the marked A4 paper under a well-solidified gradient plate. Push the broader side of a 100 µL pipette tip into the semisolid medium surface at the marked position. Press the pipette tip until it reaches the bottom of the upper-layer medium.

3.1.3. When the tip touches the bottom, apply no vertical force to the tip; gently rotate the tip

to isolate the content of the cylindrical well.

3.1.4. Horizontally move the pipette tips along a very small distance to allow airflow into the narrow place set aside. Press the tip with the index finger to block the gas flow inside the tip while holding the pipette using the thumb and middle finger.

3.1.5. Pull the tip out vertically, keeping the well content in the tip while pulling it out.

NOTE: If the well content slips, apply slightly more pressure with the index finger to completely seal the tip.

3.1.6. Repeat steps 3.1.2 to 3.1.5 in every marked position. Cover the swarm plate before inoculation.

3.2. Gradient plate inoculation and incubation

3.2.1. Adjust the overnight growth culture density to OD_{600nm} = 1.0.

3.2.2. Pipette 80 µL of the overnight growth culture into every well. Do not spill the bacterial culture outside the wells.

3.2.3. Wrap the plates with sealing film. For long-term observation (3–5 days), wrap the plates with sterile laboratory rubber tape.

NOTE: Rubber tape is less likely to break.

3.2.4. Place a beaker filled with ddH_2O in the incubator to maintain humidity inside the incubator. Incubate the gradient swarm plates at 37 °C.

NOTE: Do not incubate the swarm plates upside-down; this will cause the bacterial culture to leak from the wells.

3.2.5. Image the swarm plate immediately after inoculation, recording this as the **0 h** time point.

4. Imaging bacterial surface swarming

4.1. Take the swarm plates out, one at a time, from the incubator every 12 h, holding the plate horizontally, and place them in the gel imaging system (see the **Table of Materials**).

NOTE: Do not leave fingerprints on the surface of plates; hold the side of the swarm plate with clean gloves.

4.2. Select **gel imaging** mode; expose the swarm plate to white light; and adjust the focal length to give the clearest view of swarms.

NOTE: Use the same focal length for all plates in a given batch.

4.3. Enhance the brightness of the swarms for clear observation by adjusting the **exposure time** to **300 ms**. Adjust the **threshold** to minimize interference from the background light.

NOTE: Threshold is adjusted on the operating interface of the gel imaging system. Increase values on the left to minimize interference from background light; decrease values on the right to enhance the brightness of the swarms. In this protocol, the threshold usually ranges from 6,000 to 50,000.

4.4. Save the image file for further analysis. Record the imaging time, inducer type, gradient orientation, and strains in a **.txt** file.

5. Quantify the swarm area using ImageJ software

5.1. Import the image file acquired using the gel imaging system.

5.2. Set the scale bar using ImageJ software and apply it to all the images.

5.2.1. Create a line segment marking the length of the board by clicking on the line tool.

5.2.2. Click **Analyze | Set Scale** to open the **Set Scale** window.

5.2.3. Type the actual length in **Known distance** and **Unit of length**.

NOTE: Because 13 x 13 cm square Petri dishes were used in this work, the actual length is '130', and the unit of length is 'mm.'

5.2.4. Check the **Global** box.

5.2.5. Insert a scale bar by clicking **Analyze | Tools | Scale Bar**, type **Width in mm**, **Height in pixels**, **Font size**, and select **Color**, **Background**, and **Location** from the dropdown menu. Alternatively, choose **Bold Text**, **Hide Text**, **Serif Font**, and **Overlay** by ticking the checkboxes.

NOTE: The choice of those parameters is determined by users and the properties of the images. In this protocol, **Width in mm** was set to **25**, **Height in pixels** to **20**, **Font size** to **80**, and the scale bar was placed in the lower right corner by selecting **Location | Lower Right**. Other parameters can be chosen by the user.

5.3. Click **Process | Shadows** to enhance the sharpness of the image, especially the boundaries (**Supplemental Figure S2A**). Click **Process | Batch** to process images.

NOTE: The purpose of this step is to provide more precise boundary demarcation.

5.3.1. Process an image as a reference by clicking on **Process | Shadows | South**.

5.3.2. Click **Process | Batch | Macro** to open the **Batch Process** window. Look for the following commands displayed in the window:

```
run("South");
run("Save");
close();
```

5.3.3. Type the folder address of the original images and the output file address by clicking **Process** in the **Batch Process** window.

NOTE: It is recommended to export images with shadows to another folder and retain a copy of the original images.

5.4. Use **Wand (tracing) tool** to select swarms individually and adjust the tolerance (double-click **Wand (tracing) tool**) until the generated line fits the swarm boundary correctly (**Supplemental Figure S2B**).

NOTE: First, click **Wand (tracing) tool** and select a swarm on one image. If the boundaries have not been depicted correctly, double-click the **Wand (tracing) tool** to open the **Wand Tool** windows, where the **Tolerance** can be adjusted.

5.5. Click on **Analyze | Measure** to export the area value.

5.6. Repeat steps 5.1.1–5.1.5 until all swarms are measured, save the results to a .csv file for further analysis.

REPRESENTATIVE RESULTS:

The workflow consisting of the preparation of gradient swarm plates, inoculation, and incubation is shown in **Figure 1B**. To generate gradient swam plates, the bottom-layer medium is poured into propped-up dishes at ~4.3° from the horizontal plane (**Supplemental Figure S3**), followed by pouring the upper-layer medium after the bottom layer is completely solidified. The composition of the double-layer medium is shown in **Table 1**. Then, bacterial culture cultured overnight is pipetted into the test wells and incubated at 37 °C, maintaining appropriate levels of humidity. Multiple test concentrations are set in one gradient swarm plate with two or three replicates (**Figure 1C**). The alternative option of a 3D printing model of the cover lid with the columnar protrusion on the test points is shown in **Supplemental Figure S1**.

Two engineered species, *E. coli* K12-YdeH and *P. aeruginosa* PAO1-YdeH, were tested on arabinose gradient plates. **Figure 2A** shows the surface swarming process test in five wells, with overlap occurring between adjacent wells. Three test wells were set in one plate, as shown in **Figure 2B,C**, which enabled the formation of nonoverlapping boundaries. Bacterial swarming

motility was promoted with an increase in the arabinose concentration from the lowest concentration but was gradually restricted with higher arabinose concentrations. *E. coli* K12 wild-type strains were tested on resveratrol gradient plates (**Figure 2C**), within the concentration range of 0–400 µM. A modest restriction of the swarming motility was observed with increasing resveratrol concentration. Swarm areas were quantified by ImageJ software (**Supplemental Figure S2**). The swarming curve displays the multiple concentration responses (**Figure 3**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of inducer gradient swarm plate preparation, inoculation, and incubation. (**A**) Overnight growth of bacterial culture at 37 °C. (**B**) Workflow of double-layer inducer gradient swarm plate. **i)** Prop up square Petri dishes. **ii)** Pour bottom-layer medium and solidify at room temperature. **iii)** Lay the square Petri dishes flat, and pour upper-layer medium. **iv)** Plate curing. **v)** Make wells corresponding to the marked positions. **vi)** Pipette bacterial culture into wells. **vii)** Wrap plates using sealing film or rubber tape. **viii)** Place swarm plates in a 37 °C incubator. **C)** Sketch map of wells on A4 paper or 3D printing model. Three wells with **I)** three replicates and **II)** two replicates.

Figure 2: Surface swarming processes on inducer gradient plates. Bacterial surface swarming processes are captured using a gel imaging system within 5 days of inoculation. (**A**) Arabinose induced surface swarming process of *E. coli* K12-YdeH. (**B**) Arabinose induced surface swarming process of *P. aeruginosa* PAO1-YdeH. (**C**) Surface swarming process of *E. coli* K12 wild-type strain on resveratrol gradient plate.

Figure 3: Surface swarming curves represent multiple concentration responses in inducer gradient plates. Every quantifiable data point consists of three replicates. (**A**) Arabinose-induced swarming motility of *P. aeruginosa* PAO1-YdeH; approximate concentrations within test wells are 0.17% (w/v), 0.25% (w/v) and 0.42% (w/v). (**B**) *E. coli* K12 wild-type strain swarming on resveratrol gradient swarm plate; approximate concentrations within test wells are 67 µM, 200 µM, and 335 µM.

Table 1: Double-layer swarm medium specifications.

Supplemental Figure S1: 3D printing models of swarm plate lid. (**A**) 3 x 3 wells including three wells and three replicates. (**B**) 3 x 2 wells including three wells and two replicates. (**C**) Curing plate with 3D printing models.

Supplemental Figure S2: Quantification of surface swarm area using ImageJ software. (**A**) Add 'shadows' to original images (**Process | Shadows**) to generate quantifiable swarms with clear boundaries. (**B**) Set scale bar (**Analyze | Set Scale**), select swarms, and export swarm area using **Wand Tools** (**Analyze | Measure**).

Supplemental Figure S3: Propped-up square Petri dish for pouring bottom-layer medium. The angle of inclination is ~4.3 °.

DISCUSSION:

Investigation of bacterial swarming motility on semisolid gradient plates can be a challenging task[18-20], as it involves multiple factors such as substrate viscosity, humidity, and medium components. Among these factors, agar concentration plays a decisive role in determining microbial reversion to either swimming or swarming motility. As the agar concentration increases from 0.3% (w/v) to 1% (w/v), bacterial swimming motility will be restricted and gradually shift to surface swarming, and agar concentration above 1% (w/v) will prohibit both swimming and swarming motility[7]. The agar concentration was fixed at 0.7% (w/v) based on preliminary experiments, as it showed better performance than other concentrations.

This agar concentration was also previously employed to study microbial chemotaxis[21]. Decreased agar concentration results in a larger swarm area, accompanied by overlaps between adjacent wells, increasing the difficulty of quantifying swarm area due to unclear boundary demarcation. Relatively high agar concentration results in a small swarm area, decreasing the possibility of overlaps. However, excessive agar (>1.0%) can prevent bacterial surface swarming. Hence, it is essential to select an appropriate agar concentration that can be applied to all test species to generate comparable results with a standard viscosity.

Isometrically arranged wells provide equal and appropriate space for bacteria to swarm. The arrangement of wells can be different depending on the needs of gradient swarm plates. Surface swarm can overlap due to insufficient distance between test wells (**Figure 2A**), hindering the quantification of the swarm area, especially in a prolonged study. Three test wells were set within one swarm plate to test multiple concentration responses while preventing colony overlap (**Figure 2B,C**).

Compared to an inoculating needle[22], holding wells prepared in these semisolid plates provide a standardized inoculation volume. Holding wells were also found to sustain the bacterial culture, preventing bacterial spread observed in other methods such as pipetting [23]. Holding wells made by inserting 3D print models present clearer and standardized inoculation start sites. Although these wells require additional preparation, they reduce the deviation between the test point and the marked position on the template. Additionally, the exact bacterial counts in each holding well can be calculated, improving the reproducibility of the data. As a precaution, hasty or careless preparation of wells could result in the cracking of the wells, resulting in variation of the surface swarming during migration, as the microbes are inclined to move through the cracks.

Care must be taken to avoid splattering of the microbial starter culture during the preparation of the loading well and sample loading, especially in low-viscosity swarm plates. Further, the volume of the bacterial culture loaded must be optimized to minimize the time needed for the microbes to scale the inner walls of each holding-well while preventing the possibility of spillage during plate transportation (for imaging purposes). In this work, we decreased the bacterial culture loading volume to minimize the possibility of spillage, resulting in a delay in bacterial migration that is commonly mistaken for swarm lag[24].

It is necessary to adapt the medium formulation due to the differences in viscosity and required nutrient sources between motile species. For some species (e.g., *Bacillus subtilis*[25]), surface swarming can be rapid; therefore, the imaging interval should be shortened. Computer-assisted swarm area quantification gives more precise information than distance measurement of radius[26]. To generate clear boundaries for calculating swarm area by ImageJ software, a built-in method was used in this protocol that adds shadows to the original images acquired using the gel imaging system. If a boundary of the swarm merges with the adjacent one, migration toward the border will be inhibited, where these communities prefer to migrate toward the unoccupied areas of the swarming plates (**Figure 2A**). These limiting factors resulting from the overlapping boundaries present a significant challenge in determining the migratory distance of the surface swarming, where these values cannot be integrated into the free swarming process.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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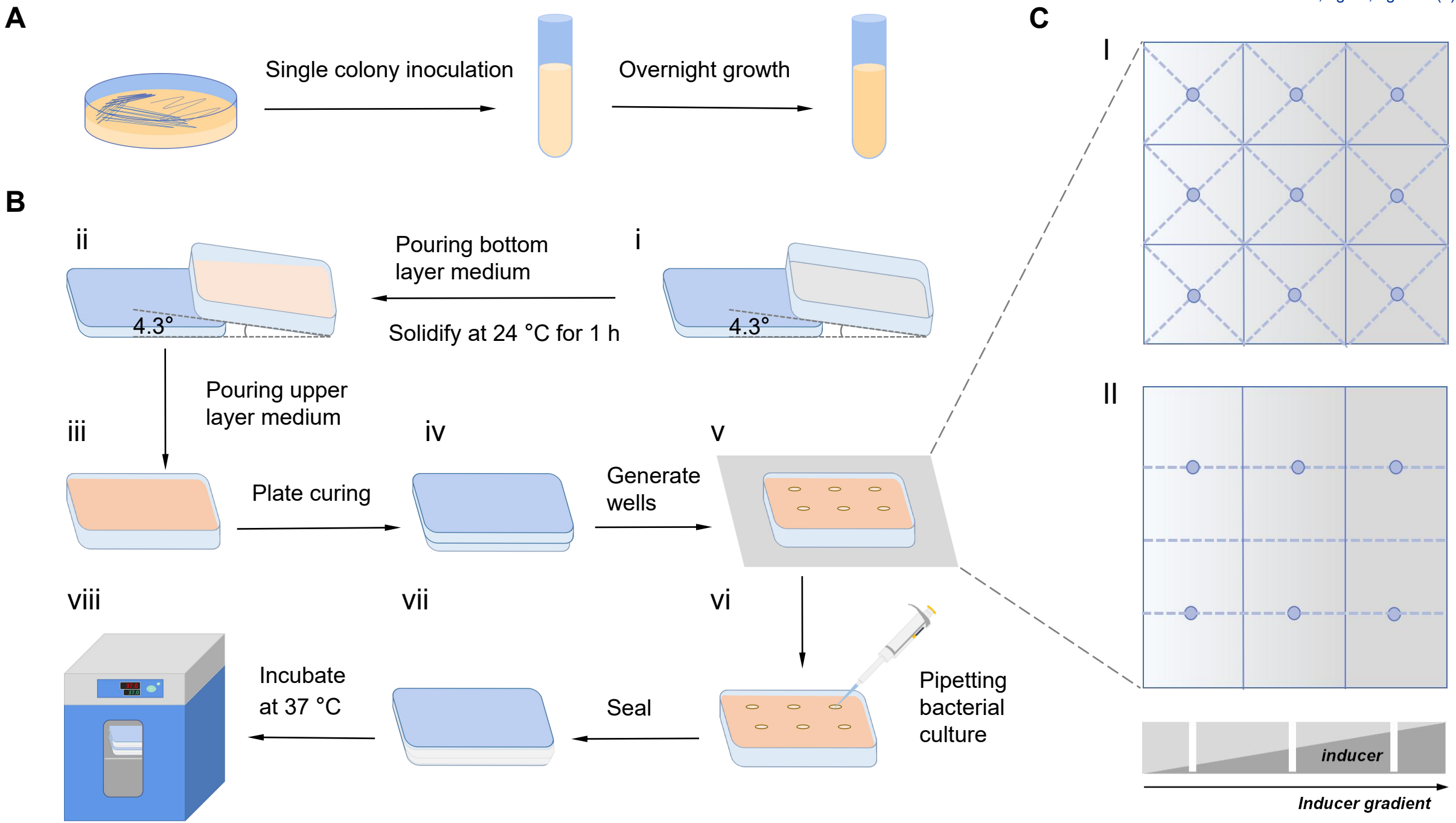
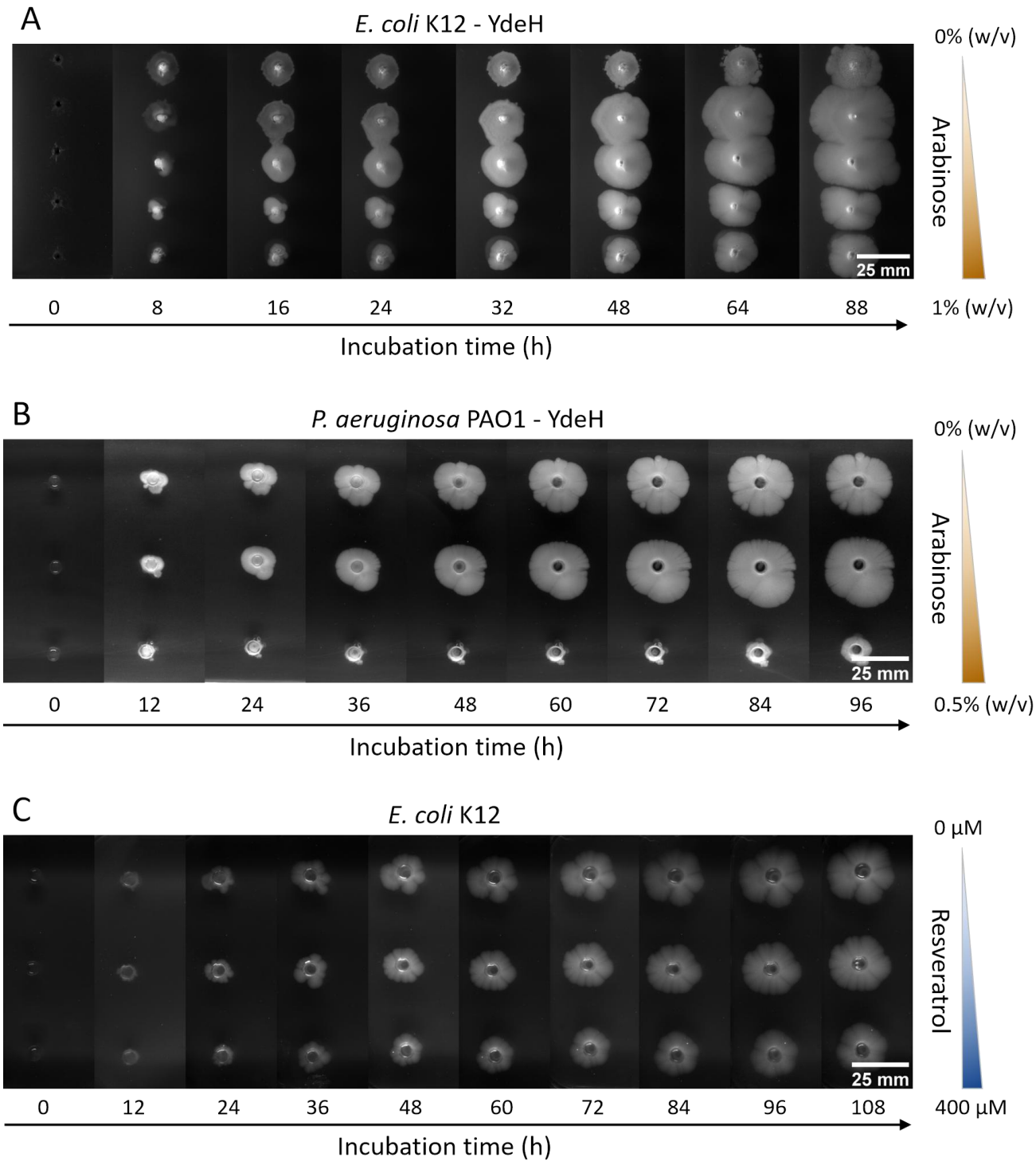
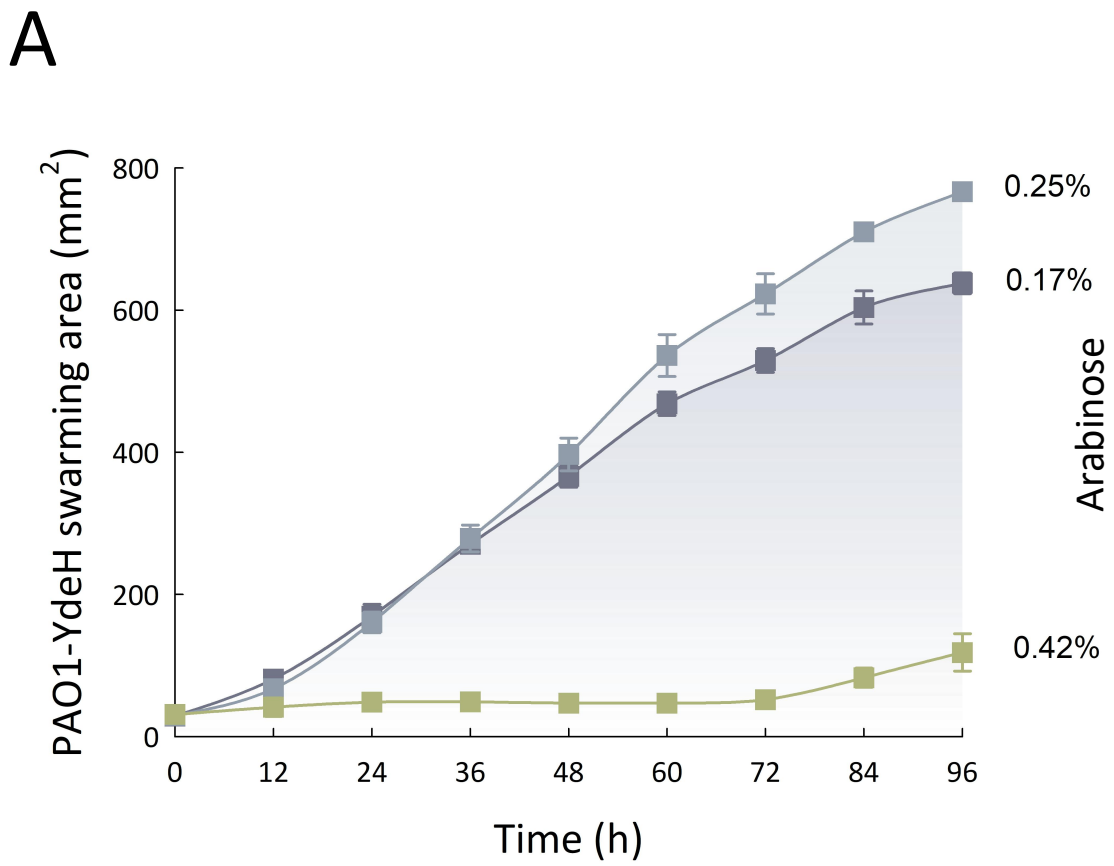
26 Wang, S. et al. Coordination of swarming motility, biosurfactant synthesis, and biofilm matrix exopolysaccharide production in *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology.* **80** (21), 6724–6732 (2014).

Figure





Figure



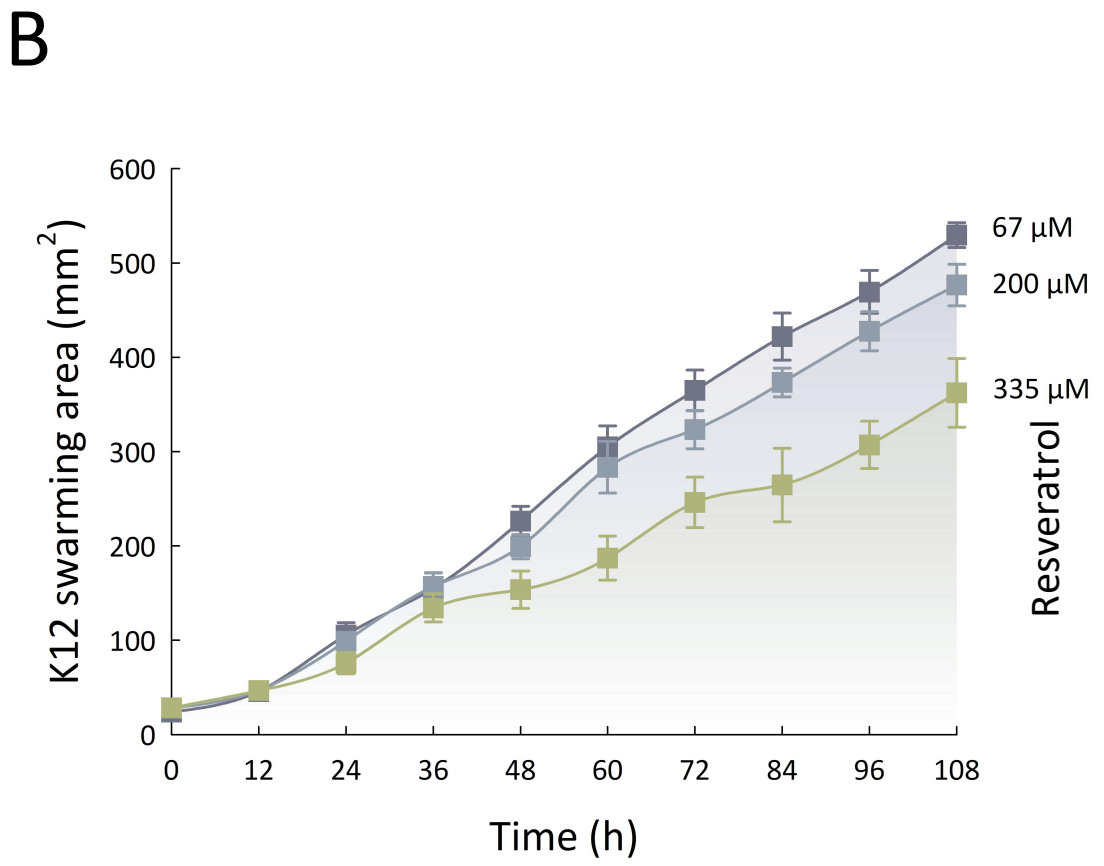


Table 1

Upper layer medium/lysogeny broth medium (*per 100 mL*)		
Tryptone: 1 g Sodium chloride: 1 g Yeast extract: 0.5 g Agar: 0.7 g		
Bottom layer medium/Inducer-containing medium (*per 100 mL*)		
Tryptone: 1 g Sodium chloride: 1 g Yeast extract: 0.5 g Agar: 0.7 g Inducer: - Resveratrol stock solution: 400 µL - Arabinose stock solution: 2.5 mL or 5 mL	Working concentration: - Resveratrol: 400 µM - Arabinose: 0.5% (w/v) or 1% (w/v)	Stock solution concentration: - Resveratrol: 100 mM - Arabinose: 20% (w/v)

Replies to the editor's comments

Journal of Visualized Experiments-63382-R2, "Quantifying bacterial surface swarming motility on inducer gradient plate."

We thank the editor for the helpful and critical comments. We have adopted many of these suggestions to better improve the readability of the manuscript, and the revised manuscript is much improved as a result of the constructive comments given.

The following are the replies to specific comments from the editor.

Line 237:
Even highlighting the quantification section will not push you over the 3-page limit for highlighted text but consider removing the highlighting from such steps UNLESS these are crucial to remind even in the video. They will remain in the text. Not spilling, wrapping the plates (you can include sealing film wrapping in the video) with tape and keeping water in the beaker don't seem very crucial to film.

Response: Thanks for editor's comments. We have removed those steps from the highlighted protocol.

Line 266:
Please add settings and any other guidance: for example, increase values to minimize or decrease values to minimize interference from background light but don't go above x value etc. Please add more information to guide the user.

Response: Thank editor for the comments. We have added more detailed information in NOTE after 4.3.

Line 269-272:
NOTE: Threshold is adjusted on the operating interface of the gel imaging system. Increase values on the left to minimize interference from background light, decrease values on the right to enhance brightness of swarms. In this protocol, threshold region is usually between 6000 to 50000.

Line 277:
Please include important steps from this section in your window. Your title is "quantifying…" so this is an important section. Moreover, please ensure you include steps on the shadow formation to guide the user about the last paragraph in your discussion (drawing shadows for correct quantifying of swarming).

Response: Thank editor for the comments. We have modified our protocol steps "Quantify the swarm area using ImageJ software". Some steps have been added (step 5.2.5), some NOTEs have been added to explain the settings and detailed processes (NOTE after step 5.2.5; 5.3.3; 5.4), some statements have been revised to give clearer expression (step 5.2.2; 5.3; 5.3.3; 5.4). We have also highlighted some steps to be in

video (5.3, 5.4 and 5.5). We have included steps to describe how to add shadows on swarm images, and how to quantify them (5.3, 5.4, 5.5).

Line 286:
5.2.2 Click **Analyze | Set Scale** to open the Set Scale window.

Line 295-302:
5.2.5 Insert scale bar by clicking **Analyze | Tools | Scale Bar**, type **Width in mm**, **Height in pixels**, **Font size**, select **Color**, **Background** and **Location** in the dropdown menu. You can also choose **Bold Text**, **Hide Text**, **Serif Font** and **Overlay** by clicking checkboxes.

NOTE: The choice of those parameter is determined by users and the properties of images. In our protocol, we set 25 in **Width in mm**. We also set 20 in **Height in pixels**, 80 **Font size** and put it in lower right by selecting **Location | Lower Right**. Other parameters can be determined by user's own choice.

Line 304:
5.3 Click **Process | Shadows** to enhance the sharpness of the image, especially the boundaries (**Supplemental Figure S2A**). Click **Process | Batch** to process images.

Line 318-322:
5.3.3 Type the folder address of the original images and the output file address by clicking **Process** in **Batch Process** window.

NOTE: It is recommended to export images with shadows to another folder, and retain a copy of original images.

Line 324-330:
5.4 Use **Wand (tracing) tool** to select swarms individually and adjust the tolerance (double-click **Wand (tracing) tool**) until the generated line fits the swarm boundary nicely (**Supplemental Figure S2B**).

NOTE: First click **Wand (tracing) tool** and select a swarm on one image, if the boundaries haven't been depicted nicely, double-click **Wand (tracing) tool** to open **Wand Too**l windows, you can adjust **Tolerance** here.

Line 281:
Please give settings, values…what you used and recommended ones. What does the scale bar do? Zoom in or zoom out?

Response: Thank editor for the comments. We have added new settings in 5.2.5, which describe how to insert scale bar using ImageJ software, and we have added NOTE following 5.2.5, which give some of our parameters that set in **Scale Bar** window. The actual length of square petri dishes in our protocol have been given in

NOTE after 5.2.3, for other values like color, location, etc. It is dependent on user's own choice, so we didn't give a recommendation.

Line 295-302:

5.2.5 Insert scale bar by clicking **Analyze | Tools | Scale Bar**, type **Width in mm**, **Height in pixels**, **Font size**, select **Color**, **Background** and **Location** in the dropdown menu. You can also choose **Bold Text**, **Hide Text**, **Serif Font** and **Overlay** by clicking checkboxes.

NOTE: The choice of those parameter is determined by users and the properties of images. In our protocol, we set 25 in **Width in mm**. We also set 20 in **Height in pixels**, 80 **Font size** and put it in lower right by selecting **Location | Lower Right**. Other parameters can be determined by user's own choice.

Line 293:

I added box. Please see if you want to add more guidance here for the user…should the scale be a certain value? Do you recommend staying in a particular range? If so, what?

Response: Thank editor's comments. We have added more guidance on how to set scale bar in 5.2.5 and following NOTE. We think it's hard to give all recommend parameters because some of them are dependent on user's own choice, however we added some of the parameters that we use in this protocol as a reference (Like height in pixels, front size and location).

Line 362:

Please do this in the figure also.

Response: Thank editor for the comments. We have changed labels in Figure 1.

Line 390:

Please add the space between 120 and mm in S3.

You have all three images in one Word file. Please upload the three figures as separate PDF files and keep the legends here.

Response: Thank editor for the correction. We have modified Supplemental figure S3. And we have uploaded figures as separate PDF files.

Discussion:

Please add some limitations of this method.

Response: Thank editor for the comments. We have described some of the limitations of this method in discussion part: First is the overlap between adjacent swarms that have been discussed in Line 417 to Line 419:

Line 417-419:

Surface swarm can overlap due to insufficient distance between test wells (**Figure 2A**), hindering the quantification of the swarm area, especially in a prolonged study.

Second is the additional preparation of wells that could bring more factors leading experiment failure, in line 429:

Line 429-431:

As a precaution, hasty or careless preparation of wells could result in the cracking of the wells, resulting in variation of the surface swarming during migration, as the microbes are inclined to move through the cracks.

Line 444:

I chose the original text that I edited. Consider adding these steps (built-in method) to the protocol [imperative tense: click this to add shadow. Etc] and include them in the video. That way, this will be clear because of demonstration.

Response: Thank editor for the comments. We have included such descriptions in protocol step 5.3. Some of the steps have been highlighted to be included in the video.

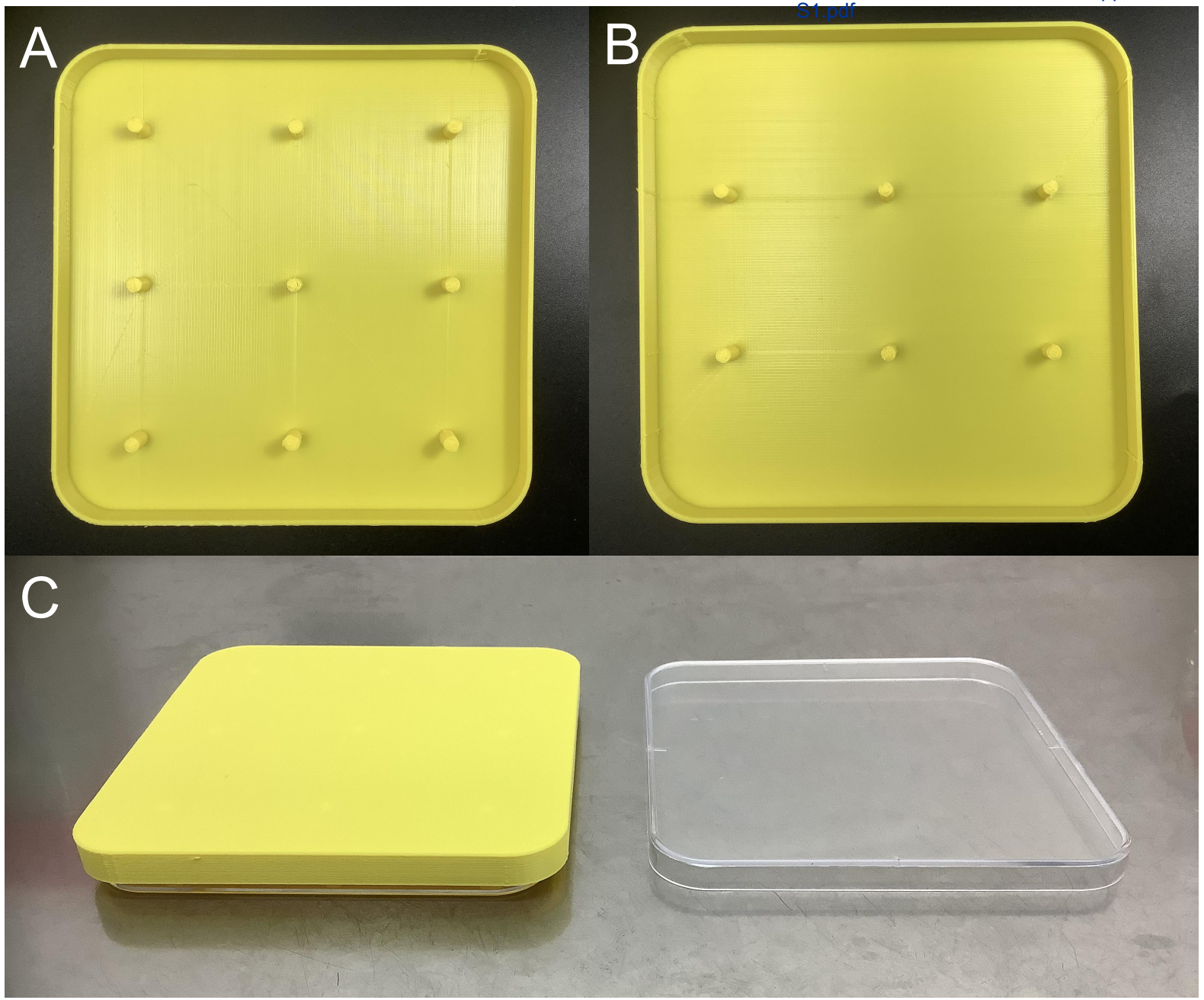
Line 462:

Please note that the journal title must be fully written. No abbreviations

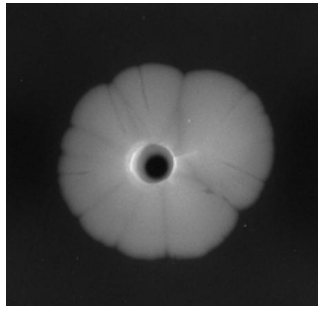
Response: Thank editor for the comments. We have modified reference style with full written of journal title.

Figure S1PDF

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental Figure S1.pdf

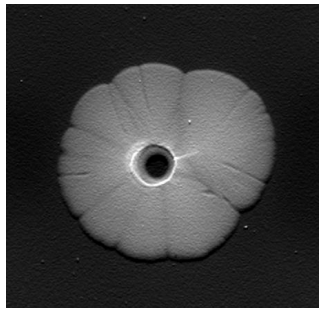


A



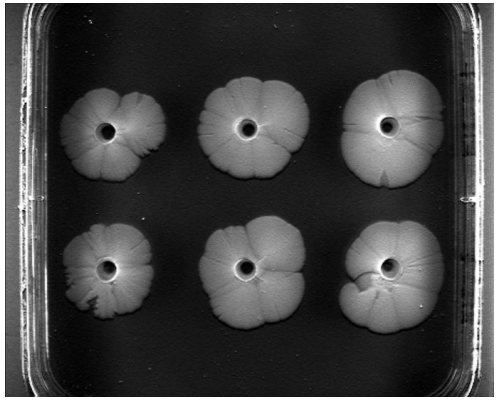
Original swarm morphology

Process | Shadows | North



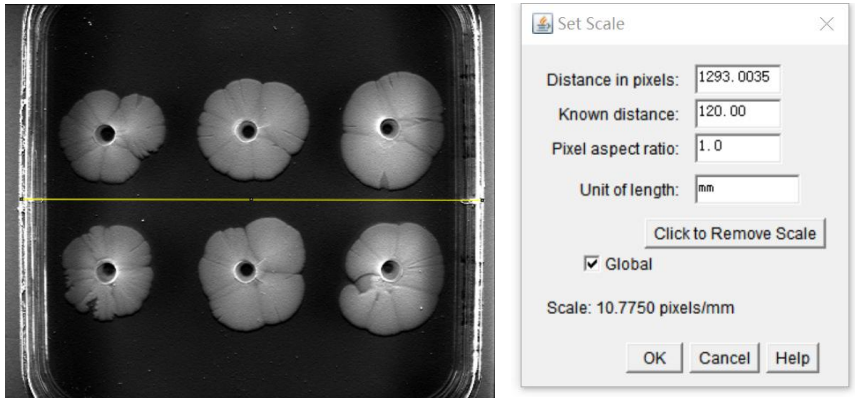
Swarm with “Shadows”

B



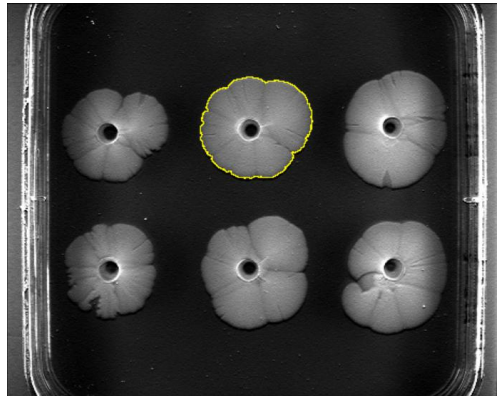
Import swarm image

Analyze | Set Scale



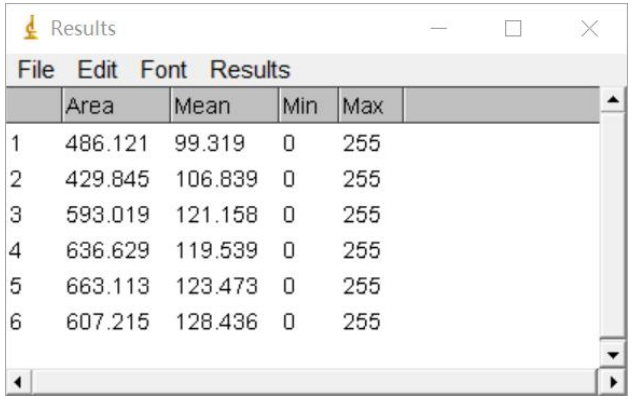
Set scale bar

Wand (tracing) Tools



Select swarms

Analyze | Measure

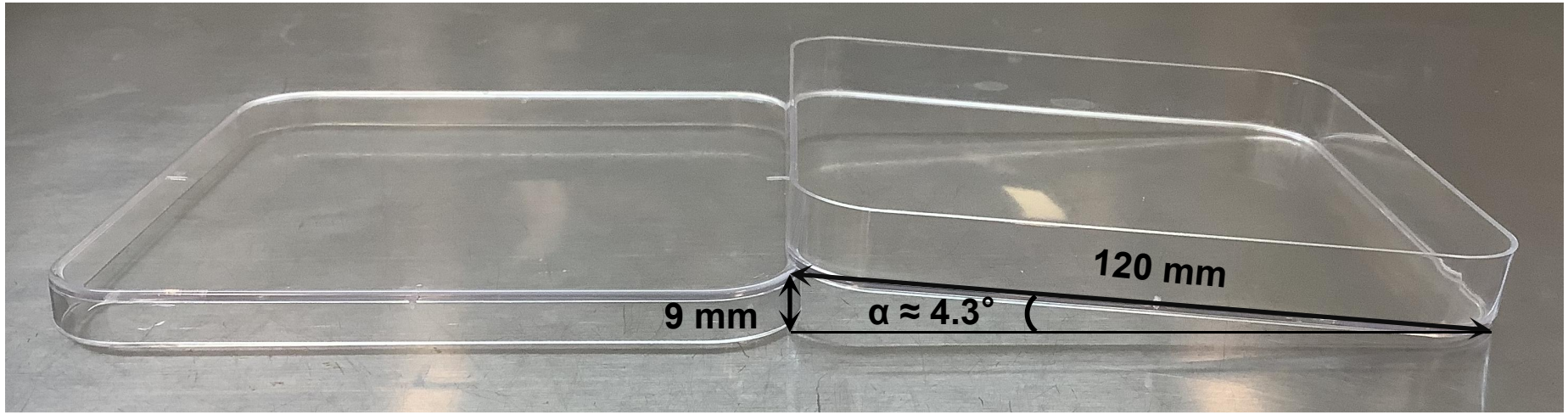


	Area	Mean	Min	Max
1	486.121	99.319	0	255
2	429.845	106.839	0	255
3	593.019	121.158	0	255
4	636.629	119.539	0	255
5	663.113	123.473	0	255
6	607.215	128.436	0	255

Export swarm area

Figure S3PDF

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental Figure S3.pdf



3D printing model 1

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Supplemental Coding Files

renamed_cea06.STL

3D printing model 2

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renamed_2e941.STL

3D printing model 3

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