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Dear Dr. Upponi,

Enclosed please our manuscript "Time-lapse imaging of bacterial swarms and the collective stress response" for consideration for publication in *Journal of Visualized Experiments.*

In this work, we present a simple method to produce high resolution time-lapse movies of *Pseudomonas aeruginosa* swarms and their response to bacteriophage and antibiotic stress using a flatbed document scanner, automation software, and ImageJ. We used this technique to produce the bulk of the data in our upcoming publication (Bru et al., "PQS produced by the *Pseudomonas aeruginosa* stress response repels swarms away from bacteriophage and antibiotics, *J. Bacteriology*, In press). This procedure is a fast, simple, and cost-effective method of monitoring swarming dynamics and may be adapted to study the motility and growth of other bacterial species. We believe that it will appeal to your readership.

We thank you for your consideration and look forward to hearing back from you soon.

Sincerely,

Nina M. Høyland-Kroghsbo

Albert Siryaporn

TITLE:

Time-Lapse Imaging of Bacterial Swarms and the Collective Stress Response

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KEYWORDS:

Pseudomonas aeruginosa, swarming, bacterial danger communication, quorum sensing, PQS, antibiotic stress, bacteriophage

SUMMARY:

We detail a simple method to produce high-resolution time-lapse movies of *Pseudomonas aeruginosa* swarms that respond to bacteriophage (phage) and antibiotic stress using a flatbed document scanner. This procedure is a fast and simple method for monitoring swarming dynamics and may be adapted to study the motility and growth of other bacterial species.

ABSTRACT:

Swarming is a form of surface motility observed in many bacterial species including *Pseudomonas aeruginosa* and *Escherichia coli*. Here, dense populations of bacteria move over large distances in characteristic tendril-shaped communities over the course of hours. Swarming is sensitive to several factors including medium moisture, humidity, and nutrient content. In addition, the collective stress response, which is observed in *P. aeruginosa* that are stressed by antibiotics or phage, repels swarms from approaching the area containing the stress. The methods described here address how to control the critical factors that affect swarming. We introduce a simple method to monitor swarming dynamics and the collective stress response with high temporal resolution using a flatbed document scanner, and describe how to compile and perform a quantitative analysis of swarms. This simple and cost-effective method provides precise and well-controlled quantification of swarming and may be extended to other types of plate-based growth assays and bacterial species.

INTRODUCTION:

Swarming is a collective form of coordinated bacterial motility that increases antibiotic resistance

and production of virulence factors in the host[1–3]. This multicellular behavior occurs on semi-solid surfaces that resemble those of mucous layers covering epithelial membranes in the lungs[4, 5]. Biosurfactants are commonly produced by swarming populations to overcome the surface tension on surfaces and the production of these is regulated by complex cell-cell signaling systems, also known as quorum sensing[6–8]. Many species of bacteria are capable of swarming, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*[9–12]. The swarming patterns created by bacteria are diverse and are affected by the physical and chemical properties of the surface layer including nutrient composition, porosity, and moisture[13, 14]. In addition to surface properties, growth temperature and ambient humidity affect several aspects of swarming dynamics, including swarming rate and patterns[12–15]. The growth variables that affect swarming create challenges that impact experimental reproducibility and the ability to interpret results. Here, we describe a simple standardized method to monitor the dynamics of bacterial swarms through time-lapse imaging. The method describes how to control critical growth conditions that significantly affect the progression of swarming. Compared to traditional methods of swarm analysis, this time-lapse imaging method enables tracking the motility of multiple swarms concurrently during extended periods of time and with high resolution. These aspects improve the depth of data that can be gained from monitoring swarms and facilitate the identification of factors that affect swarming.

Swarming in *P. aeruginosa* is facilitated through the production and release of rhamnolipids and 3-(3-hydroxyalkanoyloxy)alkanoic acids into the surrounding area[6, 16]. The introduction of stress from sub-lethal concentrations of antibiotics or infection by phage virus impacts the organization of swarms. In particular, these stresses induce *P. aeruginosa* to release the quorum sensing molecule 2-heptyl-3-hydroxy-4-quinolone, also known as the *Pseudomonas* quinolone signal (PQS)[17, 18]. In swarm assays that contain two populations of swarms, PQS produced by the stress-induced population repels untreated swarms from entering the area containing the stress (**Figure 1**). This collective stress response constitutes a danger communication signaling system that warns *P. aeruginosa* about nearby threats[18, 19]. The effects of stress on *P. aeruginosa,* the activation of the collective stress response, and the repulsion of swarms can be visualized using the time-lapse imaging method described here. The protocol described here explains how to: (1) prepare agar plates for swarming, (2) culture *P. aeruginosa* for two types of assays (traditional swarming assays or collective stress response assays) (**Figure 1**), (3) acquire time-lapse images, and (4) use ImageJ to compile and analyze the images.

Briefly, *P. aeruginosa* from an overnight culture is spotted in the middle of a swarming agar plate while *P. aeruginosa* that are infected with phage or treated with antibiotics are spotted at the satellite positions. The progression of *P. aeruginosa* swarming is monitored on a consumer document flatbed scanner that is placed in a humidity-regulated 37 °C incubator. The scanner is controlled by a software that automatically scans the plates at regular intervals over the swarm growth period, typically 16–20 h. This method yields concurrent time-lapse videos of up to six 10 cm swarming plates. The images are compiled into movies and the repulsion of swarms by stress-induced populations is quantified by using freely available ImageJ software. Special consideration is given to ensure consistency and reproducibility between different swarming experiments.

PROTOCOL:

1. Preparing swarming agar plates for *P. aeruginosa* swarming time-lapse imaging

1.1) Prepare 1 L of 5x M8 minimum media in a glass bottle by adding 64 g of $Na_2HPO_4 \bullet 7H_2O$, 15 g of KH_2PO_4, and 2.5 g of NaCl in 500 mL double-distilled water (ddH_2O). Adjust the final volume to 1 L with additional ddH_2O. Autoclave to sterilize and store liquid media at room temperature.

1.2) Prepare 100 mL of 1 M $MgSO_4$ (magnesium sulfate) in a glass bottle by adding 24.6 g of $MgSO_4 \bullet 7H_2O$ in 50 mL ddH_2O. Adjust the final volume to 100 mL with additional ddH_2O. Autoclave to sterilize. Store at room temperature.

1.3) Prepare 100 mL of 20% casamino acids in a glass bottle by adding 20 g of casamino acids in 50 mL ddH_2O. Adjust the final volume to 100 mL with additional ddH_2O. Autoclave to sterilize. Store at room temperature.

1.4) Prepare 100 mL of 20% glucose in a glass bottle by adding 20 g of glucose in 50 mL ddH_2O. Adjust the final volume to 100 mL with additional ddH_2O. Sterilize by filtration with 0.22 µm filter. Store at room temperature.

1.5) To make 10 swarming agar plates, add 1 g of agar in 100 mL of ddH_2O and adjust the final volume to 160 mL with additional ddH_2O in a 250 mL Erlenmeyer flask. Sterilize by autoclaving.

1.5.1) Immediately after autoclaving, place the agar solution in a 55 °C water bath for 15 min.

1.5.2) Remove the agar solution from the water bath and add 40 mL of 5x M8 minimum media, 200 µL of 1 M $MgSO_4$, 2 mL of 20% glucose, and 5 mL of 20% casamino acids[15]. Proceed to step 1.6 immediately after mixing.

NOTE: The final concentrations are 0.5% agar, 1 mM $MgSO_4$, 0.2% glucose, and 0.5% casamino acids.

1.6) Using a 25 mL pipette for consistent volume, add 20 mL of the swarming agar solution per 10 cm diameter Petri dish.

NOTE: A fixed volume of agar solution is important, as the volume affects the drying time and moisture content of the agar. Avoid bubbles when making the swarming agar plates.

1.7) Allow the agar to solidify by placing the swarming agar plates in a single stack with lids on for 1 h on the bench at room temperature. Turn on the dehumidifier to decrease relative humidity of the room to 40–50% 1 h prior to the next step.

1.8) Dry the swarming agar plates for an additional 30 min with the lids off in a laminar flow hood at 300 cubic ft./min with 40–50% relative humidity at room temperature. Dry the interior of the

lids by placing them face up in the laminar flow hood. Store swarming agar plates at 4 °C for up to 24 h.

1.9) Prepare black 10 cm Petri dish lids for imaging by smoothing the inside of the lid with sandpaper. Put the lids inside a packaging box and place the packaging box under a chemical hood. Spray inside the lids using black spray paint. Allow the lids to dry.

NOTE: Black lids may be re-used for additional experiments. It is important that the lids are painted so that they do not reflect light during scanning.

2. Growth of *P. aeruginosa* and plating conditions

2.1) Prepare 400 mL of lysogeny broth (LB) by adding 10 g of LB-Miller powder mix into 400 mL ddH_2O. For 2% LB-agar Petri dishes, add an additional 8 g of agar. Autoclave to sterilize.

2.2) Pour 20 mL of molten LB-agar medium into 10 cm diameter Petri dishes and allow them to solidify at room temperature overnight. Store liquid media at room temperature and agar plates at 4 °C.

2.3) Streak *P. aeruginosa* on an LB-agar Petri dish from a frozen stock stored at -80 °C using sterile loops or wooden sticks. Incubate the Petri dish upside-down overnight at 37 °C. Store LB-agar plate at 4 °C for up to 1 week.

2.4) Pick a single colony from the Petri dish with a sterile loop or wooden stick, inoculate it into 2 mL LB medium, and incubate the culture to saturation overnight (16–18 h) at 37 °C in a roller drum set at 100 rpm.

2.5) Pipet 5 µL of overnight culture from step 2.4 using a p20 pipet and spot at the center of the swarming agar plate by approaching the pipet tip at an angle (10–45°) 2.5 cm above the spotting area, pipetting down to the first stop, and touching the agar with only the liquid drop.

2.5.1) Avoid touching the agar with the pipet tip as it damages the agar (**Figure 1B**). Use a template in order to position the spot consistently across different swarming agar plates (**Supplementary Figure S1**).

2.5.2) For traditional swarming assays, use only the center spot and skip to step 2.8. For collective stress response assays continue to step 2.6 (for phage infection) or step 2.7 (for antibiotic stress).

2.6) For phage infection, mix 30 µL of overnight culture of *P. aeruginosa* from step 2.4 with 6 µL of 1 x 10^{12} pfu/mL phage DMS3vir[20]. Proceed immediately to the next step.

2.6.1.) Pipet up 6 µL of the *P. aeruginosa*-phage mixture from step 2.6 using a P-20 pipet and spot at 6 equidistant satellite positions on a 5.8 cm radius concentric circle that is centered at the Petri dish by approaching the pipet tip at an angle (10 to 45°) 2.5 cm above the spotting area, pipetting

down to the first stop, and touching the agar with only the liquid drop.

2.6.2) Avoid touching the agar with the pipet tip as it damages the agar (**Figure 1C**). Use a plating template for consistency (**Supplementary Figure S1**). Proceed to step 2.8.

2.7) For antibiotic treatments, mix 30 µL overnight culture *P. aeruginosa* from step 2.4 with 6 µL of 3 mg/mL gentamycin, 10 µL of 100 mg/mL kanamycin, or 7.5 µL of 100 mg/mL fosfomycin. Proceed immediately to the next step.

2.7.1) Pipet 6 µL of antibiotic treated *P. aeruginosa* from step 2.7 using a p20 pipette and spot at 6 equidistant satellite positions on a 5.8 cm radius concentric circle about the center of the dish by approaching the pipet tip at an angle (10 to 45°) 2.5 cm above the spotting area, pipetting down to the first stop, and touching the agar with only the liquid drop.

2.7.2) Avoid touching the agar with the pipet tip as it damages the agar (**Figure 1D**). Use a plating template for consistency (**Supplementary Figure S1**). Proceed to step 2.8.

2.8) Replace the clear Petri dish lids with black lids made in step 1.9 (**Figure 2A**).

2.9) Place the swarming agar plates on a scanner in an incubator set at 37 °C with a 10 L water bath to maintain humidity at 75% (**Figure 1E**, **Figure 2B**).

CAUTION: Do not disturb spotted cells on the swarming agar plates. Keep plates facing up at all times.

3. Image acquisition with scanner

3.1) Decrease the ambient lighting of the Petri dishes by attaching black matte fabric to a rack 40–60 cm above the flatbed document scanner. Secure it using zip ties (**Figure 2B**).

3.2) The scanner will be controlled using a scanning software and an automatic scripting software.

3.2.1) In the scanning software, select **Home Mode** (**Figure 3A**). Capture images in color by selecting **Color** under **Image Type**. To set the image quality, select **Other** under **Destination** and adjust the **Resolution** to 300 dpi. Keep the standard size for the images by selecting **Original** for **Target Size**. Leave all options under **Image Adjustments** unchecked for standard image quality.

NOTE: Target Size is set to Original by default. To select other options for Target Size, click on **Preview** first.

3.3) Set the saving path of images by clicking on the folder icon to the right of **Scan** to open **File Save Settings** (**Figure 3A**).

3.3.1) Select the folder destination for saving images by selecting **Other** under **Location** and click on **Browse**. Choose a folder to save the images.

3.3.2) Name the images in the **Prefix** text box. Set **Start Number** 001 to begin naming sequence for the images. Set the file format to JPEG by choosing JPEG (*.jpg) for **Type** under **Image Format** and click on **Options** to adjust for **Details**. Set the image format quality by adjusting **Compression Level** to 16, **Encoding** to Standard, and check **Embed ICC Profile**. Click **OK** to close the window (**Figure 3B**).

3.3.3) Leave the first option unchecked ("Overwrite any files with the same name") and check the 3 next options ("Show this dialog box before next scan", "Open image folder after scanning", and "Show Add Page dialog after scanning"). Click **OK** to close the window

3.3.4) Check the image quality by clicking on **Preview**. The preview window appears, and the **Scan** icon becomes functional (**Figure 3C**).

3.4) Use the scripting software to automate the image acquisition. The provided script clicks on **Scan** in the Scan window and **OK** in the **File Save Settings** window at 30-min intervals.

3.4.1) Import the script by clicking on **Task | Import** and select both **Single_scan.tsk** and **Idle_scanning.tsk** (TSK files provided as **Supplemental Files 1 and 2**). See **Figure 3D**.

NOTE: *Single_scan.tsk* clicks on the **Scan** button in the Scan window and **OK** in the File Save Settings window. *Idle_scanning.tsk* activates *Single_scan.tsk* every 30 min. One may change the scan frequency by changing the activation of *Idle_scanning.tsk*.

3.4.2) Enable automatic scanning at 30 min intervals by selecting both **Idle scanning (imported)** and **Single scan (imported)**, right clicking on **Idle scanning (imported)**, and left clicking on **Enabled** (**Figure 3D**, **Supplementary Figure S2**).

NOTE: Automatic scanning runs until the user manually stops the script. To stop the script, select **Idle scanning (imported)**, right click **Idle scanning (imported)**, and left click on **Enabled**. The check mark will be removed.

4. Compiling time-lapse images and measuring swarm repulsion

4.1) Perform movie editing and image analysis using ImageJ.

4.2) Import all the scanned images to ImageJ by clicking on **File | Import | Image Sequence** and select the images. In the **Sequence Options** window, check **Convert to RGB** to keep images in color. **Number of images** indicates the number of images selected.

4.3) Keep **Starting image** at 1 to start from the first picture in the folder and **Scale images** at 100% to conserve original size of the images. Leave **Use virtual stack** unchecked. Click **OK** and wait for

images to load (**Figure 4A**).

4.4) Set the video compression level to 100 by clicking on **Edit | Options | Input/Output…** and adjust **JPEG quality** to 100.

4.5) Save the file as an .avi by clicking on **File | Save As | AVI**. Adjust **Compression** to JPEG and **Frame Rate** to 5 fps (**Figure 4B**). Save the .avi time-lapse in the desired folder.

4.6) To quantify swarm repulsion distances, open an image near the end of the swarming period in ImageJ. Click on **File | Open** and select the image. Adjust the scale by clicking on **Analyze | Set Scale** and setting **Distance in pixels** to 118, **Known distance** to 1, **Pixel aspect ratio** to 1.0, and **Unit of length** to cm (**Figure 4C**). Leave **Global** unchecked. Click **OK** to close the window.

4.7) Click on the **Straight icon** and measure from the center of the colony at the satellite position to the edge of the swarming population. Select **Analyze | Measure** to make a new window appear with the measurements (Length) (**Figure 4D**).

NOTE: Use "+" to zoom in closer and "-" to zoom out.

REPRESENTATIVE RESULTS:

The steps to grow *P. aeruginosa*, stress the cells, and image the swarming agar plates are represented in **Figure 1**. We inoculated a single colony of wild-type *P. aeruginosa* UCBPP-PA14 strain from an LB-agar plate in 2 mL of LB broth overnight at 37 °C and spotted 5 µL in the center of the swarming agar plate. Time-lapse imaging of this plate reveals initial growth in the form of a colony at the center and then spreading of tendrils radially from the colony (**Video 1**). For collective stress response assays, in addition to spotting *P. aeruginosa* at the center, 30 µL of the same overnight culture is mixed with 6 µL of 1 x 10^{12} pfu/mL DMS3vir or 6 µL of 3 mg/mL gentamycin at a ratio of 5:1 and 6 µL is spotted at the satellite positions. Swarms move from the center of the swarming agar plates to the periphery and are repelled by a stress signal emitted by the bacteria that were infected with phages (**Video 2**, top left plate) or treated with gentamycin (**Video 2**, top right plate). Phages (**Video 2**, bottom left plate) or gentamycin (**Video 2**, bottom right plate) spotted alone at the satellite positions do not cause swarming populations to avoid these areas.

FIGURE LEGENDS:

Figure 1: Schematic of the *P. aeruginosa* swarming assay and collective stress response. (**A**) *P. aeruginosa* cells are grown overnight (16–18 h to OD_{600} of approximately 1.5) in LB broth at 37 °C and (**B**) spotted in the middle of the swarming agar plate. Overnight cultures are mixed with (**C**) phages or (**D**) antibiotics and spotted at the satellite positions for collective stress response assays. (**E**) Up to 6 plates are imaged on a scanner at 30 min intervals for 16–18 h at 37 °C. After 18 h, *P. aeruginosa* swarming populations avoid (**F**) cells infected with phage or (**G**) cells treated with antibiotics (gentamycin). (**H**) *P. aeruginosa* populations swarm across the swarming agar plate.

Figure 2: Scanner setup inside the incubator. (**A**) Black Petri dish lids constructed in section 1. These lids are used during scanning to reduce light reflections and replace clear Petri dish lids. (**B**) The flatbed document scanner is placed in an incubator set at 37 °C. Six plates with black lids are placed on the scanner (left image). Black matte fabric is attached to the rack 60 cm above the scanner to further reduce reflections and stray light (right image).

Figure 3: Automated image acquisition from the flatbed document scanner using the scanning and automatic scripting software. (A) Screenshot of main Scan window. Selection of Image type (Color) and Resolution (300 dpi). The red square indicates the folder icon to open File Save Settings window. Note the Preview button can be pressed but the Scan button is disabled. (B) Screenshot of File Save Settings window to set folder destination for saving images, naming the images, and choosing the format of the images (left). The Plug-In Settings window is used to set the image format quality (right). (C) Screenshot of Scan window after clicking on Preview. The Scan button is clickable after a preview has been acquired. The program can now be automated using the scripting software (Materials). (D) Screenshot of the scripting software windows indicating the Import button used to import the automation scripts (left). Once Single_scan.tsk and Idle_scanning.tsk are imported, these appear as tasks in the main window (right). After selecting both tasks and right clicking them, the Enabled button appears. Left clicking Enable starts the scripts to automatically scan at 30-min intervals (right).

Figure 4: Image analysis of swarming avoidance using ImageJ. (**A**) Steps to import an image sequence from the time-lapse scanner images. Clicking on File | Import | Image Sequence in the main ImageJ window (left) brings up the Sequence Options window (right) and opens all the scanned images. The red square indicates the checked option to load images in RGB format. All other options are left as default. (**B**) Steps to save the time-lapse video in AVI format. Selecting File | Save As | AVI brings up the Save as AVI window. Compression is set to JPEG and Frame Rate to 5 fps. (**C**) Setting the scale units for images. Selecting Analyze | Set Scale bring up the Set Scale window. For 300 dpi images, the appropriate scale is 118 pixels/cm. (**D**) Measurement of avoidance from swarming populations. A yellow line is drawn from the center of the stressed colonies to the edge of the tendrils. Selecting Analyze | Measure reports the length of the line in a new window labeled Results. Ctrl + M is a keyboard shortcut that performs the measurement without selecting the menu items.

Figure 5: Representative swarms of *P. aeruginosa*. *P. aeruginosa* swarming populations on swarming agar plates that are (**A**) dry, (**B**) normal, (**C**) moist, and (**D**) extra moist. Dry swarming agar plates inhibit the swarm rate of *P. aeruginosa* and reduce the number of tendrils. Moist swarming agar plates cause formation of large tendrils. Under extra moist conditions, tendrils form unevenly throughout the swarming agar plates. Drying times in the laminar flow hood and ambient humidity have significant effects on swarming plate moisture content. The dishes are 10 cm Petri dishes.

Video 1: Time-lapse movie of swarming. Wild-type *P. aeruginosa* were spotted at the center of the swarming plate and were imaged on the scanner over the course of 22 h.

Video 2: Time-lapse movie of the collective stress response. Wild-type *P. aeruginosa* were spotted at the center of the swarming plate. Satellite positions were spotted with *P. aeruginosa* that are mixed additionally with (upper-left) phage or (upper right) gentamycin, or spotted solely with (lower-left) phage or (lower-right) gentamycin. White dots indicate the center of the spots. Plates were imaged over the course of 16 h.

Supplementary Figure S1: Plating template for spotting *P. aeruginosa* cells. The middle black dot represents the spotting area of 5 µL overnight *P. aeruginosa* culture. The inner circle is 5.8 cm away from the center of the plate. The intersection between the inner circle and the straight lines across the outer circle indicates the spotting area of 6 µL of stressed *P. aeruginosa*, phage infected or antibiotics treated cells. The outer circle represents the circumference of 10 cm Petri dish.

Supplementary Figure S2: Macro commands to periodically start scanning using a scripting software. (**A**) The macro commands in Single_scan.tsk moves the cursor to Scan in Scan window, clicks on Scan, moves to OK in File Save Settings window, and clicks on OK. (**B**) Commands to scan in 30-min intervals. The task Idle_scanning.tsk starts Single_scan.tsk and is set to activate in 30-min intervals.

DISCUSSION:

This protocol focuses on minimizing the variability in swarming agar plates and providing a simple and low-cost method to acquire time-lapse images of *P. aeruginosa* swarming and responding to stress. This procedure can be extended to image other bacterial systems by adapting the media composition and growth conditions. For *P. aeruginosa*, although M9 or FAB minimal medium can be used to induce swarming[16, 21], the protocol presented here uses M8 medium with casamino acids, glucose, and magnesium sulfate[6]. *P. aeruginosa* swarming is sensitive to medium composition such as iron availability and nutrient sources including amino acids[22–24]. Therefore, the selection of media for swarming agar plates illustrates an important aspect of assaying swarming motility.

Controlling for the humidity and temperature in an open laboratory area represents one of the largest challenges for consistency of swarm assays. Seasonal changes contribute to variability in the swarming agar plates moisture, which can significantly impact swarming patterns. Therefore, constant control of the relative humidity is required to ensure optimal plate quality. Starting the dehumidifier 1 h prior to drying the swarming agar plates under the laminar flow hood will control the relative humidity to a constant 45%, keeping drying time to 30 min. If ambient moisture cannot be controlled, increasing the drying time is a potential simple solution to compensate for humid environments. During swarming, relative humidity should stay at 70% in the 37 °C incubator to prevent the agar plates from drying out. An uncapped bin of water in the incubator can serve as a water reservoir. Dry swarming agar plates slow down the progression of swarming populations and reduce the number of tendrils while moist plates cause broad tendril structure (**Figure 5A–C**). Extra moist swarming agar plates prevent clear tendril formation and cause the tendrils to spread in an uneven pattern (**Fig 5D**). The method described here can be used to

maintain a constant humid environment that will ensure consistency of swarming on plates (**Figure 5B**, **Video 1**). Additionally, plate size and agar thickness play a role in retaining moisture in the plate. We have used 10 cm diameter Petri dishes and added 20 mL of swarming agar solution per plate to ensure consistency. Pouring plates without measuring volumes is not recommended. Due to the many variables that affect the swarming assay, we recommend optimizing the assay to local laboratory conditions and we stress the importance of performing multiple biological replicates on separate batches of plates to observe consistent and comparable swarming patterns.

The advantage of the time-lapse imaging method to record swarming motility is the ability to observe the progression of motility without the need to disturb the swarms. Our method conveniently creates time-lapses of 6 plates concurrently under the same conditions, which provides both a controlled environment for the simultaneous assessment of multiple strains, multiple experimental conditions, or biological replicates. The use of six satellite positions on each plate additionally facilitates statistical analysis and the use of ImageJ enables the quantification of swarming repulsion.

The procedure described here is a simple method to study the interaction between sub-populations of *P. aeruginosa*: a healthy swarming population and stressed cells. Beyond DMS3vir and gentamycin, additional types of phages, antibiotics, and competing bacteria or fungi can be used to study stress signaling. Although this method focuses on *P. aeruginosa* swarming motility, other bacterial species such as *S. aureus* and *E. coli* also exhibit swarming patterns, but they require adapted media to swarm[10, 11]. By optimizing media compositions and plate conditions, this method can be applied to analyze swarming, swarming interactions between bacterial strains, and stress responses.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

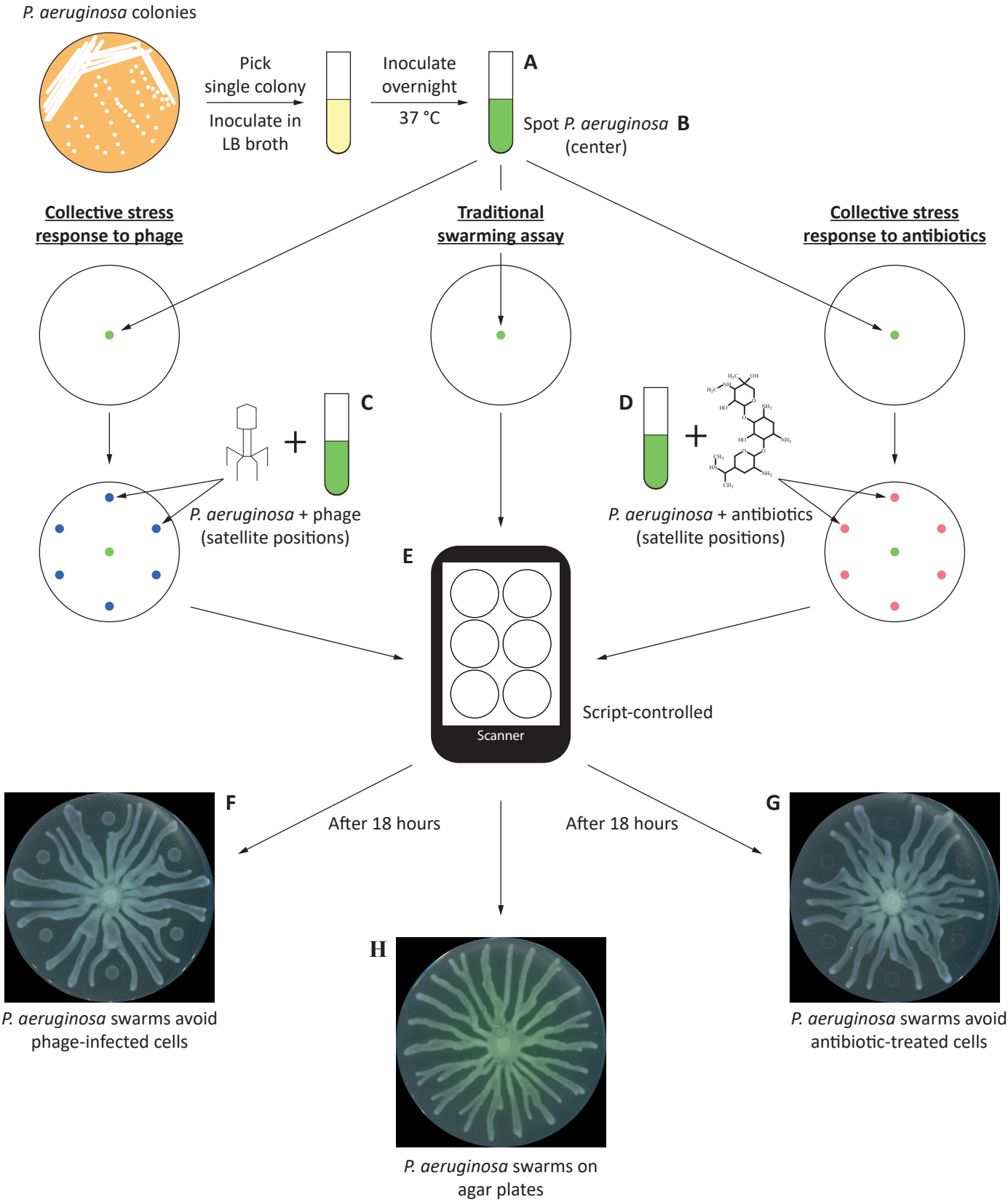

Figure 2

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A

B

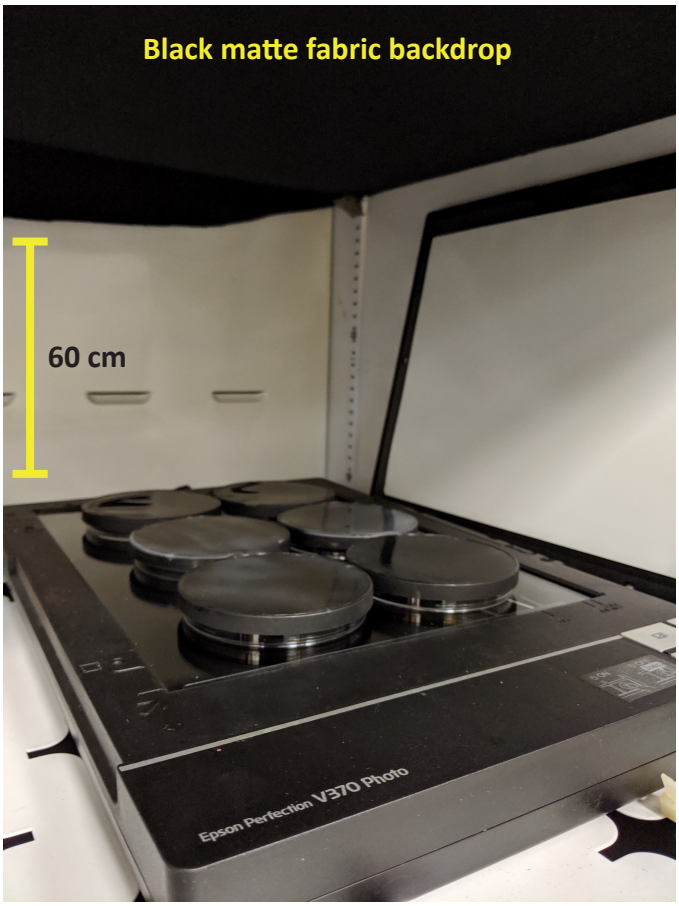

Figure 3

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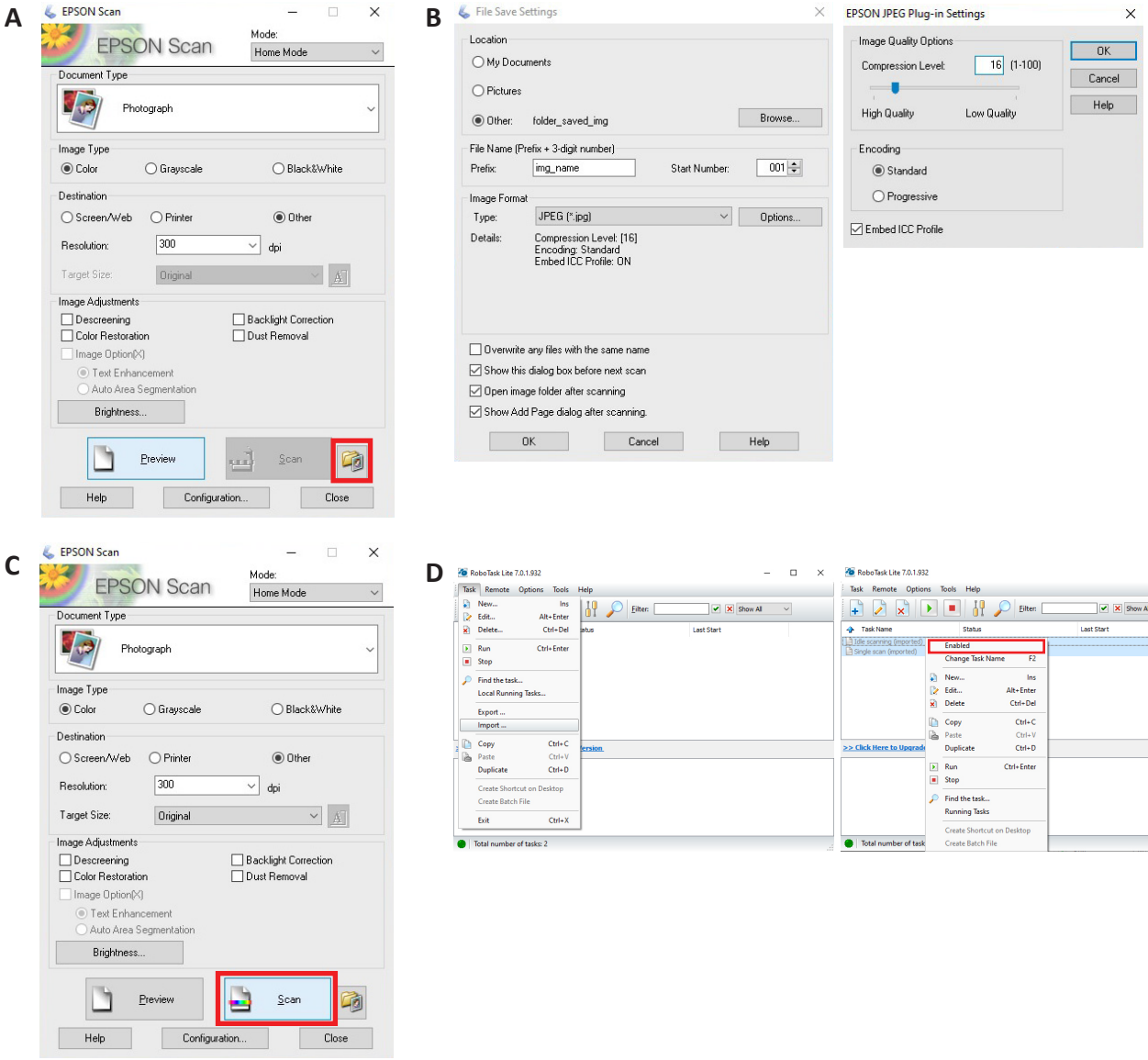

Figure 4

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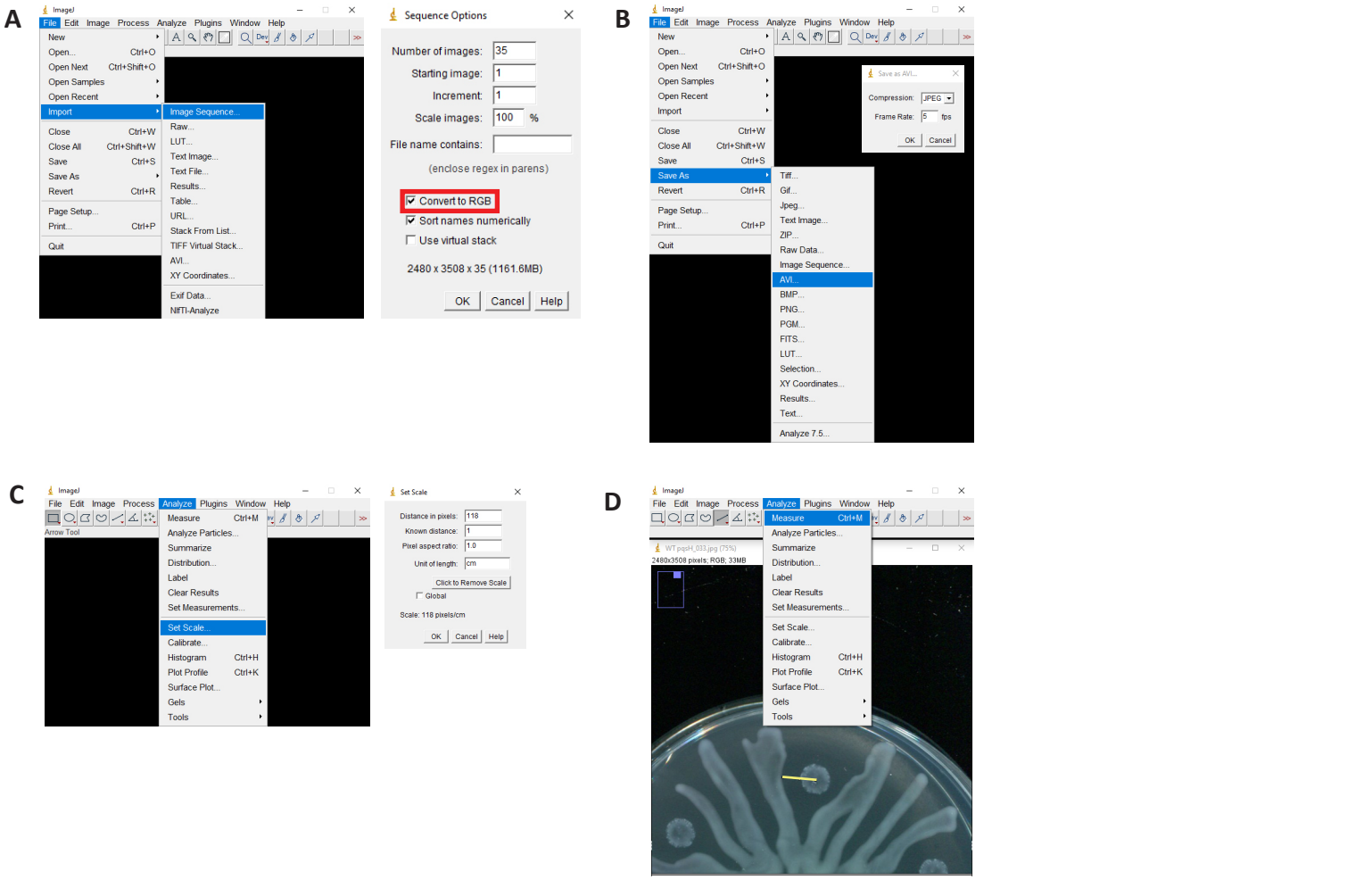
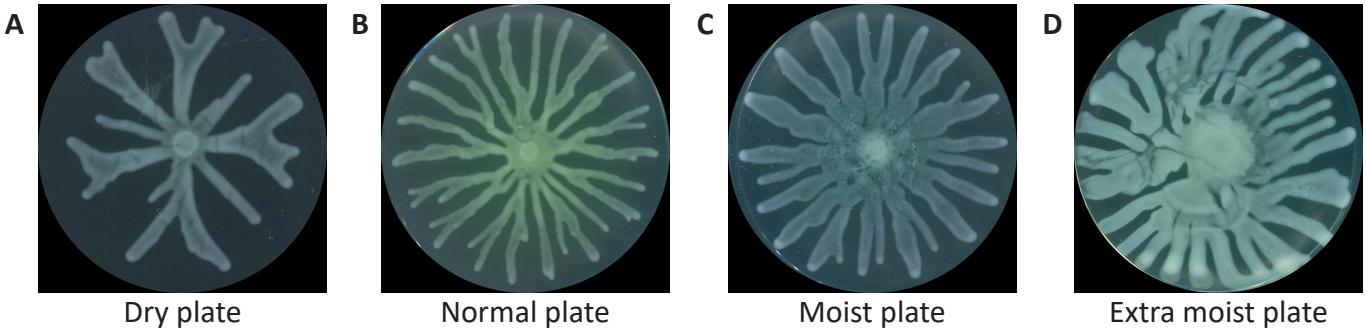


Figure 5



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Video 2

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Name	Company	Catalog Number	Comments/Description
Reagents			
Bacto agar, dehydrated	BD Difco	214010	For LB-agar plate and swarming agar plate
Casamino acids	BD Difco	223050	For swarming media
D-Glucose	Fisher Chemical	D16500	Dextrose. For swarming media
Fosfomycin disodium salt	Tokyo Chemical Industry	F0889	Stock concentration: 200 mg / mL. Dissolved in ddH_2O
Gentamycin sulfate	Sigma-Aldrich	G1914	Stock concentration: 3 mg / mL. Dissolved in ddH_2O
Kanamycin sulfate	Sigma-Aldrich	60615	Stock concentration: 100 mg / mL. Dissolved in ddH_2O
LB-Miller	BD Difco	244620	For LB broth and LB-agar plates
Magnesium sulfate heptahydrate	Sigma-Aldrich	230391	For swarming media
Potassium phosphate monobasic	Sigma-Aldrich	P0662	For 5X M8 media
Sodium chloride	Sigma-Aldrich	S9888	For 5X M8 media
Sodium phosphate dibasic	Fisher Chemical	S373	For 5X M8 media
Strains			
Pseudomonas aeruginosa	Siryaporn lab	AFS27E.1[18]	PA14 strain
DMS3vir	O'Toole lab	DMS3vir[20]	Bacteriophage
Supplies			
Aluminium oxide sandpaper	3M	150 Fine	For black lids
Black fabric	Joann	PRD7089	Black fabric
Black spray paint	Krylon	5592 Matte Black	For black lids
Erlenmeyer flask	Kimax	26500	250 mL
Glass storage bottles	Pyrex	13951L	250 mL, 500 mL, 1000 mL
8 inches zip ties	Gardner Bender	E173770	For attaching black matte fabric
Petri dishes (100 mm x 15 mm)	Fisher	FB0875712	100 x 15 mm polystyrene plates
Wooden sticks	Fisher	23-400-102	For streaking and inoculating bacteria
Equipment			
Autoclave	Market Forge Industries	STM-E	For sterilizing reagents
25 mL pipette	USA Scientific, Inc.	1072-5410	To pipet 20 mL for swarming agar plates
Dehumidifier	Frigidaire	FAD704DWD 70-	For maintaing room relative humidity at about 45%
ImageJ	NIH	v1.52a	Software for image analysis
Incubator	VWR	89032-092	For growth of bacteria at 37 °C

Isotemp waterbath	Fisher	15-462-21Q	For cooling media to 55 °C
Laminar flow hood	The Baker Company	SG603A	For drying plates
P-20 pipet	Gilson	F123601	Spotting on swarming agar plates
Pipette Controller	BrandTech	accu-jet	To pipet 20 mL for swarming agar plates
Roller Drum	New Brunswick	TC-7	For growth of bacteria at 100 rpm
Scanner	Epson	Epson Perfection	Scanner for imaging plates
Scanner automation software	RoboTask Lite	v7.0.1.932	For 30-min internals imaging
Scanner image acquisition software	Epson	v9.9.2.5US	Software for imaging plates

Response to reviewers:

We thank the reviewers and the editor for a constructive review process. Our point-by-point response to each concern is addressed below in red.

Editorial comments:
General:

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

We have fully checked the entire manuscript for these issues.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of 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: Epson

We have removed any mention of commercial language or company name before an instrument or reagent in the main text. There is no more mention of Epson or RoboTask Lite.

Protocol:
1. For each protocol step/substep, 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. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

We have re-read the protocol and ensured that we have fully addressed “how” each step is done. We have added an additional reference that describes specifically the way an M8 swarming plate is made.

Specific Protocol steps:
1. 2.6, 2.7: The ‘next step’ is unclear-it could mean either 2.6.1/2.7.1 or 2.7/2.8.

We have changed step 2.6 and 2.7 to respectively 2.5 and 2.6. Step 2.8 and 2.8.1 are now 2.7 and 2.7.1 respectively. We have specified exactly which step to continue after step 2.4. Step 2.5 now directs specifically to step 2.5.1, then step 2.7. Step 2.6 now directs to step 2.6.1, then step 2.7.

We have also removed step 3.2 (“Acquire time-lapse images of swarming agar plates using EPSON Scan and RoboTask Lite.”) and shifted all the following steps one number down.

Figures:
1. Please remove ‘Figure 1’ etc. from the Figures themselves.
2. Figure 2C is cited in the manuscript (step 2.8.1) but is not present in the figure.

We have removed the ‘Figure’ label from the figures themselves and have removed the mention of Figure 2C.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

We have ensured that the Table of Materials is complete. We added 3 new pieces of equipment: autoclave, 25 mL pipette, and pipette controller.

Reviewers' comments:
Reviewer #1:

Manuscript Summary: This paper reported a robust procedure to generate agar plates suitable for swarming assay and documented detailed steps on how to perform the swarming assay, imaging the swarming phenotype overtime using a simple and lost cost imaging system with scanner, and the analysis steps with ImageJ. The authors have done a great job in detailing the potential problems in this assay and strategies to troubleshoot them. A good example is given regarding how stresses such as phage challenge and antibiotic treatment can affect the swarming process. I think the procedure provided by the paper will benefit many labs performing or planning to perform swarming assay. The paper is well written. I recommend publishing the paper as it is.

Major Concerns: N/A

Minor Concerns: N/A

We thank the reviewer for their positive comments.

Reviewer #2:

Manuscript Summary:
The protocol described here under the title, "Time-lapse imaging of bacterial swarms and the collective stress response" is a simple procedure that is designed to be not only quick but reproducible. Swarming is highly sensitive to assay conditions such as temperature, humidity, and media composition. A precise protocol that controls for these conditions appropriately such as described here is very useful to many microbiology labs that study swarming and/or motility.

Major Concerns:

None

Minor Concerns:
It would be nice to elaborate a bit on other potential uses of this protocol in the discussion.

We thank the reviewer for their comments and suggestion. We have elaborated on potential other uses at the end of the discussion (line 422-428).

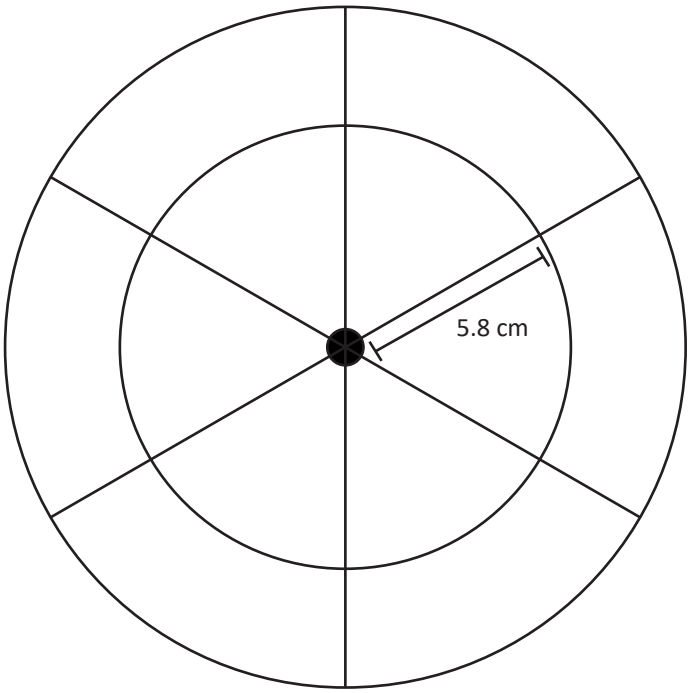

Figure S1. **Plating template for spotting *P. aeruginosa* cells**. The middle black dot represents the spotting area of 5 µL overnight *P. aeruginosa* culture. The inner circle is 5.8 cm away from the center of the plate. The intersection between the inner circle and the straight lines across the outer circle indicates the spotting area of 6 µL of stressed *P. aeruginosa*, phage infected or antibiotics treated cells.The outer circle represents the circumference of 10-cm Petri dish.

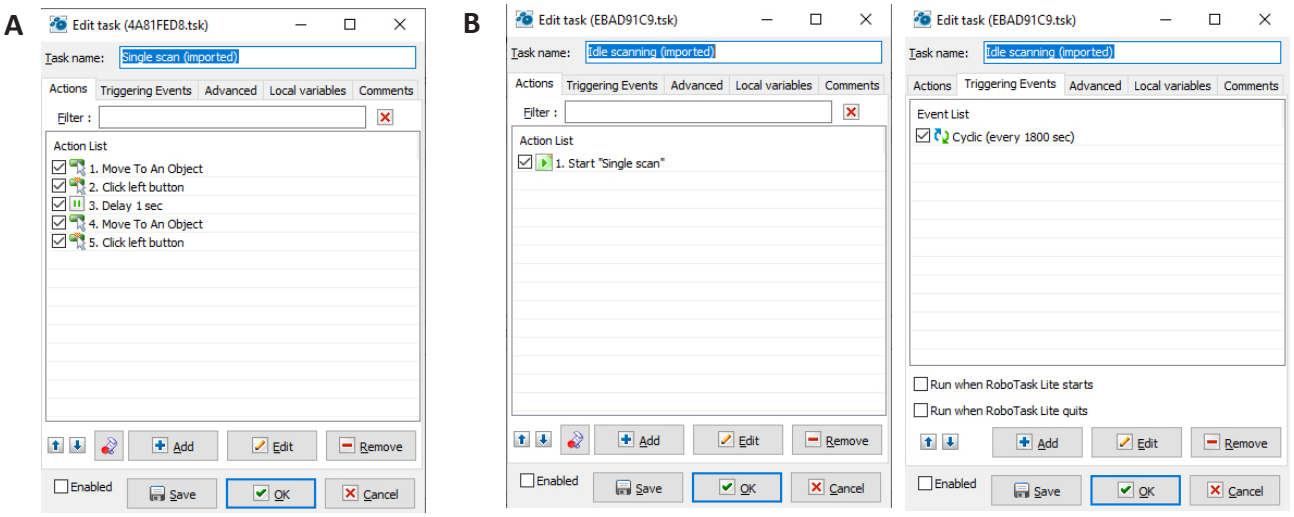

Figure S2. **Macro commands to periodically start scanning using a scripting software**. (A) The macro commands in Single_scan.tsk moves the cursor to Scan in Scan window, clicks on Scan, moves to OK in File Save Settings window, and clicks on OK. (B) Commands to scan in 30-min intervals. The task Idle_scanning.tsk starts Single_scan.tsk and is set to activate in 30-min intervals.

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Author(s):	Jean-Louis Bru, Albert Siryaporn, and Nina Molin Høyland-Kroghsbo

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