FINAL SCRIPT: APPROVED FOR FILMING

Submission ID #: 67761
Scriptwriter Name: Pallavi Sharma
Project Page Link: https://review.jove.com/account/file-uploader?src=20667153

Title: High-Throughput Method for Observing Motility Phenotypes in *Pseudomonas aeruginosa*

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

   If **Yes**, we will need you to record using screen recording software.

   We recommend using the screen capture program OBS. JoVE's tutorial for using OBS Studio is provided at this link: https://review.jove.com/v/5848/screen-capture-instructions-for-authors?status=a7854k

   As these files are necessary for finalizing your script, please upload all screen-captured video files to your project page as soon as possible: **https://review.jove.com/account/file-uploader?src=20667153**

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length
Number of Steps: 25
Number of Shots: 54

Introduction

AUTHORS: The introduction statements are restricted to a 30-word limit as per the updated guidelines. The statements have been edited accordingly

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Océane Goncalves:** We study bacterial physiology using systems biology and genomics to identify infection-relevant genes, aiming to discover new antibiotic targets and alternative treatments.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What significant findings have you established in your field?

1.2. **Océane Goncalves:** We developed a high-throughput protocol for genome-wide analysis of *Pseudomonas aeruginosa* motility, uncovering molecular mechanisms linked to biofilm formation, colonization, and host defense evasion. This adaptable method applies to various assays and species.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:2.14*

How will your findings advance research in your field?

1.3. **Océane Goncalves:** Bacterial motility studies are often limited by throughput. Our high-throughput protocol enables genome-wide analysis, identifying all motility-related genes and uncovering mechanisms of bacterial infection.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:4.2*

What new scientific questions have your results paved the way for?

1.4. **Océane Goncalves:** Our findings highlight new questions *about Pseudomonas aeruginosa* motility, its role in biofilms, and pathogenesis, while exploring environmental influences and potential antimicrobial strategies targeting motility genes.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:4.1*

Videographer: Obtain headshots for all authors available at the filming location.

2. High-Throughput Motility Experiment to Study the Motility Behaviors of *Pseudomonas aeruginosa*

Demonstrator: Océane Goncalves

~~2.1. To begin, prepare twitching plates and swarming plates **[1-TXT].** Pour 25 milliliters of media into each plate **[2].** Gently lift each corner of the plate, two times, to evenly distribute the media **[3-TXT].** Replace the lid back onto the plate **[4].**~~

~~2.1.1. WIDE: Talent in front of twitching and swarming labelled plates. **TXT: Refer manuscript for plate preparation**~~

~~2.1.2. Shot of media being poured into each plate.~~

~~2.1.3. Shot of the plate corners being lifted and media being distributed.~~

~~2.1.4. Talent places the lid back on to the plate.~~

~~2.2. Once the plates have dried at room temperature, place them in hermetic bags for storage of upto 3 weeks **[1].**~~

~~2.2.1. Shot of the plates being placed in hermetic bags.~~ NOTE: Not filmed

2.3. To begin, take the source plates of the *Pseudomonas aeruginosa* transposon mutant library **[1]** and leave them flat on a surface at room temperature for approximately 1 hour to cool down **[2-TXT]**.

2.3.1. Talent removing the plates from a 4°C fridgeg. .

2.3.2. Talent placing the plate on a flat surface. **TXT: Remove lid condensation with a delicate wipe to avoid water droplets on plates**

2.4. For the desired assay, select M9-glucose 0.5% agar plates for swarming, and LB agar 1% agar plates for twitching **[1]**.

2.4.1. Close-up shot of the two types of plates labeled for their respective assays.

2.5. To configure the replicator for accurate colony transfer, turn on the replicator **[1]**. In the section **Select an operate mode**, choose **Select and Run Stored Programs [2]**. In the section Select source plates, select **PlusPlate 384 Agar** *(Plus-Plate-Three-Eighty-Four-Agar)* **[3].**

2.5.1. Talent interacting with the replicator and turning it on.

2.5.2. Main screen of the replicator showing the option Select and Run Stored Programs being selected.

2.5.3. SCREEN: 67761_Steps-2-5-to-2-9.mp4: 00:23-00:33

2.6. Then, in the Select target plates section, select **PlusPlate 384 Agar [1].** In the section **Select pads**, select **Short Pin 384 [2]**. In **Singer Programs**, select **Replicate Many** then go to Replicate program options and choose **General, Recycle,** and **None [3].**

2.6.1. SCREEN:67761_Steps-2-5-to-2-9.mp4: 00:43-00:45

2.6.2. SCREEN: 67761_Steps-2-5-to-2-9.mp4: 00:47-00:52

2.6.3. SCREEN: 67761_Steps-2-5-to-2-9.mp4: 00:58-01:10

2.7. In the section **Source**, select **Offset,** then **Random** and **Default Radius [1]**. In the section **Target**, select **Pinning** and adjust the pressure to 2%. Leave all other settings as default **[2]**.

2.7.1. SCREEN: 67761_Steps-2-5-to-2-9.mp4: 01:17-01:25

2.7.2. SCREEN: 67761_Steps-2-5-to-2-9.mp4: 01:27-01:34

2.8. Then, insert the **Short Pin RePads 384** into the appropriate compartment **[1]**.

2.8.1. Talent inserting the Short Pin RePads 384 into the designated compartment of the replicator.

2.9. Position the source and target plates on the designated platform of the replicator **[1]**. Place the 384-density source plates and the empty motility plates onto the platform **[2]**. Press **Run Using the Selected Parameters** to begin the transfer process **[3]**. The microbial array pinning robot will transfer colonies from the 384-density source plates to the motility plates **[4]**.

2.9.1. Talent positioning the source and target plates on the replicator platform.

2.9.2. Close-up of the 384-density source plates and empty motility plates being placed.

2.9.3. SCREEN: 67761_Steps-2-5-to-2-9.mp4: 01:48-01:50

2.9.4. Shot of the pinning robot transferring colonies onto the motility plates.

2.10. Afterward, place the inoculated motility plates into plastic bags **[1]**. Carefully place the bags containing the plates into the incubator set to 37 degrees Celsius for 18 hours **[2]**.

2.10.1. Talent placing the motility plates inside the plastic bags.

2.10.2. Talent placing the bags into the incubator set at 37 degrees Celsius.

2.11. After the incubation period, turn on the Phenobooth **[1]** and click on the **icon** corresponding to the Phenobooth software **[2]**.

2.11.1. Talent powering on the Phenobooth.

2.11.2. SCREEN: Computer screen showing the Phenobooth software icon being clicked. NOTE: Use the missing media title card

2.12. When the **Select Project** page opens, click **New Project,** followed by **Colony Counting [1]**. In the **Location** field, choose the desired file path to store the images. In the **Resolution** field, select **5626 x 4220** and click **OK** to confirm **[2]**.

2.12.1. SCREEN: "New Project > Colony Counting" being selected. NOTE: Use the missing media title card

2.12.2. SCREEN: The file path is entered in the Location field, 5626 x 4220 is selected in the Resolution field, the OK button is clicked, and a confirmation message is displayed. NOTE: Use the missing media title card

2.13. After removing the lid, insert the plate into the reader with the medium side facing up **[1]**.

2.13.1. Talent inserts the plate into the reader with the medium side facing up.

2.14. In the **Lighting** tab, set the parameter to **white light** and click **Preview** to check the image **[1]**. Adjust the exposure settings in the **PhenoBooth settings** tab to obtain optimal parameters for observing motility phenotypes **[2]**.

2.14.1. SCREEN: "White light" option selected in the Lighting tab. Image preview displaying the plate. NOTE: Use the missing media title card

2.14.2. SCREEN: Exposure settings being adjusted for optimal observation.NOTE: Use the missing media title card

2.15. Then, click **Acquire** to capture the images. The captured images will appear on the left side of the screen **[1]**. Once finished, close the software, and all captured images will be saved in the previously chosen file path from the **Location** tab **[2]**.

2.15.1. SCREEN: Acquire button being clicked, and the capture process starting. NOTE: Use the missing media title card

2.15.2. SCREEN: Software being closed after the image capture process is completed.NOTE: Use the missing media title card

3. Quantitative Assessment of Bacterial Motility Using ImageJ and Manual Validation Assays

3.1. After performing the motility assay and imaging the motility plates **[1],** run the macro in ImageJ **[2]**. Open the folder containing the images to load the batch for analysis **[3]**.

3.1.1. WIDE: Talent in front of the computer with the acquired images in the frame.

3.1.2. SCREEN: 67761_Steps-3-1-to-3-5.mp4: 00:11-00:15

3.1.3. SCREEN: 67761_Steps-3-1-to-3-5.mp4: 00:16-00:24.

3.2. When prompted, modify the angle using the **Preview** option until the plate is straight to adjust the plate's rotation **[1]**. When satisfied, click the **OK** button **[2]**.

3.2.1. SCREEN: 67761_Steps-3-1-to-3-5.mp4: 00:31-00:39

3.2.2. SCREEN: 67761_Steps-3-1-to-3-5.mp4: 00:40-00:43

3.3. Then, move the rectangle tool around the colonies to crop the portion of the plate containing the colonies **[1]**. When ready, click **OK** to complete the cropping **[2]**.

3.3.1. SCREEN: 67761_Steps-3-1-to-3-5.mp4: 00:50-00:56

3.3.2. SCREEN: 67761_Steps-3-1-to-3-5.mp4: 01:00-01:03

3.4. Define the grid used to draw the **Region of Interest (ROI)** around each colony and measure the motility area **[1]**. Select the appropriate colony density and adjust the parameters as needed to ensure that each circle is positioned around a colony **[2]**.

3.4.1. SCREEN:67761_Steps-3-1-to-3-5.mp4: 01:09-01:13

3.4.2. SCREEN: 67761_Steps-3-1-to-3-5.mp4: 01:19-01:26

3.5. When ready, activate the **Okay?** *(Okay)* box and click the **OK** button **[1]**. The motility area for each colony will be measured, and a CSV file containing the data will be saved in the designated folder **[2]**.

3.5.1. SCREEN: 67761_Steps-3-1-to-3-5.mp4: 01:45-01:49

3.5.2. SCREEN: 67761_Steps-3-1-to-3-5.mp4: 01:50-01:52

3.6. Combine the CSV file containing the motility data with the plate legend, also organized by rows, to identify the motility area corresponding to each mutant **[1]**.

3.6.1. SCREEN: CSV file containing motility data being opened alongside the plate legend. **TXT: Lower integrated density indicates reduced or non-motile mutants** NOTE: Use the missing media title card

3.7. For motility assay, autoclave an agar solution containing M9, glucose, casamino acids, and magnesium sulfate **[1]**. Pour 25 milliliters of the cooled, mixed agar into each Petri plate **[2]**.

3.7.1. Talent placing the agar solution into the autoclave.

3.7.2. Talent pouring the cooled agar mixture into individual Petri plate.

3.8. Then, inoculate each plate with 2.5 microliters of overnight bacterial culture **[1]** and incubate at 37 degrees Celsius for 18 hours with the lids facing up to observe motility phenotypes **[2]**.

3.8.1. Talent using a micropipette to add 2.5 microliters of bacterial culture to the center of the plate.

3.8.2. Talent placing the plate inside the incubator with the lids facing up.

3.9. After autoclaving a solution of LB with 1% agar, pour 25 milliliters of the cooled, mixed LB 1% agar into Petri plates **[1]**.

3.9.1. Talent pouring the cooled LB agar mixture into Petri plate.

3.10. Stab-inoculate bacteria to the bottom of the twitching plates containing 1% agar **[1]**. Incubate the plates at 37 degrees Celsius for 48 hours, and then at room temperature for an additional 48 hours **[2]**. After incubation, carefully remove the agar from the plates **[3]**. Visualize the twitching zone by staining the Petri dish with 1% crystal violet solution **[4-TXT]**.

3.10.1. Talent using an inoculation needle to stab-inoculate bacteria into the twitching plate.

3.10.2. Talent placing the inoculated plate inside the incubator set to 37 degrees Celsius.

3.10.3. Talent removing the agar from the plate.

3.10.4. Talent applying 1% crystal violet to the Petri dish for staining the twitching zones. **TXT: Photograph the stained Petri dishes using the PhenoBooth**

Results

4. Representative Results

4.1. The twitching motility assay showed that *Pseudomonas aeruginosa* mutants with T4P machinery gene deletions exhibited significantly smaller halo regions compared to the wild type, indicating reduced twitching motility **[1].**

4.1.1. LAB MEDIA: Figure 3. Video editor: *Highlight the green dots corresponding to pilO, pilF, and pilG in figure 3B and ΔpilO, ΔpilF, and ΔpilG panels in figure 3C*

4.2. Swarming motility analysis revealed that deletion mutants of the PA14 *(P-A-Fourteen)* library had significantly reduced halo regions with no protrusions, confirming defective swarming ability **[1].**

4.2.1. LAB MEDIA: Figure 4

1. Pseudomonas aeruginosa
Pronunciation link:
No entry in Merriam-Webster; reliable pronunciation available at HowToPronounce.com: https://www.howtopronounce.com/pseudomonas-aeruginosa How To Pronounce
IPA (American): /ˌsuːdəˈmoʊnəs ˌɛərəˌdʒɪˈnoʊsə/
Phonetic spelling: soo-duh-MOH-nuhs air-uh-jin-OH-suh

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2. Pseudomonas
Pronunciation link:
Merriam-Webster: https://www.merriam-webster.com/dictionary/pseudomonas Merriam-Webster
IPA (American): /ˌsuːdəˈmoʊnəs/
Phonetic spelling: soo-duh-MOH-nuhs

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3. Aeruginosa
Pronunciation link:
Combined with the genus above (HowToPronounce.com covers aeruginosa pronunciation) YouTube+15How To Pronounce+15YouTube+15
IPA (American): /ˌɛərəˌdʒɪˈnoʊsə/
Phonetic spelling: air-uh-jin-OH-suh

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4. Transposon
Pronunciation link:
Merriam-Webster: https://www.merriam-webster.com/dictionary/transposon OpenMD+14Merriam-Webster+14How To Pronounce+14
IPA (American): /træn(t)s-ˈpoʊzɑn/
Phonetic spelling: trans-POH-zon