Journal of Visualized Experiments

Kinetic visualization of single-cell interspecies bacterial interactions

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61376R1
Full Title:	Kinetic visualization of single-cell interspecies bacterial interactions
Section/Category:	JoVE Immunology and Infection
Keywords:	Microscopy, imaging, fluorescence microscopy, bacteria, bacterial interactions, interspecies interactions, motility, Pseudomonas, Staphylococcus
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Iowa City, Iowa, USA

TITLE:
Kinetic Visualization of Single-Cell Interspecies Bacterial Interactions

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SUMMARY:
This live-bacterial cell imaging protocol allows for visualization of interactions between multiple bacterial species at the single-cell level over time. Time-lapse imaging allows for observation of each bacterial species in monoculture or coculture to interrogate interspecies interactions in multispecies bacterial communities, including individual cell motility and viability.

ABSTRACT:
Polymicrobial communities are ubiquitous in nature, yet studying their interactions at the single-cell level is difficult. Thus, a microscopy-based method has been developed for observing interspecies interactions between two bacterial pathogens. The use of this method to interrogate interactions between a motile Gram-negative pathogen, *Pseudomonas aeruginosa* and a non-motile Gram-positive pathogen, *Staphylococcus aureus* is demonstrated here. This protocol consists of co-inoculating each species between a coverslip and an agarose pad, which maintains the cells in a single plane and allows for visualization of bacterial behaviors in both space and time.

Furthermore, the time-lapse microscopy demonstrated here is ideal for visualizing the early interactions that take place between two or more bacterial species, including changes in bacterial species motility in monoculture and in coculture with other species. Due to the nature of the limited sample space in the microscopy setup, this protocol is less applicable for studying later interactions between bacterial species once cell populations are too high. However, there are

several different applications of the protocol which include the use of staining for imaging live and dead bacterial cells, quantification of gene or protein expression through fluorescent reporters, and tracking bacterial cell movement in both single species and multispecies experiments.

INTRODUCTION:

Polymicrobial communities are ubiquitous in nature, spanning a variety of environmental[1-3] and human niches[4,5]. Some of the most notorious polymicrobial infections in human disease include dental biofilms[6], chronic wounds[7,8], and respiratory infections in individuals with chronic obstructive pulmonary disease[9], ventilator-associated pneumonia[10], and the genetic disease cystic fibrosis (CF)[11,12]. These infections are often composed of diverse microbial species; however, a recent focus on the interactions between the Gram-positive bacterium *Staphylococcus aureus* and the Gram-negative bacterium *Pseudomonas aeruginosa* has emerged. Studies including patients coinfected with these organisms and in vitro analyses reveal both competitive and cooperative interactions that can have profound and unexpected influences on disease progression, microbial survival, virulence, metabolism, and antibiotic susceptibility (reviewed in detail by Hotterbeekx et al. 2017[13] and Limoli and Hoffman 2019[3]).

The growing interest in interspecies interactions during infection has yielded a variety of methods for studying polymicrobial community behaviors. Typically, these studies have utilized plate or broth-based assays to investigate the phenotypic differences between monoculture and coculture. For example, coculturing *P. aeruginosa* and *S. aureus* on solid surfaces has allowed for visualization of growth inhibition or changes in colony phenotype, pigment, or polysaccharide production[14-16]. Mixed species biofilms, on biotic or abiotic surfaces, as well as coculturing bacterial species in liquid culture also has enabled measurement of changes in growth, metabolism, antibiotic tolerance, competition and viability, in addition to gene and protein expression[17,18]. While these bulk culture experiments have revealed insight into how *P. aeruginosa* and *S. aureus* might influence one another on the community-scale, they are unable to answer important questions about the interactions that take place at the single-cell level. The method presented here adds to the approaches for studying interspecies interactions by focusing on the changes in movement, cell viability, and gene expression of single cells within a cocultured community over time.

Single-cell interactions can widely differ from the interactions that take place in a bulk community of cells. Through single-cell analysis, heterogeneity within a community can be quantified to study how spatial placement of cells influence community dynamics or how gene and protein expression levels change within individual members of a group. Tracking cells can also provide insight into how single cells move and behave over time, through multiple generations. By tracking cell movement and changes in gene expression concurrently, correlations can be made between gene fluctuations and corresponding phenotypes. Previous protocols for studying individual bacterial species at the single-cell level have been described. These studies take advantage of live-imaging cells over time in a single plane, and have been useful for observing phenotypes like cell division and antibiotic susceptibility[19,20]. Additional live-imaging microscopy has been utilized to monitor growth, motility, surface colonization and biofilm formation of single

bacterial species[21-23]. However, while these studies have been insightful for understanding the physiology of bacteria in monoculture, experiments for observing single-cell behavior of multiple bacterial species over time in coculture are limited.

Here, previous protocols used for single-species imaging have been adapted to study interactions between *P. aeruginosa* and *S. aureus*. These organisms can be differentiated under phase contrast based on their cell morphologies (*P. aeruginosa* are rod-shaped and *S. aureus* are coccus-shaped). Development of this method recently enabled the visualization of previously undescribed motility behaviors of *P. aeruginosa* in the presence of *S. aureus*[24]. *P. aeruginosa* was found to be capable of sensing *S. aureus* from a distance and responding with increased and directional single-cell movements towards clusters of *S. aureus* cells. *P. aeruginosa* movement towards *S. aureus* was found to require the type IV pili (TFP), hair-like projections whose coordinated extension and retraction generate a movement called twitching motility[25].

These studies demonstrate the utility of this method for interrogating earlier interactions between species. However, imaging at high cell densities at later interaction time points is difficult given that single layers of cells can no longer be identified, which mostly poses issues during post-imaging analysis. Given this limitation, the method is best suited for earlier interactions that could then be followed up with traditional macroscopic assays at higher cell densities representative of later interactions. Additional limitations of this method include the low-throughput nature, since only one sample can be imaged at a time and the cost of the microscope, camera, and environmental chamber. Moreover, fluorescence microscopy poses risks to the bacterial cells like phototoxicity and photobleaching, therefore limiting the frequency that fluorescence images can be acquired. Lastly, the agarose pads used in this method are highly susceptible to changes in the environment, making it critical to control for conditions like temperature and humidity, given that the pads can start to shrink or expand if the conditions are not correct. Finally, while this method does not mimic the host environment, it provides clues for how different bacterial species respond on surfaces, which can be followed-up in assays designed to mimic environmental/host conditions.

This method differs from previous studies tracking single-cell movement, in that cells are inoculated between a coverslip and agarose pad, restricting cells to the surface. This enables cell tracking over time in a single plane; however, limits cycles of transient surface engagement observed when cells are submerged in liquid[26]. An additional benefit of imaging bacteria under an agarose pad is that it mimics macroscopic plate-based sub-surface inoculation assays classically used to examine *P. aeruginosa* twitching motility[25]. In this assay, bacterial cells are inoculated between the bottom of a Petri dish and the agar, keeping the cells in a single plane as they move across the bottom of the dish outward from the point of inoculation, much like this microscopy protocol.

The time-lapse microscopy protocol for visualizing interspecies interactions presented here consists of 1) preparing the bacterial sample and agarose pad, 2) selecting microscope settings for imaging acquisition and 3) post-imaging analysis. Detailed visualization of cell movement and tracking can be performed by acquisition of images at short time intervals by phase contrast.

Fluorescence microscopy can also be utilized to determine cell viability or gene expression over time. Here, we show one example of adaptation for fluorescence microscopy through the addition of viability dyes to the agarose pads.

PROTOCOL:

NOTE: A full description and catalog numbers for all supplies in this protocol can be found in the Table of Materials.

1. Preparation of M8T minimal media

1.1. Prepare 1 L of M8 minimal salts base (5x) by dissolving 64 g of $Na_2HPO_4 \cdot 7H_2O$, 15 g of KH_2PO_4, and 2.5 g NaCl in 800 mL of ultrapure water (UPW, resistivity 18 MΩ/cm) in a 2 L bottle. pH to 7.6. Complete volume to 1 L with UPW. Autoclave for 45 min.

1.2. Prepare 500 mL of a glucose solution (20% w/v) by dissolving 100 g of glucose in 400 mL of UPW in a 1 L bottle. Complete solution to 500 mL with UPW. Autoclave for 45 min.

1.3. Prepare 500 mL of a tryptone solution (20% w/v) by dissolving 100 g of tryptone in 400 mL of UPW. Complete to 500 mL with UPW. Autoclave for 45 min.

1.4. Prepare 1 L of M8 + 10% tryptone (M8T) minimal media by adding 200 mL of 5x M8 minimal salts (1x final), 10 mL of 20% glucose (0.2% final), 1 mL of 1 M $MgSO_4$ (1 mM final), and 50 mL of 20% tryptone (1% final) to 600 mL of UPW. Complete to 1 L with UPW. Filter through a 0.2 μm sterile filter into a sterile 500 mL media storage bottle.

Day 1:

2. Preparation of bacterial overnight cultures

2.1. Inoculate 5 mL of M8T minimal media with a single colony of *P. aeruginosa* or *S. aureus* (including antibiotics when appropriate) and incubate overnight at 37 °C with aeration for no longer than 16 h.

NOTE: The bacterial pathogens *P. aeruginosa* and *S. aureus* were used for this method, because they are commonly coisolated from chronic infections, and studying their interactions is important to understand how they contribute to patient outcomes during polymicrobial infections. Other bacterial species could be used depending on the focus of the study.

Day 2:

3. Subculture of bacterial strains

3.1. Subculture *P. aeruginosa* 1:500 and *S. aureus* 1:1000 in 5 mL of fresh M8T (include antibiotics when appropriate). Incubate with aeration at 37 °C until cultures reach mid-log phase (OD_{600} = ~0.3 - 0.5).

4. Preparation of materials for pad molds

4.1. Prepare metal spatulas by heating the end of a flat, rounded laboratory spatula with a Bunsen burner until half of the flat end can be bent to a 90° angle. Heat the end of another flat, rounded laboratory spatula and slightly bend the last 10 mm to a 45 °C angle.

4.2. Cut off the four corners of the silicone molds so that the molds fit inside a 35 mm dish.

4.3. Sterilize the spatulas and a pair of tweezers by adding 70% ethanol and passing them through the flame of the Bunsen burner.

4.4. Clean the dish and silicone molds with 70% ethanol and dry them with lint-free wipes.

5. Preparation of agarose pads

NOTE: The pads are prepared with the M8T minimal media as the nutrient source for the bacteria used in this protocol. However, the nutrients used in the pads can be modified for different organisms.

5.1. Melt 2% low-melt agarose in 10 mL of M8T in a clean 50 mL Erlenmeyer flask. Microwave in short intervals (2-5 s) until the agarose is in solution in order to prevent the contents of the flask from boiling over. Once melted, let cool in a 50 °C water bath for at least 15 min.

5.2. Prepare molds by aligning the silicone cutout with the opening in the 35 mm dish and tap lightly with spatula to secure the silicone to the dish, and to remove all air bubbles between the mold and the dish.

5.3. Once cool, pipette 915 µL of molten agarose into the mold. Leave the lid ajar and let the pad dry at room temperature for 30 min.

5.4. Cover the dish with the lid and leave at room temperature for an additional 2 h.

5.5. Prepare humidity wipes by tightly rolling up a lint-free wipe. Place the rolled wipe inside a sterile Petri dish and evenly add 500 µL of sterile water across the wipe. Warm to 37 °C for 1 h.

5.6. Warm the pad and a sterile 35 mm dish to 37 °C for 1 h.

6. Preparation of bacterial cells and inoculating pads

6.1. Measure the OD_{600} of each subculture and dilute *P. aeruginosa* to an OD_{600} = 0.03 and *S.*

aureus to an OD_{600} = 0.10 in M8T prewarmed to 37 °C. Mix *P. aeruginosa* and *S. aureus* in a 1:1 ratio and vortex.

NOTE: If strains require antibiotics, the antibiotics can be added to the overnight and subculture, but should not be added when mixing species for imaging if it affects the other species in the coculture. Plasmid stability and the effects of antibiotics on all species in the coculture will need to be determined for each organism/plasmid being used.

6.2. Pipette 1 µL of coculture evenly across the bottom of a pre-warmed, sterile 35 mm glass coverslip dish.

6.3. Remove the silicone cutout from the mold using sterile tweezers.

6.4. Remove the pad from the dish using sterile spatulas.

6.4.1. Slip the slightly bent spatula under the edge of the pad, while holding the mold upside down, to drop it out onto a sterile Petri plate lid.

NOTE: Take care not to force the pad out or it will rip. Keep track of which side of the pad is the bottom.

6.5. Transfer the pad to the dish with the bacterial cells, bottom-side-down, by sliding the 90° angled spatula under the pad and placing it on top of the inoculated coverslip. Use the 90° angled spatula to make the pad flush against the coverslip and gently press out any air bubbles.

6.6. Remove excess moisture from moist wipes then place around the edge of the dish, making sure it does not touch the pad. The sample is now ready for imaging.

7. Setting up the microscope for live imaging

7.1. Warm environmental chamber to 37 °C at least 2 h prior to experimental set-up.

7.2. Turn on all components including microscope, computer, and transilluminated and fluorescence light sources.

7.3. Open the imaging software and make sure that light sources are connected and running.

7.4. Perform Köhler illumination[27] to align all image planes.

7.4.1. First, bring marked coverslip into focus with a 20x objective using a “dummy” dish. Make sure the condenser turret is set to “open” position.

NOTE: Köhler illumination should be performed for each unique objective/sample to properly align image planes. However, focus and alignment with the “dummy” dish on lower magnification

expedites set-up on live samples at higher magnification to limit time between set-up and experiment start time.

7.4.2. Focus the condenser.

7.4.2.1. Close the field diaphragm.

NOTE: An octagon-shaped aperture should appear. If the condenser is completely out of focus, the entire field of view (FOV) will appear dark.

7.4.2.2. Rotate the condenser focusing knobs until the octagon edges are crisp.

NOTE: The light intensity will increase as the image planes approach the correct alignment.

7.4.3. Align the condenser.

7.4.3.1. Center the field condenser by adjusting the aligning knobs.

NOTE: The octagon should be centered to the middle of the FOV. The aligning knobs differ depending on the microscope. For example, some microscope condensers have knobs, while others have screws requiring a screw driver.

7.4.4. Focus the lamp filament and adjust the condenser aperture.

7.4.4.1. Place a phase telescope or Bertrand lens into the light path to observe the back focal plane of the objective.

NOTE: There should be two concentric circles.

7.4.4.2. Turn the Bertrand lens focus knob until the rings look crisp.

7.4.4.3. Remove the Bertrand lens from the light path.

7.4.5. Open the field diaphragm until the octagon is just outside of the FOV.

7.4.6. Change to the 100x objective and slide the matching phase ring into place before adding a drop of immersion oil, and place the prepared sample dish on top.

7.4.7. Perform Köhler illumination on the 100x objective with the bacterial sample dish.

7.5. Focus on the bacteria using the fine adjustment only. Once the bacteria in the FOV are focused through the eyepiece, switch the light path to the camera by pressing the camera button on the microscope.

7.6. Click the **Phase** option in the imaging software.

7.7. Adjust the percentage of DIA LED light emitted by selecting the light source in the software and either manually entering the desired percentage of light to be used or sliding the bar on the light percentage scale.

7.8. Adjust the DIA LED light exposure time by clicking on the light source and either manually entering the desired exposure time or selecting an exposure time from the provided drop-down menu.

NOTE: The exposure time will vary depending on the camera being used.

7.9. If using fluorescence, adjust the camera settings in each corresponding channel (i.e., TxRed, GFP), by clicking on the fluorescent channel of interest.

7.9.1. Set the percentage of fluorescence light emitted, then adjust the exposure time (as performed in steps 7.7 and 7.8 for DIA LED light).

7.9.2. Alternatively, change the bit depth to adjust the dynamic range by selecting one of the other bit depth options in the visualization controls drop-down menu.

7.10. Choose the XY positions of interest by clicking on the XY option in the acquisition controls menu.

7.10.1. Move the stage position with the joy stick, or by clicking and dragging the FOV on the screen, and click on the empty box to save the X and Y coordinates of a specific position.

NOTE: Selection of no more than three different XY positions, as close together as possible, is recommended for time-lapse acquisition.

7.11. Turn on the perfect focus system (PFS) by either clicking the PFS box on the XY tab of the acquisition control menu or pressing the PFS button on the joy stick control panel.

7.12. Rotate the fine adjustment knobs to focus on the bacterial cells.

7.13. Click the PFS button for each XY position, once cells are in the desired plane of focus.

NOTE: PFS compensates for drift in the Z-axis during time-lapse experiments. This is necessary to maintain focus of bacterial cells over time. Different manufactures have different compensation systems.

7.14. Choose the image acquisition conditions including acquisition interval and frequency for each channel (e.g. phase contrast and each fluorophore) by selecting from the options in the acquisition controls menu.

NOTE: For the experiments presented here, phase contrast images are acquired at intervals every 5-10 s while fluorescence images in the GFP and TxRed channels are acquired every 20 min.

7.15. Begin imaging once the microscope and acquisition settings are set up.

8. Optional: Modifications for live/dead imaging

8.1. Melt 2% agarose in 10 mL of M8T and let cool in 50 °C water bath for at least 15 min.

8.2. Add 1 mM of propidium iodide to the molten agarose.

8.3. Once cooled, pipette 915 µL of agarose in the prepared mold.

8.4. Leave the lid ajar and let pad dry at room temperature for 30 min. Protect from light.

8.5. Cover the dish with the lid and leave at room temperature for an additional 2 h.

8.6. Prepare humidity wipes.

8.6.1. Roll a lint-free paper wipe up tightly.

8.6.2. Place the wipe in a sterile Petri dish and add 500 µL of sterile water to the wipe.

8.6.3. Incubate at 37 °C for 1 h.

8.7. Incubate the pad and a sterile 35 mm dish at 37 °C for 1 h.

8.8. Proceed to inoculate the dish as indicated in section 6: Preparation of bacterial cells and inoculating pads.

9. Data analysis

9.1. Identification of cells

9.1.1. Open image file in the imaging software and crop the file to only include the frames to be used for tracking, zoomed in to the cells of interest, and only in the phase contrast channel.

NOTE: The cropped file can be saved as a new file in which tracking data can be stored without interfering with the original file.

9.1.2. Select the option to choose regions of interest (ROI) in the analysis controls menu and define ROIs by tracing either individual bacterial cells or clusters of cells in the first frame to be used for analysis.

NOTE: The ROIs can either be defined manually or as binaries.

9.1.2.1. Manually define ROI.

9.1.2.1.1. Trace the perimeter of each individual bacterial cell or clusters of cells to manually define the ROIs.

NOTE: In the analysis software, *P. aeruginosa*, or other rod-shaped cells, can be manually traced by selecting the **Ellipse** ROI and drawing an ellipse adjusted to the size of the bacterial cell. Alternatively, the **Polygon** ROI option can be selected to trace non-traditional-shaped ROIs, such as clusters of cells.

9.1.2.2. Identify binary ROIs

9.1.2.2.1. Click the **Binary to ROI** option in order to define binary ROIs.

NOTE: Objects are defined in a binary layer based on separation of the darker-pixelated bacterial cells from the lighter-pixelated background in the phase contrast channel.

9.1.2.2.2. To threshold the cells, select the **Threshold** option in the analysis controls menu. Select the channel of interest and slide the bars in the fluorescence histogram to adjust the threshold interval values.

NOTE: Binary object identification for ROIs can also be defined in fluorescent images by thresholding the bacterial cells. Thresholding establishes which fluorescence intensities are considered objects and what fluorescent intensities constitute the background.

9.2. Cell tracking

9.2.1. To manually track ROIs, select the next frame in the imaging sequence and adjust the position of the ROIs by clicking and dragging each ROI to align with the new position of the original bacterial cell.

NOTE: If the bacterial cells have not changed position, the ROIs do not need to be moved.

9.2.2. Repeat in all sequential frames for which cells are to be tracked.

9.2.3. As cells divide, define the daughter cells as new ROIs, as described in step 9.1, and begin tracking the newly divided cells.

NOTE: If cells are identified as binaries, use the **Track Binaries** function to automatically track ROIs in selected frames.

9.2.4. Once cells have been tracked through all selected frames, export the data to be analyzed.

9.2.5. Open the data spreadsheet and identify the measurements required for analysis (i.e., object speed, acceleration, or path length).

NOTE: In the case of measuring directedness, the Path Length and Line Length measurements are required. The Line Length is a measurement of the Euclidian distance, or the straight distance from the track origin to the edge of the *S. aureus* colony. The Path Length is a measurement of the accumulated distance, or the sum of track segments from all frames. Directedness can be calculated as a ratio of the Euclidian distance, $D_{(E)}$, (Line Length), over the accumulated distance, $D_{(A)}$, (Path Length).

9.3. Fluorescence quantification

9.3.1. Define ROIs for fluorescent bacterial cells as described in step 9.1.

9.3.2. Repeat tracing or movement of ROIs for bacterial cells or clusters in the remaining fluorescent frames.

9.3.3. Export the generated table to a spreadsheet file for analysis of fluorescent intensity.

9.3.4. In the data spreadsheet, look for the column with "Mean Intensity" which represents the average fluorescence intensity for the traced bacterial cell(s) ROI.

9.3.5. Graph the Mean Intensity values to look at the changes in fluorescence over time.

NOTE: The changes in fluorescence over time represent the fluctuation of gene expression for the fluorescently labeled gene of interest.

REPRESENTATIVE RESULTS:

Successful use of the described method will result in a series of frames that generate a video in which the interspecies interactions can be observed over time. The schematic in **Figure 1** provides a visual to highlight the key steps involved in preparing materials for imaging. Use of this method has allowed the demonstration of *P. aeruginosa* cells exhibiting different behaviors in monoculture versus in coculture with *S. aureus*. Compared to the *P. aeruginosa* cells in monoculture that remain grouped in rafts, when in coculture with *S. aureus*, *P. aeruginosa* has increased single-cell motility towards *S. aureus* colonies (**Figure 2A-2B**). Fluorescently labeled bacterial strains also allow for visualizing mixed populations of bacterial species. Using GFP-labeled *P. aeruginosa* permitted the observation that after *P. aeruginosa* single cells increase in motility, they will surround and eventually invade *S. aureus* colonies (**Figure 2C**). Utilization of fluorescently-marked cells also allows for visualization of *P. aeruginosa* cell invasion into the *S. aureus* colonies for the first time (**Figure 2C**, bottom).

While the protocol yields high quality images for observing interspecies interactions, there are several common problems that can lead to poor quality frame sequences. Inconsistent humidity over the duration of the experiment and incorrectly dried pads are two common problems that lead to drift resulting from the pad shifting and dragging the cells with it out of the FOV (**Figure 3A**). Changes in humidity affect the dryness of the agarose pads. Increased humidity makes the pads too wet, allowing moisture to settle between the pad and the bottom of the glass-bottom dish. The moisture leaves a layer of liquid thick enough for motile cells to swarm or swim through and does not keep bacterial cells in a single plane. Meanwhile, decreases in humidity dry out the pad faster, which cause cells to drift early on. Another common mistake when using this method with fluorescence is acquiring fluorescent images too frequently or exposing bacterial cells to fluorescent light for too long. Short acquisition intervals for fluorescent images repeatedly excite fluorophores with high intensity light of a specific wavelength. Excited fluorophores then react with oxygen, causing degradation of the fluorophore. The resulting photobleaching depletes fluorescence until more of the fluorophore can be expressed and folded, but does not harm the bacterial cells themselves (**Figure 3B**). Loss of fluorescence, however, does interfere with measurements of gene/protein expression, leaving the cells markerless until the fluorophore can be fully synthesized and excited again. Moreover, the oxygen interacting with excited fluorophores can form reactive oxygen species (ROS). These ROS radicals then damage bacterial cells, ultimately becoming toxic and resulting in cell death within a few cell divisions (Figure 3C). The processes of photobleaching and phototoxicity can be easily seen as cells will first lose their fluorescence completely, and in subsequent frames, the cells will stop dividing and may eventually lyse (**Figure 3B-3C**). One final common issue when setting up the experiment is starting with too many cells per FOV or with cells too close to each other, which is generally less than 20 µm apart. Crowded cells in the first frame will yield clusters of dividing cells that simply merge into each other as they grow rather than interact. Additionally, cells that begin too close in proximity may not have sufficient time to establish a gradient of secreted signals to which the other species can respond (**Figure 3D**) whereas distant bacterial cells may not have the chance to encounter one another within the duration of the experiment.

Figure 4 shows an example of how bacterial cell viability can be visualized with this protocol by adding propidium iodide to the agarose pads. Propidium iodide is impermeable to live cells, but can diffuse through the membrane of dead cells and intercalate with the DNA. Here mid-log GFP-labeled WT *S. aureus* were treated with media alone or with 70% ethanol and imaged immediately following treatment. Three different channels were imaged: Phase, TxRed and GFP. Green cells indicate live cells actively expressing GFP as seen for the *S. aureus* treated only with medium (**Figure 4A**), and red cells indicate dead propidium iodide-stained *S. aureus* cells, after being treated with 70% ethanol (**Figure 4B**). While only one time point is shown, this method can be adapted to determine cell viability during time-lapse live imaging by adding propidium iodide to the molten agarose before pouring the pad.

Several post-imaging analyses can be performed to quantify aspects of interspecies interactions. For example, cell tracking can provide measurements for the directedness of *P. aeruginosa* single cell movements towards a cluster of *S. aureus.* The movements of individual *P. aeruginosa* cells are tracked from the frame a cell leaves the raft through the frame in which the cell reaches the

S. aureus cluster (**Figure 5A**). The distance between the *P. aeruginosa* raft and the *S. aureus* cluster provide the Euclidian distance, $D_{(E)}$, while the total track lengths provide the accumulated distance, $D_{(A)}$ (**Figure 5B**). Directedness of each cell is calculated as a ratio of $D_{(E)}/D_{(A)}$. In the coculture experiments, WT *P. aeruginosa* moved towards WT *S. aureus* with significantly higher directedness than towards *S. aureus* Δ*agrBDCA*, a mutant lacking Agr-regulated secreted factors, previously determined to be necessary for directional motility towards *S. aureus*[21] (**Figure 5C**).

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic of the imaging setup protocol. Overview of critical steps for preparation of bacterial cultures and agarose pads. Created with BioRender.com.

Figure 2. Time-lapse, live-imaging microscopy shows differences in *P. aeruginosa* behavior when cocultured with *S. aureus.* Representative snap shots of *P. aeruginosa* (rods, green) in monoculture (**A**) and in coculture with *S. aureus* (cocci, unmarked) (**B**). (**C**) Fluorescently-labeled bacteria allow for visualization of *P. aeruginosa* single cells invading *S. aureus* clusters. Phase contrast and GFP channel overlay (top) and GFP channel alone (bottom).

Figure 3. Representative snap shots of common imaging issues that lead to poor image acquisition. (**A**) Improperly dried pads and inconsistent humidity lead to drifting of cells across the FOV over the duration of imaging. The position of the founding cell is marked in each frame (green rod). (**B**) Photobleaching from exposure to light for too long will deplete detectable levels of fluorescence for a period of time, but does not kill the cells. (**C**) Phototoxicity as a result of frequent exposure to light leads to cell death. The first signs of phototoxicity are seen when cells stop fluorescing and fail to divide. (**D**) High initial inoculum crowds cells in the FOV and prevents observation of interspecies interactions.

Figure 4. Comparison of live versus dead *S. aureus* cells. Representative snap shots for GFP-labeled WT *S. aureus* treated with either medium alone (**A**) or medium with 70% ethanol (**B**). Cells were immediately imaged following treatment. Live cells (green) actively express GFP and exclude propidium iodide, while dead cells (red) lose GFP fluorescence and membrane permeabilization allows propidium iodide to diffuse into the cell and bind DNA.

Figure 5. Cell tracking analysis of *P. aeruginosa* in coculture with *S. aureus*. Previously, this method was used method to perform cell tracking of WT *P. aeruginosa* in coculture with either WT or Δ*agrBDCA S. aureus*. (**A**) Representation of *P. aeruginosa* single-cell tracks in a coculture with *S. aureus* Δ*agrBDCA*. (**B**) Schematic for the Euclidian distance ($D_{(E)}$) and accumulated distance ($D_{(A)}$) measurements used to determine directedness (($D_{(E)}/D_{(A)}$). (**C**) Directedness measurements of single WT *P. aeruginosa* cells in coculture with WT and Δ*agrBDCA S. aureus*. This figure has been modified from Limoli et al. 2019[21].

DISCUSSION:

The methods presented here describe a protocol for live-cell imaging of bacterial species interactions at the single-cell level with modifications for other applications including cell tracking

and monitoring cell viability. This method opens new avenues for studying single-cell behaviors of microorganisms in coculture with other species over time. Specifically, the protocol demonstrates the usefulness of this coculture method in observing bacterial surface behaviors, particularly when studying organisms that have both surface and liquid-associated appendages for motility. For example, by limiting bacterial movement to a surface in a single plane of focus, the increased and directional pili-mediated motility of *P. aeruginosa* in response to *S. aureus* can be visualized.

As previously mentioned, achieving optimal results with this imaging method requires consideration of several conditions, including temperature and humidity of the sample and imaging instruments (i.e., objectives and stage). For more tips and troubleshooting, see the **Supplemental File 1**. A critical step in successful use of the protocol is preparation of the agarose pads, as inadequately drying pads is one of the most common problems. As shown in **Figure 3A**, if the pads are not dried long enough, drift appears at the beginning of the time-lapse imaging, whereas pads that are dried for too long start to shrink and the cells drift out of the FOV a few hours into imaging. Ensuring that all materials are pre-warmed and maintained at uniform temperature and humidity over the duration of the experiment, through use of a stage-top incubator and damp lint-free wipes, will help reduce drift. It is also advised that a backup pad is always made in case the first pad is not dried properly or tears during transfer from the mold to the sample dish. Additionally, it is important to use a low-autofluorescence medium, both for growing bacterial cultures and for making the pads in order to minimize the background fluorescence from the medium when imaging the cells. It is recommended to use minimal media for microscopy, since rich media often have high autofluorescence. Starting with a low-density inoculum and even spatial distribution of cells in the FOV are also key factors in this method. Specifically, in prior studies evaluating *P. aeruginosa* and *S. aureus* interactions, this allowed the bacteria to generate a sufficient gradient of secreted factors that can then be detected by the other species present (**Figure 2B**).

Despite the benefits of this method, there are also limitations including its price, low-throughput nature, fluorescence restrictions, and high dependence on controlling environmental conditions. **Figure 3B-3C** show the major limitations of using fluorescence microscopy. As discussed in the results section, if the fluorescent images are captured in short intervals, photobleaching and phototoxicity can occur. In order to avoid these two outcomes, fluorescent image intervals should be taken far enough apart and with as low fluorescent light exposure time as possible to still adequately visualize the fluorophore. Additionally, when determining the interval for fluorescence imaging, it is important to consider the maturation time of each fluorophore. The fluorophores used in the *P. aeruginosa* and *S. aureus* strains shown in **Figures 2-4**, for example, have a maturation time of about 20 minutes and can therefore be excited every 20 minutes without concern of potential photobleaching effects. Meanwhile, another disadvantage of this method is that it does not allow for observations of late interspecies interactions once cells reach a high cell density. In order to visualize individual bacterial cells, they must remain in a single plane of focus. However, once the population reaches a high cell density, the cells begin to grow in more than one plane.

This method can be modified to study different phenotypes such as cell viability (**Figure 4**) and expression of genes of interest (data not shown). **Figure 4** shows an example of how the method has been adapted to visualize bacterial viability by adding propidium iodide to the agarose pads. Another application of this method is measuring bacterial gene/protein expression in coculture with another organism through fluorescent reporters. For example, multiple fluorophores can be incorporated into a plasmid vector or the bacterial chromosome to simultaneously study the expression of different genes or proteins. Here it is important to select fluorophores that do not have overlapping excitation and emission spectra. Lastly, the use of bacterial cell tracking in post-imaging analysis enables the directionality (**Figure 5**), speed and acceleration, among other measurements, to be calculated as well[24].

Overall, this coculture imaging method adapted from previously described monoculture protocols enhances the ability to visualize behaviors of multiple bacterial species in coculture. This method offers the opportunity to study microbes from a mixed-culture perspective, which will increase understanding of how each species alters its behaviors in a single-cell manner, ultimately providing new insight into how bacterial species interact in polymicrobial environments.

ACKNOWLEDGMENTS:

This work was supported by funding from the Cystic Fibrosis Foundation Postdoc-to-Faculty Transition Award LIMOLI18F5 (DHL), Cystic Fibrosis Foundation Junior Faculty Recruitment Award LIMOLI19R3 (DHL), and NIH T32 Training Grant 5T32HL007638-34 (ASP). We thank Jeffrey Meisner, Minsu Kim, and Ethan Garner for sharing initial protocols and advice for imaging and making pads.

DISCLOSURES:

The authors declare that they have nothing to disclose.

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Figure 1

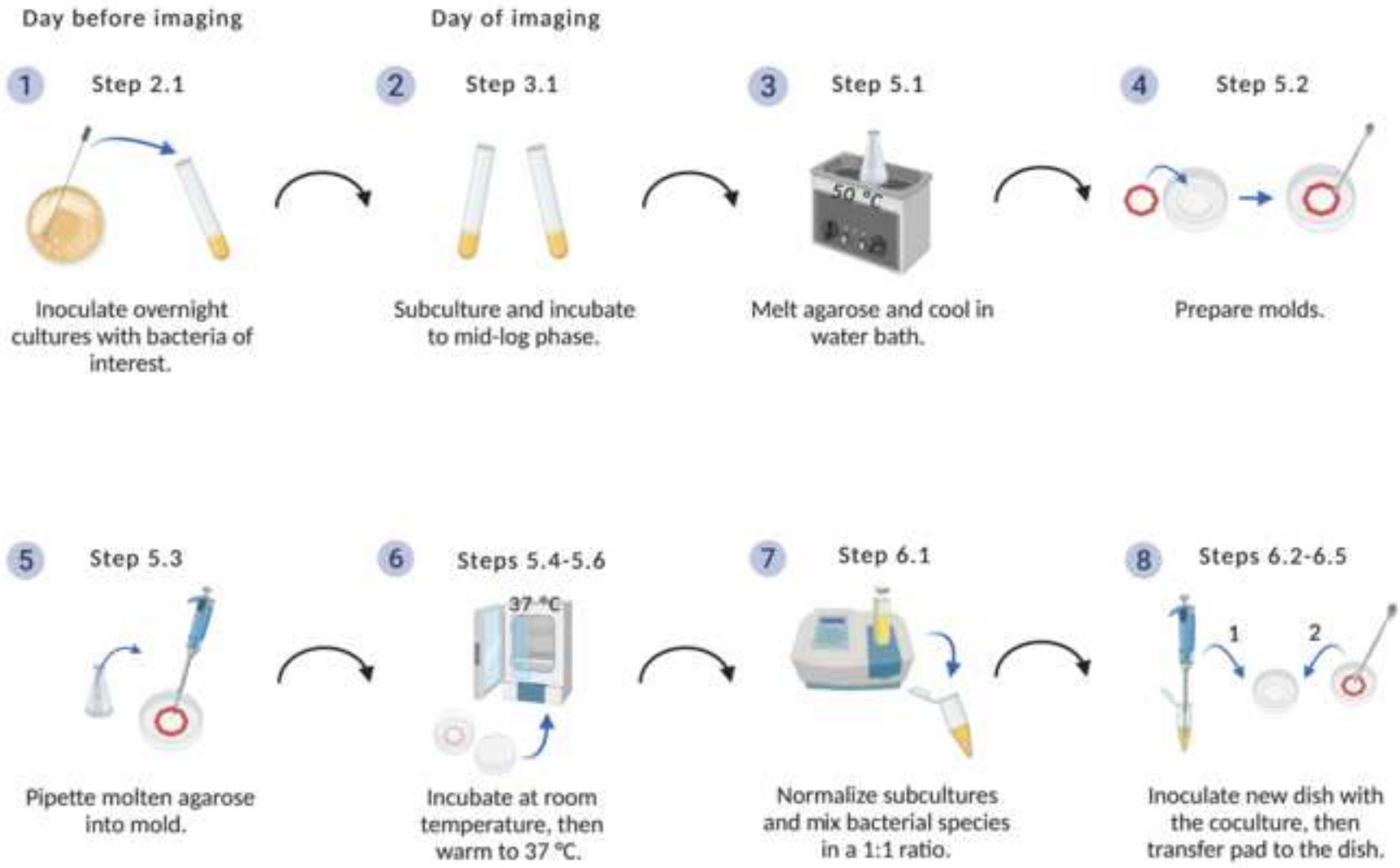


Figure 2

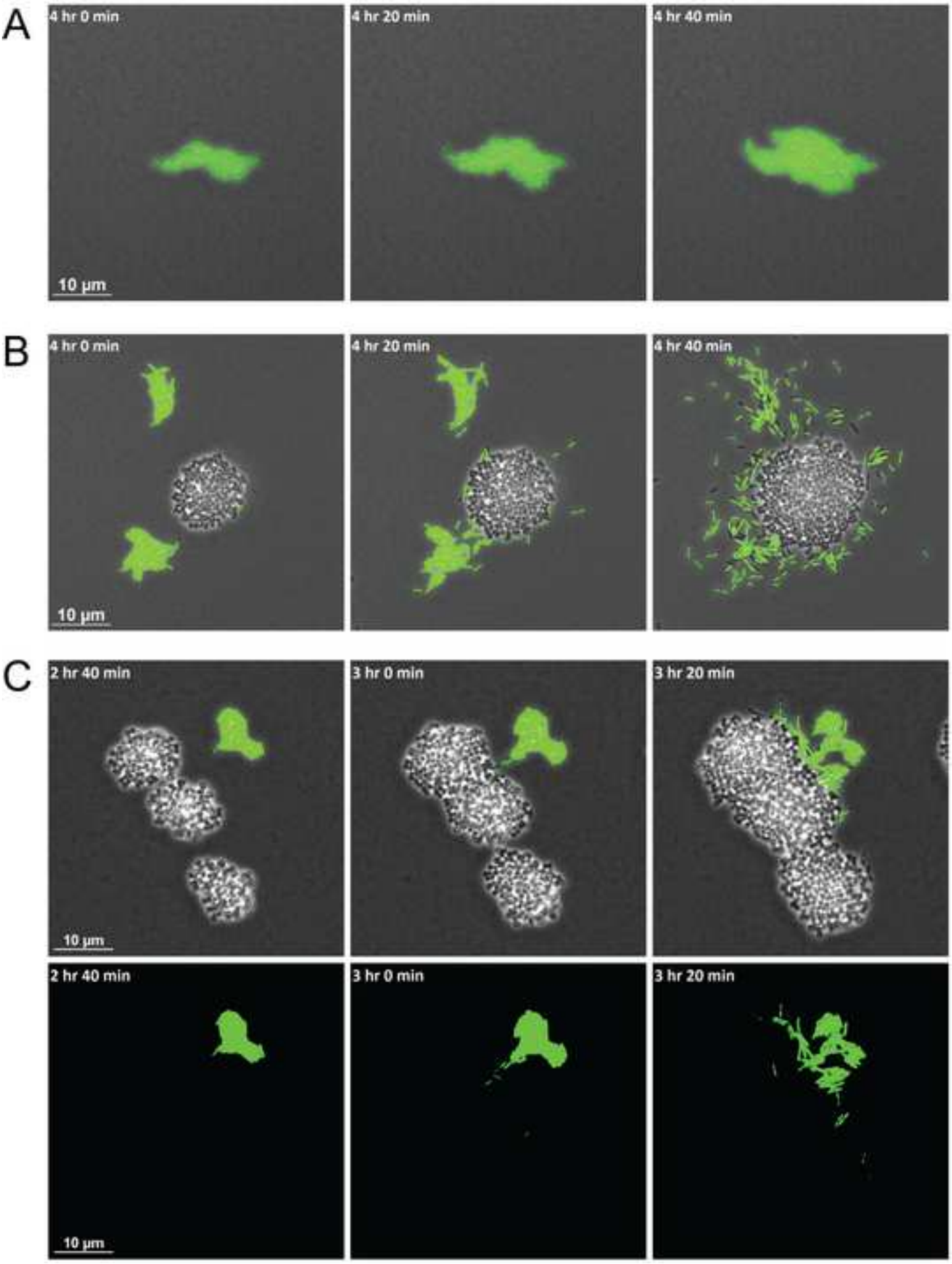


Figure 3

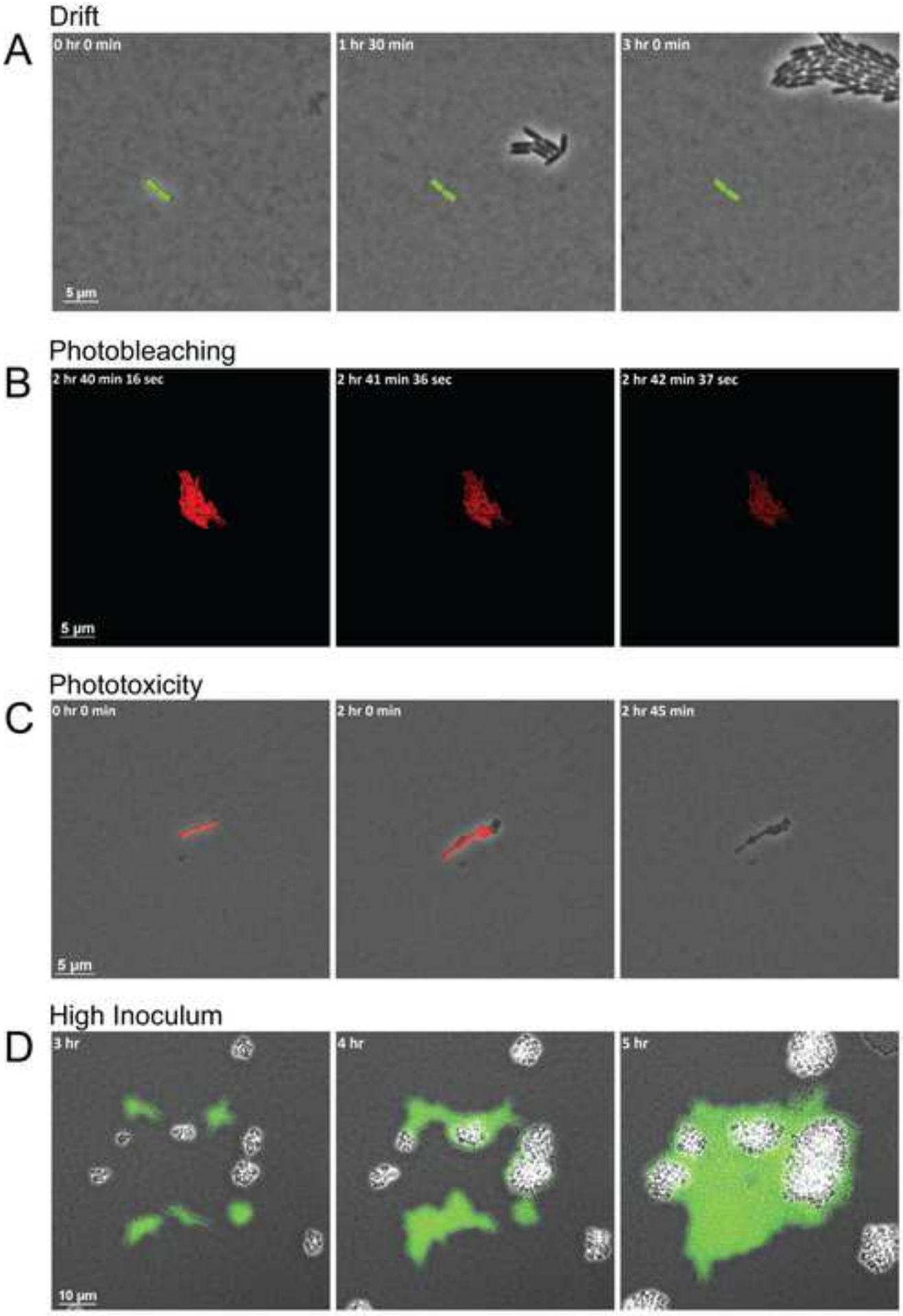


Figure 4

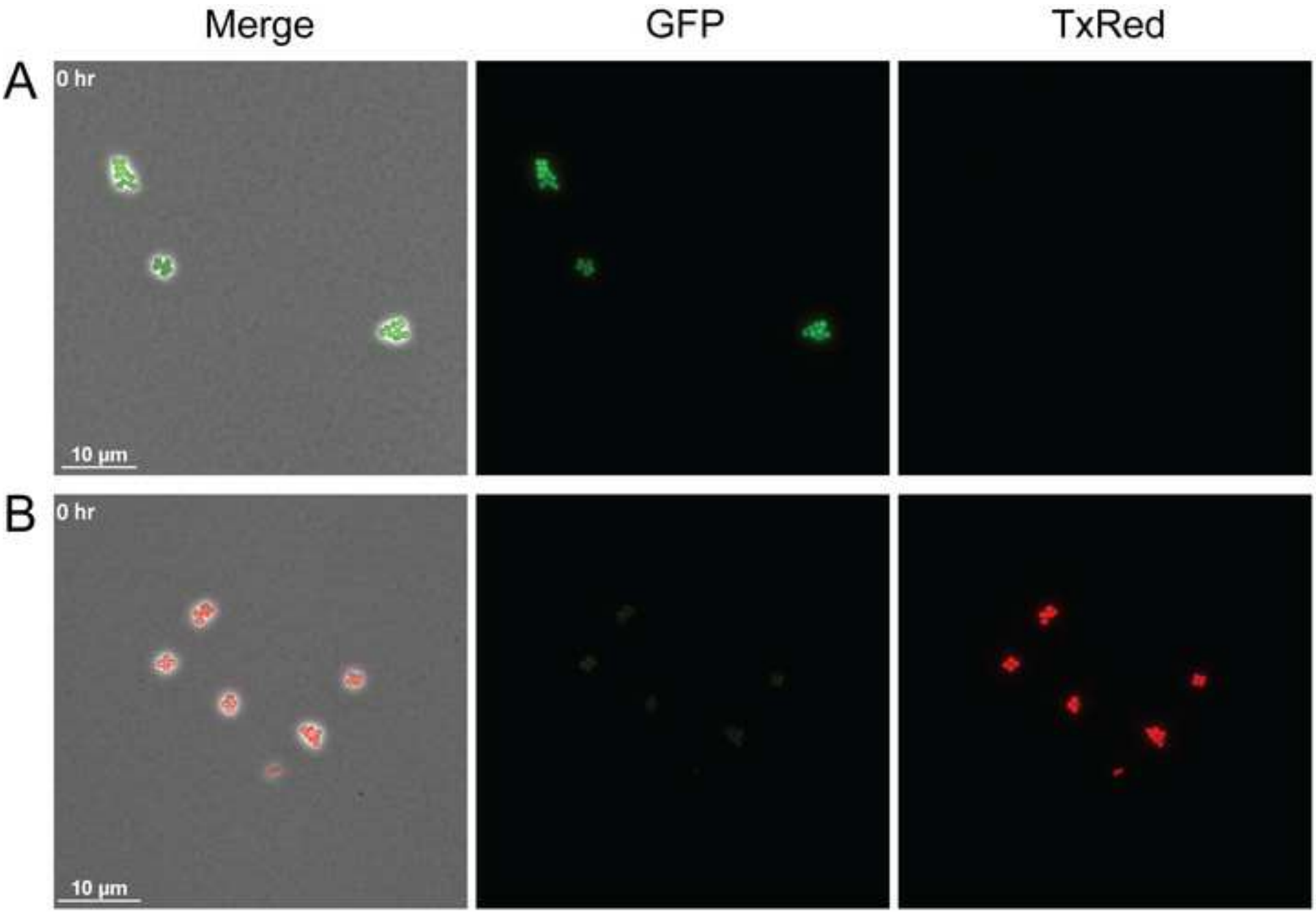
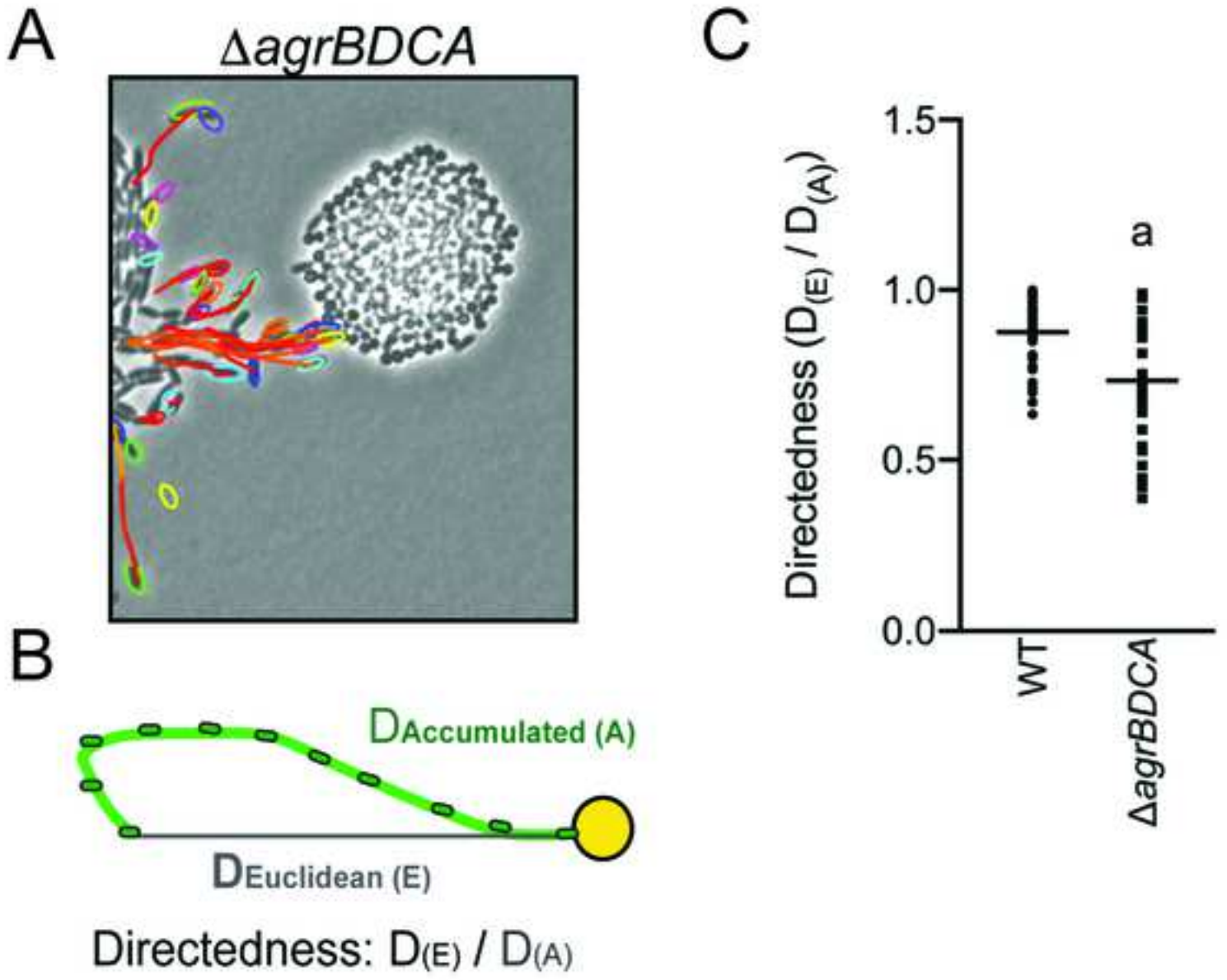


Figure 5



Name of Material/Equipment
Agarose pads
35 mm Glass Bottom Dish with 20 mm Micro-well #1.5 Cover Glass
KimWipes
Low-Melt Agarose
Round-Bottom Spatulas
Round-Tapered Spatulas
Silicon Isolators, Press-to-Seal, 1 well, D diameter 2.0 mm 20 mm,
Sterile Petri Plates, 85 mm
Tweezers
M8T Minimal Media
D (+) Glucose
KH_2PO_4
$MgSO_4$
NaCl
$Na_2HPO_4.7H_2O$
Tryptone
Microscope
Andor Sona 4.2B-11
Filter Cube GFP
Filter Cube TxRed
H201-NIKON-TI-S-ER
Nikon NIS-Elements AR with GA3 and 2D and 3D tracking
Nikon Ti2 Eclipse
CFI Plan Apo λ20x objective (0.75NA)
CFI Plan Apo λ100x oil Ph3 DM objective (1.45NA)
ThermoBox with built-in fan heaters
Bacterial Strains
Pseudomonas aeruginosa PA14 (WT)
Pseudomonas aeruginosa PA14 (WT) pSMC21 (*Ptac-GFP*)
Pseudomonas aeruginosa PAO1 (WT) p*PrpoD-mKate2*
Staphylococcus aureus USA300 LAC (WT)
Staphylococcus aureus USA300 LAC (WT) pCM29 (*sarAP1-sGFP*)
Staphylococcus aureus USA300 LAC Δ*agrBDCA*
Viability Stain
Propidium Iodide

Company	Catalog Number
Cellvis	D35-20-1.5-N
Kimberly-Clark Professional	06-666A
Nu-Sieve GTG/Lonza	50081
VWR	82027-492
VWR	82027-530
Sigma-Aldrich	S6685-25EA
Kord-Valmark /sold by RPI	2900
VWR	89259-944
RPI	G32045
RPI	P250500
Sigma-Aldrich	208094
RPI	S23025
Sigma-Aldrich	230391
BD Biosciences	DF0123173
Andor	77026135
Nikon	96372
Nikon	96375
Okolab	77057447
Nikon	77010609, MQS43110, 77010603, MQS42950
Nikon	Model Ti2-E
Nikon	MRD00205
Nikon	MRD31905
Tokai Hit	TI2TB-E-BK
	PMID: 7604262
	PMID: 9361441
	PMID: 26041805
	PMID: 23404398
	PMID: 20829608
	PMID: 31713513
Invitrogen	L7012

Comments/Description
One for agarose pad molds, one for experiment
For making agarose pads
For agarose pad molds
Camera. 4.2 Megapixel Back-illuminated sCMOS, 11 µm pixel, 95% QE, 48 fps, USB 3.0, F-mount.
Filter cube
Filter cube
Stagetop incubator
Software for data analysis
Microscope
Objective
Objective
Enclosure
Non-mucoid prototroph
USA300 CA-Methicillin resistant strain LAC without plasmids
LIVE/DEAD™ BacLight™ Bacterial Viability Kit

Response to reviewers' comments

We are very grateful for the editor and reviewers' time and helpful feedback on our manuscript. We have addressed each comment below and edited the text accordingly. We feel that these edits have put this protocol in context of previously published studies and increased the clarity of the manuscript.

Below we have included the response to the comments. The editor and reviewers' comments are in italics in order to differentiate them from our responses. We have also uploaded both a tracked-changes edited copy of the manuscript and a clean version. It should be noted that the line numbers we refer to here, are from the marked document. Please do not hesitate to contact us if further concerns, questions or requests arise.

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

We thank the editor for all these helpful comments. We have addressed all of the editorial comments, and we specified the line numbers when necessary.

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.*

2. *Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.*

3. *JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.*
   For example: Nikon PFS, NIS-Elements, etc.

4. *The Protocol should contain only action items that direct the reader to do something. Please move the materials to the table of materials instead.*

5. *Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."*

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

7. *Please use complete sentences throughout the protocol section. Also, we cannot have paragraph of texts in the protocol section.*

8. *Please ensure you answer the "how" question, i.e., how is the step performed? For this please include all the mechanical actions, knob turns, button clicks in the software etc.*

9. *5: Significance of using these bacterial strains?*

Under the Section 2, step 2.1 of the protocol: "Preparation of bacterial overnight cultures", a note was included to address the significance of the bacterial strains used in this manuscript.

Lines 370-373

10. 5.3.1: Please convert this to a note instead.

We converted this step to a note. In the current manuscript, the note is under the step 6.1.

Lines 432-435

11. 6.4.3: How is this done? Citations if any?

In order to enhance clarity, we have included the steps to perform the Köhler illumination in steps 7.4 through 7.4.7, and have provided a reference.

Lines 489-543

12. 6.7. Set to what? How do you adjust the dynamic range and to what?

We have further clarified how to adjust the dynamic range in step 7.9.2.

Lines 567-568

13. 8: For the data analysis, please include all the button clicks in the software and command lines used if any (if large scripts please include as a supplementary file) to show how the steps are performed.

We have clarified important button clicks and options to select in the software for data analysis steps, which is now section 9.

Lines 658-763

14. Tips and troubleshooting cannot be a part of the protocol section. Please either move it to the discussion section in the paragraph style or make a separate table in .xlsx format and

upload it separately to your editorial manager account.

We have deleted the tips and troubleshooting section from the protocol, and have uploaded a spreadsheet in the .xlsx format with this section instead.

15. *There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.*

We have highlighted around 2.5 pages of the protocol identifying the steps for the video.

16. *Please ensure that the representative result is presented with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title.*

We have addressed how the representative results enable the observation and interpreation of bacterial interactions at the single-cell level.

17. *Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."*

Figures 1 through 4 are new and do not require copyright permission. Figure 5 is from a previous publication and the link to the editorial policy to allow re-print of this figure is found in the figure legend of Figure 5.

18. *As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:*
    a) Critical steps within the protocol
    b) Any modifications and troubleshooting of the technique
    c) Any limitations of the technique
    d) The significance with respect to existing methods
    e) Any future applications of the technique

19. *Please sort the materials table in alphabetical order.*

Reviewers' comments:

Reviewer #1:
Manuscript Summary:

Yarrington and colleagues present a system in which to study early stage microbial interactions. The protocol is succinct and well written.

Major Concerns:
The protocol is currently confusing as to when the agarose pads should be prepared. The day before (when overnight culture is set up), or the day of? If day before how are they stored? can they be stored? A schematic of timing for each part of the setup would be really useful if you want to skip the video and go straight to the printed methods.

We thank the reviewer for suggestions for clarification. We also agree that a schematic would be helpful to provide better direction on the timing of the preparation of the agarose pads. The pads need to be prepared the day of imaging. We have uploaded a schematic as Figure 1 that provides the description of each step. In the schematic we made clear that the overnight culture of the bacteria is prepared the day before imaging.

Minor Concerns:
How tunable are the pads in regard to nutrition? The authors discuss the application of looking at live/dead or gene/protein expression and regulation - many of these are influenced by metabolism. Can the pads be modified for different organisms. Additional data showing a reporter would be nice.

Yes, the pads are easily tunable in regard to nutrition to support the growth of other organisms of interest and examine metabolism or gene expression. However, many rich media have background autofluorescence, thus if fluorescence imaging is desired, the user would need to take this into account. We agree that it would be interesting to demonstrate how gene expression changes in response to varying nutrient conditions. However, here we have chosen a few representative examples of the utility of this method and mention examples of adaptation for other applications, such as monitoring gene expression.

We have clarified these points in the manuscript on lines 398-400.

Reviewer #2:
Manuscript Summary:
This manuscript describes a method to visualise interactions between S. aureus and P. aeruginosa during early colonisation using time-lapse fluorescent microscopy. This method offers some very interesting and important insights into early bacterial interactions that could shape biofilm formation.

Major Concerns:
The study focusses on S. aureus and P. aeruginosa, with the CF lung given as an example. It would be nice to see the context elaborated upon a little more - these bacteria are commonly co-isolated from other chronic infections, including wounds, and so this data has broader reaching impact. I notice there are few references. There are a large number of studies that have looked at interaction between SA and PA, and it would be good to see those reflected here,

and the findings from this study put into context in this regard.

We thank the reviewer for this suggestion and have broadened the contextual discussion of coinfection with *P. aeruginosa* and *S. aureus* in terms of the range of diseases the organisms are found in coinfection, the physiological consequences of such infections, and how single-cell imaging builds on these studies and enhances our understanding of their interactions.

This information is discussed in the introduction.

Minor Concerns:
in point 5.6 maybe replace "your cells" with "the cells"

We have edited this line to say “the bacterial cells” in step 6.5

Lines 450-452

The summary mentions that this model could be used to quantify gene expression, but this study does not include this aspect. Could it be made clear that this is the case? As it stands, this gives the impression that this study includes gene expression analysis.

We thank the reviewers for pointing out this confusion. Indeed, this protocol can be used to monitor gene expression; however, we have chosen not to include these data here, as these experiments are a topic of a future manuscript. We have edited the summary to more accurately reflect the representative data presented here. Further, we modified the introduction and the discussion to focus only on the adaptations shown in the manuscript.

Summary:
Line 35-36

Introduction:
Lines 276-277

Discussion:
Lines 1063-1064

Reviewer #3:
Manuscript Summary:
This is an interesting manuscript in which the authors propose a time-lapse microscopy protocol for observing interactions between motile Pseudomonas aeruginosa and non-motile Staphylococcus aureus. As the authors state, these organisms are relevant in that they are found in a variety of environments including pulmonary infections associated with cystic fibrosis. While the potential future applications are very intriguing, it is worth noting that some earlier work employing video microscopy in relation to biofilm formation has been done. As well, there are some earlier investigations of the interactions between the two organisms used in this study

(P. aeruginosa and S. aureus), that may explain the chemotaxis results. I have raised these issues and some other issues needed for clarification in my comments below:

Major Concerns:
1. What is the pH of the media?

The pH of our media is around 7.6 (neutral). We specified this as a note in the protocol, under section 1: Preparation of M8T minimal media, step 1.1.

Line: 288

2. I would recommend that the authors reference Korgaonkar et al., PNAS 110: 1059-64, 2013. In this study, the authors show that P. aeruginosa uses peptidoglycan and specifically N-acetyl glucosamine as a stimulant for the virulence factor pyocyanin. P. aeruginosa chemotaxis towards S. aureus may be explained in part by this study.

We agree with the reviewer and also had the same hypothesis based on the studies by Korgaonkar *et al*. However, we have tested peptidoglycan and N-acetyl glucosamine and did not see an influence on *P. aeruginosa* chemotaxis or motility. Instead, we identified *S. aureus* phenol soluble modulins as the primary signal necessary for this response. These data are described in a previous publication (Limoli *et al.* 2019) and the mechanism investigated in more detail in an additional manuscript in preparation. We feel the description of each of these studies to be outside the scope of the current methods paper and refer the readers to reviews on the many excellent manuscripts previously published that have laid the foundation for our understanding of interactions between *P. aeruginosa* and *S. aureus* (lines 70-73).

3. For context, there have been a number of previous studies involving video microscopy including work by JR Lawrence, DR Korber and colleagues (e.g. J. Bacteriol. 174: 5732-5739, 1992; Appl. Environ. Microbiol. 60: 1166-1173, 1994; Appl. Environ. Microbiol. 60: 1421-1429, 1994; Microb Ecol 18: 1-19, 1989)

We thank the reviewer for these helpful references. We have added them to the manuscript in lines 146-148.

4. Some of the methodology (possibly the highlighted material) would need to be displayed for clarity.

We agree with this comment. The highlighted material will be displayed in the video.

5. The adjustments for fluorescence microscopy (steps 6.6, 6.7, 6.7.1 and 6.7.2) are vague.

We have edited these steps to include more details in order for the reader to be able to successfully adjust the light source settings.

In the current manuscript, these steps are 7.9-7.9.2.

Lines: 561-568

6. While a number of experimental parameters would need to be determined empirically, how reproducible are the images and data? The manuscript would be greatly enhanced by some type of statistical analysis.

We have addressed this concern by adding an additional figure (Figure 5) from a recent manuscript from our laboratory (Limoli *et al.* 2019) that shows the cell tracking analysis of *P. aeruginosa* in coculture with *S. aureus*.

Minor Concerns:
I would recommend the use of high resistance water (18 MΩ / cm) rather than MilliQ (MQ) water as there are a variety of manufacturers in addition to Millipore that produce ion exchange resins capable of generating high resistance water.

We have clarified this point by referring to the water as “ultrapure water (resistivity 18 MΩ/cm)”.

Tips and Troubleshooting
Keep all experimental materials at the same temperature
Maintain humidity
Minimize drift
Optimize starting cell inoculum
Select acquisition interval frequency

Optimize acquisition interval frequency for bacterial cell tracking
Optimize fluorescence exposure time

Description
Make sure all media, dishes, pads, and environmental chamber are equilibrated to the temperature of the experiment in advance. This will ensure that bacterial cells do not respond to a temperature change at the initiation of the experiment and prevents the accumulation of moisture within the sample dish. A full stage enclosure is recommended to maintain consistent temperature of all components, including the stage and objective, to minimize drift. Warming pads to the temperature of the experiment before use will also reduce drift.
Changes in humidity cause one of the biggest problems in using this protocol. Adaptations of this protocol have been optimized for experiments performed in the summer and winter to minimize drift and maintain humidity, but other users of this protocol will need to optimize the humidity and pad dry times based on the environment in which experiments are being performed. A stage-top incubator is recommended to help maintain even humidity. The humidity of the environmental chamber will differ based on the geographical location, season, and building conditions that the experiments are being performed in. For example, pads that are dried for the same amount of time will show more drift early on in time-lapse imaging under more humid conditions. In order to maintain even humidity throughout the duration of time-lapse experiments, damp lint-free paper wipes are placed around the inside edge of the 35 mm dish, being careful to avoid contact with the agarose pad.
The most common problem in time-lapse imaging is bacterial cells drifting out of the field of view. In addition to changes in humidity, inadequate drying of the agarose pads leads to major cell drifting. Failure to dry the pads either results in pads that are too wet or too dry, both of which lead to unfortunate drift over the duration of the time-lapse. Pads that are too dry stick to the mold and are much more difficult to drop out. Furthermore, dry pads will show drift after a few hours of the time-lapse, towards the end of imaging acquisition. On the other hand, wet pads will still have visible moisture on them when dropped out of the mold and will cause drift right away during imaging acquisition. It is always helpful to pour an extra agarose pad and keep molten agarose warmed at 50 °C
Because cells will divide over the course of time-lapse imaging studies, it is crtical to start with a low cell inoculum in the sample dish. Starting with 2-4 single cells (per 18 mm field of view) allows for enough space between cells to prevent cell clusters from growing into one another while still visualizing the interspecies interactions. Too many cells in the starting inoculum will result in clusters of cells that are too close to each other and therefore do not allow for observation of interactions because the cell density is too high within a few cell divisions. On the other hand, starting with too few cells can be a problem if the cells are not close enough together to allow interactions to take place within the duration of the experiment. Starting with 1-2 cells of each species in a coculture experiment with cells spaced 20-30 µm apart yields the best images for clear observation of interactions between
Capturing interspecies interactions will highly depend on how frequently the images are acquired. Shorter acquisition intervals will increase the likelihood of capturing interactions that happen quickly or within a short time. However, shorter acquisition intervals may not be the most useful in the earlier time points when cells are growing and dividing, but not yet interacting. Therefore, it may be useful to use longer acquisition intervals earlier in the time-lapse and then switch to shorter acquisition intervals as bacterial species begin to interact. The other caveat with selecting frequency is that shorter acquisition intervals not only limit the number of XY positions that can be imaged in a single experiment, but also determine how close the selected field of views must be.

Cell tracking analysis requires much shorter acquisition intervals in order to keep track of motile bacterial cells as they move across the field of view. The acquisition interval length will depend on how quickly cells move, but cell tracking works best when frames are acquired 3 s or less apart. One consideration when obtaining frames for cell tracking is that due to the short acquisition intervals, the number of XY positions that can be imaged is highly limited. Typically, only one XY position is imaged per experiment when intervals of less than 5 s are used.
Each fluorophore has a unique excitation and emission wavelength, but will also require an optimized light exposure time to maximize fluorescence captured while minimizing photobleaching and phototoxicity to the bacterial cells. If cells are exposed to light for too long, then the fluorophore will bleach, yielding an inaccurate fluorescence intensity. This is especially critical when gene expression is being measured with fluorescence markers. Additionally, a high exposure time for measuring gene expression that increases over time may max out the fluorescence intensity that can be measured, which would also yield inaccurate gene expression measurements. Furthermore, phototoxicity can occur in bacterial cells that are exposed to fluorescence light too long, which is easily detected by bacterial cells that stop dividing early on. In contrast, too short of an exposure time will fail to detect