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TITLE:

Tools for the Real-time Assessment of a *Pseudomonas aeruginosa* Infection Model

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SUMMARY:

Synthetic cystic fibrosis sputum medium (SCFM2) can be utilized in combination with both confocal laser scanning microscopy and fluorescence-activated cell sorting to observe bacterial aggregates at high resolution. This paper details methods to assess aggregate populations during antimicrobial treatment as a platform for future studies.

ABSTRACT:

Pseudomonas aeruginosa (Pa) is one of the most common opportunistic pathogens associated with cystic fibrosis (CF). Once *Pa* colonization is established, a large proportion of the infecting bacteria form biofilms within airway sputum. *Pa* biofilms isolated from CF sputum have been shown to grow in small, dense aggregates of ~10–1,000 cells that are spatially organized and exhibit clinically relevant phenotypes such as antimicrobial tolerance. One of the biggest challenges to studying how *Pa* aggregates respond to the changing sputum environment is the lack of nutritionally relevant and robust systems that promote aggregate formation. Using a synthetic CF sputum medium (SCFM2), the life history of *Pa* aggregates can be observed using confocal laser scanning microscopy (CLSM) and image analysis at the resolution of a single cell. This *in vitro* system allows the observation of thousands of aggregates of varying size in real time, three dimensions, and at the micron scale. At the individual and population levels, having the ability to group aggregates by phenotype and position facilitates the observation of aggregates at different developmental stages and their response to changes in the microenvironment, such as antibiotic treatment, to be differentiated with precision.

INTRODUCTION:

Pseudomonas aeruginosa (Pa) is an opportunistic pathogen that establishes chronic infections in immune-compromised individuals. For those with the genetic disease, CF, these infections can span the course of a lifetime. CF causes the buildup of a viscous, nutrient-rich sputum in the airways, which becomes colonized by a variety of microbial pathogens over time. *Pa* is one of the most prevalent CF pathogens, colonizing the airways in early childhood and establishing difficult-to-treat infections[1]. *Pa* remains a significant clinical problem and is considered a leading cause of mortality in those with CF, despite improved therapy regimens in recent years[2,3]. This persistence phenotype and increasing antibiotic tolerance have earned *Pa* a place in a group of pathogens identified by both the Centers for Disease Control (CDC) and the World Health Organization (WHO) as research priorities for the development of new therapeutic strategies—the ESKAPE pathogens[4].

Like other ESKAPE pathogens, acquired antibiotic resistance is common in *Pa*, but there are also many intrinsic properties that contribute to *Pa* antimicrobial tolerance. Among these is the ability of *Pa* to form aggregates—highly dense clusters of ~10–1,000 cells, which can be observed in multiple infections, including CF patient sputum[5,6]. Similar to *Pa* studied in other biofilm systems, *Pa* aggregates display clinically relevant phenotypes such as increased resistance to antibiotics and activation of cell-cell communication (quorum sensing (QS)). For example, aggregates of *Pa* have been shown to use QS-regulated behaviors to combat other microbes as well as tolerate antimicrobial treatments such as the production of pyocyanin[7]. The ability to study such behaviors offers an exciting insight into bacterial ecosystems in an environment similar to the one in which they exist in the human body.

One of the biggest challenges to studying how *Pa* aggregates respond to the changing sputum environment is the lack of nutritionally relevant and robust systems that promote aggregate formation. Much of what is known about *Pa* has been discovered using *in vitro* systems in which cells grow planktonically or in a characteristic surface-attached, “mushroom” architecture that has not been observed *in vivo*[8]. While classical biofilm growth models, such as flow cells or solid agar, have yielded extensive and valuable knowledge about bacterial behaviors and mechanisms of antibiotic tolerance, these findings do not always translate *in vivo.* Many *in vitro* models have a limited ability to mimic the growth environment of the human infection site, necessitating costly *in vivo* studies. In turn, many *in vivo* models lack the flexibility and resolution afforded by *in vitro* techniques.

Synthetic cystic fibrosis sputum (SCFM2) is designed to provide an environment for *Pa* growth similar to that experienced during chronic infection in the CF lung. SCFM2 includes nutritional sources identified in expectorated CF sputa in addition to mucin, lipids, and DNA. *Pa* growth in SCFM2 requires a near identical gene set to that for growth in actual sputum and supports natural *Pa* aggregate formation[9,10]. After inoculation, planktonic cells form aggregates that increase in size through expansion. Individual cells (referred to as migrants) are released from aggregates, migrate to uncolonized areas, and form new aggregates[10]. This life history can be observed using CLSM and image analysis at the resolution of a single cell. Aggregates of *Pa* formed in SCFM2 are of similar sizes to those observed in the CF lung[10]. This model allows the observation of multiple aggregates of varying size in real-time and in three dimensions at the micron scale. Time-lapse microscopy allows the tracking of thousands (~50,000) of aggregates in one experiment. The use of image analysis software allows the quantification of aggregate phenotypes from micrographs, including aggregate volume, surface area, and position in three dimensions to the nearest 0.1 µm, both at the individual aggregate and population levels. Having the ability to group aggregates by phenotype and position allows the differentiation of aggregates at different developmental stages with precision, as well as their response to a changing microenvironment[6,11].

The application of SCFM2 to study *Pa* aggregates in low volume and high-throughput assays make it a flexible, cost-effective model. As a defined medium, SCFM2 offers uniformity and reproducibility across multiple platforms, providing a nutritionally and physically relevant method to study *Pa* aggregates *in vitro*[9]. Applications include its use in combination with CLSM to observe spatial organization and antibiotic tolerance at high resolution (as described in this methods paper). The ability to perform experiments that provide real-time, micron-scale data allows the study of intra-species and inter-species interactions as they may occur *in vivo*. For example, SCFM2 has previously been used to study the spatial dynamics of cell-cell communication in aggregate populations via a network of systems utilized by *Pa* to regulate multiple genes that contribute to virulence and pathogenesis[6].

[Insert **Figure 1** here]

Here, the utility of SCFM2 to study the impact of antibiotic treatment on *Pa* aggregates in real time is demonstrated, followed by the use of a cell-sorting approach to isolate populations of aggregates with distinct phenotypes for downstream analysis (**Figure 1**).

PROTOCOL:

1. Prepare synthetic cystic fibrosis medium (SCFM2)

NOTE: Preparation of SCFM2 comprises three main stages outlined below (**Figure 2**). For full details and references, see[9,10,12].

[Insert **Figure 2** here]

1.1. Sterilization of porcine mucin

1.1.1. Prepare sterile mucin at a final concentration of 5 mg/mL in SCFM2. For example, for a 5 mL volume of SCFM2, weigh 25 mg of Type II mucin in a sterile Petri dish, and place into an ultraviolet (UV) sterilizer for 4 h, gently agitating every hour.

1.1.2. After 4 h, transfer the UV-treated mucin into autoclaved 1.7 mL tubes under sterile conditions, and store at -20 °C.

1.1.3. To confirm complete sterilization, dissolve a sample of mucin in a sterile liquid, such as water or Luria Bertani (LB) broth, and observe under a microscope.

NOTE: Sterilized mucin can be stored at -20 °C for up to 6 months.

1.2. Preparation of buffered base

1.2.1. Prepare salt and amino acid stock solutions by adding the appropriate amounts by weight to deionized water as listed in **Table 1**. Filter-sterilize all stock solutions using a 0.22 µm filter, wrap in foil to protect from light degradation, and store at 4 °C for up to one month.

1.2.2. Prepare buffered base by combining 190 mL of deionized water with amino acid and salt stock solutions by volumes listed in **Table 1**. Adjust the solution to pH 6.8, and increase to a final volume of 250 mL. Filter-sterilize using a 0.22 µm filter, and store at 4 °C for up to 30 days.

1.2.3. On the evening before the experiment, aliquot the desired amount of buffered base into a glass culture flask, and add mucin (5 mg/mL as described in step 1.1.1) and purified salmon sperm DNA (0.6 mg/mL). Agitate gently, wrap in foil, and leave at 4 °C overnight to allow mucin and DNA to dissolve into solution.

NOTE: Salmon sperm DNA aliquots should be thawed on ice, vortexed, and added to buffered base and mucin. Tryptophan, asparagine, and tyrosine stock solutions must be prepared in solutions of NaOH (see **Table 1** for concentrations) instead of deionized water. Keep buffered base and all stock solutions wrapped in foil to protect from light exposure. Most stocks will be stable for up to a month. Stocks that become discolored should not be used and should be replaced before use.

1.3. Addition of supplemental stocks

1.3.1. On the day of the experiment, add the stocks listed in **Table 2** to the buffered base containing mucin and DNA.

NOTE: Prepare a fresh $FeSO_4$ solution on the day of the experiment, but all other stocks can be made ahead of time and stored for 30 days at 4 °C. 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) contains chloroform. Handle with caution, and do not use near open flames. After the addition of DOPC, incubate SCFM2 at 37 °C with shaking (250 rpm) for at least 20 min (for 5 mL culture). This incubation period allows the chloroform in the DOPC to evaporate. The flask should not be airtight; instead, cover the flask opening loosely with foil.

2. Real time assessment of antimicrobial tolerance in bacterial aggregates

2.1. Prepare overnight cultures

2.1.1. On the evening before the experiment, inoculate 5 mL of LB broth with several colonies of *Pa* PAO1-pMRP9-1[13] from an LB agar plate containing antibiotic (carbenicillin 300 µg/mL). Grow overnight at 37 °C with agitation at 250 rpm.

NOTE: Grow overnight cultures with the addition of antibiotics required for the selection of required plasmids (here, the green fluorescent protein (GFP) expression plasmid, pMRP9-1). Note that *Pa* cells are to be washed before SCFM2 is inoculated. LB may be substituted for other rich laboratory media for overnight cultures. All bacterial isolates should be handled using appropriate BSL-2 guidelines throughout this protocol.

2.2. Inoculate SCFM2

2.2.1. On the day of the experiment, back-dilute overnight cultures of *Pa* 1:10 (culture: liquid media) by inoculating 500 µL into 5 mL of fresh LB broth. Grow cells until log phase (60–90 min) at 37 °C with agitation at 250 rpm.

2.2.2. Centrifuge log phase cultures at 10,000 × *g* for 5 min. Wash the cells by removing the supernatant and resuspending in 3 mL of filter-sterilized phosphate-buffered saline (PBS, pH 7.0). Repeat twice, and resuspend the pellet in a final volume of 1 mL of PBS.

2.2.3. Measure the absorbance of the washed cells using a spectrophotometer at 600 nm (OD_{600}), and calculate the volume of culture required for a starting OD_{600} of 0.05 in 5 mL of SCFM2. Inoculate *Pa* into the SCFM2, and vortex gently to distribute cells throughout. Pipette 1 mL of the inoculated SCFM2 into each chamber of a 4-well, glass bottom, optical dish, and incubate for 4 h statically at 37 °C.

NOTE: The doubling time of *Pa* cultures is dependent on the strain and the availability of oxygen. In SCFM2, under the conditions described here, the doubling time of *Pa* is ~1.4 h[10].

3. Visualizing aggregates during antibiotic treatment with confocal laser scanning microscopy (CLSM)

NOTE: This section describes the use of confocal laser scanning microscope and image capture software for the imaging of *Pa* aggregates in SCFM2. The goal is to observe and characterize the remaining (tolerant) bacterial biomass after treatment with antibiotics. The steps outlined can be performed with success on other confocal microscopes, although the instrument operating manual should be referenced for specific guidance.

3.1. Image *Pa* cultures using either a heated chamber or a heated microplate fitted on the microscope stage to maintain an ambient temperature of 37 °C. Start the **Incubation** module at least 2 h prior to the beginning of the experiment to allow all apparatus to reach the desired temperature, and reduce further expansion and movement during data collection.

3.2. After 4 h, transfer SCFM2 cultures containing *Pa* cells to the heated microscope stage. Designate 3 out of 4 wells as technical replicates for antibiotic treatment, and consider the 4th well as a no-treatment control, containing only *Pa* cells in SCFM2, without antibiotic. Identify aggregates using brightfield microscopy within the **Locate** tab prior to any excitation of fluorescent reporters. Define an area for imaging within each well, and store its position (x-y-z coordinates) using the **Positions** module in the imaging software.

3.3. Use a 63x oil-immersion objective to visualize *Pa* cultures containing the GFP expression plasmid pMRP9-1 in SCFM2 with an excitation wavelength of 488 nm and emission wavelength of 509 nm. Take images using the z-stack option within the **Acquisition** module at 1 μm intervals (total of 60 slices). Use the **Line-averaging** module to reduce background fluorescence in the GFP channel within the total volume of the 60 μm z-stack images (1,093.5 mm^3)· Take control images of uninoculated SCFM2 by using identical settings to determine the background fluorescence for image analysis.

NOTE: Images are acquired by producing 512 pixels x 512 pixels (0.26 μm x 0.26 μm pixels) 8-bit z-stack images that are 60 μm from the base of the coverslip.

3.4. Use the time series option within the imaging software to capture 60 slices at each position (well) at 15-min intervals over a period of 18 h. Use the definite focus strategy within the software to store a focal plane for each position, which is returned to at each time point throughout the experiment.

3.5. After a total 4.5 h of incubation, image each position using the above settings to determine the aggregate biomass within each of the four wells before the addition of antibiotic.

3.6. After 6 h of total incubation, add antibiotic at 2x-below minimum inhibitory concentration (MIC) to each replicate. Pipette directly and gently into the middle of the well, just below the air-liquid interphase. Maintain all cultures in the heated confocal chamber.

NOTE: Here, colistin sulfate at a concentration of 140 μg/mL was used.

3.7. Begin imaging post-antibiotic treatment by clicking on the **Start Experiment** option within the imaging program.

NOTE: Antibiotic concentration used is dependent on the antibiotic, the *Pa* isolate, and whether the user would like to examine killing or tolerance effects. This experiment uses a single dose. Additional doses can be added without disrupting cultures if needed.

4. Propidium iodide staining of *Pa* aggregates

NOTE: Propidium iodide (PI) is commonly utilized as a staining reagent to identify non-viable (dead) bacterial cells in culture. Here, it is used to identify aggregates sensitive to antibiotic treatment applied in section 3. Throughout this protocol, the expression and detection of GFP in *Pa* cells is used as the main proxy for cell viability. This final step allows confocal imaging to be used once more to identify the spatial positioning of live/dead aggregates in relation to each other. Additionally, aggregates are identified as live/dead for further downstream cell sorting in section 5.

4.1. After 18 h, add PI to each well of the four-chambered optical bottom dish containing SCFM2 cultures. Follow the manufacturer's instructions for the volume of PI and incubation time (*e.g.*, ~2 µL per mL of culture, ~20–30 min).

5. Isolating live cells from aggregates using a FACS approach

NOTE: FACS presents a powerful platform to sort and isolate groups of cells according to a fluorescently tagged phenotype. Here, FACS is used to isolate live (antibiotic tolerant) aggregates from non-viable aggregates.

5.1. After staining with propidium iodide, remove the cultures from incubation, and transfer to a FACS instrument in an insulated container to maintain 37 °C.

5.2. Run 1 mL aliquots of SCFM2 containing Pa aggregates at the lowest flow rate.

NOTE: Each aliquot will contain ~15,000 aggregates.

5.3. To detect GFP, illuminate the cells with a 488 nm laser, and record the fluorescent signal height at 530/30 nm. Visualize the PI staining by excitation with a 561 nm laser, and record the fluorescent signal height at 610/20 nm. Perform sorting using a 70-u nozzle.

NOTE: Sorted aggregates can be pooled in multiple ways depending on the user's application. In this case, FACS was used to sort viable *Pa* aggregates for downstream RNA sequencing. Alternative applications are discussed below.

6. Image analysis

NOTE: Time-lapse microscopy generates large amounts of data. An 18-h experiment for the observation of *Pa* aggregates in SCFM2 identifies ~50,000 aggregates over time, which have the potential to be characterized for volume and spatial positioning. Use an image analysis software to quantify aggregate dynamics in SCFM2:

6.1. For aggregate studies in SCFM2, quantify the background GFP fluorescence by creating a histogram of counts in the GFP channel that is produced for uninoculated SCFM2 and SCFM2 inoculated with *Pa* strain PAO1 carrying pMRP9-1. To ensure that detected GFP voxels correlate to *Pa* biomass, define a GFP+ voxel as ≥1.5x the GFP background count value.

NOTE: The background fluorescence is defined as the highest voxel value of three randomly chosen positions, averaged. Background counts are, as a standard measure, subtracted from all pixels in experimental images by the image analysis software.

6.2. After background subtraction in the **Surpass** module, produce isosurfaces for all remaining voxels.

6.3. To detect individual aggregates, enable the **Split Objects** option, and define aggregates as objects with volumes of ≥5 μm^3. Use the **Vantage** module in the image analysis software to calculate the volume, x-y-z, and sum of GFP voxels for each individual object. Export this data to an external statistical platform.

NOTE: Some image analysis software permit the export of multiple quantitative phentoypes at once, allowing for correlations to be calculated.

6.4. Filter data exported from the **Vantage** module by size to ensure that no objects of <0.5 μm^3 (dispersed biomass) remain. For each individual object within the image, calculate the distance from itself in relation to other objects (aggregates) using the **Vantage** module of the image analysis software or manually with the following equation.

$$d = \mathrm{sqrt}((x_2 - x_1)^2 + (y_2 - y_1)^2 + (z_2 - z_1)^2) \qquad (1)$$

6.5. Use SUM and AVERAGE calculations to find the total biomass and average aggregate volume. Alternatively, export data into other statistical platforms or scripts, *e.g.*, the distribution of aggregates across biomass as discussed in the representative results (script unpublished in collaboration with the Whiteley laboratory, Georgia Institute of Technology).

REPRESENTATIVE RESULTS:

This work details methods to observe *Pa* aggregates at a high resolution and in an environment similar to that of chronic infection of the CF lung[9,10,12]. SCFM2 provides an *in vitro* system that promotes natural aggregation of *Pa* cells in sizes similar to those observed during actual infection[10]. The adaptability of SCFM2 as a defined medium can be leveraged to approach many research avenues. The goal of this work is to highlight a workflow that involves a combination of microscopy and FACS, with the intention of encouraging its use for future research, collaborations, and applications.

The use of CLSM allows temporal, high-resolution imaging that can be used to study the dynamics of developing bacterial populations. This work demonstrates several potential ways by which *Pa* aggregates can be characterized after treatment with antibiotic. **Figure 3A** provides a broad view of viable *Pa* aggregates after antibiotic treatment. Four hours after the application of antibiotic, multiple aggregates remain; color ranges can be applied to represent different characteristics of each individual aggregate. Examples of quantifiable characteristics include volume, area, voxel intensity (for use of fluorescent reporters), and position in either of the x-y-z axes.

Image analysis using dedicated software allows for the fully customizable observation of data, identifying each aggregate as an individual object, wherein quantitative data can be exported for further analysis. **Figure 3B** provides an example of how exported data can be used. Here, the total biomass of aggregate populations can be calculated over time. In these representative data, treatment with antibiotic significantly reduces biomass in comparison to untreated aggregates. **Supplemental Video S1** is a powerful representation of how time-series microscopy can be used to physically observe aggregates within these biomass data after antibiotic treatment. Each aggregate has been color-coded to represent the calculated volume (μm^3), allowing a visual record of aggregate populations, which can also be used for further spatial analysis using applications, both within the image analysis software and other resources (not discussed here[10,15]).

Experiments developed to identify mechanisms of antibiotic tolerance in *Pa* aggregates produce large amounts of data. Each replicate produces physical data of ~15,000 aggregates (~60,000 aggregates in total). A new approach to handling this data has involved the production of aggregate heat maps (**Figure 3C**). By calculating how aggregates of different sizes contribute to the overall population, patterns of how aggregates respond to antibiotic treatment in relation to their size, shape, and position in relation to each other can be identified.

[Insert **Figure 3** here]

Spatial analysis at this stage involves using the expression of GFP by *Pa* as a proxy for viability in the presence of antibiotic. The addition of PI after 18 h allows the identification of the position of non-viable aggregates in relation to viable aggregates (**Supplemental Figure S1**). The overarching goal of this method is to identify spatial patterns between sensitive and tolerant aggregates after treatment with antibiotics. Future studies will include multiple time points in addition to the end-point assay described above.

The final stage of the workflow utilizes a FACS-based approach to sort aggregates in SCFM2. This work demonstrates FACS' ability to separate viable cells from the remaining population of aggregates (including non-viable). **Figure 4** demonstrates the use of FACS to pool viable aggregates for further experiments such as high-throughput sequencing, *e.g.*, RNA-sequencing (RNA-seq). After treatment with antibiotics (**Figure 4A** represents one time point (18 h), aliquots of SCFM2 containing *Pa* aggregates can be successfully separated according to their fluorescent features. Here, GFP has been identified for tolerant aggregates. **Figure 4B** provides an example of one such sorting event, where GFP-expressing cells are separated from the remaining culture (PI-treated aggregates). In addition, aggregates can be clearly identified from SCFM2, which produces its own fluorescent profile. The ability to sort cells in this way has many applications (**Figure 5**).

[Insert **Figure 4** here]

[Insert **Figure 5** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Graphical depiction of the main experimental steps. (**A**) SCFM2 is inoculated with *Pa* cells and allowed to form aggregates in a glass-bottomed culture dish. (**B**) Aggregates are

transferred to the confocal microscope, and antibiotic is added. Depicted are three technical replicates (chambers 1–3) and a control well (4) of inoculated SCFM2 without antibiotic treatment. Aggregates are imaged using CLSM over the course of 18 h. (**C**) After the initial 18-h imaging, aggregates are treated with propidium iodide to visualize dead cells and imaged using CLSM (**D**) Aggregates with desired phenotype are separated from SCFM2 using FACS. Abbreviations: SCFM2 = synthetic cystic fibrosis sputum medium; *Pa* = *Pseudomonas aeruginosa*; CLSM = confocal laser scanning microscopy; FACS = fluorescence-activated cell sorting.

Figure 2: Preparation and inoculation of SCFM2 medium. (**A**) Buffered base is prepared using salts and amino acids listed in **Table 1** and **Table 2**. Buffered base can be stored at 4 °C for up to 30 days, but must be protected from light exposure. (**B**) Mucin and DNA are added to an aliquot of buffered base and dissolved into solution overnight at 4 °C. (**C**) Lipid and additional stocks are added to the overnight solution and incubated at 37 °C with agitation at 250 rpm for $\geq$20 min. SFCM2 is then inoculated with washed, log phase cells at an $OD_{600} = 0.05$. Abbreviations: SCFM2 = synthetic cystic fibrosis sputum medium.

Figure 3: Examples of aggregate data analysis. (**A**) Overview of remaining aggregate biomass after antibiotic treatment. Isosurfaces of corresponding GFP voxels have been rendered and color-coded according to volume (μm^3). Scale bar = 30 μm. (**B**) Total biomass (μm^3) of *Pa* aggregate populations over time in SCFM2. Blue line represents untreated aggregates, red line represents aggregates treated with the antibiotic, colistin (140 μg/mL). Presented data include 3 biological replicates (± SEM). (**C**) Aggregate volume heat maps representing the contribution of individual aggregates by volume (μm^3, x-axis) to total biomass (μm^3, second y-axis) over time (h, y-axis). Representative data include *Pa* aggregates in the presence and absence of antibiotic (as in **Figure 3B**), where 0 h represents time of antibiotic addition after 6 h aggregate growth. Data include three biological replicates (~50,000 total aggregates). Abbreviations: GFP = green fluorescent protein; SCFM2 = synthetic cystic fibrosis sputum medium; SEM = standard error of the mean; *Pa* = *Pseudomonas aeruginosa*.

Figure 4: FACS of *Pa* aggregates in SCFM2. (**A**) Diagram of a FACS instrument used to separate viable and non-viable *Pa* aggregates from SCFM2. (**B**) Representative plot of aggregates separated in SCFM2 generated by FACS software. Three quadrants indicate live aggregates (GFP), remaining culture containing non-viable cells (RFP is used as an alternative to propidium iodide staining here, both excitable by the 488 nm laser), and SCFM2 control. Data represent 1 of 3 biological replicates containing ~15,000 events (aggregates). Abbreviations: FACS = fluorescence-activated cell sorting; SCFM2 = synthetic cystic fibrosis sputum medium; *Pa* = *Pseudomonas aeruginosa*; GFP = green fluorescent protein; RFP = red fluorescent protein; PE-Texas Red-H = peak height for phycoerythrin-Texas Red conjugate stained cells; FITC-H = peak height for fluorescein isothiocyanate-stained cells.

Figure 5: Potential experimental applications utilizing SCFM2. (**A**) Exposure of *Pa* aggregates to repeated antibiotic doses. (**B**) Co-culture of *Pa* aggregates with other bacterial species. (**C**) Exposure of *Pa* aggregates to host immune cells, *e.g.*, PMNs. **A**, **B**, and **C** can be combined with CLSM imaging methods and FACS approach for downstream RNA-seq, proteomics, or 3D spatial

analysis. Abbreviations: SCFM2 = synthetic cystic fibrosis sputum medium; *Pa* = *Pseudomonas aeruginosa*; PMNs = polymorphonuclear leukocytes; CLSM = confocal laser scanning microscopy; FACS = fluorescence-activated cell sorting; RNA-seq = RNA sequencing; 3D = three-dimensional; LC-MS/MS = liquid chromatography/tandem mass spectrometry.

Table 1: Preparation of salt, amino acid, DNA, and mucin stocks required for buffered base of synthetic cystic fibrosis sputum medium, SCFM2.

Table 2: Preparation of additional stocks required for buffered base of synthetic cystic fibrosis sputum medium, SCFM2.

Supplemental Figure S1: *Pa* aggregates in SCFM2. A rendered confocal micrograph of viable (green) and non-viable (red) *Pa* aggregates formed in SCFM2. Scale bar = 5 μm**.** Abbreviations: SCFM2 = synthetic cystic fibrosis sputum medium; *Pa* = *Pseudomonas aeruginosa*.

Supplemental Video S1: Aggregates in SCFM2 after treatment with antibiotic. *Pa* aggregate images captured every 30 min in SCFM2 using CLSM. Aggregates are labelled individually by colors representing aggregate volume (μm^3, color scale not shown as representative image). Scale bar = 5 μm. Abbreviations: SCFM2 = synthetic cystic fibrosis sputum medium; *Pa* = *Pseudomonas aeruginosa*; CLSM = confocal laser scanning microscopy.

DISCUSSION:

This work has introduced methodologies that can be combined to study bacterial aggregate populations in the presence and absence of antibiotic treatment. High-resolution CLSM allows the visualization of changes in aggregate biomass and the structural orientation of aggregates over real time when exposed to antibiotics. In addition, physical and structural features of the biomass that remain after treatment with antibiotics can be quantified, with the goal to correlate these observations with future gene expression studies utilizing RNA-seq. While the use of GFP-expressing cells allows visualization of the collective and individual behavior of antibiotic-tolerant cells, it provides only part of the overall picture. Much can be learned from the spatial positioning of cells that did not survive antibiotic treatment, both individually and in relation to the surviving and tolerant biomass.

Time-lapse microscopy generates large amounts of data. An 18-h experiment for the observation of *Pa* aggregates in SCFM2 identifies ~50,000 aggregates over time, which have the potential to be characterized for volume and spatial positioning. The CLSM used here (see the **Table of Materials**) captures high-resolution images of developing aggregates for analysis. Spatial positioning of an individual aggregate (relative to surrounding aggregates) in three dimensions (± 0.1 μm) can be used to allow for precise measurements of the distance between aggregates. Physical phenotypes of bacterial aggregates can be determined using a combination of analysis approaches. Imaging software provides surface rendering and three-dimensional (3D) positioning. In-house-generated scripts can be used to sort aggregates by phenotype (*e.g.*, volume) and calculate distribution of size-binned biomass. Additional resources are available to segment individual cells within aggregates (*e.g.*, to support the use of transcriptional reporters[14]).

Combined, these analysis tools provide a broad assessment of aggregate phenotypes, with the flexibility to modify analyses for emerging interests as data are generated.

After the initial 18-h imaging, aggregates were treated with PI, a technique often referred to as live/dead staining. PI enters cells with a porous (compromised) membrane, allowing the visualization of dead or severely stressed cells in contrast to live cells expressing GFP. The spatial organization of bacterial communities in the CF lung as aggregates can provide information about bacterial community behaviors. Identifying viable and non-viable cells, which are sequestered within an aggregate, facilitates the identification of the distribution of cells within the community that may be more sensitive to antibiotic treatment. Quantifying the physical changes that aggregates undergo in response to antibiotics as well as identifying genotypic features of antibiotic-tolerant aggregates could inform future therapies against *Pa*.

The use of PI also has downstream applications in the FACS approach of the workflow, although it should be noted that it is not always necessary, as viable cells in this experiment can be differentiated by GFP expression. However, PI staining allows spatial information to be gained from aggregates at the end of each assay. By highlighting the use of FACS to sort aggregates, this methods paper demonstrates 1) that SCFM2 can be used in a FACS machine without causing damage or clogs despite its viscosity, 2) FACS can be used to separate single cells from an aggregate and group them into distinct populations (phenotypes), and 3) PI staining has utility for separating dead cells from a population with FACS, particularly if the other cells of interest are not expressing a fluorescent marker. The use of live-cell staining techniques highlights the application of this protocol for the use of clinical isolates wherein genetic manipulation is often difficult. Although optimization is likely required strain-by-strain , aggregate phenotypes can be identified at the same resolution by using many commercially available fluorophores.

Compatibility of SCFM2 with FACS is a huge advantage and now allows investigators to isolate specific single cells from an aggregate into distinct populations. This itself has many applications for future studies containing mixed populations of cells. For example, studying aggregation with other species or heterogeneity within single species aggregates[6]. This exciting, high-resolution sorting process is widely available in many institutions and has many downstream applications. Viable cells from the aggregates in this study were sorted for future RNA-seq experiments. Other applications could include proteomics; resampling of aggregates for evolutionary studies, such as assessing the effects of repeated antibiotic exposure; co-culturing with other species; or co-culturing with human immune cells (**Figure 5**). Combining these methods with CLSM imaging experiments allows the correlation of observed bacterial traits and behaviors with quantitative data, with the potential to identify mechanisms of bacterial communities, intra-species relationships, or resistance to antimicrobials.

Alongside the many advantages of these methodologies, there are some pitfalls that should be addressed. This study uses PI as an end-point marker of cell viability; ideally in the future, this would be replaced with cell activity models to differentiate cells over multiple time points. However, these data establish the use of SCFM2 to culture aggregates that can be sorted in multiple ways other than live/dead. The primary advantage is that aggregates can be transferred directly to the FACS machine without washing, and potential disruption of any spatial structure developed over the course of an experiment. Overall, this is an exciting platform that could be used by many labs to foster collaborative projects with those interested in *Pa*, CF, and microbial behaviors.

ACKNOWLEDGMENTS:

S.E.D is supported by start-up funds provided by the Department of Molecular Medicine, The University of South Florida, as well as a CFF research grant (DARCH19G0) the N.I.H (5R21AI147654 – 02 (PI, Chen)) and the USF Institute on Microbiomes. We thank the Whiteley lab for ongoing collaboration involving data sets related to this manuscript. We thank Dr. Charles Szekeres for facilitating FACS sorting. Figures were created by A.D.G and S.E.D using Biorender.com.

DISCLOSURES:

The authors have no conflicts of interest.

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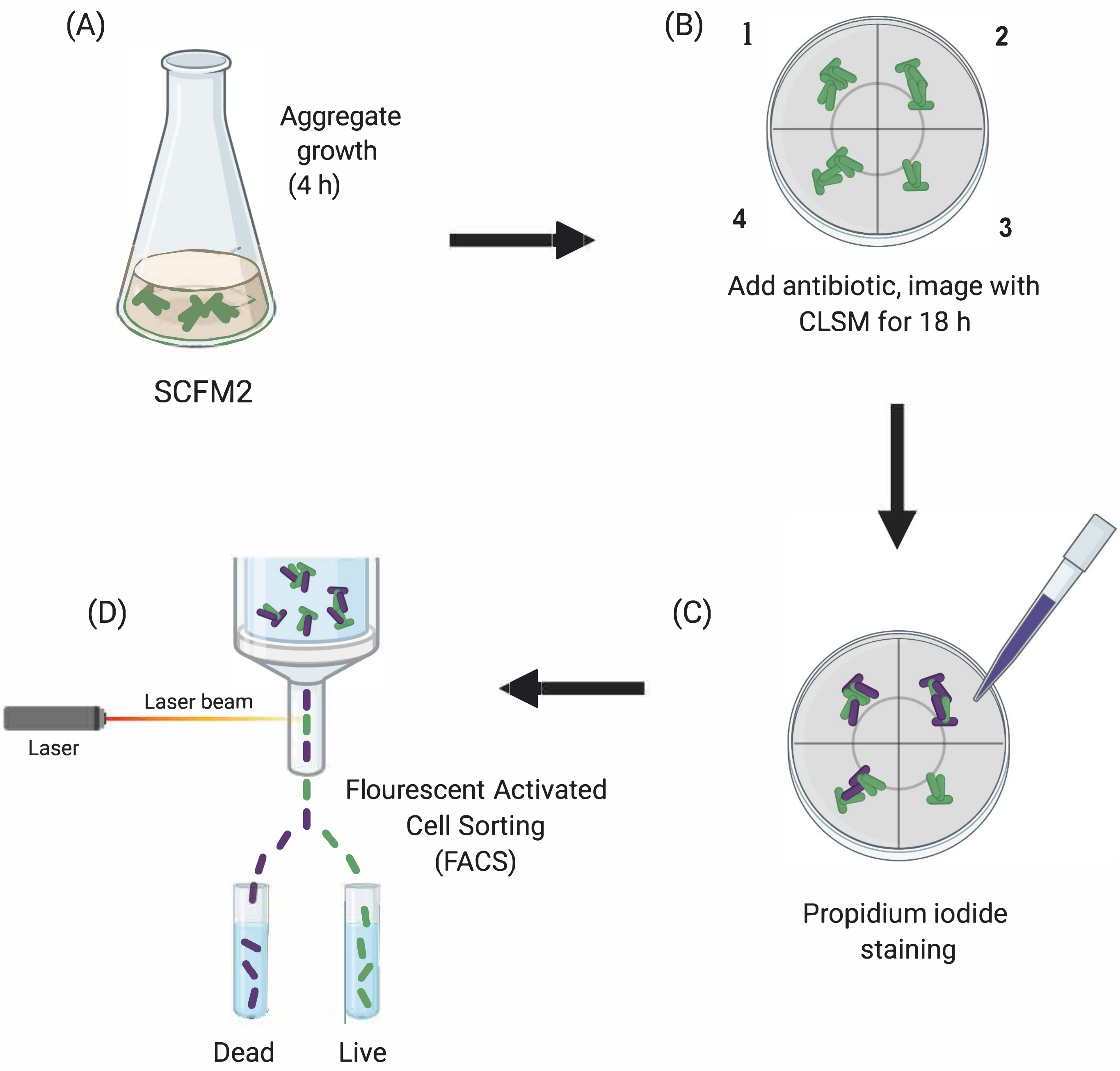

(A)
Aggregate growth (4 h)
SCFM2
(B)
1
2
3
4
Add antibiotic, image with CLSM for 18 h
(C)
Propidium iodide staining
(D)
Laser beam
Laser
Flourescent Activated Cell Sorting (FACS)
Dead
Live

Figure 2

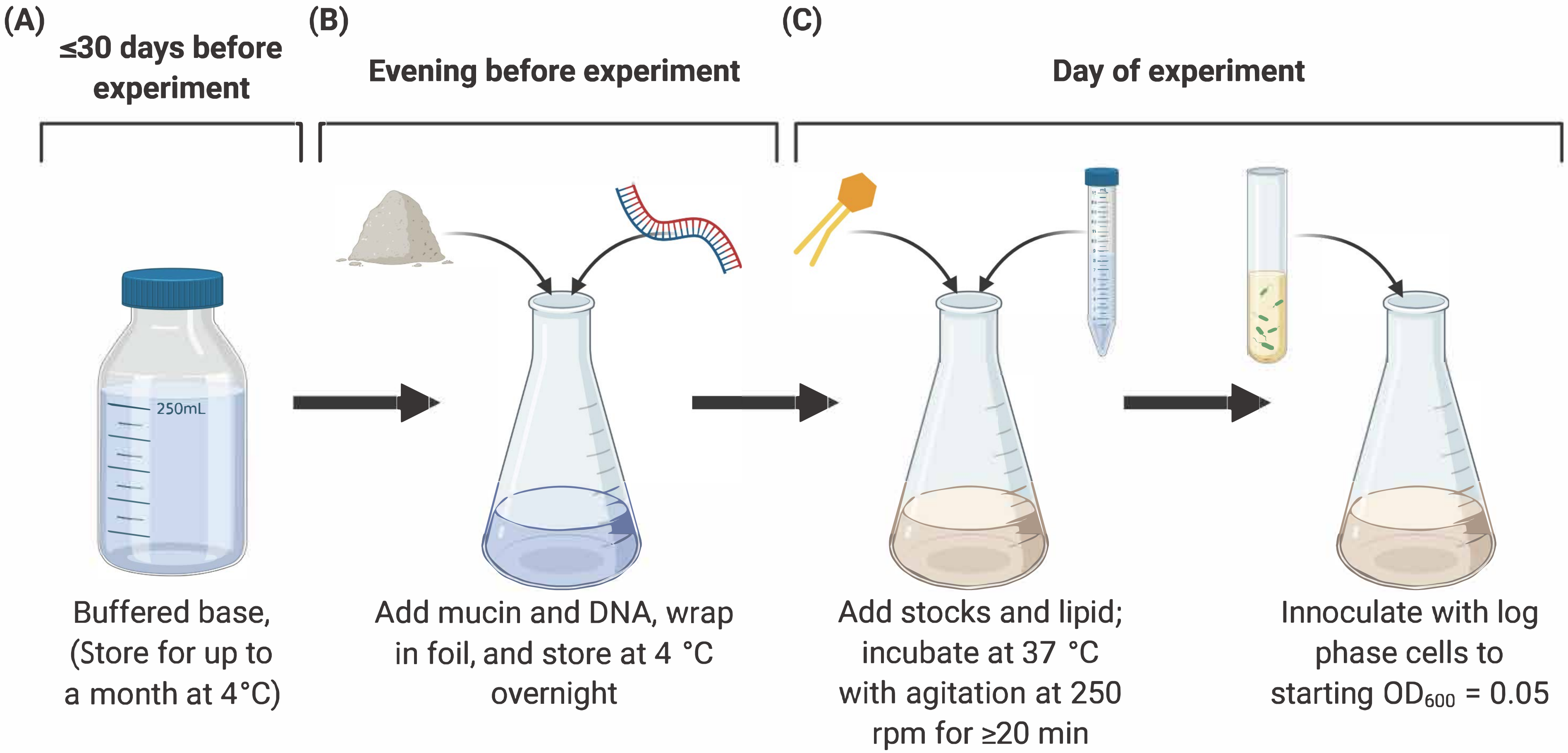

(A)
≤30 days before experiment
(B)
Evening before experiment
(C)
Day of experiment
250mL
Buffered base, (Store for up to a month at 4°C)
Add mucin and DNA, wrap in foil, and store at 4 °C overnight
Add stocks and lipid; incubate at 37 °C with agitation at 250 rpm for ≥20 min
Innoculate with log phase cells to starting OD600 = 0.05

Figure 3

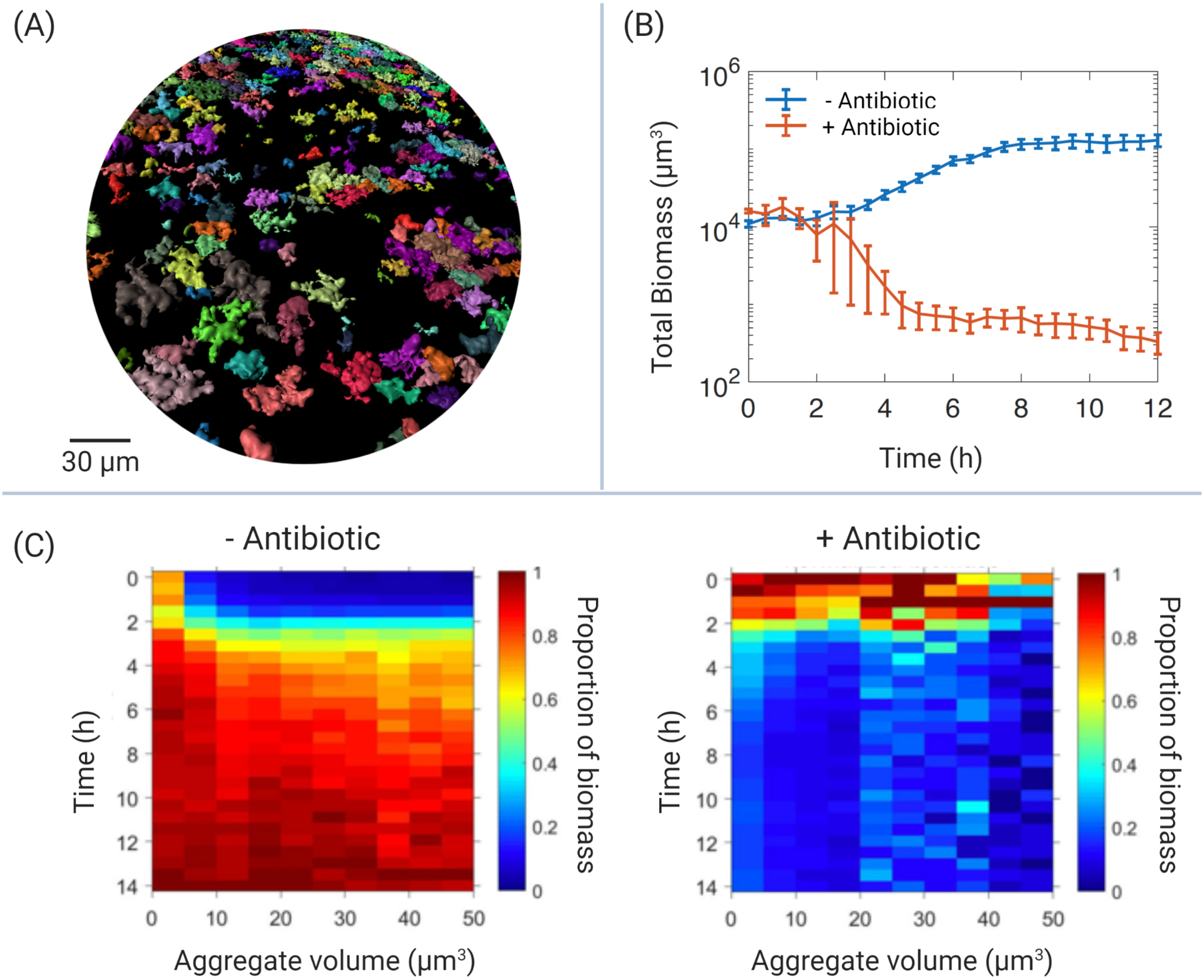
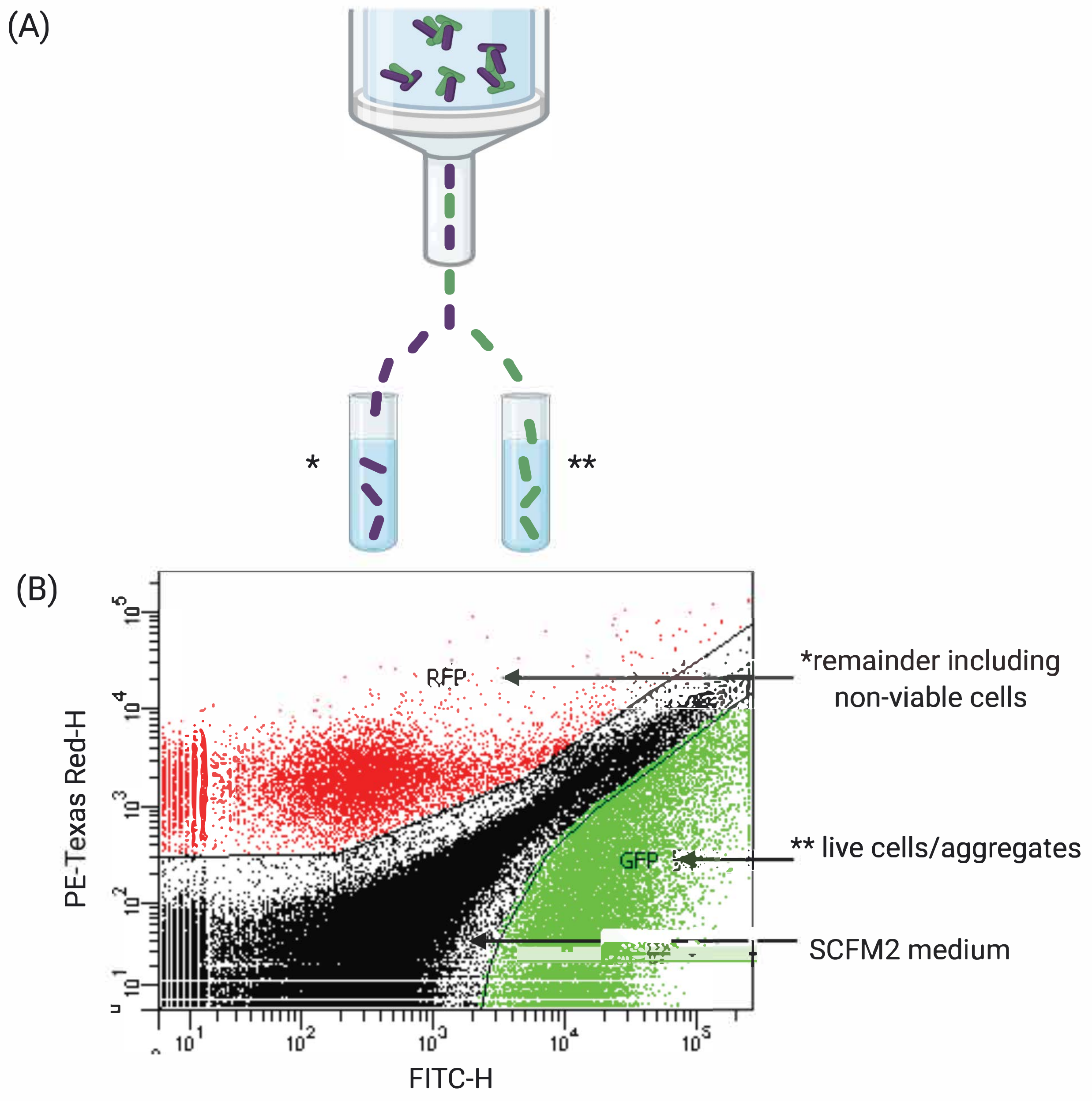

(A)
30 µm
(B)
- Antibiotic
+ Antibiotic
Total Biomass (µm³)
10⁶
10⁴
10²
0
2
4
6
8
10
12
Time (h)
(C)
- Antibiotic
+ Antibiotic
Time (h)
0
2
4
6
8
10
12
14
0
10
20
30
40
50
Aggregate volume (µm³)
Proportion of biomass
0
0.2
0.4
0.6
0.8
1

Figure 4



*remainder including non-viable cells

** live cells/aggregates

SCFM2 medium

Figure 5

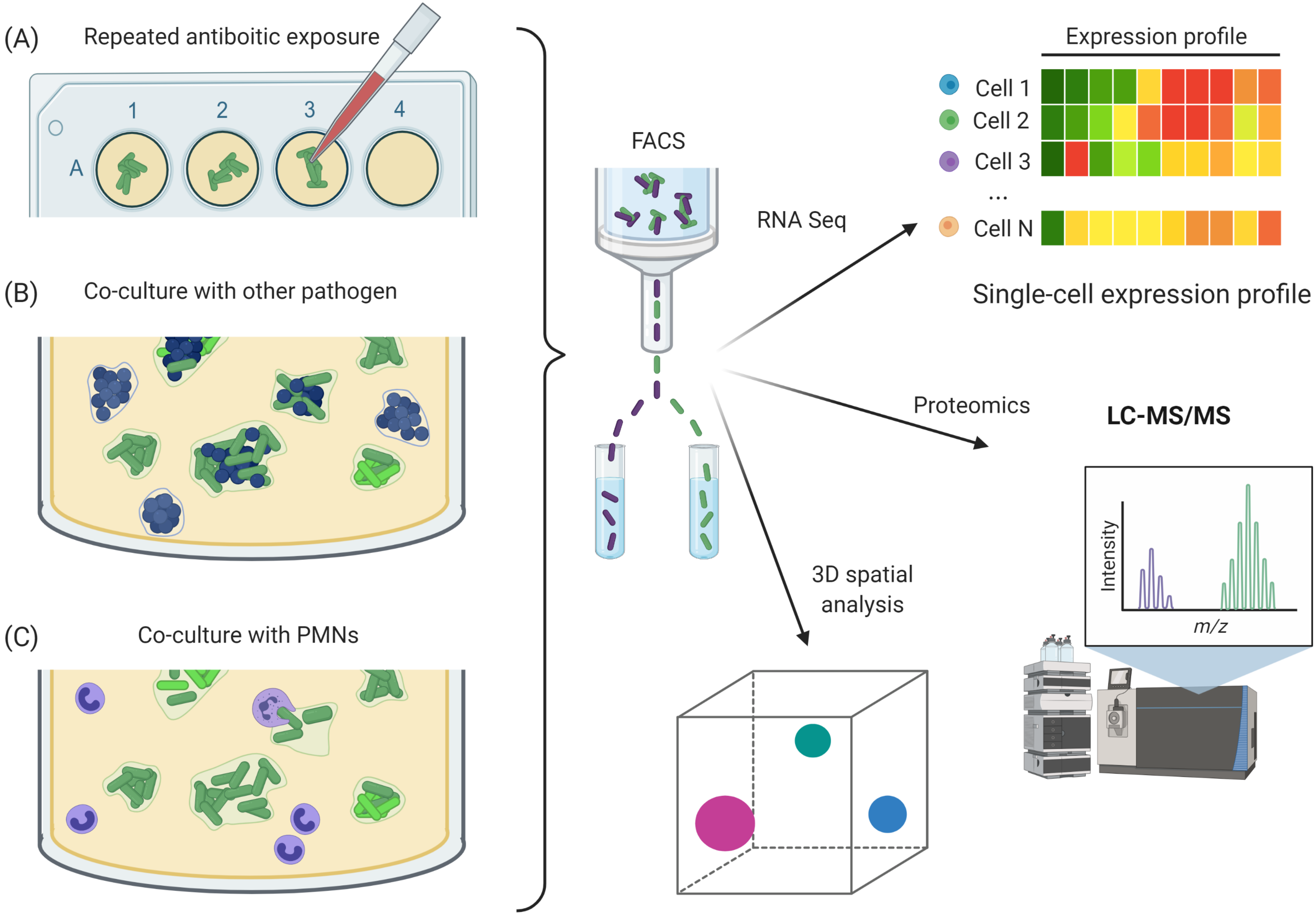

(A)
Repeated antiboitic exposure
1
2
3
4
A
(B)
Co-culture with other pathogen
(C)
Co-culture with PMNs
FACS
RNA Seq
Expression profile
Cell 1
Cell 2
Cell 3
...
Cell N
Single-cell expression profile
Proteomics
LC-MS/MS
Intensity
m/z
3D spatial analysis

Table 1

Chemical	Stock Concentration (M)	Final concentration (mM)
NaH_2PO4	0.2	1.3
Na_2HPO4	0.2	1.25
KNO3	1	0.35
K_2SO4	0.25	0.27
NH_4Cl		2.28
KCl		14.94
NaCl		51.85
MOPS		10
Ser	0.1	1.45
Glu HCl	0.1	1.55
Pro	0.1	1.66
Gly	0.1	1.2
Ala	0.1	1.78
Val	0.1	1.12
Met	0.1	0.63
Ile	0.1	1.12
Leu	0.1	1.61
Orn HCl	0.1	0.68
Lys HCl	0.1	2.13
Arg HCl	0.1	0.31
Trp	0.1	0.01
Asp	0.1	0.83
Tyr	0.1	0.8
Thr	0.1	1.07
Cys HCl	0.1	0.16
Phe	0.1	0.53
His HCl H_2O	0.1	0.52
Salmon Sperm DNA		0.6 mg/mL
Porcine Mucin		5 mg/nL

Notes
Add solid directly to buffered base
Add solid directly to buffered base
Add solid directly to buffered base
Add solid directly to buffered base
Prepared in 0.2 M NaOH
Prepared in 0.5 M NaOH
Prepared in 1.0 M NaOH

Chemical	Stock concentration (M)	Final concentration (mM)	Notes
Dextrose (D-glucose)	1	3	
L-lactic acid	1	9.3	*Adjust to pH 7.0 with NaOH*
$CaCl_2*2H_2O$	1	1.75	
$MgCl_2*6H_2O$	1	0.61	
$FeSO4*7H_2O$	1 mg/mL	0.0036	*Make fresh daily*
N-acetylglucosamine	0.25	0.3	
1,2-dioleoyl-sn-glycero-3-phosphocoline (DOPC)	25 mg/mL	100 µg/mL	*Incubate for at least 20 min at 37 °C after addition*

Name of Material/ Equipment	Company	Catalog Number
	Amino acids	
Alanine	Acrōs Organics	56-41-7
Arginine HCl	MP	1119-34-2
Asparagine	Acrōs Organics	56-84-8
Cystine HCl	Alfa Aesar	L06328
Glutamic acid HCl	Acrōs Organics	138-15-8
Glycine	Acrōs Organics	56-40-6
Histidine HCl H_2O	Alfa Aesar	A17627
Isoleucine	Acrōs Organics	73-32-5
Leucine	Alfa Aesar	A12311
Lysine HCl	Alfa Aesar	J62099
Methionine	Acrōs Organics	63-68-3
Ornithine HCl	Alfa Aesar	A12111
Phenylalanine	Acrōs Organics	63-91-2
Proline	Alfa Aesar	A10199
Serine	Alfa Aesar	A11179
Threonine	Acrōs Organics	72-19-5
Tryptophan	Acrōs Organics	73-22-3
Tyrosine	Alfa Aesar	A11141
Valine	Acrōs Organics	72-18-4
	Antibiotic	
Carbenicillin	Alfa Aesar	J6194903
	Day-of Stocks	
$CaCl_2 * 2H_2O$	Fisher Chemical	C79-500
Dextrose (D-glucose)	Fisher Chemical	50-99-7
1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)	Fisher (Avanti Polar Lipids)	4235-95-4
$FeSO_4 * 7H_2O$	Acrōs Organics	7782-63-0
L-lactic acid	Alfa Aesar	L13242
$MgCl_2 * 6H_2O$	Acrōs Organics	7791-18-6
N-acetylglucosamine	TCI	A0092
	Prepared solids	
Porcine mucin	Sigma	M1778-100G
Salmon sperm DNA	Invitrogen	15632-011
	Stain	
Propidium iodide	Alfa Aesar	J66764MC
	Salts	
K_2SO_4	Alfa Aesar	A13975

KCl	Alfa Aesar	J64189	
KNO_3	Acrōs Organics	7757-79-1	
MOPS	Alfa Aesar	A12914	
NaCl	Fisher Chemical	S271-500	
Na_2HPO_4	RPI	S23100-500.0	
NaH_2PO_4	RPI	S23120-500.0	
NH_4Cl	Acrōs Organics	12125-02-9	

Consumables

Conical tubes (15 mL)	Olympus plastics	28-101	
Conical tubes (50 mL)	Olympus plastics	28-106	
Culture tubes w/air flow cap	Olympus plastics	**21-129**	
35 mm four chamber glass-bottom dish	CellVis	NC0600518	
Luria Bertani (LB) broth	Genessee Scientific	11-118	
Phosphate-buffered saline (PBS)	Fisher Bioreagents	BP2944100	
Pipet tips (p200)	Olympus plastics	23-150RL	
Pipet tips (p1000)	Olympus plastics	23-165RL	
Serological pipets (5 mL)	Olympus plastics	12-102	
Serological pipets (25 mL)	Olympus plastics	12-106	
Serological pipets (50 mL)	Olympus plastics	12-107	
Ultrapure water (RNAse/DNAse free); nanopure water	Genessee Scientific	18-194	
Syringes (10 mL)	BD		794412
Syringes (50 mL)	BD		309653
0.22 mm PES syringe filter	Olympus plastics	25-244	
PS cuvette semi-mico	Olympus plastics	91-408	

Software

Biorender	
FacsDiva6.1.3	Becton Dickinson, San Jose, CA
Imaris	Bitplane
Zen Black	

Equipment

FacsAriallu	Becton Dickinson, San Jose, CA
LSM 880 confocal laser scanning microscope	Zeiss

Comments/Description

Prepared in 0.5 M NaOH

Prepared in 0.2 M NaOH
Prepared in 1.0 M NaOH

shake 15-20 min at 37 °C
to evaporate chloroform

this stock equals 1 mg/mL,
MUST make fresh

pH stock to 7 with NaOH

UV-sterilize

add solid directly to buffered base

add solid directly to buffered base

add solid directly to buffered base

add solid directly to buffered base

Nanopure water used for preparation of solutions in Table 1

To prepare the figures

version 9.6

USF Health

February 23rd 2021

Dear JOVE Editor,

Attached please find our revised manuscript JoVE62420 entitled “Tools for the real-time assessment of a *P. aeruginosa* infection model” that we wish to have considered for publication in JOVE.

We have addressed all comments received from both reviewers and the editor, included is a point-by-point response as requested.

Thank you for considering our manuscript.

Sincerely,

Sophie E. Darch, Ph.D. – Assistant Professor
DEPARTMENT of MOLECULAR MEDICINE – INTERNAL MEDICINE
MORSANI COLLEGE of MEDICINE
University of South Florida | 12901 Bruce B Downs Blvd | Tampa, FL 33612
sdarch@usf.edu | www.darchlab.com

Response to reviewers JoVE6242 (Real time assessment of antimicrobial tolerance in bacterial aggregates)

We thank the reviewers for their positive responses towards our manuscript. We have addressed each comment individually below.

Reviewer #1:

In Real time assessment of antimicrobial tolerance in bacterial aggregates by Alexa Gannon and Sophie Darch, the group proposes a model to observe the growth of different developmental stages of *Pseudomonas aeruginosa* aggregates in a synthetic CF sputum media using confocal laser scanning microscopy. They are able to analyze the aggregates by volume, surface area, and location or position of the aggregates. They present data from aggregate populations during antibiotic treatment and use cell sorting to separate the aggregates for downstream analyses.

We thank Reviewer #1 for their review and suggestions for improving the manuscript. We have addressed each comment individually.

Major Concerns:

1. The goal of recreating physiologically relevant conditions with artificial sputum media is worthwhile. The media alone may lack some important features, especially oxygen - CF sputum may be quite rich in many nutrients, with growth limited by oxygen. The word oxygen does not appear in the manuscript, and oxygen gradients may have critical impact on Pseudomonas physiology and antibiotic response, driving the formation of smaller aggregates, for example.

The reviewer makes a valid point about oxygen levels in this system. Recapitulating an infection like environment is something we are striving to achieve. How oxygen effects aggregation is a research question we are extremely interested in, but outside the scope of this methods paper. Our previous study (Darch et al. 2018 PNAS) provides data that shows oxygen limitation does not occur in this experimental set up for up to 18 hours (determined by a cytochrome oxidase (cbb3) fluorescent reporter) – therefore is unlikely to impact effects of antibiotic treatment measured here. Assessing environmental gradients and their effects within synthetic sputum is a worthy application of this system and an example of the type of questions we hope to enable others to investigate.

2. For the sterilization of porcine mucin, have you verified that the UV sterilization has sterilized the mucin?

We have verified that UV sterilizes mucin (after following the included protocol), by incubating sterile mucin in LB media overnight and assessing for visual growth and turbidity. The general practice in our lab is to use brightfield microscopy to observe a wet mount slide of the buffered base +/- mucin before use to ensure there is no contamination. We have now added this additional note to the protocol (Line 188).

3. Are there any concerns of the sample drying out after 18 hours of incubation on the heated microscope stage?

The Zeiss LSM880 set up allows us to humidify both the heated chamber and/or heated plate stage. Throughout the experiment, the lid of the multi-well petri dish remains in place without *interfering with the imaging process. Both aspects limit evaporation to a negligible level.*

Minor Concerns:

1. How did you dissolve the purified salmon sperm DNA?

Salmon sperm DNA is obtained as liquid aliquots at a concentration of 10 mg/mL (stored at -20 ℃.1 ml aliquots are thawed on ice, vortexed and added to buffered base and mucin at 0.6 mg/ml. SCFM2 is then stored at 4 ℃ overnight to allow time to go into solution. This results in a homogenous distribution of DNA throughout the media. We have added some additional details to the protocol (line 208).

2. Which antibiotic treatment and concentration did you use?

For the experiment described here, we used Colistin sulfate at 140 ug/ml. This was previously calculated as 2 x below the MIC of colistin against our *P. aeruginosa* strain. We have added these details to the manuscript (line 347).

Line 175 - please clarify the yellow highlighting

We have clarified yellow highlighting, now including first step of SCFM2 preparation.

Line 227 - The doubling time of Pa is strain dependent and oxygen dependent, not just SCFM2 dependent. Please clarify

This has now been clarified in the manuscript at line 261.

Line 394: induvial > individual (typo fix)

This has been corrected.

Line 399 - PI may stain cells with compromised membranes that are not dead. Consider describing them as stressed/dead cells

We have incorporated this suggestion, it can now be found on line 717.

Figure 5 panels A, B and C would be more clear with headers, as the other figures have

We have amended the figure to incorporate this suggestion.

Reviewer #2:

The manuscript by Gannon and Darch details the necessary protocols to grow *Pa* aggregates and analyze them using confocal microscopy. This process runs from initial aggregate growth in synthetic CF septum medium, analysis with microscopy (3D & temporal), FACS analysis, and necessary downstream processing.

Overall this work is an excellent candidate for a JOVE video, and will be beneficial for the field. Indeed, I am now planning on incorporating these techniques in my lab and looking forward to the video! I support this excellent publication with some minor changes and suggestions detailed below.

We thank Reviewer #2 for their very positive and encouraging review. We have addressed each comment individually.

Major Concerns:

none

Minor Concerns:

- More (any) detail should be provided on the data processing in the results, rather than referencing unpublished scripts. While I realize that it may be published elsewhere, at least discussing how the data was processed may provide value for the reader.

Additional details have been added to the image analysis section – highlighting the available options and processing methods for confocal data generated using this protocol (line 484).

- Figure 3 - why is the 0 time point different between the 2 samples? Should it actually be a later point (i.e. if 0 is addition of antibiotic, how long until first image taken), or is it just a scaling issue between the two samples and both show a distribution skewing toward the smallest aggregate size as in the - condition?

We apologize for the confusion. Figure 3 represents data after antibiotic addition (0 time point = addition of antibiotic after 6 hours of aggregate growth). We have clarified this in the figure legend (line 674).

- Figure 4 - why is it labeled as RFP in the area - i.e. is there actually RFP being measured, or if it is a different abbreviation that should be explained (or changed) as the reader would likely conclude it is a GFP/RFP pair. Alternatively, if this is previously published data from a different experiment w/RFP this should be explained in text or caption.

We agree with the reviewer that this is not clear. For the protocol we describe the use of propidium iodide, but the figure provides representative data of excitement of RFP for separation from GFP expressing cells. This has now been clarified in the figure caption (line 681).

Potential suggestions (publication still supported without these changes)

- It may be worth expanding the breadth of the title. As the authors detail in the text this approach would be applicable to more research questions than just antimicrobial tolerance.

We agree with the reviewer and have modified our title to:

Tools for the real-time assessment of a *P. aeruginosa* infection model

- It may be worth discussing applicability to samples w/o GFP expression (e.g. patient samples).

We agree with the reviewer that this would be a nice addition to the discussion. We have now added some details on this potential application (line 741).

Editor:

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

These revisions have been made.

2. Please revise the following lines to avoid previously published work: 58-59, 60-61, 320-321, 323-324.

These revisions have been made.

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

We have removed all personal pronouns from the manuscript.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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. For example: Sigma, Invitrogen, Ibidi, Zeiss LSM 880, FacsDiva6.1.3, Becton Dickinson, FacsAriaIlu, Imaris, Bitplane.

All commercial language has been removed from the manuscript and is now only in the Table of Materials.

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.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc. (Lines: 230-255, 299-301,308-334)

We added an additional sentence to section 4.1 to state the use of BSL-2 guidelines for the handling of P. aeruginosa in this protocol. We believe this covers the safety requirements throughout, as have no additional safety protocols outside of BSL-2 for FACS or image analysis methods (line 242).

6. Line 297: Please include the details of the concentration of aggregates used.

The protocol uses the entire 18-hour culture from the microscopy experiment. After 18 hours of growth each aliquot will contain ~15,000 aggregates (line 424).

7. Please highlight up to 3 pages 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have highlighted lines 176-551.

8. Please move the Figure Legends section to the end of the Representative Results.

We have moved the figure legends section (now at line 667).

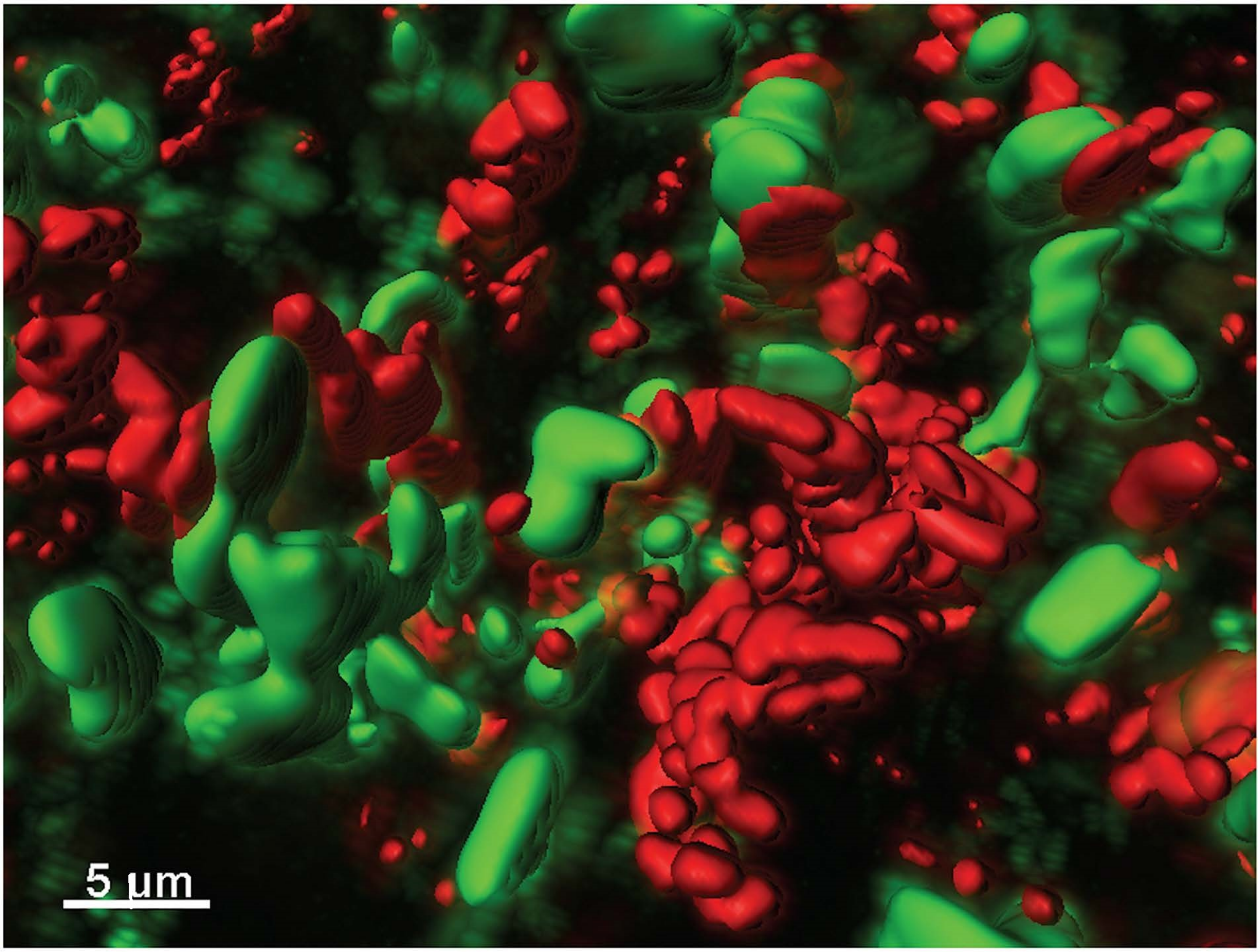
9. Please do not abbreviate journal titles and book titles.

We have ensured that all literature cited complies with the JOVE referencing style available for ENDNOTE.

10. Figure 3: Please ensure that standard abbreviation of units is used. Please replace “hours” to “h” in the X-axis of Figure 3B and Y axis of 3C, 3D.

These revisions have been made.

Supplemental Figure S1



Video S1

Click here to access/download

Supplemental Coding Files

Video S1.mp4