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 °C.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 °C 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, FacsAriallu, 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.