**Response to reviewers’ comments**

• *JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.*

*• Please make sure that your references comply with JoVE instructions for authors. In-text formatting: corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text of the manuscript. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then et al.): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source.****Volume****(Issue), FirstPage – LastPage, doi:DOI (YEAR).]*

We have now included all available DOI for the references and they are in proper format.  
  
• *Protocol section 1 was un-highlighted as these steps cannot be filmed.*

We are in agreement with this.  
  
• *5.2 and 5.2 Note: The http link was moved to the Table of Materials. Please provide the http details (along with authors (if applicable), publication year etc.) in the reference section and cite the reference here.*

We are in agreement with move of the http link to the Table. The reference to COMSTAT is already included in reference 18 and 19.   
  
•*Formatting:  
-3.1 – Should be rpm, not “rotations”.*

This has been changed.

-*Please submit figures as individual files in the preferred format, and not as Word files.*

We will do this as suggested.

-*Please cite figure panels in the text as “(Figure 2A)” for example, rather than (2A).*

This has been corrected.  
  
•*Grammar: Line 266 – “The use of the live-dead stain as per manufacturers protocols have”*

This has been corrected.

•*Additional detail is required: Is sputum used in step 3.3? Please clarify and highlight, otherwise it is confusing to have a section on sputum processing but then not use it.*

This is now indicated in step 3.3. All of these experiments can be done in sputum or not, or with antibiotics or not. Filtrates from other bacteria can be prepared similarly to the sputum and used in the system.

•*Branding should be removed from the 5.2 note – “SYTO9”*

This has been removed from the text.

•*Results: Please define the error bars (SD, SEM, etc.) and the scale bars in the figure legends for figures 1 & 2.*

This has now been added to the legends.

**Reviewer 1:**

*Major Concerns:*

1. *The title of the manuscript suggests that the focus of the paper is on methodology to visualize how host derived factors affect biofilm formation. While this manuscript focuses on sputum supernatants, the manuscript should include the use of other host factors in general, and describe the use of sputum as one example only. It was difficult to follow when the focus was inconsistent among the steps. The authors should revise to describe a protocol that readers could easily follow and adapt for use with other bacteria and alternative host factors.*

-We understand the reviewer’s concerns. To clarify the protocol and to specify the exact experiments that we have previously done, we have changed the title to “Visualizing the effects of sputum on biofilm development using a chambered coverglass model” as this is the specific host factor that we tested. We have included other potential bacteria that could be used for these experiments and have detailed the sputum supernatant step more clearly in step 3.3 and throughout. As we have not tested other host factors, we have not included examples of these in the protocol.

2. *It is very unclear from the Protocol, Results and Figure 1 when and how host factors (sputum supernatants) should be added to the biofilms. There is a brief mention of 10% sputum supernatants addition on lines 145-146 in the Protocol and line 225 in the Figure 1 legend. As the primary focus of the manuscript, it is very unclear when and how host factors should be added to the biofilms. The authors should provide and discuss multiple different options for the addition of host factors, dependent on the desired outcomes or hypothesis to be tested. This information should be included in both the Protocol section and schematic in Figure 1.*

-We have now added more details regarding when and how sputum in added to the biofilms in step 3.3 as well as Figure 1. As discussed above, we have not included details of other host factors given that we have not tested these.

Added: 3.3 Dilute the culture from step 3.2 to 1/100 in desired media, with 10% sputum filtrates or without (as control). Other concentration of sputum filtrate can be tested (i.e. 50% or 100% sputum filtrates).

3. *The procedure for image collection by confocal microscopy is generally equipment specific. Section 5 "Visualizing biofilms with confocal microscopy" reads very specific to the equipment and organism used by this author. Most users who perform confocal microscopy are familiar with the setup and process specific to their microscopy. As such, a brief general overview of methods used should provide enough information for the typical confocal user.*

-These details were added on request of the editors at JoVE with the first manuscript review.

a. *Step 5.4 Why should the images be collected at every 0.8 uM in the Z-plane? This is a very specific number and the z-plane resolution will vary based on multiple factors, such as the type of microscope, the lenses used, the pinhole size, and the bacterium utilized.*

-We thank the reviewer for this question. We have added a range (0.5-1μm) in which the images can be collected. To further explain why we used this range, we have included comments on this subject in the Discussion in lines 290-297 on page 7:

The acquisition of images is important prior to the analysis of data from these experiments. Care must be taken to not over saturate images and when determining the microscope setup. Depending on the level of detail required, and the microscope available, a number of different objectives (10X, 25X, 40X and 63X) can be used to acquire images, though we find that 25X objective gives nice images. The thickness of the Z-stack can also affect overall image quality and level of detail. Having images taken every 0.5-1μm seems to provide clear images at 25X objective, while keeping the Z-stack images at a size that can be analyzed by COMSTAT on standard computer systems.

b. *Step 5.3 Why should a 25x water objective be used as opposed to a 40x or 63x, these factors may need to be adjusted or selected on a case-by-case basis.*

-Although we agree with the reviewer, we were asked by the editor at JoVE to be very specific to what we did. We agree that you can use another objective if it suits your purpose.

4. *More information should be provided with regards to image analysis. Software programs, such as COMSTAT and Volocity are mentioned in the abstract and introduction. Methods for biofilm image analysis should be included, or at least briefly discussed, in the Protocol section. Specifically, it would be helpful to list which analysis metrics are relevant to the analysis of statically grown biofilms that have been treated with host factors.*

-Details regarding the analysis metrics have now been include in step 5.7:

5.7) Once images are imported into COMSTAT, parameters such as average thickness, biomass and surface coverage for each channel (Red and Green) are analyzed.

In addition, given that COMSTAT is a free software program, we have also provided the references so that individuals can look up the specifics related to their experiments.

5. *The manuscript refers to the use of chamber slides to seed and grow biofilms, yet the Materials table lists chambered coverglass. It should be made clear whether chambered slides or chambered coverglass were used for these experiments, as they are inherently different in the way the biofilms are processed and visualized by confocal microscopy. Additionally, line 271 states that the bacteria attach to a plastic surface; however, in the case of both chambered slides and chambered coverglass, the substrate would be glass. Please correct this statement accordingly.*

-Thank you for this comment. Changed to chambered coverglass and 1.5’ borosilicate glass.

6. *In Figure 2 panel D there is very little difference in the percentage dead cells between the intermediately resistant strain treated with 1 mg/mL and 2 mg/mL tobramycin, yet in panel A there is visually a large difference in the amount of red cells between these tobramycin concentrations. This leads this review to wonder what method was used to determine percentage dead and if this method accurately reflects what is occurring in each of these biofilms.*

-Given that the reviewer wishes us to focus on the effects of sputum supernatants on biofilms, we have deleted this figure and left in the figure showing the effects of sputum supernatant on *B. cepacia* complex biofilms. We have added figure 3 which shows the effects of antibiotic on sputum treated bacteria. The dead:live ratio in figure 3C represents the mean of 3 independent experiments where 12 images were analyzed from each experiment (4 views from 3 wells for each experiment). Thus each bar represents the mean of 36 images. Because of the variability seen in biofilms from experiment to experiment, there will be variability between images and the mean represented by the dead:live ratio. Because this is a measure that reflects what is seen it is still a useful measure.

7. *It is unclear why antibiotic susceptibility was tested and shown in Figure 2. There is no mention of the use or addition of antibiotics in the Protocol. Methodology used to test the effect of antibiotics on biofilm development need to be included in the Introduction and Protocol sections if they are to be shown and discussed as Representative Results.*

-We have removed Figure 2 but believe it is important to include the effects of antibiotics on biofilms in the presence of sputum supernatant as this is a barrier to antimicrobial efficacy and have added a figure (figure 3) to show this. We have included more details on the addition of antibiotics in lines 164-166 on page 4 and in Figure 1:

3.8) To study the effect of sputum supernatants on biofilm antibiotic susceptibility, antibiotics are added to the media following 24 hours of biofilm growth and are maintained in the media until staining and imaging of biofilms.

*Minor Concerns:*

1. *Overall, the manuscript contains many spelling and grammatical errors that make the writing unclear and should be corrected.*

-We thank the reviewer for the comment. We have gone through the manuscript to correct the mistakes.

2. *The sentence at lines 86-88 is redundant and unclear. As written, it suggests that it is the biofilm viability kit that has been "subjected to host or other factors." It is likely that the authors meant to say that the biofilms, and bacteria within, were subjected to host or other factors.*

-This sentence has been changed to:

This method allows for rapid visualization of biofilms exposed to sputum containing host factors using a commercial biofilm viability kit that has been.

3. *On line 203, it is stated that this protocol allows visualization of "biofilm growth and viability over time", yet no time course is shown or described as all data represent single time points only.*

-We have clarified that this means that biofilms can be grown for different periods of time prior to visualization, such as 24, 48 or 72 hours in lines 220 on page 5:

-The use of this protocol provides a convenient method to visualize the changes in biofilms grown for different periods of time (eg. 24, 48 or 72 hours).

4. *There is an inconsistency in the volume used to seed the biofilms. Line 148 states 250 uL and line 223 states 200 uL, figure 1 also states 200 uL. The volumes for media replacement (line 152) and staining (line 167) are also inconsistent. Please correct the volumes given.*

-This has been corrected to 200 microliters.

5. *Lines 208 - 211 discuss estimation of percentage of dead biofilm. The ratio of the signal intensity in the red channel compared to the signal intensity of both channel combined does not provide an accurate estimation of the percentage of dead vs. live bacteria. Many factors influence signal intensity and not all bacteria take up SYTO9 or propidium iodide equally. Relative density, fluorophore quenching and detector gain setting can all affect fluorescent signal intensity. The alternative method of comparing the volume or biomass of red (dead) vs. green (live) bacteria is a much better method for the determination of relative viability in a biofilm. Please adjust or correct these lines for clarity.*

-We have acknowledged that an alternative method is an estimation of the biomass in lines 220:

Alternatively, it is also possible to derive a ratio of the volume of the biomass of cells stained red as a ratio of total volume of biomass.

We have also added a sentence in the Discussion of the limitation of percent dead as a method in line 280-284 on page 7:

A number of factors can affect the florescence ratio of the images (including dye uptake of bacteria, relative density of biofilm and detector gain settings), however this method should relate to what is observed in the images. Other measures may be used to represent the relative amount of dead/live biofilm, such as the biomass from the dead cells as a ratio of the total biomass present in the biofilm (as derived from COMSTAT).

6. *The authors should discuss the use (or non-use) of a fixation step following staining of the biofilms, as fixation is a common technique used in confocal microscopy and visualization of in vitro biofilms.*

-Although we agree that fixation has been used by other investigators, we have not mentioned it because we have not used it ourselves and don’t have the experience with this method. We prefer to visualize the biofilms promptly and without alteration by fixatives as this may affect the biofilm itself.

7. *The authors should include the methods of analysis and statistics that were used in Figure 2 and Figure 3.*

-Figure 2 has been removed. Statistical methods have been added to Figure 3.

8. *The authors should include discussion of and reference to previous work published in the Journal of Visualized Experiments by Jurcisek J et al, which describes a very similar method for generating, staining and visualizing biofilms grown in 8-well chamber slides. [J. Vis. Exp. (47), e2481, doi:10.3791/2481 (2011)].*

-We thank the reviewer for this comment. We have added this reference and explained how this manuscript differs in the addition of sputum supernatants and antibiotics to bacterial biofilms in lines 323-329 on page 9:

This is an expansion of the model described by Jurcisek et al. in 2011.20 This method uses slide chamber growth similar to what is described, and uses formalin to fix biofilms prior to visualization. Alternatively, in this method we visualize biofilms immediately after staining and do not use a fixative. Additionally, by adding exogenous factors to the system and testing the effect of antibiotic on biofilms formed in these chambers these models have the potential to considerably further our understanding of bacterial biofilms in human disease.

*Reviewer 2*

*Major concerns*

*None.*

*Minor Concerns:*  
*Line 154-155: I think it would be helpful to detail if biofilm has to be washed after each medium replacement.*

- This has been replaced with 3.6) After 4 hr, remove media and gently wash biofilm 1X with fresh media. Replace with 200 μL fresh media.

*Lines 159-160: washing biofilms..very critical issue. The authors should indicate how many times the biofilm has to be washed.*

4.1) Following desired growth time (24-48 hr works best), remove media from chamber wells and gently wash 2X with 300 μL of sterile PBS.

*Lines 165: manufacturer recommend to use water for preparing staining mixture. BUT, since water could lyse cells, is it possible to use other solvents? Do the Authors have some experience at this regards?*

-We use the same media with which we grew the biofilm in (media solution of dye, mentioned in line 165). We have not noticed interference with phosphates, though it is a possible concern. Other solvents can be tested, but we have not tried them.

*Line 177: the authors stated that biofilms have to be observed IMMEDIATELY after staining. What does it mean? In the sense that...how much the observation can be delayed without having problems at observation?*

-Once the stain is removed, we visualize the biofilm on the microscope immediately and visualize all wells within an hour of staining. This has been added to Step 5.1.

*Line 189: Z-stacks to model biofilm. The authors took images every 0.8 um. I think it depends on the resolution desired/needed. Could the authors be more clear at this regard? Maybe it could be more adequate to refer at a range of values.*

-We thank the reviewer for this question. We have added a range (0.5-1μm) in which the images can be collected. To further explain why we used this range, we have included comments on this subject in the Discussion in lines 290-297 on page 7:

The acquisition of images is important prior to the analysis of data from these experiments. Care must be taken to not over saturate images and when determining the microscope setup. Depending on the level of detail required, and the microscope available, a number of different objectives (10X, 25X, 40X and 63X) can be used to acquire images, though we find that 25X objective gives nice images. The thickness of the Z-stack can also affect overall image quality and level of detail. Having images taken every 0.5-1μm seems to provide clear images at 25X objective, while keeping the Z-stack images at a size that can be analyzed by COMSTAT on standard computer systems.

*Line 198: please replace "COMTAT" with "COMSTAT".*

-This has been corrected.

*Figures 2 and 3: please add SD bars, and p values and the statistical analysis used in the legend.*

-Figure 2 has been removed and Figure 3 has been corrected.

*line 252: It is proposed to filter sputum to remove resident microbiota. I think this method is not always easy to perform considering how dense is the sputum. Further, filtration could significantly modify chemical composition of sputum. Exposure to UV light could be more practical, conservative, and reproducible to decontaminate the sample.*

-We thank the reviewer for this comment and agree that this may be a useful option but we have not tested this methodology. We have diluted sputum in 2X sputum volume with PBS prior to centrifugation and supernatant filtering. It has been possible to filter even the most purulent sputum in this manner. We have not tried UV sterilization but this may be something to try in the future.

*Line 257: Authors suggest to use a nutrient RICH media in evaluating the effects of exogenous factors on biofilm development. I do not agree. A rich media could be able to mimicry physico-chemical conditions observed at CF lung. I think that using minimal medium would be more realistic.*

-We completely agree with the reviewer that the choice of media is important and each media has its advantages and disadvantages. The choice of appropriate media for experimentation is noted in the protocol in note after 3.1. We have added some discussion regarding this in lines 272-275 on page 7:

This will allow sufficient nutrition for growth while determining how exogenous factors may affect the biofilm. Other media, such as minimal or defined media, can be used to better mimic certain conditions, and have been used with good results in this system (data not shown).