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Visualizing the effects of sputum on biofilm development using a chambered coverglass model

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Abstract:	Biofilms consist of groups of bacteria encased in a self-secreted matrix. They play an important role in industrial contamination as well as in the development and persistence of many health related infections. One of the most well described and studied biofilms in human disease occurs in chronic pulmonary infection of cystic fibrosis patients. When studying biofilms in the context of the host, many factors can impact biofilm formation and development. In order to identify how host factors may effect biofilm formation and development, we used a static slide chamber method to grow biofilms in the presence of host-derived factors in the form of sputum supernatants. Bacteria are seeded into Lab-Tek chamber slides and exposed to sputum filtrates. Following 48 hours of growth, biofilms are stained with a commercial biofilm viability kit prior to confocal microscopy and analysis. Following image acquisition, biofilm properties can be assessed using different software platforms from PerkinElmer (Volocity) or software such as COMSTAT. This method allows us to visualize key properties of biofilm growth in presence of different substances including antibiotics.
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Question	**Response**
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July 22nd, 2016

Dear Editor,

We sincerely thank the reviewers for their comments in the review of our manuscript. Please find attached the revised form of our paper and the responses to the reviewers comments.

We thank you for suggestions and hope we have addressed any concerns.

Sincerely,
Trevor Beaudoin

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TITLE:
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KEYWORDS:
Biofilms, Chambered coverglass, Microscopy, Sputum, Microbiology, Viability Staining, Cystic Fibrosis

SHORT ABSTRACT:
This protocol describes the visualization of biofilm development following exposure to host-factors using a slide chamber model. This model allows for direct visualization of biofilm development as well as analysis of biofilm parameters using computer software programs.

LONG ABSTRACT:
Biofilms consist of groups of bacteria encased in a self-secreted matrix. They play an important role in industrial contamination as well as in the development and persistence of many health

related infections. One of the most well described and studied biofilms in human disease occurs in chronic pulmonary infection of cystic fibrosis patients. When studying biofilms in the context of the host, many factors can impact biofilm formation and development. In order to identify how host factors may affect biofilm formation and development, we used a static chambered coverglass method to grow biofilms in the presence of host-derived factors in the form of sputum supernatants. Bacteria are seeded into chambers and exposed to sputum filtrates. Following 48 h of growth, biofilms are stained with a commercial biofilm viability kit prior to confocal microscopy and analysis. Following image acquisition, biofilm properties can be assessed using different software platforms. This method allows us to visualize key properties of biofilm growth in presence of different substances including antibiotics.

INTRODUCTION:

Bacterial biofilms are groups of microorganisms that are attached to one another and encased in a self-secreted matrix.[1, 2] Classically, they represent bacteria physically attached to an abiotic or biotic surface formed under conditions of flow. Biofilms have also been shown to grow in static conditions (absence of flow) and distal from surfaces, such as at the air-liquid interface of thermal pools or pellicles formed in test tubes. These biofilms have long been recognized in the environment and are a major detriment to industrial processes, as they can form in water reservoirs or in pipes, resulting in biofouling, corrosion and blockages.[3, 4]

Biofilms are also critical in healthcare settings, as they have been shown be involved in catheter related infections, pulmonary infections in cystic fibrosis patients, as well as in numerous other infections[5, 6] One of the hallmarks of biofilm infections is the decreased susceptibility of bacteria to antibiotics and impaired clearance by the innate immune system.[7-9] The most well studied, clinically relevant scenarios involving biofilm-based infection occurs in patients with cystic fibrosis (CF), who are chronically infected with *Pseudomonas aeruginosa* biofilms. *P. aeruginosa* can undergo a number of changes during establishment of chronic infection that make it very difficult to treat.[10, 11] Biofilms can differentially activate innate immunity and drive inflammation.[12-14] As these infections lead to increased morbidity and mortality in CF patients, it is crucial to understand factors that can affect biofilm development in this context.

A recent study suggests that host-factors are critical in the formation of *P. aeruginosa* biofilm aggregates.[15] These biofilms contribute to reduced susceptibility to antibiotics and host defense mechanisms. The presence of host-derived factors, such as neutrophil elastase, as well as secreted products from microorganisms present in the CF lung, have the potential to greatly modulate biofilm formation and development.[16] Additionally, biofilms interact with the host to modulate expression of numerous pathways and initiate inflammation. While high throughput methods, such as the standard crystal violet assay, can provide some information with regards to the biofilm process, visualization of the biofilm in response to these factors provide more in-depth information.

In this manuscript we describe a method for using factors from the sputum of patients with CF to study the development of biofilms *in vitro.* This method allows for rapid visualization of biofilms exposed to sputum containing host factors using a commercial biofilm viability kit. This

technique can be used to visually identify changes that occur during biofilm growth in the presence of exogenous products, and represents an improved method to analyze the changes in biofilm development under various conditions.

PROTOCOL:

Note that Research Ethics Board (REB) is required to collect and store sputum samples from human subjects. These studies were approved by the Hospital for Sick Children REB#1000019444.

1. Preparing CF sputum samples.

1.1) Collect sputum sample from patients during routine visits to the cystic fibrosis clinic and keep on ice.

1.2) Transport sputum sample on ice within the first hour of collection, to the research laboratory, to undergo processing.

2. Sputum Processing

2.1) Record the volume of the sputum sample obtained. Add phosphate buffered saline (PBS) to 2X the volume of the sample (i.e. 2 parts PBS, 1 part sample).

2.2) Mix the sample well with a transfer pipette. Vortex the sample on the highest setting for 1 min to mix completely.

2.3) Aliquot 1 mL of the above mixture into the appropriate number 1.5 mL microcentrifuge tubes and spin down at 5000x g for 20 min at 4 ^{o}C.

2.4) Following centrifugation, remove the supernatant and discard the pellet.

2.5) Filter sterilize the supernatant through a 0.22 µm filter and collect in a clean microcentrifuge tube.

Note: Sterility of filtrate is tested by plating on LB agar and inoculating liquid media.

2.6) Store sputum supernatant at -80 ^{o}C for future use.

Note: Sputum from multiple patients can also be pooled following filtration.

2.7) Prior to use, dilute sputum filtrate 1/10 v/v (100 µl of sputum, 900 µl of media) in desired media.

Note: Here, standard lysogeny broth (LB) media was used.

3. Chambered coverglass method for biofilm formation

3.1) Grow bacterial isolate of interest overnight in desired media at 37 ^{o}C with shaking (200 rpm).

Note: A number of different bacteria were used, including clinical isolates *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Burkholderia cepacia* complex and *Achromobacter xylosoxidans.* Choice of media depends on strains and conditions of interest, however LB media can be used for initial experiments.

3.2) From overnight culture, place 40 µL of culture into 4 mL of fresh media and grow for 3-4 h at 37 ^{o}C with shaking (200 RPM) to obtain a culture with an optical density at 600 nm (OD_{600}) of approximately 0.5-0.6.

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

3.4) Use 200 µL of the dilution to seed wells of slide chambers.

3.5) Allow bacteria to attach for 4 h at 37 ^{o}C without shaking.

3.6) After 4 h, remove the media and gently wash the biofilm with 1X fresh media. Replace with 200 µL fresh media.

Note: To study the effects of sputum on the biofilm, the fresh media should contain sputum supernatant.

3.7.) Allow biofilms to grow for desired amount of time at 37 ^{0}C without shaking, replacing media every 12 h, without washing until the time for microscopy.

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

4. Staining biofilms and confocal microscopy

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

4.2) Prepare staining mixture for biofilm by mixing 1 µL of each dye (provided in the viability kit) for each mL of solution needed. Make dye in water or media solution.

Note: Water is recommended by the manufacturer.

4.3) Add 200 µL of dye mixture to each well of chambered coverglass and incubate at room temperature, in the dark for 45 min.

4.4) Remove staining mixture from chambers and wash each well with 300 µL of sterile PBS. Remove PBS and replace with fresh water or media.

4.5) Proceed with visualization of biofilms via confocal microscopy.

5. Visualizing biofilms with confocal microscopy

5.1) Read stained biofilms in chambers immediately after staining (within 1 h). Minimize delay in visualization of the slides by staining 1 to 2 8-well chambers at a time.

5.2) Perform imaging using confocal microscope with lasers for excitation and filter sets for acquisition.

Note: Here, the spinning disk confocal system with spectral borealis lasers (Green: 491nm, Red: 561nm) were used for excitation. Emission filter sets of 515/40 and 624/40 were used to visualize the stains from the biofilm viability kit.

5.3) Take images using a 25x water objective on confocal microscope with camera.

5.4) Use Z- Stacks to model the biofilm. Take images every 0.5-1 µm starting from the first in-focus plane to the last in-focus frame of the biofilm (typically spanning 30-80 µm for 48 h biofilms)

5.5) Take 3-5 images from each well.

Note: Thus for an 8 well chambered coverglass, 24-40 images will be generated.

5.6) Save images for analysis.

Note: Images should be saved as OME-TIFF files to be analyzed using COMSTAT[18, 19]. Instructions for biofilm image analysis can be found at http://www.comstat.dk/. Once images are imported, parameters such as average thickness, biomass and surface coverage for each channel (red and green) can be analyzed.

REPRESENTATIVE RESULTS:

The overall design of the experiment is represented in Figure 1. The use of this protocol provides a convenient method to visualize the changes in biofilms grown for different periods of time (e.g. 24, 48 or 72 h). Importantly, exogenous signals, such as sputum filtrates, can be added to visualize the changes in biofilm development. As seen in Figure 2, the presence of 10%

sputum filtrates can change the architecture of the biofilm (Figure 2A, lower panels).[16] These images can be analyzed using COMSTAT software to obtain key biofilm matrices, including average thickness of the biofilm, total biomass and surface coverage. This is reflected in an overall increase in biofilm thickness (Figures 2B, and 3).[16] The effects of antibiotics on biofilms in the presence sputum is shown in Figure 3. By visualizing the changes in biofilm development, one can better appreciate how different factors can affect biofilm growth, to a much better extent than traditional biofilm assays, such as crystal violet staining.

Figure Legends:

Figure 1. Overall design of experiment: Flow diagram of basic protocol. An overnight (O/N) culture is diluted 1/1000 and allowed to grow to a final OD_{600} of 0.5. This is further diluted 1/1000 and 200 μL is seeded into well of chambered coverglass and allowed to attach for 3-4 h. Following this, media is removed and replaced with fresh media. Biofilms are allowed to grow for desired time. Media, exogenous products or antibiotics can be added to the biofilms. Following growth, media is removed, biofilms are stained and confocal imaging is performed.

Figure 2. Representative images of biofilm development following exposure to sputum filtrates: A) Representative images of *Burkholderia cepacia* complex *(*BCC) clinical isolates following 48 h of growth in chambered coverglass with media alone (top panels) or in the presence of 10% sputum filtrates (lower panels) followed by staining with biofilm viability kit. 1 scale unit represent 19.68 μm. B-C) Average thickness of isolates (B) and dead:live ratio (C) of multiple images of BCC isolates grown for 48 h in slide chambers in the absence (white bars) or presence (black bars) of sputum filtrates . Each bar represents the mean of 45 images plotted with the standard error of the mean. **p<0.001 compared to control (media alone) using Kruskal-Wallis test. Figure adapted from Kennedy et al [16].

Figure 3. Representative images of antibiotic treatment on biofilms exposed to sputum filtrate: A) Images of *Burkholderia vietnamensis* clinical isolates following 48 h of growth in chambered coverglass with media alone (left) or in the presence of 10% sputum filtrates (right) with or without 1000 μg/mL of tobramycin. Biofilms were grown for 24 h in media alone or media supplemented with 10% (v/v) sputum filtrates. After 24 h, media was removed and replaced with media (+/- sputum) containing antibiotics. 1 scale unit represent 19.68 μm. B-C) Average thickness of isolates (B) and dead:live ratio (C) of multiple images (n=9) of *B. vietnamensis* isolates grown for 48 h in slide chambers in the absence or presence of sputum filtrates . Each bar represents the mean of 45 images plotted with the standard error of the mean. **p<0.001 compared to control (0 μg/mL) using Kruskal-Wallis test. Figure adapted from Kennedy et al.[16]

DISCUSSION:

The methods described herein allow for visualization of bacterial biofilms grown in the presence of exogenous products. Not surprisingly, the production of the exoproducts is of importance when using this type of system. For instance, Dithiothreitol (DTT), is often used on human sputum samples to help liquefy the samples. However, the effect of DTT alone can decrease biofilm development and viability (data not shown). Thus, proper controls for all

conditions are necessary. Furthermore, the addition of human sputum products creates inherent variability on the experiment due to the fact that the sputum of each patient has a unique microbiome. To adjust for this, we have used pooled sputum samples to avoid patient specific results. Additionally, the choice of appropriate media is important when using any model system. Standard media for biofilm growth of the intended organism are recommended for initial setup of the system. If the goal of the experiment is to identify how exogenous products are affecting biofilm formation, a nutrient rich media that provides adequate biofilm formation is suggested. 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. Other media have been used with good results in this system (data not shown). The attachment of the biofilms to the chambered coverglass is robust, however great care must be taken when removing/adding media or during washing steps of the biofilms to prevent disrupting the biofilms.

The use of the live-dead stain as per manufacturer's protocol has yielded good results for the system described. A number of factors can affect the fluorescence 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). Using bacterial counts to confirm biofilm viability in repeated experiments is recommended to confirm the visual observations of this model. Due to inherent heterogeneity of biofilms, multiple images from each chamber and multiple chambers should be used for each condition in an experiment. This adds to the cost and duration of these experiments.

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 better images. The thickness of the Z-stack can also affect overall image quality and level of detail. Having images taken every 0.5-1 μm 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.

These experiments are more costly and time consuming than other attachment assays, and cannot be done in a high throughput manner. In this procedure, bacteria attach to a borosilicate surface, which is not reflective of an in vivo condition and may affect biofilm formation and development. However, they provide additional information and can generate hypotheses for mechanism related to biofilm antibiotic resistance and physiological response to exogenous signal. If the goal of the experiment is to understand how biofilms change in response to different factors, rather than identifying anti-biofilm compounds using high-throughput methods for instance, the additional information gained using this technique makes the method worthwhile. Thus the current method is a significant advancement over previous limited attachment assays such as the crystal violet assay, which is most commonly used to

study biofilms.

Critical steps to this procedure include: 1) Ensuring timely processing of sputum samples to avoid degradation; 2) Being gentle with media changes on the biofilms to avoid disruption of the biofilm and 3) Doing repeated measurements of biofilm imaging to ensure an accurate representation of the biofilm thickness due to the heterogeneity of biofilm formation.

Once the system is set-up for a given bacterial species, one can test a number of different conditions, including the effects of exogenous factors on multiple clinical isolates. This system has been used to study the effect of antibiotics on biofilms exposed to human sputum (Kennedy et al.) and to compare highly resistant and intermediately resistant clinical isolates (Tom et al).[16,17] Modifications to the chambered coverglass, such as coating the bottom with mucin or collagen micro-scaffold (e.g. puracol), can further extend the range of experiments possible. It may be possible to adapt this system to study direct interactions, such as those between epithelial cells and bacteria, or between different bacterial species. This is an expansion of the model described by Jurcisek et al. [20] The method uses slide chamber growth similar to that described here 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. In addition, we add exogenous factors to test the effect of antibiotic on biofilms. This model thus has the potential to considerably further our understanding of bacterial biofilms in human disease.

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DISCLOSURES:
None.

REFERENCES:
1. Beaudoin, T. & Waters, V. Infections with biofilm formation: selection of antimicrobials and role of prolonged antibiotic therapy. *Pediatr.Infect.Dis.J.*, doi:10.1097/INF.0000000000001144 [doi] (2016).
2. Donlan, R.M. Biofilms: microbial life on surfaces. *Emerg.Infect.Dis.* **8** (9), 881-890, doi:10.3201/eid0809.020063 (2002).
3. Hobley, L., Harkins, C., MacPhee, C.E. & Stanley-Wall, N.R. Giving structure to the biofilm matrix: an overview of individual strategies and emerging common themes. *FEMS Microbiol.Rev.* **39** (5), 649-669, doi:10.1093/femsre/fuv015 (2015).
4. Katharios-Lanwermeyer, S., Xi, C., Jakubovics, N.S. & Rickard, A.H. Mini-review: Microbial coaggregation: ubiquity and implications for biofilm development. *Biofouling.* **30** (10), 1235-1251, doi:10.1080/08927014.2014.976206 (2014).
5. Donlan, R.M. Biofilm formation: a clinically relevant microbiological process. *Clin.Infect.Dis.* **33** (8), 1387-1392, doi:CID010382 (2001).
6. Bjarnsholt, T.*, et al*. The in vivo biofilm. *Trends Microbiol.* **21** (9), 466-474, doi:10.1016/j.tim.2013.06.002 (2013).
7. Mah, T.F., Pitts, B., Pellock, B., Walker, G.C., Stewart, P.S. & O'Toole, G.A. A genetic basis for

Pseudomonas aeruginosa biofilm antibiotic resistance. *Nature.* **426** (6964), 306-310, doi:10.1038/nature02122 (2003).
8. Mah, T.F. Biofilm-specific antibiotic resistance. *Future Microbiol.* **7** (9), 1061-1072, doi:10.2217/fmb.12.76; 10.2217/fmb.12.76 (2012).
9. Beaudoin, T., Zhang, L., Hinz, A.J., Parr, C.J. & Mah, T.F. The biofilm-specific antibiotic resistance gene ndvB is important for expression of ethanol oxidation genes in Pseudomonas aeruginosa biofilms. *J.Bacteriol.* **194** (12), 3128-3136, doi:10.1128/JB.06178-11; 10.1128/JB.06178-11 (2012).
10. Beaudoin, T., Aaron, S.D., Giesbrecht-Lewis, T., Vandemheen, K. & Mah, T.F. Characterization of clonal strains of Pseudomonas aeruginosa isolated from cystic fibrosis patients in Ontario, Canada. *Can.J.Microbiol.* **56** (7), 548-557, doi:10.1139/w10-043; 10.1139/w10-043 (2010).
11. Vidya, P.*, et al*. Chronic infection phenotypes of Pseudomonas aeruginosa are associated with failure of eradication in children with cystic fibrosis. *Eur.J.Clin.Microbiol.Infect.Dis.*, doi:10.1007/s10096-015-2509-4 (2015).
12. Beaudoin, T., Lafayette, S., Nguyen, D. & Rousseau, S. Mucoid Pseudomonas aeruginosa caused by mucA mutations result in activation of TLR2 in addition to TLR5 in airway epithelial cells. *Biochem.Biophys.Res.Commun.* **428** (1), 150-154, doi:10.1016/j.bbrc.2012.10.030; 10.1016/j.bbrc.2012.10.030 (2012).
13. Beaudoin, T.*, et al*. The level of p38alpha mitogen-activated protein kinase activation in airway epithelial cells determines the onset of innate immune responses to planktonic and biofilm Pseudomonas aeruginosa. *J.Infect.Dis.* **207** (10), 1544-1555, doi:10.1093/infdis/jit059 (2013).
14. LaFayette, S.L.*, et al*. Cystic fibrosis-adapted quorum sensing mutants cause hyperinflammatory responses. *Sci.Adv.* **1** (6), e1500199. Epub 2015 Jul 31, doi:e1500199 (2015).
15. Staudinger, B.J.*, et al*. Conditions associated with the cystic fibrosis defect promote chronic Pseudomonas aeruginosa infection. *Am.J.Respir.Crit.Care Med.* **189** (7), 812-824, doi:10.1164/rccm.201312-2142OC (2014).
16. Kennedy, S.*, et al*. Activity of Tobramycin against Cystic Fibrosis Isolates of Burkholderia cepacia Complex Grown as Biofilms. *Antimicrob.Agents Chemother.* **60** (1), 348-355, doi:10.1128/AAC.02068-15 (2015).
17. Tom, S.K., Yau, Y.C., Beaudoin, T., LiPuma, J.J. & Waters, V. Effect of High-Dose Antimicrobials on Biofilm Growth of Achromobacter Species Isolated from Cystic Fibrosis Patients. *Antimicrob.Agents Chemother.* **60** (1), 650-652, doi:10.1128/AAC.02240-15 (2015).
18. Heydorn, A., et al. Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology, **146** (Pt 10): p. 2395-407, doi:10.1099/00221287-146-10-2395 (2000).
19. Vorregaard, M. Comstat2 - a modern 3D image analysis environment for biofilms, in Informatics and Mathematical Modelling. *Technical University of Denmark: Kongens Lyngby,* Denmark (2008).
20. Jurcisek, J. A., Dickson, A. C., Bruggeman, M. E., Bakaletz, L. O. *In vitro* Biofilm Formation in an 8-well Chamber Slide. *J. Vis. Exp.* **47**, e2481, doi:10.3791/2481 (2011).

Figure 1

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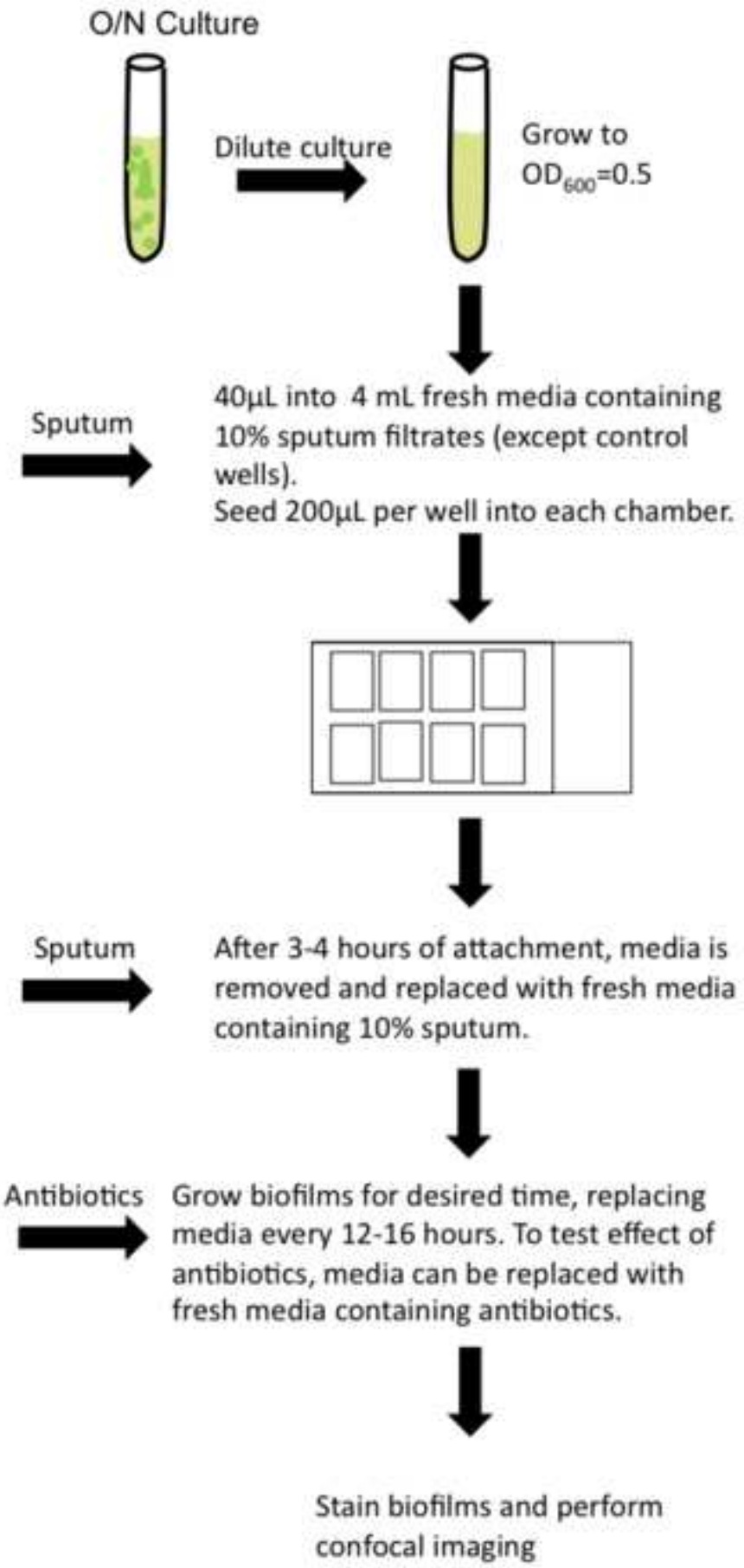

Figure

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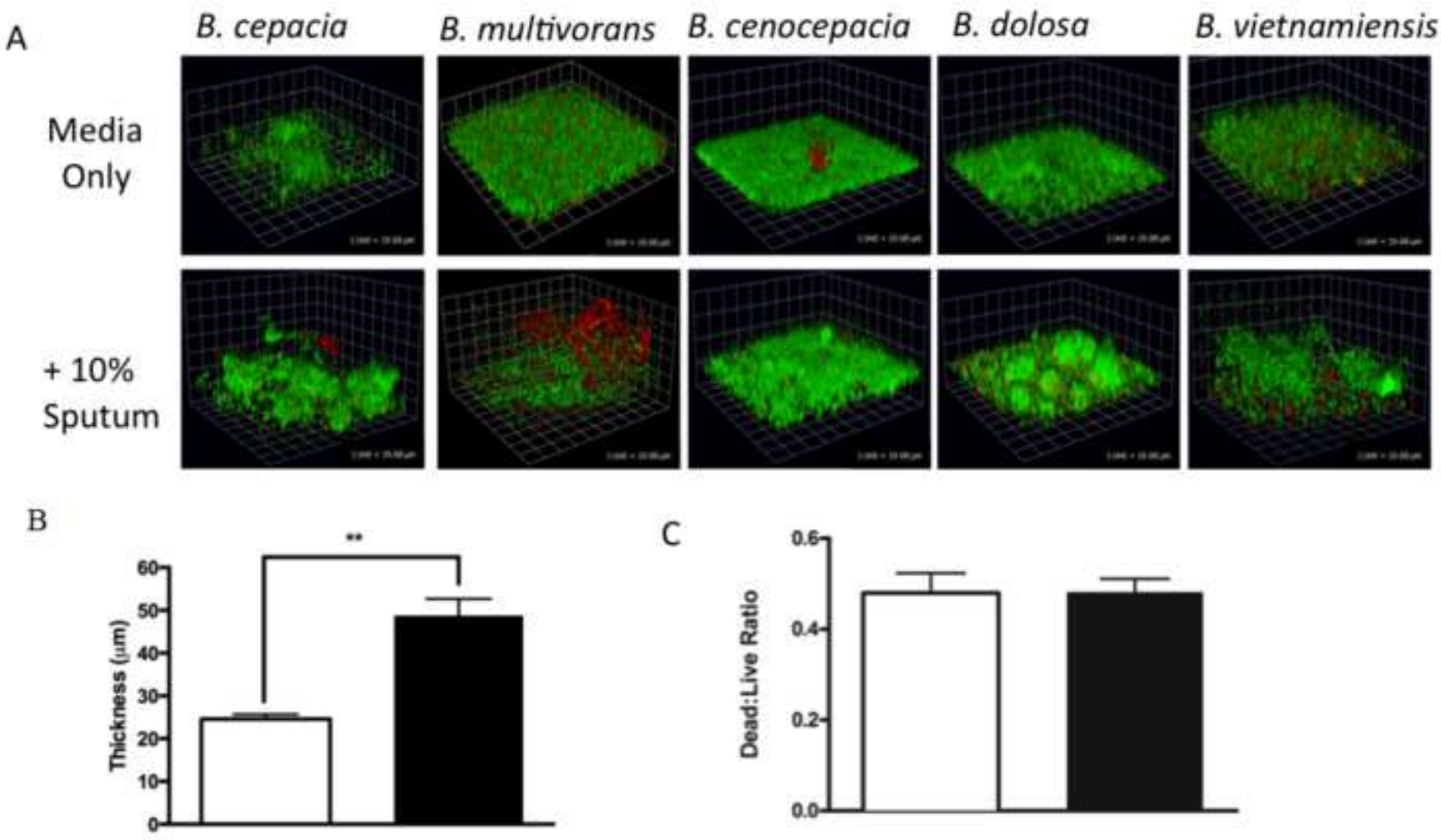
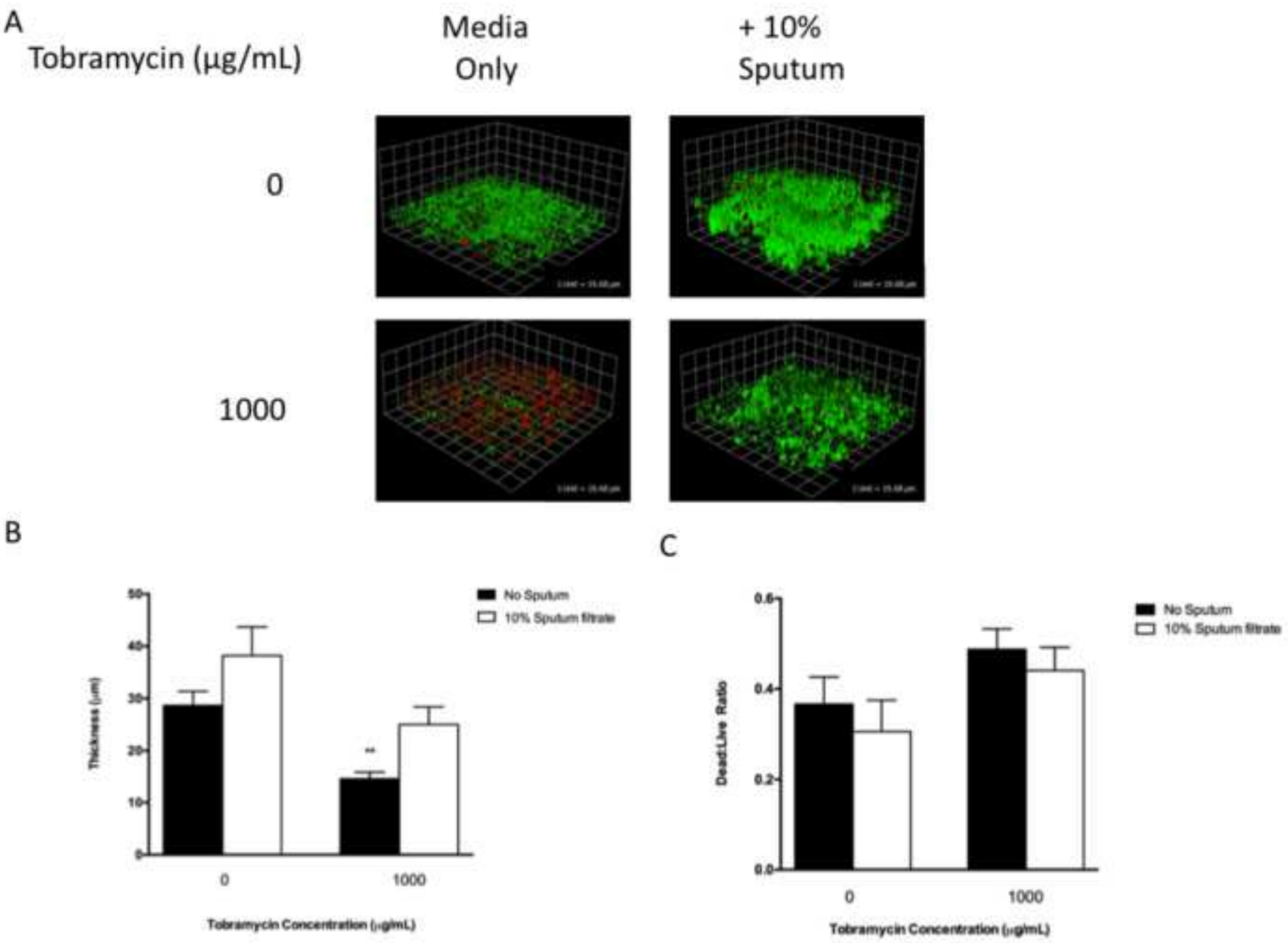


Figure 3



Excel Spreadsheet- Table of Materials/Equipment

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Name of Reagent/ Equipment	Company	Catalog Number
Lab-Tek II Chambered coverglass, #1.5 borosilicate, 8-well	Thermo Sicher Scientific	155409
Filmtracer Live/Dead Biofilm Viabilty Kit	Thermo Fisher Scientific	L10316
Blood agar plates	Thermo Fisher Scientific	R10215
COMSTAT	Availble software online	
Millers LB Broth	Thermo Fisher Scientific	12780-052
Millex-GV Syringe Filters	Millipore	SLGV013SL
Phosphate Buffered Saline (Dulbecco A)	Oxoid	BR0014G
Zeiss AxioVert 200M	Carl Zeiss	
Hamamatsu C9100-13 EM-CCD	QS Technologies Inc.	
Spectral Borealis	Qs Technologies Inc.	
Perkin Elmer Volocity	QS Technologies Inc.	

Comments/Description

Confirming viability via CFU counts or selecting colonies for innoculation

COMSTAT is software to analyze biofilm images. Available **www.comstat.dk**

Standard media for overnight gowth/biofilm growth

Filtering of sputum supernants

Washing of biofilm chambers after media removal

Instructions for this software can be found at: http://cellularimaging.perkinelmer.com/pdfs/manuals/VolocityuserGuide.pdf

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Signature:	
Date:	March 30, 2016

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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).