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Visualization of Pseudomonas aeruginosa within the Sputum of Cystic Fibrosis Patients

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TITLE:

Visualization of *Pseudomonas aeruginosa* within the Sputum of Cystic Fibrosis Patients

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KEYWORDS:

sputum, *Pseudomonas aeruginosa*, cystic fibrosis, confocal microscopy, Psl, antibodies, tissue clearing, Fluorescence in situ hybridization

SUMMARY:

This protocol provides methods for visualization of bacterial cells and polysaccharide synthesis locus (Psl) polysaccharide within the sputum of cystic fibrosis patients.

ABSTRACT:

Early detection and eradication of *Pseudomonas aeruginosa* within the lungs of cystic fibrosis patients can reduce the chance of developing chronic infection. The development of chronic *P. aeruginosa* infections is associated with a decline in lung function and increased morbidity. Therefore, there is a great interest in elucidating the reasons for the failure to eradicate *P. aeruginosa* with antibiotic therapy which occurs in approximately 10-40% of pediatric patients. One of many factors that can affect host clearance of *P. aeruginosa* and antibiotic susceptibility is variations in spatial organization (such as aggregation or biofilm formation) and polysaccharide production. Therefore, we were interested in visualizing the in situ characteristics of *P. aeruginosa* within the sputum of CF patients. A tissue clearing technique was applied to sputum samples after embedding the samples into a hydrogel matrix to retain the 3D structures relative to host cells. After tissue clearing, fluorescent labels and dyes were

added to allow visualization. Fluorescence in situ hybridization was performed for the visualization of bacterial cells, binding of fluorescently labeled anti-Psl-antibodies for the visualization of the exopolysaccharide and DAPI staining to stain host cells to obtain structural insight. These methods allowed for the high-resolution imaging of *P. aeruginosa* within the sputum of CF patients via confocal laser scanning microscopy.

INTRODUCTION:

In this study, experiments were designed to visualize the in vivo structure of *Pseudomonas aeruginosa* within the sputum of pediatric cystic fibrosis (CF) patients. *P. aeruginosa* infections becomes chronic in 30-40% of the pediatric CF population; once chronic infections become established, they are almost impossible to eliminate[1]. *P. aeruginosa* isolates from patients with early infection are generally more susceptible to antimicrobials, therefore, these are treated with anti-pseudomonal antibiotics to prevent the establishment of chronic infection[2]. Unfortunately, not all *P. aeruginosa* isolates are effectively cleared from the lung following antibiotic therapy. The precise mechanisms associated with antibiotic failure have not been fully elucidated. Previous studies have shown that variations in biofilm cell density, aggregation, and polysaccharide production can affect antibiotic efficacy[3]. *P. aeruginosa* produces three extracellular polysaccharides: Pel, Psl, and alginate[4]. Most strains of *P. aeruginosa* have the genetic capacity to express each of the exopolysaccharides, though often one type of polysaccharide is expressed predominantly[5]. The exopolysaccharide alginate is associated with chronic infections in the CF lung, resulting in a mucoid phenotype[6,7]. The polysaccharides Pel and Psl have multiple functions including aiding initial attachment and the maintenance of biofilm structure, and conferring antibiotic resistance[8].

Methods aimed at visualizing in vivo structures of tissues have been developed for a variety of sample types[9–11]. More recently, they have been tailored to visualize in vivo microbial communities within sputum from CF patients[12]. The optimization of a tissue clearing protocol specifically for the identification of microbial communities within sputum was developed by DePas et al., 2016[12]. The term MiPACT, which stands for *m*icrobial *i*dentification after *Pa*ssive *C*LARITY *t*echnique was coined for the clearing of CF sputum[11,12]. For tissue clearing techniques, the specimens are first fixed, then rendered transparent while leaving their inherent architecture intact for staining and microscopic visualization[11]. Fixing and clearing CF sputum samples allow researchers to answer questions related to biofilm structure, bacterial cell density, polymicrobial associations, and associations between pathogens and host cells. The advantage of directly examining bacteria which have been preserved within the sputum is that they can be analyzed and visualized in a host-specific context. Although in vitro growth of clinical isolates in the laboratory for experimentation can be very informative, such methods are unable to fully recreate the CF lung environment, resulting in a disconnect between laboratory results and patient outcomes.

The methods presented here can be used to fix and clear sputum to visualize bacteria, whether from CF patients or patients with other respiratory infections. The specific type of staining and microscopic analysis described herein is fluorescence in situ hybridization (FISH), followed by anti-Psl-antibody binding within the hydrogel, and subsequent analysis via confocal laser

scanning microscopy (CLSM). Following tissue clearing, other immunohistochemistry and microscopy methods can also be applied.

PROTOCOL:

Research Ethics Board (REB) approval is required to collect and store sputum samples from human subjects. Studies presented herein were approved by the Hospital for Sick Children REB#1000058579.

1. Sputum Collection

1.2. Store expectorated sputum in a sterile collection cup and immediately store at 4 °C for a maximum of 24 h prior to the fixation.

NOTE: Leaving the sputum too long at 4 °C without fixation can lead to cellular degradation, particularly degradation of white blood cells. Fixation as soon as possible is preferred.

1.3. Transfer the sputum samples to a sterile 15 mL tube.

1.4. Add an equal volume of 4% paraformaldehyde (PFA) to the sputum samples. For example, if the sputum sample is 0.5 mL, add 0.5 mL of 4% PFA. Mix by gentle inversion.

1.5. Incubate the sputum overnight at 4 °C.

1.6. Wash the sample by adding 5 mL of phosphate buffered saline (PBS) to each 2 mL of fixed sputum.

NOTE: There is no centrifugation required for this washing step.

1.7. Carefully remove the supernatant with a pipette.

NOTE: Avoid sucking up the sputum with the pipette by pointing the tip away from the sputum and very slowly aspirate off the surface liquid. The washing step does not involve centrifugation to not disturb the structural integrity of the sputum plugs.

1.8. Repeat steps 1.6-1.7 two more times.

1.9. Resuspend the pellet in 2x the volume of PBS with 0.01 % (w/v) sodium azide.

1.10. Store the sputum at 4 °C.

2. MiPACT (tissue clearing technique) processing of sputum

2.1. Degas the hydrogel components (30% 29:1 acrylamide:bis-acrylamide, hardner, and PBS) in a sealed container containing an anaerobic pack prior for 72 h.

NOTE: The anaerobic pack and sealed container are necessary because oxygen inhibits acrylamide polymerization. Alternatively, oxygen can be removed by performing this step in an anaerobic hood, by applying a vacuum to a sealed container, or by bubbling N_2 gas through the acrylamide mixture.

2.1.1. Add 2 mL of a 30% 29:1 acrylamide:bis-acrylamide solution to a 15 mL tube with the cap off inside the sealed anaerobic container.

2.2.2. Make a concentrated stock of 10% (w/v) hardener by adding 0.5 g of hardener to 5 mL of PBS in a 15 mL tube. Leave the cap off the tube and store in the sealed anaerobic container.

2.2.3. Leave a few mL of sterile PBS in a tube, with the cap off, inside the anaerobic container.

2.2. Make 5 mL solution of hydrogel with a final concentration of 0.2% hardener and 4% 29:1 acrylamide:bis-acrylamide in PBS in a 15 mL tube. Mix by inversion and filter sterilize.

2.3. Remove sputum samples from the fridge, then cut into small sections (roughly 5 mm in diameter) under sterile conditions with a scalpel.

NOTE: If the sputum is quite fluid then this step can still be performed, however, it takes increased patience and practice to remove the sputum from the storage solution with tweezers prior to using the scalpel to separate the desired fractions.

2.4. Place the cut sputum samples inside a well of an 8 chambered coverglass slide.

2.5. Add 300 µL of filter sterilized hydrogel solution from step 2.2 to each of the wells that contain sputum.

2.6. Place the 8 chambered coverglass inside a sealed container containing an anaerobic pack for 3 h at 37 °C.

NOTE: Once polymerized the hydrogel with embedded sputum should be the consistency of firm gel.

2.7. Transfer the solidified hydrogel sputum samples to a 15 mL culture tube containing 5 mL of 8% sodium dodecyl sulfate (SDS), pH 8, and allow samples to clear for 3-14 days at 37 °C (with or without shaking), until the sputum becomes transparent.

NOTE: Clearing times depend on sputum composition and can generally be decreased with shaking. However, take care to not increase the shaking speed to the point of disrupting sample integrity. More DNA-rich samples take longer to clear compared to mucus rich samples.

2.8. Decant the 8% SDS solution into a waste collection container. Using sterile tweezers transfer each hydrogel-embedded sample to a sterile 50 mL conical tube. Add 10 mL of PBS to each of the 50 mL conical tubes to wash the hydrogels and let the solution sit for 30 min to 1 h before decanting. Repeat this 2x more times.

NOTE: This washing step does not involve centrifugation. Excess fluid and supernatant are carefully removed with a pipette.

2.9. Store washed samples in PBS with 0.01% (w/v) sodium azide and 1x RNase inhibitor at 4 °C.

3. Hydrogel fluorescent in situ hybridization (FISH) protocol

3.1. Remove the hydrogel samples from their storage solution using sterile tweezers and place the samples on a sterile surface (such as a glass slide or Petri dish).

3.2. Using a sterile scalpel cut the hydrogels into ~ 1 mm thick slices.

3.3. Place the 1 mm sections of hydrogel inside sterile 1.5 mL tubes.

3.4. Prepare 1 mL the hybridization buffer (25% formamide, 0.9 M NaCl, 20 mM Tris-HCl [pH 7.6], 0.01% SDS, purified and deionized H_2O) in a 1.5 mL tube.

3.5. Add the fluorescently labeled PseaerA probe (150.7 nM[12]) to the hybridization buffer and mix by inversion.

NOTE: Formalin solution should be kept away from an open flame. The hybridization buffer can be prepared in advanced and stored in aliquots at -20 °C[13].

3.6. Add 200-500 µL of hybridization buffer to each ~1 mm section of hydrogel and ensure the entirety of the hydrogel sample is submerged.

3.7. Allow the PseaerA probe to hybridize with the hydrogel samples by placing them in the dark for ~ 18-24 h, at 46 °C, without shaking.

3.8. Decant the hybridization buffer into a waste collection container.

3.9. Rinse samples once with filter sterilized wash buffer (337.5 mM NaCl, 20 mM Tris-HCl, 5 mM EDTA (Ethylenediaminetetraacetic acid) [pH 7.2], 0.01% SDS, and purified and deionized H_2O) by adding 1 mL of wash buffer to each of the 1.5 mL tubes and then remove it.

NOTE: The wash buffer can be made in advance and stored at room temperature (RT).

3.10. Add 1 mL of fresh wash buffer to the tubes, then incubate the samples in the dark for 6 h at 48 °C, without shaking.

4. Hydrogel and Psl0096 antibody binding

4.1. Sterilely remove the wash buffer using a 1 mL pipettor.

4.2. Rinse the samples with a 2% BSA (w/v) in PBS solution by adding and removing 1 mL of the 2% BSA solution.

4.3. Add 500 µL of the 2% BSA/PBS solution to the hydrogel samples to block non-specific protein binding. Then incubate the samples overnight, in the dark, at RT, without shaking.

4.4. Sterilely remove the blocking solution using a 1 mL pipette.

4.5. Prepare the Psl0096-Texas Red antibody solution by diluting the antibody to a final concentration of 0.112 µg/mL in 500 µL of fresh 2% BSA/PBS.

4.6. Add the 500 µL antibody solution to the hydrogel samples and incubate them at RT for 6 h, protected from light, without shaking.

5. DAPI (4',6'-diamidino-2-phenylindole) staining

5.1. Prepare the refractive index matching solution (RIMS) by adding 40 g of non-ionic density gradient medium, 30 µL of Tween20, 3 µg sodium azide, and 30 mL of PBS to a flask containing a magnetic stir bar. Stir the solution for 15 min on a magnetic stirrer, or until completely dissolved.

5.2. Filter sterilize the solution into a 50 mL conical tube using a 10 mL syringe and a sterile 0.2 µm filter.

NOTE: The solution can be stored at 4 °C for several months.

5.3. Remove the Psl0096-Texas Red antibody solution with a sterile 1 mL pipette.

5.4. Rinse the hydrogel samples by adding and then removing 1 mL of PBS.

5.5. Incubate the hydrogel samples with 250 µL of RIMS solution and 10 µg/mL of DAPI at RT with gentle shaking, in the dark, overnight.

5.6. Prior to confocal imaging mount the samples onto 0.9 mm or 1.7 mm perfusion chambers and seal with a glass coverslip.

NOTE: After FISH and/or immunohistochemistry and before immersion in RIMS, fluorescent lectin stains can be applied if visualization of sputum mucous is desired[12].

6. Imaging

6.1. Perform confocal laser scanning microscopy imaging using standard techniques at 25x, 40x, 63x, or 100x magnifications.

REPRESENTATIVE RESULTS:

The overall design of the experiment is summarized in **Figure 1** and **Figure 2**. **Figure 1** provides a summary of the sputum processing and sputum clearing protocols. Sputum processing and clearing may take up to 17 days. Though, the protocol may be stopped, and samples can be stored after fixation with PFA (day 2) or following tissue clearing (days 5-17 depending on clearing time). In **Figure 2**, the FISH and antibody binding protocols are summarized. The FISH and antibody staining protocols take 4 days to complete but should be completed and confocal images taken once started. Using the above protocols, high resolution 3D images of *P. aeruginosa* cells can be obtained with their in-situ structure within sputum visualized.

The clearing of sputum and subsequent application of fluorescent stains used in this protocol allows for detailed visualization of *P. aeruginosa* within the samples. The sputum samples shown in **Figure 3** and **Figure 4** were collected from a pediatric CF patient (17 years old) with a new-onset *P. aeruginosa* infection. In **Figure 3A**, an aggregate of cells was seen within a sputum sample; the appearance of yellowish-green rods was due to the overlap of all three fluorophores. Although we do not have cell counts for this sputum sample, we found the probe detection limit to be 10^4 cells/mL (see **Supplemental Figure 1**). In **Figure 3B**, individual rod-shapes were seen in green from the species-specific binding of the PseaerA-488 probe to *P. aeruginosa* cells. The Psl0096-Texas Red antibody seen in red illustrates where the pseudomonal exopolysaccharide was located within the sputum; in this case the Psl0096-antibody appeared to overlap mostly with the *P. aeruginosa* cells (**Figure 3C**). This method also allowed for the visualization of pseudomonal cells within the sputum in relation with other bacterial cells and host structures. In **Figure 4**, a cluster of *P. aeruginosa* cells was seen phagocytosed within a eukaryotic cell and other small coccal cell clusters were observed in the vicinity. It has been demonstrated that the Psl0096 antibody can also bind to Psl produced form planktonic *P. aeruginosa* (see **Supplemental Figure 2**).

FIGURE AND TABLE LEGENDS:

Figure 1: Flow diagram illustrating the sputum processing and MiPACT protocols. In

summary, sputum is fixed prior to being embedded within a polyacrylamide solution. Once cleared the hydrogel can either be stored at 4 ˚C or used with the FISH and antibody binding protocol. This image was created with BioRender.com.

Figure 2: Flow diagram denoting the fluorescence in situ hybridization and antibody staining protocols. Once the sputum has completely cleared within the hydrogel matrix staining methods can be applied. This image was created with BioRender.com.

Figure 3: Immunofluorescence image of a sputum sample collected from a patient with an early *P. aeruginosa* infection embedded into a hydrogel matrix. The hydrogel sample was hybridized with a PsearA-Alexa488 probe (green), a Psl0096-Texas Red antibody (red), and DAPI (blue). (**A**) sputum sample viewed under all 3 channels, (**B**) sputum under only the green channel indicating where the PseaerA-488 probe bound, and (**C**) sputum under only the red channel where the Psl0096-Texas red binding occurred. Images were taken at 100x magnification.

Figure 4: Immunofluorescence image of a sputum sample collected from a patient with an early *P. aeruginosa* infection embedded into a hydrogel matrix. The hydrogel sample was hybridized with a PsearA-Alexa488 probe (green), a Psl0096-Texas Red antibody (red), and DAPI (blue).

Supplementary Figure 1: Fluorescence *in situ* hybridization of a planktonic culture of PAO1 with the PseaerA-488 probe.

Supplementary Figure 2: *P. aeruginosa* stained with DAPI and the Psl0096-Texas Red antibody. (A) Strain PAO1-Δpsl, and (B) strain PAO1.

DISCUSSION:

The purpose of this protocol is to allow a glimpse into the in-situ organization of *P. aeruginosa* cells in sputum from CF patients. Sputum samples should be stored at 4 °C until processed if they cannot be immediately fixed. It has been demonstrated that *P. aeruginosa* cell numbers in sputum do not change significantly if processed at 1 h, 24 h, or 48 h, when stored at 4 °C, though if left at 25 °C for 24 or 48 h, bacterial cell counts will significantly increase as a result of bacterial growth[14]. For this study, sputum samples were stored at 4 °C up to a maximum of 24 h after expectoration. It should be noted that inflammatory cell counts have been shown to decrease in sputum if left at 4 °C and processed more than 9 h later[15]. Therefore, it is important to consider the specific cells and markers one wishes to visualize in sputum when deciding on a cut-off time for sample processing.

Sample processing in this method begins with the fixation of sputum samples in 4 % PFA. Paraformaldehyde will cross-link bacterial cells and their extracellular matrix, preserving their structures for microscopic visualization and analysis[11,16]. Unfortunately, if the aim is to get total cell count on certain inflammatory cells in sputum, PFA has been shown to decrease the counts of these cells thus other fixatives should be considered[17]. Another limitation of this

study is that it can be time consuming and may take over two weeks to perform. Thus, it may not be suitable for development into a diagnostic method requiring time sensitive treatment decisions. Furthermore, for understanding the total microbial diversity within sputum samples, this method would not be suitable, but could be paired with other high-throughput microbial detection methods such a qPCR.

The composition of the acrylamide hydrogel can be altered depending on tissue type and application[11]. For unstable specimens like CF sputum, it is necessary to provide structural support with a 29:1 acrylamide:bis-acrylamide mixture (instead of just acrylamide). Including paraformaldehyde in the hydrogel can further stabilize the structure, with the trade-off of longer incubation times to allow diffusion of probes and antibodies[9]. Adding formaldehyde to the hydrogel can also prevent tissue swelling during the clearing process if that effect is undesirable[11].

The current method specifically targets *P. aeruginosa* cells within the sputum of CF patients. Alternative methods and modifications to this protocol can be considered to guide optimization of visualization of other bacteria. By applying species and genus-specific FISH and hybridization chain reaction (HCR) probes, other CF pathogens such as *Staphylococcus aureus*, *Streptococcus* sp., and *Achromobacter xylosoxidans* can be identified[12]. In our study, we targeted the pseudomonal exopolysaccharide Psl. Other targets, including alginate or Pel can be examined with fluorescent antibodies specific for these exopolysaccharides in future experiments. Applying the MiPACT method along with FISH and antibody staining for CLSM takes a couple weeks to complete. If the research question does not concern the 3-dimensional spatial visualization of the sputum, there are more rapid methods to visualize bacteria present. Previous methods used to visualize bacteria within sputum samples utilize thin sectioning or smearing and include: FISH[18], Gram stain, and immunohistochemistry techniques that apply primary antibodies and counterstains to allow the visualization of biofilm exopolysaccharides and bacterial cells[19, 20].

There are several potential future applications of these types of imaging techniques. The ability to visualize different bacterial organisms and their interaction with host cells, such as phagocytes, may further our understanding of why some *P. aeruginosa* strains are effectively cleared from CF airways whereas other strains are not. Imaging bacteria within respiratory specimens may also be used as a measure of antimicrobial efficacy and as a study outcome for new anti-biofilm drugs[21]. In addition, visualizing the spatial relationship between *P. aeruginosa* and other organisms within the CF lung microbiome, such as *Staphylococcus aureus*, may help to elucidate the role of co-infection/colonization in the pathogenesis of pulmonary exacerbations, and their response to antibiotic treatment. *In vivo* imaging of bacteria can be applied to other infections as well, including those with ventilator-associated pneumonias or chronic wound infections[22]. The insights gained can thus be used to guide future therapeutic development.

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DISCLOSURES:

None.

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Figure 1

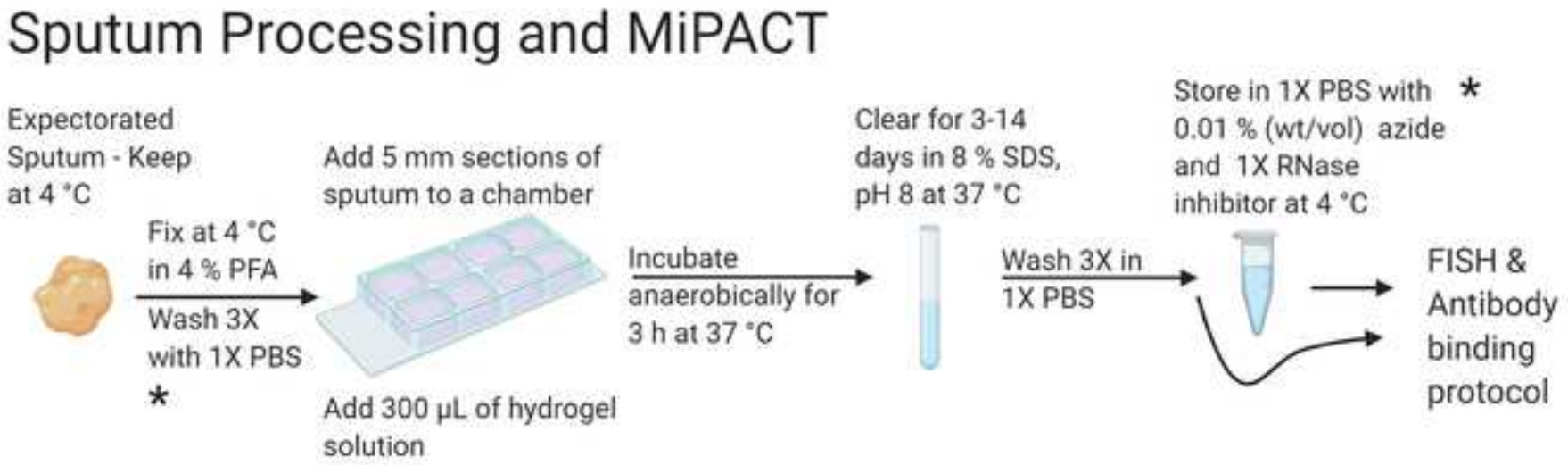

Figure 2

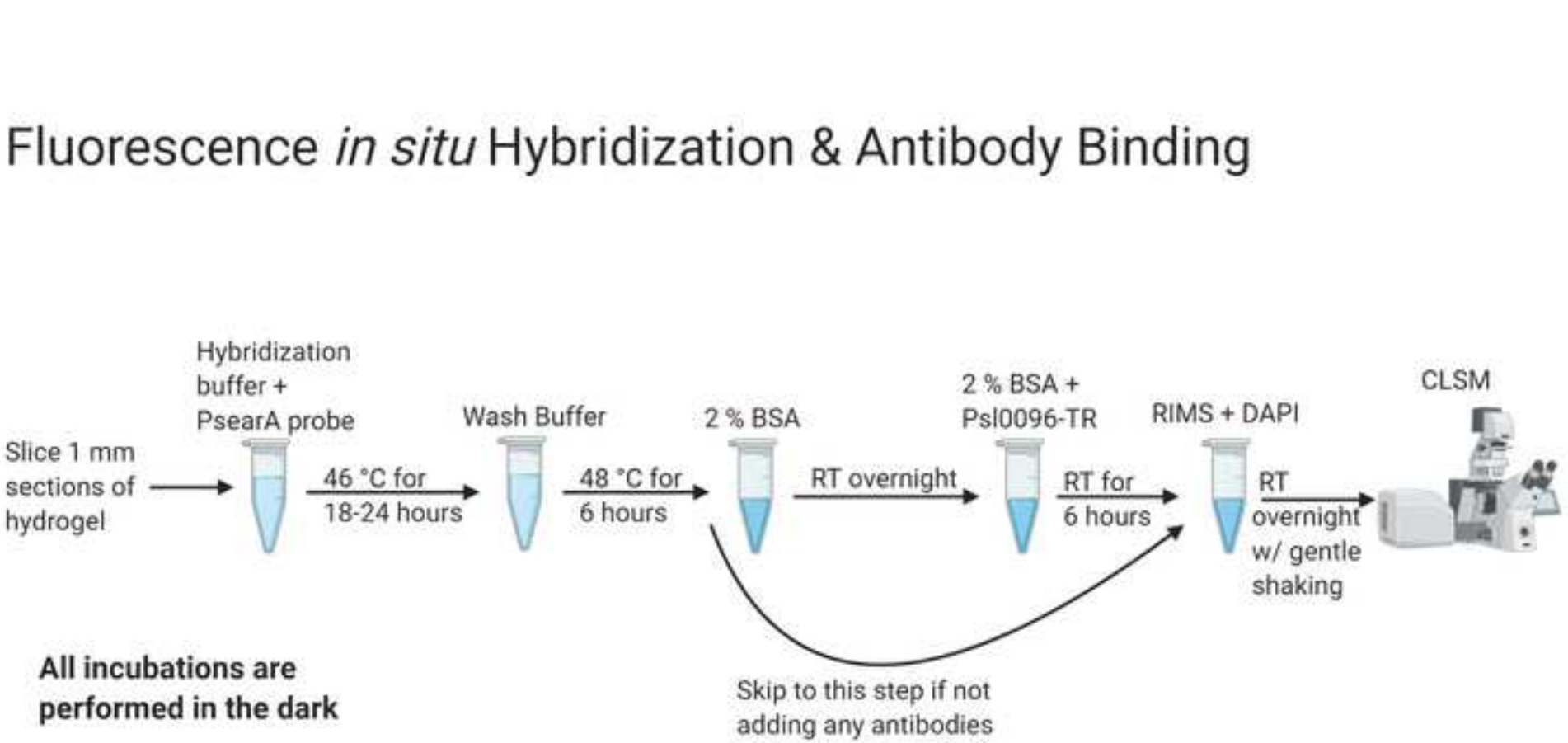

Figure 3

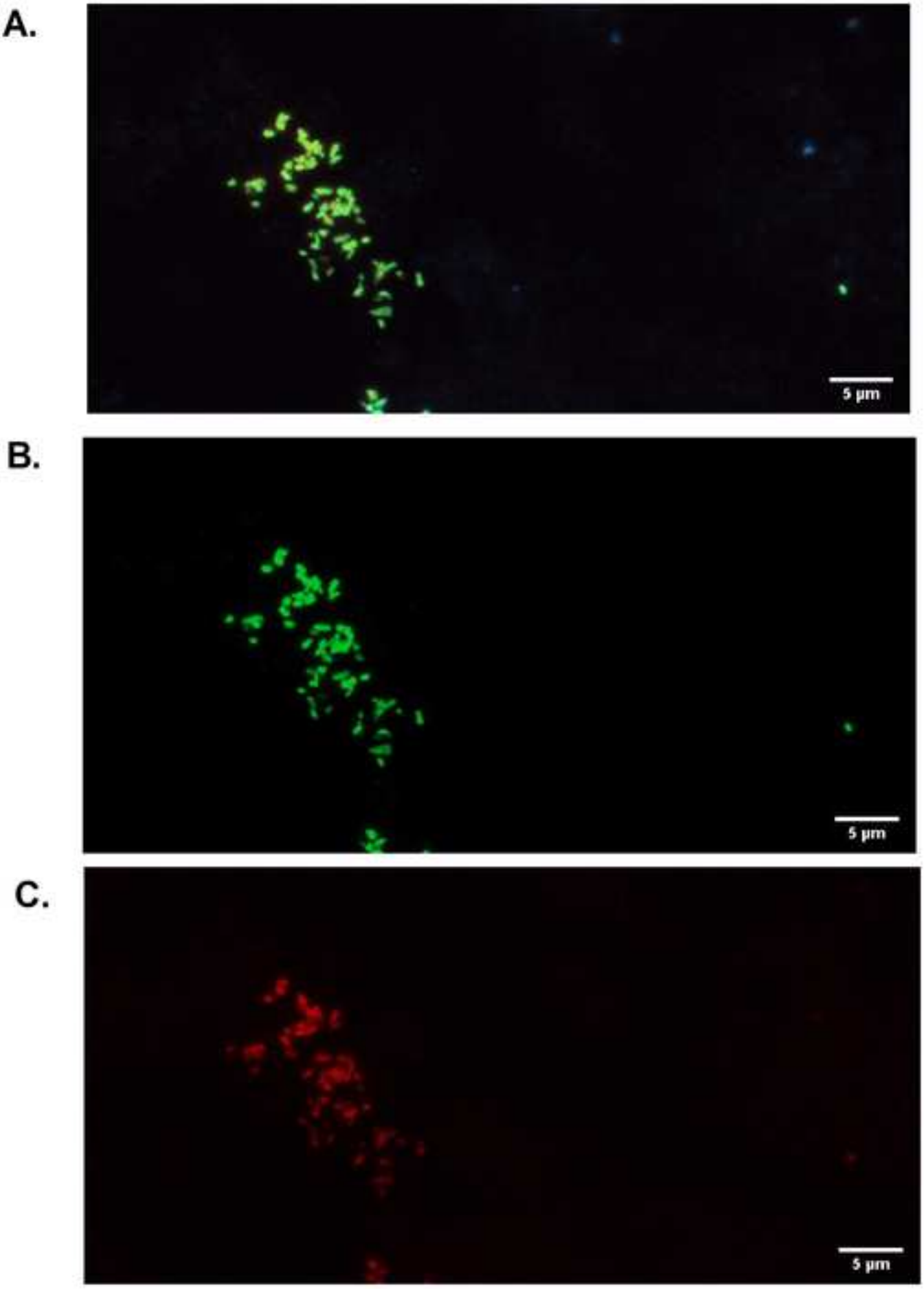

Figure 4

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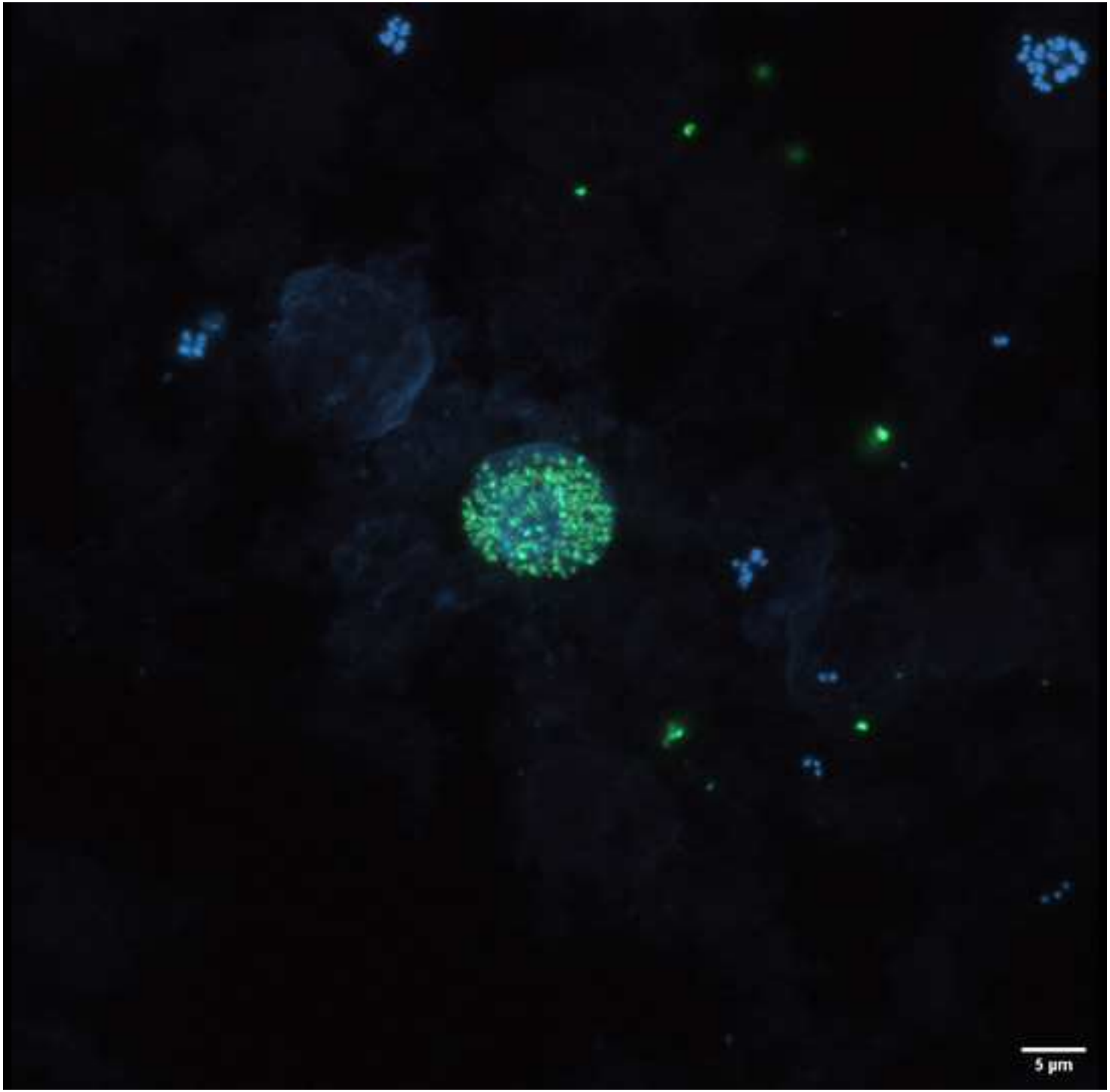

Name of Material/ Equipment	Company	Catalog Number
29:1 acrylamide bisacrylamide, 30 % solution	BioRad	161-0146
8-Chambered Coverglass Nunc Lab-Tek	ThermoFischer Scientific	155411
Anaerogen2.5L	Oxid Inc.	35108
Coverwell perfusion chambers	Electron Microscopry Sciences	70326 -12/-14
HistoDenz	Sigma	D2158
Protect RNA Rnase Inhibitor	Sigma	R7387
PseaerA - GGTAACCGTCCCCCTTGC	Eurofins	
Psl0096-Texas Red	Medimmune	
VA-044 Hardener	Wako	27776-21-21

Comments/Description

Order Details: Product: Modified DNA Oligo; Name: PseaerA; Sequence: [Alexa488]GGTAACCGTCCCCCTTGC; Synthesis: 50 nmol; Purificatior
The Psl0096-Texas red antibodies were a gift kindly provided by Medimmune and the company should be contacted for order inquiries.

ı: HPLC; Ship state: Full yield (dry)

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8. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.
9. Please ensure you answer the "how" question, i.e., how is the step performed?
10. 1.4, 1.5, 2.8: How do you perform the wash? Do you centrifuge- what speed, time and temp? Do you rinse? Please include all actions.
11. 1.7: Please check the step numbers.
12. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
13. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
14. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols.
15. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
16. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the table in alphabetical order.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This is a very nice addition to the literature and describes experimental details on how to image Pseudomonas in sputum. Although it is claimed that biofilms are imaged, what is imaged is small clusters of Pseudomonas. No evidence is provided that the clusters are biofilms. This is an over statement and should be modified to imaging Pseudomonas in sputum.

Major Concerns:
Although it is claimed that biofilms are imaged, what is imaged is small clusters of Pseudomonas. No evidence is provided that the clusters are biofilms. This is an over statement and should be modified to imaging Pseudomonas in sputum.

Thank you for pointing out this concern. We have now removed the inaccurate description of biofilms throughout the text (Abstract, Intro, Discussion). Instead we mention visualization of P. aeruginosa, bacterial cells, or P. aeruginosa cells and/or aggregates.

See new lines: 30, 40-42, 44, 47, 49, 53, 69, 81, 299, 300, 306, 307, 309, 351, 394, 400

Minor Concerns:
There are a number of minor mistakes that should be corrected, and a few issues to be clarified. These include:

Line 121- 1.7 Repeat steps 2.4 & 2.5 two more times. I think the authors mean steps 1.4 to 1.6.

Fixed. See new line – 128.

Line 123 - Resuspend the supernatant in 2 mL of PBS with 123 0.01 % (wt/vol) sodium azide. Supernatant?? Don't you mean the sputum pellet?

Yes; changed to pellet. Thank you. See new line – 130.

Line125 - Divide the sputum samples into 0.5 mL aliquots and store in sterile 1.5 mL eppendorf
126 tubes at 4°C. Will this disrupt the architecture of the sputum? CF sputum is thick and sticky. Please expand on this point.

It is unnecessary to aliquot the sputum at this stage and a more detailed description of carefully cutting the sputum into small sections is provided in step 2.3 and the note following step 2.3, thus I removed the mention of aliquoting sputum here. See new line 133.

Line 130 - Degas the hydrogel components in a sealed container containing an anaerobic pack
prior for 72 hrs. Should describe the hydrogel components first.

Thank you. I added the hydrogel components that are described in the substeps of 2.1 in brackets to be more descriptive and ensure better understanding of what is meant by hydrogel components here. See new line 138-139.

Line 157-do you mean Hydrogel or polyacrylamide solution?

I mean the hydrogel solution. I have fixed this. See new line 169.

Line 157 Add 300 µL of filter sterilized polyacrylamide solution from step 3.2.2 to each of the
wells that contain sputum. Do you mean 2.2

Yes. Fixed. See new line 169.

Line 166 - Transfer the solidified hydrogel sputum samples to a 15 mL culture tube containing 5
mL of 8% sodium dodecyl sulfate (SDS, pH 8 and allowed to clear for 3-14 days at 37 °C
(with or without shaking). 3-14 days-how do you decide how long to wait-vague-please clarify.

To clarify when the clearing is complete the statement "until sputum becomes transparent" has been added. See new line 180.

Line 184 2.8.4 Repeat step 3.8.3 two more times. Do you mean step 2.8.3.

Yes, thank you. Fixed this. See new line 197.

Line 197 - Place the 1 mm sections of hydrogel were (delete were) inside a sterile 1.5 mL eppendorf tubes.

Done. See new line 213.

Line 202 - Keep the biosafety cabinet lights off when adding the fluorescently labelled PseaerA Probe. What does the probe label-please describe?

Thank you for this comment. I have double checked with the manufacturer at Eurofins, and they said that there is no specification to keep the probe protected from light. I will adjust this part of the protocol. See new line 219.

Line 336 - application (REF). Please provide reference.

Reference provided. See new line 369.

Reviewer #2:
Manuscript Summary:
This method article by Jackson and colleagues guides the experimenter through a method to visualize Pseudomonas aeruginosa in sputum samples, and to preserve the sample architecture. The literatures lacks in situ visualization of Pseudomonas, in sputum samples as presented here, but also in lung models where mucus is also present, for example in transwells grown primary lung cells, lung on chip models or even infected lungs. Therefore the technical advances presented in this method article will benefit the community, and have an excellent degree of "recyclability" for other applications. The method is nicely described, comprehensive and easy to follow, and will be helpful for the community.

Minor comments:
Line 106: the final PFA concentration will be 2% (after diffusion exchange with the sample), which halves the standard 4% concentration. Have you tested higher PFA concentrations, and if yes, can you comment on the differences of fixation?

We've shown that 2% PFA is adequate for fixing cultured bacterial cells, and 2%-4% PFA (depending on the volume of the tissue relative to the PFA liquid volume) is generally suitable for tissue. In general, the major risk of underfixation is decreased structural integrity, which can be particularly problematic for less stable sputum samples before they are embedded in the hydrogel. The major risk of overfixation is that too many cross links can block probes from binding to their molecular targets.

Line 112: unlike at line 106 where you provide a volume ratio PFA:sputum, here you provide an example with a define volume. Why not providing also a ratio instead? And if you prefer to keep the example of a defined sputum volume, maybe you could keep using the example provided on line 10, i.e. a 1 mL PFA-sputum sample + 2.5 mL PBS.

A ratio has been provided for step 1.8 as suggested. See new line 130.

Line 121: there is probably a mistake when you refer to steps "2.4 & 2.5", do you mean "1.5 & 1.6"?

Fixed. See new line 128.

Line 125: can you describe how you "divide the sputum sample"? Maybe this will be illustrated in the video, but, with the text only, I'm wondering how this step is exactly achieved with minimal disturbance of the sputum plug architecture.

It is unnecessary to aliquot the sputum at this stage and a more detailed description of carefully cutting the sputum into small sections is provided in step 2.3 and the note following step 2.3, thus I removed the mention of aliquoting sputum here. See new line 133.

Line 145: as you highlight the importance of removing the oxygen to allow for proper acrylamide polymerization, with the same logic shouldn't the PBS used for the hydrogel also be oxygen free?

PBS has been added to the hydrogel components to be degassed in substep 2.1.3. See new lines 152-153.

Line 149: "Thaw frozen sputum samples": you did not mention before that the sputum samples could be frozen. In case they can be frozen, please mention it earlier in the method. But as you said that samples should be stored at 4°C (line 125), I would recommend to start mentioning the usage of fresh sample, and optionally write a note that there is the less recommendable possibility to start from frozen samples.

Removed the thawing directive. See new line 159.

Line 155: could you provide the reference for the 8-chambered coverglass slide?

The reference for the 8-chambered coverglass has been added to the materials list. Please see updated document.

Line 157: once again, the numbering seems to have been changed, as the reference to step "3.2.2" is most likely actually referring to step 2.1.

Fixed. See new line 169.

Line 166: although I'm not an expert in clearing procedures, I've only used method with successive clearing reagent bath. Can you comment on whether it would improve the clearing to do so? If not, just ignore the comment. Besides, "3 to 14 days" seems to be a very wide interval, can you comment on why this is so inconsistent? Does it mostly depends on the sample size? Or the patient? Or other factors? What should people expect?

A larger volume of SDS (up ~ 50 mL) will facilitate clearing of some samples. Refreshing smaller volumes would also work fine in lieu of using a larger volume. The major variables affecting how long sputum takes to clear is size and composition. More DNA-rich samples take longer (towards the higher end of the estimate) compared to mucus rich samples.

Please see modification to the note on clearing in the new line 185.

Line 193: is there is specific safety reason why the experimenter should work in a safety cabinet (toxic fumes…)? Many BLS2 labs simply work on the bench, and use other means to work under sterile conditions. If there is not special reason, I would delete this specification (also on line 202), and would replace it by a more general statement about the sample light protection.

Thank you for this question, I have removed the specification for the biosafety cabinet. I have put a special note though that formalin solution (found in the hybridization buffer) should not be used near an open flame. See new line 219.

Line 197: remove "were". Also "1.5 mL tubes" is understandable, and mentioning the Eppendorf brand is unnecessary. Same on line 200.

Removed "were" and the Eppendorf brands throughout the text. See new lines 216 and 238.

Line 209: consider changing "entirety […] are submerged" to "entirety […] is submerged"

Change made as suggested. See new line 228.

Line 225: please provide the reference in the Material list for the Psl-0096 antibody. Have you tested and compared other Psl antibodies? If yes, can you comment on that, this would be helpful for the reader.

The Psl0096 antibody has been added to the materials list.

Yes, previous work in our lab has tested antibodies to 3 different Psl epitopes (psl0096, wapR001 and wapR0016, MedImmune) that all demonstrated the capability to bind to Psl. Our reason for using Psl0096 in these experiments was because experiments with all 3 antibodies (unpublished data) on 14 cystic fibrosis isolates from SickKids[1] and 63 isolates from the EPIC trial[2] Psl0096 antibody showed significant differences in biofilm aggregation between persistent and eradicated P. aeruginosa isolates. Although we have tested the different antibodies against Psl in biofilms, we have not yet done that in the hydrogels so cannot comment on their effectiveness in this specific protocol, though we can imagine they would similarly bind in the hydrogel matrix as they do in biofilms.

Line 229: homogenize the style of "w/v" throughout the text (e.g. line 123: "wt/vol"). In the same line, you write "1 mL pipettor" on line 227, and 1 mL pipette just after on line 236. Also, the Psl antibody is referred to sometimes as Psl-0096, sometimes as Psl0096. Same for "mL" and "ml".

Done. All instances of "wt/vol" have now been changed to "w/v".

Line 251: 0.45µm filters are not adapted for sterilization, as small bacteria can go through, and 0.2 µm should be used instead. If 0.45 µm filters are adapted for this step, consider changing the sentence to "Filter the solution...".

The filter size has been changed to 0.2 µm. See new line 272.

Line 327: "◦C" should be replaced by "°C"

Done. See new line 357.

Line 336: reference missing.

Reference added. See new line 369.

Line 355: reference not formatted and does not appear in the reference list.

Removed reference. See new line 389.

General minor comments:

-It may be helpful to comment on the total duration of the protocol, and advice the reader on how to optimize their schedule (what can be achieved on each day, where to stop, when samples can be stored at 4°C or -20°C and the experiment resumed later).

This is a good point. We have mentioned the storage temperatures at steps 1.9 and 2.9, lines 133 and 202-203, respectively. As per your suggestion we have made additions to the manuscript, in the results section. Please see addition to the manuscript on lines 297-302.

-As the method focuses on preserving the 3D architecture for imaging, it would be nice to provide for example a movie of a 3D representation of Figure 4, or show this movie in the Video method, or provide the raw data of Figure 4 as a supplemental file.

A supplemental file of the raw data for figure 4 has been provided. See supplemental material "Supplemental File_Figure 4.czi".

-How good is the anti-Psl-antibody staining against planktonic Pseudomonas, as EPS are mostly synthetize after hours of surface exposure?

P. aeruginosa produces Psl in the planktonic state[3] as well as in biofilm. We have done a control experiment with planktonic cultures of PAO1 and PAO1-ΔPsl (a mutant incapable of Psl polysaccharide production) and saw no anti-Psl0096-texas red antibody staining in these mutant strain. We will include this control in the supplemental material. See supplemental material "Supplemental_Figure 2.png".

Can you comment about the interest and feasibility of a double EPS staining (e.g. Pel + Psl), to avoid the absence of staining of clinical isolates devoid of Psl EPS?

Unfortunately, we cannot comment on feasibility of double EPS staining at this stage as we have not tested this. The research question was focused on the variation in Psl production (presence or absence). However, this is a very good question and would be excellent to test in future experiments to see variations in all of the Pseudomonal polysaccharides produced in new onset infections.

-The authors used Biorender to generate figures. Just to make sure JoVE or the authors comply with the regulation, shouldn't the author mention they used Biorender to generate figure? And do you have a license?

Thank you for pointing this out. Figures 1 and 2 now have the tag "created with biorender.com" at the end of the figure caption.

Formatting comment: "Pseudomonas aeruginosa" should be in italic in the references.

Pseudomonas aeruginosa occurrences in the references have now been italicized.

Material list: there is a typo in Microscopy in "Electron Microscopry services" and isn't the company name "ELECTRON MICROSCOPY SCIENCES"?

Yes, thank you. The company name has been fixed to read "Electron Microscopy Sciences".

Reviewer #3:
Manuscript Summary:
Authors aim at fixing patient sputum samples and then using the MiPACT method for tissue clearing to allow for FISH, DAPI and Fluorescent antibody labeling of the Psl component of *P. aeruginosa* biofilm.
Overall an interesting and well written manuscript.

Major Concerns:
1. Would like to see the use of controls for the staining. Something like the reference strain PAO1 or PA14 can be used as controls.

Thank you, we can provide the controls in the supplemental materials (see "Supplemental_Figure 2.png") and in the results section see new lines 311 and 312. We have tested the anti-Psl antibody binding in planktonic PAO1 and PAO1-ΔPsl strains and demonstrated an absence of anti-Psl antibody staining in the PAO1-ΔPsl mutant.

Also, would a mucoid strain that produces a large amount of alginate block any of the antibody binding to Psl?

The Psl0096-antibody has been used in the lab with P. aeruginosa mucoid isolates collected from CF patients and we have seen anti-body binding (unpublished data). However, we have not specifically tested an alginate overproducer and the Psl0096 antibody binding.

2. Authors should show the sensitivity and specificity of the assay, ie. what the detection limit is using a molecular and/or viable cell count as a control.

The PseaerA probe detection limit using FISH was found to be around 10^4 cells per mL. See new figures in supplemental material "Supplemental_Figure 1". Also see manuscript addition on new lines 302 and 303.

3. This paper generalizes Psl as being biofilm which is a product of Psl, Pel, alginate as well as eDNA and other cellular debris. Since this method is specific to Psl, false-negative stains might be a result of strains of Pseudomonas that preferentially produce the other exopolysaccharides over Psl.

Thank you for pointing this out. Reviewer 1 made a similar comment. Thus, to address this we removed the mention of a biofilm here and said we visualized P. aeruginosa cells or aggregates instead. Please see all the sections of text where mention of biofilms were removed, lines 30, 40-42, 44, 47, 49, 53, 69, 81, 299, 300, 306, 307, 309, 351, 394, 400.

4. This method is being coined as a diagnostic method. Yet this is a qualitative assessment of the samples and not an exact quantitative method to measure biofilm production/presence in the sample as the authors are suggesting. If the method is to be used as a measurement tool more work is needed to prove the efficacy of this measurement. Also, the 3D spatial architecture in the sputum sample might may necessarily translate to the lung therefore studying co-infections through this method may not be a true indicator.

We apologize if this was not clear as this is not meant to be a validated diagnostic clinical test but rather a research tool to investigate the presence and behaviours of organisms.

Minor Concerns:
1. DAPI acronym is never defined.

The acronym has now been defined. Please see new line 264.

2. The confocal microscope acronym is misspelled in line 87 and in the diagram in Figure 2.

Fixed. Please see new line 90 and 383.

3. Line 70. a misplaced ")" at the end of the sentence

Fixed. Please see new line 72.

4. Figure 3 and 4 legends don't have a bolded title. (Lines 309/ 315).

Fixed. Please see lines 337 & 338 for figure 3. Please see lines 344 & 345 for figure 4.

5. RT acronym not defined. (line 304).

The acronym has now been defined. Please see new line 240.

Comments:
Not sure if the use of a reducing agent like Dithiothreitol (DTT) would help with breaking the sputum plug for ease of manipulation.

1. Vidya, P. *et al.* Chronic infection phenotypes of Pseudomonas aeruginosa are associated with failure of eradication in children with cystic fibrosis. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology*. **35** (1), 67–74, doi: 10.1007/s10096-015-2509-4 (2016).

2. Mayer-Hamblett, N. *et al.* Pseudomonas aeruginosa phenotypes associated with eradication failure in childrenwith cystic fibrosis. *Clinical Infectious Diseases*. **59** (5), 624–631, doi: 10.1093/cid/ciu385 (2014).

3. Yang, S. *et al.* Differential production of Psl in planktonic cells leads to two distinctive attachment phenotypes in Pseudomonas aeruginosa. *Applied and Environmental Microbiology*. **84** (14), 1–18, doi: 10.1128/AEM.00700-18 (2018).

4. da Silva, R.M., Zimermann Teixeira, P.J., da Silva Moreira, J. The clinical utility of induced sputum for the diagnosis of bacterial community-acquired pneumonia in HIV-infected patients: A prospective cross-sectional study. *Brazilian Journal of Infectious Diseases*. **10** (2), 89–93 (2006).

5. Hogan, D.A. *et al.* Analysis of lung microbiota in bronchoalveolar lavage, protected brush and sputum samples from subjects with Mild-To- Moderate cystic fibrosis lung disease. *PLoS ONE*. **11** (3), 1–23, doi: 10.1371/journal.pone.0149998 (2016).

6. DePas, W.H., Starwalt-Lee, R., Van Sambeek, L., Kumar, S.R., Gradinaru, V., Newman, D.K. Exposing the three-dimensional biogeography and metabolic states of pathogens in cystic fibrosis sputum via hydrogel embedding, clearing, and rRNA labeling. *mBio*. **7** (5), doi: 10.1128/mBio.00796-16 (2016).

Supplemental Material

Files

The original file for figure 4 has been provided. The file is titled “Supplemental File_Figure 4.czi”

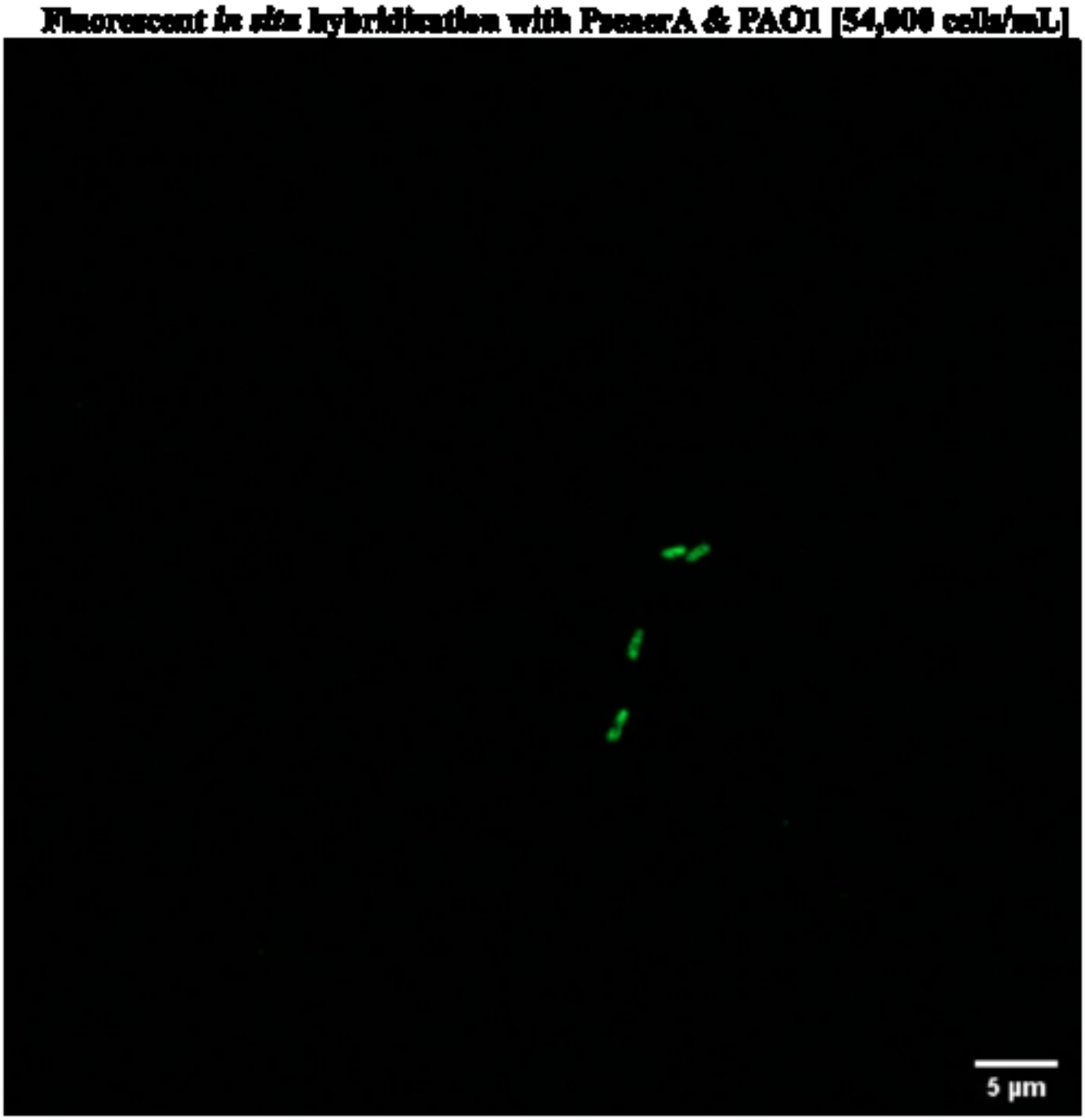
Figures

Figure 1. *P. aeruginosa* stained with DAPI and the anti-Psl0096-Texas Red Antibody. A) *P. aeruginosa* strain PAO1-Δpsl, and B) *P. aeruginosa* strain PAO1.

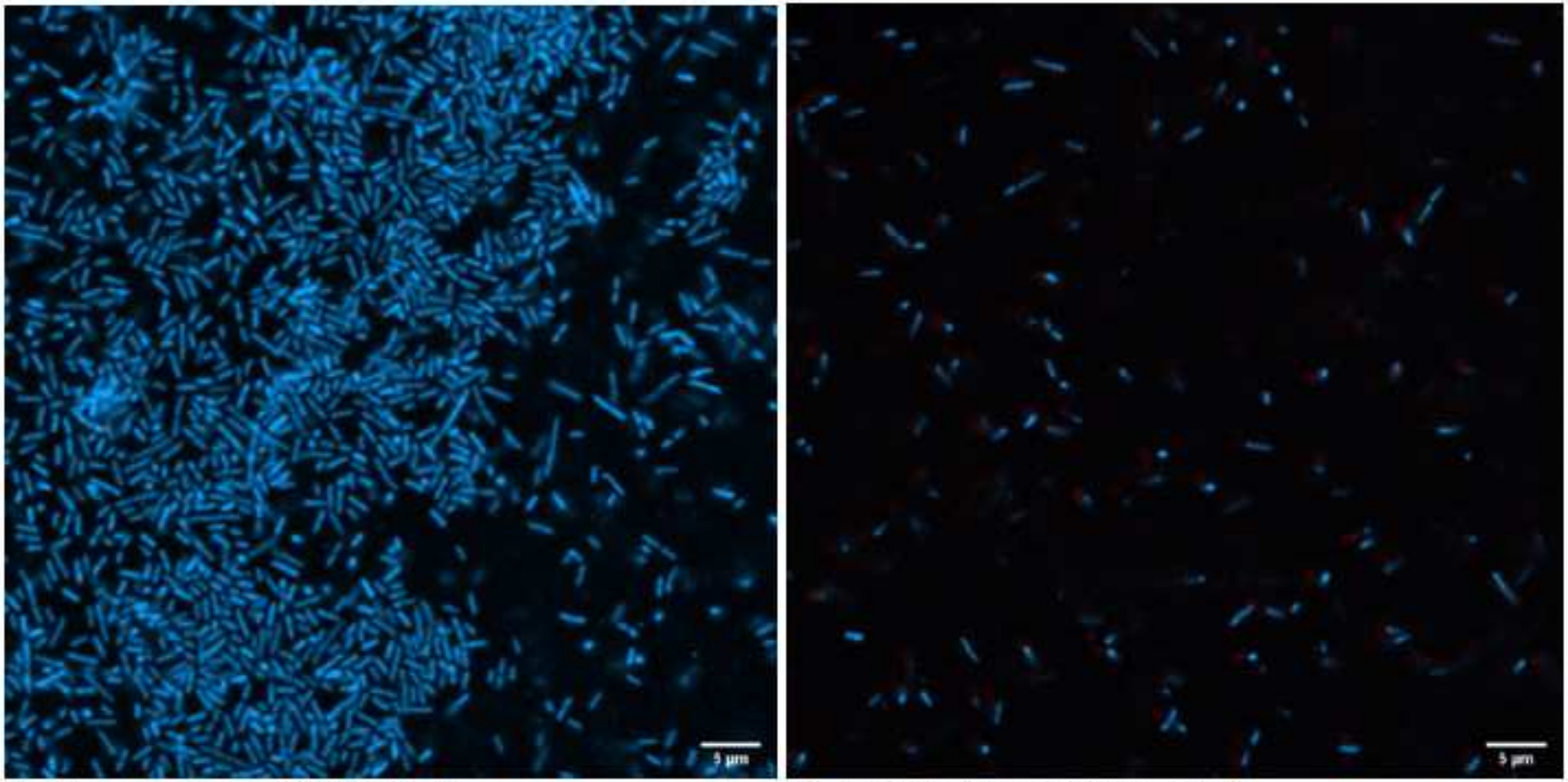
Figure 2. Fluorescence *in situ* hybridization with *P aeruginosa* PAO1 hybridized to the PseaerA probe and visualized with confocal scanning laser microscopy. The image shows the detection limit of the probe at a concentration of 54,000 CFU/mL. Lower cells counts may be detectable, though we found that at lower cell densities the fluorescent green spots on the slides were not as clearly pseudomonal rods.

Supplemental File (Figures, Permissions, etc.)

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental Figure 1.png



P. aeruginosa stained with DAPI and anti-Psl0096-Texas Red Antibody



A. _P. aeruginosa_ PAO1-Δpsl

B. _P. aeruginosa_ PAO1-Δpsl

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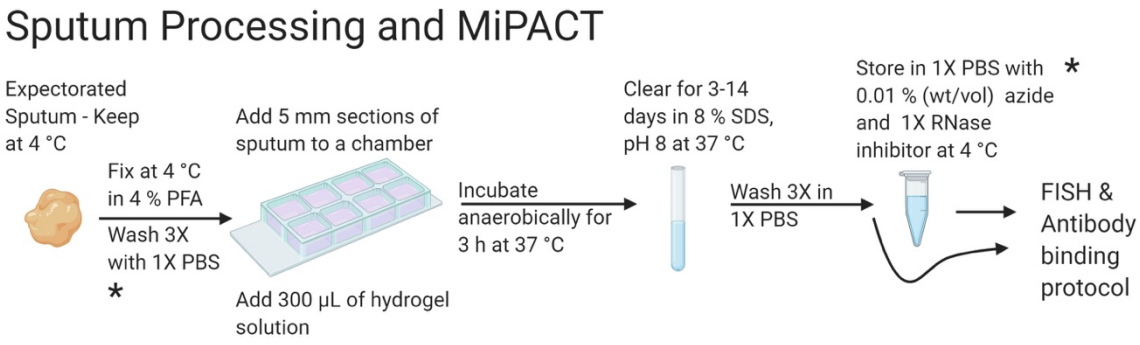
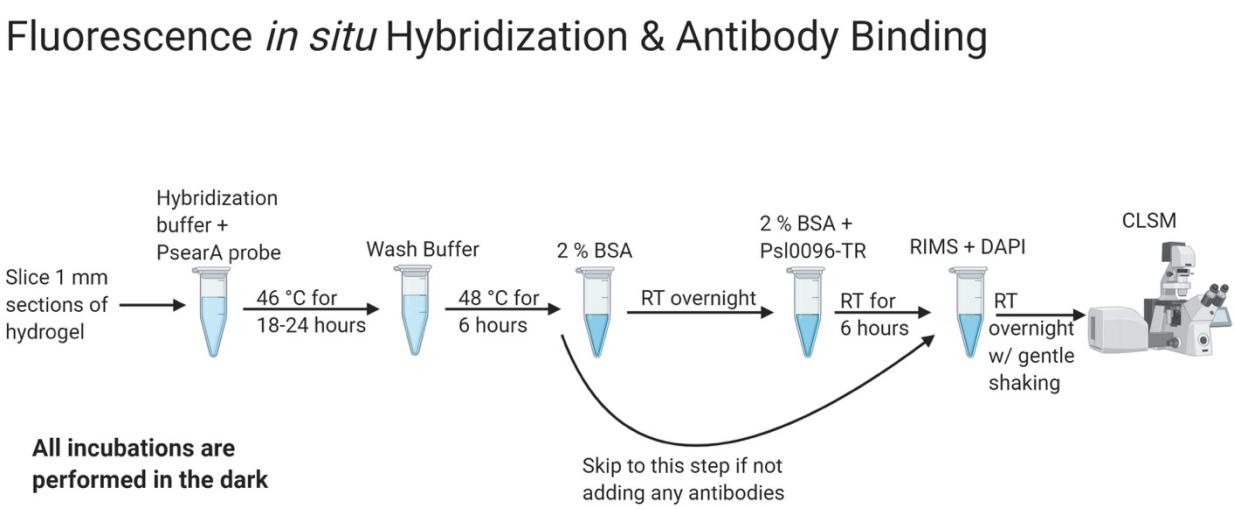
1. **Figure 1 Flow diagram illustrating the sputum processing and MiPACT protocols, Lindsay Jackson**



2. **Figure 2 Flow diagram denoting the fluorescence in situ hybridization and antibody staining protocols, Lindsay Jackson**



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May 26, 2020