Video Article

Co-culture Models of *Pseudomonas aeruginosa* Biofilms Grown on Live Human Airway Cells

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Abstract

Bacterial biofilms have been associated with a number of different human diseases, but biofilm development has generally been studied on non-living surfaces. In this paper, we describe protocols for forming *Pseudomonas aeruginosa* biofilms on human airway epithelial cells (CFBE cells) grown in culture. In the first method (termed the Static Co-culture Biofilm Model), *P. aeruginosa* is incubated with CFBE cells grown as confluent monolayers on standard tissue culture plates. Although the bacterium is quite toxic to epithelial cells, the addition of arginine delays the destruction of the monolayer long enough for biofilms to form on the CFBE cells. The second method (termed the Flow Cell Co-culture Biofilm Model), involves adaptation of a biofilm flow cell apparatus, which is often used in biofilm research, to accommodate a glass coverslip supporting a confluent monolayer of CFBE cells. This monolayer is inoculated with *P. aeruginosa* and a peristaltic pump then flows fresh medium across the cells. In both systems, bacterial biofilms form within 6-8 hours after inoculation. Visualization of the biofilm is enhanced by the use of *P. aeruginosa* strains constitutively expressing green fluorescent protein (GFP). The Static and Flow Cell Co-culture Biofilm assays are model systems for early *P. aeruginosa* infection of the Cystic Fibrosis (CF) lung, and these techniques allow different aspects of *P. aeruginosa* biofilm formation and virulence to be studied, including biofilm cytotoxicity, measurement of biofilm CFU, and staining and visualizing the biofilm.

Video Link

The video component of this article can be found at https://www.jove.com/video/2186/

Protocol

1. Static Co-culture Biofilm Model

1. The Static Co-culture Biofilm Model[1] uses CFBE41o- cells (CFBE cells), which are immortalized cells originally developed from an individual with CF homozygous for the ΔF508-CFTR mutation [2,3,4]. CFBE cells should be seeded at a concentration of 10^6 cells/well in a 6-well tissue culture plate or 2 X 10^5 in a 24-well tissue culture plate in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 2mM L-glutamine, 50 U/mL penicillin, and 50 µg/mL streptomycin. We use 1.5 mL medium per well in 6-well plates and 0.5 mL medium per well in 24-well plates.
2. Cells should be grown at 37°C and 5% CO_2-95% air for 7-10 days to form a confluent monolayer before inoculation with bacteria. The medium must be changed every 2-3 days. These conditions have been shown to lead to formation of a confluent monolayer and tight junctions.
3. Grow P. *aeruginosa* in 5 mL LB for 18 hours at 37°C on an incubator shaker at 200 rpm. Under these conditions, *P. aeruginosa* cultures will typically reach a density of 5x10^9 CFU/ mL.
4. For bacterial inoculation, remove the medium from CFBE cells and add an equal volume of MEM without phenol red, supplemented with 2 mM L-glutamine (Microscopy medium). Confluent CFBE monolayers are inoculated with *P. aeruginosa* at a multiplicity of infection of approximately 30:1 relative to the number of CFBE cells originally seeded. This equates to 2 X 10^7 CFU/mL in 1.5 mL MEM/well for 6-well plates and 1.2 X 10^7 CFU/mL in 0.5 mL MEM/well for 24-well plates.
5. Incubate plates for 1 hour at 37°C and 5% CO_2-95% air.
6. Following the 1 hour incubation, the supernatant should be removed and replaced with fresh Microscopy medium supplemented with 0.4% arginine.
7. Incubate at 37°C and 5% CO_2-95% air for various time points (up to approximately 8 hours) and analyze the integrity of the CFBE monolayer using phase contrast microscopy. If airway cells are non-confluent, *P. aeruginosa* will rapidly (within minutes) gain access to the basolateral surface of the cells and destroy cellular integrity. Inoculation with *P. aeruginosa* strains that constitutively express GFP results in fluorescent biofilm microcolonies which can be visualized by epifluorescence or confocal microscopy.
8. The bacterial CFU in the biofilm can be determined by washing the co-culture 2-3 times with phosphate-buffered saline (PBS) to eliminate planktonic bacteria. Following the wash, treat with 0.1% Triton X-100 in PBS or MEM for 10-15 minutes to lyse the epithelial cells and disperse the biofilm.

9. Vortex for 3 minutes and prepare serial dilutions of the lysate. Plate these dilutions onto LB agar and incubate overnight at 37°C. Count the colonies the next day to determine the CFU.

2. Flow Cell Co-culture Biofilm Model

1. The Flow Cell Co-culture Biofilm Model (flow assay) involves modification of the standard biofilm flow cell apparatus to accommodate CFBE cells[5].
2. Place several 40-mm diameter glass coverslips into a clean 100-mL beaker. Cover tightly with 2 layers of aluminum foil and autoclave on a dry cycle for 20 min.
3. Working in a cell culture hood, grab one sterile coverslip from the beaker using ethanol washed forceps and place into a sterile 60-mm diameter plastic dish.
4. Add 3 mL of pre-warmed cell growth medium, pressing on the coverslip with the tip of the pipette to remove any bubble trapped underneath and to force the coverslip to the bottom of the plastic dish.
5. Seed $2x10^6$ cells per dish and shake the dish gently back and forth. Avoid swirling to prevent centrifuging the cells against the sides of the dish.
6. Place the dish in a 5% CO_2-95% air incubator at 37°C and feed the cells every other day with 3 mL fresh growth medium for 8 to 10 days. The cells will form a confluent monolayer on the glass coverslip.
7. Grow *P. aeruginosa* in 5 mL LB for 18 hours at 37°C on an incubator shaker at 200 rpm. Under these conditions, *P. aeruginosa* cultures will typically reach a density of $5x10^9$ CFU/mL. We use *P. aeruginosa* strain PAO1 carrying the pSMC21 plasmid for constitutive expression of GFP[6].
8. Add 1 mL of bacterial culture into a sterile microcentrifuge tube. Centrifuge at 6000 rpm for 3 min, and wash the bacterial pellet twice in 1 mL of Microscopy medium.
9. Dilute 0.5 mL of washed and resuspended bacteria into 4.5 mL Microscopy medium to achieve a concentration of ~$5x10^8$ CFU/mL.
10. Observation of live cells in real time requires an imaging chamber coupled to a peristaltic pump (to ensure a flow of nutrients for extended lengths of time) and a temperature controller. We use the Bioptechs FCS2 (Focht Live-Cell) chamber connected to a low-flow micro-perfusion pump with 1/16th C-flex tubing cut to appropriate lengths and autoclaved for sterility, and temperature-regulated via the FCS2 chamber controller. We keep the input source of medium in a 37°C water bath located right next to the microscope.
11. Assemble the flow chamber. The FCS2 chamber includes a self-locking base (which sits on the stage adapter during imaging) and an upper half connected to the perfusion tubes and the chamber controller. The chamber is assembled upside-down before being flipped over and placed onto the stage adapter. Hold the upper half of the chamber upside down so that the perfusion tubes are visible, and align the clearance holes of a 0.75-mm thick rubber gasket onto the perfusion tubes. Stack the microaqueduct slide provided with the chamber on top of the rubber gasket, making sure the grooved side is up, and place another rubber gasket on top of the slide. The thickness and internal geometry of this second gasket will determine the volume of the chamber. We typically work with a 0.75-mm thick rubber gasket with a 30-mm round internal geometry.
12. Add 1 mL of pre-warmed (37°C) Microscopy medium at the center of the slide.
13. Retrieve a 60-mm dish from the cell culture incubator, remove the spent medium and wash the cells once with 3 mL of pre-warmed Microscopy medium. This step ensures the elimination of the phenol red and antibiotics present in the cell growth medium. Phenol red is mildly fluorescent and can interfere with the imaging, while antibiotics can eradicate *P. aeruginosa* bacteria before they can establish biofilms.
14. Using ethanol-washed forceps, retrieve the coverslip from the dish and lower it upside down onto the bead of Microscopy medium placed onto the chamber. The coverslip is now resting on the second rubber gasket and the monolayer of airway cells is facing downward.
15. Holding the assembled components in one hand, place the base of the chamber on top of the stack, and turn the chamber over swiftly so that everything is right side up. Lock the base into place by turning the ring.
16. Connect the inlet tube to the low-flow micro-perfusion pump and start the flow at a rate of 20 mL/h; this flow rate is within the swimming speed capability of *P. aeruginosa*. A second piece of tubing links the pump to a flask of Microscopy medium placed in the 37°C water bath located right next to the microscope.
17. Attach sterile pre-cut 1/16th C-Flex tubing to the inlet and outlet perfusion tubes of the chamber, connect the temperature controller, then place the assembled chamber onto the microscope stage of an inverted fluorescence microscope.
18. Using a 1-mL disposable syringe, inject the previously prepared bacterial suspension into the chamber, using a two-way valve placed in-line between the pump and the chamber. In our particular setting, it takes a 0.6 mL volume of bacterial suspension to reach the chamber. Adjust this volume according to your particular tubing length. We also find it useful to place an in-line disposable 0.22 µm filter between the pump and the two-way valve to prevent bacteria from swimming upstream and contaminating the input flask.
19. To allow bacteria to attach to the airway cells, stop the pump for 2h. The flow can then be reinitiated and maintained at 20 mL/h for the rest of the experiment.
20. Monitor the integrity of the airway cells by differential interference contrast (DIC) microscopy throughout the experiment to check for signs of damage to the monolayer. Simultaneously, follow the development of GFP-labeled *P. aeruginosa* biofilms at the apical surface of airway cells by acquiring images with an inverted confocal or wide-field fluorescence microscope.

3. Representative Results

We use a strain of *P. aeruginosa* containing the pSMC21 plasmid which allows for constitutive expression of GFP[6]. For this reason, GFP-labeled biofilms growing on a CFBE monolayer can be visualized by epifluorescence microscopy. Alternatively, visualization of the biofilm can be achieved by staining unlabeled *P. aeruginosa* with a 1% calcofluor white solution for 1 hour at 37°C[1].

For imaging biofilm formation on CFBE cells in the flow assay, we use a custom-made stage adapter, but several companies can now provide specially fitted stage adapters. We work with an Olympus IX70 inverted microscope equipped with an ORCA-AG deep cooling CCD camera and an x60 oil-immersion objective (numerical aperture 1.40). The filter wheel is equipped with a 480/40 nm band pass excitation filter and a 535/30 nm band pass emission filter. Digital images were acquired with the OpenLab 4.0.3 software package (Improvision) and volumes were

deconvolved by iterative restoration using the Volocity 3.5.1 software (Improvision). Quantitative analysis of 3D biofilm structures was achieved with the COMSTAT image analysis software package[7,8].

For any co-culture model considered, one must pay particular attention to the cytotoxicity developing between the components of the model. In both the static and the flow cell assays, we found that the CFBE monolayer could withstand the presence of *P. aeruginosa* for up to 8 hours after inoculation without any sign of alteration. Epithelial monolayer integrity can be assessed by phase-contrast microscopy using an inverted microscope[1] (Figure 1A) or by differential interference contrast (DIC) microscopy throughout the experiment[5]. Over time, *P. aeruginosa* will produce toxins and virulence factors which accumulate and can damage the epithelial cell monolayer fully or in sections (Figure 1B-1C). Cytotoxicity can be quantitatively assessed using various kits to measure the release of lactate dehydrogenase (LDH) from the epithelial cells. LDH is a stable cytosolic enzyme released in the extracellular milieu upon cell lysis or cell death.

When the integrity of the airway monolayer is not compromised (typically for ~8 h following inoculation), *P. aeruginosa* biofilms can successfully form and develop at the apical surface of airway cells in both co-culture models described (Figure 2A-2B). Following 3D reconstruction (Figure 2C) and quantification, biomass accumulation can be accurately determined. We have also used these co-culture biofilms in several phenotypic assays. For instance, as mentioned above, biofilm CFU can be easily determined by lysing the epithelial cells, serially diluting the lysate, and plating on agar plates. We have found this technique advantageous in determining the resistance of different strains to antibiotic treatment. Furthermore, biofilm toxicity toward the epithelial cells can be measured using commercially available LDH detection kits. In this manner, the role of virulence factors in toxicity of biofilms can be assessed. These model systems also support a number of gene expression applications, including promoter fusion studies, RT-PCR, and microarray analysis[1,5,9].

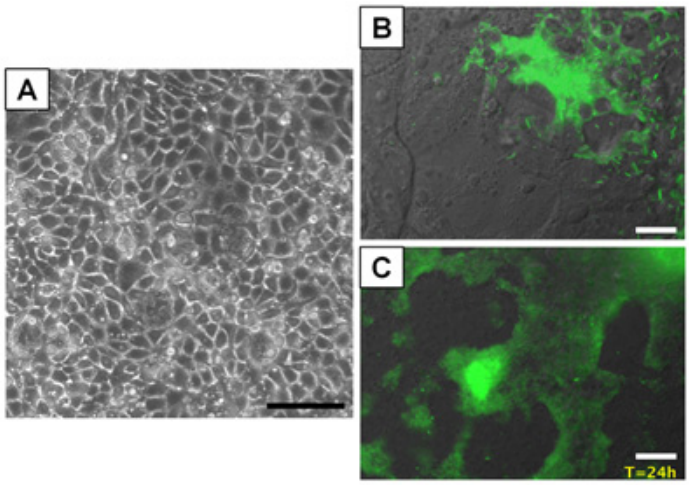

Figure 1. Monolayer of CFBE cells and representative images of compromised and damaged airway cell monolayers due to *P. aeruginosa* biofilm growth. (A) Representative image of a confluent monolayer of CFBE cells grown in tissue culture plates, assessed by phase-contrast microscopy. Scale bar, 120 µm. (B) Example of a compromised CFBE monolayer. Even though the monolayer does not display obvious visible signs of damage yet, *P. aeruginosa* bacteria are seen spreading between the tight junctions of the epithelial cells and gaining access to the basolateral membranes. Biofilm formation is typically not achieved under these conditions due to the monolayer deteriorating. Scale bar, 20 µm. (C) Example of an overgrown *P. aeruginosa* biofilm observed 24 h post-inoculation. After successfully supporting biofilm formation, the CFBE monolayer was damaged beyond repair and is now virtually absent. Residual biofilm, growing as a flat layer of bacteria, is shown attaching to the glass coverslip. Scale bar, 20 µm.

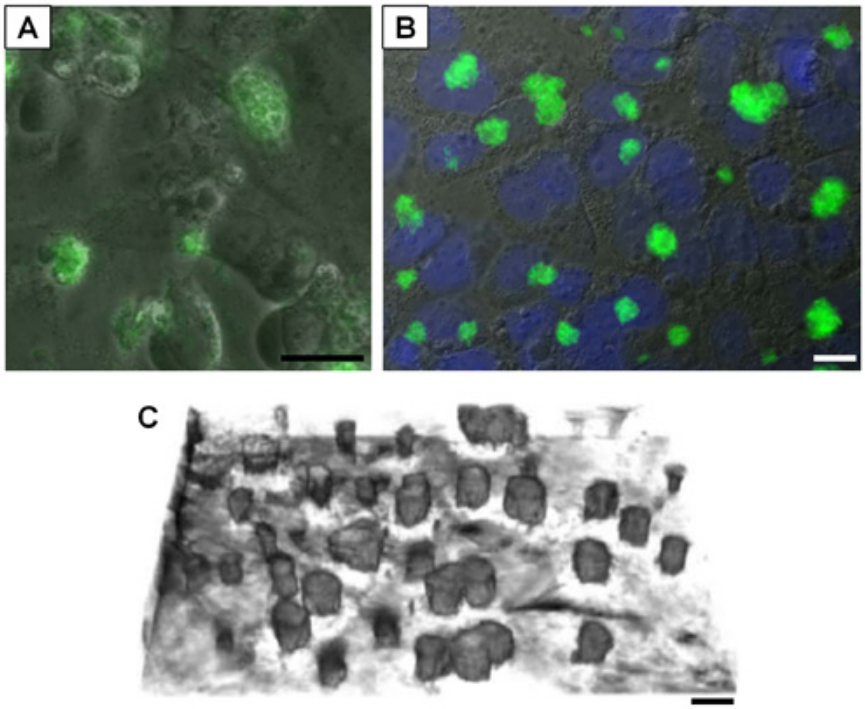

Figure 2. *P. aeruginosa* biofilms grown at the apical surface of confluent CFBE cells using the Static Co-culture Biofilm Model and the Flow Cell Co-culture Biofilm Model. (A) Representative image of a GFP-expressing *P. aeruginosa* biofilm grown on a confluent monolayer of CFBE cells using the Static Co-culture Biofilm Model, assessed by epifluorescence microscopy. Image is an overlay of the phase contrast channel and the fluorescence channel. Scale bar, 35 µm. (B) Representative image of a GFP-labeled *P. aeruginosa* biofilm grown for 6 h on a confluent monolayer of CFBE cells using the Flow Cell Co-culture Biofilm Model. To facilitate the visualization of the airway monolayer, nuclei were stained with 10 µg/mL Hoechst 33342 (Molecular Probes) for 30 min prior to inoculation with *P. aeruginosa*. Merged and pseudocolored images were viewed by differential interference contrast (DIC), and the corresponding fluorescent images are shown. Biofilms, presenting as green clumps attached to the apical surface of the CFBE cells, are dispersed across the airway cells. Scale bar, 20 µm. (C) Three-dimensional reconstruction of z-series image stacks showing the typical mushroom-like structures of 6 h-old *P. aeruginosa* biofilms forming on a CFBE cell monolayer. Scale bar, 10 µm.

Discussion

Biofilms are communities of bacteria that form in response to environmental stimuli. These environmental signals lead to global regulatory changes within each bacterium, resulting in binding to a surface, aggregation, production of exopolysaccharides, and other phenotypes such as increased antibiotic resistance[10]. Over the last couple of decades, much evidence has supported the hypothesis that biofilms play a large role in the pathogenesis of chronic infections. For instance, it is well accepted that *P. aeruginosa* is able to establish chronic infections in the lungs of Cystic Fibrosis (CF) patients by forming biofilms[11]. CF is a genetic disorder, where mutations in the CFTR gene lead to improper chloride secretion[12]. CF patients typically experience altered airway physiology leading to thick mucus plugs in the airways and, concurrently, to microbial infection[13]. By late adolescence, the dominating infectious agent of CF airways is *P. aeruginosa*, leading to a chronic biofilm infection that is resistant to antibiotics[14].

The protocols described in this paper have been developed to act as model systems for studying biofilm formation on living lung cells. Bacterial biofilms are implicated in many disease states and, yet, have mostly been studied on non-living solid surfaces, such as glass and plastic[15,16]. By studying biofilm formation and growth on abiotic surfaces only, one is missing important interactions between pathogen and host that can only occur with a model system such as described in the protocols above. Specifically, the Static Co-culture Biofilm Model and the Flow Cell Co-culture Biofilm Model take advantage of the ability of *P. aeruginosa* to interact with and bind to the epithelial surface of the lung. Using these models, we have shown that the response of the co-culture biofilms to antibiotic treatment is unique and that these models are more likely to accurately reflect the infectious state[1,5]. In this regard, we have reported that the resistance to tobramycin increases by >25-fold when *P. aeruginosa* biofilms are grown on airway cells compared to biofilms grown on abiotic surfaces such as glass[5]. It is likely that these techniques reflect the events that occur during early colonization of the lung[17]. Therefore, the co-culture model systems represent innovative tools for understanding early infection of the CF airway epithelium by *P. aeruginosa*[1].

Tissue culture systems have commonly been employed to understand interactions between host and pathogen. We have taken these interactions a step further by forming biofilms on live human epithelial cells. In the future, modified versions of the models described here could potentially be used to investigate biofilm formation of different bacteria in other infectious states.

Disclosures

No conflicts of interest declared.

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References

1. Anderson, G. G., Moreau-Marquis, S., Stanton, B. A. & O'Toole, G. A. *In vitro* analysis of tobramycin-treated *Pseudomonas aeruginosa* biofilms on cystic fibrosis-derived airway epithelial cells. *Infect Immun* 76, 1423-1433 (2008).
2. Bruscia, E. *et al.* Isolation of CF cell lines corrected at DeltaF508-CFTR locus by SFHR-mediated targeting. *Gene Ther* 9, 683-685 (2002).
3. Cozens, A. L. *et al.* CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 10, 38-47 (1994).
4. Hentchel-Franks, K. *et al.* Activation of airway cl- secretion in human subjects by adenosine. *Am J Respir Cell Mol Biol* 31, 140-146 (2004).
5. Moreau-Marquis, S. *et al.* The DeltaF508-CFTR mutation results in increased biofilm formation by *Pseudomonas aeruginosa* by increasing iron availability. *Am J Physiol Lung Cell Mol Physiol* 295, L25-37 (2008).
6. Kuchma, S. L., Connolly, J. P. & O'Toole, G. A. A three-component regulatory system regulates biofilm maturation and type III secretion in *Pseudomonas aeruginosa*. *J Bacteriol* 187 (2005).
7. Heydorn, A. *et al.* Experimental reproducibility in flow-chamber biofilms. *Microbiology* 146 (Pt 10), 2409-2415 (2000).
8. Heydorn, A. *et al.* Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* 146 (Pt 10), 2395-2407 (2000).
9. Anderson, G. G., Yahr, T. L., Lovewell, R. R. & O'Toole, G. A. The *Pseudomonas aeruginosa* magnesium transporter MgtE inhibits transcription of the type III secretion system. *Infect Immun* 78 (2010).
10. Costerton, J. W. Overview of microbial biofilms. *J Ind Microbiol* 15, 137-140 (1995).
11. Hoiby, N., Frederiksen, B. & Pressler, T. Eradication of early *Pseudomonas aeruginosa* infection. *J Cyst Fibros 4 Suppl* 2, 49-54 (2005).
12. Ko, Y. H. & Pedersen, P. L. Cystic fibrosis: a brief look at some highlights of a decade of research focused on elucidating and correcting the molecular basis of the disease. *J Bioenerg Biomembr* 33, 513-521 (2001).
13. Gibson, R. L., Burns, J. L. & Ramsey, B. W. Pathophysiology and management of pulmonary infections in cystic fibrosis. *Am J Respir Crit Care Med* 168, 918-951 (2003).
14. Furukawa, S., Kuchma, S. L. & O'Toole, G. A. Keeping their options open: acute versus persistent infections. *J Bacteriol* 188, 1211-1217 (2006).
15. Merritt, J. H., Kadouri, D. E. & O'Toole, G. A. Growing and analyzing static biofilms. *Curr Protoc Microbiol Chapter* 1, Unit 1B 1 (2005).
16. O'Toole, G. A. & Kolter, R. Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol* 28, 449-461 (1998).
17. Gomez, M. I. & Prince, A. Opportunistic infections in lung disease: Pseudomonas infections in cystic fibrosis. *Curr Opin Pharmacol* 7, 244-251 (2007).

