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Microfluidic-based Growth and Imaging of Bacterial Biofilms

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[Abstract] Biofilms are a ubiquitous form of growth for bacteria on surfaces in most environments, natural or manmade. Here we present a protocol using the Bioflux microfluidic system to investigate the organized structure and development of these multicellular communities. Microfluidic systems present an opportunity to grow biofilms in a stable, physiologically maintained environment that is readily observable via time-lapse microscopy.
Keywords: Biofilms, Microfluidics, Bioflux, Microscopy, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, eDNA, EPS, Matrix, Expolysaccharides, Extracellular DNA

[Background] The Bioflux microfluidic system is a commercially available system that consists of a series of input and output wells linked by microfluidic channels set in a standard microplate format. Growth medium is flowed through the channel by application of controllable pneumatic pressure. The system allows for high throughput, reproducible growth of biofilms whilst avoiding the lengthy preparation required for 'home constructed' microfluidic set-ups.

The complex and heterogenous biofilms formed by bacteria have long proved challenging to cultivate and visualize. Traditional methods of biofilm growth, such as the use of abiotic surfaces placed in a bacterial growth medium suffer from myriad artifacts such as nutrient depletion and disturbance of the biofilm. Home-made flow systems alleviate many of these issues but remain labor intensive and unwieldy. The nature of the traditional flow systems renders them difficult to use in combination with time lapse microscopy, particularly as the large channel sizes means the continuous use of stains is costly. As a result, the structure and architecture of bacterial biofilms has so far been probed using 'snapshots' at arbitrary time points.

Here, we describe a simple protocol for the effective use of the Bioflux microfluidic systems, with a focus on the use of fluorescent probes to examine changes in the structure and architecture of the model biofilm-forming bacteria, *Pseudomonas aeruginosa* and *Staphylococcus aureus.*

This protocol can be easily adapted to different fluorescent stains, bacterial species, including complex multispecies biofilms such as oral biofilms. Data obtained are reproducible and biofilms are in line with the architectural features and dimensions reported in the literature.

Materials and Reagents

1. 48 or 24 well Bioflux plate (Fluxion Biosciences, catalog numbers: 910-004 and 9210-009)
2. Fluorescent dyes and stains

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To stain the biofilms several different fluorescent markers can be employed. Possible options include:

a. Fluorescent proteins. This protocol was developed using the red and green fluorescent proteins mCherry and eGFP (Zacharias *et al.*, 2002; Shaner *et al.*, 2004)
b. Live/dead BacLight dyes (Syto9 and Propidium iodide) (Invitrogen, catalog number: L7007)
c. Concanavalin A (Thermo Fisher, catalog number: C860), used at a concentration of 50 µg/ml. Stock solutions prepared to 1 mg/ml in PBS
d. Wheat germ agglutinin (Thermo Fisher, catalog number: W849), used at a concentration of 10 µg/ml. Stock solutions prepared to 1 mg/ml in PBS
e. YOYO-1 (Thermo Fisher, catalog number: Y3601), used at a concentration of 0.1 µM. Stock solution prepared to 10 µM in PBS
f. HHA and WFL (EY labs, R-8008-1 and F-3101-2), used at a concentration of 50 µg/ml. Stock solutions prepared to 1 mg/ml in PBS at room temperature with gentle agitation

3. Glycerol stock of *Pseudomonas aeruginosa* (*P. aeruginosa*). The protocol was developed using the strain PAO1-N
4. Glycerol stock of *Staphylococcus aureus* (*S. aureus*). The protocol was developed using the strains SH1000 and USA300 JE
5. $Na_2HPO_4 \cdot 7H_2O$ (Acros Organics, catalog number: 424380010)
6. KH_2PO_4 (Sigma-Aldrich, catalog number: P0662)
7. NaCl (Sigma-Aldrich, catalog number: S7653)
8. NH_4Cl (Sigma-Aldrich, catalog number: 11209)
9. $MgSO_4$ (Sigma-Aldrich, catalog number: 20894)
10. Glucose (Sigma-Aldrich, catalog number: G5400)
11. $CaCl_2$ (VWR, catalog number: IB110)
12. Tryptic soy broth (TSB) (Oxoid, catalog number: CM0129)
13. 70% v/v ethanol (Fisher Scientific, catalog number: 10517694)
14. M9 salts (see Recipes)
15. M9 minimal medium (see Recipes)

Equipment

1. Bioflux 200 system (Fluxion Biosciences, see https://bioflux.fluxionbio.com/systems)
   Note: This consists of an interface, moisture trap, tubing and pump.
2. Bunsen burner (Fisher Scientific, catalog number: 12842154) or flow hood (Fischer Scientific, catalog number: 10795194)
   Note: All manipulations of the Bioflux plate with the interface detached should be performed under aseptic conditions (i.e., manipulations should be performed inside a flow hood or adjacent to a lit Bunsen burner).
3. Heating stage (included with Bioflux 200 kit or bespoke temperature hood (see the webpage for

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guidance, https://www.microscopyu.com/references/live-cell-imaging-and-perfusion-chambers)

4. Appropriate personal protective equipment (PPE)
   Note: PPE should be worn throughout and consist of a lab coat (Fisher Scientific, catalog number: 12841388), safety goggles (Fisher Scientific, catalog number: 11952815), and nitrile gloves (Starlab microflex supreno su-int-l).
5. Inverted fluorescent microscope (we use a Nikon Ti Eclipse, https://www.microscope.healthcare.nikon.com/en_EU/products/inverted-microscopes/eclipse-ti2-series)
   Note: An inverted fluorescent microscope with the appropriate filter sets, a 20x objective and a motorized stage.
6. Computer (Hewlett Packard model z440)
   Note: A computer connected to the microscope system and running the listed software for control, acquisition and image analysis.
7. Incubator (Panasonic, model MIR-154_PE)
   Note: For the bacteria used in this protocol, temperature of 37 °C was used, and both a shaking and a static incubator would be required for overnight cultures and plates respectively.
8. Autoclave (Prestige, model: Medical ED2A Classic, https://www.prestigemedical.co.uk/products/classic-standard-121)

Software

1. ImageJ (https://imagej.nih.gov/ij/) or its packaged version FIJI (https://fiji.sc/)
2. NIS elements (Nikon)
3. Bioflux control software (Fluxion Biosciences)

Procedure

This protocol is for the growth of either *P. aeruginosa* or *S. aureus* in the Bioflux system. Where differences in the protocols are used, those for *S. aureus* are listed in square brackets.

1. Prepare all solutions, growth medium and reagents at least a day in advance of the experiment.
2. Place Bioflux plate and growth medium into a 37 °C incubator overnight to degas. Degassing is important to prevent formation of air bubbles in the microfluidic channels during the experiment.
3. Prepare cultures from a single colony on an agar plate to grow for 16 h at 37 °C with 250 rpm shaking.
4. The next day, pipette 100 µl M9 minimal medium [TSB] into the center of the outlet well (Figure 1) of a Bioflux plate in sterile conditions. Care should be taken to avoid introduction of air bubbles into the system when pipetting.

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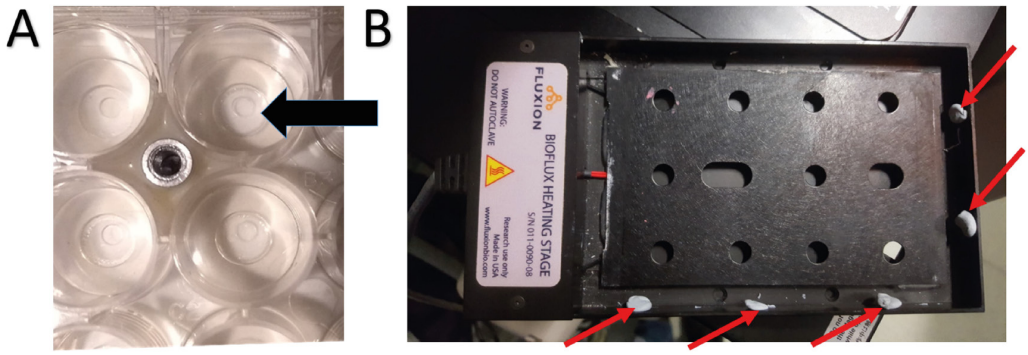

Figure 1. Layout and securing of a Bioflux plate. A. Growth medium should always be pipetted directly into the inner section of the outlet and inlet wells as indicated by the black arrow. This prevents entry of air bubbles into the system as the medium will completely cover the channel entrance. B. The positions to attach Blu tack to secure the Bioflux plate to the heated stage are indicated by the red arrows.

5. Sterilize the interface by wiping with 70% v/v ethanol and attach to the Bioflux plate using the screws.
6. Attach the Bioflux unit tubing to the appropriate port on the interface gasket.
7. Start the Bioflux control software (Figure 2), and flow from the outlet well at a shear rate of 2 dyn/cm^2 for 4 min. A droplet of media should form in the inlet wells.
8. Dilute the overnight culture to OD_{600} = 0.05 [0.1] in 0.5 ml growth medium. Pipette 50 µl of the inoculum into the outlet well. Add 200 µl M9 minimal medium [TSB] to the inlet well(s).
9. Re-attach the interface to the plate and then to the tubing.
10. Seed the channel by flowing from the outlet well at a rate of 2 dyn/cm^2 for 4 s. Care should be taken to ensure the bacteria being seeded does not contaminate the inlet well. Incubate the plate at 37 °C for a minimum of 45 min.

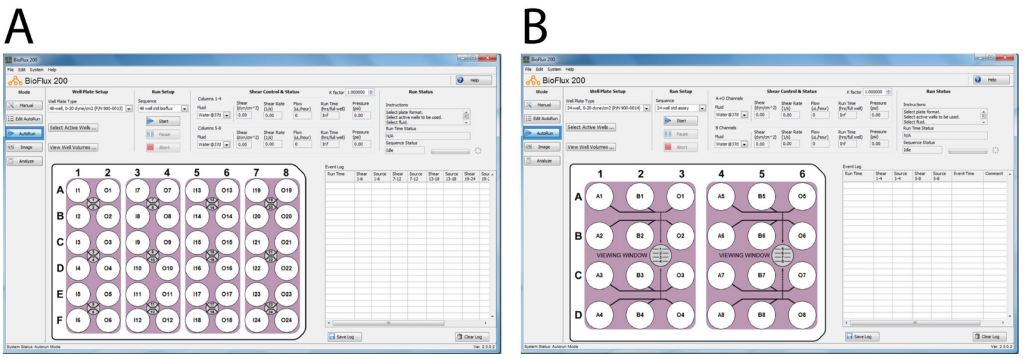

Figure 2. The Bioflux control software interface. Screenshot of the Bioflux software for the 48-well plate (Panel A) and also the 24-well plate (Panel B).

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11. Add the appropriate concentrations of fluorescent stains to the growth medium as indicated in the Materials and Reagents. The volume of media required per well varies by the experimental design and can be calculated by multiplying the shear rate by the number of hours the experiment will be run. For example, a typical experiment with *P. aeruginosa* run at 0.25 dyn/cm^2 for 16 h would require a minimum of 400 µl medium. However, to ensure the well does not run dry it is recommended to supplement the calculated volume with 100-200 µl extra medium. Place the prepared medium at 37 °C.
12. After 45 min, remove the interface from the Bioflux plate and pipette the prepared growth medium into the inlet wells being used. Sterilize the interface with 70% ethanol prior to attachment.
13. Securely fasten the Bioflux plate to the heated stage (or to the stage if using a temperature hood), ensuring the Bioflux plate sits flat on the stage.
14. Ensure the tubing connecting the Bioflux to the pump is free of obstruction and unlikely to get snagged during the experiment.
15. For a simple growth experiment using the 48-well plates, use the Bioflux control software to set a flow rate of 0.25 dyn/cm^2 [0.6 dyn/cm^2]. Run for 16 h, growth of microcolonies should be obvious. The run length can be altered depending on how rapidly a strain grows as a biofilm.
    Note: More complex experiments such as antibiotic-killing of the biofilm can be performed using the Bioflux 24 well plates:
    a. *Prepare growth medium containing antimicrobial agent of interest or other supplements as well as the required dyes as Step 11.*
    b. *Pipette this medium into inlet well 2 at Step 12.*
    c. *The Bioflux control software can be used to set up an automated program of flow. Flow from inlet well 1 at 0.25 dyn/cm^2 for the first 12 h, then set to run from inlet well 2 at 0.25 dyn/cm^2 for the remainder of the experiment.*
16. Set the microscope to acquire images every 20 min for 16 h with the appropriate filter sets. Figures 3 and 4 illustrate the structures and staining that can be obtained using these techniques.
17. Return periodically to ensure plane of focus of the microscope is maintained. This is important if only a heated stage is being used due to temperature fluctuations over time.
18. At the conclusion of the experiment, stop the Bioflux flow. Image acquisition automatically ends if the program is set up to end. Detach the interface and sterilize with 70% v/v ethanol.
19. Analysis of the data can be performed in ImageJ. For example, if doing live/dead analysis, the images obtained can be converted to an 8-bit binary image. Thresholds for images can set to remove the background fluorescence. The ratio of live to dead cells can also be presented for each time point.

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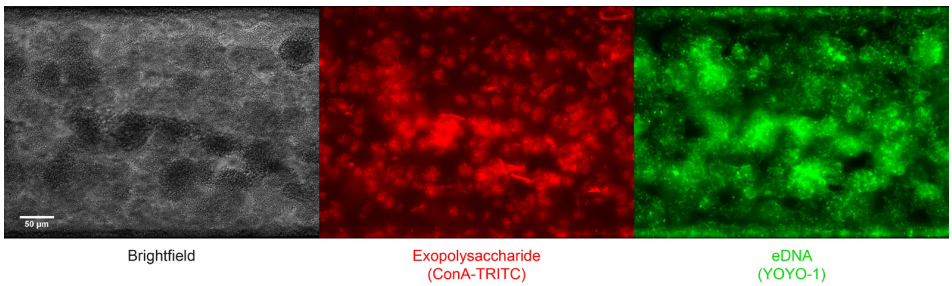

Figure 3. *Pseudomonas aeruginosa* PAO1 biofilm grown in M9 minimal media with the Bioflux for 16.5 h and visualized using Concanavalin A-TRITC and YOYO-1

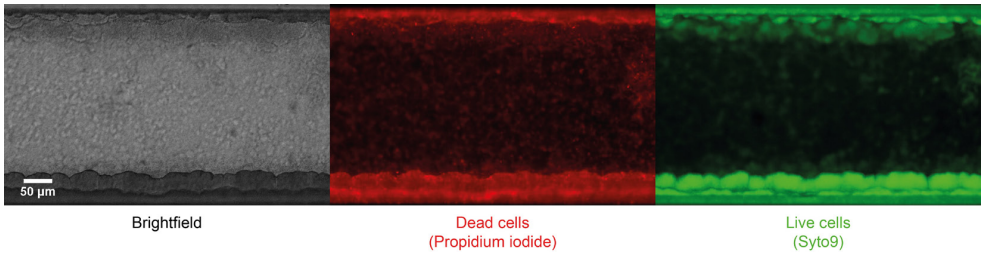

Figure 4. *Staphylococcus aureus* SH1000 biofilm grown in TSB with the Bioflux for 16 h and visualized using the Live/Dead stains Syto9 and propidium iodide

Notes

1. -80 °C freezer stocks of bacterial strains were prepared in 25% v/v glycerol.
2. Autoclaving of materials and reagents was at 121 °C for 15 min.
3. Constitutively expressed fluorescent proteins provide the easiest means to track the cells within a biofilm. Newer generation fluorescent proteins are recommended (Barbier and Damron, 2016).
4. The system can be used to explore gene expression in a biofilm through the construction of promoter fusions to a fluorescent protein such as GFP.
5. The stains Syto 9 and propidium iodide enables staining of dead and live cells. An alternative is to pair propidium iodide with a strain constitutively expressing GFP.
6. The lectin Concanavalin A is specific for α-D-mannose and α-D-glucose residues. Binds to the *P. aeruginosa* exopolysaccharide, Psl (Polysacharide synthesis locus) can be conjugated to a range of different fluorophores.
7. The lectin Wheat Germ Agglutinin is specific for sialic acid and N-acetylglucosamine residues found in the Gram-positive cell envelope and in the *S. aureus* exopolysaccharide, poly-N-acetylglucosamine.
8. YOYO-1 is a cell impermeant double stranded DNA dye that can be used to stain extracellular DNA with minimal staining of dead cells.

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9. The lectins HHA and WFL are specific to the *P. aeruginosa* exopolysaccharides Psl and Pel respectively. The lectins that can be conjugated to different fluorophores, but commercially available conjugated to TRITC and FITC.
10. A small amount of medium is added to the inlet wells at Step 8 to counter the effect of gravity on the flow of the bacteria from the outlet well during seeding.
11. Blu tack (Bostik) can be used to ensure the Bioflux plate fits securely on the microscope stage. Apply small amounts of the blu tack to the sides of the plate and not the base to ensure the plate is level.
12. A higher shear rate of 0.6 dyn/cm^2 is used for *S. aureus* experiments to prevent blockage of the microfluidic channel.

Recipes

1. M9 salts
   64 g $Na_2HPO_4 \cdot 7H_2O$
   15 g KH_2PO_4
   2.5 g NaCl
   5.0 g NH_4Cl
   Adjust to 1,000 ml with distilled H_2O
   Sterilize by autoclaving
2. M9 minimal medium
   Measure ~700 ml of dH_2O (sterilized by autoclaving)
   Add 200 ml of M9 salts
   Add 2 ml of 1 M $MgSO_4$ (sterilized by autoclaving)
   Add 20 ml of 20% w/v glucose (or other carbon sources: sterilized by autoclaving)
   Add 100 µl of 1 M $CaCl_2$ (sterilized by autoclaving)
   Adjust to 1,000 ml with dH_2O (sterilized by autoclaving)

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Competing interests

A small number of Bioflux plates were provided by Fluxion Bioscience.

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