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Establishment of Experimental Biofilms Using the Modified Robbins Device and Flow Cells

Luanne Hall-Stoodley, Joanna C. Rayner, Paul Stoodley, and Hilary M. Lappin-Scott

1. Introduction

1.1. *Properties of Biofilms*

Recent studies have shown that biofilms (a complex organization of bacterial cells present at a surface or interface, which produces a slime-like matrix) represent the principal form of bacterial growth in all environments studied to date (1). There are numerous advantages to bacteria growing in biofilms. These include extended protection against environmental changes, antimicrobial agents such as chemical disinfectants and antibiotics (2) and grazing predators such as amoebae (3), as well as providing increased access to limited nutrients (4). Biofilms are of interest in medical, industrial, and natural environments for several reasons. For example, they can act as reservoirs from which the dissemination of pathogens may occur. *Legionella pneumophila* has been shown to be harbored within biofilms formed within drinking water pipelines (5). Similarly, it is well established that biofilms can colonize numerous types of medical implants (6). In industrial systems, detrimental effects may occur following biofilm growth such as reductions in heat-transfer efficiency and flow capacity. Biofouling may also markedly increase corrosion (7). Finally, biofilms represent a bacterial architecture that may support genetic transfer, nutrient utilization, and biodegradation (8).

1.2. *Establishing Experimental Biofilms*

A major problem associated with the investigation of environmental systems is the inherent degree of complexity within a system. To facilitate the study of biofilms in the laboratory, simplified model systems have been devel-

oped that enable the growth of biofilms, along with the analysis of several defined parameters, under conditions that can be replicated. There are several model systems in current use (*see* Chapter 19). Among these, the modified Robbins device (MRD) and flow cells have many advantages and are readily adaptable to individual experimental systems. The major advantage is that they allow the study of biofilms under flowing conditions with controlled hydrodynamics.

The MRD was initially developed to study biofouling in industrial pipelines (8). It has since been modified and used to investigate biofilms from a number of environmental habitats. The primary advantage associated with the system is the number of colonized sampling ports available for analysis. This allows for several samples to be taken simultaneously as well as sampling more than a single time point in the development of the biofilm. Quantification of several aspects of the biofilm, such as viable and total cell counts, and total protein and carbohydrate content is therefore possible. Microscopic analysis is possible using conventional staining techniques of slide-mounted samples or electron microscopy of the colonized surfaces. The MRD is also relatively inexpensive. It can be used in both batch (recirculating) and flow-through culture systems and can be connected to a chemostat if close monitoring of growth conditions is required. Disadvantages of the MRD system include the inability to visualize the biofilm *in situ*, the possibility of nutrient gradients existing along the length of the device, and the possibility of compromised hydrodynamics around the sampling stud. Finally, the MRD is prone to the drawback shared by many systems utilized in the study of biofilms. For quantitative analysis of the biofilm to be carried out, destructive sampling techniques are required. Conventional techniques such as viable cell counts, total cell counts, and total protein or carbohydrate content analysis usually all involve disruption of the biofilm.

The continuous and nondestructive monitoring of biofilms is essential in understanding biofilm processes (9). There are several different types of flow cells suitable for many different experiments (10). Flow cells can also overcome many of the drawbacks of the MRD. Firstly, they allow *in situ* visualization of the biofilm in its hydrated form when used in association with computer-enhanced image analysis or a television camera. This is particularly advantageous because alteration of the biofilm by fixation or desiccation, which may have unknown effects on biofilm structure, are avoided. Also, using image analysis, accumulation rates can be calculated by comparing captured images with those at the outset of the experiment, allowing quantification of the growth

kinetics of the biofilm. Qualitative information regarding surface colonization is also possible. Second, the flat plate reactor (developed at the Center for Biofilm Engineering, Bozeman, Montana) can accommodate various surfaces so that they may be compared. The surface can be removed at the end of the experiment to enable either quantification of the biofilm by scraping and/or sonication of the surface, as in the MRD, or analysis using scanning electron microscopy (SEM).

A disadvantage of the flat plate reactor is that it is constrained by the channel thickness. This type of flow cell must necessarily be thin owing to the limitations of the working distance of the microscope objectives. Thus, there is a tradeoff between magnification and hydrodynamics (11). Another type of flow cell, the square glass tube reactor, overcomes this problem by facilitating higher magnification of the biofilm, allowing the flow cell to be viewed from above, and the channel depth is not restricted. This flow reactor was designed as a model to study biofilm biofouling in industrial pipelines. Most flow reactors that allow direct microscopic observation operate at low, laminar flow rates. However, this system can be operated at high, fully turbulent flow rates which are often more industrially relevant. The system can be operated using two parallel flow cells through which the flow rates can be independently controlled, allowing the influence of flow on biofilm structure and biofouling to be determined. The hydrodynamics of the square tube reactors have been well characterized using the relationship between the friction factor and the Reynolds number and fit well to established equations describing laminar and turbulent flow through a smooth pipe (12). They are also easy to make and adapt to particular experimental conditions. However, larger bore tubing requires thicker glass, thereby restricting magnification. Nevertheless, depending on the experimental conditions, there are several flow devices that permit analysis of biofilms.

2. Materials

2.1. Modified Robbins Device

1. Ethylene-oxide gas sterilized MRD (Fig. 1A) fitted with removable studs to which the surfaces of choice have been fitted.
2. Sterile replacement studs.
3. Bacterial culture reservoir (usually a glass flask) with an outflow connector and filtered air inlet.
4. Sterile medium reservoir with an outflow connector and filtered air inlet (*see Note 1*).
5. Sterile flask for waste collection.
6. Sterile silicon rubber tubing for connection of the MRD to the medium reservoir, bacterial culture reservoir, and waste flasks (*see Note 2*).

7. Peristaltic pump calibrated to give required flow rate (*see Note 3*).
8. Sampling equipment consisting of sterile scalpel blades, buffer solution, waste jar containing bleach or disinfectant, sterile test tubes containing diluent (e.g., 0.9 mL sterile buffer solution), forceps, scalpel blade holder, 70% alcohol, 5-mL pipet tips, and 5-mL Gilson pipet.

2.2. Flow Cells

1. Sterile closed channel reactor with an observation window consisting of a 24 mm × 60 mm glass cover slip held in place by a rubber gasket and metal flange (*see Note 4*).
 2. Sterile flask with outflow connector, filtered air inlet, tubing, flow breaks, and connectors for attachment of the flow cell.
 3. Sterile waste reservoir including inflow connector and filtered air outlet, tubing with flow break, and connectors for attachment to the flow cell.
 4. Peristaltic pump calibrated to desired flow rate.
 5. Water bath or heating or cooling units, if necessary, to keep test cultures at temperatures other than room temperature.
 6. Microscope.
 7. Camera (*see Note 5*).
 8. Computer with Framestore board (*see Note 6*).
 9. Image analysis software (*see Note 7*).
-
1. Flow cells made from sections of square glass tubing (S-103 Camlab, Cambridge, UK) 3 mm wide and 3 mm deep and 20 cm long (**Fig. 1C [13]**).
 2. Sterile nutrient reservoir.
 3. Peristaltic pump delivered with a recycle flow rate (*see Note 8*) controlled with a vane head pump (Masterflex, Cole-Parmer, Niles, IL).
 4. Flow meters (McMillan Flo-sensor model 101T #3724 and 3835 supplied by Cole-Parmer).
 5. Pressure transducers (RS Components, Corby, Northants, UK, model 286-686).
 6. Waste reservoir.
 7. Polycarbonate holder mounted on the stage of an upright microscope with epifluorescence capabilities. By positioning the flow cells on the holder, the biofilm can be imaged *in situ* without interrupting flow.
 8. Camera (*see Note 5*)
 9. Computer with Framestore board (*see Note 6*).

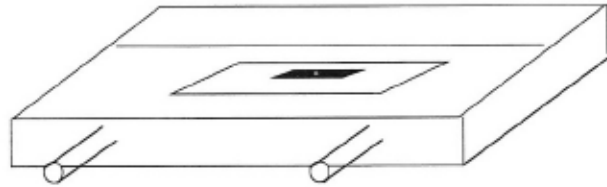
2.3. Suppliers

All of the described flow devices can be found on the following Web pages: for information on MRDs, contact Environmental Microbiology Research Group at Exeter University at <http://www.ex.ac.uk/biology/>

A Modified Robbins Device



B Flat Plate Reactor



C Square Glass Tube Reactor



Fig. 1. Three types of devices to study biofilms under flow conditions.

resrch.html#DrHMLappin-Scott; for information on flat plate reactors, contact BioSurface Technologies Corp. at <http://www.imt.net/~mitbst/flowcell.html>.

3. Methods

3.1. Modified Robbins Device

1. Cut silastic rubber, black backing discs using a 0.85-mm cork borer.
 2. Attach the surfaces, e.g., silastic rubber, glass, or plastic, of a known diameter to the black backing discs using a strong adhesive or waterproof sealant (*see Note 9*).
 3. Wipe the fitted surfaces with 70% alcohol solution and lint-free tissue and allow to air dry.
 4. Fit the studs into the MRD so that the surfaces for colonization lie flush with the central lumen.
 5. Wipe the MRD with 70% alcohol and seal in gas-permeable bags.
 6. Package 25 replacement studs in batches of approx 4 studs per bag to prevent contamination during the course of the experiment.
 7. Sterilize the MRD using ethylene oxide gas (*see Note 10*).
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1. Remove the MRD from the gas-permeable bag and check for loose surfaces.
 2. Remove any surfaces, that have become detached during sterilization.

3. Connect the MRD to the culture, medium, and waste reservoirs using wide-bore, sterile silastic rubber tubing (*see Fig. 2* for experimental setup).
4. Inoculate the culture reservoir to give an appropriate planktonic viable cell count, e.g., 3% v/v exponential bacterial broth culture and incubate for 18 h or overnight.
5. Inoculate the MRD surfaces. Turn on the peristaltic pump and ensure that the inoculated culture is moving through the MRD system and into the waste jar. After the initial biofilm has been formed (e.g., by inoculating the surfaces for 24 h), quickly switch the system to flowthrough with only the sterile medium and control agent (antibiotic or biocide), by changing the open and closed clamps.
6. Maintain a low rate of flow during the switchover to prevent backflow of liquid through the system.
7. Remove any air bubbles from the MRD by turning it upside down and tilting at a 45° angle for a few minutes while under the normal flow conditions.

1. Switch off the pump at the appropriate time period, and clamp the silastic tubing at either end of the MRD.
2. Remove a stud (determine using random number tables) and immediately replace it with a sterile replacement stud.
3. Hold the removed stud above a pot containing disinfectant or bleach solution. Rinse to remove any nonadherent bacteria by pipetting 10 mL of sterile buffer solution gently onto the side of the stud, so that the flow is not directed at the immediate colonized surface (*see Note 11*).
4. Place both the scalpel blade and the scraped surface into a test tube containing sterile buffer solution (for techniques for the analysis of biofilm formation *see Subheading 3.2.*).
5. Sonicate the scalpel blade and surface for approx 5 min to disperse the biofilm and any clumps of cells.
6. Place the used MRD stud into the pot of disinfectant.
7. Repeat for appropriate number of samples.
8. Spray the MRD with 70% alcohol after sampling, wipe, and remove the clamps.
9. Switch the pump back on and turn the MRD upside down to remove any air bubbles formed during sampling.

1. Empty the tubing and the MRD by tipping the reservoirs while maintaining the pump rate so that no liquid is taken up into the system.
2. Spray the tubing with 70% alcohol and disconnect from the reservoirs and the MRD.
3. Seal all open ends of tubing with aluminum foil and autoclave.
4. Soak the MRD in bleach or disinfectant for approx 12–24 h (increase the time of disinfection depending on thickness/viscosity of biofilm). Do not soak for longer than 48 h.

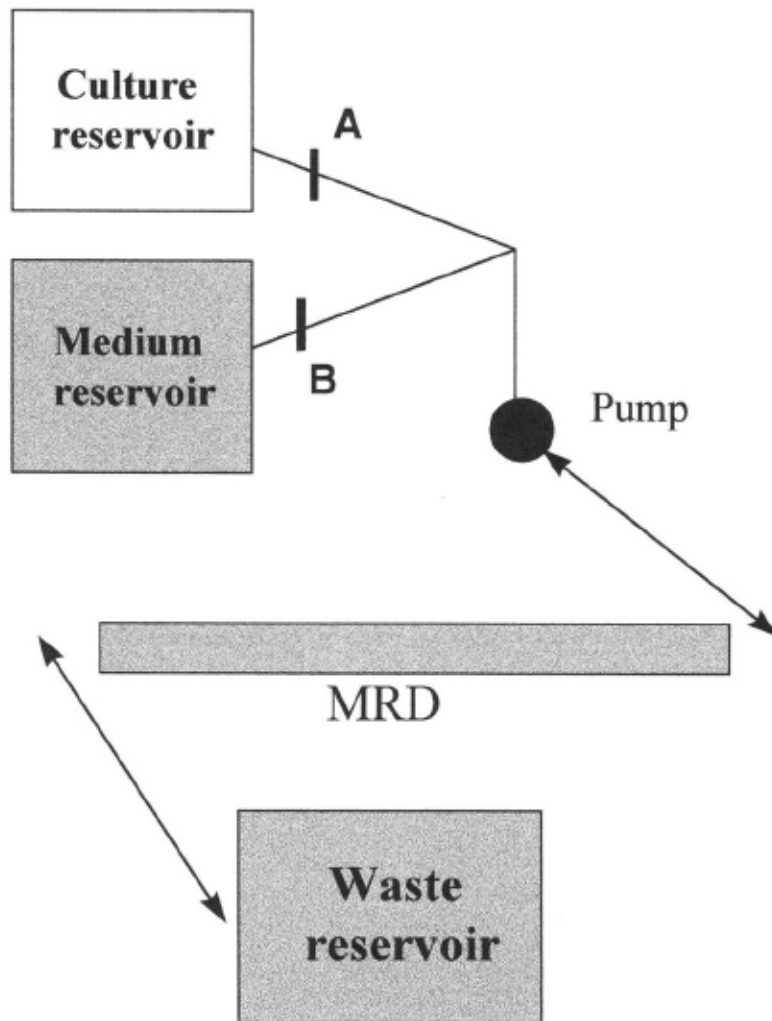


Fig. 2. Schematic diagram of MRD experimental setup. (A) and (B) indicate clamps: B closed = inoculation of surfaces, and A closed = flowthrough with only steril medium.

5. Connect to a tap and rinse in cold, running water for 8–12 h to remove residual disinfectant.
6. Soak the used studs in bleach or disinfectant for approx 8 h and then rinse in continuous running water for a further 8-12 h.
7. Allow the MRD and studs to dry prior to reassembling.
8. Autoclave all medium, waste, and inoculum reservoirs as appropriate (increase autoclaving time for large volumes).

3.2. Experimental Measurements

1. Vortex the tube containing the surface and scalpel blade for 15–20 s after sonication (**Subheading 3.1.3.**).
2. Serially dilute in buffer and plate out on an appropriate solid growth medium.
3. Calculate the numbers of viable cells per square centimeters of surface using the following equation:

$$\text{Number of bacterial colonies} \times 10 \text{ (biofilm removed is assumed to constitute 0.1 mL)} \times Df \times 1/As = \text{bacteria/cm}^2$$

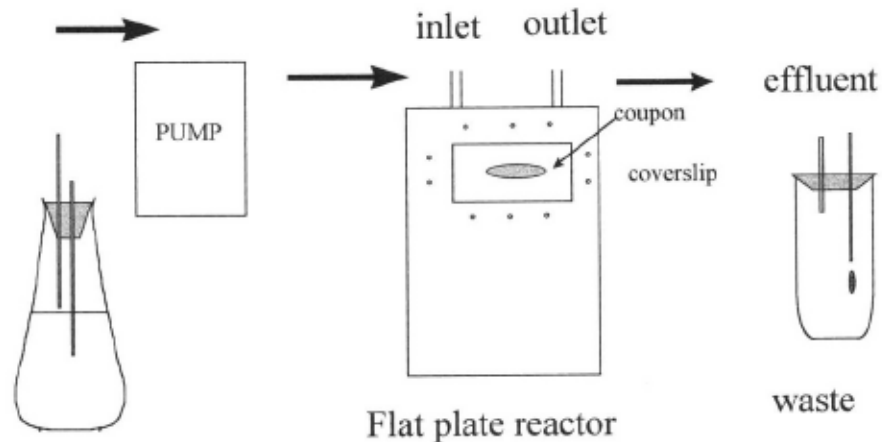
where Df = dilution factor and As = area of surface in square centimeters (*see Note 12*).

1. Fix the surface at room temperature for 2 h or overnight at 4°C (**Subheading 3.1.3.**).
2. Remove from the buffer and use a successive ethanol series to dehydrate the sample: start with a concentration of 30% and work through 50, 70, and 100% ethanol for 3 min.
3. Remove the solution after 3 min and discard, then replace with the next solution in the series.
4. Place the stud onto an SEM mount, coat with silver using a palladium catalyst, and view using SEM (*see Note 13*).

1. Scrape one to three surfaces into glutaraldehyde cacodylate buffer (*see Note 11*).
2. Fix at room temperature for 2 h or overnight at 4°C. Samples may also be frozen and processed at a later date.
3. Serially dilute the sample in buffer solution and filter 3–5 mL onto a black 0.2- μm polycarbonate membrane. Apply and then release the vacuum.
4. Stain with 1 to 2 mL (enough to cover membrane) of a 0.1 mg/mL acridine orange solution (in phosphate buffer) for 3 min.
5. Reapply vacuum and while running destain with 1.5 mL of isopropyl alcohol.
6. Remove filter and air dry on filter paper, view using a $\times 100$ oil immersion lens under epifluorescence microscopy, with a calibrated eyepiece graticule, a mercury lamp and acridine orange filter block (emission wavelength of 488–514 nm).
7. Count the fluorescently stained cells in approx 10 fields of view and average to calculate the total cell count.

3.3. Flat Plate Reactor

1. Calibrate pump to desired flow rate by volumetric displacement prior to autoclaving all tubing and connectors.



Culture or nutrients

Fig. 3. Example of flat plate reactor system with nutrients and waste reservoir.

2. Autoclave the flow cell (Fig. 1B) after fitting with the coupon or test material, and cover with a rubber gasket, glass cover slip, and metal covering.
3. Grow the bacterial culture to the desired density and attach to pump, flow cell, and waste reservoir via sterile silicon rubber tubing (see Fig. 3).
4. Initiate flow (see Note 14) and monitor flow cell, tubing, and connectors for leaks. Tighten seals if necessary.
5. Monitor the biofilm at various time points depending on the experimental design (see Note 15).
6. Autoclave the tubing, reservoirs, and flow cell at the end of the experiment, and rinse well in running water to remove any biofilm residue.
7. Replace any tubing if necessary.
8. Clean the flow cell with 70% ethanol to remove any remaining residue, and fit with a new surface before autoclaving in preparation for the next experiment.

3.4. Square Glass Tube Reactor

The square glass tube reactor flow system (see Fig. 4) was designed to have laminar flow in one flow cell and turbulent flow in the other.

1. Measure the flow rate through each of the flow cells (Q_f) using flow meters controlled independently by tightening or loosening clamps on the inlet tubing. The average flow velocity (u) is calculated from:

$$u = Q_f / CSA \quad (1)$$

where CSA is the cross sectional area (in this case $9 \times 10^{-6} \text{ m}^2$). The Reynolds number is found from:

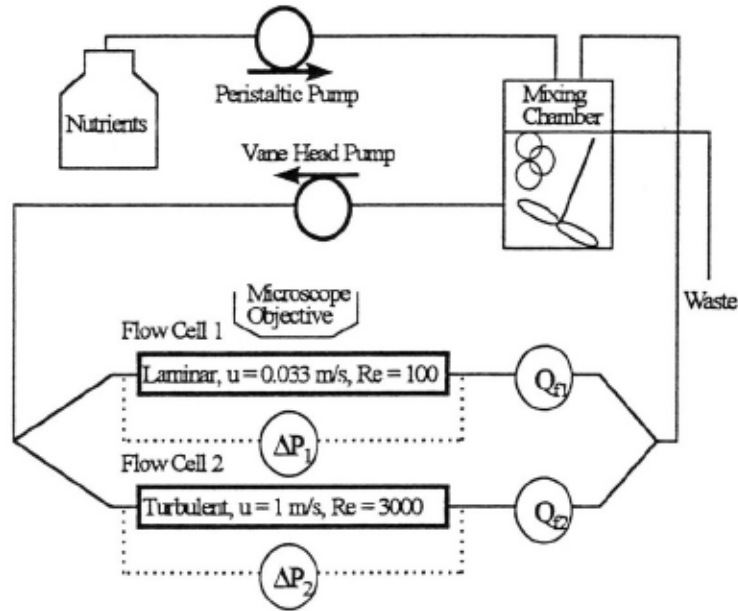


Fig. 4. Example of biofilm reactor system consisting of parallel flow cells in a recycle loop attached to a mixing chamber. The mixing chamber was aerated and the level maintained by overflow to waste.

$$Re = uD_h/\nu \quad (2)$$

where ν is the kinematic viscosity of the media (for low-nutrient media the value for water can be used), D_h is the characteristic length, which in this case is the hydraulic diameter calculated from:

$$D_h = 4CSA/WP \quad (3)$$

WP is the wetted perimeter of the flow cell, $2(\text{width} + \text{depth})$. For these flow cells, $D_h = 3 \times 10^{-3}$ m. The Reynolds number is a dimensionless number commonly used by engineers to characterize flow conditions. It is particularly useful because it predicts whether flow will be laminar or turbulent and can be used as a comparative parameter for a diverse range of flow systems.

- Determine the pressure drop (ΔP) across each flow cell using differential pressure transducers. ΔP can be used to find the Fanning friction factor (f):

$$f = (\Delta P \times D_h)/2l_p\rho_w u^2 \quad (4)$$

where ρ_w is the density of liquid media and l_p is the distance between pressure ports (14). f is also a dimensionless number and can be used as an indicator of biofouling. The predicted f for laminar flow through a smooth (clean) pipe (from the Hagen–Poiseuille equation) is:

$$f = 16/Re \quad (5)$$

and in the turbulent region f is predicted from the Blasius formula:

$$f = 0.0791/Re^{0.25} \quad (6)$$

The relationship between Re and f for 20-cm long flow cells showed that the transition between laminar and turbulent flow occurred at $Re = 1200$ ($Q_f = 3.15 \text{ cm}^3/\text{s}$). To increase the sensitivity of the ΔP measurement, the flow cells can be lengthened.

3. At the end of the experiment, biofilms can be fixed with 1% paraformaldehyde (30 min) and stained with nucleic acid stains such as propidium iodide (0.4%, at 25°C for 30 min).
4. Biofilms can be imaged *in situ* using confocal laser scanning microscopy, transmitted light microscopy, or epifluorescent ultraviolet microscopy, all of which can be used in conjunction with image analysis.
5. Biofilm accumulation can be routinely monitored (e.g., by obtaining surface area coverage data) and related to changes in pressure drop.
6. Metabolic activity of the biofilms can be examined using the metabolic stains such as 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) in which flow cells are removed from the reactor system and stained with CTC (0.04 % w/v) for 30 min at 25°C in a shaker incubator.

4. Notes

1. Antibiotics or biocides may be added to the medium reservoir after autoclaving if testing susceptibility of biofilms to antimicrobial agents.
2. Insert metal or thick-walled glass tubing into rubber bungs and place them into the sterile medium, waste, and inoculation reservoir flasks. After sterilization, the silastic rubber tubing can then be attached to the reservoirs via the tubing.
3. Precalibrate the pump to the required flow rate by using a nonsterile system with water in place of the culture or growth medium.
4. Certain designs allow a desired surface to be fitted with various test materials.
5. We use a COHU 4612-5000 charge-coupled device (Cohu, San Diego, CA).
6. We use a Scion VG-5 PCI (Scion, Frederick, MD).
7. We use the NIH-Image 1.59 program from the National Institutes of Health, available from the Internet by anonymous FTP from zippy.nimh.nih.gov or floppy disk from the National Technical Service, Springfield, VA, part no. PB95-500195GEI.
8. The volume (V) of the mixing chamber and recycle loop, including the flow cells, was approx 175 mL. The nutrient influent flow rate (Q_n) was 4.3 mL/min, giving a resulting residence time ($\theta = V/Q_n$) of 40 min.
9. When cutting surfaces, ensure that the diameter of the surface does not exceed that of the sample port; otherwise, this may interfere with removal of the stud from the MRD and the surfaces may become detached.
10. Autoclaving and chemical disinfectants damage the MRD and O-rings used to ensure a tight seal where the studs are fitted into the MRD (*see Fig.1A*). Check

for leaks on the MRD and in the areas where the tubing is joined to the MRD. Spray with 70% alcohol, wipe, and then seal with a quick-drying waterproof aquarium sealant where necessary.

11. If sampling for SEM, place the rinsed surface in 3–5% glutaraldehyde buffer (25% SEM grade glutaraldehyde diluted in 0.1 M cacodylate buffer). If sampling for viable cell counts, total cell counts, total protein, or carbohydrate, use a sterile scalpel blade to scrape the biofilm from the colonized surface (7–10 times should be sufficient to remove the adherent cells).
12. The planktonic viable cell count in the system can also be monitored in order to check sterility and cell growth by removing 0.1-mL samples, carrying out serial dilutions, and plating out on appropriate growth medium.
13. Alternatives to the use of the ethanol series include critical point and air drying of the sample. Once dehydrated and coated, samples can be stored for 1 to 2 wk until required.
14. Determine planktonic cells at the initiation of flow. A sampling port near the effluent interrupted by a flow break to reduce the possibility of contamination allows easier access.
15. For example, initial colonization events may be monitored in the first 24 h, or a biofilm of a certain thickness may be grown before examination. Biofilm thickness may be measured microscopically by focusing on the substratum of the cell cluster and then on the surface of the cell cluster and noting the difference on calibration on the fine focus adjustment (12). It is important to determine the optimal working distance between the microscope objective and the flow cell and to use the appropriate objective lens. Once this is established, surface area, as well as heights and areas of cell clusters can be compared to previous images. The appropriate software allows for images to be linked and provides a virtual record documenting changes over time. Length and width of cells may be measured, and all the assays outlined for the MRD are possible, but at only one time point. Focusing on a single area of the biofilm enables a cell cluster or groups of clusters to be monitored with time. Such images may be animated to provide a real-time record of cell attachment, aggregation, and sloughing, as well as the evolution of the biofilm with time.

The flat plate reactor flow cell is easily disassembled at the end of the experiment and the coupon can be removed and subjected to the same experimental measurements and quantitative sampling used in the MRD, that is rinsing and sonication of the coupon resulting in disruption of the sessile organisms to yield viable cell counts (*see Subheading 3.1.*) However, viable cell determination of the colonized surface and SEM is available only at the final time point to maintain sterility.

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