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Bursting the bubble on bacterial biofilms: a flow cell methodology

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The flow cell biofilm system is an important and widely used tool for the *in vitro* cultivation and evaluation of bacterial biofilms under hydrodynamic conditions of flow. This paper provides an introduction to the background and use of such systems, accompanied by a detailed guide to the assembly of the apparatus including the description of new modifications which enhance its performance. As such, this is an essential guide for the novice biofilm researcher as well as providing valuable trouble-shooting techniques for even the most experienced laboratories. The adoption of a common and reliable methodology amongst researchers would enable findings to be shared and replicated amongst the biofilm research community, with the overall aim of advancing understanding and management of these complex and widespread bacterial communities.

Keywords: biofilm; flow cell; bubble trap; *Pseudomonas aeruginosa*; method; protocol

Introduction and background

It is now well established that microbial populations exist in sessile biofilm communities. Biofilms are complex structures consisting of a high density of bacterial populations embedded in a self-produced polymeric matrix, often on submerged surfaces or as flocs in the water column of aquatic environments (Costerton et al. 1995; Hall-Stoodley et al. 2004). Though the importance of biofilms was realised many decades ago (Costerton et al. 1999), the last two decades have seen an exponential growth in biofilm research.

Laboratory methods for culturing surface attached biofilms are varied and their use depends upon the question being asked. Batch culture methods, such as growth within microtitre plates, enable high throughput but do not allow for detailed microscopy of the biofilm (Merritt et al. 2005; Peeters et al. 2008). Abiotic surfaces, which can be placed in a bacterial growth medium such as glass, enamel or steel coupons allow for visualisation by microscopy but suffer from the artifice of nutrient depletion (Coenye and Nelis 2010). The rising profile of biofilms, especially in a medical context, raises the need for direct microscopic examination of biofilms and hence the development of flow cells with glass surfaces.

Flow cell methods allow for growth of mature biofilms in the absence of planktonic cells which are removed by flow. When coupled with fluorescence microscopy, such systems enable the researcher to observe, non-invasively, the growth, structure and physiology of a live, hydrated, adherent population over time (Pamp et al. 2009). Though they provide a powerful tool to perform detailed investigations of multiple biofilm parameters, such experiments are difficult to assemble and perform. This paper provides a detailed user guide for constructing and running the biofilm flow cell system and in addition, incorporates novel modifications which result in a marked reduction in the formation of problematic air bubbles.

The current flow cell 'gold standard'

The most widely favoured apparatus is the flow cell, constructed of polystyrene mounted with a microscope slide and connected by tubing to an inlet medium vessel and an outlet waste container (Wolfaardt et al. 1994). The flow of liquid medium is achieved by a multichannel peristaltic pump, usually positioned upstream of the flow cell. The flow cells are inoculated with a suspension of microorganisms from a syringe and following incubation, are mounted directly on a microscope for the microscopic observation and imaging of the biofilm. This system is well suited for the study of flow conditions and allows for the control

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of growth parameters such as nutrient composition of the medium, incubation temperature and flow rate.

As biofilms are complex three-dimensional structures, traditional light microscopy fails to provide optimal images. There is a loss of resolution in thick biofilms due to the contribution from unfocused parts of the viewing field. The direct non-invasive and non-destructive examination of biofilms by confocal laser scanning microscopy (CLSM) is therefore preferred. Digital images are acquired by photomultiplier tube detection of fluorescence excited by a laser source. By collecting fluorescent light from only the thinnest focal plane afforded by the objective lens and by scanning several planes interspersed by short distances, it is possible to reconstruct virtual 3D images of the biofilm structure tens of microns thick, aided by powerful image-analysis programmes such as IMARIS (Bitplane) and ImageJ (free and open source software). Furthermore, the digital data captured can be used to calculate biofilm parameters using software programmes such as COMSTAT (Heydorn et al. 2000) or PHLIP (Mueller et al. 2006).

The merits of the flow cell system make it a popular *in vitro* model and whilst pre-assembled systems can be purchased, devices constructed in-house are considerably less expensive and offer the researcher greater versatility. For the novice researcher however, the system can be technically challenging to assemble. Furthermore, users often struggle with the problem of frequent air bubble formation within the system. Air bubble formation can be a consequence of: (i) changes in the temperature of the liquid medium, (ii) pressure changes due to changes in diameter of the tubing and (iii) the actions of the peristaltic pump.

Air bubbles lead to the destruction of developing biofilm architecture. Based on the authors' experience with flow cell work, it is estimated that the incidence of bubble formation is approximately 1 in 3 experiments when the conventional flow cell systems are run at 37°C. This is severely limiting as experiments in which bubble formation is recorded have to be discarded and possibly repeated. Measures to reduce bubble formation include the use of upstream bubble traps and pre-inoculation agitation of the flow cells to dislodge bubbles. Some researchers operate their system at room temperature or 30°C, as there is usually no bubble formation at these lower temperatures. However, these approaches have limitations as the use of bubble traps may open the system to contamination and ambient temperatures may not be representative of human infections. In the authors' experience, the modified system described here does not suffer from bubble formation at 37°C and therefore a large saving in time and effort is made by the researcher.

It became apparent that analogous flow equipment is used in the process of renal dialysis, in which a patient's blood is circulated *via* an ex*vivo* system of tubing and a dialysis membrane to remove toxins and waste products. It is imperative in such a system that no air bubbles are introduced as these would lead to 'air embolus' formation which impedes the circulatory flow of blood to an organ and leads to tissue infarction, potentially fatal in the brain or heart. Advice was sought from the local renal dialysis team as to how their experience could be applied to the flow cell biofilm system. The description presented here, of the steps and materials needed to construct a flow cell system, incorporates these modifications, which have helped the authors to minimise bubble formation in their flow cell systems. This is illustrated with rendered CLSM-captured images.

Flow cell experimental procedure

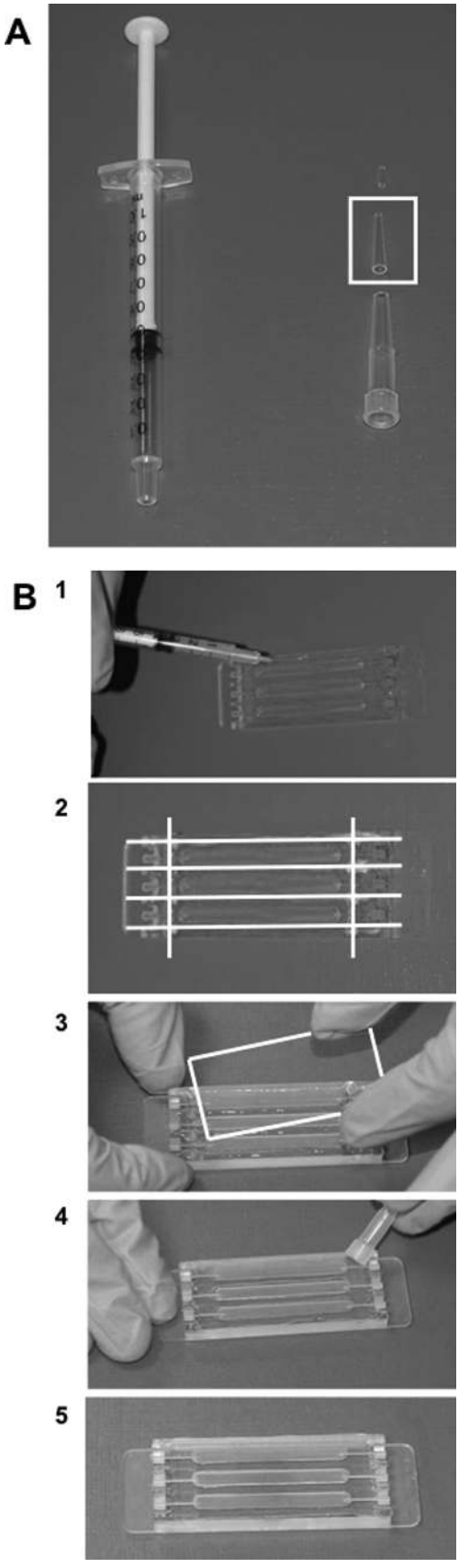
Construction of a flow cell

Whilst it is possible to mill a flow cell in-house from polycarbonate sheeting using a tooling or drilling machine (Tolker-Nielsen and Sternberg 2011), machine-made flow cells can be purchased (eg from DTU Systems Biology, Technical University of Denmark) and were used in preference as they represent a standardised product and thus data from biofilms grown in different flow cells can be compared and pooled.

A flow cell is composed of three parallel channels machine-cut in perspex (poly[methyl methacrylate]), covered with a no. 1 24 × 50 mm glass coverslip (SLS Ltd) which serves as the biofilm substratum. Each channel has a dimension (length × width × height) of 40 × 4 × 4 mm and was cleaned with 96% (v/v) ethanol prior to use (Figure 1).

To assemble the flow cell, a thin continuous layer of clear silicone gel (RS Silicone Rubber Compound – Flowable Fluid, RS 692 542) was applied 'sausage-like' between the channels using a 2 ml syringe (Terumo)

Figure 1. Representation of a 3-channel flow cell base. Each channel measures 40 × 4 × 4 mm. The 1 × 3 mm silicone tubing attaches directly to the inlet and outlet channel as shown (design copyright Biocentrum-DTU 2005).



with a cut 200 μl pipette tip inserted as a nozzle. The silicone glue was applied as very thin threads between each channel and along the perimeter of the top of the base, with care taken to avoid holes in the glue threads, which would lead to liquid leakage from the flow cell (Figure 2). The glass coverslip was placed on top of the silicone and carefully pushed down (the handle of the syringe piston provides an excellent tool for this purpose) until the silicone covered the whole area of perspex between the channels, without entering the channels themselves. If areas with insufficient gluing were observed, they were re-sealed by applying extra glue outside the flow cell adjacent to the potential leak. The silicone was allowed to dry overnight before use. Silicone tubing (Versilic) with the dimensions 1 mm inner diameter (ID) and 3 mm outer diameter (OD), was then connected to each end of the flow channel.

Assembly of flow cell system

The traditional flow cell system usually comprises an inlet autoclavable medium vessel (Nalgene Company) housing the sterile medium appropriate for the micro-organisms and type of biofilm being grown, a peristaltic pump (Ismatec) to drive the flow of the liquid medium, bubble traps (DTU Systems Biology, Technical University of Denmark), the flow cell within which the biofilms are cultivated and subsequently viewed and an outlet waste container. These individual components were connected by silicone tubing (Versilic) and different sized plastic connectors and T-connectors (Cole Parmer) except for the portion of tubing that passed through the peristaltic pump, for which the stronger Marprene tubing, ID 0.88 mm (Watson Marlow Ltd, Cornwall, England) was used. (See Figure 3A.)

Modifications to minimise bubble formation

In order to minimise air bubble formation, a number of modifications were made to the traditional flow cell system (Figure 3B). Firstly, the inlet growth medium bottle was rendered airtight with silicone glue around

Figure 2. Construction of the flow cell. The silicone gel (RS Silicone Rubber Compound – Flowable Fluid, RS 692 542) dispenser is composed of a 1 ml syringe (Terumo BS–01T) and the middle part of a 200 μl pipette tip cut into three parts (A). The dispenser is used to place a grid of 'sausage-like' silicone lines on the flow cell along the edges of each channel and across the ends (B1,2). The coverslip is carefully placed on top of the silicone (B3). Gentle pressure can be applied using the end of the pipette tip (B4) until the silicone has reached the edges of the channels but does not protrude into them (B5). The silicone is left to set overnight.

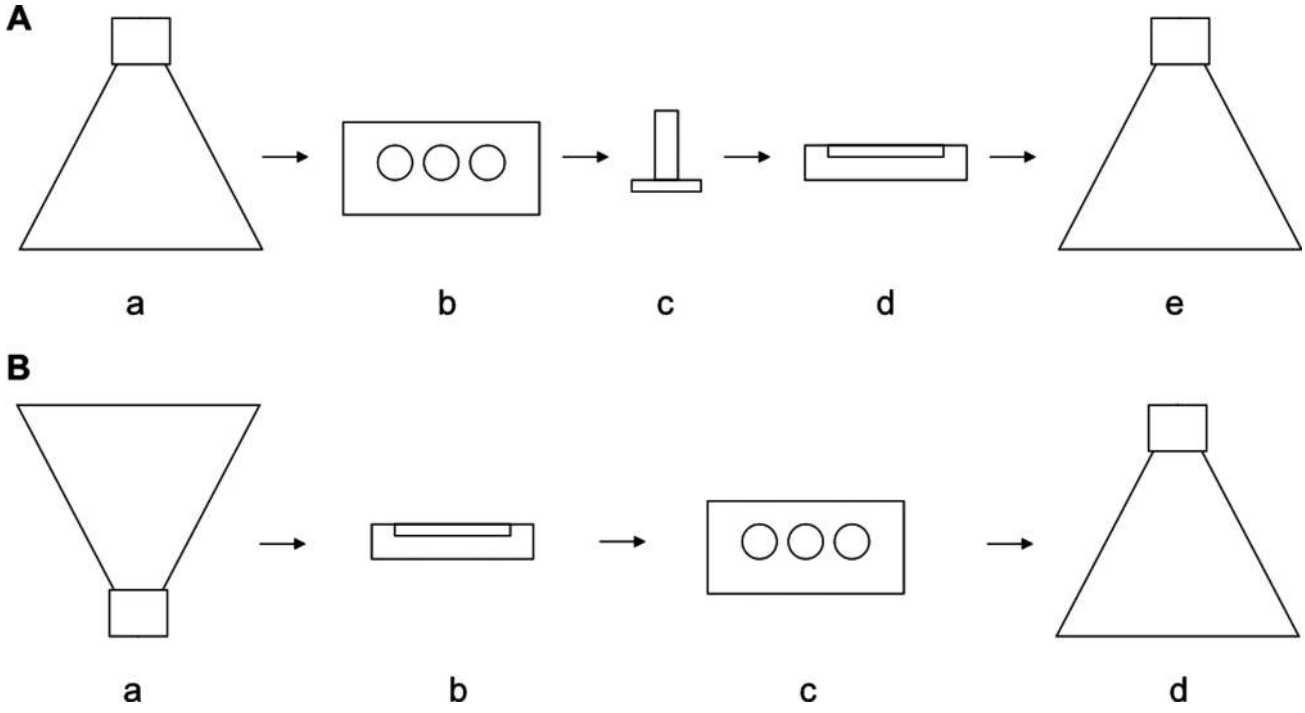


Figure 3. Schematic diagram of the components of a flow cell system. (A) The traditional order of connection used in previous studies comprises a. medium bottle; b. peristaltic pump. c. syringe bubble trap; d. flow cell; e. waste bottle connected *via* flexible silicone tubing providing a closed system. Arrows represent the direction of flow. (B) The modified system comprises a. inverted medium bottle; b. flow cell; c. peristaltic pump; d. waste bottle. The bubble trap has been removed from the design.

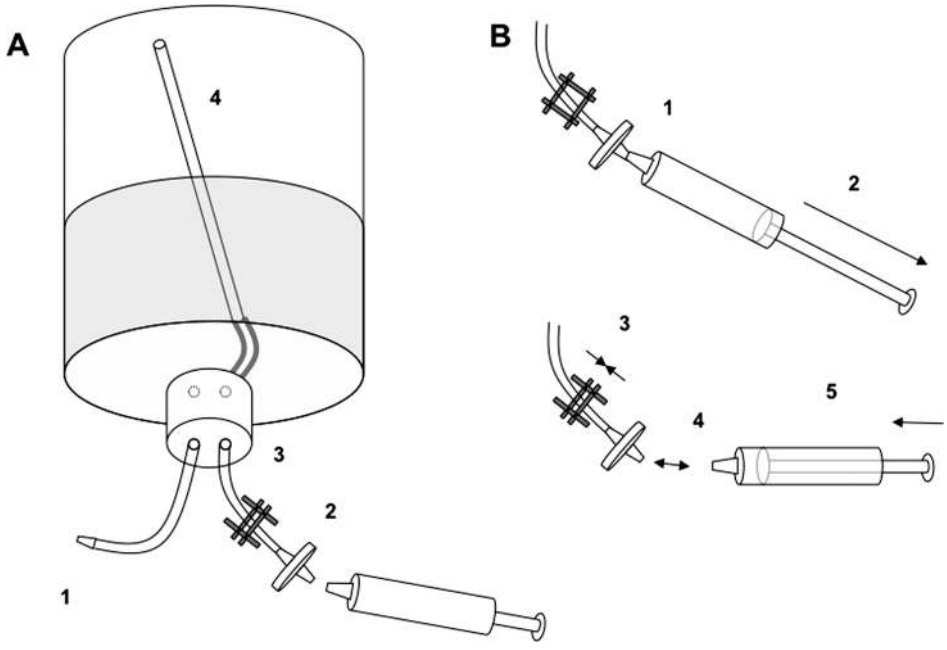


Figure 4. Construction of medium vessel (A) and creating a negative pressure gradient from the inside to the outside of the vessel to de-gas the medium (B). Growth medium flows out through silicone tubing and a connector (A1). The connector can be wrapped in tin foil before autoclaving to maintain sterility. Filtered air enters the vessel through a filter and silicone tubing connected to the vessel (A2). The junction between the tubing and the outside of the vessel can be sealed with silicone glue to prevent leaking (A3). Filtered air is carried to the top of the vessel by silicone tubing and a rigid glass tube (eg Pasteur pipette, A4). A pressure gradient can be created by attaching a 50 ml syringe to the filter (B1), drawing air out (B2) and then clamping the air inlet tube (B3). Once the syringe is detached and the air within released, these steps can be repeated to increase the negative pressure gradient. Small bubbles of gas may be observed escaping from the liquid medium. Note the clamp on the air inlet tube should be released before resuming flow.

its entry ports and fitted with an internal non-collapsible tube connected to a venting air filter (Midistart 2000 0.20 μl pore, Sartorius). The vessel was then inverted and suspended above the level of the flow cell on a retort clamp stand, allowing the flow of medium by gravity, thus reducing the work of the peristaltic pump. This also served to reduce the negative pressure gradient created within the tubing by the pulling action of the pump, which itself can lead to air bubbles being drawn out of solution. This is in keeping with the manner in which intravenous fluids are usually administered in a healthcare setting.

Prior to running the system, the growth medium outlet port was clamped off and using a 50 ml syringe, air within the inlet vessel above the medium was drawn off *via* the air filter in order to create a negative pressure gradient within the vessel above the growth medium. This allowed any dissolved gases within the medium to be drawn out of solution. In order for this measure to be effective, the seal on the inlet vessel had to be airtight and there could not be any gaps in the silicone seal between the lid and the inlet/outlet tubes. The system was then allowed to equilibrate prior to running. (See Figure 4.)

A further action to reduce air bubble formation was to prevent the liquid medium from cooling after autoclaving by placing it immediately at the correct temperature for the experiment. If the medium is colder than the ambient temperature of the experiment, air bubbles tend to emerge throughout the system if it is running as the temperature of the medium rises.

It was observed that the multi-channel peristaltic pump itself could introduce bubbles into the system, which were able to enter and lodge within the flow cells when the pump was positioned upstream of the flow cells. This occurred despite the incorporation of bubble traps between the pump and the flow cells. Hence, the pump was moved downstream of the flow cells and the bubble traps were removed from the design. This modification, whilst greatly simplifying the design and assembly of the system and removing a potential source of contamination, had the added advantage of allowing the pump to gently pull non-attached free-floating biofilm material distally out of the channel so that it did not interfere with the subsequent microscopy. (See Figure 5.)

Sterilisation and saturation of flow system

Sterilisation of the flow system was performed by pumping 1 l of 0.5% (v/v) sodium hypochlorite bleach

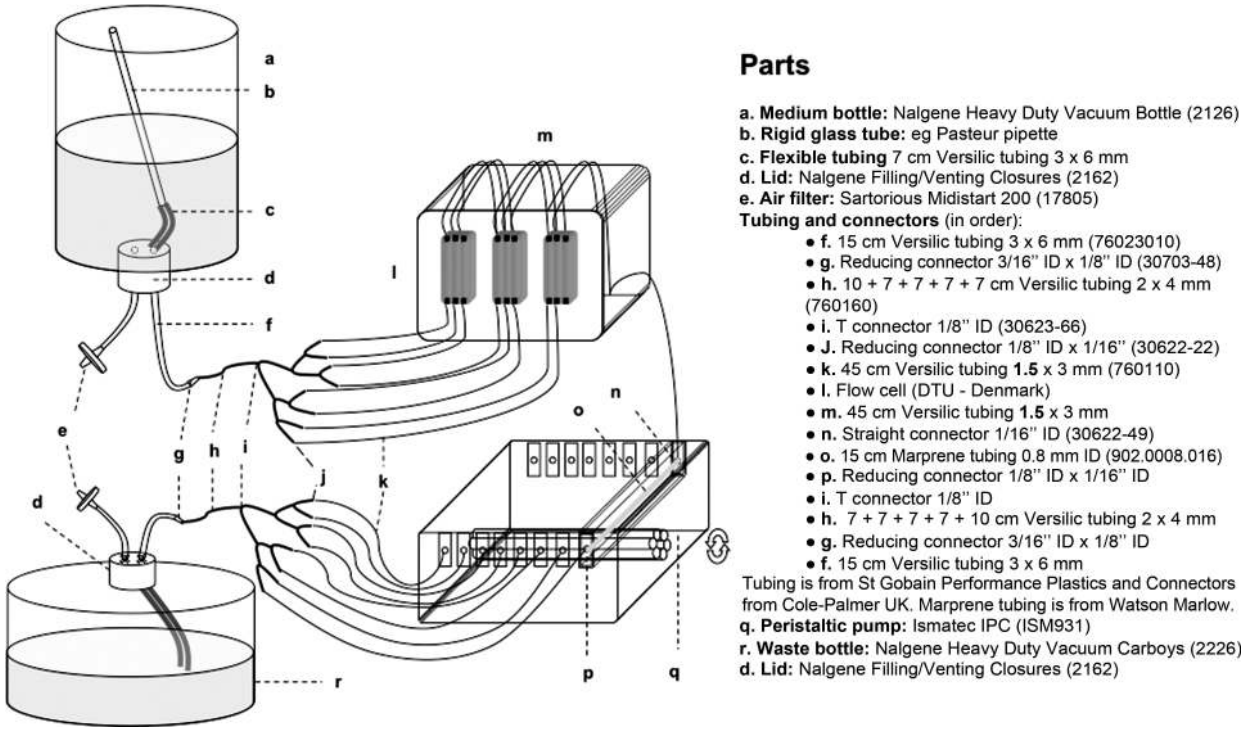


Figure 5. Diagram of the entire flow cell circuit including details of the parts. Important: Note that the medium bottle is inverted on a retort stand and the waste bottle is placed lower down usually on two tiers of a trolley for easy transfer between incubation and imaging. The flow cells are vertically suspended with the inlet at the bottom to reduce the accumulation of bubbles in the channels.

through the system over 3 h at a pump setting of 0.45 ml min^{-1}. The flow system was then washed to remove the hypochlorite by filling and emptying the system two to three times with 1.5 l of sterile distilled water (dH_2O). The flow system was then filled with sterile liquid medium pre-warmed to 37°C and the pump was calibrated to produce a flow rate of 3.3 ml h^{-1} $channel^{-1}$. The system was then allowed to run overnight at 37°C to saturate the silicone tubing before inoculation.

Preparation of bacterial inoculum

A dilution of a fresh overnight culture should be prepared. The dilution depends on the strain and experiment, for example, for *Pseudomonas aeruginosa* a starting OD_{600} of 0.1 is suitable for most experiments.

Inoculation of flow cells

Before inoculation of the flow cells, the flow was stopped and the tubing between the flow cell and inlet growth medium vessel was clamped off. The effluent container was placed at a level higher than the flow cells to prevent air being drawn into the system when the tubing was breached. The pump clamps were removed and the tubing at the flow cell inlet was sterilised with 96% ethanol. A 1 ml insulin syringe (Sterilin) was filled with prepared bacterial culture and air bubbles expelled from the syringe. The syringe needle was inserted into the tubing as near as possible to the flow cell inlet and the bacterial cells carefully injected into the channel (Figure 6). After inoculation, the tubing was sterilised with 96% (v/v) ethanol and the injection hole sealed by applying a thin layer of silicone. At this point the pump clamps were re-applied. Then, to allow the bacterial cells to establish on the glass coverslip substratum, the flow cell was inverted with the glass coverslip surface facing downwards and incubated at 37°C for 1 h. Following this, the tubing was unclamped, the media flow resumed and the flow cells were suspended vertically to ensure that any air bubbles that did enter rose to the distal end of the channel and passed out of it.

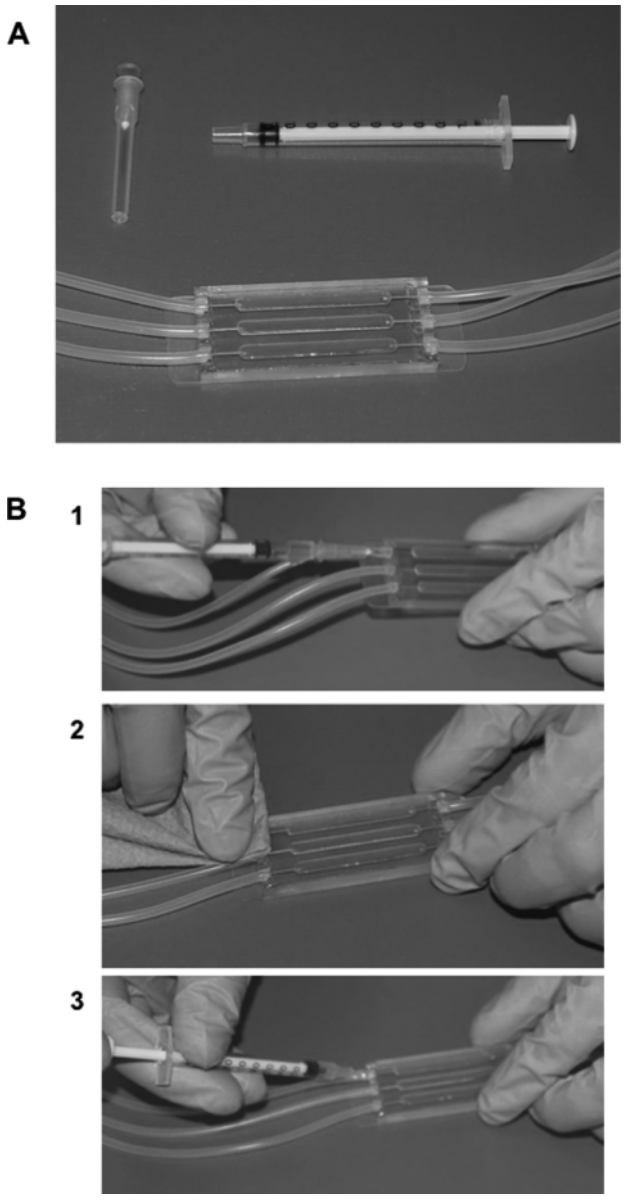


Figure 6. Inoculation of the flow cell. Before inoculating, clamp the tubing 10 cm upstream of the flow cell, place the waste bottle at a level higher than the rest of the flow system and release the peristaltic pump clamps. The flow cell is inoculated using a sterile 1 ml syringe and accompanying needle (A). The silicone tubing is wiped with ethanol, pierced near the entrance to the flow cell and the bacterial suspension injected (B1). The tubing is wiped with ethanol again (B2) and sealed using silicone dispenser as in Figure 2 (B3).

Incubation of biofilm flow cell system

Flow cells were generally incubated at 37°C for a number of days depending on the experimental set up. During this time, upstream fouling of the tubing did occur. This bacterial back growth was cut out on a daily basis to prevent the influence of upstream biomass on the flow chamber biofilms. This was performed by clamping off the tubing either side of the flow cell and sterilising the upstream tubing with 96% ethanol. This upstream tubing was then cut with a sterile scalpel immediately above the backgrowth and the tubing containing the backgrowth removed. The upstream tubing was then reconnected aseptically to the flow cell ensuring that no bubbles were introduced into the channel. The clamps were then removed and the flow resumed.

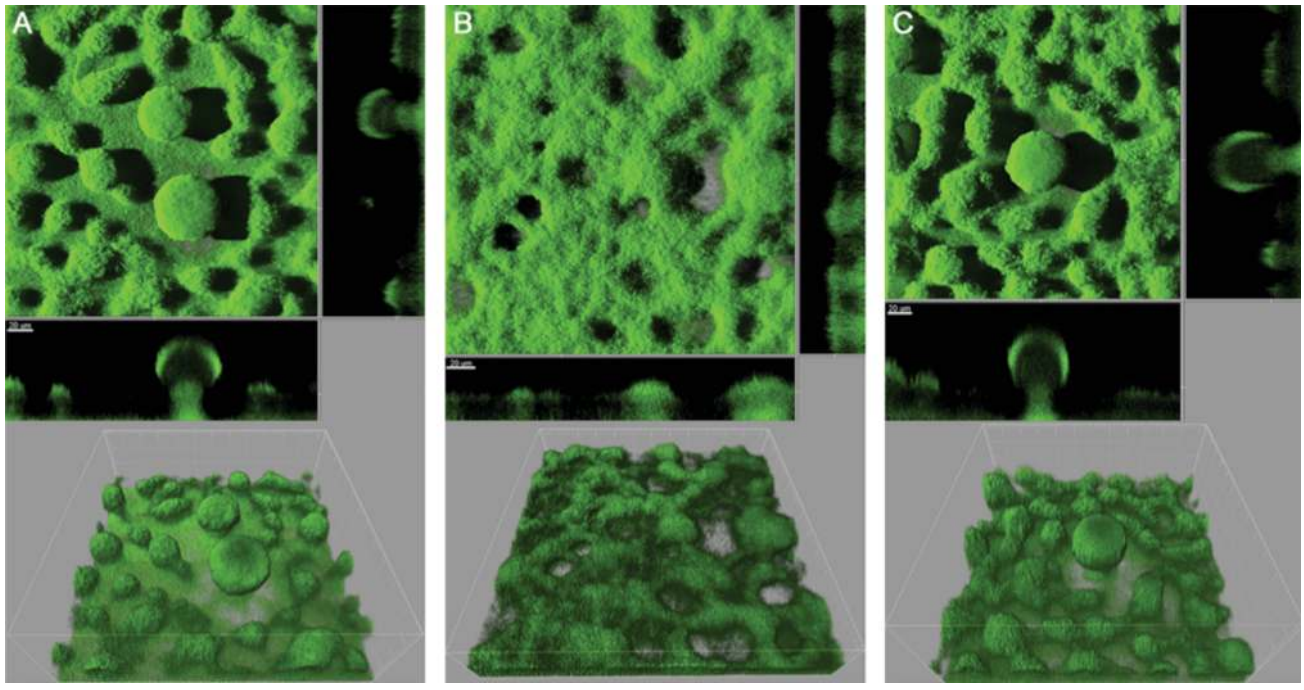


Figure 7. Biofilms formed by *P. aeruginosa* in flow cells in the modified and traditional systems. (A) Example of the characteristic mushroom-shaped microcolonies formed by *P. aeruginosa* in flow-cells in the traditional system in the absence of air bubbles. (B) Example of a biofilm formed by *P. aeruginosa* in a traditionally constructed flow-cell in the presence of damaging air bubbles which prevent normal microcolony formation. (C) Example of *P. aeruginosa* biofilm formed in the modified system demonstrating that characteristic microcolony formation occurs. All images are from 4-day-old biofilms grown with glucose as the carbon source. The top images display a central top-down view of the biofilms with flanking vertical cross-sections. The lower images display 3D-rendered depictions of the biofilms. The images were taken using a CLSM with a set up for detecting GFP-tagged bacteria. Scale bars = 20 μm.

Microscopy and data collection

Microscopy techniques will vary depending on the experiment being performed. Generally, CSLM was undertaken with the flow cell mounted directly on the microscope stage and the system manipulated on site (for example flow stoppage, injection of reagents in to the flow channels, changing the flow rate or switching the medium). Images were recorded at desired time points or upon manipulation of the system.

To demonstrate the method, biofilms were grown in the modified system described here and the traditionally constructed system (Figure 7). It was observed that whilst biofilms formed in the modified system appeared morphologically similar to those in the traditional system, the reduced incidence of bubble formation in the modified system suggested less variable experimental outcomes due to air bubble-induced architectural damage. The detrimental effects of bubble formation in flow cell systems can also be quantified by COMSTAT analysis (Heydorn et al. 2000). Such quantification shows, as expected, that disturbed biofilms on average contain less biomass and are thinner compared to undisturbed biofilms, due to detachment of parts of the microcolonies upon passing of the bubbles (Table 1).

Table 1. COMSTAT analysis of undisturbed *P. aeruginosa* biofilms and *P. aeruginosa* biofilms disturbed by bubbles.

	Total biomass (μm^3 μm^{-2})	Maximum thickness (μm^2)
Undisturbed	65.72 (11.01)	104.3 (19.1)
Disturbed	35.91 (8.38)	49.8 (8.6)

Displayed are the average values with SDs (in brackets), from analysis of 6 CLSM micrographs taken in different 4-day-old *P. aeruginosa* biofilms grown as described in the legend to Figure 7.

Disassembly and cleaning of flow cell

At the conclusion of the flow cell experiment, the system was emptied and then rinsed with 1 l of 0.5% (v/v) sodium hypochlorite bleach. All tubing was detached and the upstream portion was discarded. The downstream effluent tubing was massaged to dislodge adherent waste matter, flushed with water,

autoclaved and recycled. The glass coverslip substratum was carefully removed from the flow cell base with a scalpel (and is inevitably broken in the process). Any remaining silicone glue was removed from the base using 96% ethanol and scrubbed with a toothbrush.

Summary

The widespread occurrence of bacterial biofilms in the human body and natural and industrial settings has significant clinical, environmental and economic impacts. Thus there is a huge impetus for research aimed at understanding and tackling these sophisticated and tenacious microbial communities. The flow cell system is an integral *in vitro* analytical tool, which in conjunction with CLSM, enables the detailed study of the growth of live, fully hydrated biofilms over a number of days, with the ability to control parameters such as growth medium composition, flow rate and incubation temperature. The use of fluorescent probes to differentially label strains and mutants renders the system even more powerful.

Though the method described here is often used to culture bacterial biofilms, it can also serve as a general flow system in which some of the associated problems of flow, such as bubbles, have been alleviated. For instance any microbial species able to attach to glass could be cultured in such a system or indeed eukaryotic cells, the study of which has also been advanced greatly by the use of continuous flow culture. It is possible to use any type of attachment surface that can be secured to the perspex flow chambers, although microscopy requires optically transmitting materials. Researchers can also customise the flow regimen by implementing a peristaltic pump and operating programme that suits the needs of their experiment.

Whilst the flow cell system has a relatively simple design, the process of its assembly and operation is detailed and lengthy. In addition, the major drawback of the traditionally constructed flow cell system is that random bubble formation and uncontrolled destruction of the biofilm architecture occurs when the system is run at 37°C. However, lessons can be drawn from the experience of renal dialysis technology, where there is an imperative to avoid bubble formation within the tubing of a system running at body temperature. The modified flow cell system described here should be useful to biofilm researches as it enables the performance of flow cell experiments at 37°C without the formation of damaging bubbles.

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