Video Article

# *Pseudomonas aeruginosa* and *Saccharomyces cerevisiae* Biofilm in Flow Cells

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## Abstract

Many microbial cells have the ability to form sessile microbial communities defined as biofilms that have altered physiological and pathological properties compared to free living microorganisms. Biofilms in nature are often difficult to investigate and reside under poorly defined conditions[1]. Using a transparent substratum it is possible to device a system where simple biofilms can be examined in a non-destructive way in real-time: here we demonstrate the assembly and operation of a flow cell model system, for *in vitro* 3D studies of microbial biofilms generating high reproducibility under well-defined conditions[2,3].

The system consists of a flow cell that serves as growth chamber for the biofilm. The flow cell is supplied with nutrients and oxygen from a medium flask via a peristaltic pump and spent medium is collected in a waste container. This construction of the flow system allows a continuous supply of nutrients and administration of e.g. antibiotics with minimal disturbance of the cells grown in the flow chamber. Moreover, the flow conditions within the flow cell allow studies of biofilm exposed to shear stress. A bubble trapping device confines air bubbles from the tubing which otherwise could disrupt the biofilm structure in the flow cell.

The flow cell system is compatible with Confocal Laser Scanning Microscopy (CLSM) and can thereby provide highly detailed 3D information about developing microbial biofilms. Cells in the biofilm can be labeled with fluorescent probes or proteins compatible with CLSM analysis. This enables online visualization and allows investigation of niches in the developing biofilm. Microbial interrelationship, investigation of antimicrobial agents or the expression of specific genes, are of the many experimental setups that can be investigated in the flow cell system.

## Video Link

The video component of this article can be found at https://www.jove.com/video/2383/

## Protocol

### 1. Assembly of the Flow Cell System with All Components

The assembled flow system includes: autoclavable tubing, bubble traps, medium/waste bottle and flow cells as shown in Figure 1. All these parts can be reused between experiments.

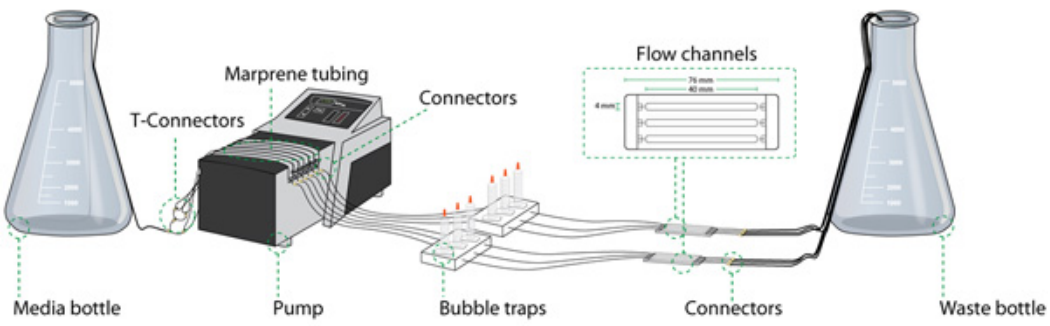

**Figure 1.** The flow cell system setup (essential components of the setup). The flow cell system consists of several components: a medium bottle, a peristaltic pump, bubble traps, the flow cell, a waste bottle, and diverse sections of tubing interconnected by various connectors. Figure kindly provided by Rune Lyngklip.

## 2. Assembly of the Flow Cell

1. The flow cell (Figure 2a) is treated with thin lanes of silicone glue, using a syringe (Figure 3).
2. Place a cover slip on top of the silicone lines (Figure 3). Glass cover slips are used as substratum for *P. aeruginosa* while PVC cover slips are applied for *S. cerevisiae* biofilm.

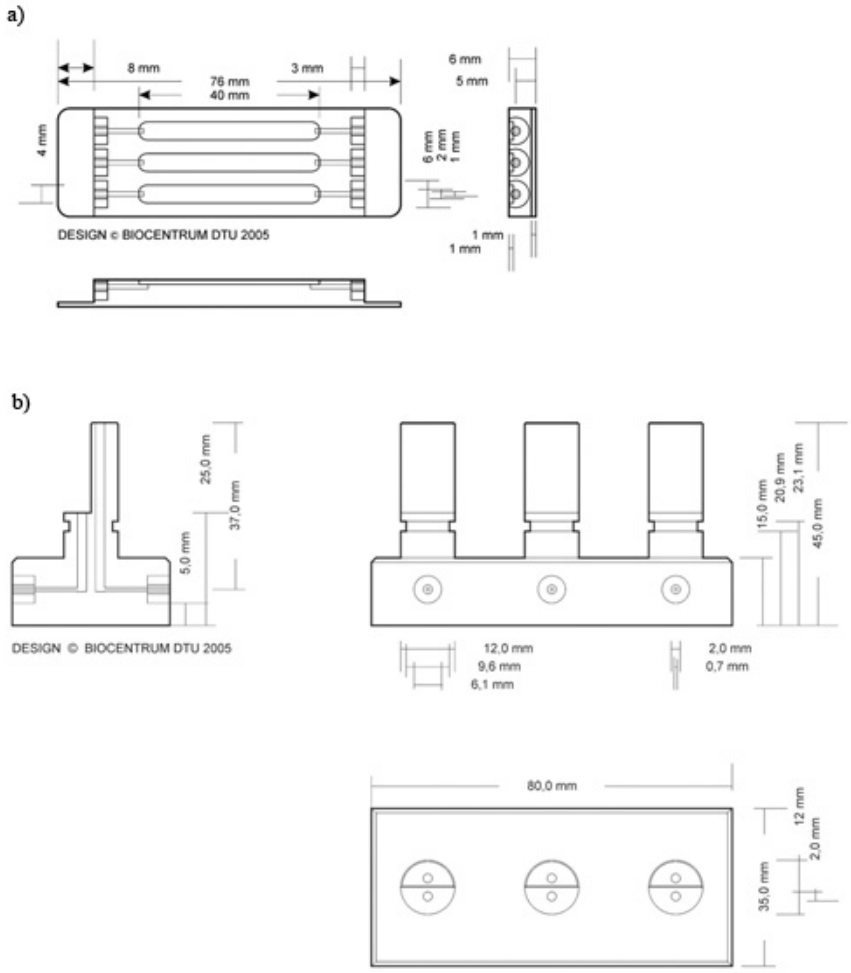


**Figure 2.** Schematic drawing of flow cell and bubble trap[2]. Detailed description of the dimensions used for the production of a) flow cell b) bubble trap, DTU Systems Biology. Reprinted with permission of John Wiley & Sons, Inc. (DTU Systems Biology was formerly entitled Biocentrum, as depicted in the figure)

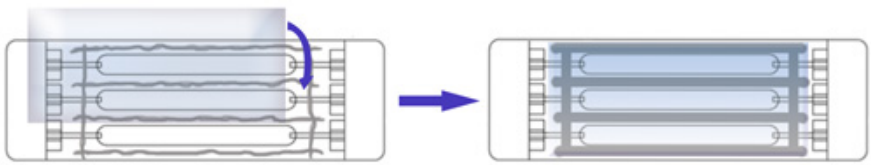

**Figure 3.** Illustration of the silicone glue application lines for attachment of the glass substratum. The indicated cover glass is placed over the silicone glue to attach it to the flow cell.

3. Turn the flow cell over and place it on a flat surface with the cover slip side down. Press gently on the back of the flow cell to push the cover slip onto the base of the flow cell. Turn the flow cell over and inspect for areas that are not sealed by the silicone. The handle (piston) part of a syringe can be used as a tool to gently press the glass and flow cell together.

## 3. Medium Bottle

1. Place a feeding silicone tube (2 mm inner diameter) in a medium bottle and insert a straight connector at the other end.
2. Add medium to the bottle (for medium content see "media" paragraph)
   Cover connector and bottle with metal foil and autoclave the medium. Make sure that the end of the supply tube is fixed above the liquid level in the medium bottle or a siphon effect may empty the medium bottle when autoclaving.

## 4. Connecting the Bubble Trap, Flow Cell and Pump

Assemble all tubing according to the outline in Figure 1. Use silicone tubing except for the part that goes through the peristaltic pump where Marprene tubing is applied.

1. In order to connect the tube from the medium bottle to all individual flow chambers, split a tube into the required number of inlets applied in the experiment. Use T-connectors to make the desirable number of connection tubes from the feed tube to the Marprene tubes in the pump (see Figure 1, T-connector). It is generally a good idea to keep the same sequence order of tubes and flow cells throughout the system, to facilitate identification of components in case of faults in the system.
2. Connect each individual feeding tube (2 mm in diameter) to Marprene tubes in the pump using straight connectors. Connect the Marprene tubing to a bubble trap (Figure 2b) via intermediate silicone tubing (1 mm in diameter). Make sure that the pump is connected to the inlet at the tallest part of the bubble trap.
3. Connect the resulting outlet tube of the bubble traps to the flow cell inlet (1 mm in diameter), make sure that the length of these tubings allows the flow cell to be moved to the stage of the confocal microscope (typically 1 m).
4. Place 5 mL syringes on top of the bubble traps. Close the tops with suitable caps.
5. On the flow cell outlet connect a short, approximately 40 mm piece of (1 mm in diameter), tubing and use a "reducing" straight connector to attach a waste tube (2 mm in diameter) of needed length. Place waste tubes in the waste container.
6. Importantly, the waste container must always be placed at the same level as the flow cells, never below flow-cell level. Also, make sure that the end of the waste tubing is fixed above the expected level of waste liquid to avoid flush-back due to a siphon effect when handling the flow cells.

## 5. STERILIZING AND WASHING THE FLOW SYSTEM

1. Remove bubble trap caps and place them in 70% ethanol to keep them sterile.
2. Run at highest pump speed to fill the system with 0.5% (v/v) sodium hypochlorite in water.
3. Place the bubble trap caps back on when the bubble traps are completely filled.
4. Tap the flow cells to remove bubbles in the flow chamber. Take care not to damage the fragile cover glass.
5. Allow the system to sterilize for 3-4 h at a flow rate of 3 mL/h/channel (0.2 mm/s linear flow rate).
6. Wash the system 2-3 times to wash out all the hypochlorite. Fill and empty the system with sterile water. Bubble traps must be emptied completely between each wash. This can be done by pumping in air until bubble traps have been emptied. After emptying, remove caps from the bubble traps before refilling the system. Replace caps after the bubble traps have been completely filled with liquid. Repeat as required.
7. Run sterile water through the system at a low flow rate (1-3 mL/h/channel) over night or proceed to the next step.
8. Connect the medium bottle to the inlet and flush the system with medium over night at low flow rate (3 mL/h/channel) at the temperature where the experiment will be performed. Note: bubble traps must be emptied for water before the system is filled with medium.

## 6. INOCULATION OF THE FLOW CELL

1. From an overnight culture make a dilution to a desired optical density (for *P. aeruginosa* e.g. 0.001 $OD_{600nm}$ and 0.1 $OD_{600nm}$ for *S. cerevisiae*).
2. Use a 0.5 mL syringe with a 27G needle to load enough inoculum to fill the chamber. 250 µL is sufficient for the flow chambers having the dimensions specified in this work (Figure 2., 40 mm x 4 mm x 1 mm).
3. Stop the peristaltic pump.
4. Clamp off the silicone tubing leading to the flow cell to prevent back flow into the system.
5. Sterilize the inoculation site on the silicone tubing by wiping it with 70% ethanol.
6. Insert the needle into the silicone tube and introduce the tip into the inlet of the flow cell. Slowly inject the inoculum into the chamber (be careful not to inject air bubbles).
7. Remove the needle and wipe the injection site with 70% ethanol followed by immediate sealing of the hole using silicone glue over the injection site.
8. Turn over the flow cell and let the micro-organisms adhere to the substratum for 1 hour without flow through the flow cell.
9. Turn the flow cell, start the medium pump (3 mL/h/channel) and take the clamp off the silicone tube.
10. The system is placed for incubation, at 37°C in the case of *P. aeruginosa* and 30°C in the case of *S. cerevisiae*.
11. Biofilm in the flow chambers can now be visualized by CLSM.

## 7. STAINING OF BIOFILM FOR MICROSCOPY

1. Make a dilution of the appropriate staining (e.g. 1:1000 Syto 9 live stain for *S. cerevisiae*)
2. Stop the peristaltic pump.
3. Clamp of the silicone tubing leading to the flow cell.
4. Sterilize inoculation site on the silicone tubing by wiping it with 70% ethanol.

5. Use a 0.5 mL syringe with a 27G needle to load enough staining solution to fill the chamber. 250 µL is sufficient for the flow chambers used here.
6. Insert the needle into the silicone tube and introduce the tip into the inlet of the flow cell. Slowly inject the staining solution into the chamber (be careful not to inject bubbles).
7. Remove the needle and wipe the injection site with 70% ethanol followed by immediate sealing of the injection site.
8. Leave the flow cell without flow for 15 minutes.
9. Take off the clamp and start the flow (3 mL/h/channel)
10. Acquire data with the CLSM

## Discussion

We have demonstrated a flow cell system that represents a powerful tool in biofilm investigations. Combined with 3D imaging by confocal microscopy, the system has a range of advantages in comparison to other methods of analyzing microbial biofilms by means of more traditional microscopic techniques. This system allows 3D visualization of living microbial biofilm communities without disturbance of the community. Light microscopy will not provide detailed information about niches of the biofilm and while electron microscopy provides nanoscale resolution of the biofilm, it does not allow live cell imaging.

Using the described flow channel system we have previously elucidated the spatial distribution of bacterial cells sensitive to several antibiotics[5-8] (Figure 4a), distribution of extracellular compounds, e.g. DNA[9-11] and, the distribution of motile and non-motile cells of the same species within a bacterial community[4,6,9] (Figure 4c). We envision that the flow cell system can be used to study aspects of yeast biofilms. This may be the spatio temporal distribution of yeast biofilm in response to environmental factors such as fungicides as well as identification of genes involved in yeast biofilm development. Though yeast is not known to differentiate into motile and non-motile cells, other aspects of biofilm diversification may be studies such as the morphological shift from yeast to pseudohyphal cells and the shift from haploid to diploid cells.

We have shown a system that comply with several microbial species and will work with several staining techniques. A variety of different staining probes and fluorescent proteins, such as GFP, enable specific niche investigations in the developing biofilm and is an efficient tool in analyzing the effect of antimicrobial agents or other environmental factors. The information that can be gained is very detailed (Figure 4) and features in the biofilm can be quantified with computer programs such as COMSTAT[12,13].

Overall, the most critical aspect of the protocol is the fact that it is a time-consuming process. It is also a limitation that the cells need to be able to grow on a non-fluorescent, transparent surface. Since the biofilm formed is analyzed using a confocal microscope, the depth that can be investigated is limited to a few hundred micrometres[14].There are further technical limitations inherent in the design: the system is not suited for high throughput screening, as an experienced researcher can handle at most about 15 channels per experiment, which in turn can take several days to prepare. However, antibiotics or mutants that are considered relevant for biofilm studies can initially be mass screened with other methods such as crystal violet staining before the most interesting candidates are transferred to the flow cell system. The cover glass sheets are very thin and break easily, and care should be taken when handling the systems. In addition the tubing should be examined daily during the run of an experiment; as considerable "back-growth" in the inlet tubes just upstream of the flow cells can occur. Such contamination can be solved by removing several centimeters of silicone tube from the inlet side of the flow cells, using sterile technique.

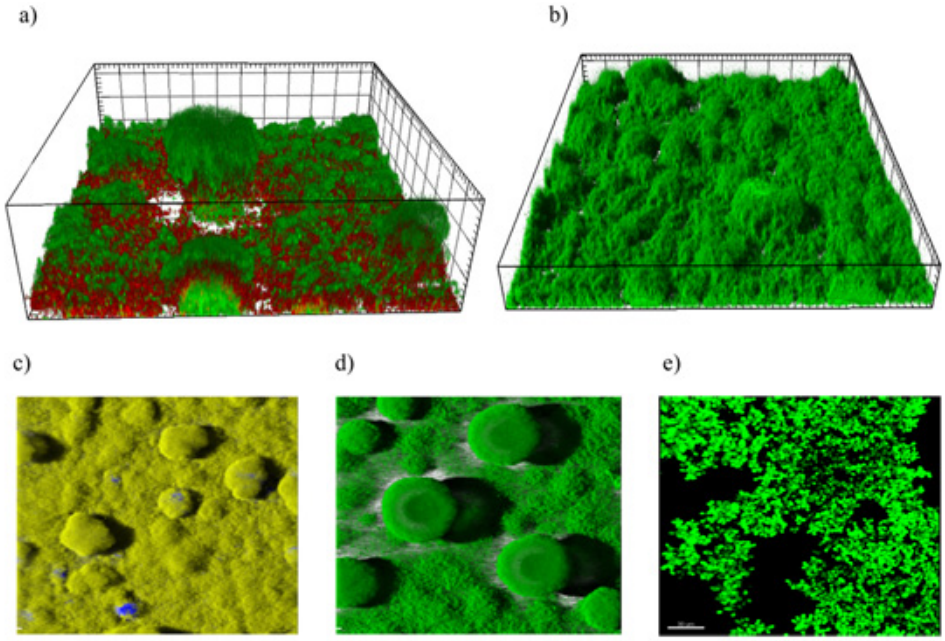


**Figure 4.** a) 4 day old PAO1 - GFP biofilm treated for 24h with Colistin and Propidium iodide for dead staining (red stain) b) 3D presentation of a three day old *P. aeruginosa* PAO1 (*P. aeruginosa* wild type) - GFP biofilm[6] c) 3D picture presentation of a PAO1 - CFP pilA mutant (blue) with an PAO1 wild type YFP (yellow) d) 5 day old PAO1 - GFP biofilm presented as a 3D picture e) 26 h *S. cerevisiae* (PTR3 mutant in CEN.PK background) biofilm stained with Syto-9[15].

## Disclosures

No conflicts of interest declared.

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