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Biofilm responses to smooth flow fields and chemical gradients in novel microfluidic flow cells

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Abstract

We present two novel microfluidic flow cells developed to provide reliable control of flow distributions and chemical gradients in biofilm studies. We developed a single-inlet microfluidic flow cell to support biofilm growth under a uniform velocity field, and a double-inlet flow cell to provide a very smooth transverse concentration gradient. Both flow cells consist of a layer of polydimethylsiloxane (PDMS) bonded to glass cover slips and were fabricated using the replica molding technique. We demonstrate the capabilities of the flow cells by quantifying flow patterns before and after growth of *Pseudomonas aeruginosa* biofilms through particle imaging velocimetry, and by evaluating concentration gradients within the double-inlet microfluidic flow cell. Biofilm growth substantially increased flow complexity by diverting flow around biomass, creating high- and low-velocity regions and surface friction. Under a glucose gradient in the double-inlet flow cell, *P. aeruginosa* biofilms grew in proportion to the local glucose concentration, producing distinct spatial patterns in biofilm biomass relative to the imposed glucose gradient. When biofilms were subjected to a ciprofloxacin gradient, spatial patterns of fractions of dead cells were also in proportion to the local antibiotic concentration. These results demonstrate that the microfluidic flow cells are suitable for quantifying flow complexities resulting from flow-biofilm interactions and investigating spatial patterns of biofilm growth under chemical gradients. These novel microfluidic flow cells will facilitate biofilm research that requires flow control and in situ imaging, particularly investigations of biofilm-environment interactions.

Keywords

Microfluidic flow cell; biofilms; flow-biofilm; physicochemical environment; chemical gradients; environment-biofilm interaction

Introduction

Biofilm-resident cells have a drastically different mode of growth from planktonic cells, and heterogeneity is a defining characteristic in biofilm ecology (Lens et al. 1993; Xu et al. 1998; Prigent-Combaret et al. 1999). Biofilm structure and activity depend on multiple environmental factors, including the nutritional environment (Patrauchan et al., 2007; Shrout et al., 2006) and surrounding flow conditions. Flow conditions affect biofilms by controlling delivery of substrates and nutrients to biofilm-resident cells (Stewart, 2012), and by exerting

shear stresses over the biofilm surface (Stoodley et al., 1999). Hydrodynamic shear affects biofilm morphology (Purevdorj et al., 2002) and detachment (Choi and Morgenroth, 2003). Shear-induced mechanical failure is considered the primary mechanism of biofilm detachment in many model studies (Alpkvist and Klapper, 2007; Böl et al., 2009; Picioreanu et al., 2001). Detachment or dispersal of biofilm-resident cells is an important stage in the biofilm life cycle in both biomedical and ecological contexts, as dispersal contributes to pathogen transmission and recolonization (Hall-Stoodley and Stoodley, 2005).

Chemical cues such as oxygen and signaling molecules can govern biofilm formation, spatial distribution and virulence by triggering gene expression and protein synthesis (Davies et al., 1999; Boriello et al., 2004; Ng and Bassler, 2009). Internal gradients of nutrients, signaling molecules and antimicrobial exist within biofilm (Xu et al., 1998; Stewart, 2003; Tseng et al., 2013). These local chemical gradients change with time as a result of coupled hydrodynamic transport and microbial metabolism (Stewart and Franklin, 2008), but the response and adaptation of biofilms to external chemical gradients has not been well explored. Several flow cell designs are available to culture biofilms and assess growth and biofilm physiology under controlled conditions. Drip-flow reactors are widely used to culture biofilms in slow flow, free-surface conditions (Goeres et al., 2009). This type of system is suitable for microsensor monitoring (Xu et al., 1998), but does not support in situ imaging, because the biofilms are grown on microscope slides that must be removed from the reactor to be examined. Rotating disk reactors support biofilm growth under controlled hydrodynamic shear. The rotating disk reactor consists of a disk with removable coupons and a magnetic stirrer, both of which are placed inside a glass reactor vessel. As the liquid medium circulates through the reactor vessel, the disk is rotated by a magnetic stirrer, and fluid shear is generated across the coupon surfaces. This system is suitable for evaluating biofouling, antifouling materials, and biofilm susceptibility to antibiotics (Hentzer et al. 2001; Singh et al. 2002; Boles et al. 2004). However, the biofilm studies using the drip-flow or rotating disk reactors were often invasive and destructive, as microsensors may be inserted, and biofilms are scraped off or sonicated from the coupons for analyses.

Studies of interplay between biofilms and the surrounding fluid environment require nondestructive, in situ imaging of biofilms in flow cells that provide controlled hydrodynamic conditions and environmental heterogeneity. In situ imaging of biofilms grown in aqueous medium and on a transparent substratum with confocal microscopy has provided detailed 3D, spatially explicit information about biofilm architecture and physiology by labeling cells and proteins with fluorescent probes (Tolker-Nielsen and Sternberg, 2006). Applications include redox probes for directly observing cellular respiration (Nielsen et al., 2003; Rodriguez et al., 1992), fluorescent probe for staining EPS component (Wang et al., 2013), constitutively expressed GFP to directly measure morphology (Bester et al., 2010; Boles and Horswill, 2008) and live/dead staining to quantify antimicrobial susceptibility (Benoit et al., 2010).

More recently, interest in interactions between environmental complexity and biofilm heterogeneity has spurred development of several flow cell designs that can be used to observe biofilm growth under controlled environmental gradients. The effect of bulk fluid flow on biofilm growth has been observed in circular pipes (Horn et al., 2003), semicircular pipes (Teodósio et al., 2011), square or rectangular channels (de Beer et al., 1994; Pereira et al., 2002; Stoodley et al., 2002) and under Couette-Taylor flow (Ochoa et al., 2007). Multi-channel flow cells made of Plexiglas have been widely used, since they can fit on a microscope stage and enable noninvasive imaging of biofilms (Christensen et al., 1999; Wolfaardt et al., 1994). Flow cells made of reusable, autoclavable parts are still used widely. Multi-channel unidirectional flow cell designs provide good flow control and yield highly

reproducible results, but do not support user-defined environmental heterogeneity. We previously developed a device for culturing biofilms under a variety of user-specified planar flow configurations (Zhang et al. 2011). This planar flow cell enables biofilm culturing under a user-specified flow field with varying fluid velocities, which is useful to directly assess the influence of hydrodynamics on biofilm growth and detachment. However, such unidirectional and planar flow cell devices do not support exact control of flow distributions and chemical gradients at the microscale.

As local flow-biofilm interactions are responsible for biofilm detachment events, there is still a need for devices that enable control and assessment of the velocity field around biofilms at the microscale rather than the bulk flow scale (Graba et al., 2013). Microfluidic flow cells offer the potential for the desired degree of flow control at the biofilm scale. The development of soft lithography manufacturing methods fostered the microfluidic approach due to the use of biocompatible polymers, flexible prototype design, and low cost (Weibel et al., 2007). Recently, the geometry of microfluidic channels has been shown to influence *P. aeruginosa* biofilm growth (Kim et al., 2013). Many microfluidic channel designs reported previously have square or rectangular cross-sections with aspect ratios of less than 5 (Kim et al., 2012b; Lee et al., 2008). However, devices with such small aspect ratios (<5) produce complex three-dimensional flow fields that make it difficult to evaluate feedbacks between biofilm growth and flow conditions (Lecuyer et al., 2011).

Numerous microfluidic platforms that enable both linear and non-linear concentration gradients, under flowing and stagnant conditions have been developed, as reviewed by Ahmed et al. (2010) and Kim et al. (2012a). Studies of planktonic population dynamics, chemotaxis, and quorum sensing under diverse environmental conditions have benefited from microfabricated devices (Cho et al. 2007; Englert et al. 2009; Lanning et al. 2008; Boedicker et al. 2009; Park et al. 2003). Furthermore, microfluidic approaches have recently been used to investigate quorum sensing in biofilms (Janakiraman et al., 2009) and high-throughput screening of biofilms (Benoit et al., 2010; Eun and Weibel, 2009). Recently, Kim et al. (2012b) reported a microfluidic device for studying biofilms under diverse chemical conditions. Pre-mixing channels were used in this device to produce a linear distribution of discrete solute concentrations in eight parallel flow chambers. However, none of these devices have been used to support biofilm growth under user-defined hydrodynamic conditions and microscale chemical gradients.

Here we present two new microfluidic flow cell designs that provide well-defined flow conditions and smooth chemical gradients for studies of biofilm-environment interactions. These flow cells are designed with larger chamber dimensions than most microfluidic devices in order to achieve well defined vertical velocity profiles over a large area of the flow cell, as well as to support observations of the feedbacks between biofilm growth and environmental gradients. We show that the new microfluidic devices produce well-defined flow fields and chemical gradients, and present results on the interactions of *P. aeruginosa* biofilms with the flow field and with imposed nutritional and antimicrobial gradients.

Microfluidic flow cell design and construction

The two flow cell designs are shown in Figure 1. The single-inlet microfluidic flow cell is comprised of a PDMS layer and an irreversibly sealed glass cover slip. The chamber has a length of 23 mm, width of 13 mm, and height of 0.24 mm. The double-inlet flow cell is constructed similarly, but has two inlets to enable introduction of two distinct fluids in order to generate well-controlled concentration gradients within the flow cell. The gradient formation is perpendicular to the direction of the flow and the gradient relaxes in the downstream distance as a result of molecular diffusion. This generates very smooth concentration gradients in the transverse and downstream directions.

Both flow cells were constructed using the replica molding technique (Duffy et al., 1998; Weibel et al., 2007) with the commercially available elastomer polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning, Midland, MI). The flow cells are comprised of glass cover slips mounted on PDMS bodies. Channels and chamber designs are completely formed in PDMS, while glass cover slip provides a flat, rigid and transparent surface suitable for imaging. The replica molding procedure is shown in Figure 1e. To create a mold, three sheets of Mylar with adhesive backing (0.002-inch thick, McMaster-Carr) were layered to create a thickness of 240 μm and then cut to the flow-cell design using a CAD-based programmed CO₂ laser (X2-660, Universal Laser Systems Inc., Scottsdale, AZ). To make frames for PDMS molding, acrylic sheets (0.25-inch, McMaster-Carr, Elmhurst, IL) were laser-cut into rectangles with void interior of 60 $\times$ 24 mm. PDMS monomers and curing agent were mixed in a 10:1 ratio by weight and then cast into the mold, degassed, and finally cured overnight under room temperature and pressure. After being cured, PDMS was peeled off the mold to yield the flow cell body. The inlets and outlets were created by punching holes of 1.50 mm internal diameter with a tissue punch (Harris Uni-core 1.50, Ted Pella, Redding, CA). The PDMS layer was then autoclaved at 121 $^{\circ}\text{C}$ for 15 minutes to increase rigidity. The PDMS layer and 60 $\times$ 24 mm glass cover slip (Fisher Scientific, Itasca, IL) were treated with oxygen plasma for 1 minute (PC2000 Plasma Cleaner, South Bay Technology, San Clemente, CA), and then gently pressed to make an irreversible bond. Stylus profilometry (Dektak stylus profilometer, Bruker Corporation, Tucson, AZ) was performed on an epoxy casting of the chamber interior to confirm that the chamber dimensions followed the design specification. The chambers were observed to have the desired rectangular configuration, and there was no sagging in the PDMS layer.

Characterization of flow field

The physical performance of the flow cells was tested by observing the flow fields generated within each design, and solute transport in the double-inlet design. Velocity distributions in the single-inlet flow cell were measured by particle-image velocimetry (PIV) at multiple transverse and longitudinal locations under influent flow rates of 0.03 ml/min and 0.4 ml/min. The flow was imposed by means of a high-quality peristaltic pump (Minipuls-3, Gilson Inc., Middleton, WI). Transverse velocity distributions at $x = 6, 12$ and 18 mm were resolved by introducing fluorescent microbeads (FluoSpheres® Carboxylate-Modified Microspheres, 1.0 μm , Orange Fluorescent (540/560), 2% solids, Molecular Probes Inc., Eugene, OR) to the influent and imaging them by epifluorescence microscopy with a $4\times$ objective lens (Zeiss Axiophot, Carl Zeiss SMT, Peabody, MA). Movies of particle motion were acquired at a frame rate of 4.8 Hz using a CMOS camera (pco.1600, PCO AG, Kelheim, Germany). Higher-resolution transverse profiles were obtained near the side walls to characterize the wall boundary layer. Vertical velocity distributions were measured by confocal microscopy, which provides precise control of the imaging plane. 1.0 μm Red Fluorescent (580/605) FluoSpheres® were imaged using a Leica TCS SP5 confocal microscope at a frame rate of 1.0 Hz with a $63\times$ objective lens (Leica Microsystems CMS GmbH, Mannheim, Germany). Vertical velocity profiles spanning $z = 0$ to 90 μm were obtained under a flow rate of 0.01 ml/min.

Fluorescence images were pre-processed using ImageJ (Schneider et al., 2012) by subtracting the first acquired image from all subsequent images in the same image set in order to eliminate background fluorescence. We measured flow fields around biofilms using particle image velocimetry, which entails acquiring time-series images of tracer particles seeded in the flow and processing the images to obtain flow vector information (Maas et al., 1993). We performed optimization-based calculations using the Streams 2.00 software package (Maxworthy and Nokes, 2007). Mean fluid velocities at each measurement location were calculated from averaging at least 4,200 discrete measurements of particle velocity.

The vertical velocity profile in the flow cell is expected to follow the analytical solution for Poiseuille flow between parallel plates, because the flow cell width (13 mm) is much greater than the vertical gap spacing (0.24 mm). The vertical velocity distribution between parallel plates is:

$$u_p(z) = \frac{1}{2\mu} \left(\frac{\partial P}{\partial x} \right) (z^2 - Hz) \quad (1)$$

where u_p is the x-component of the fluid velocity, H is the vertical spacing, μ is the viscosity of the fluid medium, and $\partial P / \partial x$ is the pressure gradient in the downstream direction. This vertical velocity profile is related to the volumetric flow rate, Q , by integration over the vertical gap spacing. The subscript u_p specifically indicates velocities predicted for Poiseuille flow, while $u_b = Q/A_c$ defines the average velocity over the flow cell cross-section.

Biofilm growth and flow-biofilm interactions

The effects of biofilm growth on the flow field were observed in the single-inlet flow cells. We used *Pseudomonas aeruginosa* PAO1-gfp with chromosomally expressed green fluorescent protein (GFP) in these studies of flow-biofilm interactions. *P. aeruginosa* is a common biofilm-forming organism in nature, is frequently used as a model organism for biofilm studies (Mann and Wozniak, 2011), is an opportunistic pathogen (Costerton et al., 1999) and has available a wide genetic library with favorable properties for experimentation. *P. aeruginosa* PAO1-gfp was grown on a Luria-Bertani (LB) agar plate, and a colony was transferred to 3 ml of sterile LB broth in a culture tube and the culture was shaken overnight at 225 rpm and 37°C. 50 μ l of this culture was transferred to 3 ml of sterile tryptic soy broth (TSB), grown to a mid-log culture at 225 rpm and 37 °C, and then diluted with sterile deionized water to $OD_{600} = 0.01$ for inoculation to the flow cells. Inoculated bacteria were allowed to attach to the flow cell cover slip under stagnant conditions (no flow) for one hour, and then supplied with 1% TSB medium continuously pumped into the flow cell for 3 days. These experiments were repeated at flow rates of 0.01, 0.02, and 0.04 ml/min. Extracellular polymeric substances (EPS) were visualized by staining with Concanavalin A conjugated with Alexa Fluor 633 (Molecular Probes Inc., Eugene, OR) (Johnsen et al., 2000). The stain was introduced to the flow cell and incubated for 30 minutes, and then the normal medium flow was resumed to wash out the residue.

The resulting three-day-old biofilms were imaged using a Leica TCS SP5 confocal microscope with a 63 $\times$ objective lens. Three-dimensional renderings of the confocal micrographs were generated using Volocity (PerkinElmer Inc., Waltham, MA). Morphological parameters such as biofilm thickness were calculated using a custom-developed MATLAB code. The flow field around and over the biofilm was characterized by confocal PIV as described previously. The particles introduced for PIV did not disturb the biofilm morphology.

Formation of concentration gradients in double-inlet flow cell

Solute concentration gradients in the double-inlet microfluidic flow cell were measured by introducing a solution of 50 μ g/ml Rhodamine B dye to one inlet and autoclaved deionized water in the other inlet, each at an influent flow rate of 0.01 ml/min. Rhodamine concentration profiles were imaged along the centerline of the flow cell at downstream distances of 4, 8, 12, 16, and 20 mm. Rhodamine B fluorescence was imaged with epifluorescence microscopy using a 4 $\times$ objective lens using a Rhodamine filter set (Carl Zeiss SMT, Peabody, MA) and recorded with a pco.1600 video camera (PCO AG, Kelheim, Germany). An analytical solution of the advection-diffusion equation (ADE) was fit to the

obtained fluorescence data in order to evaluate if the concentration gradients can be described by a constant diffusion coefficient. Observed Rhodamine concentration profiles were fit by least squares optimization to an analytical solution to:

$$c(y, t) = c_0 \left\{ 1 - \operatorname{erf} \left(\frac{y}{\sqrt{4Dx/U}} \right) \right\} \quad (2)$$

where c is concentration, c_0 is the maximum concentration, D is the diffusion coefficient of Rhodamine B in water, and U is the mean downstream velocity.

Biofilm growth under imposed glucose gradient

P. aeruginosa PAO1-gfp biofilms were also grown under a nutritional gradient in the double-inlet microfluidic flow cell. For glucose gradient formation, modified FAB medium with 0.6 μM glucose (Heydorn et al., 2000; Shrout et al., 2006) was introduced in one inlet, while FAB medium without any added carbon source was introduced in the other inlet. The medium flow rate was 0.03 ml/min per inlet. The spatial distribution of biofilm growth was imaged at day 3 using the methods described above.

Biofilm killing under antimicrobial gradient

3-day old *P. aeruginosa* PAO1-gfp biofilms were grown in 1% TSB as previously described. To observe patterns of biofilm killing under an antimicrobial gradient, 50 $\mu\text{g/ml}$ ciprofloxacin was introduced in one inlet for 2 hours. The influent flow rate was 0.01 ml/min per inlet, identical to the dye visualization case. After the ciprofloxacin treatment, 300 μM propidium iodide (BacLight Bacterial Viability Kit, Molecular Probes Inc., Eugene, OR) was introduced, incubated for 15 minutes and flushed for live/dead staining. The fraction of dead cells for each location was determined using a custom-developed image processing code in MATLAB.

Results

Flow characterization in single-inlet flow cell

The longitudinal, vertical and transverse velocity distributions observed in the single-inlet flow cell chamber are plotted in Figure 2. u/u_b had a mean value of 0.97 and a standard deviation of 0.13 throughout various locations in the flow cell chamber (Figure 2a). This result confirms that the designed fluid velocities are achieved and the velocity distribution is consistent throughout the chamber. The measured vertical velocity distribution closely followed the analytical solution for Poiseuille flow ($R^2 = 0.95$) (Figure 2b). We investigated the effects of the side walls by measuring the transverse velocity profile, to determine the lateral extent of the region where Poiseuille flow occurred. The wall boundary layer thickness was 375 μm at influent flow rate of 0.03 ml/min (Figure 2c). Thus the wall effects were found to be limited to a narrow region comprising < 6% of the flow cell width, and the desired Poiseuille vertical velocity profile was maintained across 12.3 mm of the width.

Flow-biofilm interactions

The cell clusters in the biofilm showed dome-like architecture. Fiber-like extracellular polymeric substances (EPS) enveloped the cell clusters, as identified by EPS staining (Supplementary Figure S1). Biofilm growth substantially modified the flow field (Figure 3). The flow initially had unidirectional streamlines, but after biofilm colonization, the streamlines deviated around the biofilm clusters (Figure 3a). The microbeads used for PIV did not penetrate the biofilm, so the flow measurements were restricted to regions not occupied or enclosed by biomass. As noted by other works (de Beer et al., 1994; Stewart,

2003; Tseng et al., 2013), the velocities within the biofilm clusters are expected to be negligible. The presence of cell clusters thus created spatial variability in local velocities as the fluid flowed around the biofilm.

Biofilm colonization also resulted in deviation of the vertical velocity profile from Poiseuille flow (Figure 4). The bulk flow rate was identical before and after colonization since it was driven by a peristaltic pump. However, biofilm growth decreased velocities near the surface and increased velocities away from the biofilm. This deviation from the Poiseuille velocity distribution indicates that the fluid flow was substantially redistributed by the presence of biomass on the substratum. Further, the steepened vertical velocity gradient indicates that the biofilm increased fluid shear and thus friction and drag forces over the surface.

Characterization of chemical gradient in double-inlet flow cell

The measured solute gradients in the double-inlet microfluidic flow cell fit very well the 2D ADE (Figure 5). The transverse solute concentration profile was steep near the inlet, and gradually relaxed with downstream distance and associated travel time (Figure 5). As the flow regime was laminar (Reynolds number = 0.05), molecular diffusion dominated lateral transport, and the diffusion-based chemical gradient was well characterized with a single diffusion coefficient. The five concentration profiles agreed well with the ADE solution (R^2 values between 0.82 and 0.96). The least-squares fit to the five concentration profiles yielded an estimate of the diffusion coefficient of Rhodamine B in water, $6.25 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, similar to the value reported in the literature, $3.77 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, (Culbertson et al. 2002; corrected to the temperature of 20°C used here).

Biofilm growth under glucose gradient

The biofilm morphology observed under an imposed glucose gradient was again dome-like, consistent with the glucose-supplemented *P. aeruginosa* morphology reported by Shrout et al. (2006). The steepest transverse gradient of biovolume distribution was observed nearest the inlet ($x=4 \text{ mm}$) and it gradually relaxed downstream, following the solute concentration profiles. At a downstream location of 4 mm, average biofilm thickness ranged from $18.4 \mu\text{m}$ in the region of high glucose concentration to $0.015 \mu\text{m}$ in the region of low glucose concentration (Figure 6). Further downstream, 12 mm from the inlet, the distribution of biomass became much more homogeneous, with average biofilm thickness ranging from $7.72 \mu\text{m}$ in the region of high glucose concentration ($y=0.5 \text{ mm}$), $5.58 \mu\text{m}$ at the centerline location corresponding to the mixing interface ($y=0.0 \text{ mm}$) to $1.37 \mu\text{m}$ in the region of low glucose concentration ($y=-0.5 \text{ mm}$). While glucose concentrations could not be measured directly in situ, the observed spatial patterns in biofilm growth clearly reflect the combined effects of hydrodynamic transport and mixing of glucose and the metabolism of glucose by biofilm-resident cells.

Biofilm killing under ciprofloxacin gradient

Spatial patterns of killing after the exposure to ciprofloxacin gradient were related to the local antimicrobial concentrations (Figure 6b). With an exception of an unusually high killing fraction at the location $x=8 \text{ mm}$, $y=0.0 \text{ mm}$, high fractions of dead cells were found in regions with high local concentrations of ciprofloxacin. The transverse pattern of killing corresponded to the imposed ciprofloxacin gradient. The killing fraction was also higher near the inlet, and relaxed downstream as the antibiotic gradient relaxed.

Discussion

The microfluidic flow cell devices presented here are advantageous for answering several important questions in biofilm research and environmental microbiology. The single-inlet

flow cells have fully characterized hydrodynamics, enabling specific and highly detailed quantification of flow-biofilm interactions. As the flow cell chambers presented here have high width-to-height ratio, the flow field in the bulk of the chamber resembles Poiseuille flow between parallel plates, with minimal side wall effects. Relative to other available devices for culturing biofilms under fluid flow, this flow cell provides a much wider region of well-characterized flow, enabling investigations of larger-scale feedbacks between biofilm growth and the flow field. Flow-biofilm feedback is an important process in several stages of biofilm development – attachment (Lecuyer et al., 2011), maturation (Rochex et al., 2008) and detachment (Garny et al., 2008; Lee et al., 2008). The fluid flow and the shear forces applied on the biofilm are known to influence biofilm structure by inducing erosion, oscillatory movement or development of streamers (Stewart, 2012). While advective nutrient delivery enhances biofilm growth, taller biofilms experience heightened shear stress and therefore are more susceptible to shear-induced detachment. Using our novel flow cell device, we observed that *P. aeruginosa* PAO1 biofilm growth substantially modifies flow in an initially uniform flow field. We found that biofilms strongly increase the complexity of the flow field by producing three-dimensional flows around colony structures. Local observations at the biofilm scale signified that the velocities were retarded at the fluid-biofilm interface, and the streamlines flowed around the biofilm clusters. Biofilm growth caused the vertical velocity distribution to deviate from the initial Poiseuille flow condition. Relative to the Poiseuille flow distribution, velocities decreased near the biofilm surface, and increased in the bulk flow away from the biofilm.

While the significance of fluid-phase transport of substrates and nutrients to biofilm growth is well recognized, little is known on the mechanics of biofilm detachment under hydrodynamic shear. Biofilm detachment and dispersal mechanisms are thought to be closely linked to the flow distribution around the biofilm and the resulting shear stress field (Böl et al., 2009; Duddu et al., 2009; Woodcock et al., 2013). As the flow cell device presented here provides a well-characterized flow field and supports in situ imaging, it can be used as a platform for addressing research questions in flow-biofilm interactions and biofilm detachment due to fluid shear. In addition, the open flow configuration of the single-inlet microfluidic flow cell is advantageous for studies of microbial ecology, such as interactions of multiple species within biofilms. Natural biofilms almost always occur as multi-species communities under complex habitat conditions (Stoodley et al., 2002b; Sutherland, 2001). As a result, advective and diffusive transport, chemical transformations and inter-species interactions occur concurrently within and around complex biofilm communities. In order to elucidate questions in biofilm ecology, such as spatiotemporal dynamics of microbial diversity (Rochex et al., 2008) and inter-species signaling (Lee et al., 2007), the microfluidic flow cells presented here provide a platform for conducting controlled experiments of mixed species biofilms under flow conditions, and imaging them in situ. Further, the microfluidic flow cells presented here have much more localized wall effects and smaller volumes than annular or disk reactors, which is advantageous in maintaining continuous cultures over long times.

Finally, the double-inlet flow cell supports biofilm growth under highly controlled diffusion-based chemical gradients with continuous flow. This provides a considerable advantage for observing the effects of diverse chemical concentrations on biofilms. Many different chemical factors influence microbial processes. Biofilm structures become differentiated under various carbon sources (Shrout et al., 2006), levels of oxygen availability (Skolimowski et al., 2010), and calcium cations (Sarkisova et al. 2005). Signaling molecules, such as acyl-homoserine lactones for *P. aeruginosa* (Dickschat, 2010) and indole for *Escherichia coli* biofilms (Lee et al., 2007) significantly affect biofilm virulence and motility. The double-inlet microfluidic device is convenient for studies of chemical interactions with biofilms, as it enables biofilm growth to be observed under a continuous

concentration gradient in a single chamber. In addition, the device is uniquely suited for investigating how biofilm growth and morphology respond specifically to chemical gradients, which are known to be important in cell motility (e.g. chemotaxis) and signaling. The device also enables new investigations of biofilm-environment interactions, such as alteration of chemical gradients by microbial metabolism, co-development of biological and chemical patterns, and efficiency of use of limited environmental resources in heterogeneous systems. Further advances in visualizing chemical distributions and microbial metabolism in situ, such as visualization of tobramycin penetration in biofilms (Tseng et al., 2013) and interspecies interactions (Yang et al., 2011) will improve the quality of data that can be obtained from the double-inlet device and enable investigations of additional processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) Single-inlet microfluidic flow cell design: 3D rendering of the chambers and channels. The coordinates and the origin are indicated in the figure: x is the longitudinal, y the transverse and z is the vertical direction. (b) Double-inlet microfluidic flow cell design: 3D rendering of the chambers and channels. The coordinates and the origin are identical to those used for single-inlet flow cells. (c) Top-down view of the single-inlet microfluidic flow cell design. The flow cell chamber has a width of 13 mm, a length of 23 mm and a height of 0.24 mm, and is designed to fit on a standard glass cover slip. (d) Replica molding procedure.

Figure 2.

Comparison of measured and predicted velocity distributions in single-inlet flow cell. (a) Bulk velocities measured by epifluorescence PIV (u) matched predicted bulk velocities (u_b) throughout the flow cell. Mean $u/u_b = 0.97$, standard deviation = 0.13 ($Q = 0.03$ and 0.4 ml/min). (b) The vertical velocity profile measured by confocal PIV follows the analytical solution for Poiseuille flow ($Q = 0.01$ ml/min). (c) Transverse velocity profile measured by epifluorescence PIV showing that the wall boundary layer was approximately $375 \mu\text{m}$ wide ($Q = 0.03$ ml/min).

Figure 3.

Velocity distribution around a biofilm cell cluster. (a) Horizontal velocity distributions were measured at vertical distances of 20, 30, 40 and 50 μm around a biofilm cluster under a flow rate of 0.02 ml/min. The maximum height of the cluster was approximately 45 μm . (b) Histograms of the velocities in each horizontal plane. (c) Velocity vector fields observed in each horizontal plane overlain on a confocal micrograph of the cell cluster taken at $z = 0$. Note that the fluid flow diverted around the biofilm cluster at $z = 20, 30$ and $40 \mu\text{m}$.

Figure 4.

Vertical velocity profiles around a 3-day old *P. aeruginosa* biofilm cluster (red diamonds) under flow rates of (a) 0.01 ml/min, (b) 0.02 ml/min and (c) 0.04 ml/min compared with analytical solutions for Poiseuille flow. The measured velocity profile without biofilms is included in (a) for comparison.

Figure 5.

(a) Dye concentration gradient achieved in two-inlet microfluidic flow cell. Open circles indicate observed dye concentrations, and the solid lines indicate the analytical solution to the advection-diffusion equation with a diffusion coefficient of $6.25 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$. The background shading is for illustrative purposes only. (b) Comparison of fitted curves of dye concentration gradients at $x = 4, 8, 12, 16, 20 \text{ mm}$ show how the concentration profile relaxes downstream as a result of transverse diffusion.

Figure 6.

(a) Spatial distribution of average biofilm thickness under glucose gradients. Biofilm growth depended strongly on local glucose delivery, resulting in regular patterns of biomass accumulation that mirror the imposed glucose concentration gradient. As the concentration gradient relaxes in the downstream direction, biofilm growth becomes more homogeneous across the flow cell. (b) Spatial distribution of cell killing under ciprofloxacin gradient. Biofilm killing followed the antimicrobial gradient, as high fractions of dead cells were observed in regions of high ciprofloxacin concentration. (c) 3D images of biofilm clusters grown under the glucose gradient. (d) Horizontal and vertical sections of live (green) and dead (red) cells in biofilm clusters subjected to the ciprofloxacin gradient. In (c) and (d), the longitudinal location was 8 mm and the transverse locations were 0.5, 0 and -0.5 mm.