Artificial Teeth: Dental Biofilm Analysis on a Chip

by

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ABSTRACT

In this thesis, an “artificial teeth” microfluidic device is developed that provides unprecedented control over the conditions required to simulate the growth of complex dental biofilm. Dental plaque formation is not only a precursor to tooth decay, but also induces more serious systemic health problems such as cardiovascular disease, pre-term labor, and diabetes. Therefore, understanding the conditions promoting colonization and subsequent biofilm development involving complex bacteria coaggregation is particularly important. The requirement of the continuous culture and analysis systems for large quantities of growth media and reagents has pushed the move toward microfluidics – the miniaturization and chip-based control of fluidic operations.

Microfluidic oxygenation is necessary to regulate the cellular gas condition of culture medium, especially for mixed population biofilms consisting of both anaerobic and aerobic cell populations. A double-layer gas perfusion network structure fabricated above the cell culture regions is developed for culture oxygenation. Throughout the modeling and analysis of the oxygen transfer in microfluidic oxygenators, design strategies for such devices are proposed for different configurations. Various designs of oxygen-nitrogen mixer networks providing parallel oxygenation with differential or tunable oxygen concentrations are described and verified experimentally to test the corresponding applicability in microbiological culture.

The microfluidic “artificial teeth” platform, integrated with the microfluidic oxygenators, functions as an effective and inexpensive analysis tool to dynamically adjust critical growth parameters such as bacteria population, growth medium composition, medium flow rate and dissolved oxygen levels. The first single-chamber “artificial tooth” chip is developed for long-term dental biofilm culture with better medium handling, such as mixing, humidification and automated growth medium replenishment. This device is also compatible with different analysis techniques using optical microscopy in order to determine the biofilm thickness, the ratio between viable and dead cells, and the visualization of spatial distribution of different dental bacteria in the biofilm.

Furthermore, the single-chamber design is extended to a device containing up to 128 chambers. This “artificial teeth” chip is developed to achieve high-throughput parallel
biofilm culture and analysis with a matrix of different growth conditions that can contribute to the quantitative studies of the physiology of dental biofilms. The artificial teeth device is applied to investigate the response of two key dental bacteria, *Streptococci* sp. and *Fusobacterium nucleatum*, in the biofilm under different microenvironments, including their growth under different gas conditions and their adherence properties with different sucrose concentrations. This work demonstrates a successful application of microfluidics to long-term biofilm culture applications.

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Cambridge, Massachusetts
2010
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Dental plaque has a complicated formation process involving multiple microorganisms and extracellular matrix, including protein, lipids, and long-chain polysaccharides. Conceptually, plaque development can be viewed as a complex biological co-aggregation of microbes, which grow and evolve in a symbiotic environment that is sensitive not only to the local biofilm composition, but also to the surrounding oral environment. The development of oral microbial communities involves competition as well as cooperation among more than 500 species. Understanding the conditions that promote colonization and subsequent biofilm development is particularly important, as dental plaque formation not only acts as a precursor to tooth decay, but also induces more serious systemic health problems such as cardiovascular disease, pre-term labor, and diabetes especially when left untreated. Dental bacteria that have entered the bloodstream through bleeding gums can set off a cascade of events that can cause serious systemic problems. Chronic gum infection has been linked to the development of clogged arteries and blood clots, putting people at an increased risk of heart attack or stroke.

Under proper physiological conditions (e.g. nutrients and oxygen concentrations), the primary adherents in biofilms form micro-colonies providing the foundation for subsequent co-colonization with other species. As biofilm develops, bacteria secrete extracellular polymeric substances that act as a strong glue to enhance the mechanical stability of the biofilm and shield its inhabitants from adverse environmental changes. Complex dental biofilms, commonly referred to as plaque, require strong mechanical scraping for removal, and, once established, are not affected by phenol-based mouthwashes such as Listerine. Classic studies have outlined two stages in such microbial adhesion: (1) the reversible sorption, which is an instantaneous attraction of micro-organisms to tooth surfaces in the early colonization stage, and (2) the following irreversible sorption, which involves biologically specific reactions providing firm adhesion to the surfaces during biofilm development. Many coaggregation adhesins have been identified on the cell surfaces of dental plaque bacteria and the majority of such coaggregation adhesins identified so far are synthesized by Streptococci species and Actinomyces naeslundii.

Dental biofilms have been extensively studied and characterized through in vitro laboratory experiments, which investigate the co-colonization of bacteria on artificial matrices. In vitro microcosms involving culture of the mixed oral flora in a biofilm reflect
plaques and appear to be a realistic approach to some applications. To reproduce the dental biofilm formation, local environmental conditions for *in vitro* cell culture should be tightly regulated. However, it is non-trivial to create *in vitro* systems for simulating oral conditions. Minor variations in the composition or the ionic strength of the suspending medium may alter the adhesive properties in the system. With regard to temperature, pH fluctuations and atmospheric conditions, most early dental plaque model systems failed to mimic the oral environment mainly because they lacked consistent reproducibility. In the past thirty years, a number of the model systems have been reported and applied to some specific applications. The multiple artificial mouth (MAM) system developed by Sissons *et al.* was employed for the long-term growth of five plaque samples sharing the same simulated oral environment generated by computer-controlled facilities. As a platform for biofilm culture, MAM is probably the best existing option among the various developed systems. However, its inflexible hardware settings and incapability on growth detection make it suboptimal as a surrogate dental biofilm culture system.

The challenges of maintaining and operating continuous bioreactors or culture systems, including the requirement for large quantities of growth media and reagents, have pushed the move toward miniaturization and chip-based control. Moreover, the considerable diffusion time, induced by macro-size length scale, limits the gas (e.g. oxygen and carbon dioxide) transfer capacity, of conventional bioreactors. Stirred tanks in bioreactors are an effective way to enhance gas and liquid transfer in media; however, their operation is often incompatible with many culture applications, especially for ones that require a steady microbial environment.

Microfluidics, consisting of molded or machined micron-scale channels to manipulate nanoliter sample volumes, is an interesting technology to scale down and potentially automate culture-based platforms. With the ability to run dozens to hundreds of assays in parallel in a low-cost, disposable polymeric chip, microfluidics has the potential to revolutionize the ability to perform procedures such as high-throughput drug screening, biological and chemical sensing, and genetic analysis. The ability to process samples at nanoliter volumes significantly reduces dosage requirements for testing, cutting down on both cost and waste. The length scale of microfluidic systems increases the speed of common experimental techniques, such as protein and DNA electrophoresis, by orders of magnitude. As a tool for cellular culture, microfluidics is a tool that enables the bench researchers to spatiotemporally regulate the environment around cell populations at different scales, ranging from single cells to thousands/millions of cells.

Silicone rubber-based chemostats, bioreactors, and other microfluidic platforms containing multiple cell chambers have been successfully applied in the microbial cell culture applications in recent years. These microfluidic devices, consisting of optically-transparent polydimethylsiloxane (PDMS), were fabricated using a casting process from silicon wafer molds containing photoresists with positive-relief channel patterns. The fabrication is based on both standard lithographic techniques for a single layer of channel networks, and multilayer soft lithography, which uses stacked 2D networks of microchannels to add functional valves and pumps on chip, for example.

PDMS is an ideal structural material for cell culture, as it can be easily molded with soft lithography and exhibits high gas permeability. In miniaturizing culture systems with
microfluidic chips, the volume of the culture chambers can be reduced from from the mL to nL scale. As microfluidic devices only require a minimal amount of media and culture samples, biological waste can be greatly reduced. The pneumatic valving mechanism for PDMS microfluidic chips enables gas regulation, nutrient/chemicals insertion, and waste removal on a chamber-by-chamber basis. Using simple programming interfaces (e.g. Java and C++), single chamber regulation can be easily achieved, providing a higher level of spatiotemporal culture control that can not be obtained using bulk culture tools.

While previous studies of dental biofilm have enhanced our understanding of adhesion and organization properties, setting up a matrix of experimental conditions to mimic the microenvironments present in human mouth remains a laborious process. In this thesis, the microfluidic “artificial teeth” platform has been developed for the culture of dental bacterial isolates to study in vitro biofilm formation on saliva-coated substrates contained within individual microchambers. The multilayer elastomeric microfluidic devices support the spatiotemporal control of multiple parameters, such as nutrients concentration, oxygen level and microorganism composition. The corresponding throughput has obtained significant improvement in chamber density (>10^2 chambers) vs. existing state-of-the-art biofilm model systems (<10 chambers).

1-2 OBJECTIVES

The overall goal of this thesis research was to develop a microfluidics-based platform to support the ongoing need to study biofilm formation that leads to plaque development and subsequent tooth decay in the mouth. The artificial teeth devices fabricated contain up to 128 culture chambers, enabling the analysis of biofilm culture dynamics under environmental changes mimicking the oral cavity, including fluctuations in sucrose and oxygen concentrations. The proposed multilayered microfluidic platform uses a series of elastomeric microchambers for bacterial colonization and subsequent biofilm formation. The platform provides the ability to dynamically adjust critical parameters such as bacteria stains, growth medium composition, medium flow rate and dissolved oxygen level. With the functionality of controllable input oxygen concentration, solution flow rate and supplied nutrients, this microfluidic platform functions as a very powerful, inexpensive tool to study differential biofilm formation under a matrix of environmental conditions.

The specific aims of the research outlined in the thesis are:
1. To develop a high-throughput chemostat-type dental biofilm system, which can perform growth characterization and be further applied to general biofilm analyses with no/minimal modifications;
2. To provide a digitally-controllable environment (steady or transient) for the co-culture of multiple bacterial species;
3. To study how the variations in oral environment, such as sucrose and oxygen levels, affect the dental biofilm formation;
4. To evaluate the growth process of biofilm, formation especially in the early stage, including the ratio of viable/dead cells and the evolution of the biofilm thickness as it matures; and
5. To identify subset of the major colonizers in an in vitro dental biofilm.
Overall, the work contained in this thesis provides a roadmap for the development of a microfluidic platform for the multiplexed culture and analysis of a model dental biofilm. Having a microlfuidic tool to study the complex and dynamic environmental conditions driving dental biofilm formation, the platform opens the door for future follow-up work, including screening mechanisms to disrupt the biofilm formation through techniques such as small molecule inhibitors and surface chemistry modifications.

1-3 THESIS OUTLINE

This thesis is divided into two principal research thrusts: (1) Development and modeling microfluidic oxygenation systems on a chip, and (2) “artificial teeth” devices for in vitro dental biofilm development and analysis. The following chapter first describes about the fundamental technologies and concepts, including also the literature reviews.

The works on microfluidic oxygenation are split into three chapters. Chapter 3 introduces the concept and basic structure of oxygenator, as well as the related measurement techniques. Chapter 4 analyzes the oxygen transfer in oxygenator and outlines the device design strategies. Chapter 5 can be viewed as the extension of oxygenation to generate differential and tunable dissolved oxygen levels. Chapter 6 discusses about the issues on long-term culture using microfluidic devices, including the regular medium replacement and long-term oxygenation approaches. It also includes verification experiments on the long-term culture of several dental bacteria. Chapters 7 and 8 focus on the design and operations of “artificial teeth” devices. Chapter 7 first describes the design and analysis techniques of the device unit for dental biofilm culture – the single chamber “artificial tooth” chip. Following this chapter, two versions (32-chamber and 128-chamber) of multiple chamber “artificial teeth” devices are described in chapter 8. This chapter also includes the results on parallel dental biofilm culture and analyses for different cellular environments. Finally, the thesis is summarized in chapter 9. Potential applications and further development are also proposed in this chapter.

REFERENCES

2-1 DENTAL BIOFILM FORMATION

Dental biofilm is best characterized as a complex multispecies mat of bacteria adhering to and growing on tooth surfaces (Fig. 2.1). It often develops with the aid of polymers secreted from the wide variety of dental bacteria, including two major colonizing groups. The early colonizers, principally spherical coccal bacteria like Streptococci, dominate the biofilm over the first 24 hours. The relative number of viable micro-organisms is lower in the early (<4 hr) (vs. the late (>24 hr)) phase of plaque formation, probably due to the exposure of salivary antimicrobial factors against early colonizers. As an early colonizer, Streptococcus sp. adheres to the teeth and acts as a template that defines the specificity of subsequent microbial colonization in early plaque formation. As the biofilm ages, filamentous bacteria like Actinomyces sp. and Fusobacterium sp. co-colonize with the Streptococcus sp. and give the biofilm a pronounced corn-cob-like morphology. Eventually, high levels of gram-negative anaerobic bacteria join the biofilm, fermenting sugars and creating noxious by-products, as found in halitosis.

Van Loosdrecht et al. schematically illustrated microbial adhesion to tooth surfaces as a four-stage sequence, and suggested that the four stages may be studied separately in a laboratory environment. The first adhesion stage involves the initial transport of a bacterium to the tooth surface. Random contact may occur through Brownian motions, sedimentation of micro-organisms, liquid flow, or active movement to the micro-organisms. Afterwards, the initial and reversible adsorption is established by the interplay between attractive van der Waals’ forces and repulsive electrostatic interaction. These interactions are influenced by factors such as the pH and ionic strength of the suspension medium. The adhesion of early colonizers initiates dental plaque formation. Streptococci first bind to various components in the pellicle coated on tooth surface, including praline-rich proteins and enzymes such as α-amylase. Biologically-specific microscopic characteristics of the pellicle-covered tooth and microbial surfaces become determinant for the attachment. Streptococcus sp. is found to be the only genus of oral bacteria that demonstrates extensive intrageneric co-aggregation as well as intergeneric co-aggregation. The ability to bind to other early colonizers and small molecules (e.g. sugars and peptides) on the tooth surfaces provide an advantage that Streptococci exploit in establishing early dental plaque. S. gordonii and S. ganguis were shown to
be primary colonizers that promote adhesion to tooth surfaces. Actinomyces sp., such as A. naeslundii, then attaches to Streptococci and form the major primary colonized community of the tooth surface. The interactions between Streptococci and Actinomyces, and the anchoring tooth substrate provide the scaffolding for the early biofilm community. Once the initial colonizers have attached to the surface, cell-cell communication comes into play. F. nucleatum is extremely important in this stage by co-aggregating with all of the early colonizers and the late colonizers, such as P. gingivalis and P. intermedia. (However the later colonizers generally do not co-aggregate with each other.) In the absence of F. nucleatum, many other secondary colonizers cannot become part of the dental plaque community. Additionally, anaerobic secondary colonizers cannot survive in the planktonic state unless coaggregated to F. nucleatum. In the last stage, surface colonization and biofilm formation continues until a saturated state is achieved, where the thickness is a function of both the biofilm composition and the local environmental resources (e.g. oxygen and nutrients).

Figure 2.1. Diagrammatic representation of the human oral bacterial accretion on tooth surfaces (adapted from ref. 16).
2-2 EXISTING ARTIFICIAL MOUTH SYSTEMS

Many dental biofilm culture systems, often called "artificial mouths", with the control of multiple environmental factors have been developed over the past thirty years\(^\text{17}\) for various aspects on the biofilm analysis and characterization. In some basic applications, flow cells with saliva-coated surfaces are particularly useful for short-term studies of biofilm formation and observation\(^\text{18,20}\). In terms of "artificial mouth" systems, Sissons has summarized the recent model systems into four major categories (Table 2.1): chemostat-based systems, growth rate-controlled biofilm fermenters (GRBFs), constant-depth film fermenters (CDFFs) and multiple artificial mouth (MAM) models.\(^\text{21}\)

The chemostat-based system\(^\text{22-25}\) supports the growth of microorganisms in a physiological steady state. It consists of a continuous-flow culture chamber. Culture medium is removed while fresh medium is being applied during operation. The steady growth rate of bacteria can be controlled by varying the medium flow rate. GRBFs\(^\text{26,27}\) were developed to regulate the development rate of biofilm. They can be viewed as the extended version of chemostat-based systems with real-time cell counting capability. The growth of cell communities were regulated by a limited medium supply depending on the transient growth rate estimated by the number of freshly divided daughter cells at the medium outlet. The CDFF\(^\text{27,28}\) provides a set of multiple culture regions with a defined allowable height. When the dental biofilm grows beyond the target height, the excessive portion of biofilm surface will be swept off by a Teflon blade. Such a culture environment is not realistic mimic of the human cavity as the bacteria removal by sweeping generates an extremely high shear over the entire community, and may cause serious mechanical damage, even on the inner part of biofilm. The MAM\(^\text{29-31}\) was specifically designed to mimic the human oral environment with computer control of (i) three or more independent simulated oral fluid supply, and (ii) data acquisition for continuous measurement (e.g. pH) up to two weeks.\(^\text{21}\) However, its inflexible hardware settings and incapability of detecting biofilm growth indicate that there is room for improvement in dental biofilm systems.

<table>
<thead>
<tr>
<th>Table 2.1. Summary of dental biofilm systems</th>
</tr>
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<tbody>
<tr>
<td>Throughput</td>
</tr>
<tr>
<td>------------</td>
</tr>
<tr>
<td>Culture duration</td>
</tr>
<tr>
<td>Shear</td>
</tr>
<tr>
<td>Flow controllability</td>
</tr>
<tr>
<td>Thickness</td>
</tr>
<tr>
<td>Growth measurement</td>
</tr>
<tr>
<td>Environmental control/detection</td>
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</table>

The chemostat-based system, while a potential candidate to handle the high-throughput biofilm research, is constrained by the need to provide continuous flow, which impedes primary colonization. This problem can be solved by adopting a programmable flow control scheme that provides a more stable liquid environment and enables control of the flow rate...
and direction. With an optimized configuration of the dynamic flow control, such a system can provide adequate nutrient supply, and at the same time, negligible shear and a static microenvironment for cell attachment. The modified chemostat-based approach is also scalable, enabling parallel culture processes in multiple chambers can also be controlled by a shared medium supply. For growth measurement, chemostat-based systems can be manufactured with an optical transparent material, with dynamic biofilm analysis monitored using optical microscopy.

2-3 MICROFLUIDICS FOR MICROBIOLOGY RESEARCH

Research efforts in microfluidics – an enabling technology to perform operations on fluid using sub-microliter volumes – have rapidly expanded in terms of both applied and theoretical applications over past two decades. Microfluidic device engineering has become a cross-disciplinary effort, often involving different aspects of science and engineering (e.g. optics, biology, chemistry, mechanics and material science). Some of the major advantages of the device miniaturization are the reduction in biological and chemical wastes production, precise fluid handling, faster analysis and molecular-based detection, and the capability to carry out parallel or high-throughput operations.

Microfluidic devices can handle clearly handle small volume of fluids, extending down to attoliter-scale volumes in the case of droplets, which can be applied a large variety of biological solutions (i.e. enzymes, nucleic acids, cells). Such devices offer excellent benchtop controllability and provide a tool to manipulate molecules or cells in microchannels/chambers with dimensions on the order of ~10 – 100 μm. Some examples of applied microfluidic research in microbiology include the quantification of bacteria in environmental samples, toxin detection in food samples, saliva-based detection of infectious diseases, and characterization of bacterial transport parameters. Microfluidic devices have also been used to precisely control the in vitro biofilm environment. In particular, a device was developed to study the biofilm morphology forming under shear by imposing different flow velocities. An integrated microfluidic chip has also been used for monitoring cell culture, including the monitoring of culture density over time and the probing cellular functions at a single-cell level.

The implementation of traditional laboratory work with microfluidic chips is often called “micro total analysis systems (μTAS)” or “lab-on-a-chip (LOC)”, and the biocompatibility and biological/chemical stability of the device material is a serious issue. While silicon-based microdevices manufactured with methods developed in the semiconductor industry were some of the earliest examples of microfluidics-based research, there has been a shift over the last decade towards polymer-based devices, using materials such as Teflon, thermoset polyesters, silicon elastomer photoresist, SU-8 photoresist, polydimethylsiloxane (PDMS) and poly-methylmethacrylate (PMMA).

While there have been many good examples of microfluidic tools introduced for cell typing, primarily using modified immunoassay protocols, organism identification using nucleic-acid based probes, while potentially faster and easier to develop for a specific strain, is still challenging. Nucleic-acid based assays, using techniques such as fluorescence in situ
hybridization (FISH), have broad applications that extend beyond typing, including gene expression monitoring in individual cells\textsuperscript{48}, and the chromosomal analysis for mammalian cells\textsuperscript{49}. The optimization of FISH assays for biofilm analysis is particularly interesting, as probes targeting the highly-conserved 16s rRNA, for example, can be used to identify species variants within a film.

2-4 SPECIFICATIONS FOR IN VITRO DENTAL BIOFILM CULTURE

A microfluidic dental biofilm system developed to culture dental bacterial isolates and study dental biofilm formation \textit{in vitro} should be scalable, gas permeable to enable dissolved oxygen levels to be modified to support the culture of both aerobic and anaerobic \textit{sp.}, temperature-regulated, and optically clear to dynamically monitor biofilm formation and growth.

Using multilayer soft lithography (MLS) and PDMS rubber, a scalable microfluidic device architecture can be readily developed for parallel operation, creating a miniaturized version of the chemostat-based biofilm culture system with dozens to hundreds of chambers. MLS provides a facile fabrication method to incorporate integrated functional elastomeric components into microfluidic devices for cell biology (e.g. pneumatic microvalves, micropumps and multiplexors\textsuperscript{53,54}) that enable not only sample compartmentalization, but also control of nutrient delivery and waste removal. As an example of a proof-of-concept microfluidic device with a scalable architecture for biofilm analysis, an “artificial teeth” device with up to 128 chambers was developed (with its target specifications outlined in Table 2.2). Each culture chamber has sufficient capacity and depth (>150 \textmu m) to accommodate the salivary film (pellicle) (~10 \textmu m\textsuperscript{50}) and dental biofilm (~80 \textmu m\textsuperscript{51,52}). The microfluidic platform functions as a very powerful, inexpensive tool to study differential biofilm formation under a matrix of environmental conditions, and establishes an important baseline for other similar \textit{in vitro} platforms that provide a tailored application-specific environment for cell culture and analysis.

Gas regulation is also a critical feature in a dental biofilm chip, of PDMS is also a good choice for cell-based microfluidic devices, as both the composition of a dental biofilm and its growth rate depend on the level of dissolved oxygen (DO) in the surrounding saliva. In human oral cavity, the volumetric ratio of oxygen in daytime is around 16 (exhaled) – 20 % (inbreathed)\textsuperscript{55-57}. While in a deep sleep stage, the tongue often relaxes and naturally falls towards the back of throat. Such action prevents substantial amount of oxygen to reach the back of the mouth during overnight hours and so it induces a microaerobic or even anaerobic environment. However, most of the reported dental biofilm systems provide solely an aerobic\textsuperscript{6,58,59} or anaerobic environment\textsuperscript{29,60}. To improve on prior efforts, and create a more realistic model system for biofilm growth, the artificial teeth chip described in this thesis enables dynamic tuning of the DO environment by exploiting the gas permeable nature of the PDMS substrate (as presented in Chapters 3 – 5).

Temperature regulation is essential for a microfluidic \textit{in vitro} dental biofilm system, as parameters such as cell growth and dissolved oxygen levels ultimately depend on temperature. For work in this thesis, temperature regulation was carried out using a
commercial regulated plexiglass enclosure (Weather Station™, Olympus) that fits over a microscope stage, for microscopes. Because the temperature in oral cavity is steady over time, analyzing the bacterial growth behavior under different local temperature was not the major interest in this work. In case a tunable temperature control scheme is needed for particular applications, embedded micro-heaters can be fabricated underneath the artificial teeth chip.

**Table 2.2. Specifications of the proposed artificial teeth platform.**

<table>
<thead>
<tr>
<th>Requirement</th>
<th>Reasons</th>
</tr>
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<tbody>
<tr>
<td>Culture duration</td>
<td>~Days</td>
</tr>
<tr>
<td>Throughput</td>
<td>≥10²</td>
</tr>
<tr>
<td>Chamber size</td>
<td>Height: &gt;150 μm, Diameter: &gt;&gt;height</td>
</tr>
<tr>
<td>Medium insertion</td>
<td>Continuous or regular replacement</td>
</tr>
<tr>
<td>Medium flow rate</td>
<td>0 – 0.01 μL/min with accurate control</td>
</tr>
<tr>
<td>Substrate material</td>
<td>Compatible with salivary protein deposition</td>
</tr>
<tr>
<td>Temperature</td>
<td>~37 °C with negligible variation</td>
</tr>
<tr>
<td>Sucrose concentration</td>
<td>0 – 10 %, changeable</td>
</tr>
<tr>
<td>DO content</td>
<td>0 – 21 %, changeable</td>
</tr>
<tr>
<td>Shear stress</td>
<td>&lt;5 mPa</td>
</tr>
<tr>
<td>Detection scheme</td>
<td>Capable of measuring biofilm thickness, and the spatial distributions of species and living/dead cells (optional)</td>
</tr>
</tbody>
</table>

Finally, optical transparency (and low fluorescence background) of the microfluidic substrate is desirable for both biofilm growth analysis and cell labeling studies. PDMS has excellent transparency and background characteristics, enabling the dynamic monitoring of the biofilm dynamics using conventional optical microscopy platforms.

REFERENCES

3-1 BASIC DESIGN OF OXYGENATOR

Monitoring and controlling the dissolved oxygen (DO) concentration in medium are critical for biological culture and tissue engineering applications. Cellular growth, especially biofilm formation, involves the complex correlations of growth environment\textsuperscript{1-2} and cell-cell communications among cellular species\textsuperscript{3-4}. For cellular growth analysis, including the single cells/small cell cluster\textsuperscript{5} monitoring, precise control of the cellular environment is clearly desirable. Several microscale silicone-based chemostats\textsuperscript{6-8}, bioreactors\textsuperscript{9-12}, and other microfluidic platforms\textsuperscript{13-14} containing multiple cell chambers have been developed for this purpose. Such platforms were engineered to provide moderate to long-term control (on the order of hours to days) of the microenvironment, including elements such as temperature, pH value, dissolved gas concentration, nutrient delivery and waste removal. Because of the excellent reproducibility of soft lithography\textsuperscript{15}, the structural material choice of many microfluidic platforms is polydimethylsiloxane (PDMS), which is biocompatible and has an oxygen diffusivity ($DO_{2-PDMS} \approx 6 \times 10^{-5} \text{ cm}^2\text{s}^{-1}$)\textsuperscript{16} on the same order as water at standard temperature and pressure (STP) (20 °C and 101.325 kPa)\textsuperscript{17}.

Several oxygenation methods have been recently reported that regulate medium DO using integrated microfluidic gas channels\textsuperscript{8,18-21}. Oxygenation can be achieved through an electrode array to regulate the local oxygen levels along a series of culture chambers\textsuperscript{22}. In such approach, oxygen was generated using water electrolysis in a channel fabricated below the medium channel. While very effective, the method utilized a complex fabrication protocol and required precise control of the electrolysis process. Controlling local DO levels in PDMS microfluidic devices can be also achieved by flowing oxygen through dedicated gas microchannels that are in close proximity to the fluid-filled microchannels. Using conventional soft lithography methods\textsuperscript{15,23}, separation distances between gas and fluid microchannels on the order of tens of microns can readily be achieved. A double-layer gas perfusion network structure fabricated above the cell culture region was designed for parallel mammalian cell culture\textsuperscript{24}. Using a continuous oxygen supply, identical DO levels were maintained within an array of wells via passive gas diffusion.

Due to the low aspect ratio (height to width) of the microchannels fabricated by multilayer soft lithography, the mass transfer for the double-layer microfluidic oxygenator
can be modeled as 2-D geometry as shown in Fig. 3.1. The oxygenator contains two channel layers with an upper oxygen reservoir over the cell culture chamber (height: \(H_2\)). A thin PDMS membrane (thickness: \(H_3\)) separated the channel layers, allowing only diffusion for the gas transfer across the membrane. The PDMS substrate is bonded on a glass slide to close the medium channel, so the bottom surface of medium channel is oxygen-impermeable. During cell culture, a layer of biofilm (thickness: \(H_1\)) can form on the lower surface along channel over continuous/regular supply of culture medium. Under steady fluid flow with very low Reynolds number, there is a locally fully-developed velocity profile \(u(y)\) along the cell culture chamber.

The PDMS membrane should be sufficiently thin to obtain a short diffusion time. However, it should also provide sufficient stiffness against the deformation by gas pressure or hydraulic pressure of medium, setting a constraint on the membrane thickness. For a long oxygenation channel, the maximum possible deflection of membrane \(\delta b\) can be approximated as a simply supported beam model (in the channel width direction) under an equivalent pressure difference:

\[
\delta b = \frac{5W_b^4 \Delta P}{32E_{\text{PDMS}}H_b^3}
\]

where \(E_{\text{PDMS}} (= 750 \text{ kPa})\) is the Young’s modulus of PDMS; \(W_b\) and \(H_b\) are the width and thickness of membrane; and \(\Delta P\) is the pressure difference between input gas and medium. For an oxygenation channel with \(W_b = 100 \mu\text{m}\) and \(H_b = 20 \mu\text{m}\), a culture channel can be designed with thickness 20 \(\mu\text{m}\) with negligible membrane deflection (~1 \(\mu\text{m}\)) under the operation with gas pressure \(\sim 0.5 - 1.5 \text{ kPa}\) and medium flowrate \(\sim 0.01 \mu\text{l/min}\) (equivalent to hydraulic pressure \(\sim 1 \text{ kPa}\)).

**3-2 VALIDATION OF VELOCITY PROFILE**

The velocity profile of medium mentioned in the previous section can be modeled as the planar Poiseuille flow. Validation of the flow profile is useful in the analysis of the molecular diffusion of oxygen from the gas channel to cell layer in the developing region.
Considering the Navier-Stokes equations of the medium layer with a chamber height (100 μm – 800 μm) much smaller than its length (3 mm) and width (3 mm), Reynolds number (Re << 1) is very small (<< 1) under an imposed medium velocity of <1 mm/s. It is assumed that the rate of change of the medium flowrate is sufficiently small such that the quasi-steady assumption holds. In the ideal case, the velocity profile \( u(y) \) is parabolic along the vertical direction, described by

\[
    u(y - h) = \frac{6Q}{WH^2} (Hy - y^2), \quad \text{for } H_1 \leq y \leq H_2
\]

where \( Q \) is the flow rate of culture medium, \( W \) is the chamber width and \( H_e = H_2 - H_1 \) is the effective chamber height.

Particle Image Velocimetry (PIV) was applied to validate the steady velocity field along the effective region of microchannel \((H_1 < y < H_2\) in Fig. 3.1). In the experiment, fluorescent beads (Molecular Probes’ yellow-green-fluorescent Fluospheres beads (F8852), 1 μm diameter, Invitrogen) were first seeded with distilled water \((Q = 1 \mu L/min \text{ at } Re = 0.003)\) in a microchannel without a cell layer \((H_1 = 0 \mu m, H_e = 500 \mu m \text{ and } W = 100 \mu m)\) fabricated by soft lithography. A relatively high aspect ratio (= 5) was used to generate a parabolic velocity profile with respect to channel width, which is equivalent to the velocity profile with respect to channel height direction of microchannel with aspect ratio 0.2 \((H_e = 100 \mu m \text{ and } W = 500 \mu m)\). Hence, this experiment can also validate the developed velocity profile \( u(y) \) along the medium channel (aspect ratio: <0.2) in Fig. 3.1. A series of microscopic images was then photographed with a GFP fluorescence filter at a fixed spot on the channel. The mean local fluidic velocity of each image sub-region, segmented with fixed length (in pixel) along width and height, was calculated by the cross-correlation of image intensity. The detailed calculation procedures are described as follows:

1. Consider a series of consecutive grayscale images of fluorescent particles \( F_k(i, j) \),

\[
    F_k(i, j), \quad \text{for } k = 1, 2, \ldots, N + 1; i = 1, 2, \ldots, X; j = 1, 2, \ldots, Y
\]

where \( k \) is the image index, \( N + 1 \) is the number of images, \( X \) and \( Y \) are the image width and height in pixel, respectively.

2. Since particles seldom exist at a particular image pixel over a long period of image capture, the background intensity \( I(i, j) \) can be estimated by

\[
    I(i, j) = \frac{1}{N + 1} \sum_{k=1}^{N+1} F_k(i, j), \quad \text{for } i = 1, 2, \ldots, X; j = 1, 2, \ldots, Y.
\]

3. Removing background noise, obtain \( f_k(i, j) \) by sampling the updated intensity in a segmented region with lower left corner \((X_w, Y_w)\) and upper right corner \((X_w + X/N_x, Y_w + Y/N_y)\), where \( N_x \) and \( N_y \) are the number of segments along image width and height, respectively:

\[
    f_k(i, j) = F_k(X_w - 1 + i, Y_w - 1 + j) - I(X_w - 1 + i, Y_w - 1 + j),
\]

for \( k = 1, 2, \ldots, N + 1; i = 1, 2, \ldots, X/N_x; j = 1, 2, \ldots, Y/N_y \)

4. Calculate the cross-correlation of each image sub-region \( \Phi_k(u, v) \) by
\[ \Phi_k(u,v) = \sum_{i=1}^{X/N_k} \sum_{j=1}^{Y/N_k} f_k(i,j) \cdot f_{k+1}(i+u, j+v), \text{ for } k = 1, 2, \ldots, N. \] (3.6)

The peak location of \( \Phi(u,v) \) is the estimate of mean velocity in a particular image region [pixel/(time between two consecutive images)], calculated by

\[ \Phi(u,v) = \frac{1}{N} \sum_{k=1}^N \Phi_k(u,v). \] (3.7)

The result (Fig. 3.2a,b) shows that the medium velocity basically follows a parabolic profile, as expected.

![Image](image.png)

**Figure 3.2.** (a) Measured velocity field using PIV, and (b) its comparison with the analytical parabolic profile \( u(z)/u(W/2) \) along channel width \( (z-) \) direction.

### 3-3 GAS MIXING MICROCHANNEL

The double-layer oxygenator effectively regulates the medium DO, emphasizing the importance of the design of the gas exchange system. The preparation of gases with a particular concentration of oxygen can be achieved by mixing pure nitrogen and oxygen in different ratios. To scale down the process to a chip level operation, the basic microfluidic gas mixing element was designed as a microchannel structure composed of two inlet
channels and one outlet channel as shown in Fig. 3.3a. By designing sufficiently long inlet channels, nitrogen and oxygen are locally fully mixed along them. The validity of such assumption is supported by the low scaled Peclet number, defined by the ratio between convection and diffusion, in the gas microchannels, i.e. $Pe^* = \frac{UW}{D-W/L} << 1$, with diffusion dominating over convective fluxes. For a gas mixer having two inlets with different flow rates and oxygen concentrations, the corresponding oxygen concentration $C$ after mixing can be estimated based on the conservation of mass:

$$C = \frac{Q_1C_1 + Q_2C_2}{Q_1 + Q_2}$$  \hspace{1cm} (3.8)

where $Q_1$ and $Q_2$ are the flow rates of the channel inlets; and $C_1$ and $C_2$ are the corresponding oxygen concentrations.

Practically speaking, the flow rates of gases along channels are regulated by the supply pressure of the gases at inlets ($P_1$ and $P_2$) and outlet ($P_o$). For given inlet and outlet pressure, the flow rates along each channel can be predefined by the fluidic resistance along inlet ($R_1$ and $R_2$) and outlet ($R_o$) channels, given by

$$Q_1 = \frac{P_1 - P_o}{R_1}, \hspace{1cm} (3.9)$$

$$Q_2 = \frac{P_2 - P_o}{R_2}, \hspace{1cm} (3.10)$$

$$Q_o = \frac{P_o}{R_o}$$  \hspace{1cm} (3.11)

where $Q_o$ is the flow rate of outlet channel, and $P_o$ is the gage pressure at the channel connection defined by

$$P_o = \frac{P_1/R_1 + P_2/R_2 + P_o/R_o}{1/R_1 + 1/R_2 + 1/R_o} \hspace{1cm} (3.12)$$

In microchannels with length scale $\sim 100 \mu m$ flowed with gases at velocity $\sim 1 \text{ ms}^{-1}$, the corresponding scaled Reynolds number ($Re^* = \frac{\rho UL}{\mu \cdot H^2/L}$), which is defined as the ratio between inertial and viscous effects, are typically in the range $\sim 10^{-3} - 10^{-1}$. Under dominating viscous effect, the velocity profile of gas flow is locally fully developed and the fluidic resistance $R$ for a straight rectangular microchannel can be estimated as

$$\frac{1}{R} = \frac{WH^3}{12\mu L} \left\{1 - \frac{192H}{\pi^5 W} \sum_{n=0}^{\infty} \tanh[(2n+1)\pi W / 2H] \right\} \hspace{1cm} (3.13)$$

where $\mu$ is the viscosity of fluid along channel; $L$, $W$ and $H$ are the channel length, width and height, respectively.

The folded channels were designed to be much narrower ($\sim 20 \mu m$) than the channels for flow connection ($\sim 100 \mu m$). So, the resistance of folded channels dominates over other sections in the mixer, and the overall resistance can be regulated by the length of folded channels. Eq. 3.13 indicates that the fluidic resistance is determined by channel dimensions and the viscosity of flowing gas mixture. Due to the different viscosity between N$_2$ and O$_2$,
such viscosity of gas mixture $\mu_m$, depending on the volumetric ratio of oxygen and nitrogen, can be approximated as

$$\mu_m \approx \frac{1}{C_{O_2}/\mu_{O_2} + C_{N_2}/\mu_{N_2}}$$

(3.14)

where $C_{O_2}$ and $C_{N_2} (= 1 - C_{O_2})$ are the volumetric concentration of oxygen and nitrogen; $\mu_{O_2} (= 2 \times 10^{-4} \text{ cm/s})$ and $\mu_{N_2} (= 1.8 \times 10^{-4} \text{ cm/s})$ are the viscosity of oxygen and nitrogen, respectively.

The performance of mixer was validated by a series of experiments using the fabricating mixing channel with different inlet resistances ($R_1$ and $R_2$). Pure nitrogen and oxygen gases were applied at the inlets with the same pressure ($P_1 = P_2 = 1 \text{ kPa}$), while the outlet is connected to an oxygen sensor. (Details of the oxygen measurement are discussed in section 3.4.) Result (Fig. 3.3b) shows that the experimental oxygen levels correlated well with the expected values calculated by Eqs. 3.8 – 3.14.

![Diagram of microfluidic gas mixer](image)

**Figure 3.3.** (a) The structure of microfluidic gas mixer and (b) the result of the validation experiment. Experimental values are plotted against analytical volumetric ratios of oxygen, scaled from 0 to 1. The error bars represent the standard deviation of each data point calculated from 5 repeated measurements.
3-4 POLYMERIC OXYGEN SENSOR

Measurement of partial pressure of oxygen (pO2) in oxygenated channel is essential for the performance characterization of a particular microfluidic oxygenation technique. For precise control of pO2, this can also be used as the feedback signal for the dynamic adjustment of medium DO. While Clark-type electrodes have been used for nearly half a century to measure DO levels in aqueous media\textsuperscript{26-27}, these devices irreversibly convert oxygen molecules to hydroxide ions that are potentially harmful to cells. Moreover, miniaturizing and integrating such electrodes into microscale flow devices is challenging, limited by fabrication complexity, noise, and run-to-run signal drift. Non-contact optical sensors represent a better alternative for the DO measurement in biofluidic systems\textsuperscript{28-30}. They typically consist of an oxygen-permeable polymer film embedded with a sensing material, e.g. Pt/Pd-porphyrin complexes\textsuperscript{31}, polyaromatic hydrocarbon complexes\textsuperscript{32} or ruthenium dimines\textsuperscript{33}. The operation principle is based on the reduction in luminescent intensity of sensing material due to the oxygen quenching of the emitting excited electronic state. Recently, a method to fabricate Pt-porphyrin complexes as patterned polymeric films with consistent film thickness and a high signal-to-noise ratio has been presented\textsuperscript{34}. Porphyrin-based sensors are a practical choice for real-time microfluidic oxygen sensing, providing the benefits of reversible quenching, high sensitivity and biocompatibility\textsuperscript{35-36}.

Real-time oxygen concentration measurement is achieved by an optical oxygen-sensing system. Platinum (II) octaethylporphine ketone (PtOEPK) was selected as the optical sensing element due to its long lifetime, high photostability and low photobleaching rate among other fluorescent dyes. The excitation (570 nm) and emission (760 nm) wavelengths of PtOEPK induce a large Stokes shift to reduce the signal-to-background ratio\textsuperscript{37-38}. A schematic diagram of the oxygen-sensing scheme is illustrated in Fig. 3.4a. The excitation light is generated by a yellow light emitting diode (LED) with a bandpass color filter (CVI laser, BG-39) placed between the LED and the microfluidic oxygenator chip. The PtOEPK dye, embedded in polystyrene films integrated into the fluid channels of the oxygenator, re-emits light with an intensity corresponding to the oxygen concentration. The emitted light is detected by a photodiode (OPT101) with a longpass color filter (CVI laser, LP-720). After further signal processing, the signal is fed to computer via a data acquisition unit.

The oxygen sensor was prepared by wet-etching the sensor pad regions on a glass substrate followed by deposition of a PtOEPK film. To initiate the process, a sacrificial layer of AZ4620 photoresist (10 μm) was spin-coated on glass surface and patterned by photolithography. The exposed sensor regions were then etched with buffered hydrofluoric acid (7 H\textsubscript{2}O : 1 HF) for 15 min. After etching, the protective photoresist layer was stripped with acetone, and a droplet (~1 μL) of PtOEPK dye solution was applied using a pipette tip to each sensor region. The stock PtOEPK dye solution in the polymer matrix was prepared by mixing PtOEPK (1 mg) with polystyrene (50 mg) and toluene (950 μL). After applying the dye droplets, the solvent rapidly evaporated, leaving behind a thin film (2 – 4 μm) of dye-embedded polymer. The glass substrate can then be aligned and boned to the PDMS part patterned with the channel structures using oxygen plasma bonding machine.
To minimize the oxygen measurement sensitivity to ambient light, we applied an oscillating voltage to drive the LED instead of a direct current (DC) voltage. The photodiode receives an oscillating intensity with a frequency matched to the driving signal. By choosing a high oscillating frequency (on the order of kHz), the ambient intensity can be filtered out by a high-pass signal filter (cutoff frequency 1.6 kHz). Afterwards, the amplitude of the extracted oscillating signal is converted to a steady voltage by the signal conditioning circuit containing a rectifier, low-pass filters and amplifiers. The modified signal is fed into the serial port of a computer, where the mean signal output voltage is correlated with the oxygen concentration using a data acquisition module (DI-194RS, DataQ Instruments). The packaged oxygen sensing system is shown in Fig. 3.4b. The overall sampling rate of the oxygen sensing system is 240 Hz, which is sufficient given the equilibration time of DO in
the embedded sensors (~1 – 3 min, depending on the media flow rate). Post-analysis of the measured data was carried out using a script written in Visual C++.

Circuits for light excitation and detection (Fig. 3.5) were designed based on the mechanism of oxygen measurement shown in Fig. 3.4a. The square-wave oscillating signal (~0.3 – 3 kHz) was generated by an electronic oscillator and a rectifier. The oscillating emission intensity from PtOEPK dye, which corresponded to a certain oxygen level, was extracted through high-pass filtering and rectification. Afterwards the amplitude of signal was converted to a DC voltage by low-pass filters. By regulating the rheostat in amplifier B, the output signal, $V_{out}$, can be scaled to obtain a better resolution of data acquisition.

Figure 3.5. Circuit schematics of (a) light excitation module and (b) infrared detection module.

Experiments were conducted to calibrate the sensor parameters for the array of PtOEPK-polystyrene films in the microfluidic oxygenator chip with the channel design shown in Fig. 3.6a,b. In the gas detection experiment, nitrogen (0 % O$_2$), air (~20 % O$_2$) and
pure oxygen (100 % O2) were used for calibration purposes, with the identical concentration added to both inlet ports with pressure 1 kPa. On the other hand, the calibration in aqueous solution was conducted by applying oxygenated/partially-oxygenated water along a fluidic channel with a flow rate of 0.01 μL/min. Water samples with different DO levels were obtained by mixing different volumetric ratios (4:0, 3:1, 2:2, 1:3 and 0:4) of oxygenated and de-oxygenated water, which were respectively prepared by bubbling oxygen and nitrogen into distilled water for 15 min. The sensor response of each representative sample sensor for gaseous or aqueous state measurement is presented in Fig. 3.6c,e. The results show the emitting signal intensity (I), in terms of output voltage as a function of time, for a panel of oxygen/nitrogen ratios ranging from nitrogenated to fully-oxygenated. After changing the oxygen condition of liquid inside the channel, the sensor intensity is allowed to stabilize over a period of ~3 s for gases and ~3 min liquids, based on the diffusivity of oxygen in water, pdms membrane and the polystyrene sensor matrix.

Using the stabilized intensity readings (defined in terms of the output voltage from the infrared detection module) for the array of sensors, DO concentration was correlated with the emission intensity of the PtOEPK dye through the Stern-Volmer relation:

\[ \frac{I_0}{I} = 1 + K_{SV}pO_2 \]  

(3.15)

where I is the emitting fluorescence intensity; \( I_0 \) is the intensity in deoxygenated state; and \( K_{SV} \) is the Stern-Volmer constant. The Stern-Volmer constant and the deoxygenated state intensity of PtOEPK are unique for each sensor due to the thickness variation of evaporated PtOEPK-polystyrene films. Consequently, in the fabricated test module, every DO sensor in the oxygenator was individually calibrated to determine their corresponding sensor parameters (\( I_0 \) and \( K_{SV} \)) for the least-square-error corresponding to the DO levels in water samples. Fig. 3.6d,f show that the pO2 in both gas and water has a good agreement with the Stern-Volmer relation.
Figure 3.6. (a) Fabricated device and (b) its design layout for sensor calibration. Calibration results: output signal (voltage) versus time and dimensionless output sensor intensity versus DO levels of oxygen sensor under (c) – (d) gaseous and (e) – (f) aqueous environments.

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CHAPTER
FOUR

OXYGEN TRANSFER IN OXYGENATOR

4-1 GOVERNING EQUATIONS FOR THE MICROFLUIDIC OXYGENATOR

The double-layer microfluidic oxygenator design with low aspect ratios (height to width) of its membrane and flow layers has been widely used in the cell culture and analysis applications\(^1\text{--}^5\). Studying the mass transfer among the multiple layers of heterogeneous materials (e.g. PDMS and water) in the oxygenators enables the selection of device dimensions for different design configurations, culture conditions and target cells. Because of the low aspect ratios of gas and medium channels, the corresponding oxygen transfer dynamics can be often modeled as a 2-D geometry as discussed in section 3-1. A more detailed cross-section sketch of the device with the double layer channel structure of upper oxygen gas reservoir and lower cell culture chamber separated by a thin oxygen-permeable PDMS membrane is shown in Fig. 4.1. Along the culture chamber, a layer of biofilm grows over the bottom chamber surface with a measurable oxygen uptake, while the cell density in medium is low and the corresponding uptake is negligible. During culture oxygenation, a convective medium flow is imposed from the inlet of cell culture chamber to provide fresh nutrients for the growth of cells located on the lower chamber surface. The governing equations for the transient DO concentration \([\mu\text{M}]\) in cell layer \((c_1(x, y, t))\), medium \((c_2(x, y, t))\) and PDMS membrane \((c_3(x, y, t))\) are expressed as follows:

\[
\frac{\partial c_1}{\partial t} = D_1 \nabla^2 c_1 - OCR, \text{ for } 0 \leq y \leq H_1
\]  
\[\text{(4.1)}\]

\[
\frac{\partial c_2}{\partial t} = D_2 \nabla^2 c_2 - u(x) \cdot \nabla c_2, \text{ for } H_1 \leq y \leq H_1 + H_2
\]  
\[\text{(4.2)}\]

\[
\frac{\partial c_3}{\partial t} = D_3 \nabla^2 c_3, \text{ for } H_1 + H_2 \leq y \leq H_1 + H_2 + H_3
\]  
\[\text{(4.3)}\]

where \(H_1\) is the cell layer thickness, \(H_2\) is the effective channel height or culture medium layer thickness, and \(H_3\) is the PDMS membrane thickness separating the gas and medium channels; \(t\) is time; \(x\) and \(y\) are the horizontal and vertical position, respectively; \(D_i\) and \(S_i\) \((i = 1, 2, 3)\) are the diffusivity and solubility of their corresponding materials (cell, medium and
PDMS, respectively) as summarized in Table 4.1; \( u(y) \) is the developed velocity profile of medium mentioned previously in Eq. 3.2; \( OCR(x, y, t) \) is the oxygen consumption rate of cells [\( \mu M/s \)] based on Michaelis-Menten kinetics:

\[
OCR = \frac{V_{\text{max}} P_{\text{cell}}}{K_m + c_i} \tag{4.4}
\]

where \( V_{\text{max}} (= 1.42 \times 10^{-5} \mu M/s)^7 \) is the maximum respiration rate per aerobic cell volume [cm\(^3\)], \( \rho_{cell} \) is the volumetric cell density [cm\(^3\)/mL], and \( K_m (= 5.42 \times 10^{-2} \text{ mM})^7 \) is the Michaelis-Menten constant. Depending on cell types, Eq. 4.4 can have a different Michaelis-Menten constant, \( K_m \). For negligible \( K_m (<< c_i) \), OCR can be approximated by \( V_{\text{max}} \rho_{cell} \). The initial conditions of oxygen concentration were initially set to be 0 \( \mu M \) over the material domains such that the simulated response of \( pO_2 \) can be viewed as the upper bound of diffusion time.

Under continuous flow of nitrogen-oxygen gas mixture, the partial pressure of oxygen, \( pO_2 \), on the upper surface of the PDMS membrane was defined as constant (Eq. 4.10), equal to that of the supplied gas. The bottom glass substrate is oxygen-impermeable so there is no mass flux into the bottom glass substrate (Eq. 4.5). Applying Henry's law, \( pO_2 \) at interfaces
between heterogeneous materials should be continuous (Eqs. 4.6, 4.8)\textsuperscript{15}, while the mass flux of oxygen should be consistent across interface at \(y = H_1\) and \(H_1 + H_2\) (Eqs. 4.7, 4.9). Hence, the boundary conditions (for \(t > 0\)) of Eqs. 4.1 – 4.3 are

\[
\frac{\partial \epsilon_1}{\partial y} = 0, \text{ at } y = 0
\]  

(4.5)

\[
\frac{c_1}{S_1} = \frac{c_2}{S_2}, \text{ at } y = H_1
\]  

(4.6)

\[
D_1 \frac{\partial c_1}{\partial y} = D_2 \frac{\partial c_2}{\partial y}, \text{ at } y = H_1
\]  

(4.7)

\[
\frac{c_2}{S_2} = \frac{c_3}{S_3}, \text{ at } y = H_1 + H_2
\]  

(4.8)

\[
D_2 \frac{\partial c_2}{\partial y} = D_3 \frac{\partial c_3}{\partial y}, \text{ at } y = H_1 + H_2
\]  

(4.9)

\[
c_3 = S_3 p, \text{ at } y = H_1 + H_2 + H_3.
\]  

(4.10)

### 4-2 Finite Difference Solution of Oxygen Transfer

The characterization of the oxygen transfer dynamics was performed to investigate the oxygenation efficacy as a function of device dimensions, cell type and density. A numerical solution using finite difference method was developed as Matlab scripts, enabling the concentration profiles to be quickly calculated by setting material dimensions as simulation parameters. Considering Henry's gas law, oxygen concentration in Eqs. 4.1 – 4.3 was first converted to \(p_{O2} (p_i = c_i/S_i\) for \(i = 1, 2, 3\) for higher accuracy of the numerical solution on the continuous \(p_{O2}\) profile. The oxygen concentration can then be easily resolved by scaling the \(p_{O2}\) solution in every material domain. The finite difference solution of \(p_{O2}\) was implemented using Cartesian 2D grids. The material domains were discretized along channel (x-) direction with length \(L\) and \(m + 1\) grids, and along height (y-) direction with \(n_1, n_2 - m\) and \(n_3 - n_2\) grids for cell layer, medium and PDMS, respectively. The three finite difference equations for Eqs. 4.1 – 4.3 using Crank-Nicolson method are

\[
\frac{p_{i-1,j}^{t+1} - 2p_{i,j}^{t+1} + p_{i+1,j}^{t+1}}{(\Delta x)^2} + \frac{p_{i-1,j}^{t} - 2p_{i,j}^{t} + p_{i+1,j}^{t}}{(\Delta x)^2} = \frac{D_i}{2} \left( \frac{p_{i-1,j}^{t+1} - 2p_{i,j}^{t+1} + p_{i+1,j}^{t+1}}{(\Delta y)^2} + \frac{p_{i,j-1}^{t+1} - 2p_{i,j}^{t+1} + p_{i,j+1}^{t+1}}{(\Delta y)^2} \right) \frac{OCR(i \Delta x, j \Delta y, \Delta t)}{S_i}
\]

for \(i = 1, 2, \ldots, m; j = 1, 2, \ldots, n-1\)  

(4.11)
\[
\frac{p_{i,j}^{*+1} - p_{i,j}^*}{\Delta t} = \frac{u_j}{2} \left( \frac{p_{i+1,j}^{*+1} - p_{i-1,j}^{*+1}}{\Delta x} + \frac{p_{i+1,j}^* - p_{i-1,j}^*}{\Delta x} \right) + D_2 \left( \frac{p_{i+1,j}^{*+1} - 2p_{i,j}^{*+1} + p_{i-1,j}^{*+1}}{(\Delta x)^2} + \frac{p_{i,j}^* - 2p_{i,j}^* + p_{i,j}^{*+1}}{(\Delta x)^2} \right) + \frac{D_3}{2} \left( \frac{p_{i,j+1}^{*+1} - 2p_{i,j}^{*+1} + p_{i,j-1}^{*+1}}{(\Delta y)^2} + \frac{p_{i,j}^* - 2p_{i,j}^* + p_{i,j}^{*+1}}{(\Delta y)^2} \right),
\]
for \( i = 1, 2, \ldots, m; j = n_1+1, n_1+2, \ldots, n_2-1 \) \hspace{1cm} (4.12)

\[
\frac{p_{i,j}^{*+1} - p_{i,j}^*}{\Delta t} = \frac{D_3}{2} \left( \frac{p_{i,j+1}^{*+1} - 2p_{i,j}^{*+1} + p_{i,j-1}^{*+1}}{(\Delta y)^2} + \frac{p_{i,j}^* - 2p_{i,j}^* + p_{i,j}^{*+1}}{(\Delta y)^2} \right),
\]
for \( i = 1, 2, \ldots, m; j = n_1+1, n_1+2, \ldots, n_2-1 \) \hspace{1cm} (4.13)

where \( n = \lfloor t/\Delta t \rfloor \) is the discrete time (or the number of iteration); \( \Delta t \) is time step; \( \Delta x \) and \( \Delta y \) \((i = 1, 2, 3)\) are grid size along \( x \) and \( y \) directions in material domain \( i \), respectively; and \( u_j \) is the sampled local medium velocity at grid points, defined as \( u(y) \) at \( y = (j - n_i) x H_j / (n_j - n_i) \) in Eq. 3.2. The derivative of \( p_0^2 \) in culture medium (Eq. 4.12) was approximated by centered difference to obtain second order accuracy in space.

Based on Eqs. 4.5 – 4.10, the boundary conditions for the upper PDMS surface imposed with steady \( p_0^2 \) and the oxygen impermeable glass surface below biofilm are \( p_{n_1} = P_i \) (Dirichlet) and \( p_{n_1} = p_{n_1}^* \) (Neumann), respectively. The governing equations along the material interface are

\[
\frac{p_{i,j}^{*+1} - p_{i,j}^*}{\Delta t} = \frac{D_2}{(\Delta y_1 + \Delta y_2)} \left( \frac{p_{i,j+1}^{*+1} - p_{i,j-1}^{*+1}}{\Delta y_1} + \frac{p_{i,j}^{*+1} - p_{i,j}^*}{\Delta y_1} \right) + \frac{D_3}{(\Delta y_2)} \left( \frac{p_{i,j+1}^{*+1} - 2p_{i,j}^{*+1} + p_{i,j-1}^{*+1}}{(\Delta y_2)^2} + \frac{p_{i,j}^* - 2p_{i,j}^* + p_{i,j}^{*+1}}{(\Delta y_2)^2} \right) + \frac{\Delta y_1}{\Delta y_1 + \Delta y_2} \frac{OCR}{S_1},
\]
for \( i = 1, 2, \ldots, m; j = n_1 \) \hspace{1cm} (4.14)

\[
\frac{p_{i,j}^{*+1} - p_{i,j}^*}{\Delta t} = \frac{D_3}{(\Delta y_2 + \Delta y_3)} \left( \frac{p_{i,j+1}^{*+1} - p_{i,j-1}^{*+1}}{\Delta y_2} + \frac{p_{i,j}^{*+1} - p_{i,j}^*}{\Delta y_2} \right) + \frac{\Delta y_2 D_2 + \Delta y_3 D_2}{(\Delta y_2 + \Delta y_3)} \left( \frac{p_{i,j+1}^{*+1} - 2p_{i,j}^{*+1} + p_{i,j-1}^{*+1}}{(\Delta y_2)^2} + \frac{p_{i,j}^* - 2p_{i,j}^* + p_{i,j}^{*+1}}{(\Delta y_2)^2} \right) + \frac{\Delta y_2}{\Delta y_2 + \Delta y_3} \frac{OCR}{S_2},
\]
for \( i = 1, 2, \ldots, m; j = n_2 \) \hspace{1cm} (4.15)

Moreover, the fluxes across the leftmost and rightmost interface in Fig. 4.1 are set to zero, except that the inflow medium were assumed to be constant (i.e. \( p_{in} = P_i \) for \( m < j < n_2 \)).

A simulation example \((L = 5 \text{ mm}, W = 5 \text{ mm}, H_1 = 5 \text{ \mu m}, H_2 = 195 \text{ \mu m} \text{ and } H_3 = 50 \text{ \mu m}, \Delta x = 5000/50 \text{ \mu m}, \Delta y_1 = 5/5 \text{ \mu m}, \Delta y_2 = 150/50 \text{ \mu m}, \Delta y_3 = 50/25 \text{ \mu m}, Q = 0.05 \text{ ml/min}, P_i = 0.2 \text{ atm and } Q_{cell} = 7.28 \times 10^{-4} \) is shown in Fig. 4.2. Culture medium flowing from the left inlet was set to anaerobic \((P_i = 0)\) for the estimation of entrance length. The result shows that after applying oxygen along the top gas channel, oxygen diffused from the
membrane layer to sensor layer (4.2a-c) and the diffusion eventually reached a steady state (Fig. 4.2d). The steady state pO$_2$ profile indicates an entrance length region of oxygen diffusion, in which pO$_2$ cannot reach the target level. Fig. 4.2e shows the corresponding oxygen concentration profile scaled by the solubility for different materials.

![Image of oxygen profiles and concentration profile]

Figure 4.2. Development of the pO$_2$ profile in a microfluidic culture channel. The profiles at simulation time (a) 0.4 s, (b) 2 s, (c) 10 s and (d) 50 s demonstrate the O$_2$ diffusion during oxygenation. (e) The developed oxygen concentration profile obtained by scaling pO$_2$ with different solubility in material domains.
4-3 OXYGEN PRESSURE DROP ALONG CELL LAYER

Analytical solution

The gas condition of many PDMS-based microfluidic chips for cell culture has been often regulated by placing the devices in a defined gas environment\(^ {16} \). For aerobic cell culture, it was expected that oxygen could diffuse into the culture regions because of the oxygen permeability of the structural material. However, the typical device PDMS thickness (\(\sim 5 \) mm) overhead culture regions greatly reduce the efficiency of passive oxygen transfer by diffusion. The total oxygen uptake of aerobic cells during culture can be sufficient (especially for dense cell population) to induce a locally oxygen pressure drop (OPD) along the cell layer, even in the fully developed region. The OPD can be expressed in terms of cell properties and device configurations.

Considering the steady state of oxygen transfer in the fully-developed region in an oxygenator described in Fig. 4.1, the oxygen fluxes across each material layer are identical, as oxygen consumption within the heterogeneous material stack is zero. Such oxygen fluxes can be expressed as

\[
q = \frac{D_y S_2}{H_2} \left( P_{2-3} - P_{2b} \right) = \frac{2D_y S_2}{H_2} \left( P_{2-3} - P_{2b} \right) = \frac{2D_y S_2}{H_2} \left( P_{2b} - P_{1-2} \right),
\]

where \( P_{2-3} \) is the pO\(_2\) at the interface between PDMS membrane and medium channel, \( P_{2b} \) is the bulk pO\(_2\) in medium, and \( P_{1-2} \) is the pO\(_2\) along top cell surface. If we correlate \( q \) with the cellular oxygen uptake rate at the equilibrium state, \( q \) can be expressed as

\[
q = D_y \frac{\partial c_1}{\partial y} \bigg|_{y=H_w} = \int_0^{H_w} \frac{V_{\text{max}} P_{\text{ed}} c_1}{K_m + c_1} dy = \frac{V_{\text{max}} P_{\text{ed}} H_1 P_{\text{ed}}}{K_m / S_1 + P_{\text{ed}}},
\]

where \( P_{\text{ed}} \) is the pressure value satisfying the relation in Eq. 4.17. In general, \( P_{\text{ed}} \) is very close to the average pO\(_2\) in cell layer. Thus, the exact solution for the dimensionless oxygen pressure drop (\( \text{OPD} = (P_g - P_{\text{ed}})/P_g \)) between the cell layer and gas reservoir can be simplified in following form:

\[
\text{OPD} = \frac{P_g - P_{\text{ed}}}{P_g} = \frac{1}{2} \left( 1 + \Phi + \Theta \right) - \frac{1}{2} \sqrt{(1 - \Phi - \Theta)^2 + 4\Phi},
\]

\[
\Phi = \frac{K_m}{P_g S_1} \quad \text{and} \quad \Theta = \frac{V_{\text{max}} P_{\text{ed}} H_1}{P_g} \left( \frac{H_3 + H_2}{D_y S_3} + \frac{1}{3} \right)
\]

The value \( \Phi \) is a cell-type specific constant while \( \Theta \) lumps all of the other general parameters into a single value. The surface plot of \( \text{OPD} \) as a function of \( \Phi \) and \( \Theta \) is shown in Fig. 4.3. For significant oxygen supply (\( P_g >> K_m S_1 \)), \( \text{OPD} \approx \Theta \). In particular, a higher cell density \( q_{\text{cell}} \) induces the larger parameter \( \Theta \). This implies that \( \text{OPD} \) can become a problem during cell culture, even when there has been initially only negligible \( \text{OPD} \). For instance, the
implementation of mammalian cell culture should avoid the anoxic (pO2 ≈ 0 mmHg) or hypoxic (pO2 5 – 15 mmHg) environments leading to necrosis or stress on aerobic mammalian cells\(^\text{17}\) that has also been shown by the cell response under different oxygenation conditions using an oxygen gradient oxygenator\(^\text{2}\). (This result is available in Appendix A.)

![Figure 4.3. Surface plot of OPD as a function of variables \(\Phi\) and \(\Theta\).](image)

**Experimental validation**

Experiments were performed to verify that OPD becomes significant as cell density increases. As an experimental test bed, aerobic bacteria were cultured in a single layer microfluidic device. Single layer PDMS devices containing three culture channels were fabricated as described in Fig. 4.4a-b. The microchannels were designed with an equivalent structure as described in Fig. 4.1, with PDMS thickness \((H_3) \sim 5\) mm, channel height \((H_2)\) 200 \(\mu\)m and width \((W)\) 5 mm. As a parallel experiment, oxygenators with double layer structures \((H_2 = 200\) \(\mu\)m, \(H_3 = \sim 100 – 800\) \(\mu\)m) were also used. However, no measurable OPD values were obtained in the double layer devices (data not shown).

The PDMS channel structures were fabricated by pouring 10:1 PDMS monomer/harder mixture on a Si/SU-8 mold patterned with the culture medium channels. Prior to O\(_2\) plasma bonding to the PDMS substrate, a glass slide deposited with PtOEPK/polystyrene sensors was fabricated per the method described in section 3-4, except that the HF etching step was skipped and an extra layer of \(\sim 8\) \(\mu\)m PDMS was spin-coated over the sensor-patterned glass substrate (4 \(\mu\)m thick sensor layer) to create an additional barrier between the cells and the PtOEPK dye, whose cytotoxic effects are unknown. PtOEPK does not consume oxygen, so there is no mass flux of oxygen along the bottom cell surface.
In the experiment, each oxygen sensor was calibrated before the microfluidic devices were sterilized by flushing medium channels with 70% ethanol followed by baking at 80 °C for 2 hr. The saliva sample was prepared by mixing it with brain heart infusion broth until a cell density \( \sim 10^5 - 10^8 \text{ cells/cm}^2 \) was achieved. The bacteria sample was subsequently seeded into culture channels by a syringe pump (PicoPlus, Harvard Apparatus) at flow rate 0.05 \( \mu l/min \) for 10 min and cultured in a humidified incubator (\( \sim 20 \% \) oxygen supply) without medium flow for 10 hr, allowing cells to deposit on the glass surface. After culturing, the volumetric cell density [cm\(^3\)/mL] was estimated by microscopic images at sensor regions (Fig. 4.4c) (A calibration experiment was performed to relate the area ratio of cell [cm\(^2\)/cm\(^2\)] to \( \rho_{cell} \)). The \( pO_2 \) in cell layer was measured by the sensing system as described in section 3-4.

Figure 4.4. (a) Photograph and (b) cross-section schematic diagram of a device for OPD validation. (c) Bacteria samples were cultured along microchannels with different \( \rho_{cell} \) and \( H \), during experiment.

The results indicated that the measured \( OPD \) (~0, ~0, 0.017 ± SD 0.005 and 0.05 ± 0.008) increased with the cell density \( \rho_{cell} \) (0.0069 ± SD 0.0012, 0.038 ± 0.009, 1.5 ± 0.2 and 4.76 ± 1.3 [\( \times 10^8 \) cells/cm\(^2\)], respectively). Each measured value of each \( OPD \) was calculated from three individual measurements. The corresponding experimental maximum cellular respiration rate \( V_{max} \) (~0.5 \( \times 10^{-5} \) \( \mu M/s \), calculated from Eq. 4.18) was within 3x of the reported value (1.42 \( \times 10^{-5} \) \( \mu M/s \))\(^7\) that the experimental results suggest that \( OPD \) could become a critical problem for the cell applications using microfluidic device with thick (~5 mm) overhead PDMS substrate, particularly as cell culture density increases over the culture period. Therefore, designing an oxygenator with a thin (~10 – 200 \( \mu m \)) overhead PDMS membrane is suggested to increase oxygen transfer to the microfluidic cell culture and significantly reduce the \( OPD \) of the system.
Transient gas environments are necessary for specific biofilm formation including dental biofilm development\textsuperscript{18-20}. In culturing bacterial biofilms, which consist of a mixture of aerobic and anerobic sp, a microfluidic oxygenator should enable tunable pO$_2$ levels during operation to mimic the transition from an aerobic to an anerobic environment as the biofilm evolves. Using a thin PDMS layer ($\sim$20 – 100 $\mu$m) as a separation barrier between the culture channel and the overhead gas supply not only minimizes the OPD, but also maximizes the diffusive flux (and minimizes the diffusion time) of gas ($D_{3S3}/H_3(P_1 - P_2)$ in Eq. 4.16) into the culture channel. To investigate the diffusion time for different oxygenator configurations, experiments were performed to measure the dynamic DO profile inside an oxygenated microchannel after an abrupt change in pO$_2$ along the gas perfusion channel.

Microfluidic chips (Fig. 4.5a) containing arrays of double layer structures with different layer thicknesses were designed as the structure shown in Fig. 4.5b,c. The fabrication of the PDMS substrate was based on the multilayer soft lithography\textsuperscript{21} for PDMS with defined thicknesses. A glass slide was etched and deposited with the PtOEPK/polystyrene DO sensors using the method described in section 3-4. Afterwards, the PDMS and glass substrates were bonded by oxygen-plasma.

\textbf{Figure 4.5.} (a) Photograph and schematic diagrams of (b) top view and (c) cross-section of a PDMS chip for validation of diffusion time.
In the diffusion time measurement devices, no cells were used. Rather, the model layer of dental biofilm (as shown in Fig 4.1) was replaced by an oxygen-sensitive platinum (II) octaethylporphine ketone (PtOEPK)-polystyrene layer (thickness: ~4 μm). The governing equations for the modified oxygenator have no OCR term (due to zero steady state oxygen consumption of sensor material) and different values of diffusivity ($D_1 = 5.7 \times 10^{-7} \text{cm}^2/\text{s}$) and solubility ($S_1 = 0.42 \text{mM/atm}$) for the base layer. Furthermore, the sensor layer has a negligible effect on the diffusion profile and the time for the DO to reach equilibrium (<1 % of measured values) due in part to its negligible thickness ($H_1 << H_2$).

To measure the time-varying dissolved oxygen profile at the sensor layer, oxygen was continuously supplied along gas layer. Real-time measurement was achieved by a detection method aforementioned in section 3-4. In the experiment, the medium channel was initially filled with fresh medium under ambient environmental partial pressure and oxygen was then applied along the gas channel. The corresponding numerical oxygen profiles were also calculated using the approach described in section 4-2. Comparisons of the computational and experimental dynamic sensor responses indicate that the devices had similar pO2 profiles (Fig. 4.6) and response/rise time as numerical solution.

![Graphs](image)

**Figure 4.6.** Sample sensor responses in the validation experiment of diffusion time and the corresponding simulation results for sensor thickness ($H_1$) 3 μm, effective medium channel height ($H_2$) ~100 μm and membrane thickness ($H_3$) (a) 20 μm, (b) 100 μm, (c) 300μm, and (d) 1250 μm.
To investigate the efficiency of oxygenation, the experimental and simulated rise time $T_r$, defined by the time required for the sensor pO$_2$ to reach 90% of its stabilized value, was plotted against different channel height and membrane thickness (Fig. 4.7) with reasonable agreement. Results show that $T_r$ was basically determined by the medium channel thickness for a thin PDMS membrane ($H_3 << H_2$), while it increased significantly as the square of PDMS membrane thickness (i.e. $T_r \propto H_3^2$) for $H_3 >> H_2$. In particular, for a typical single layer microfluidic device with 5 mm thick PDMS substrate and a 100 $\mu$m tall microchannel, the generation of dynamic oxygen microenvironment would be challenging because of the relatively long rise time ($T_r = 2.93$ hr). In other words, implementing cell culture in such microfluidic devices in a controlled dynamic gas condition may lead to an unexpected growth response of cells.

![Figure 4.7. Numerical Solution (NS) and experimental values (Exp) of rising time ($T_r$) as a function of effective channel height ($H_2$) and PDMS membrane thickness ($H_3$). Each data point represents the mean value of at least three independent measurements except that only one measurement was made for $H_2 = 800 \mu$m and $H_3 = 4000 \mu$m because of the long rise time. The error bars indicate the standard deviations of data points.](image)

4-5 EFFECTIVE CHANNEL LENGTH

Along an oxygenated microchannel, medium flowing from the inlet requires a certain characteristic length to obtain the fully developed oxygen concentration profile. Ideally, cells should be cultured in the fully developed region. The effective channel length $L^*$, which
represents the ratio of oxygen diffusion to convection along the medium channel, has been defined to determine whether a chamber region is fully developed \((L^* \gg 1)\). When both the cell and PDMS layers are sufficiently thin \((H_1/D_1S_1 \gg H_2/D_2S_2)\), we may let \(L^* = \frac{x}{PeH_2}\) as proposed in ref. (20). For thick cell and PDMS layers, the variation of \(pO_2\) across medium (along \(y\) direction) can be smaller than supply \(pO_2\) in gas layer, so \(L^*\) should be scaled according to the \(pO_2\) in different material layers. The transition region of oxygen diffusion is assumed to be the position where oxygen starts diffusing to the bottom side of cell layer with negligible OUR and so the pressure scale in each layer can be related to the diffusive resistance, i.e. \(H_1/D_1S_1\), \(H_2/D_2S_2\) and \(H_3/D_3S_3\) for cell, medium and PDMS layers, respectively. \(L^*\) can then be defined as

\[
L^* = \frac{x}{PeH_2} \left[ \left( \frac{H_1}{D_1S_1} + \frac{H_3}{D_3S_3} \right) \frac{D_2S_2}{H_2} + 1 \right].
\]  

(4.19)

The effective channel length reflects whether the \(pO_2\) of an oxygenation region is fully developed by the condition \(L^* \gg 1\). Fig. 4.8 shows the numerical dimensionless \(pO_2\) along biofilm \(P^{cell}_{*} (= P^{cell}/P)\) as a function of \(L^*\), calculated with different effective channel height \((H_2 \approx 50 - 500 [\mu m])\) and membrane thickness \((H_3 = 50 - 5000 [\mu m])\) as shown in Fig. 4.8a and 4.8b respectively. Other simulation parameters are \(Pe = 3\) and \(\rho_{cell} = 5 \times 10^6\) cells/mL. It can be observed that the transition region of \(pO_2\) along cell layer \(P^{cell}_{*}\) is around \(L^* \sim 1\), where the diffusive effect is comparable to the convective one. The \(pO_2\) of biofilm becomes fully developed, i.e. \(P^{cell} = (1 - OPD)P_{in}\), after the transition region with \(L^* \gg 1\). Therefore, designing the culture region at the location with \(L^* \gg 1\) can ensure a uniform gas microenvironment for oxygenated cell culture.

![Figure 4.8](image_url)

**Figure 4.8.** Plots of \(P^{cell}_{*}\) against \(L^*\) under variations of (a) effective channel height \((H_2)\) and (b) membrane thickness \((H_3)\) representing oxygen diffusion at biofilm layer along medium channel with the constant Peclet number and cell density.
4-6 SUMMARY OF OXYGENATOR DESIGN

In this chapter, the oxygen diffusion process in the typical configuration of double layer microfluidic oxygenator for biofilm culture has been investigated through modeling, characterization and experimental validation. The design parameters including \( T_r \), \( \text{OPD} \) and \( L^* \) have been proposed to quantify the oxygenation performance through characterization of the device configuration, while the validation of \( T_r \) and \( \text{OPD} \), which also reflect the validity of governing equations, were performed by experiments.

To maintain an adequate amount of oxygen supply to satisfy confluent cell cultures, the oxygenator should be optimized with respect to the design parameters. Specifically, the overhead PDMS membrane should be designed with minimal thickness for fast diffusion response (or small \( T_r \)) and a small \( \text{OPD} \), which includes parameters \( \Phi \) depending on the cell type and \( \Theta \) as a unified value of the device configurations aforementioned in Eq. 4.18. For the case of device configurations that induce a large \( \text{OPD} \), the \( pO_2 \) in the the gas layer should be increased to obtain adequate oxygenation levels for aerobic cultures.

The condition \( L^* >> 1 \) may be challenging to achieve in some practical implementations such as the typical single layer PDMS devices \( (H_3 \sim 5 \text{ mm}) \), the tissue culture applications with significant cell layer thickness \( (H_1 > 200 \mu\text{m}) \), and the analysis of cell behavior under high shear \( (Pe \sim 99.2 \text{ with medium velocity } \sim 2.78 \text{ mm/s}^{23-25}) \). Such limitations can be overcome by modifying the oxygenator with extended gas and medium channels prior to the culture chamber to pre-oxygenate the medium.

Overall, oxygen transfer modeling is useful as a design tool for designing micro-oxygenator configuration, leading to a better understanding of how physical parameters such as device dimensions, gas partial pressure and flow rate define the local culture environment. Microfluidic oxygenators are likely to find important future applications that extend beyond general cell culture, providing a valuable tool to understand how single cells and cell networks are affected by defined changes in oxygen levels.

REFERENCES

CHAPTER

FIVE

GENERATION OF DIFFERENTIAL AND TUNABLE OXYGEN LEVELS

5-1 OXYGEN GRADIENT GENERATOR USING CASCADE MIXER ARRAYS

In this section, we present a PDMS microfluidic oxygenation system that provides a step-function gradient of DO concentrations across parallel microchannels for the monitoring of culture growth dynamics versus medium oxygen levels\(^1\). The ability to vary and tune pO\(_2\) in a microfluidic environment has practical applications in areas such as microbiology and cancer research\(^2\), where fluctuations in dissolved oxygen concentration impact not only cell viability but also the regulation of key biochemical pathways. The multilayer microfluidic device consists of a gas-based analog of a microfluidic solution gradient generator\(^3\) similar to the design utilized by Polinkovsky et al.\(^4\) with a network of branching gas-filled microchannels that overlap the underlying microfluidic culture channels. Like the chemical solution gradient generator, which has been applied in chemotaxis studies\(^5\) and continuous cell culture\(^6\), gases like oxygen and nitrogen are mixed like liquids, with a parallel output of streams containing a step-wise gradient of oxygen concentrations. By varying the dimensions of the individual microchannels within the mixer network, the output oxygen concentration(s) can be fine-tuned for the target application. Oxygenation of the culture media is achieved by the double-layer gas perfusion channel structure along the cell culture region. While the Polinkovsky platform used an inverted fluorescent microscope to monitor oxygenation with a solution-based fluorescent dye, monitoring in our platform is achieved with an array of optical (Pt-porphyrin) oxygen sensors embedded in each culture channel that provides a real-time medium DO measurement with low-cost light emitting diodes.

The PDMS microfluidic oxygenator consists of an array of eight microchannels (20 µm (height) x 100 µm (width)) that provides differential DO concentrations (channel-to-channel) for cell culture. The chip has a double-layer channel structure, with the design layout illustrated in Fig. 5.1a. Gas channels flowed with oxygen-nitrogen gas mixture are located overhead the culture chambers. Several methods to regulate the volumetric ratio of oxygen such gas mixture using integrated microfluidic gas channels have been recently reported\(^8-11\).
In this section, the volumetric ratios of oxygen determining the pO₂ of culture regions has been achieved by cascade arrays of the gas mixing channels described in section 3.1. A double-layer gas perfusion network structure was fabricated above the cell culture region with a small separation between gas and fluid microchannels on the order of tens of microns. Because of the specific geometric configuration, such a mixer can be fabricated using conventional soft lithography methods with a single layer SU8-silicon mold. The mixer contains two gas inlets flowed with pure nitrogen and oxygen and multiple gas outlets with different oxygen-nitrogen ratios, which can be adjusted by exploiting the different fluidic resistances of of the mixing microchannel network. Controlling local DO levels in PDMS microfluidic devices can be achieved by flowing oxygen through dedicated gas microchannels that are in close proximity to the fluid-filled microchannels. Using continuously flowing oxygen and nitrogen supplies, stable identical DO levels were maintained within an array of wells via passive gas diffusion.

**Fabrication**

The fabrication process was based on previously reported multilayer soft lithography methods. The mold with medium channel patterns was prepared by patterning two layers of 10 μm thick positive AZ4620 photoresist (AZ Electronic Materials) on a 3” silicon wafer (James River Semiconductor), followed by a one minute reflow at 150 °C. Photolithography (12 s × 3 exposure) was performed using a high resolution transparency mask (~20,000 dpi). SU-8 negative photoresist was selected for the gas channel mold. A 40 μm thick SU-8 (Microchem SU-8 50) layer was spin-coated on a 3” wafer and patterned by photolithography (Karl Suss Mask Aligner MJB3, 75 s exposure). Afterwards, the molds were silanized with a high molecular weight trichloro-perfluorooctyl saline (Aldrich) for ~5 min to facilitate PDMS mold release. The silanization process reduces the adhesion of PDMS to Si/SU-8 and Si/AZ4620 surfaces to increase the mold lifetime.

The glass/oxygen sensor array was prepared by wet-etching the sensor pad regions on the glass surface followed by deposition of the PtOEPK substrate. To prepare the glass for wet-etching, a sacrificial layer of AZ4620 photoresist (10 μm) was spin-coated on the surface and patterned by photolithography. The exposed sensor regions were etched with buffered hydrofluoric acid (1 HF : 7 H₂O) for 15 min. After etching, the protective photoresist layer was stripped with acetone, and a droplet (~1.0 μL) of the PtOEPK dye solution was applied using a pipette tip to each sensor region. The stock PtOEPK dye solution in the polymer matrix was prepared by mixing PtOEPK (1 mg) with polystyrene (50 mg) and toluene (950 μL). After applying the dye droplets, the solvent rapidly evaporated, leaving behind a thin film (2 – 4 μm) of dye-embedded polymer.
Figure 5.1. (a) Schematic diagram of microfluidic oxygenator. The device consists of two PDMS layers (gas and medium) containing molded microchannels. The multiplexor and $O_2$ gradient generator is contained in the gas layer, while the DO sensors are contained in the medium channels. (b) Fabricated microfluidic oxygenator. (c) Micrograph of multiplexor and oxygen concentration gradient generator.
The molding and assembly of the gas and fluid channels networks was achieved via multilayer soft lithography\textsuperscript{12}. 10:1 A:B two-part PDMS (Sylgard 184, Dow Corning) was mixed and poured onto the SU-8/silicon mold containing a multiplexor\textsuperscript{14} and gas channels to a thickness of \( \sim 6 \) mm. The mold was subsequently degassed in a vacuum bell jar for \( \sim 10 \) min before it was baked in an oven for 1 hr at 80 °C. For the fluid channel mold, 10:1 PDMS was spin-coated (2300 rpm, 50 s) to a thickness of \( \sim 40 \) μm and baked for 10 min at 80 °C. After the initial bake, both molds were removed from oven for alignment. The \( \sim 6 \) mm thick PDMS gas mold replicate was released from the mold and cut to size with a razor blade. A blunt-tipped 20G surgical steel Luer stub was used to punch gas inlet and outlet holes in the PDMS. After punching, an isopropyl alcohol wash was applied to remove debris followed by drying under a nitrogen stream. The processed thick PDMS gas layer was then aligned over the spin-coated fluid layer under a dissecting scope (Olympus SZX9). To bond the two layers, the composite PDMS substrate was post-baked in an oven for >2 hr at 80 °C. The devices were then cut from the flow mold and the fluid layer inlet/outlet holes were punched as previously described. The assembled PDMS was subsequently bonded to the prepared glass substrate containing sensor film using oxygen-plasma (Plasmod, Tegal Corporation, 600 mTorr) for 15 s, with the composite device shown in Fig. 5.1b,c.

**Design of cascade mixer arrays**

The gas layer in microfluidic oxygenator is composed of microchannels with a constant height (40 μm) and variable width (20 μm – 2 mm), and a summary of the equivalent resistances is listed in Table 5.1. Different oxygen levels are generated by flowing gases continuously with constant input pressures.

Modeling each individual channel as the fluidic equivalent of an electrical resistor, the gas layer network is simplified to an equivalent circuit as illustrated in Fig. 5.2. In the circuit model, electrical voltage represents gas pressure while current represents gas flow rate. The gas supplies were regulated to the same gage pressure. By adjusting the effective fluidic resistance of each individual channel, a gradient generator requiring low input gas pressures, i.e. \( P_1 = P_2 = 1 \) kPa, can be achieved. The resistances of folded channels, \( R_s \), are set to be much larger than the common resistance of interconnecting channels, \( R_h \), such that a linear distribution of oxygen concentrations at the respective series of microchannel outlets can be obtained by adjusting only \( R_s \). The values of the \( R_s \) were calculated by assuming the pressure drop for a particular folded channel row is consistent. Because oxygen-nitrogen mixer was fabricated as a single layer structure along flow layer, all the folded channels were defined with the same height (40 μm). Considering the requirement of small \( Pe^* < 0.1 \), the folded channels were designed with the fixed channel width (20 μm) and variable length (\( \sim 2000 \) μm) as shown in Table 5.2, in order to generate differential oxygen levels with the same incremental change in O\textsubscript{2} ratios \( (\Delta P_{O2}/(P_{O2}+P_{N2})) \) from \( C_{out1} (0\%\ O_2) \) to \( C_{out8} (100\%\ O_2) \).
Table 5.1. Dimensions of channels in gas layer, and the corresponding fluidic resistances, scaled (*) Reynolds ($Re^* = \rho u L/\mu H^2$) and Peclet numbers ($Pe^* = u W/D\cdot W/L$).

<table>
<thead>
<tr>
<th></th>
<th>Dimensions $W \times H \times L$ [$\mu m^3$]</th>
<th>Max. Resistance $[Ns/m^3]$</th>
<th>Max. $Re^*$</th>
<th>Max. $Pe^*$</th>
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<tbody>
<tr>
<td>$R_{in}$</td>
<td>$100 \times 40 \times 7000$</td>
<td>$3.51 \times 10^{11}$</td>
<td>0.023</td>
<td>0.14</td>
</tr>
<tr>
<td>$R_{out}$</td>
<td>$100 \times 40 \times 18000$</td>
<td>$9.02 \times 10^{11}$</td>
<td>0.003</td>
<td>0.03</td>
</tr>
<tr>
<td>$R_v$</td>
<td>$20 \times 40 \times \sim 2000$</td>
<td>$2.19 \times 10^{12}$</td>
<td>0.009</td>
<td>0.08</td>
</tr>
<tr>
<td>$R_b$</td>
<td>$100 \times 40 \times 500$</td>
<td>$2.51 \times 10^{10}$</td>
<td>0.267</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Table 5.2. Length of folded channels in oxygen-nitrogen mixer

<table>
<thead>
<tr>
<th>Row</th>
<th>Channel Length [$\mu m$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2000</td>
</tr>
<tr>
<td>2</td>
<td>2000</td>
</tr>
<tr>
<td>3</td>
<td>2000</td>
</tr>
<tr>
<td>4</td>
<td>2000</td>
</tr>
<tr>
<td>5</td>
<td>2000</td>
</tr>
<tr>
<td>6</td>
<td>2000</td>
</tr>
</tbody>
</table>
Applying Eq. 3.8-13, the oxygen ratios along outlet channels (\(C_{\text{out1}}\) to \(C_{\text{out8}}\) in Fig. 5.2) varied linearly, calculated as 0 %, 14.2 %, 28.49 %, 42.82 %, 57.18 %, 71.53 %, 85.81 % and 100 %, respectively. Alternative sets of monotonically increasing/decreasing oxygen levels can also be designed by such approach with a different set of \(R_s\).

**Numerical validation of gas ratios**

The calculated output oxygen levels described in the previous section were also been validated by computational software using the same input parameters. The excellent agreement between the computational and analytical model confirms that the simplified circuit model is adequate for designing microfluidic oxygen gradient generators.

The concentration profile of gases (nitrogen and oxygen) in the gas gradient generator was numerically solved by a commercial computational fluid dynamics (CFD) software package (STAR-CD version 3.15a, CD-adpaco) based on finite volume method (FVM), as shown in Fig. 5.3a. The mesh size in the 3D model is typically \(<8 \text{ um} \times 8 \text{ um} \times 8 \text{ um}\), while the mesh size over the folded channels is \(\approx 8 \text{ um} \times 4 \text{ um} \times 8 \text{ um}\), maintaining a reasonable representation of the velocity profile (Fig. 5.3b). Steady state solutions of the momentum and continuity equations were obtained by the Semi-Implicit Method for Pressure Linked Equation (SIMPLE) algorithm\(^{15,16}\) with a convergence tolerance of \(1.0 \times 10^{-7}\). The tolerance is defined as the norm of the relative change of the velocity field in the instantaneous iteration. The typical local velocity is \(\approx 10^{-1} \text{ ms}^{-1}\), and the mean accuracy of this simulation is \(\approx 10^{-8} \text{ ms}^{-1}\). At each of the two gas inlets, a flat velocity profile was first imposed at a uniform flow velocity of \(0.2 \text{ ms}^{-1}\) and the velocity profile was modeled as fully-developed within the inlet channel, due to the low scaled Reynolds number \((10^{-3} < R_e^* < 10^{-1})\) of the flow. The pressure boundary conditions at the eight channel outlets were set as atmospheric \((1.02 \times 10^{5} \text{ Pa})\). The solution of continuity equation was applied for every combination of gas mixtures, as the sum of mass fractions of nitrogen and oxygen is always unity. We applied one-way coupling of fluidic flow to the concentration field, as oxygen and nitrogen gases have similar molecular weights. The mass transfer of the two source gases (a set of convection-diffusion equations) was solved (tolerance on gas ratios: \(\approx 10^{-8}\)) using the pre-computed flow fields. The simulated oxygen ratios at the outlets (0.04 %, 14 %, 28.4 %, 42.8 %, 57.2 %, 71.6 %, 86.1 % and 99.6 %) are within 1 % of those calculated by the aforementioned circuit model.
Experimental validation for dissolved oxygen

The distribution of DO concentrations along cell culture channels under continuous flow was investigated experimentally. In each measurement, the multiplexor valve array was used to open a single medium channel with steady flow rate controlled by a syringe pump (PicoPlus, Harvard Apparatus). The culture channels are located at the middle sections in the diffusion region as shown in Fig. 5.1a. The scaled Peclet number along the culture channels is <0.03 (Table 5.1). Consequently, the medium DO level will be fully-diffused within ~100 \( \mu \text{m} \), generating steady DO levels in the downstream cell culture and sensor regions. With the DO sensors located outside the culture region, the test chip enables simultaneous pO\(_2\) monitoring and cell density analysis. The experimental results (Fig. 5.4) show that the oxygenator can generate different DO levels along channels which correlate with the oxygen concentrations from the gradient generator mentioned in the previous section. Additionally, repeatable results were obtained with a low variation (\( R^2 > 0.99 \)) between separate runs.
Figure 5.4. Experimental values of DO concentrations in diffusion channels. The regression $R^2$ was calculated by the average of four individual experimental data.

5-2 OXYGEN GRADIENT GENERATOR USING PARALLEL MIXERS

The mixing component discussed in the previous section was designed to generate gases with different volumetric ratios of oxygen. This can be alternatively achieved by a set of parallel gas mixing channels with higher flexibility and robustness for the output $pO_2$, as shown in Fig. 5.5. The length of folded channels in the mixers was determined by the relation described in section 3.1. Because the mixing channels function individually, a defect along an individual mixing channel only affects its corresponding mixing channel, but not all other gas outputs as the gradient generator structure described in section 5.1. Output levels of $pO_2$ can also be defined arbitrarily for every outlet, based on the mixing channel geometry, while the cascade mixer array only generates the monotonically increasing/decreasing pattern across channels. The parallel mixer structure (blue channels in Fig. 5.5) requires a pair of gas inlets for each condition, of which each can be connected to a common source. To create unified input channel for each gas supply, channel routing becomes an important issue. To connect each mixer pair to the common gas lines, an additional layer (red channels in Fig. 5.5) for channel routing was added to the design of parallel mixing channels, with holes on PDMS membranes at the overlapping regions (highlighted with green squares in Fig. 5.5) between the mixer and routing layers for flow connection. The experimental validation of the parallel oxygenation method applied in a long-term cell culture device will be discussed in section 6.4.
Parallel mixer fabrication

The basic fabrication process of parallel mixers consists of two layers of soft lithography on PDMS. For each mold for the channel layers, a 20 μm thick SU-8 layer (SU-8 10) is spin-coated (1500 rpm, 30 s) on a 3” silicon wafer and patterned by photolithography (∼4 mW/cm², 70 s). After silanization of the molds, the PDMS substrate (ratio of monomer to curing agent: 10:1) with two layers of channels are combined using multilayer soft lithography. The flow connection between the layers is made by cutting rectangular holes on PDMS membranes in the overlapping regions using a razor blade (Bard-Parker stainless steel surgical blade, Becton Dickinson and Company) under a stereo microscope (SZX9, Olympus). Afterwards, the PDMS oxygenator layers are aligned and covalently bonded either using passive, secondary thermal bonding (80°C, 2 hours) or oxygen plasma.

5-3 TUNABLE OXYGEN-NITROGEN MIXER

An adjustable oxygenation source is desirable to mimic particular cellular environments, and to culture cell types with different oxygen requirements. For instance, the human oral cavity has an aerobic environment (DO ~ 8 ppm) in the daytime and anaerobic environment at night, due to the close oral cavity blocked by tongue during sleeping (as aforementioned in section 2-4). In the last section, an array of nitrogen-oxygen mixers was proposed to provide different oxygen levels along multiple gas streams. A tunable oxygen-nitrogen mixer can be achieved by dynamically connecting a shared gas channel to only one of the gas mixers corresponding to different oxygen levels, determined by a microfluidic multiplexor. Such oxygenator with an adjustable oxygen level can also be applied in the universal culture devices, i.e. the dimensions of folded channels do not need to change upon oxygen requirements of different species. In this section, a testing chip with the tunable gas mixer providing sixteen oxygen levels ranging from 0 – 100 % of oxygen content was developed to verify the feasibility of such tunable mixer design. The number and range of oxygen levels can vary depending on the accuracy requirement of applications. The tunable mixer was connected to a PtOEPK oxygen sensor (Fig. 5.6) in the testing device in order to achieve
real-time measurement for calibration purpose. The integration of oxygen sensor can be eliminated in the microfluidic oxygenator design for other applications.

**Fabrication**

The tunable gas mixer chip has three layers of structures: control layer, flow layer and sensor film as illustrated in Fig. 5.6a. The mold for control layer was fabricated by patterning 20 μm thick SU-8 on a 3” silicon wafer using photolithography. The mold for flow layer contains two layers of structures, with SU-8 patterns (height: 20 μm) for gas mixer and reflowed AZ4620 photoresist (height: 10 μm) for flow channel region overlapping with overhead microvalves. AZ4620 photoresist was spin-coated on a 3” silicon wafer, patterned by photolithography, reflowed and cured in oven at 130 °C for >30 hr. The following layer SU-8 containing the folded channel patterns was then fabricated on the AZ4620 layer by photolithography.

For the device fabrication, the PDMS substrate with the control layer on top of the flow layer was fabricated by the traditional multilayer soft lithography. The oxygen sensor was deposited on a cavity on the base glass substrate, wet-etched by buffered hydrofluoric acid (1:7 H2O). The sensor film was then prepared by applying ~1 μL of solution containing ~95 % (w/w) toluene, ~5 % polystyrene and 0.1 % PtOEPK on the etched sensor region. Afterwards the tunable gas mixer (Fig. 5.6b) was fabricated by bonding the PDMS substrate on the glass substrate using oxygen-plasma treatment, with the alignment under a stereo microscope (Olympus SZX9).

**Experimental results**

Performance of the tunable mixer was investigated based on its capability to generate a predefined transient oxygen concentration profile. Pure oxygen and nitrogen were applied by gas tanks (1 kPa gauge) during operation. The valves were actuated with ~13 kPa compressed air, and the multiplexor was used to regulate the ratio between gasses. The target concentration profile (blue in Fig. 5.6c) was set as a sine function over time, which was discretized based on the available oxygen levels in the tunable mixer, ranging from anaerobic to pure oxygen (blue in Fig. 5.6c). For an effective measurement, the minimal switching time should be longer than the response time of sensor (~3 s) Consequently, the period of the sine-wave concentration profile was set to 16 min and the time for each change of oxygen ratio was 15 s. Experimental result (red in Fig. 5.6c) shows that the tunable gas mixer can generate varying transient oxygen concentrations close to the target profile. The resolution of possible oxygen levels can be further improved by increasing the number of gas mixers. The switching time in this implementation example (~2.8 – 4.9 s) is sufficiently short for most cell culture applications (typical doubling time: >>15 min).
Figure 5.6. (a) Design layout and (b) photograph of tunable oxygen-nitrogen mixer. (c) Sample transient output of tunable mixer that generates oxygen level a sine function over time.

5-4 APPLICATION TO CELL CULTURE

Experimental

To validate the oxygenation approach for cell culture applications, the growth characteristics of bacteria with different DO requirements, including *Escherichia coli* (facultative anaerobe), *Actinomyces viscosus* (aerobe) and *Fusobacterium nucleatum* (strict anaerobe), have been analyzed as a function of eight discrete DO concentrations ranging from anaerobic to fully saturated using the microfluidic device described in section 5-1. *A. viscosus*
and F. nucleatum were chosen based on their important roles in the coaggregation of dental biofilm; while E. coli was chosen as the reference case.

For the preparation of bacterial samples, E. coli (NCTC 86) and A. viscosus (ATCC 19246) cells were continuously cultured in Brain Heart Infusion (BHI) broth (Hardy Diagnostics R20). Culturing was performed in an incubator with a rotary shaking platform (200 rpm) at 37 °C for 20 hr. F. nucleatum (ATCC 10953) cells were cultured in Pre-Reduced and Anaerobically Sterilized (PRAS) chopped meat media (Hardy Diagnostics AS811) inside a GasPak anaerobic jar (BD 26026). Gas-generating sachets (BD 260678) were used to maintain the anaerobic environment.

Because of the branched growth pattern of A. viscosus, microbial clusters are often found in the culture, causing inconsistent cell densities during cell loading. A cluster seeded into a culture channel will induce large variations in the cell count. To fix such problem, additional procedures for cell extraction were performed to select only the distributed A. viscosus cells. The bulk culture of A. viscosus was first re-suspended by pipetting (>50 times) to scatter the aggregated cells into smaller communities. The cells were then cultured at 37 °C for 30 min without shaking to allow sedimentation of the cell clusters. Samples extracted from the top portion of culture were seeded into culture channels as other species.

To pre-condition the microfluidic channels, the device was sterilized by flushing fluid channels with 70 % ethanol followed by baking at 80 °C for 2 hr. After baking, the fluid channels were rinsed with 1x phosphate-buffered saline (PBS), pH 7.4, and degassed by forcing trapped air through the walls of the gas-permeable oxygenator with pressurized PBS buffer. Confluent bacteria cultures of each species (OD600 0.95) were diluted in their respective media to a cell density of ~10^7 cells/mL. Following dilution, cells were loaded into the oxygenator at a flow rate of 0.01 μL/min for 3 min. Prior to on-chip oxygenation, the microfluidic devices for E. coli and A. viscosus were placed in a 37 °C aerobic incubator for 2 hr to promote adhesion between the bacteria and glass microchannel walls, while the devices for F. nucleatum were anaerobically cultured by flowing pure nitrogen through the gas layer microchannel network.

During on-chip cell culture, oxygen and nitrogen (supply pressure: ~1 kPa), humidified by bubbling through water reservoirs, were flowed through the gradient generator to generate different DO levels in the underlying medium-filled channels (0 – 42 ppm). A syringe pump (Harvard Apparatus) was used to continually supply fresh medium (flow rate: 0.003 μL/min) to each fluid channel in turn, switching channels every one minute under the control of an integrated microfluidic multiplexor. This operation provides a consistent medium supply along every channel, even when there were inconsistencies in the channel cross-sections and fluidic resistances developed by differential cell growth rates.

Results and image analysis

To estimate the cell density of bacteria for different culture periods, phase-contrast microscopic images (20X) of culture channels were obtained as shown in Fig. 5.7a. In the culture region, bacterial communities appeared darker in the phase-contrast microscopic images. Their corresponding pixels were extracted by a serious of image processing steps. The culture region was first specified individually in the microscopic images. The
background pattern of culture region was estimated by convolution of the image with Gaussian mask (size: 20 pixel × 20 pixel, radius: 10 pixel) as a low pass image filter. After subtracting the image intensity with the background image, thresholding was performed (cutoff intensity: 12/256) to identify bacterial cells as shown in Fig. 5.7b. The cell surface coverage was then calculated by counted the area of cells in the culture region and the corresponding cell density was determined by comparing to the control images in which the cell densities (10^6 - 10^8 cells/mL) were measured by a hemocytometer. After hours of culture, incremental growth was observed. The doubling time \( T_d \) of species could be estimated with two successive cell counts \( N_1 \) and \( N_2 \) at \( t_1 \) and \( t_2 \) \( (t_2 > t_1) \), respectively, by

\[
T_d \approx \frac{(t_2 - t_1) \ln 2}{\ln(N_2 / N_1)}.
\]

\[ (5.1) \]

Figure 5.7. Procedures of growth estimation. Each (a) phase-contrast microscopic image was filtered and thresholded as (b) a homochromatic image. At the end of cell culture, the cell growth was estimated by comparing the increment of black dots in the threshold images.

All culture experiments showed reasonable results for the testing species (Fig. 5.8). E. coli (Fig. 5.8a) cells grew under both aerobic and anaerobic conditions, with the shortest doubling time \( (T_d = 1.9 \text{ hr}) \) under ambient condition \( (pO_2 \sim 12 \text{ ppm}) \). A. viscosus (Fig. 5.8b) grew only under aerobic conditions, with the shortest doubling time \( (T_d = 14.1 \text{ hr}) \) at \( pO_2 \sim 18 \text{ ppm} \). F. nucleatum (Fig. 5.8c) exhibited maximum growth under anaerobic conditions \( (T_d = 9.67 \text{ hr}) \), with some growth observed up to \( pO_2 \sim 12 \text{ ppm} \).
Figure 5.8. Cell density of (a) E. coli, (b) A. viscosus and (c) F. nucleatum. The data points were obtained by averaging the results of five individual experiments. The minor reduction of cell densities observed in A. viscosus (at 42 ppm) and F. nucleatum (at 30 ppm) was due to cell detachment under continuous medium flow.
Discussion

The application of a microfluidic differential oxygenator system to the culture of bacteria with different oxygen demands has been described. Integrating the multiplexor, oxygen-nitrogen gas mixer and double-layer diffusion channels, the oxygenator generates a step-function of repeatable DO concentrations in an array of parallel microchannels containing aqueous media. Integrated polymeric oxygen sensors provide a robust method for real-time monitoring of the DO levels in culture media within the microchannels. To validate its potential for the culture of both eukaryotic and prokaryotic cells, on-chip growth profiles of anaerobic and aerobic bacteria, and even a model mammalian cell line (3T3) described in Appendix A was demonstrated. The culture experiments showed differential cellular growth response verses DO concentrations. Microfluidic oxygenator chips, representing a robust and low-cost method to regulate DO levels in culture, are anticipated to be of wide appeal not only to cancer researchers, but also public health laboratories for bacteria that are difficult to be cultured using established microbiology protocols.

REFERENCES


CHAPTER SIX

DESIGN OF MICROBIOREACTOR FOR LONG-TERM CULTURE

6-1 HUMIDITY CONTROL USING WATER JACKET

The oxygenation using a gas channel flowed with defined pO$_2$ overhead culture chambers have been shown an effective oxygen transfer due to the small length scale of diffusion (~10 – 100 μm). However, convective flow in the gas channel also enhances the transfer of moisture from the culture channel through the PDMS separation membrane, such that the culture medium evaporates quickly (within minutes). Evaporation can be reduced by using pre-humidified (saturated) gases, but convection still leads to the eventual dessication of the culture. Experiments showed that the corresponding evaporation time could be improved to ~5 – 8 hr, which still fails to fulfill the requirement of long term cell culture for days/weeks. Moreover, any significant moisture loss over time, even at a reduced rate, changes the relative osmolarity in the culture media, leading to cell stress and death.

Applying a design similar to the microfluidic gas exchange channel network, an alternative approach to sustained culture hydration is achieved by fabricating a layer of water jacket channel between gas and medium layers to define a saturated humidity zone above culture regions.¹ The water jacket acts as a sacrificial layer of moisture under the gas stream. For cell culture implementation, the water jacket was connected to a water reservoir driven by a low pressure gas source (~1 kPa) to replentish water lost to evaporation. Simulation was performed to analyze a prototype oxygenator containing a cylindrical chamber (diameter: 1 mm, height: 210 μm), a gas reservoir (diameter: 18 mm, height: 20 μm) and a water jacket (diameter: 16 mm, thickness: 20 μm) separated by two PDMS layers above and below water jacket with thickness ~40 μm and ~300 μm, respectively.

Figs. 6.1a and b show the numerical pO$_2$ and humidity profiles, respectively, of the prototype by solving the corresponding diffusion equations in cylindrical coordinates based on the axisymmetric prototype design. The normalized pO$_2$ above the device ($P^*_a$) was predefined to be 0, while the normalized pO$_2$ around the gas layer ($P^*_g$) was set to 1 and no diffusion flux across the bottom PDMS-glass interface. Based on the linear solution properties of diffusion equation, this simulated profile $p^*(r; \xi, \theta), 0 \geq p^* \geq 1$, can be scaled upon different atmospheric ($P_a$) and imposed ($P_g$) pO$_2$ as $P_a + p^*(P_g - P_a)$. Furthermore, the
numerical moisture profile was represented by the normalized value $m^*(r, \theta)$, with different configurations of boundary conditions. Considering the water jacket and culture chamber are filled with liquid, the normalized moisture level at their boundaries ($M_w^*$ and $M_c^*$) were both set to 1 as indicated in Fig. 6.1b. The normalized ambient humidity ($M_a^*$) and the boundaries around gas layer ($M_g^*$) were set to 0 and there was no diffusion into gas-impermeable glass substrate. Analogous to $pO_2$, the actual humidity profile can be calculated by $M_a^* + m^*(M_w - M_a)$, where $M_a$ is the ambient humidity and $M_w$ is the moisture level around water jacket, which is also equal to the level around chamber ($M_c^*$).

According to the simulated profiles, most the region around chamber is nearly saturated, so the gradient of humidity level around chamber is negligible. Such approach of the addition of water jacket under a humidified environment can greatly reduce the moisture loss of the double-layer microfluidic oxygenator. The performance of water jacket can be further improved by flowing humidified gases. The practical implementation indicated the corresponding evaporation time could be extended to $>20$ hr. The configuration discussed in this section can be viewed as the basic oxygenator design for the long-term culture chips. In the following sections, this prototype design has been adopted in different culture chips including the long-term oxygenator and the artificial teeth devices.

**Figure 6.1.** Normalized profiles of spatial (a) $pO_2$, $p^*(r, \theta)$, and (b) humidity, $m^*(r, \theta)$, over a prototype microfluidic oxygenator containing a water jacket.
MODIFIED OXYGENATOR DESIGN PARAMETERS

Because of the additional water jacket in oxygenator for long-term culture, the design parameters discussed in chapter 4 are modified as illustrated in Fig. 6.2a. Oxygen from the gas channel has to diffuse through both the upper and lower PDMS membranes and the water jacket to reach culture media, instead of a single PDMS layer. The typical pO₂ profile at the fully-developed region is shown in Fig. 6.2b. Due to the same material along the two PDMS layer, and the similar diffusivity and solubility of medium and water, the gradients of pO₂ over the y direction ($\frac{\partial p_{O2}}{\partial y}$) are the same at the fully developed region for the two PDMS layer ($i = 3$ and 5), and for the water jacket ($i = 4$) and medium layer ($i = 2$).

Figure 6.2.(a) Oxygenator structure and (b) typical pO₂ profile along vertical direction (y) in the fully developed region. (Not in scale) (c) Diffusive resistance model. Under oxygenation, the OCR of biofilm is often much smaller than the imposed pO₂. So, at the transition region of pO₂, the ratios of pressure difference between material interfaces can be approximated as the ratios of their vertical diffusive resistances.

Considering the change in diffusive resistance (Fig. 6.2c) along y direction, the term $H_3/D_3S_3$ in Eqs. 4.16 and 4.18 are replaced by $(H_3+H_5)/D_3S_3+H_4/D_2S_2$. Therefore, the oxygen pressure drop (OPD) for oxygenators containing a water jacket can be calculated as
\[
\text{OPD} = \frac{P_{x} - P_{w}}{P_{x}} = \frac{1}{2} \left( 1 + \Phi + \Theta \right) - \frac{1}{2} \sqrt{\left( 1 - \Phi - \Theta \right)^2 + 4 \Phi},
\]
\[
\Phi = \frac{K_{w}}{P_{x}}, \quad \text{and} \quad \Theta = \frac{V_{max} \rho_{at}^2}{P_{x}} \left( \frac{H_{4} + H_{5} + H_{5}}{D_{2}S_{2}} + H_{4} \right) + \frac{1}{3}
\]  
(6.1)

where \(H_{4}\) and \(H_{5}\) are the thicknesses of water jacket layer and upper PDMS membrane, respectively. The \(\text{OPD}\) (as a function of \(\Phi\) and \(\Theta\)) in Eq. 6.1 has the identical mathematical structure as in Eq. 4.13, so the parameter \(\Theta\) still summarizes the device dimensions. The relation \(\text{OPD} \approx \Theta\) also holds when \(K_{w} \ll P_{x}\).

Fig. 6.2c shows the equivalent diffusive resistance model describing the ratios of pressure difference across the material layers at the transition region \((L^{*}(x) = 1\) by definition). Applying the same modification to the horizontal diffusion terms, the dimensionless effective channel length \(L^{*}\) is

\[
L^{*} = \frac{x}{PeH_{2} \left[ \left( \frac{H_{4} + H_{5}}{D_{2}S_{2}} \right) + \frac{D_{2}S_{2}}{H_{2}} + \frac{1}{D_{2}S_{2}} \right]^{1/2}}.
\]  
(6.2)

The relative dimensions of the thin water \((H_{4})\) and PDMS \((H_{3} \text{ and } H_{5})\) layers determine the transition position, \(x\), where the culture media becomes full developed with respect to the DO profile.

To determine the rise time \(T_{r}\) of oxygen diffusion, the thickness of water jacket and membrane are lumped into an equivalent membrane thickness \(H_{3}\) as

\[
H_{3}^{*} = H_{3} + \frac{D_{2}S_{2}}{D_{2}S_{3}} H_{4} + H_{5}.
\]  
(6.3)

Applying \(H_{3}^{*}\) as the PDMS membrane thickness (analogous to \(H_{3}\) in Fig. 4.7), the corresponding \(T_{r}\) can be approximated using Fig. 4.7 for different device configurations. In the modified oxygenator design, both PDMS layers are required sufficiently thick (>100 \(\mu\)m by Eq. 3.8) to maintain membrane rigidity upon pressure along gas channel and water jacket. Because the overall microfluidic device with the water jacket is thicker than its non-jacketed analog (PDMS membrane thickness: ~20 \(\mu\)m), the corresponding \(T_{r}\) is relatively longer. For example, a 100 \(\mu\)m tall microchannel oxygenated with a water-jacketed membrane \((T_{r} \sim 1 \text{ min})\) requires around ten times the diffusion time of the non-jacketed design \((T_{r} \sim 6 \text{ s})\). The larger rise time places important constraints on cell culture applications with the water-jacketed architecture, particularly for cell culture applications that require dynamic tuning of the culture DO levels over time. To mitigate the problem, the culture channel should be extended in length, with the targeted culture region occurring in the regime where \(L^{*} \gg 1\).

### 6-3 MEDIUM REPLACEMENT APPROACHES

Considering the nutrient consumption levels of living cells, medium supply is the key requirement for successful cell culture, especially for long-term implementation. This can be achieved by continuously flowing fresh medium along the culture microchannel. Such an
approach as a flow cell has been widely used for biofilm studies\textsuperscript{2-5} including both prokaryotic and mammalian cell culture, with cells pre-attached on the channel substrate. However, the induced shear and the rapid removal of cells in a continuously pumped flow cell detrimentally affect biofilm formation and cell culture in general. Growth of suspended cultures is infeasible and, for adherent cells, the convective flow of media confounds initial cell attachment to the solid substrate. Therefore, other medium replacement methodologies for more general cell culture applications have been developed as described in the following subsections.

\textbf{Regular medium insertion with partial chamber volume}

Instead of continuous flow, medium can be inserted regularly with a defined volume to support the growth of cells in a culture chamber. Implementation of this approach involves periodically flowing fresh medium for a fraction of the overall culture period (~1 – 3 min every 30 – 60 min). Operating under this condition, combining static flow conditions with brief periodic media changes, enables initial colonizing of the substrate in the case of bacterial biofilms, and creates an ideal environment for miniaturized biofilm studies like the artificial teeth platform.

For the implementation of regular medium replacement, a flow line was designed around the culture chamber as shown in Fig. 6.3a. Microvalves were utilized to switch the flow either along chamber or alternative flow line. Prior to medium insertion, fresh medium was used to initially prime the flow line (Fig. 6.3b) using a peristaltic micropump\textsuperscript{6}. This pre-flowing scheme ensured that fresh medium was present at the inlet of chamber. After switching the active path of flow to culture chamber, fresh medium partially filled the chamber with a defined volume determined by the number of cycles of peristaltic pumping (Fig. 6.3c). The path of flow was then switched back to the flow line, isolating the chamber again. Flushing PBS/DI water along flow line, cells outside culture region were removed (Fig. 6.3d) to prevent cells growing in the downstream regions near the waste outlets.

The above protocol promotes cell growth in the microfabricated culture chamber through periodic partial replenishment of the culture media. Considering the growth rate of cells $\kappa$ [s\textsuperscript{-1}] under culture in log phase, fresh medium should be supplied to culture chamber (with volume $V$) with a limited rate of inflow ($<\kappa V$), which is equal to the rate of outflow of old medium. Furthermore, the time between two consecutive medium insertions should be sufficiently longer than the diffusion time of nutrients in chamber, estimated by $4R_o^2/D_{H2O}$ where $R_o$ is chamber radius and $D_{H2O}$ is the diffusivity of water/medium (due to the fact that the maximum diffusion length is the distance from chamber inlet to outlet). Therefore, the selection of a sufficiently small culture chamber is important for this design. In particular, for the culture of cells with relatively short doubling time ~30 min in an ~1 mm diameter chamber, the transient concentration profile of nutrients before fully development will cause an uneven growth of cells with doubling time ~30 – 60 min.
Cell trapping using ring shape valve

The partial medium replacement in culture region can also be achieved by isolating part of the region using a ring shape valve. By embedding the valve (inner diameter 150 μm and outer diameter 350 μm) in a culture chamber (diameter 600 μm) as shown in Fig. 6.4a, the center portion of cells can be trapped (Fig. 6.4b) and fresh medium can then flow along the outer chamber region (Fig. 6.4c). Medium can diffuse in chamber after releasing the ring shape valve (Fig. 6.4d).

The gas pressure required (ΔP) to ensure "trapper" closure can be roughly approximated as a 1D fixed-fixed beam model. The deflection of PDMS trapper membrane (with Young’s modulus $E_{\text{PDMS}} = 750$ kPa) should be equal to or larger than the chamber height for complete closure, hence

$$\Delta P \geq \frac{32E_{\text{PDMS}}H_c^2H_e}{W_b^4}.$$  \hspace{1cm} (6.4)

The characteristic membrane length ($W_b$) can be set as the width of ring valve, which is the distance between the outer ($R_{\text{outer}}$) and inner ($R_{\text{inner}}$) valve radii (= 100 μm). For instance, a
trapper with 20 μm membrane thickness \((H_b)\) on a 20 μm tall \((H_c)\) chamber requires a driving pressure \((\Delta P)\) of \(\geq 38.84\) kPa for its closure.

A better estimation would be the fixed-fixed thin plate model in cylindrical coordinates, described by

\[
\nabla^2 \left[ \frac{E_{pdms} H^2_b}{12(1-\nu^2_{pdms})} \nabla^2 (\delta h) \right] = \Delta P
\]

where \(\nu_{pdms} (= 0.5)\) is the Poisson's ratio of PDMS.

Integrating Eq. 6.5, we can obtain \(\delta h\) as a function of radial position \(r\):

\[
\delta h(r) = C_0 r^4 + C_1 r^2 \ln r + C_2 r^2 + C_3 \ln r + C_4,
\]

where \(C_0 = 3(1-\nu_{pdms})\Delta P/(16E_{pdms} H^2_b)\), \(C_1, C_2, C_3\) and \(C_4\) are constants. The corresponding boundary conditions for this axisymmetric problem are zero deflection and deflection gradient at the outer and inner valve radii, expressed as

\[
\delta h(R_{inner}) = \delta h(R_{outer}) = \nabla(\delta h)|_{r=R_{inner}} = \nabla(\delta h)|_{r=R_{outer}} = 0.
\]

Combining Eq. 6.7 and Eq. 6.6 as linear equations to solve for the constant variables, we can obtain

\[
\begin{bmatrix}
R^2_{inner} \ln R_{inner} & R^2_{inner} & R^2_{inner} & 1 & C_1
R^2_{outer} \ln R_{outer} & R^2_{outer} & R^2_{outer} & 1 & C_2
2R^2_{inner} \ln R_{inner} + R^2_{inner} & 2R^2_{inner} & 1/R_{inner} & 0 & C_3
2R^2_{outer} \ln R_{outer} + R^2_{outer} & 2R^2_{outer} & 1/R_{outer} & 0 & C_4
\end{bmatrix}
\begin{bmatrix}
R^4_{inner}
R^4_{outer}
4R^3_{inner}
4R^3_{outer}
\end{bmatrix}
= C_0.
\]

Adopting the same dimensions as for Eq. 6.4, the constants \(C_1 = -2.90, C_2 = -24.78, C_3 = -1.77 \times 10^{-8}\) and \(C_4 = -1.84 \times 10^{-7}\). The radial position for maximum deflection \((R_{max})\) is 123 μm, obtained by solving \(r\) for \(d(\delta h)/dr = 0\) using Newton’s method. Hence, the driving pressure of the trapper requires ~51 kPa to obtain the target deflection, \(\delta h(R_{max}) = 20\) μm. The hydraulic pressure induced by liquid flow was typically <5 kPa, so the driving pressure was normally set to be 60 – 80 kPa (or ~9 – 12 psig) to guarantee the trapping operation.

This cell trapping approach can shorten the required diffusion time of medium by geometrically modifying the arrangement of fresh and old media. The fresh medium would diffuse from outer to inner part of chamber, meaning that the typical diffusion length can be estimated as the inner radius of the ring shape valve (~75 μm in this work), instead of chamber diameter (600 μm from chamber inlet to outlet). Considering the diffusion time is proportional to the square of diffusion length, the diffusion time for trapper design can be reduced to <10 % of the time for regular chambers. Such reduction of diffusion time can greatly increase the allowable chamber diameter, which provides a larger culture region for more consistent biofilm growth. However, this design has its limitations, including a constraint on chamber height for full valve closure and a fixed volume of liquid insertion for each replenishment operation. Furthermore, the chamber height sets a constraint on the overall cell layer thickness, which limits its applicability to both biofilm growth analysis and the culture of larger cells (e.g mammalian cells with a diameter ~10 – 15 μm). When
constrained by a fixed volume of liquid replacement, the trapper and chamber dimensions may need to be redesigned for different target cells and applications. The additional insertion of other solutions and biochemical molecules for further operation and analysis would also be challenging. Fig. 6.4e demonstrates the trapping of food dye. The transparent color of trapper areas during the closure of trappers indicates the isolation of liquid between inner and outer portions of chambers. For the culture implementation, the dye and water can be considered as the bacteria under culture and fresh medium, respectively. The medium insertion procedure is demonstrated in Fig. 6.4f. Food dye was perfectly trapped in parallel while water was flowing along flow channel after closing the trappers.

![Image](image_url)

**Figure. 6.4. Design and Operation of cell trapper.** (a) Ring shape valve embedded inside chamber to trap the partial population of suspending cells. (b) Pressurized valve which deforms and attaches to the base of chamber, insulating the center portion of cells. (c) Replacement of outer region. (d) Restoration of fluidic connections in chamber after releasing trapper. Isolation of medium was demonstrated by the pattern of color dye (e) before and (f) after flowing with water while trapper was being actuated.

Additionally, the parallel trapping operation was also demonstrated using a prototype design consisting of a matrix of chambers (Fig. 6.5). Ring shape valves in control layer (red) were located overhead all chambers in flow layer (blue). The device was fabricated by the typical double layer soft lithography with a thickness 20 μm for both the control and flow layers. The device was designed with 120 culture chambers and trappers along the single flow channel. For the parallel operation of all chambers, a shared “Close All” valve was used
to close all chamber inlets and outlets, while the “Trapper” valve was connected to all trappers. The medium replacement procedures start with the actuation of the trapper valve to isolate the center part of medium, followed by the release of medium into the flow channel. Fresh medium is then routed along the outer regions of all of the chambers. At the moment of trapper deactuation, the outlet valve (indicated in Fig. 6.5a) was closed while the device inlet was open, allowing additional fresh medium to replenish the displaced volume caused by the movement of ring shape valve. The chamber was then closed again with the “Close All” valve for a steady environment of cell culture. An experiment (Fig. 6.4c) was performed to trap red color dye within the ring structures in multiple chambers, validating the feasibility of the design for high throughput applications with the regular partial replacement of medium in parallel chambers.

![Diagram](image)

Figure 6.5. (a) Design layout and (b) fabricated device with an array of chambers with overhead trappers. (c) Photograph showing red color dye could be trapped in chambers.
6-4 IMPLEMENTATION OF LONG-TERM CULTURE DEVICE

Device design

A microfluidic oxygenator containing a matrix of $5 \times 8$ culture chambers was developed to investigate the long-term growth of cells. The design layout and arrangement of different layers are shown in Fig. 6.6a. The oxygen level was regulated by the eight columns of overhead gas channels (green) flowed with different volumetric ratios of oxygen and nitrogen, with each column of five chambers receiving the same gas mixture. The gas mixer architecture was an adaptation of the parallel mixer design outlined in section 5-2, designed with oxygen ratios ranging from anaerobic to fully aerobic (The levels can be modified upon different applications). To minimize culture medium evaporation, a water jacket (purple) was embedded between the gas and culture layers. The connecting channels of the parallel mixers were located in the same layer as the water jacket to reduce the fabrication effort to one fluidic channel layer. For the actuation and switching of medium flow, the control layer containing valves and a peristaltic pump were fabricated right on top of the fluidic layer. A multiplexor was used to selectively activate one of the eight channel columns during operation. Moreover, the medium replacement in this device applied the partial insertion approach with an additional flow channel around each chamber (section 6-3). The accessibility of flow channel and chamber was controlled by the “Flush” and “Loading” valves, respectively. The ring shape trapping design mentioned in the previous section may not be practical as taller chambers were required to accommodate prolonged cell growth and division under long term culture. The chamber height was achieved by fabricating PDMS cavities with the target height ($\sim$200 $\mu$m) as the chamber bases. Three inlets for the devices, controlled by integrated microvalves, permit the separate introduction of cell samples, DI water and fresh medium.

Fig. 6.6b illustrates the detailed geometry of channel layers in each chamber region. The diameters and the gaps between gas channel, water jacket and culture chamber were the same as presented in section 6-1 to obtain a similar performance as the prototype design. The gas channel and water jacket were added with PDMS pillars (white circles in Fig. 6.6b) for mechanical support against collapse under the external gas/hydraulic pressure supply. The water jacket structures were interconnected among all culture regions for humidity control, while the oxygenating channels were connected to each column of five chambers to provide parallel differential oxygenation conditions. The flow layer was composed of $\sim$10 $\mu$m tall channels (gray) for valve operation and $\sim$200 $\mu$m tall channel structures (blue) for culture chambers. Two sets of interconnected valves around all culture regions were designed to close either chambers or alternative flow channels.

Five molds for soft lithography were fabricated for the layers mentioned in Fig. 6.6a. The mold structures for flow layer was reflowed AZ4620 ($\sim$10 $\mu$m) on silicon wafers, while all other molds were fabricated with SU-8 photoresist on wafers with different thickness (100 $\mu$m for chambers and 20 $\mu$m for gas, water jacket and control layers). After mold silanization, the $\sim$5 mm thick PDMS substrate with gas channel structure was initially fabricated using its respective mold. Afterwards the stacked PDMS layers including water
jacket, control and flow layers were fabricated by multilayer soft lithography accordingly, with thicknesses of 60 \( \mu \)m, 300 \( \mu \)m and 20 \( \mu \)m, respectively. The PDMS layer with chamber structures was fabricated separately with a thickness of \( \sim 400 \) \( \mu \)m and plasma-bonded on a 75 \( \text{mm} \times 25 \) mm \( \times 1 \) mm glass slide (Cat# 16004-422, VWR) with the side of chamber cavities facing upward. After aligning and plasma-bonding the stacked PDMS substrate on the chamber patterns, the device contained multiple chambers with sufficient capacity (diameter: 1 mm, thickness: \( \sim 210 \) \( \mu \)m) for the long-term growth of cells was fabricated as shown in Fig. 6.6c.

**Implementation of long-term culture**

Three sets of experiments on *Streptococcus mutans*, *Actinomyces viscosus* and *Fusobacterium nucleatum* were performed to examine their long-term growth under different oxygen conditions. The dental bacteria were inoculated in chambers and cultured for 5 – 7 days under continuous oxygenation and humidity control by water jackets. *A. viscosus* and *F. nucleatum* were prepared with the same protocols described in section 5-4, while *S. mutans* was prepared as *A. viscosus* except that the stirring procedures were skipped.

Prior to bacteria culture, the oxygenator shown in Fig. 6.6c was baked at \( \sim 100 \) °C for \( >10 \) hr and exposed to ultraviolet (UV) light for \( >2 \) hr for simple sterilization. The water jacket and flow layers were then pre-filled with DI water and medium, respectively, using a driving pressure of \( \sim 3 \) psi at the inlets. The micropump along the outlet channel and the valves for medium and cell insertion were closed with a gas pressure of \( \sim 12 \) psi in order to block all fluidic exits for a closed cavity in flow layer. Due to the gas permeability of PDMS, the liquids could be primed into the layers by driving air out from the device. Pre-conditioning of temperature and gas conditioning were achieved by placing the oxygenator in a 37 °C incubator and applying pre-humidified oxygen and nitrogen at the gas inlets (1 psi for both gases) for \( >15 \) min. Afterwards, bacteria (density: \( \sim 10^6 – 10^7 \) cells/mL) diluted from the confluent culture were seeded into chambers on a column-by-column basis, determined by the multiplexor. In the cell seeding procedures for each column, bacteria samples were first pumped along the alternative flow channels next to chambers. In this operation, the “Loading” valves were close while the “Flush” valves were open. The valve actuation of “Loading” and “Flush” was then switched for 5 min for each chamber column, so that bacteria were flowed into the five chambers simultaneously. Switching again the actuation of valves, the flow channels were rinsed with DI water to prevent clogging due to the growth of leaked bacteria.

For long-term culture, medium was periodically inserted in all chambers for \( \sim 10 \) s every 60 min. The procedures of each medium insertion were analogous to cell loading, except for the insertion duration and the inlet liquid (fresh medium instead of cell samples). For every 1 – 3 days, the cell densities at all chamber regions were measured by taking phrase contrast microscopic images, with the oxygenator placed a \( \sim 37 \) °C water bath for temperature regulation. Post-processing of the microscopic images was performed with the same technical described in section 5-4, and the cell densities were estimated by comparing
the control images corresponding to different cell densities (~$10^6 - 10^8$ cells/mL) measured by a hemocytometer.

**Figure 6.6.** Microfluidic oxygenator designed for long-term culture. (a) It was composed of gas perfusion layer, water jacket, control layer and flow layer with flow channels and culture chambers. (b) Closer views of individual layers showing the exact design layout of each layer. The white circle shaded with blue dots represents the overlapping region of flow and chamber structures. (c) Photograph of the final fabricated device with size 30 mm (L) x 20 mm (W) x ~5 mm (H).
Results and discussion

Fig. 6.7 shows the cell densities of dental bacteria *S. mutans*, *A. viscosus* and *F. nucleatum* over 5 – 7 days of independent culture experiments. The oxygenator described in this section could obtain reasonable bacteria growth for different DO levels ranging from anaerobic to fully-oxygenated. Aerobic bacteria *S. mutans* and *A. viscosus* could grow with maximum rate under DO ~16 ppm and ~11 ppm, respectively, while Anaerobe *F. nucleatum* could only grow under an anaerobic/microaerobic (DO < 8.5 ppm) environment. After days of culture, the device obtained cell densities up to ~10^8 cells/mL for *S. mutans* and *A. viscosus*, and ~10^7 cells/mL for *F. nucleatum*. The microfluidic long term culture chip, combining regular medium replacement regiments with the the addition of alternative flow channels to only partially fill chambers in each operation, was very effective for long teerm cell culture. Furthermore, the ability to co-culture specimens under differential gas conditions enabled the monitoring of culture dynamics as a function of DO concentration. With only periodic growth medium changes, the relatively static chamber conditions enabled primary attachment to the glass substrate without the need for an adhesion promoter. The layer of water jacket was critical in the design, with the alternative flow channel substantially mitigating medium evaporation, especially when the medium flow was absent. With further device development, the platform is well suited for the dynamic monitoring for cell culture growth using optical microscopy, useful not only for biofilm formation studies, but also as a generic tool for monitoring cell-cell interactions.

REFERENCES

Figure 6.7. Cell densities of dental bacteria (a) S. mutans, (b) A. viscosus and (c) F. nucleatum in microfluidic oxygenator integrated with water jacket.
7-1 BACTERIAL ADHESION AND SUBSTRATE SELECTION

Microbial adhesion is a prerequisite to initiate dental biofilm formation, so the selection of substrate material is critical in the development of microfluidic artificial tooth device for in vitro dental biofilm culture. A substrate material should be selected for the initial adherence of primary colonizers as the foundation for subsequent biofilm development involving other co-colonizers. All surfaces in the oral cavity are coated with saliva, forming a salivary protein film that may alter the surface properties and mask the surface structures. It was reported that the surface pre-treatment of artificial substrates to promote biofilm formation can be achieved using filtered human saliva. Specific components, such as salivary glycoprotein, may serve as receptors for oral Streptococci in the salivary pellicle. Additionally, saliva also provides free calcium ions that may bridge opposing negatively charged tooth and microbial surfaces, thus promoting non-specific bonding.

In previous in vitro studies, it was reported that the salivary pellicle-coated film could be formed on various materials (glass, plastic and hydroxyapatite) to facilitate the adhesion of bacteria to their specific receptors. To mimic the surface condition of human teeth, the substrate was required to be pre-coated with filtered human saliva for >1 hr to obtain the same surface energy, zeta potential and hydrophobicity as in oral cavity. Three candidate materials (glass, polystyrene and PDMS) were considered to select the most effective material for the formation of pellicle-coated film. The testing samples were prepared by applying toluene solution with 5 % (w/w) dissolved polystyrene (gray) on glass (highlighted in yellow in Fig. 7.1a) and PDMS (green in Fig. 7.1b) surfaces. After toluene evaporated, a layer of polystyrene was formed and human saliva filtered by the filtration bottle (Cat# 8-0000-42 0803, Nalgene Labware, Inc.) was applied on the entire surface of samples for 30 min. S. sanguis, which is one of the major early colonizers in dental biofilm, was pre-cultured in brain heart infusion (BHI) broth and was then added on the sample surfaces for 5 min. (The preparation of S. sanguis was the same as S. mutans described in section 6-4.) After rinsing gently with PBS, phase contrast microscopic images were taken as shown in Fig. 7.1. The results show that S. sanguis (highlighted by red arrows) had better adherence on polystyrene than other test materials (glass and PDMS).
Based on the preliminary adhesion studies, the polystyrene coating protocol was further optimized in the artificial tooth devices. Polystyrene deposition was achieved by applying ~0.3 nL toluene solution containing 5% polystyrene (w/w) in a circular PDMS cavity with diameter ~1 mm and depth ~200 µm (cross section view in Fig. 7.2). Additional experiments were performed to determine the required time of saliva surface pre-treatment with the polystyrene-coated microchambers. Samples with polystyrene layers deposited in PDMS cavities were first pretreated with filtered saliva for 30 min. After applying *S. Sanguis* with medium broth to cover the cavities for different durations, PBS was used to rinse the samples. Phase contrast microscopic images were then taken and converted to cell coverage (unit: cells/cm²) based on the cell diameter (~0.8 µm) as shown in Fig. 7.2. The adherence of *S. Sanguis* increased as a function of primary incubation time (1–10 min). Saturated cell coverage (~3.2 × 10⁶ cells/cm²) was obtained for the treatment times of ≥10 min. For subsequent biofilm experiments in the artificial tooth devices, both the time of surface conditioning with filtered saliva and initial bacterial adhesion were carried out for 30 min to guarantee the diffusion of salivary components in substrate material and the primary attachment of *Streptococci* from bacteria samples.
Because teeth are composed of the mineral hydroxyapatite, another possible candidate is polystyrene-hydroxyapatite, prepared by putting polystyrene and hydroxyapatite in toluene and pipetting to solution on target area. The substrate of multiple materials has the potential for other dental plaque related analysis, such as dental caries by investigating the destruction of hydroxyapatite. However, the fluorescent properties of hydroxyapatite may disrupt the bacterial characterization using fluorescent-based microbial labeling techniques.

7-2 ARTIFICIAL TOOTH DESIGN AND OPERATION

Device design and fabrication

The design layout of the artificial tooth chip shown in Fig. 7.3a was based on the microfluidic elements proposed for long term culture, including the oxygenator, water jacket and alternative flow adjacent to the ~165 pL culture chamber (diameter: 1 mm, height: 210 μm). A micropump consisting of three microvalves (area: 100 μm (W) × 150 μm (L)) was placed over the outlet channel (height: 10 μm) to drive liquid flow with a switching time of 100 ms (flowrate: 30 pL/min). Furthermore, a multiplexor was used to gate the flowing liquid by opening one of the eight inlet channels (1 to 8), which were used for flushing the channels and chamber with DI water, PBS and other solutions for microbial analysis. An additional flow channel was specifically designed for culture medium. The structure of artificial tooth chip was similar to the oxygenator for long-term culture as mentioned in section 6-4, except that six microvalves were embedded around the culture chamber for
mixing after every liquid insertion. (Details of the mixer design are described in the next section.) The mixer was fabricated between the water jacket and chamber as illustrated in a layered view of the simplified device structure (Fig. 7.3b).

Figure 7.3. (a) Design layout of the artificial tooth chip. The legend box indicates the fluidic layers and their corresponding position from top to bottom. (b) The basic structure of the culture region contained a culture chamber (blue) with an embedded peristaltic mixer (red). The oxygen and humidity conditions were regulated by the gas layer (green) and water jacket (gray), respectively. (c) A photograph of the fully assembled device (22 mm (l) x 18 mm (w) x 5 mm (h)).

For device fabrication, six molds were prepared corresponding to the stack shown in the legend box in Fig. 7.3a before soft lithography of PDMS. The mold patterns of the gas control layer (scaled by 101.7%), water jacket, control and chamber layers were fabricated by photolithography using SU-8 10 (Microchem) as the mold substrate on 3” silicon wafers.
(Jame River Semiconductor) (SU-8 thickness \(h\) \(\sim\) 200 \(\mu\)m for chamber layer and \(\sim\) 20 \(\mu\)m for all other layers). The flow layer mold was fabricated with 10 \(\mu\)m thick AZ4620 photoresist (AZ Electronic Materials) patterned on a 3" silicon wafer, and was subsequently reflowed after microchannel pattern exposure and development at 150 °C on a hotplate for 1 min to smoothen the structure profiles for valve operation. The gas layer contained flow channels to both support valve operation and regulate the volumetric ratio between oxygen and nitrogen, and was fabricated using a two layer process. AZ4620 photoresist (\(h\) \(\sim\) 10 \(\mu\)m) was first patterned and reflowed on a silicon wafer (light green regions in Fig. 7.3a), followed by a hard bake for \(\sim\) 130 °C for >30 hr to promote adhesion to the wafer. SU-8 (\(h\) \(\sim\) 20 \(\mu\)m) structures were then patterned (dark green regions in Fig. 7.3a) on the AZ photoresist layer. After mold silanization, a multilayer soft lithography process with 10:1 A:B two-part PDMS (Sylgard 184, Dow Corning) was utilized to fabricate channel structures with different substrate thicknesses for gas control (\(\sim\) 5 mm), gas (\(\sim\) 30 \(\mu\)m), water jacket (\(\sim\) 60 \(\mu\)m), control (\(\sim\) 300 \(\mu\)m) and flow (\(\sim\) 20 \(\mu\)m) layers. The corresponding fabrication sequence is indicated in legend box (layers 1 to 5) in Fig. 7.3a. The chamber layer was fabricated by molding chamber structures with 400 \(\mu\)m thick PDMS, bonding the PDMS substrate on a glass coverslip by oxygen plasma with the structure side facing up, and depositing polystyrene layers into chamber cavities with the method described in the previous section. The composite fabricated artificial tooth chip, shown in Fig. 7.3c, was finished by bonding the multilayer PDMS stack (layers 1 to 5) and the chamber substrate using oxygen plasma.

Culture preparation and implementation

Several solutions, including purified saliva, cultured bacteria and culture medium, were prepared prior to the chip operation. Saliva extracted from human cavity was filtered by the filtration bottle as aforementioned in section 7-1, while the bacteria sample was prepared by human saliva extract diluted with filtered saliva to a cell density of \(\sim\) 1 \(\times\) 10^5 cells/mL. Basal medium mucin (BMM)\(^{12-13}\), which is a nutritional analogue of saliva and a proper pH buffer (pH 6.4 ± 0.1)\(^{13}\), was formulated according to the basal medium recipe of Glenister et al.\(^{14}\). It was prepared by autoclaving the solution containing 5 g/L trypticase peptone (Cat# 211921, BD), 10 g/L proteose peptone (Cat# 61001-520, VWR), 5 g/L yeast extract (Cat# 212750, BD), 2.5 g/L potassium chloride (Cat# 6858, Mallinckrodt Chemicals), 5 mg/L hemin (Cat# H5533-10G, Sigma-Aldrich), 1 mg/L menadione (MP Biomedicals), 2.5 g/L gastric mucin (Cat# M32610, Pfaltz & Bauer), 1 mmol/L urea (Cat# 8642-12, Mallinckrodt Chemicals) and 1 mmol/L L-arginine (Cat# 1.01542.0100, EMD Chemicals).

To prepare the culture experiment, the artificial tooth chip was first sterilized by baking at 100 °C (\(t\) > 10 hr) and UV-exposure (\(t\) > 2 hr). The water jacket and all control and flow channels were initially primed with pressurized DI water (\(\sim\) 3 psig head pressure). Medium, DI water and PBS were then inserted into the medium inlet, and solution inlets 1 and 3, respectively. Solution inlets 3 - 8 were reserved for further applications, such as supplementary nutrients to modify the culture condition, and chemicals for the analysis of cell characteristics. The control and flow inlet channels were operated under continuous flow (\(\sim\) 2 psig) for the duration culture experiment to eliminate the bubble generation due to minor evaporation. Afterwards, the artificial tooth device was placed on a microscope
(Olympus IX71), which had been fitted with a Weather Station cell culture chamber (Precision Control LLC) to provide a stable operating temperature of 37 °C. Oxygen and nitrogen, which were humidified by pre-flowing the gasses through water reservoirs, were connected to the gas inlets. In the preparation stage, the valve for oxygen was initially closed to generate anaerobic microenvironment (pure nitrogen) in within the culture chamber.

Prior to cell culture, the chamber was filled with human saliva for 30 min in order to form a glycoprotein-rich pellicle layer on the polystyrene substrate. After replacing saliva with BMM, dental bacteria sample was inserted into half of the chamber using the peristaltic micropump (operation time: ~2 min 45 s). All liquid insertion operations were implemented with the procedures using an alternative flow channel described in section 6-3. Peristaltic mixing with the integrated microvalves was then carried out for 30 min to obtain an even cell density within the culture chamber. Afterwards, the chamber was isolated for 15 min to allow initial cell attachment.

During biofilm culture, the microbial environment should be maintained with constant temperature and regular inflow of fresh medium (1 – 100 nL./min). The control valves for gases enable switching of the DO level between aerobic (8.5 ppm) and anaerobic (0 ppm) to mimic the gas environment in oral cavity during daytime and nighttime, respectively. The durations of aerobic and anaerobic conditions were set as 20 hr and 4 hr, respectively. (The ratio of the aerobic/anaerobic exposure conditions is based on the polymicrobial composition in the media, and will be discussed in more depth in section 8-5.) Since the overall salivary flow rate in human oral cavity (volume: ~100 mL) is ~0.22 mL/min\(^{15}\), the average medium flow rate in the microchamber should be set as ~0.363 pL/min. Moreover, the shear of medium flow should be <5 mPa. In the culture operation, BMM was pumped into the chamber for 30 s and mixed for 1 min in every 30 min, corresponding to average flow rate 0.5 pL/min and maximum shear on the biofilm of ~3 mPa. Afterwards, the growth and viability of the overall culture was assessed using BacLight Live/Dead stain and 4',6-diamidino-2-phenylindole (DAPI). (The detailed protocols are described in sections 7-4 and 7-5.) Identification of the specific species in dental biofilm was achieved by FISH with oligonucleotide probes\(^{16,17}\). The proposed artificial tooth device is capable of assessing colonization density, composition, morphology and spatial arrangement of the species in the communities as the biofilm matures.

7-3 PERISTALTIC MIXER IN CULTURE CHAMBER

Mixer design and operation

Under continuous culture operation, medium is regularly inserted into the culture chamber to partially refresh the nutrients. Without mixing, the addition of new medium through the chamber inlet sets up a concentration gradient, leading to undesirable differential growth rates of the bacteria in the chamber (heavier near the inlet). Therefore, designing a mixer to enhance the rate of medium transfer is important to avoid clogging the chamber inlet and improve the consistency of culture conditions (e.g. nutrient and cells...
concentrations) over the culture region. A culture example without mixing in the chamber (Fig. 7.4) shows a dense population of cells near inlet, which eventually clogged the inflow of medium after two days of culture.

Figure 7.4. Distribution of dental bacteria in a microchamber with medium replacement, but no mixing.

A peristaltic mixer composed of six PDMS membranes fabricated around the culture chamber was developed to enhance the nutrients transfer after each medium insertion operation. The membranes were actuated as two peristaltic pumps to generate flow inside chamber. The symmetry of flow was eliminated by switching the grouping of membranes with three different stages iteratively in different flow directions as illustrated in Fig. 7.5a-c. For culture implementation, the mixing operation was applied immediately after each medium insertion for three minutes, with 30 s for every stage and a pump actuation rate of 100 ms. A testing device with the mixer located around a 20 μm tall chamber was developed to verify the feasibility of this mixing scheme. In the verification experiment, a small amount of blue dye was initially inserted near the chamber inlet (Fig. 7.5d). As the mixer was operated, the pattern of dye started to change (a snapshot at t = 5 s is shown in Fig. 7.5e), indicating that the two peristaltic pumps could induce a flow with two symmetric circulations inside chamber. The active mixing operation not only induces a more consistent concentration of nutrients, but also more even initial bacteria distribution if it is applied right after bacteria insertion. However, different from latching valve operation, the membranes should not contact with the bottom chamber surface as the direct compression could induce high stress and mechanical damage on cells. In the actual design of artificial tooth chip, the chamber height (200 μm) was set to be larger than the possible membrane deflection (~50 μm) to avoid direct contact with the biofilm.
Figure 7.5. Operation of mixer prototype with (a) – (c) three different grouping stages. The flow actuation in each stage generated two symmetric circulations within chamber indicated by comparing (d) the initial color pattern with (e) the one after 5 s of mixing.

Integrating the mixer in the artificial tooth chip, dental bacteria grew evenly within the culture region. Snapshots of the bacteria distribution (Fig. 7.6) show growth of the cell population from 24 hr to 48 hr. With the oxygenated gas environment, regular medium injection and medium mixing by the embedded mixer, the biofilm could grow continuously in the culture chamber, enabling downstream evaluation of the biofilm growth characteristics as a function of temporal parameters that include DO levels and nutrient composition.

Simulation of membrane deflection

Computational simulation on the mixing operation was performed to investigate the characteristics of mixer in terms of the flow velocity, hydraulic pressure and induced shear on cells. To simplify the computation complexity, the model was decomposed into two computation steps: (1) determination of the membrane deflection using a simplified lumped-element model, and (2) simulation of the flow profile in the fluid domain applying the estimated membrane deflections as moving boundaries.
Figure 7.6. Snapshots of in-situ dental biofilm over two days of culture at time (a) 24 hr, (b) 32 hr, (c) 40 hr and (d) 48 hr. The artificial tooth device performed mixing operations with the embedded peristaltic mixer.

Considering the operations in each stage described in Fig. 7.5, the symmetrical nature of the induced flow enabled further simplification of the model to a half chamber with three valves (Fig. 7.7a). The major effects involved in the valve movement are the membrane stiffness of valves and the fluidic resistance along control lines. Since the chamber is closed during mixing, there is no net inflow and outflow. The simplified lumped element model can then be modeled as an equivalent electrical circuit as described in Fig. 7.7b. In the circuit model, membrane stiffness is represented by capacitance ($C_1, C_2$, and $C_3$); fluidic resistance is represented by electrical resistance ($R_1, R_2$, and $R_3$); pressure change is represented by the voltage difference ($V_1 - V_6$); fluidic flow rate is represented by electrical current ($i_1 - i_3$); and driving pressure is represented by input voltage ($P_1, P_2$, and $P_3$). The absence of net flow across the chamber boundaries (including inlets and outlets) results in zero overall membrane deflection. This enables the valves to be modeled with a common node connecting all capacitors per Kirchhoff's current law (Eq. 7.1).
Figure 7.7. The operation of mixer has been simplified as (a) a half-chamber model expressed with (b) an equivalent electrical circuit.

Therefore, the simplified governing equations are

\[ i_1 + i_2 + i_3 = 0, \quad \text{(7.1)} \]
\[ P_1 - P_3 = v_1 + v_4 - v_3 - v_6, \quad \text{(7.2)} \]
\[ P_2 - P_3 = v_2 + v_5 - v_3 - v_6, \quad \text{(7.3)} \]
\[ v_j = R_j i_j \quad \text{for } j = 1, 2, 3, \quad \text{(7.4)} \]
\[ C_j \frac{dv_j}{dt} = i_j \quad \text{for } j = 1, 2, 3. \quad \text{(7.5)} \]

Eqs. 7.1-7.3 can be express as the state space form:

\[
\begin{bmatrix}
\dot{v}_1 \\
\dot{v}_2 \\
\dot{v}_3 
\end{bmatrix} =
\begin{bmatrix}
\frac{-R_3 C_1}{R_1} & \frac{1}{R_1} & 0 \\
\frac{-R_3 C_2}{R_2} & \frac{-1}{R_2} & \frac{1}{R_2} \\
C_1 & C_2 & C_3 
\end{bmatrix}
\begin{bmatrix}
\dot{v}_1 \\
\dot{v}_2 \\
\dot{v}_3 
\end{bmatrix}
= 
\begin{bmatrix}
-1/R_1 & 0 & 1/R_2 \\
0 & -1/R_2 & 1/R_2 \\
0 & 0 & 1/R_2
\end{bmatrix}
\begin{bmatrix}
v_1 \\
v_2 \\
v_3
\end{bmatrix} + 
\begin{bmatrix}
1/R_1 & 0 & -1/R_1 \\
0 & 1/R_2 & -1/R_2 \\
0 & 0 & 0
\end{bmatrix}
\begin{bmatrix}
P_1 \\
P_2 \\
P_3
\end{bmatrix} \quad \text{(7.6)}
\]

The driving pressure \((P_1, P_2, \text{and } P_3)\) should be applied as peristaltic pumping and the transient pressure inputs for the three membranes are plotted as Fig. 7.8 (over 2 pumping cycles).
Figure 7.8. Driving pressures and duty cycles for the microvalves in the embedded mixer over two pumping cycles.

The fluidic resistances along control lines were calculated using Eq. 3.13, while the membrane stiffness was approximated using plate equations. Because of the low aspect ratio ($h/w < 0.1$) of the PDMS membrane, it is reasonable to model the rectangular valve as a thin plate (Eq. 6.5) with negligible thermal edge loads under a uniform normal pressure $P_\zeta$. When the membrane has limited displacement, the in-plane displacement can be neglected. The membrane deflection $w(x, y)$ in Cartesian coordinates is expressed as

$$\frac{E_{pdms}H^3}{12(1-\nu_{pdms}^2)} \left( \frac{\partial^4 w}{\partial x^4} + \frac{\partial^4 w}{\partial y^4} \right) = P_\zeta.$$ (7.7)

The boundary conditions for a simply supported plate with width $W$ and length $L$ are

$$w(x, y) = 0 \quad \text{at } x = 0, x = W, y = 0 \text{ and } y = L,$$ (7.8)

$$\frac{\partial^2 w}{\partial x^2} = 0 \quad \text{at } x = 0 \text{ and } x = W,$$ (7.9)

$$\frac{\partial^2 w}{\partial y^2} = 0 \quad \text{at } y = 0 \text{ and } y = L.$$ (7.10)

Solving Eq. 7.7 using the above boundary conditions, the membrane deflection can be calculated as

$$w(x, y) = \sum_{m=1,3,...} \sum_{n=1,3,...} 192(1-\nu^2) \sin(m\pi x / L) \sin(n\pi y / W) \frac{P_\zeta}{mn\pi^2EH^3[(m\pi / L)^2 + (n\pi / W)^2]}.$$ (7.11)

Integrating the above equation over the valve area to obtain the displaced volume $Q$, we can estimated the membrane stiffness $C$ as

$$Q = C \frac{dv}{dt},$$ (7.12)

$$C = \sum_{m=1,3,...} \sum_{n=1,3,...} \frac{768LW(1-\nu^2)}{m^2 n^2 \pi^4 EH^3[(m\pi / L)^2 + (n\pi / W)^2]}.$$ (7.13)
The pressure difference across membranes (Fig. 7.9a) was computed using Eq. 7.6 with the driving pressure described in Fig. 7.8 and zero initial membrane deflection. It was extracted with one pumping cycles at the periodic developed state after a long simulation time (i.e. 1.5 – 1.8 s as highlighted in Fig. 7.9a) to eliminate the error caused by the unrealistic initial states. The mixing performance highly depends on the flow driven by membrane movement. Rapid valve operation (i.e. 100 ms) produces maximal fluid displacement and efficient mixing, with sharp actuation pressure transitions, while, with longer switching times (e.g. 500 ms as shown in Fig. 7.9b), the mixing efficiency is greatly reduced.

![Figure 7.9. Pressure across membranes for the mixing operation with valve switching times of (a) 100 ms and (b) 500 ms.](image)

**Simulation of medium flow**

The simulation of fluid flow during mixing was performed by commercial finite element method based software (COMSOL 3.5). The simulation was set up as a pure fluid flow problem with isolated chamber geometry and 3D moving boundaries without net fluid inflow/outflow. The extracted pressures ($v_1$, $v_2$, and $v_3$) and rates of change of pressure ($\dot{v}_1$, $\dot{v}_2$, and $\dot{v}_3$) from the aforementioned circuit model (Fig. 7.9a) were transformed into the deflections and velocities of membranes (Eq. 7.11), respectively, with different positions and orientations on chamber. The maximal membrane deflections (<50 μm) defined the moving boundaries of the chamber and the computational mesh geometry as shown in Fig. 7.10a.
Figure 7.10. (a) Simulated dynamic valve deflections, and the corresponding (b) flow profile, (c) hydraulic pressure, and (d) shear along bottom chamber surface in the fluid domain.
Under the predefined membrane deflection, the fluid flow in chamber was computed by numerically solving the three-dimensional Navier-Stokes equations (Fig. 7.10b). The simulation was performed for 1.8 s (six pumping cycles) to obtain a stabilized periodic solution. The flow profiles, hydraulic pressures and shear at time \( T_1 = 1.5 \) s, \( T_2 = 1.6 \) s and \( T_3 = 1.7 \) s (for extreme membrane deflections) were selected as illustrated in Fig. 7.10. The results indicated the maximum flow velocity during mixing was \( \sim 90 \) \( \mu \)m/s and the difference in hydraulic pressure in chamber was \(< 0.2 \) Pa (Fig. 7.10c). This difference in hydraulic pressure was much smaller than the changes of membrane pressures \( (v_1, v_2 \text{ and } v_3 \sim 8 \) Pa), verifying that the shapes of deformed membranes obtained by Eq. 7.11 were reasonable approximations. Additionally, another major concern in the mixing operation was that the shear generated on biofilm should be \(< 5 \) mPa. The calculated fluid velocities, \( u(x, y, z) \) and \( v(x, y, z) \), were sampled with Cartesian grids \((\Delta x = 10 \) \( \mu \)m, and \( \Delta z = 1 \) \( \mu \)m). Then, the shear over the bottom chamber surface \( \tau(x, y, z, \tau) \bigg|_{\tau=0} \) was estimated by

\[
\tau(x, y, z, \tau) \bigg|_{\tau=0} \approx \mu \left( \frac{u(x, y, 0, \tau)^2 + v(x, y, 0, \tau)^2}{\Delta z} \right).
\] (7.14)

The calculation indicated that the mixing operation would generate only negligible shear on biofilm (maximum shear: \( \sim 4.8 \) mPa). Hence, the mixing operation would not induce significant mechanical disruption to the biofilm formation.

**Mixing of liquid and particles**

Experiments were performed to examine the required mixing time for medium and bacteria. Red dye, analogous to culture medium, and fluorescent beads, which are of comparable size \( (d \sim 0.5 \) \( \mu \)m) to dental bacteria, were separately inserted in mixing chambers to investigate their distributions under a continuous mixing operation as shown in Figs. 7.11b and 7.12b, respectively. Control experiments without mixing (Figs. 7.11a and 7.12a) were also conducted to show the properties of passive mixing by pure diffusion.

The dye results (Fig. 7.11) showed that the embedded peristaltic mixer could reduce the diffusion time of dye from \( \sim 5 - 8 \) min to \( \sim 45 \) s. During mixing, transfer of the dye was driven by diffusion as well as the flow induced by valve actuation \((Pe \sim 0.18 - 1.8)\). Considering the similar diffusivity coefficients of dye and nutrients in water \((\sim 10^{-10} - 10^{-11} \text{ m}^2/\text{s})\), analogous performance in anticipated for culture medium mixing. In contrast, diffusion of the fluorescent bead sample by passive diffusion was very slow as expected (Fig. 7.12a), due to the small Brownian diffusivity of beads in water \( D_{\text{bead}} \sim 4.4 \times 10^{-13} \text{ Ns/m}^2 \), described by the Stokes-Einstein equation,

\[
D_{\text{bead}} = \frac{k_B T}{6 \pi \mu R_{\text{bead}}},
\] (7.15)

where \( k_B = 1.38 \times 10^{-23} \text{ JK}^{-1} \) is Boltzmann constant, \( T \sim 300 \) K is temperature, \( \mu \) is viscosity of water and \( R_{\text{bead}} \) is radius of fluorescent bead.

Fig. 7.12b shows that the active mixing operation greatly improved the redistribution of the beads in the chamber, driven by the flow generated by the peristaltic valves \((Pe \sim 40)\). Based
on the required mixing time for dye and beads, the artificial tooth device was set with
peristaltic mixing for 30 min after bacteria insertion and 1 min after every medium
replacement as described in section 7-2.

![Image of mixing times and dye distribution](image)

Figure 7.11. Distribution of red dye in chamber at different times (a) without and (b) with mixing operation.

Figure 7.12. Distribution of fluorescent beads in chamber at different times (a) without and (b) with mixing operation.

7-4 GROWTH CHARACTERISTICS

**Dental biofilm thickness**

The dental biofilm cultured in artificial tooth chip over one week indicated a reasonable
biofilm thickness (~100 μm as shown in Fig. 7.13), comparable to previously published in
vitro biofilm platforms (thickness: ~3518 - 300 μm19). After culture, 4',6-diamidino-2-
phenylindole (DAPI) stain was applied in the chamber (concentration: 500 nM in PBS) for 30 min and flushed by DI water. The blue-fluorescent DAPI nucleic acid stain binds to double-stranded DNA or RNA, and is a good marker for non-specific cell fluorescence-based cell visualization. A stack of fluorescent microscopic images were then taken at the upper surface of biofilms (±40 μm) and processed with image deconvolution using a commercial software (Huygens Deconvolution Software, Scientific Volume Imaging) to extract slices of image features from burry images caused by the overlapping of capture intensity over the depth of field of the microscope. For each of the culture durations (1, 2, 3, 5 and 7 days), five to ten biofilms were selected for the imaging and estimation of biofilm thicknesses. The biofilm culture under two daily gaseous microenvironments (20/4 hr of aerobic and aerobic/anaerobic) had a similar incremental growth profile, possibly due to the compensated net cell growth of aerobes and anaerobes (longer duration of aerobic environment induced faster cell growth of aerobes but, at the same time, slower growth of anaerobes, and vice versa).

![Biofilm Thickness Over Time](image.png)

*Figure 7.13. Change in biofilm thickness over one week of culture experiments under two different daily gas conditions. The error bars represent the standard deviation of each measurement.*

**Distribution of viable and dead cells**

After ~60 hr of dental biofilm culture, the distribution of vital and dead cells in dental biofilms was measured per the protocol described in the previous sub-section, except that fluorescent-based BacLight LIVE/DEAD stains (L7012, Invitrogen), instead of DAPI, were applied in the chamber. The stains were composed of SYTO 9 green-fluorescent nucleic acid stain and red-fluorescent propidium iodide for the detection of living and dead cells, respectively. SYTO 9 generally labels all bacteria, while propidium iodide penetrated only bacteria with damaged membranes and reduced the intensity of SYTO 9 stain fluorescence.
In the implementation, the cultures were incubated for 30 min in 20 nM of SYTO 9 dye and 120 nM of Propidium iodide in 1x PBS. After staining, image slices were captures (±40 μm from the biofilm top surface). Sample image slices for a dental biofilm (thickness: ~40 – 50 μm) after the deconvolution processing are shown in Fig. 7.14. Inspection of the images showed that dead bacteria were typical located in the inner part of biofilm, which was not surprising as it represented the oldest region of the community.

![Image slices with z = 0 μm, 6 μm, 12 μm, 20 μm, 30 μm, and 40 μm](image)

*Figure 7.14. Sample image slices of viable (green) and dead (red) cells in dental biofilm at different vertical (z) locations: 0, 6, 12, 20, 30 and 40 μm.*

The surface densities of bacteria along different vertical (z) positions (Fig. 7.15a) showed the lower section of dental biofilm contained higher cell density than the upper section. Because bacteria grew amorphously over biofilm surface, the lower biofilm section was developed for longer time and the surface coverage was usually higher. For the same reason, the ratio of viable to dead cells (Fig. 7.15b) was lower in the lower biofilm section. The top surface of the biofilm (z = 40 – 48 μm) had a much higher viability ratio, representative of active growth and proliferation on the established biofilm scaffold.

7-5 FLUORESCENCE IN SITU HYBRIDIZATION ON A CHIP

The previous section has described the analysis techniques for the basic properties of biofilm, including the growth rate and the distribution of live/dead cells. Of greater interest is the understanding of how these biofilms self-assemble from dozens to hundreds of bacteria with very different nutrient and oxygen requirements. For example, a typical dental
biofilm consists of more than 500 different bacteria. As a first step to understanding the physiological interactions among oral bacteria in a biofilm, this section describes work undertaken using fluorescence in situ hybridization (FISH) to visualize the co-colonization dynamics of a model mixed biofilm.

![Graph showing cell surface densities of viable and dead cells and their ratios in different vertical layers.](image)

**Figure 7.15.** (a) Cell surface densities of viable and dead cells and (b) their ratios in different vertical layers.

FISH with rRNA-targeted probes is a cytogenetic technique developed to detect the presence of particular DNA sequences. Such direct genetic detection method requires no cultivation of cells and so the identification results are independent of the viability of cells. Because of its robustness, this method has been widely used to identify the existence of specific bacterium in various research areas, including single cell analysis, environmental microbiology, clinical research, and microbial ecology. FISH has also been used to visualize the bacteria on tooth surface. Applying fluorescent oligonucleotide probes with hybridization, the signal-to-noise ratio of fluorescent can be highly improved since the probes do not denature during hybridization as the nucleic acid probes, and a much higher molar concentration of probes will not induce the background intensity to an unacceptable
Recently, a microfluidic chip compatible with FISH was developed for the chromosome enumeration of the purified peripheral blood mononuclear cells. This has also demonstrated the potential of FISH using microfluidic technology in the microbiological applications.

**Implementation of FISH using artificial tooth chip**

Each of the three major steps (cell fixation, hybridization with probes and washing) in the typical FISH protocol was implemented with the artificial tooth chip with a few modifications. Following three days of the chip culture at 37 °C with partially aerobic condition (20 hr aerobic and 4 hr anaerobic gas supply per day), the temperature of the Weather Station was set to 20 °C. The culture medium in chamber was first flushed by flowing PBS (inlet pressure: 3 psi) along the chamber for 10 min to remove residual nutrients and unattached bacteria. Cell fixation on dental biofilm was performed with 4 % w/w paraforaldehyde (Sigma) in PBS at flow rate 30 pL/min for >4 hr at 20 °C. (Ethanol was not used for cell fixation because it had a very different surface tension from water.) The biofilm was washed again by PBS for 10 min. 1 % w/w lysozyme solution (Sigma) with 0.5 % EDTA (BDH314-1, VWR) and 0.01 M Tris/HCL was then applied (inlet pressure: 3 psig) for 30 min to permeabilize dental bacterial cells. The typical dehydration step using ethanol was skipped due to the surface tension issue. Adjusting temperature to 42 °C, the specimen was then incubated with the regular supply (90 pL with mixing for every 30 min) of >20 μM HPLC-purified oligonucleotide probes (Operon) in hybridization buffer (AM663, Ambion) for 24 hr. The detailed 5'-end-labeling for various target species with the corresponding fluorochromes chosen with minor spectral overlap between probes are listed in Table 7.1. Excessive probes were removed by incubating the specimen in the wash buffer containing 90 % distilled deionized water (25-055-CM, Mediatech), 10 % 20X SSC (AM9763, Ambion) and 0.5 % SDS (AM9820, Ambion) periodically (90 pL with mixing for every 30 min) for 3 hr. Afterwards, DAPI (concentration: 500 nM in PBS) was applied in the chamber for 15 min to visualize the entire biofilm. Finally, DAPI was replaced by DNA/RNA free water to reduce the background fluorescence prior to visualization.

**Table 7.1. Oligonucleotide probes for identification of species with different sequences and 5'-modifications**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Target</th>
<th>5′-3′ Sequence</th>
<th>5'-modification (λexcitation, λemission) [nm]</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>STR 405</td>
<td>Streptococcus spp.</td>
<td>TGGCCGTCCCTTTCGTT</td>
<td>Fluorescein (494, 521)</td>
<td>31</td>
</tr>
<tr>
<td>FUS664</td>
<td>F. nucleatum</td>
<td>CTTGAGTTTCG/C/TGCATCC</td>
<td>Texas Red (589, 615)</td>
<td>31</td>
</tr>
<tr>
<td>IF 201</td>
<td>A. naeslundii</td>
<td>GCTACCCGCTAACCCATT</td>
<td>Cy 5.5 (675, 694)</td>
<td>31</td>
</tr>
<tr>
<td>POGI</td>
<td>P. gingivalis</td>
<td>CAATACTCGTATCGCGGTTATT</td>
<td>IRD 800 (780, 816)</td>
<td>32, 35</td>
</tr>
</tbody>
</table>

A sample set of fluorescent images using DAPI and FISH oligonucleotide probes for four target bacteria is shown in Fig. 7.16. (Other image sets are also available in Appendix B.) The bacteria (Streptococci, Fusobacterium nucleatum, Actinomyces naeslundii and Porphyromonas gingivalis as shown in Fig. 7.16b-e, respectively) were selected based on their importance in...
dental biofilm development. *Streptococci* are the key species involved in the early colonization to support the attachment of biofilm to chamber substrate or tooth surface. *A. naeslundii* is another typical early colonizer that can attach to pellicle formed on saliva treated surfaces. *F. nucleatum* acts as the most important bridge organism because it can coaggregate with most of the both early and late colonizers. The late colonizer *P. gingivalis* it was also selected as it is the major pathogen causing periodontal diseases. In the experimental results, the existence of all the target bacteria in the *in vitro* biofilm demonstrated that the artificial tooth device and culture protocol could successfully achieve the co-culture of various bacteria species including aerobes and anaerobes. The results (from ten independent culture as presented in Appendix B) also indicated reasonable ratios of *Streptococci*, *F. nucleatum* and *A. naeslundii* (~4 : 1.6 : 0.9, respectively) comparing to the *in vivo* biofilm reported in ref. 31 (bacteria ratio: ~4.2 : 1.6 : 1). The selected bacteria covered >50% of the total cell population as in dental plaque, so this identification technique using FISH with oligonucleotide probes could reflect the properties of multi-species biofilm to a certain extent.

![Sample result on bacterial identification](image)

**Figure 7.16.** Sample result on bacterial identification. (a) The entire cell community was stained by DAPI, while the bacteria species (b) *Streptococci*, (c) *F. nucleatum*, (d) *A. naeslundii* and (e) *P. gingivalis* were identified by the corresponding FISH probes.
In summary, a single chamber artificial tooth device was successfully developed for the on-chip dental biofilm culture and analysis applications. The results on cluster thickness, distribution of cell viability, and proportion of the key dental bacteria have indicated that the culture procedures can obtain the in vitro dental biofilm with similar properties as the in vivo case. With the foundation established to culture and analyze to moderately complex biofilms in a self-contained microfluidic platform, the next intuitive steps to incorporate the scalable architecture into a multiplexed platform to look at many culture samples subjected to different environmental factors, for example. To reduce the extensive experimental efforts for such multiple cultures, the design of artificial tooth device has been further extended to a high-throughput microfluidic device containing multiple culture chambers, as described in the next chapter.

REFERENCES

CHAPTER EIGHT

PARALLEL ARTIFICIAL TEETH ON A CHIP

8-1 MICROFLUIDIC VIA-HOLES FOR MICROCHANNEL NETWORKS

Microfluidic via-holes for fluidic connection

High-throughput operation is one of the major objectives of microfluidics technology development\(^1\),\(^2\),\(^3\). Miniaturizing fluidic devices from microliters to picoliters in a multiplexed format has the potential to enable the parallel analysis of hundreds to thousands of biological samples while reducing reagent consumption. Microfluidic part fabrication based on soft lithography enables complicated and extremely compact device designs. In the fabrication process, the photolithography for mold fabrication and PDMS molding using soft lithography is a standardized process for any arbitrary 2D microfluidic network. Building stacked 2D layers through multilayer soft lithography principally relies on simple bonding of the molded layers, rather than the geometry of channel structures. Furthermore, the relative manufacturing efforts on a microfluidic chip line unit can be greatly reduced if a microfluidic chip is designed with a “unit” architecture, in which channel networks or valves can be operated in different configurations. Having flexible device architecture is particularly important for cell-based assays, which have relatively large undetermined variations and require multiple repeated experiments to statistically validate experimental hypotheses.

With a well-designed channel layout, many of the solution inlets/outlets and valve control ports can be shared among multiple device elements. Through design optimization, the number of required fluidic connections can be minimized, reducing off-chip work that includes solution preparation and connecting input (flow and control) lines into the devices. The risk of chip failure increases with the number of inputs, providing a strong motivation to put additional effort into channel routing and multiplexing strategies instead of making chips by a brute force, trial and error approach. However, routing of microchannel networks, particularly for high density co-planar designs, is non-trivial.

Considering the analogy between the channels in microfluidic devices and wires in electronics, the concept of 3-D routing using vias is an interesting strategy that can potentially solve some of the layout problems encountered in 2-D microfluidic networks. Electrical via-holes are placed between two layers of circuit patterns in electrical printed circuit boards, while fluidic via-holes can also be fabricated between two flow layers of flow
channels to provide fluidic connection in microfluidic devices. The example shown in Fig. 8.1a describes the typical usage of microfluidic via-holes in device design. In case there exists a microchannel (A) blocking the connection between two flow lines (B1 and B2), a bridging flow channel (black), which connects to the two flow lines with via-holes (red), can be fabricated in another flow layer across the blocking channel. Therefore, flow line routing of the microchannel networks can be achieved by applying via-holes to connect the blocked flow lines with bridging channels. The implementation of vias into microfluidic devices enables higher-density layouts of microfluidic circuits, which is particularly appealing for high-throughput screening applications.

![Diagram](image)

**Figure. 8.1.** (a) Example of the bridging channel design. (b) Fabrication of microfluidic via-holes on a PDMS layer.

To fabricate the microfluidic via structures, a modified PDMS fabrication process was implemented for the double-layer flow structures connected with the microfluidic via-holes. Fabrication of the upper reflowed channel layer (channel height: 10 μm) was achieved by conventional soft lithography, while a separate lower layer was fabricated using a special technique for the three-dimensional microfluidic channels. A mold for the lower flow layer was first prepared by patterning two SU-8 layers (with the 100 μm tall via-holes (red rectangles in Fig. 8.1b) on the 20 μm thick fluidic channel structures (white layer in Fig. 8.1b)) on a 3” silicon wafer. Following mold silanization, a layer of 10:1 two-part PDMS (thickness: ∼150 μm) was spin-coated on the mold. Two 5 mm thick Poly-methyl methacrylate (PMMA) sheets and two transparency films (PP2500, 3M) were then placed above and below the mold in the configuration shown in Fig. 8.1b. The stack of materials was then clamped and baked in an oven at 80 °C for >10 hr. After removing the PMMA sheets and transparency films, the SU-8/PDMS surface of the mold was aligned and bonded with the PDMS substrate with upper reflowed channel structures using an oxygen plasma machine.
blade was used to cutting along the device boundaries (red dotted lines in Fig. 8.1b). Afterwards, the PDMS substrate containing via-holes was peeled off from the mold and bonded to the remaining part of the device to finish the fabrication process.

**Prototype device for parallel differential solution mixing**

A prototype device including the via-hole structures was developed for the testing of a parallel mixing operation. The design was optimized as a parallel channel network, enabling the combination of a matrix of different nutrients/chemicals. The device layout (Fig. 8.2a) contained an array of $4 \times 4$ chambers (diameter: 600 μm), whose fluid flow was driven simultaneously by a shared peristaltic micropump. Each chamber was connected to a “row” solution inlet and a “column” inlet, determined by its corresponding row ($i$) – column ($j$) index in the chamber array. The parallel mixing device (Fig. 8.2b) consisted of control (thickness 20 μm), upper (thickness: 10 μm) and lower flow layers (100 μm tall via-holes on 20 μm thick microchannels), as indicated in the legend box in Fig. 8.2. The two upper layers were fabricated by multilayer soft lithography, while the lower flow layer and fluidic via-holes were fabricated by the aforementioned method for three-dimensional channel structures. Because each chamber was linking to two solution inlets from top and bottom layers, the characteristic length of diffusion was half of the channel height (~5 μm) that the mixing can be achieved along the mixing region indicated in Fig. 8.2b.

A preliminary experiment, applying color dyes to different solution inlets, was performed to examine the parallel mixing operation of the device. The flow of dye solutions exhibited perfect fluidic connections across the via-holes. Because of the sequence of colors from solution inlets (red, transparent, yellow and blue for both row and column inlets 1 to 4, respectively), a chamber with row – column index ($i, j$) should have a similar color as another chamber with the skewed index ($j, i$). However, dye mixtures in the chambers (Fig. 8.2c) had significant mismatch on color intensity. The inconsistency in mixture ratios indicated by such color mismatch was probably caused by the minor errors in channel dimensions and fluidic resistance, especially when the folded channel structures were absent. This result suggests that the implementation of parallel microfluidic operations is challenging, and would benefit from more optimization studies. On the other hand, the serial operation schemes often obtain more robust device performance because the fluid is driven along a unique flow path with a defined flow rate determined by the peristaltic micropump.
8-2 DEVICE DESIGN AND OPERATION

32-chamber artificial teeth

The artificial tooth chip design presented in the previous chapter has been further extended to a 32-chamber parallel culture device, by duplicating the artificial tooth unit as an array of 4 (row) × 8 (column) culture chambers deposited with polystyrene as shown in Fig. 8.3a. Each row of chambers shared the identical gas condition regulated by an oxygenator. The corresponding gas mixer channels were similar to the single chamber device, providing either an aerobic (~8 ppm O₂) or anaerobic (~0 ppm O₂) environment. A water jacket layer
was fabricated between the gas mixing channels and chambers for humidity control. The structures of oxygenator and water jacket above each chamber had the same geometry as the long-term culture device previously shown in Fig. 6b. Liquids from the solution inlets were supplied to the chambers using a multiplexed valve set to select an individual liquid, and row and control (multiplexed) valves to direct the liquid to a specific chamber. The liquid flow was driven by a peristaltic micropump fabricated along the outlet channel. This device employed the serial operations for solution pumping because driving solutions simultaneously to multiple chambers through parallel flow lines produced an inconsistent flow rate, as mentioned in the previous section. Additionally, peristaltic mixers with the parallel operation were embedded in all culture chambers. Interconnecting the six valves in each mixer to six shared control lines, all mixers could be operated simultaneously. Though the corresponding channel network became complicated, the channel routing issue was resolved by the via-hole design described in the previous section. As described in Fig. 8.3b, the control lines of mixers were fabricated as two channel layers (red and brown) connected with fluidic via-holes (yellow squares). The six common control lines (brown) linked to four sets of six intermediate connection channels (red horizontal lines), which connects all mixers of the chambers along a chamber row, by connecting each valve of the individual mixer to its corresponding intermediate connection channel.

The fabrication process of the 32-chamber artificial teeth device was similar to the single chambers version, except for the gas and control layers. (For the detailed procedures, readers may refer to the procedures described in section 7-2.) Because of the structure of the parallel gas mixers, an additional layer of channels had been designed to group the gas inlets into two common inlets for oxygen and nitrogen. Such additional channels were co-located with the layer for the water jacket to consolidate the fabrication effort to one channel layer, fabricated as described in section 5-2. Moreover, the control lines were designed as two-layer structure with interconnecting via-holes, with the upper and lower control layers fabricated using the aforementioned protocol. The composite fabricated 32-chamber (Fig. 8.3c) artificial teeth device had dimensions of 42 mm (l) × 24 mm (w) × 5 mm (b).

The bacteria sample extraction, experiment preparation and surface treatment processes were identical to the single chamber device described in section 7-1. The bacteria insertion and regular medium replacement of the artificial teeth involved routing liquids to multiple chambers in sequence using the integrated valves. The medium replacement procedures were demonstrated by a color dye experiment shown in Fig. 8.4. For each chamber column, PBS and fresh medium were initially filled along all alternative flow channels around the culture regions (Fig. 8.4a,b), driven by a 3 psi external pressure source (compressed air? Not the micropump?). Switching the valves for each alternative flow channel and chamber in sequence, all chambers in a column were inserted with fresh medium driven by the integrated peristaltic micropump (time: 20 s for each chamber) as shown in Fig. 8.4c,d. The same insertion procedures were then performed for all chamber columns (Fig. 8.4e,f). Operation time of the medium insertion step for each column of four chambers was ~1 min 30 s, so the overall medium insertion (performed every 30 min) would take ~12 min. After isolating the culture chambers, the peristaltic mixers operated in parallel (t ~ 2 min) to enhance nutrients transfer, while the flow channels were being rinsed with PBS to remove the residual bacteria (Fig. 8.4g-h).
Figure 8.3. (a) Design layout of 32-chamber artificial teeth chip. The sequence of channel layers is described with label 1-7 (top to bottom) in legend box. (b) Routing of control lines for peristaltic mixers. (c) Fabricated artificial teeth device.
Figure 8.4. Medium replacement procedures demonstrated by orange and blue dyes to represent fresh and old media, respectively.

128-chamber artificial teeth

Based on the same configuration, the multiple chamber design was further scaled up to a version consisting of 8 (row) × 16 (column) chambers as shown in Fig. 8.5. The 128-chamber artificial teeth (Fig. 8.6a) had dimensions 65 mm (l) × 45 mm (w) × 5 mm (h). The fabrication for 32- and 128-chamber artificial teeth devices was identical, although their mask designs were different. With a larger number of chambers, the 128-chamber device enabled a much higher throughput of experiments and, as such, could be utilized to investigate the properties of biofilm more efficiently under different microenvironments with a single run.

For serial microfluidic operations, the peristaltic micropump was used to route liquids along a single flow stream, so increasing the number of chamber units induced the required
processing time. Considering the number of culture chambers, the 128-chamber design required total a processing time ~50 min for medium insertion operation (20 – 30 s for each chamber). Such operation could not be implemented for every 30 min with a shared micropump. Therefore, the culture chambers were grouped into four sets of chambers arrays (8 rows × 4 columns) driven by four micropumps, reducing the required processing time to the same as the 32-chamber design. Medium insertion was then performed by pumping medium into four independent chambers simultaneously with the pumps along the four solution outlets indicated in Fig. 8.5. In the operation, the activated chambers had the same relatively location corresponding to their chamber set, because of the valve configuration of the “Column Control” multiplexor. Afterwards, the flow channels were rinsed while the parallel mixing (t ~ 4 min) was in operation.

Although four chambers were applied with medium simultaneously for the medium replacement, a specified inlet solution could also be driven into any individual chamber. An experiment was performed to demonstrate the accessibility of chambers for different inlet solutions. Dyes with different colors were applied to the predefined chambers, displaying as the characters “TEETH” (Fig 8.6b). This result verified that the configuration of flow channels could provide flexible fluidic connections from a solution inlet to a target chamber. Therefore, this multiple chamber device could be further applied to perform different parallel analysis experiments on a single chip.

Simplified target results for bacteria identification

The artificial teeth device could provide the same functionality for culture analyses, including thickness and cell viability measurements (section 7-3), and bacteria identification (section 7-4), as the single chamber design. However, considering the technical constraint on the manually-operated inverted microscope used in this research (Olympus IX71), acquiring the full set of bacteria identification results for 128 chambers was not feasible due to the limited lifetime (~2 – 3 hr) of fluorescent probes. Instead of taking images for DAPI and four FISH probes over a range of vertical (z) positions, which required at least >2000 images in total, a fluorescent image slice near chamber substrate was taken for three fluorescent probes (DAPI, STR405 and FUS664 as described in Table 7.1.) in every chamber. (However, the full image set could be potentially captured by other equipment, such as confocal microscopes with precise position control.) The model organisms (Streptococcus sp. and F. nucleatum) were selected for the proof-of-concept validation of the array chip, as Streptococcus played an important role in the early biofilm development, while the coaggregation ability of F. nucleatum was critical in the further biofilm formation.

A composite image of the fluorescently labeled bacteria co-culture in shown in Fig. 8.7 (with individual master images found in Appendix B). The shape of spherical Streptococcus and filamentous F. nucleatum can also be observed in the image. The fluorescent images for DAPI (All bacteria), STR405 (Streptococcus) and FUS664 (F. nucleatum) were combined as a single image with three color channels: blue, green and red, respectively.
Figure 8.5. Design layout of 128-chamber artificial teeth chip. The sequence of channel layers is described with label 1 – 7 (top to bottom) in legend box.
Figure. 8.6. (a) Fabricated 128-chamber artificial teeth chip. (b) Its accessibility of every culture region was tested by flowing color dyes in different defined chambers.

Figure. 8.7. Composite fluorescently labeled image of a bacteria co-culture in the 128 well chip. All bacteria (blue), Streptococci (green) and F. nucleatum (red).
**ADHESION ENHANCEMENT OF STREPTOCOCCI BY SUCROSE**

*Streptococci* are known to be the key primary colonizer species in the early development of dental biofilm, initiating the attachment of bacterial community on tooth surfaces. Therefore, the adhesion properties of *Streptococci* determine the extent and morphology of the subsequent dental plaque formation. It has been reported that the co-culture of different *Streptococcus* species (e.g. *S. mutans*, *S. sanguinis*, *S. oralis*, and *S. gordonii*) exhibits firm adhesion on salivary coated surfaces. It has been also reported that the adherence of *Streptococcus mutans* can be greatly enhanced by sucrose. *S. mutans* can synthesize adhesive glucans from sucrose using glucosyltransferases (GFTs). The produced glucans act as glue, providing receptors for other microorganisms possessing glucan-binding proteins for further attachment. Since dental biofilm is the co-aggregation of multiple species, the adhesion properties of a specific bacterium have a significant effect on the dynamics of the entire dental biofilm, especially on the biofilm firmness and the population of different species. In particular, another typical early colonizer *Streptococcus sanguinis* can induce higher adhesion of the entire community in the presence of *S. mutans* and sucrose. Since *S. sanguinis* and a few other *Streptococci* (e.g. *S. oralis* and *S. gordonii*) can adhere to *F. nucleatum*, a higher sucrose concentration may induce also the attachment of *F. nucleatum* and other late colonizers on salivary coated surfaces.

The artificial teeth device was implemented to investigate the adhesion of *Streptococci* and *F. nucleatum*. Four rows of chambers in the 128-chamber artificial teeth (sixty-four chambers in total) were used to culture saliva containing bacteria for ~6 hr with different sucrose concentrations. After surface treatment with filtered saliva on chamber substrate, human saliva samples containing dental bacteria (cell density: $10^6 - 10^7$) were mixed with 50% (v/v) basal medium mucin (prepared as described in section 7-2) and different sucrose concentrations (0%, 0.5%, 1% and 2% (w/w)). The samples were then flowed in the chambers and cultured in aerobic environment at 37°C for 6 hr. After culture, each chamber was rinse with PBS for 2 min, driven by applying external pressure from inlet (~3 psi) instead of micropumping for a faster liquid flow. Fluorescence *in situ* hybridization (FISH, as mentioned in section 7-5) was then applied to identify the target species. For the image analysis, twenty chambers with median total cell densities were extracted for each sucrose concentration. (The fluorescence images are shown in Appendix B.) The surface coverage of cells was converted from their occupied area in the image and equivalent cell diameters (~0.8 μm for *Streptococci* and ~2 μm for *F. nucleatum*). Results (Fig. 8.8a) show a significant enhancement of cell attachment with the existence of sucrose. In particular, the number of *F. nucleatum* cells attaching on the chamber surface (Fig. 8.8b) increased with increasing sucrose concentration. It has been reported that the presence of sucrose can induce the co-adhesion of *S. mutans* to other *Streptococci*, which produce the adhesin for *F. nucleatum* and facilitate its co-colonization of the biofilm. As *F. nucleatum* acts as the bridge species between the early and late colonizers, the adhesion enhancement of *F. nucleatum* may induce a higher cell population of the anaerobic late colonizers, which are the major pathogens in dental biofilm. The further experiments using the artificial teeth device, with...
the identification of more dental bacteria including the key late colonizers, may help to quantitatively investigate the mechanism on how sucrose or other sugars relate to the growth of late colonizers in dental biofilm, the cause of various human health problems.

![Bar chart showing cell coverage of Streptococci and F. Nucleatum under different sucrose concentrations.](image)

**Figure 8.8.** Cell coverage of (a) Streptococci and (b) F. nucleatum under different sucrose concentrations. The culture durations (6 hr) for all cases were identical. The stars indicate significant changes (p-value < 0.05).

### 8-4 DENTAL BIOFILM CULTURE UNDER DIFFERENT GAS CONDITIONS

The 128-chamber artificial teeth device was also applied to culture dental biofilm for three days to study the growth of aerobic Streptococci and anaerobic F. nucleatum under four different daily gas conditions (repeated every 24 hours, with thirty-two chambers for each gas condition): (1) anaerobic, (2) 20 hr aerobic and 4 hr anaerobic, (3) 16 hr aerobic and 8 hr anaerobic, and (4) aerobic. The dissolved oxygen levels with different supply durations were
regulated by eight overhead tunable oxygenators along chamber rows. Each oxygenator contained two control gases (oxygen and nitrogen) to provide aerobic and anaerobic culture conditions. All chambers were operated with the procedures described in section 7-2 with the modifications for the multiple chamber devices such as the serial medium replacement approach (section 8-2). Bacteria identification was performed by the FISH treatment (section 7-5) on chambers. For each gas condition, twenty chambers with median total cell densities were extracted to eliminate the experimental variations such as the inconsistent initial cell density during cell loading.

Fig. 8.9 shows that the proportion of *Streptococci* in dental biofilm decreased with the duration of anaerobic gas supply, while *F. nucleatum* increased with such duration. The proportion in cell coverage instead of cell density was considered because the major source of intrinsic deviation in cell density, caused by the different sizes of bacteria clusters in human saliva extracts, was hard to avoid in experiments. It can be observed in Fig. 8.9 that anaerobe *F. nucleatum* in dental biofilm could grow even under aerobic condition, with a cell density increased from \(~10^5\) to \(~10^7\) cells/mL. It was probably due to the local anaerobic microenvironment in the bottom portion of biofilm caused by the oxygen uptake of aerobes\(^{14}\). Since the artificial teeth devices were developed to simulate the dental biofilm formation with the microenvironment as the *in vivo* case, this result may explain why a larger variety of anaerobes can maintain their viability in an oral cavity for >12 hr of aerobic environment per day during a person is awake.

![Figure 8.9](image)

*Figure 8.9. Proportions in cell coverage of *Streptococci* and *F. nucleatum* under different gas conditions after 3 days of culture. The values were calculated by dividing the total area of biofilm by the area of species. The total occupied area of biofilm was measured by DAPI probe. The stars indicate significant changes (p-value < 0.05).*
REFERENCES

CHAPTER NINE

CONCLUSIONS

An "artificial teeth" microfluidic platform containing 128 culture units was successfully developed for high-throughput dental biofilm culture and analysis, achieving long-term dynamic regulation of the culture microenvironment (e.g. medium flow rate, nutrients concentrations and gaseous conditions) coupled with the development of analysis techniques to determine the biofilm properties such as thickness, viability and bacterial composition. The platform provides well-defined growth conditions to maintain the chronological viability of the entire cell communities through: (1) flow control using conventional microfluidic elements such as pneumatic microvalves, peristaltic pumps and multiplexors, (2) differential or tunable medium oxygenation using an overhead channel flowed with oxygen, (3) evaporation elimination shielded by a water jacket layer, and (4) substrate modification for biofilm adhesion by deposition of polystyrene, which is a material widely used in the conventional laboratory cell culture. The parallel dental biofilm culture and analysis system using "artificial teeth" was implemented to quantitatively investigate the effects of different gas conditions and sucrose concentrations on the populations of dental bacteria Streptococci (aerobe) and Fusobacterium nucleatum (anaerobe) co-cultured in dental biofilm. Such implementation has demonstrated its effective capability to high-throughput microbiological applications. Considering the applicability of the analysis techniques and the similar requirements on microenvironments for various single cell/biofilm cultures, the platform establishes a general framework of the microfluidic implementation of the screening of small cell populations.

To meet the target specifications for in vitro dental biofilm formation (Table 2.2), the "artificial teeth" chip was designed with multiple integrated microfluidic components, combining simulation and experimental work to validate the performance of the platform. Throughout the platform development, this research has involved several major pieces of works, which are summarized as follows.

Microfluidic oxygenator design

A significant portion of this thesis was dedicated to the design and analysis of oxygenators for microfluidic devices. As biofilm includes aerobes that require oxygen to grow, maintaining the proper dissolved oxygen (DO) levels in the culture environment is
critical for biofilm health, particularly for early aerobic colonizers that initiate biofilm formation. Using active techniques such as gas diffusion networks reported in this thesis enables stable, tunable DO levels to be supplied to individual culture microchambers. Alternatively, one could exploit the gas permeability of the structural material of the device (PDMS) to culture cells in a confined chamber with a defined pO₂. (The basic concept and implementation issues are discussed in chapter 3.) However, the typical device thickness (~5 mm) results in a relative long diffusion time (~3 hr). Due to the significant oxygen uptake of particular biofilms with high aerobic cell density, the local cellular pO₂ can be significantly lower than the pO₂ around the device.

This thesis includes the modeling and analysis of the typical oxygen transfer model in a microfluidic oxygenator used for cell culture (chapter 4). In modeling the transfer properties for the efficient oxygenation, we have proposed several design parameters: the oxygen pressure drop (OPD) to describe the relative reduction of pO₂ caused by the aerobic species in biofilms, rise time (Tr) to reflect the time for oxygen transfer, and the dimensionless effective channel length (L*) to indicate the fully developed location of gas diffusion. The general design considerations for microfluidic oxygenators include minimizing chamber and membrane thicknesses for small Tr and OPD, adjusting gas pO₂ levels to compensate a large OPD, and extending the oxygenation channel upstream of the culture region to establish a fully developed oxygen profile (L* >> 1). These strategies are very useful in the design of an effective oxygenator, especially in determining the thickness of the membrane: thin enough for effective gas transfer and thick enough to withstand gas/hydraulic pressure.

**Differential and tunable gas mixers for oxygenation**

The growth environment for some biofilms (e.g. the dental biofilm, which is the focus in this work) involves a dynamic DO condition, so there is a demand to extend the functionality of microfluidic oxygenators to support variable gas conditions. The ability to vary and tune pO₂ in a microfluidic environment has practical applications in areas such as microbiology and cancer research, where fluctuations in dissolved oxygen concentration impact not only cell viability but also the regulation of key biochemical pathways.

A differential oxygenation device has developed to generate linearly increasing step-wise DO concentrations (R² = 0.993) across eight parallel microchannels. It consists of a gas-based analog of a microfluidic solution gradient generator with a network of branching gas-filled microchannels that overlap the underlying microfluidic culture channels. Using continuous oxygen and nitrogen supplies, identical DO levels were maintained within an array of chambers via passive gas diffusion. Additionally, a modeling approach has been introduced for the gradient generator design, converting microchannel sections with Re << 1 to equivalent circuit model that can efficiently reduce the design effort. By varying the dimensions of individual microchannels within the mixer network, the output oxygen concentration(s) can be fine-tuned for the target application. This modeling methodology is analogous to the solution gradient generator, especially for the mixing of liquids with different viscosities.

A tunable gas mixer was achieved by dynamically connecting the gas perfusion channel to one of the oxygen-nitrogen mixers for different ratios under the control of a microfluidic
The design and implementation of differential and tunable oxygen-nitrogen mixers (chapter 5) enable microfluidic systems to provide dynamic gaseous microenvironments for different microbiological applications, which have been verified by the cultures experiments on aerobic and anaerobic bacteria.

Device design for long-term culture applications

The implementation of microfluidics in long-term culture is important to provide adequate time for biofilm formation and other microbiological applications. The single chamber “artificial tooth” chip was initially developed for the long-term culture of dental biofilm (chapter 7). It was integrated with several microfluidic elements designed to provide a steady microenvironment for bacterial culture (chapter 6). To maintain media for an extended period of culture time, a layer of water jacket can be added to the oxygenator (between gas perfusion channel and chamber) to create a local humidified pO2 profile (investigated by simulations) and prevent medium evaporation from direct diffusion via PDMS membrane. To prevent the clogging of flow channels due to the growth of leaked dental bacteria from culture regions, an extra flow line was designed next to the chamber so that the flow channels could be regularly cleaned while the culture chamber was isolated during culture. A peristaltic mixer composed of six pneumatic microvalves was fabricated within the culture chamber. The mixing operation can be performed after cell seeding and medium insertion to obtain more uniform concentration profiles of bacteria and nutrients in the chamber, work that has been validated experimentally. Additionally, polystyrene substrate has been shown as an ideal material as the chamber substrate for the surface pre-treatment of filtered saliva to initiate better bacterial adherence. These design implementations form a solid framework for the development of a general microfluidic platform for dental biofilm formation, and potentially for general cell/biofilm related applications with minimal modifications.

The design of “artificial tooth” was scaled up to 32 and 128 chambers as “artificial teeth” devices (chapter 8). The multiple chamber devices included the addition of microfluidic via-holes between two layers of microchannels, which increase the routing efficiency of microchannel networks as fluidic connections become more complex (i.e. very-large-scale-integrated microfluidic systems).

Microfluidics-compatible techniques for microbial analyses

The microfluidics-compatible analysis techniques using the “artificial tooth/teeth” were also successfully developed for thickness and viability measurements, three-dimensional structure visualization, and the identification of multiple species in polymicrobial biofilms (The protocols are presented in chapter 7). The microfluidic implementation of such techniques makes a significant contribution to future cell biology physiology, particularly for examining the growth and interaction of small cell populations under tightly defined environmental conditions. Protocols for Live/Dead BacLight probes and fluorescence in situ hybridization (FISH) probes have been modified and optimized for the “artificial teeth”,

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enabling both dynamic monitoring of cell health and culture composition as a biofilm matures. Using fluorescent probes, visualization of the entire cell cluster in 3D can be obtained by performing image deconvolution of microscopic images taken at various depths along the chamber.

These techniques were applied to (1) measure the 3D distribution of living and dead cells in dental biofilm that has indicated the outer viable cell layer\textsuperscript{10} (section 7.4), and (2) identify the effects of different sucrose concentrations and gas conditions in terms of the populations of \textit{Streptococci} and \textit{F. nucleatum} (chapter 8) in the dental biofilm. The adhesion of both \textit{Streptococci} and \textit{F. nucleatum} to the polystyrene teeth increased with sucrose concentration, suggesting that the production of adhesion factors and subsequent colonization is driven by environmental resources. Moreover, the bacteria displayed a symbiotic relationship under the applied differential gas conditions (aerobic and anaerobic microenvironments with different durations). While \textit{Streptococcus sp.} grew well in isolation under an ambient oxygen environment and \textit{F. nucleatum}, as an anaerobe, grew well in isolation under hypoxic conditions, \textit{F. nucleatum} in the multi-species biofilm also thrived in an aerobic environment. This result can be attributed to the microaerobic/anaerobic local cellular environment, induced by the oxygen uptake of dental aerobes\textsuperscript{11}, that is optimal for \textit{F. nucleatum}, and, in general, the numerous distinct anaerobes in human oral biofilms.

The above findings demonstrate, for the first time, the development of an “artificial teeth” system for the quantitative analysis of dental biofilms. The “artificial teeth” device can achieve high-throughput operations for cell culture, and reduce the required extensive efforts for the parallel culture and quantitative analyses of cell properties under well-controlled static/dynamic microenvironments. As a next generation tool for biofilm studies, it will enable researchers to dissect the complex polymicrobial interactions that promote biofilm formation and evolution.

REFERENCES

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**APPENDIX A**

**OXYGENATED CULTURE ON MAMMALIAN CELLS**

A-1 IMPLEMENTATION AND CULTURE RESULTS

To study the effect of dissolved oxygen (DO) concentration on mammalian cell culture, the oxygenator chip described in section 5-1 was used for parallel culture of BALB murine embryonic fibroblast cells (3T3). BALB/3T3 murine embryonic fibroblast cells (CCL-163), purchased from Advanced Type Culture Collection (ATCC), were cultured in Dulbecco's Modified Eagle Media (Gibco) containing 10 % fetal bovine serum. The cell line was manipulated under sterile tissue culture hoods and cultured in a 5 % CO₂ humidified incubator at 37 °C. Once the cells were confluent, they were trypsinized (0.25 % in EDTA) and passaged at a 1:5 sub-culture ratio.

In the experiment, the microfluidic oxygenator was first sterilized by flushing fluid channels with 70 % ethanol followed by baking at 80 °C for 2 hr. After baking, the fluid channels were rinsed with 1x phosphate-buffered saline (PBS), with pH 7.4, and degassed by forcing trapped air through the walls of the gas-permeable oxygenator with pressurized PBS buffer. The glass surface along flow channels was subsequently pre-coated with 20 mg/mL gelatin (Sigma) in 1x PBS for 1 hr to promote cell attachment. Excess gelatin was removed by rinsing with 1x PBS. In preparation for device loading, the cell line was trypsinized, spun down in a centrifuge (1000 rpm, 5 min), and reconstituted in Leibovitz’s L-15 medium (Invitrogen 11415064) at a density of ~10^6 cells/mL. To load cells into the oxygenator, a syringe pump (PicoPlus, Harvard Apparatus) was used to inject cells into each culture channel (flowrate: 0.01 μL/min, load time: 3 min), activated by an integrated microfluidic multiplexor. Cells were seeded into culture channels and pre-cultured in an incubator (~21 % O₂ and 5 % CO₂ gas supply) for one day to allow cell spreading and attachment to the treated glass surface.

During on-chip cell culture, oxygen and nitrogen (supply pressure: ~1 kPa), humidified by bubbling through water reservoirs, were flowed through the gradient generator to generate different DO levels in the underlying medium-filled channels (0 – 42 ppm). A syringe pump (Harvard Apparatus) was used to continually supply fresh medium (flowrate: 0.003 μL/min) to each fluid channel in turn, switching channels every one minute under the
control of an integrated microfluidic multiplexor. This operation provides a consistent medium supply along every channel, even when there were inconsistencies in the channel cross-sections and fluidic resistances developed by different cell growth rates. After 4 hours, the effect of DO on 3T3 cell growth in the culture region could be observed (Fig. A.1). The cells exhibited good viability and proliferation at a DO concentration of 12 ppm (Fig. A.1b). Under low pO2 (<6 ppm), 3T3 cells shrank and started detaching from the channel wall (Fig. 6a), while in high pO2 (>36 ppm), cell necrosis was observed (Fig. A.1d). A comparative experiment of 3T3 cells in traditional culture flasks incubated at 37 °C under pure nitrogen (0 % O2), air (21 % O2) and pure oxygen yielded morphological results consistent with that of the microfluidic oxygenator: 3T3 cells grown under 0 % O2 detached and pure oxygen resulted in necrosis.

![Image of 3T3 cell growth under different DO concentrations](image)

**Figure A.1. Growth of 3T3 cells under different DO concentrations:** (a) 0 ppm, (b) 12 ppm, (c) 24 ppm and (d) 42 ppm. White boxes indicate changes of cells in specific channel regions during the culture experiment.

### A-2 VALIDATION OF THE OXYGEN PRESSURE DROP

A validation experiment (as described in section 4-3) was performed to demonstrate the oxygen pressure drop (OPD) cause by the oxygen uptake of mammalian cells in the typical single layer PDMS devices. Human umbilical vein endothelial cells (HUVEC) (cell coverage: \(\sim 10^3 - 10^4 \) cells/cm², thickness: 5 µm) were chosen to be cultured in the device (Fig. A.2a,b) aforementioned in section 4-3 (channel height: 200 µm, PDMS thickness: 5 mm), under the air supply supplemented with 5 % CO2 (\(P_2 = 0.1995 \) atm) at 37 °C. (The oxygen diffusivity and solutibility in HUVEC are \(9.5 \times 10^{-6} \text{ cm}^2/\text{s} \) and 1.049 mM/atm, respectively.) HUVEC (Lonza) were cultured in Dulbecco’s Modified Eagle Media (DMEM, purchased from Gibco) containing 10 % fetal bovine serum. The cell line was manipulated into a new culture flask pre-coated with 20 mg/mL gelatin (Sigma) in 1x PBS under sterile tissue culture hoods and
cultured in a 5% CO₂ humidified incubator at 37 °C. Once the cells were confluent, they were trypsinized (0.25% in EDTA) and passaged at a 1:5 sub-culture ratio.

Each oxygen sensor along the microchannels was calibrated (section 3-4) before the microfluidic devices was sterilized by flushing medium channels with 70% ethanol followed by baking at 80 °C for 2 hr. After baking, the fluid channels were rinsed with 1x phosphate-buffered saline (PBS), with pH 7.4, and degassed by forcing trapped air through the walls of the gas-permeable oxygenator with pressurized PBS buffer. The glass surface along flow channels was subsequently pre-coated with fibronectin (Sigma, 0.1% w/w in 1x PBS) for 1 hr to promote cell attachment. Excessive fibronectin was removed by rinsing with 1x PBS. In the preparation for cell loading, the cell line was trypsinized, spun down in a centrifuge (1000 rpm, 5 min), and reconstituted in fresh DMEM at a target cell density. The cells were then seeded into the culture channels by a syringe pump (PicoPlus, Harvard Apparatus) at flow rate 0.05 μL/min for 10 min and cultured, without medium flow, in a humidified incubator (~20% O₂ and 5% CO₂ gas supply) for 6 hr to allow cell spreading and attachment to the treated glass surface. Afterwards, DO measurement was performed and microscopic images were captured at sensor regions (where the corresponding sensor output reflected the local DO levels) to determine cell density by counting the cells in the images (Fig. A.2c).

The measured OPD (0.021 ± SD 0.009, 0.072 ± 0.026, 0.157 ± 0.030 and 0.166 ± 0.033) increased linearly with the surface cell coverage \( \rho_{cell} \times H \) (0.67 ± 0.05, 1.54 ± 0.12, 3.68 ± 0.21 and 4.04 ± 0.29 [×10⁴ cells/cm²], respectively). This result has reasonable agreement with the analytical solution in Eq. 28 that \( OPD \) is proportional to the surface cell coverage for the negligible Michaelis-Menten constant of HUVEC \( (K_m = 0.5 \text{ mmHg} < < P_s \cdot S_1 = 0.21 \text{ mM})^2 \). Furthermore, the experimental maximum cellular respiration rate \( V_{max} \) (≈7.45 × 10⁻³ M/s) was in the same order of magnitude as the previously reported value in bulk culture environment (≈ 9.55 × 10⁻³ M/s)^².
REFERENCES

B-1  FOUR FISH PROBES AND DAPI

Sample 1 – 4
Sample 5 – 12

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500 μm
CULTURE FOR DIFFERENT SUCROSE CONCENTRATIONS

Sucrose 0 %

Sucrose 0.5 %

Sucrose 1 %

Sucrose 2 %
B-3  CULTURE FOR DIFFERENT DAILY GAS CONDITIONS

Aerobic condition

[Images of bacteria under different conditions]
4 hr anaerobic + 20 hr aerobic condition

- All Bacteria
- Streptococci
- F. Nucleatum

100 um
8 hr anaerobic + 16 hr aerobic condition
Anaerobic condition