Design, Fabrication, and Testing of a Multichannel Microfluidic Device to Dynamically Control Oxygen Concentration Conditions *In-Vitro*

by

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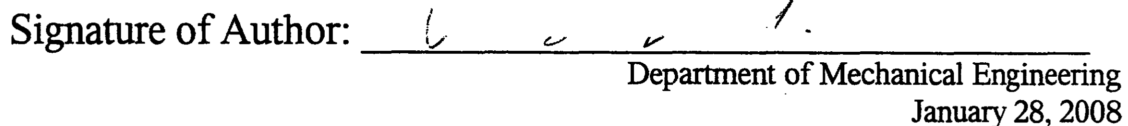

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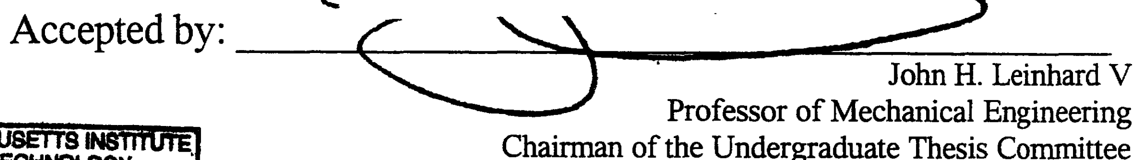

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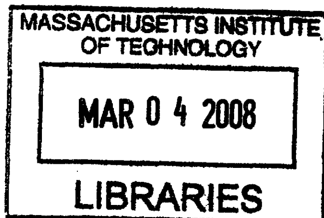

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Rosa H. Rodriguez Rivera

Submitted to the Department of Mechanical Engineering
on January 28, 2008 in Partial Fulfillment
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ABSTRACT

Multilayer microfluidic devices were designed and fabricated such that an array of different oxygen concentrations could be applied to a testing area in any desired sequence and with unconstraint application times. The principle of flow resistance dictates that a large channel length will impose a larger resistance and therefore a larger reduction in flow rate versus a shorter channel length. To exploit this feature, the microfluidic device employs a fluidic resistance network composed of an array of pre-determined variable length channels to generate different oxygen to nitrogen flow rate ratios, i.e. different oxygen concentrations. Standard lithographic techniques were used to fabricate the microfluidic devices, using highly gas permeable silicone rubber (polydimethylsiloxane (PDMS)). The stacked microchannel architecture, channel dimensions, and layer thicknesses in the device were optimized for rapid diffusion and saturation of O_2/N_2 mixtures into the testing areas. The oxygen concentration was dynamically monitored using polymeric fluorescence-based oxygen sensors integrated into the device. By altering oxygen levels over time, this device aims to selectively build up biofilms on the artificial tooth substrate as the process occurs *in-vitro*. A study concerning this application is also presented.

Thesis Supervisor: Todd Thorsen
Title: Professor of Mechanical Engineering

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Chapter 1: Thesis Introduction

Dental plaque is the general term used for the highly complex and biodiverse microbial community that builds on tooth surfaces. These communities are generally known as biofilms and are most commonly found in moist or liquid environments attached to a solid substratum, such as in the oral cavity. Although over 500 bacterial species comprise plaque, colonization follows a regimented pattern with adhesion of initial colonizers to the tooth enamel followed by secondary colonization through bacterial adhesion.[1] Understanding the conditions that promote colonization and subsequent biofilm development is particularly important as it plays an important role in the prevention and treatment of health problems resulting from dental plaque, such as tooth decay and if left untreated to more serious conditions, such as cardiovascular disease and pre-term labor.

In the study of dental plaque, *in-vivo* experimentation is complicated and limited. Apart from the ethical issues and difficulties involved in sampling collection and access, plaque's bio-diverse and heterogeneous nature causes much difficulty in controlled testing. As an alternative, there is a range of *in-vitro* model systems aiming to mimic the oral environment to some degree in order to manipulate associated variables.

A critical environmental variable affecting the composition and activity of dental biofilms, is oxygen concentration. "Exposure of bacteria to oxygen has significant impacts on sugar metabolism, acid production, stress tolerance, and factors related to persistence in dental plaque." [2] Additionally, biofilms forming in different surfaces of the mouth sustain a variety of aerobes, facultative anaerobes, and obligately anaerobic bacteria, which have different oxygen environmental requirements.

A multilayer microfluidic device capable of generating different oxygen concentrations over time has been designed and fabricated to address oxygen concentration effects. Additionally, a study was executed to survey the possibility of generating different oxygen concentrations in the device and simultaneously sustain the co-culturing of multiple bacterial species on artificial tooth substrates *in vitro*. This device has demonstrated a large potential in the investigated dental plaque application, and also in other biological micro-device applications.

The microfluidic devices, chips, were made primarily from (polydimethylsiloxane (PDMS)) cast from silicon wafer molds containing positive-relief channels patterned using standard lithographic techniques. The integration of a microfluidic platform for dynamic oxygen sensing and delivery in a flowing medium together with the exploitation of PDMS's permeability to oxygen, made it possible to help pioneer a microfluidic chip capable of generating different oxygen concentrations over time *in vitro*.

This microfluidic device not only creates the array of different oxygen concentrations, but allows the user to choose the order at which the oxygen concentrations are applied to the area of study. Nitrogen and oxygen inputs connect to the device and deliver the gasses into conduits that branch out into channels of different lengths. Due to fluidic resistance, the flow rate observed in a long channel is lower than that observed in a short channel having the same cross-sectional area. Therefore a set of pre-determined variable length channels for oxygen joint with a different set for nitrogen, grants an opportunity to select a specific combination of these so that when the flowing gasses come together, a specific oxygen concentration is achieved. To this effect, two variable length channel networks, VL channel networks, were incorporated. The device

also bears a valve system which controls which combination of channels is selected. The oxygen concentration in the gas mixture resulting from the VL network equilibrates with the testing area through a thin PDMS membrane, given the high permeability of PDMS to oxygen.

In order to test different aspects of the device relevant to its performance, three different multilayer PDMS chips were created. The first chip, Chip A, consisted of two layers: a nitrogen/oxygen mixing layer and a valve system stacked above. This chip was used to test the level of functionality of the valve system and to identify the optimum working channel width for the VL channels by varying parameters in the soft lithography fabrication process. A second one-layer chip, Chip B, was designed and fabricated to test the accuracy of the oxygen concentrations created by the VL channel combinations. Using an LED – based optical excitation and detection system the oxygen content in the microfluidic chip was monitored in real time. The final chip, Chip C, was designed and fabricated for the purposes of studying biofilm formation, specifically to selectively build up biofilms on an artificial tooth substrate located in the bacteria culture layer by dynamically imposing an array of oxygen concentrations.

Chip C's design is intended for the co-culturing of multiple bacteria species present in dental plaque. But in order to induce biofilm formation, the model system needs to be realistic; aiming to mimic the environmental conditions of plaque ecosystems during experiments is critical. A method for artificial saliva preparation and periodic supply of sucruose, i.e. meals, to replicate nutrient conditions, is presented. Also an artificial tooth substrate exhibiting the characteristic of tooth enamel is suggested to serve as the solid substratum for biofilm formation. Information is also provided on

recommended bacteria to be cultured and tested in the device. Finally, preliminary experiments conducted by Raymond Lam, testing the optimal oxygen conditions for uni-cultures grown in microfluidic devices *in-vitro* are discussed.

A more detailed account of dental plaque and the importance of its study is provided in chapter two, along with the analysis of the underlying problem being addressed and previous relevant research. Presenting a brief description of this realm of research, the evolution of the thesis objectives, and the ground work for dissertation will communicate the building blocks for a better understanding of the thesis. Chapter three outlines the theoretical work performed in the design, fabrication, and testing of the microfluidic devices and its application to the study of dental plaque. Chapter four follows a detailed account of the experimental procedures performed in the fabrication and testing of the devices and a description of possible subsequent dental plaque experiments. The results of the experiments and a respective analysis can be found in chapter five, along with concluding remarks.

Chapter 2: Groundwork for Dissertation

Dental plaque develops naturally as part of the host's defense system against exogenous microorganisms. Due to oral hygiene in humans and natural defenses the attachment, growth, and removal of bacteria in the oral cavity is a dynamic and continuous process. These multi-species microbial communities are generally known as biofilms and are highly structured with complex physiology and metabolic activity. The heterogeneous environment allows for wide variety of microorganisms to grow, thrive, and survive in the presence of one another, even if the organisms are generally incompatible. Group interactions can be beneficial or antagonistic to some of the interacting populations. Among some interbacterial interactions observed in biofilms are synergism, competition, antagonism, and environmental effects.

Dental Plaque Models

Difficulties with *in-vivo* studies along with the complexity of dental plaque have led to the development of a variety of laboratory biofilm plaque model systems. For these models to be effective in explaining plaque ecology and pathology, they must be realistic and encourage the behavior which is under investigation.[3] These systems differ in the technologies employed, their strengths and limitations, and their uses due to the inevitable compromises between the in vivo ecosystem and the simplification and control needed to gain useful results.

Aspects of Dental Plaque Relevant to Models

Many aspects of dental plaque come into play when trying to model it *in-vitro*. The biofilm of linked bacterial communities are complex and heterogeneous, site-specific, varied, evolving, yet spatially organized. The microbiota is biodiverse

experiencing microbial succession, the presence of interspecies structures, and the formation of microcolonies and clonal booms.[3] The environment is also uncontrollable, with fluctuating laminar flow fluids and the distinct, changing, interacting physcochemical gradients in the intraplaque surroundings that differ from the general oral environment.[3] Also contributing are the counter forces to plaque formation such as host immunological chemical defenses, fluid shear, abrasion, and oral hygiene procedures. [3]

Other Dental Plaque *In-Vitro* Techniques

Among the major oral biofilm culture systems are chemostat-based systems, growth rate controlled biofilm fermenter (GRBF), artificial mouths, the constant –depth film fermenter (CDFF), and the multiplaque artificial mouth. All these have distinctive features and approaches to modeling the oral environment.[3] For instance, chemostats have a controlled fluid environment and a planktonic phase with a nutritionally limited growth-rate. [3] In artificial mouths, unrestrained growth is usually observed, although, liquid shear plays a small part in limiting size.[3] When compared with chemostat systems, artificial mouths lack planktonic phases and are less controlled by the physical properties of the oral fluids. [3] In the positive sense, they have more flexibility, properties such as PH are controllable, and nutrient cycling which imitates meals are possible. These systems have their strengths, limitations, and varying applications, but they all lack in the control of the dynamic oxygen concentration in the biofilm environment.

Aspect of Dental Plaque Relevant to the Microfluidic Device

Oxygen is a vital environmental factor affecting the composition, build-up, and activity of dental plaque biofilms. Biofilms found on tooth surfaces are composed of

aerobes, facultative anaerobes, and anaerobic bacteria regardless of the rich oxygen environment in the oral cavity. "Not surprisingly, oral bacterial biofilms have relatively active oxygen metabolism and have developed defenses against the presence of oxygen and a wide variety of redox environments. "Notably, exposure of bacteria to oxygen has significant impacts on sugar metabolism, acid production, stress tolerance, and factors related to persistence in dental plaque." [2]. The development of biofilms follows a regimented pattern with adhesion of initial colonizers, mediated by specific or non specific physico-chemical interactions. Suitable conditions lead to proliferation and the formation of microcolonies. With environmental changes comes secondary colonization, through bacterial adhesion, beginning the formation of a multispecies community. Studying the effect of changing oxygen concentrations on biofilm formation among other aspects of dental plaque would be a beneficial study, with the potential to shed light on biofilm formation.

Oxygen Concentration Control - Current Techniques

Among some of the current procedures for specific oxygen concentration exposures is the culturing of bacteria in the desired oxygen concentration environment and then transferring the media while trying not to perturb the given environment.

An impressive work on oxygen concentration gradients was conducted by Jaehyun Park et al. They created a microsystem containing an array of multiple microelectrodes capable of creating accurate, consistent, and superimposed oxygen gradients. Water electrolysis was the means to generate the controlled amounts of dissolved oxygen from an array of noble metal microelectrodes embedded in silicone

microchannels.[4] This method however is not suitable for providing dynamically changing oxygen concentrations, unless the array of microelectrodes was made mobile.

Oxygen Concentration Control - Concurrent Work:

Raymond H. Lam is an MIT PhD candidate with previous and ongoing work on the generation of differential dissolved oxygen concentration for biological cell cultures *in-vitro*. He has successfully cultured the following dental plaque bacteria, *A.viscosus* and *S. mutans*, using the microfluidic oxygenator and has acquired estimates for the optimum oxygen growth conditions for these bacteria. He was a mentor for this project along with Prof. Todd Thorsen, heading the "Mouth-On-A-Chip" project working towards a microfluidic device for the co-culturing of multiple bacterial species to study biofilm formation and tooth decay under a dynamically changing oxygen environment, among other environmental factors.

Oxygen Concentration Control - Thesis Project

The main objective of the microfluidic devices fabricated was to generate an array of different oxygen concentrations applicable to a testing area within the device to simulate the constantly changing oxygen environment in the oral cavity. The device allows for the user to choose any of the oxygen concentrations and decide the order at which these are applied *in vitro* to the area of study.

The device works by manipulating nitrogen and oxygen input flows such that mixtures with different proportions of these gasses and therefore different oxygen concentrations are created. Manipulating the resistance by varying channel length is a means to control the gas flow rate. An array of pre-determined variable length channels were incorporated into the device, one for the nitrogen input and a different array for the

oxygen input. As the gasses flow through channels they experience resistance imposed by fluid flow decreasing the flow rate at which the gas travels through the channel. The election of a specific combination of an oxygen channel and a nitrogen channel results in a corresponding oxygen concentration. A valve system was used to control which combination of channels was selected in order to expose the test area to the specific oxygen concentration of choice.

The stacked chip architecture was designed such that the testing area would be located below the nitrogen/oxygen gas flow channel and separated by a thin PDMS membrane. The permeability of PDMS to oxygen permitted the nitrogen:oxygen mixture to saturate the level located below the gas channel, exposing the test area to the oxygen concentration induced by the valve system. In a biofilm application of this device, the artificial tooth substrate located in the testing channels and the medium fluid flowing through it would be exposed to the same oxygen concentration in the mixer channel above.

Oxygen Sensing - Previous work

The monitoring of the oxygen concentration inside the microfluidic device is due to the development of an integrated microfluidic platform for dynamic oxygen sensing by Adam P. Vollmer, Ronald F. Probstein, Richard Gilbert, and Todd Thorsen. This group developed a platform for real-time sensing of dissolved oxygen in a microfluidic environment using an oxygen-sensitive luminescent dye. They showed that the integrated oxygen sensor in the membrane-based micro-oxygenator device produced accurate results across a range of tested flow rates, *0.05 -5 mL/min.* [5] The incorporation of this

platform onto the biofilm microfluidic devices was possible given the flexible configuration and its ease of fabrication.

Chapter 3: Theory

Microfluidic Device Design and Components:

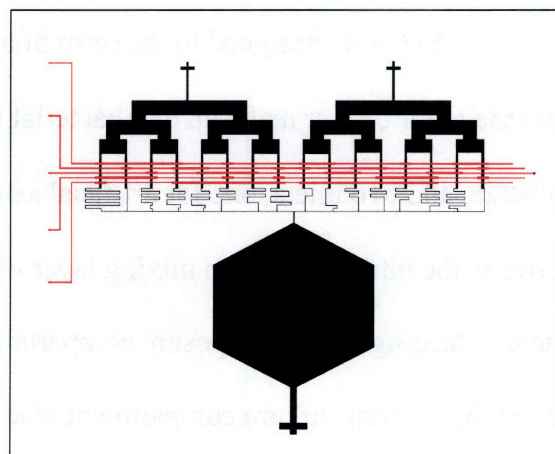
Three different sets of PDMS chips were created. The first two sets, Chip A and Chip B, were experimental versions constructed to optimize different parameters pertaining to the functionality of the third and final PDMS chip, Chip C.

Chip A was used to identify the optimal width for the variable length channels and to test the functionality of the valve system by varying parameters in the soft lithography fabrication process. Different soft lithography parameter settings affect different aspects of the final product. For instance, spin coating speed determines layer thickness and PDMS preparation parameters influence material properties. In addition, PDMS chips were made with three different widths to test for the optimum working channel width for the VL channels.

Chip A consisted of two layers: the nitrogen/oxygen mixing layer and a valve system layer. The valve system controls which variable length channel is opened for the nitrogen input and for the oxygen input. Fabrication consisted of two soft lithography procedures and plasma bonding to seal the exposed bottom layer channels to a glass slide.

Figure 1:
Experimental Chip A [Two Layers]
*No Bacteria Culture Layer
Bottom Layer: Nitrogen/Oxygen Mix Layer
- Oxygen and Nitrogen Channels
- Mixing Channel

Top Layer: Nitrogen/Oxygen Valve Control Layer
- Valve System

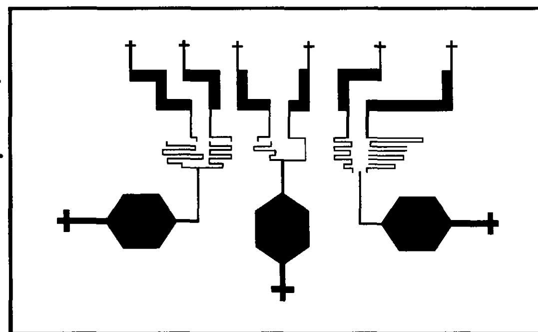


Chip B tested the accuracy of the oxygen concentrations created by the VL channel combinations. An LED – based optical excitation and detection system was used to examine the oxygen content in the microfluidic chip. The optical oxygen detection was achieved through the excitation of PtOEPK, a luminescent dye located in the oxygen sensing compartments.

Figure 2:
Experimental Chip B [One Layer]
* No Bacteria Culture Layer
* No Nitrogen/Oxygen Valve Control Layer

Bottom Layer: Nitrogen/Oxygen Mix Layer

- Oxygen and Nitrogen Channels
- Mixing Channel
- Oxygen Sensor Compartments

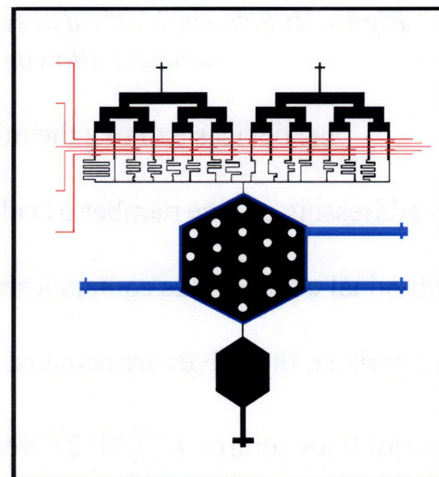


Chip B consists of only one layer containing three different test areas. For each, the oxygen and nitrogen input ports split up into the variable length channels and feed into the mixing channels and oxygen sensor compartments. Flow occurs from the small crosses towards the large cross in all the fabricated chips. Fabrication consisted of a single lithography procedure, oxygen sensor preparation, and plasma bonding to seal the microchannels onto a glass slide.

Chip C was designed in the form of a three layer structure. The bottom layer is the bacteria culture layer made up of a bacterial compartment containing an artificial tooth substrate and two inlets, one for artificial saliva and one for the growth media. Stacked above is the nitrogen/oxygen mixing layer with the VL channel network and mixing channel feeding into the exposure compartment. This exposure section is located right above the bacteria culture compartment where a thin PDMS film separates the two. Gas

diffusion occurs from the nitrogen/oxygen exposure compartment (middle layer) through the thin PDMS film into the bacteria culture compartment (bottom layer). Finally, the third and top layer contains the valve system controlling fluid flow in the middle layer. The fabrication consisted of three lithography procedures, oxygen sensor preparation, artificial tooth substrate incorporation, and plasma bonding.

Figure 3:
CHIP C [Three Layers]



Bottom Layer: Bacteria Culture Layer

- Bacteria Culture Area (Testing Area)
- Teeth Simulations
- Flow Channel

Intermediate Layer: Nitrogen/Oxygen Mixing Layer

- Oxygen and Nitrogen Channels
- Mixing Channel
- N/O area over bacterial culture area
- Support Columns
- Oxygen Sensor Compartment

Top Layer: Nitrogen/Oxygen Valve Control Layer

- Valve System

Oxygen and Nitrogen Delivery and The Valve System

A valve system was designed and implemented to control fluid flow in the nitrogen/oxygen mixing layer such that a single nitrogen feed and a pre-selected corresponding oxygen feed are opened at any point in time or alternatively the fluid flow is completely halted. These specific combinations of open feeds generate different ratios of oxygen to nitrogen flow rates which create the array of desired oxygen concentrations.

There are two inputs: the oxygen and the nitrogen input. A single input channel breaks up symmetrically to become eight input channels which then feed into the variable

length channel network. Only the two channels specified by the valve operations will experience fluid flow through the corresponding VL channels.

The following is a schematic of the valve system implemented in the microfluidic chip.

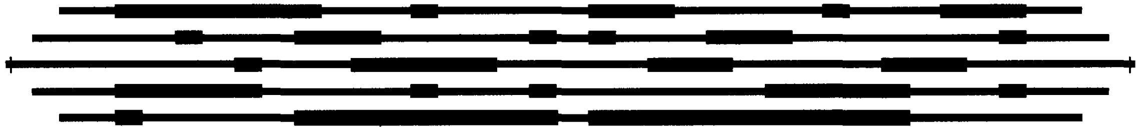


Figure 4: *Schematic of valve system used to control fluid flow for 16 vertical flow channels allowing two open channels simultaneously.*

This devices exploits the microfluidic equivalent of a combinatorial multiplexer by addressing a large number of valves with a small number of connections. The individual control lines contain a series of valves. When these control lines are pressurized, the valves are actuated and push down against the channels. N horizontal control lines control $N!/(N!/2)^2$ vertical flow lines.[6] The device was designed to have a single valve system control both the nitrogen and oxygen channels simultaneously with control lines grouped into sets of complementary valve groups such that two channels, instead of one, are opened at a time. Therefore *16* vertical flow channels called for only *5* horizontal control lines. The algorithm defining the activation of the different combination of control lines is presented in Appendix A.

Variable Length Channel Network

This microfluidic device works on the principle of flow resistance, larger channel lengths impose a large resistance on the flowing gas causing the gas's flow rate to decrease. Controlling the amount of resistance the gas experiences, controls the flowrate at which the gas travels.

Each channel in the network has a different length pattern resulting in a unique resistance. Different resistance ratios correspond to different flow rate ratios of nitrogen to oxygen. When a nitrogen feed is opened along side an oxygen feed the different resistances allow for a specific oxygen to nitrogen ratio to be achieved.

Determination of Volumetric Flow Rate and Fluidic Resistance

Equivalent resistance circuits were used in the microfluidic calculations, where Ohm's law and Kirchhoff Rules are applicable. The analogous fluidic model is as follows: hydrodynamic flow resistance corresponds to electric resistance, the flow velocity corresponds to electric current, and the pressure drop over a channel is equivalent to the voltage drop across a wire. The flow resistance was calculated in a similar fashion to that used in electric circuit theory.

Ohm's Law applied to microfluidics generates an equation relating resistance, flow rate, and pressure drop. Navier Stokes simplifies into a relation for fluid velocity, which when integrated over the cross-sectional area, results in the volumetric flow rate. An equation for channel resistance results, when this flow rate is substituted into the microfluidics version of Ohm's law.

Using Kirchoff Rules, the pre-set pressure and the calculated resistances were used to determine the final flow rates. To reduce error in the final gas flow rate and VL channel resistance calculations, the channels preceding the VL channels were designed to have a resistance at least one order of magnitude lower.

System: Characterization of Flow

The direction of fluid movement through the microchannels is defined as the x direction and the flow is modeled as steady, parallel, and one dimensional pressure driven

flow. The one dimensional parallel flow assumption is valid, given that the velocity components in the y and z direction are small compared to fluid velocity through the channel. The Reynolds numbers, ratio of inertial forces to viscous forces, calculated for the VL channels ranged from *0.006* to *0.015*, below the critical value of $Re = 1$; therefore the flow is considered laminar.

Navier-Stokes Equation

The Navier-Stokes equations, derived form viscous surface force, stress/strain constitutive equations, and Newton's second law of motion, provides the equation of linear momentum for an incompressible viscous fluid,

$$\rho \frac{D\vec{v}}{Dt} = -\nabla P + \rho\vec{g} + \mu \nabla^2 \vec{v}. \tag{1}$$

For a one-dimensional system in the x-direction, the equation is as follows:

$$\rho\left(\frac{\partial v_x}{\partial t} + v_x \frac{\partial v_x}{\partial x} + v_y \frac{\partial v_x}{\partial y} + v_z \frac{\partial v_x}{\partial z}\right) = -\frac{\partial P}{\partial x} + \rho\vec{g}_x + \mu\left(\frac{\partial^2 v_x}{\partial x^2} + \frac{\partial^2 v_x}{\partial y^2} + \frac{\partial^2 v_x}{\partial z^2}\right) \tag{2}$$

The time derivative term on the left hand side is eliminated given that the flow is steady, all non-linear terms are also eliminated since the flow is laminar, and the gravity term drops as gravity effects are negligible. Also, since the flow is considered to be fully developed, solutions should only depend on the x-coordinate setting. For this particular flow in the x-direction, the Navier-Stokes equation reduces to

$$\frac{\partial^2 v_x}{\partial x^2} = \frac{1}{\mu}\frac{dP}{dx} \tag{3}$$

where v is the velocity of the flow and μ is viscosity. The pressure gradient across the channel is constant for a steady state and was substituted as $-\Delta P/l$ where l is the length of the channel and ΔP is the pressure difference.

Integrating equation (3) twice results in the following relation

$$v_x = \frac{1}{\mu}\left[\frac{dP}{dx}\frac{x^2}{2} + C_1 x + C_2\right]. \tag{4}$$

Applying the "no-slip" boundary conditions:

$$v_x = 0 \text{ at } y = 0 \text{ and } v_x = 0 \text{ at } y = h, \tag{5}$$

the velocity distribution becomes

$$v_x = \frac{h^2}{8\mu}\left[\frac{dP}{dx}\left(4\cdot\frac{x^2}{h^2} - 1\right)\right]. \tag{6}$$

Derivation of Volumetric Flow Rate Equation (Resistance Equation):

For a rectangular channel with width *w*, height *h*, and $w \geq h$, the volumetric flow rate is calculated by integrating the velocity over the cross sectional and results in

$$Q = \frac{w\cdot h^3}{12\mu}\left|\frac{dP}{dx}\right|\left[1 - \frac{192\cdot h}{\pi^5\cdot W}\sum_{n=0}^{\infty}\frac{\tanh\left((2\cdot n+1)\frac{\pi\cdot w}{2\cdot h}\right)}{(2\cdot n+1)^5}\right] \tag{7}$$

Applying electric circuit theory, specifically the equivalent of Ohm's Law, the volumetric flow rate is written as:

$$Q = \frac{1}{R}\frac{\Delta P}{l} \tag{8}$$

R is the specific resistance and *l* is the length of the channel. The total resistance over the channel is $R_t = Rl$. Therefore the previous equation becomes

$$-\Delta P = R_t Q. \tag{9}$$

Combining equations (9) and equation (7), the following is obtained for fluidic resistance

$$\frac{1}{R}=\frac{w\cdot h^3}{12\mu\cdot l}\left[1-\frac{192\cdot h}{\pi^5\cdot w}\sum_{n=0}^{\infty}\frac{\tanh\left((2\cdot n+1)\frac{\pi\cdot w}{2\cdot h}\right)}{(2\cdot n+1)^5}\right]. \tag{10}$$

Resistance Ratio:

The ratio of resistances is analogous to the ratio of flow rates. Given the same input pressure for both the nitrogen and the oxygen,

$$-\Delta P_N = R_{t_N}Q_n \text{ and } -\Delta P_o = R_{t_o}Q_o, \tag{11}$$

the pressure drop across both systems is approximated as equal. Equating the pressure drop through the nitrogen and the oxygen channel, the following relation is obtained.

$$\frac{R_{t_N}}{R_{t_o}}=\frac{Q_N}{Q_o}, \tag{12}$$

where the ratio of resistances is equal to the ratio of flow rates.

Determination of channel lengths ratios:

Specific oxygen and nitrogen channel length combinations were determined using the resistance equation to obtain the resistance ratio needed for a specific oxygen concentration. First, an oxygen channel length with its respective calculated resistance was selected, and then iterations were executed to obtain the corresponding nitrogen channel length. A matlab program, included in Appendix B, was used to solve the resistance equation for these purposes. The channels in all the chips were 20 μm in height. The variable length channels in Chip B were 40 μm in width. The following chart contains the oxygen and nitrogen channel lengths, the corresponding resistances, the resistance ratios, and the measured concentration percentages for Chip B.

Oxygen Channel Length	Oxygen Channel Resistance	Nitrogen Channel Length	Nitrogen Resistance Length	Resistance Ratio	% p/p_0
19.58 mm	2.1946*10^13	9.08 mm	8.7851*10^12	5/7	69.03
6.09 mm	6.8260*10^12	3.50 mm	3.3863*10^12	2/3	61.13
11.30 mm	1.2666*10^13	13.00 mm	1.2578*10^13	1/2	47.09

Table 1: *Table of corresponding variable channel length to variable channel resistance and expected oxygen concentration.*

The same information for Chip C can be found in Appendix A, along with the valve system control algorithm.

Mixing Channel

Once the two gases pass through the channel network, they reach the mixing channel with the desired flow rates. The nitrogen and oxygen undergo diffusion and advection as they flow through the mixing channel.

Scaling Analysis

Transport equations can be difficult to solve directly for many real systems. The complexity of a system can be significantly reduced by addressing the notion that inherent spatial and time scales of a system constrain its behavior.[7] The comparison of processes using a single common scale provides a simple way to identify dominant or negligible processes. Applying dimensional reasoning, time and spatial scales can be determined and used to identify and predict the effects, significance, and order of magnitude of different system behaviors or parameters.

Fluid Transport

Mass transport occurs through a combination of advection and diffusion. Advection is the translation of the mass from one region to another. Diffusion, on the other hand, is the random and spontaneous net movement of particles from an area of

high concentration to an area of low concentration. The mixing channel's purpose relies on diffusion for passive mixing. Therefore, the diffusive contribution to mass transport must dominate in comparison to advection, in order for the moving mass to become well mixed. Scaling analysis was done in order to obtain estimates of an appropriate mixing channel length.

Scaling the Transport Equation

The diffusive and advective processes are additive and independent. When the total flux in the x direction including the advective and Fickian diffusion term is combined with conservation of mass, the advective diffusion equation results:

$$\frac{\partial C}{\partial t} + \nabla \cdot (uC) = D\nabla^2 C\,. \tag{13}$$

For the mixing channel, a two-dimensional system is considered in an x-y coordinate system where the time derivative term on left hand side vanishes given that the flow is steady. The length of the channel is defined as L and the width as w. Substituting the velocity in the x-direction as u and the velocity in the y-direction as v, the transport equation becomes

$$u\frac{\partial C}{\partial x} + v\frac{\partial C}{\partial y} = D\frac{\partial^2 C}{\partial^2 x} + D\frac{\partial^2 C}{\partial^2 y}\,. \tag{14}$$

In order to determine which process, if any, dominates, the magnitudes of the terms in the transport equation are compared by rewriting them in dimensionless form. First $\partial^2 C/\partial^2 x$ is eliminated, given that the flow in the x direction is considered constant and then dimensionless variables were substituted as follows

$$\frac{UC}{L}u^*\frac{\partial C^*}{\partial x^*} + \frac{VC}{w}v^*\frac{\partial C^*}{\partial y^*} = \frac{DC}{w^2}\frac{\partial^2 C^*}{\partial^2 y^*}\,, \tag{15}$$

where the dimensionless distance is $x^* = x/L$, the dimensionless concentration is $C^* = C/C_{max}$, the dimensionless x-component velocity is $u^* = u/U$, and the dimensionless y-component velocity is $v^* = v/V$. Equation *15* can further be divided by DC/L^2 to arrive at the dimensionless equation which generates the Peclet number. For the purposes of this analysis, the comparison between the advection term in the *x*-direction and the diffusion term in the *y*-direction suffices and is of interest. Setting up a comparison between the dimensionless constants preceding the derivative terms, the following realation of the advection and diffusion term results

$$\frac{U}{l} << \frac{D}{w^2}, \tag{16}$$

In order for mixing to occur, the diffusion term has to be much larger than the advection term. Substituting in the mixing channels dimensions and maximum expected velocity, the minimum length required for the gas mixture to be well mixed is approximately *0.5mm.*

The maximum velocity was calculated by modeling the entire channel configuration preceding the mixing channel using electric circuit theory. The equivalent resistance, flow rate, and pressure drop circuit was submitted to Ohm's law and Kirchhoff Rules, such that the flow rate at the beginning of the mixing channel was calculated. Given the cross sectional area of the channel, the flow rate was converted to velocity. In order to obtain the maximum velocity, the two shortest variable length channels were used in the calculation since they impose the smallest resistance.

Testing Area Exposure to Oxygen Concentrations

PDMS has one of the largest permeability coefficients of polymers to oxygen which makes it an ideal material for the purposes of this microfluidic chip. After the gas mixture flows through the mixing channel, it reaches the exposure compartment. The exposure compartment lies above the testing compartment; the two are separated by a thin PDMS film. Diffusion between the two layers occurs readily from the exposure compartment to the testing compartment. The convective mass transfer model mentioned in the previous section was used to characterize the mass transfer properties of the gas mixture in order to validate the appropriateness of the microfluidic design.

In order to guarantee a saturated bacterial compartment, the length at which the concentration boundary layer becomes fully developed must be much lower than the size of the compartment or the relative time scale of diffusion should be much lower than that of advection. The later can be assessed through the Peclet number, a dimensionless number which compares advection and diffusion measuring the ratio of the advective to diffusive mass transport, specifically the ratio of the advective and diffusive time scales. The Peclet number is defined as,

$$Pe = \frac{UH}{D_{O_2}}. \quad (17)$$

If $Pe <<< 1$, diffusion dominates. The area of the compartments is very large in comparison to that of the channels preceding and following it therefore the velocity of the fluid when flowing through the compartment is considerably lower than when flowing through the smaller channel areas. The Peclet number is clearly below 1 and therefore diffusion dominates.

Oxygen Sensing

A polymeric fluorescence-based oxygen sensor was integrated into the micro-channels. This sensor is an LED – based optical excitation and detection system capable of monitoring the oxygen concentration in the microfluidic chip. Oxygen detection is achieved through the excitation of the luminescent dye, Pt octaethyl-porphyrin-ketone (PtOEPK). This particular chemical was chosen not only because it exhibits an absorption peak of *590nm* in the visible spectrum and therefore high-intensity yellow LEDs can be used for excitation, but also because the dye has an emission peak at *760nm*, which is detectable by Si photodiodes. [5] It also exhibits long-term photostability.[8]

The Stern-Volmer equation for luminescent intensity describes the oxygen fluorescence quenching process observed in luminophores, in this case PtOEPK. Collisions between oxygen molecules and fluorophores in excited states lead to a non-radiative transfer of energy. The degree of fluorescence quenching relates to the frequency of these collisions and as a result is indicative of concentration, pressure, and temperature of the oxygen-containing media.[9] The Stern-Volmer equations for luminescent intensity are as follows

$$\frac{I_0}{I} = 1 + K_{SV}^{S}[O_2] = 1 + K_{SV}^{G} \cdot pO_2 \qquad (18)$$

Where I_0 is a reference value in the absence of oxygen and K_{SV}^{S} and K_{SV}^{G} are the Stern-Volmer constants for solution and gas, respectively.

The optical electrical apparatus for dynamic oxygen sensing used to monitor oxygen content in the microfluidic chip consisted of a yellow LED (590nm, 2500mcd, Ledtronic) placed over a sensor pad with a BG39 Schott glass (CVI Laser) filter in between. The Si photodiode (S2386-44k, Hamamatsu) was located below the sensor and

was shielded with an RG715 Schott glass long-pass filter. To amplify and filter the photodiode current signal, analog electronics were used. The signal was recorded in real time along with the reference LED signal using a USB DAQ card (PDM-1608FS, Measurement Computing). [5]

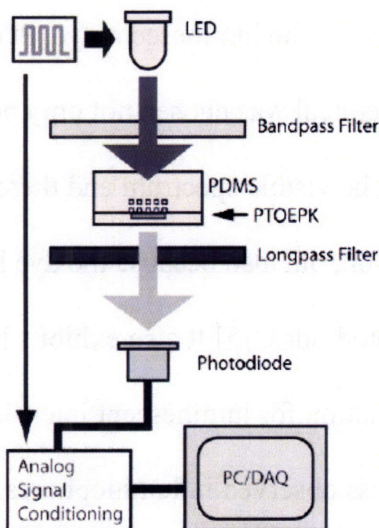


Figure 5: Schematic of the oxygen sensing apparatus displaying major components: LED, Bandpass Filter, PDMS chip with PtOEPK dye applied, Longpass Filter, Photodiode and the PC/DAQ.

Characteristic parameters are as follows: maximum sensor resolution varies between *120 ppb* across a range of dissolved oxygen concentrations ranging from *0* to *42.5 ppm* and the integrated oxygen sensor was accurate across a wide range of tested flow rates (*0.05 – 5 mL/min*). [5]

Plaque Bacteria and Experiment Theory

Bacterial Coagregation

"Coaggregation is a process where genetically distinct bacteria become attached to one another via specific molecules." [1] The adhesions have been shown to be highly specific and are typically mediated by adhesins proteins on one cell and saccharide

receptors on the other.[1] A current model of biofilm development is that successions of adhesion and multiplication events take place on the solid surface during formation.

It is thought that there are two ways coaggregation takes part in biofilm formation. One way is that single cells suspended in the moist or fluid environment, known as planktonic cells, specifically recognize genetically different cells on the developing film and adhere to them through coadhesion. Another form is that single cells coagregate while in suspension and then recognize and adhere to the developing film, also through coadhesion. [13,14] Within the oral cavity, both types are believed to be crucial for a regimented succession of bacterial adhesion events. This pairing of bacterial species within dental plaque result in mutually shared beneficial effects.[12]

Initial colonization of the pellicle on the tooth surface takes place predominantly by streptococci and gram-positive rods, followed by secondary colonization with gram-negative filamentatious anaerobes. [11,15] It has been found that primary colonizers can coaggregate with each other, but usually not with secondary colonizers. Although an exception to this accepted notion is Porphyromonas gingivalis, a secondary colonizer, which can coaggregate with Steptoccoccus gordonii, a primary colonizer. [11] Fusobacterium nucleatum is a secondary colonizer unable to coaggregate with itself. It is believed to be a bridge organism, because it can coaggregate with both primary and secondary colonizers. [10] It has also been found that anaerobic secondary colonizers need to be coaggregate to F. nucleatum in order to survive in the planktonic state.[1]

Chapter 4: Experimental Procedure

This chapter addresses the experimental work performed in the fabrication and testing of the three microfluidic devices described in chapter 3, Chip A, B, and C, followed by a description of potential procedures in the application of the device to study dental plaque biofilm formation.

Fabrication and Testing of Chip A

Chip A consists of two layers: the VL channel network layer and valve system layer above it. The valve system controls which channel is opened at the nitrogen input side and at the oxygen input side. The gases flow through these channels, meeting at the mixing channel entrance and continuing on through the chip. The purpose of this microfluidic device was to test the functionality of the valve system and to identify the optimal channel width for the VL channels.

Fabrication of Silicon Wafer Molds for the Microfluidic Chips

Photolithography techniques, used in the fabrication of the silicon wafer molds, consist of these main steps: wafer cleaning, photoresist application, soft baking, mask alignment, exposure and development, and hard baking.

In the first step the silicon wafers were cleaned using acetone, methanol, and isopropanol in order to remove particulate matter on the surface of the wafer, which could decrease adhesion between the photoresist and the silicon wafer. There are two types of photoresist: positive and negative. When a negative resist is applied to a silicon wafer and undergoes exposure to UV light, the resist in the exposed areas becomes polymerized and more difficult to dissolve. Therefore exposed areas remain on the

surface of the wafer, while the unexposed areas are removed by developer solution. The positive resists behave in the opposite manner.

SU-8(10) photoresist, a negative resist, was used to fabricate the silicon wafers used in the casting of the microfluidic chips. The resist was spin coated onto the wafer at an initial speed of *750rpm* and then ramped up to a speed of *150 rpm* for *40 s* to achieve the desired thickness of $20\,\mu m$, characteristic thicknesses at different spin speeds were found in the MicroChem Corp website.

In the next step, soft baking, the wafer was baked for *3 min* at $65\,^{o}C$ and for *7 min* at $95\,^{o}C$ to remove any remaining solvent, to stabilize the film, and induce the photoresist coating to become photosensitive. Particular care was taken with the baking time, given that over baking degrades photosensitivity by reducing developer solubility or destroying portions of the sensitizer and under baking prevents light from reaching the sensitizer.

For the mask alignment, the mask was aligned with the wafer such that the pattern was transferable onto the wafer surface. Transparency masks of the valve system and VL resistance network, inverse of the pattern to be transferred, were prepared using adobe illustrator and sent to Mikacolor Corp. for printing. An individual mask was then aligned with a *3'* silicone wafer and placed in a UV-light microscope. The exposure method used was contact printing where the resist-coated silicon wafer was brought into physical contact with a glass photomask. This contact during exposure allowed for very high resolution, although problems could result from trapped debris. The exposure time was *120 s*. The wafer was then placed on a $65\,^{o}C$ hot plate for 1 *min* and then on a $95\,^{\circ}C$ hotplate for *3 min*; the post-exposure bake continues the polymerization process. To

develop the film, PM acetate, a developer, was spin coated on to the wafer at over *200 rpm*. Finally the silicon wafer was baked for *2 hrs* to harden the photoresist and improve adhesion of the photoresist to the wafer surface.

Fabrication of PDMS microfluidic device [Chip A]

As mentioned in chapter 1, Chip A consists of the VL network and the valve system. The valve system PDMS layer was cast using soft lithography techniques, followed by slightly different techniques for the VL network channel PDMS layer.

Fabrication of Valve System PDMS Layer

Optically-transparent silicone rubber (polydimethylsiloxane (PDMS)), was used for the casting of the microfluidic chips. PDMS is a two component heat - curing system; when exposed to heat, the polymer hardens by cross linking of polymer chains. Preparation consisted of mixing the curing agent and the base at a *1:10* ratio, respectively, in a double axis centrifuge for *4 min*. The mixture was then poured onto the silicon wafer template contained within a petridish, placed in an excicator under vacuum for *15 min* for degassing, and then in an *80°C* oven for *2 hrs*. Curing at *80°C* makes the stamp shrink close to *2%*. To account for the shrinking factor, the masks were scaled by *1/102*. After baking, the stamp was pealed off from the template and it contained the channel configuration molded into it. A blade was used to cut the edges of the chips and a Harris Uni-Core – *0.75* hole puncher was used to punch holes through the chip at the input terminals of the valve control lines.

Fabrication of the Varaible Length (VL) Channel Network PDMS Layer

To prepare the middle VL channel network layer, a similar stamp procedure was followed. After mixing and degassing, the PDMS was spin coated onto the silicon wafer,

instead of being poured, at *2100rpm* for *50 s* to obtain a thickness of *40 μm*. The wafer was then allowed to stand for *15 min* while the PDMS settled and was then baked for *10 min*. The baking time for this step was monitored closely, given that over baking and under baking would compromise the proceeding step, alignment. Under baking causes excessive stickiness which makes alignment handling very difficult and damage to the channel area likely. On the other hand, the adherence to the valve system layer weakens if the PDMS hardens too much in over baking.

In order to attach and align the two layers correctly, a light microscope was used to aid in the magnification of the area to be aligned. The PDMS chip was then baked for an hour, pealed off, and holes punched in at the indicated input and output terminals. The chip was cleaned with isopropanol and dried with nitrogen gas to remove debris. Finally, it was covered with scotch tape for protection until the next step.

To seal the exposed channel configuration of the VL channel network, bottom layer, was plasma bonded onto a glass slide using a plasma preparation chamber. Both the PDMS chip and the glass slide were placed in the plasma preparation chamber with the channel etchings of the PDMS facing upward. The vacuum was activated until a pressure of *750 atm* was reached and then the oxygen supply was turned on at a medium setting for *30 s*. The PDMS chip was then carefully placed on the glass slide and baked for at least *6 hrs* or overnight. In the plasma preparation chamber, oxygen atoms are incorporated onto the PDMS surface making the PDMS hydrophilic and inducing the adherence between the micro-channel side of the stamp and the glass slip through strong ionic bonds.

Testing of PDMS microfluidic device (Chip A)

Parameters in the soft lithography fabrication process of Chip A were varied in order to observe valve performance and to optimize the valve system. Ensuring complete contact at valve restriction sites upon activation is important because incomplete contact leads to gas leakage which compromises the attainment of the target oxygen concentration.

Testing the Valve System in the PDMS Microfluidic Device

To test the functionality of the valve system, two parameters in the soft lithography fabrication process were varied: the *RPM* spin coating speed and the PDMS curing agent to base ratio. Five different *RPM* spin coat settings were used to make chips, as the rpm is increased the thickness decreases. The thinner the valve layer the easier for the valves to push down when activated, but layers too thin are susceptible to breakage, failure, and flaccid behavior. The chips were placed under a light microscope while the valve lines where activated to view the contact area of the restriction site. Activation consisted of filling the valve lines with fluid using a syringe or the Harvad syringe pump system.

Another parameter tested was the base to curing agent ratio. A larger ratio resulted in a gummier final texture of the cured polymer. Chips were made with the regular *1:10* ratio and a *1:20* ratio. The chips were placed under observation in the light microscope while valve activation took place to observe the effect of these changes on contact area.

Testing the VL channel Width in the PDMS Microfluidic Device

Once an optimized valve system was devised, the optimal channel width for the VL channels was studied. The VL network was designed with three different channel widths settings: *20*, *40*, and *50* μm, *20* μm being the minimum recommended working width. Characteristics under observation were the performance of the chip, the PDMS spin coating effects on layer integrity, and silicon wafer channel deterioration/continuity. A light microscope was used to study the resulting chips and the silicon wafers after each fabrication cycle, since flawed silicon wafers can lead to difficulties in bonding and proper valve line inflation. Chip A proved useful in determining the optimum design specs for the valve system and VL channel width.

Fabrication and Testing of Chip # B

Chip B consisted of only one layer, the VL channel network layer. This chip was designed to test the oxygen concentrations resulting from the variable length channel combinations. The fabrication and testing procedures for Chip B are as follows.

Fabrication of PDMS microfluidic device [Chip B]

The fabrication followed the same soft lithography techniques used for the valve system PDMS layer described in the previous section. The PDMS base and curing agent were prepared at a *1:10* ratio and poured onto the silicon wafer set inside a Petri dish. It was degassed and then baked for two hours.

To measure the oxygen concentration created by the variable length channel combinations, the oxygen-sensitive luminescent dye was incorporated onto the chip. A print out of the VL channel network layer, containing a schematic of the location of the dye compartment with respect to the other layer components, was placed under the glass

slide to indicate where the sensor dye had to be applied. The PtOEPK dye was prepared by dissolving *1 mg* of the dye in *1 mL of 5%* polystyrene in toluene. [5] The dye solution was then applied on the glass slide using a small pipette. A thin layer of the luminescent dye remained after the evaporation of the solvent and the edges were scratched off using a blade such that an appropriate sized square film remained. Acetone was used to clean the corners given that it has a negligible effect on the integrity of the dye.

This chip required no valve control or bacterial culture layer, therefore, the plasma boding step followed. The PDMS stamp and the glass slide with the PtOEPK dye were bonded in the plasma preparation chamber to enclose the channels following the same procedure described in the previous section. The chips were then baked over night.

The Optical Electrical Apparatus for Dynamic Oxygen Sensing:

An LED – based optical excitation and detection system was used to monitor oxygen content in the microfluidic chip. A yellow LED (590nm, 2500mcd, Ledtronic) was placed over a sensor pad with a BG39 Schott glass (CVI Laser) filter in between. A Si photodiode (S2386-44k, Hamamatsu) was placed below the sensor and was shielded with an RG715 Schott glass long-pass filter. To amplify and filter the photodiode current signal, analog electronics were used. The signal was recorded in real time along with the reference LED signal using a USB DAQ card (PDM-1608FS, Measurement Computing). [5]

Testing for Oxygen Concentration

Oxygen/Nitrogen Input

In the oxygen and nitrogen delivery scheme, gas tanks were connected to the microfluidic device using 20G steel tube segments connected to Tygon tubing, which in

turn were connected to a syringe pump (Harvard Pump 11, Harvard Apparatus), which provided a constant flow rate to the device. The gas flow into the chip was controlled by valves located on the syringes.

Oxygen Concentration Calibration and Measurement Procedure

Once the chip was connected to the nitrogen and oxygen delivery system, it was set up in the oxygen sensing apparatus. The PtOEPK dye film on the chip under study was placed below the LED. The offset error in the device was measured by blocking the sensor from the LED. This reading was subtracted from all future measurements to account for the error.

Calibration procedures for luminescent intensity versus oxygen concentration were performed by obtaining the measurements for the *0%* and *100%* oxygen references values. To provide these values, pure nitrogen and oxygen gas were separately allowed to flush the system for a minimum of *10 min*, in order for the system to stabilize, and then measurements taken.

Using the Stern-Volmer Equation for luminescent intensity and the two reference points, the Stern-Volmer constant and a calibration curve for the detection of oxygen concentration were obtained. This process was repeated for each of the microfluidic devices tested and in the case of Chip B, for all three of the PtOEPK films located on the microfluidic device. To obtain measurements, the nitrogen and oxygen gasses were flowed through the system at an input pressure of *10kPa*. A minimum flowing time of *10 min*. was carried out and any additional time needed for the sensor reading to equilibrate. Using the calibration curve the *mV* readings were converted to oxygen concentration measurements.

Fabrication of Chip C and Procedures Specific to Device Application

Chip C consisted of three layers: the bacteria culture layer, nitrogen/oxygen mixing layer, and the valve control layer. This chip is aimed at selectively building up biofilms on artificial tooth substrate located in the bacteria culture layer by dynamically imposing an array of oxygen concentration.

Fabrication of Valve System and Nitrogen/Oxygen Mixing PDMS Layer

The fabrication consisted of three lithography procedures, an oxygen sensor preparation, artificial tooth substrate incorporation, and plasma bonding. The same valve control and nitrogen/oxygen mixing layers used in Chip A were used on Chip C. Therefore the fabrication processes used are described in the Chip A fabrication section. After the preparation and alignment of the layers, the chip was baked for an hour, input and output holes were punched into the chip, and finally the chip was cleaned by washing with isopropanal, drying with nitrogen gas, and covering with scotch tape.

Fabrication of Bacteria Culture Silicon Wafer

AZ 50XT photoresist, a positive resist, was used to fabricate the silicon wafers used in the casting of the bacterial culture layer of the microfluidic chips. This fabrication process was used because the longer heating times produces a more stable surface profile and double coating with resist can have a larger effective area. This was needed, given the nature of the bacterial culture layer design which included a relatively large compartment area for the bacterial co-culturing.

For a positive resist, the resist exposed to UV light changes the chemical structure such that it becomes more soluble in the developer. The exposed resist is removed by developer solution. Transparency masks of the bacteria culture layer

containing an exact copy of the pattern which would remain on the wafer was prepared using adobe illustrator and sent to Mikacolor for printing.

AZ 50XT adhesion promoter was first spin coated on a chemically cleaned silicon wafer at an initial speed of *500 rpm* and then ramped up to *2100 rpm* for *30 s*. The spin speed of *2100 rpm* was preferred given that bubbles can be removed by high ramping speeds. The resist-coated wafer was then baked at *95 °C* for *2 min* and then at *115 °C* for *12 min*. A *45 min* delay time was carried out before the exposure to allow the wafer to cool. It was then exposed without a filter to UV light for *8* intervals of *20 s* and finally developed for *15 min*.

Fabrication of Bacteria Culture PDMS Layer

Using the silicon wafer prepared for the bacterial culture layer, the addition of the third layer onto the microfluidic chip followed. This layer was fabricated following a similar procedure to that used for the oxygen/nitrogen mixing layer. Previously prepared PDMS (*1:10* curing agent to base ratio) was spin coated onto the silicon chip at *2100rpm*, after which a *15 min* settling time was allowed. The PDMS covered silicon wafer was baked for *10 min* and then aligned with the previously prepared two layer chip using a light microscope. After baking for an hour, the chip was ready for holes to be punched out at the input and output terminals.

Finally, the chip was cleaned in order to proceed to the plasma bonding between the glass slide and the PDMS chip, which followed the same procedure described for Chip A. The final chip was then baked overnight.

Preparation for the Co-culturing of Bacteria in the Microfluidic Device

Artificial Saliva Preparation

Mimicking the environmental conditions of plaque ecosystems to the highest degree possible is critical in order for the model system to realistic and predict behavior present *in-vivo*. Nutrient conditions present in the oral cavity were simulated by the preparation and delivery of artificial saliva. The artificial saliva, basal medium mucin (BMM), prepared was based on the basal medium of Glenister et al. (1988). The ingredients were as follows: 0.5% Trypticase peptone [*5g/L*] (BBL, Becton Dickinson, MD); *1.0%* Proteose peptone [*10g/L*] (Oxoid, Unipath, Basingstoke, UK); *0.5%* yeast extract [*5g/L*] (Difco Laboratories, Detroit, MI); 0.25% KCl [*2.5g/L*]; *5mg/L* hemin; 1 *mg/L* menadione; and *0.25%* pig gastric mucin (BDH) [*2.5g/L*] (type III; Sigma Chemical Co., St Louis, MO). The suggested artificial saliva delivery is *3.6 mL/h* to each bacterial exposure area. [17]

Artificial Tooth Substrate Incorporation

Ninety-six percent of enamel, the outer surface of teeth, is made mainly of mineral, the primary one being hydroxyapatite. [16] Artificial teeth substrates can be prepared by combining *~5mg* of hydroxyapatite and 1mL solution of *5%* polystyrene in toluene.

Bacteria: Cultivation Methods and Procedures

The following bacteria *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum* subsp. *Polymorphum*, *Actinomyces viscosus* and *Steptococcus mutans* can be obtained through ATCC, The American Type Culture Collection. Product information sheets for the five bacterial organisms are provided in Appendix C. These sheets include the ATCC medium ingredients and preparation procedure, the growth conditions needed, biosafety level, and propagation procedure. The bacteria requiring

anaerobic growth conditions can be cultured using a BBL Gas Pack Anaerobic System, Becton Dickinson.

Chapter 5: Results, Discussion, and Conclusion

Valve System Optimization

Two system parameters in the lithography fabrication process for the valve system PDMS layer were varied in order to verify the functionality of the valves.

Spin Coating RPM

Batches of Chip A were fabricated using a range from *2100rpm* to the *2900rpm* spin coat speed. High rpm results in thinner layers which are more easily deformed when the valves are activated but may lack in strength. Low rpm results in thicker layers which are more difficult to displace. The maximum speed setting yielded the best restriction site contact area of all the devices, but still failed to achieve full contact from valve activation. In the following diagrams valve performance is shown for the respective spin coat speeds and the desired valve contact is shown in the lower right hand corner.

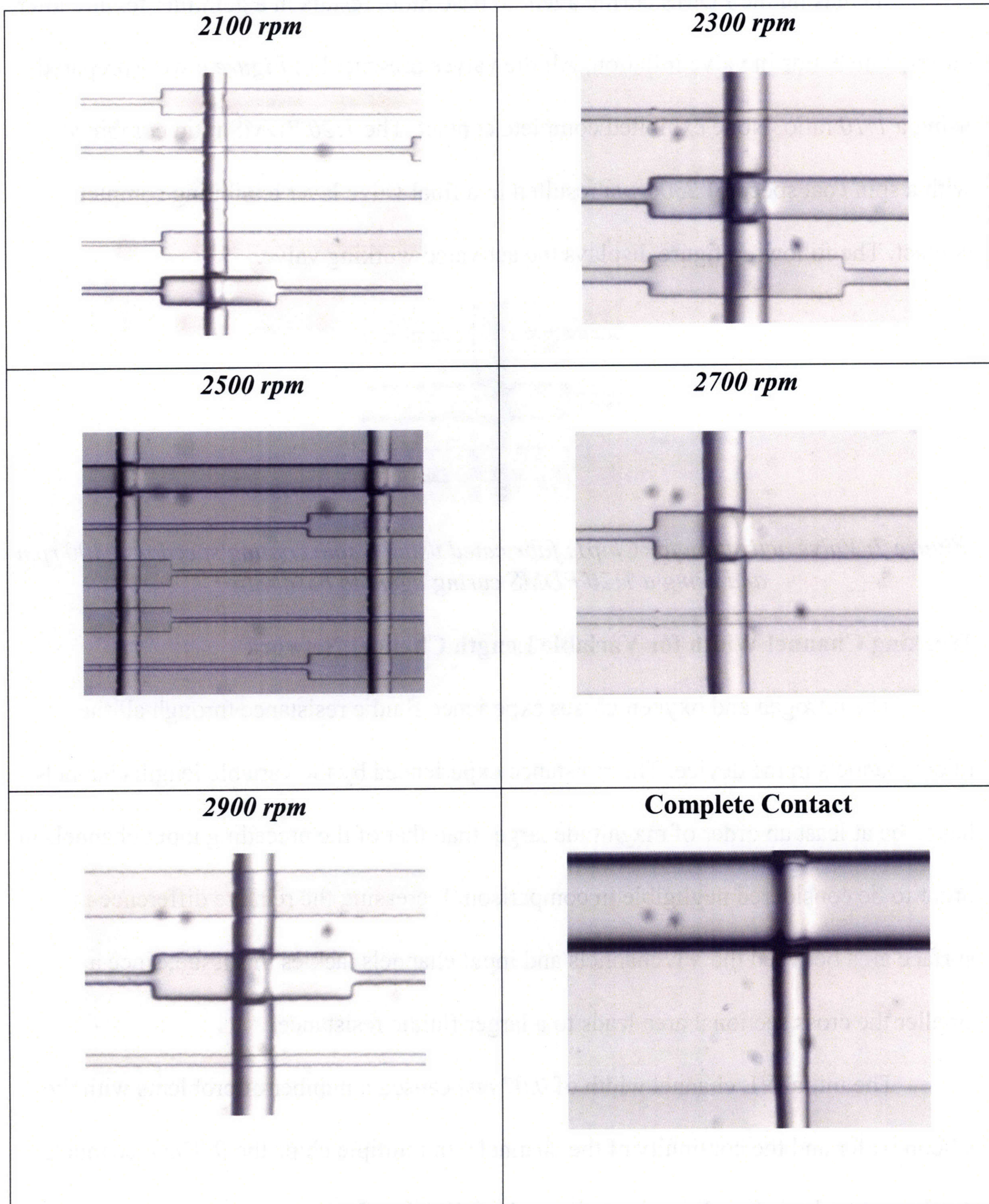


Figure 6: *Sample valve activation for different spin coat speeds used in the fabrication photolithography procedure. RPMs tested 2100, 2300, 2500, 2700, and 2900 rpm.*

PDMS Curing Agent to Base Ratio:

Increasing the PDMS curing agent to base ratio, results in a gummier texture after curing which aids in valve inflation. All the valves presented in *Figure 6* were prepared using a *1:10* ratio. None exhibited complete contact. The *1:20* PDMS ratio combined with a spin coat speed of *2300rpm* resulted in a final valve layer exhibiting complete contact. The following figure displays the activated working valve.

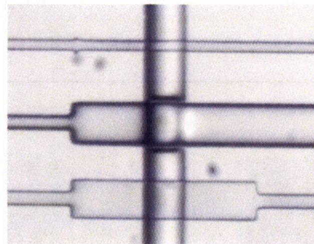


Figure 7: *Valve activation for Chip1: fabricated using a spin coating speed of 2300 rpm and using a 1:20 PDMS curing agent to base ratio.*

Working Channel Width for Variable Length Channel Network

The nitrogen and oxygen gasses experience fluidic resistance through all the microchannels in the device. The resistance experienced by the variable length channels has to be at least an order of magnitude larger than that of the preceding input channels in order to be considered negligible in comparison. Increasing the relative difference in surface area between the VL channels and input channels tackles this issue, since a smaller the cross sectional area leads to a larger fluidic resistance.

The initial VL channel width of *0.02 mm* caused a number of problems with the silicon wafer and the continuity of the channels. In multiple chips the *0.02mm* channels displayed breaks in the channel as seen in the following figure.

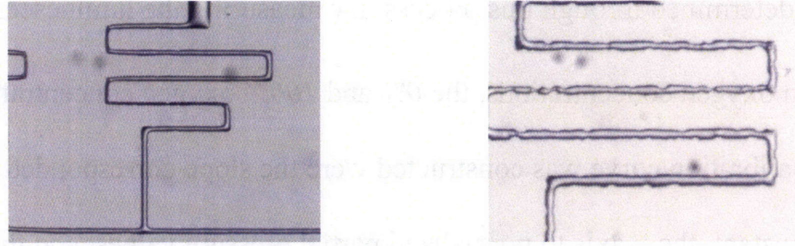


Figure 7: *Variable length channel width of 20 microns. Discontinuity in the channel is observed in both chips.*

Channel widths of *0.04mm* and *0.05mm* were tested as an alternative and both gave continuous channels in various iterations and no incongruities in the channels were observed. A width of *0.03mm* was not tested because of its proximity to *0.02mm*; a channel width that guarantees channel continuity is desired. *Figure 8* demonstrates images of both working channel widths.

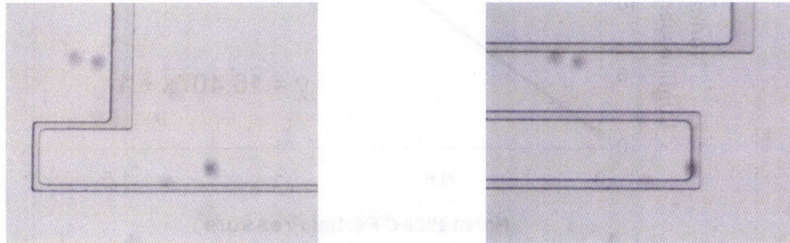


Figure 8: *The left image displays a variable channel width of 40 microns and the right image displays a variable channel width of 50 microns. No discontinuity is observed in either chip.*

Oxygen Concentration Results and Discussion

The Stern-Volmer equation, describing the oxygen quenching process occurring in luminescent dyes,

$$\frac{I_0}{I} = 1 + K_{SV}^{G} \cdot pO_2 \tag{18}$$

was used to measure the oxygen concentrations the devices produced. Each oxygen concentration procedure required calibration, because the Stern-Volmer constant for gas,

K_{SV}^{G}, was determined through this process. By measuring the luminescent intensity of two known oxygen concentrations, the *0%* and *100%* oxygen concentration reference values, a calibration curve was constructed were the slope corresponded to the Stern-Volmer constant, the x-axis to normalized partial pressure values, and the y-axis to the normalized measured luminescent intensity values. A sample calibration curve for the resistance channel combination producing a *50%* oxygen concentration mixture is shown below.

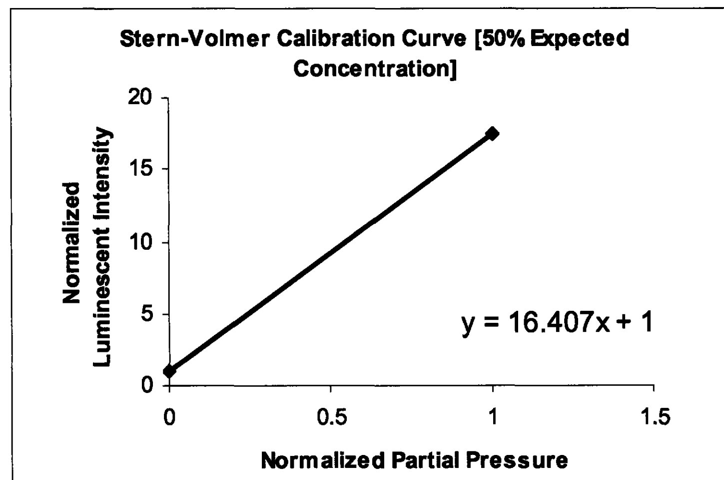


Figure 9: *Stern-Volmer calibration curve for the detection of gaseous oxygen in a 50% oxygen concentration environment. The slope represents the Stern-Volmer constant for gas in this particular calibration procedure.*

The normalized measured luminescent intensity values plotted in the respective calibration curve was used to determine the corresponding oxygen concentration, which resulted to be *47.01%* in this case. For the tested oxygen concentrations of *33%*, *50 %*, *66%*, and *77%*, the measured and expected concentration values are displayed in graph below.

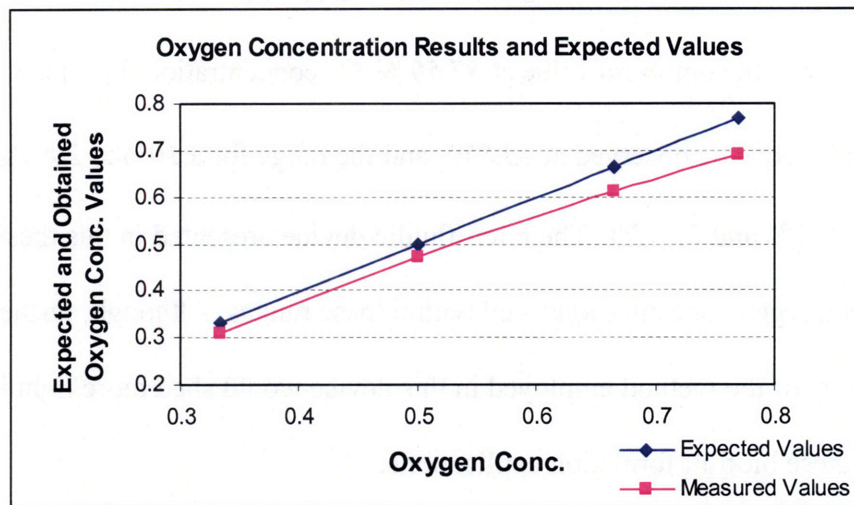


Figure 10: *Graph of expected and measured oxygen concentration obtained from four different combinations of variable channel lengths.*

Larger errors are observed at larger oxygen concentration due to the increased role of temperature and pressure vs. concentration effects on the number of collisions in the quenching process. Measured results for oxygen concentration varied $\pm 10\, in$ of the expected value.

This error in part is due to a number of factors including the truncation error in the USB DAQ card (D1-194RS, DATAQ Instruments), which has a resolution of *10-20mV*. Also the calibration's sensitivity to the *100%* oxygen reference value and measurement equilibration time. A 10 mV change in the measured reference value translates to an average *2.2%* change in the oxygen concentrations measured. Given that measurement equilibration varied $\pm 30mV$, an error as large as $\pm 8\%$ oxygen concentration can be expected, partly explaining the observed ± 10 oxygen concentration range.

Lam's uniculture experiments show optimum oxygen concentration ranges for the two bacteria to be quite large about the optimum value as shown in *Figure 11*. The channel numbers represent a seventh fraction with channel *1* corresponding to *0* oxygen concentration, channel *2* to *(1/7)* conc., channel *3* to *(2/7)* conc. and so forth. For the *A.*

viscosus, a *40%* to *65%* growth rate is observed between *42.85 %* and *100%* oxygen concentration with the optimum value at *87.50 %* O_2 concentration. For the *S. mutans* the optimum O_2 conc. is observed at *42.85%* and the range for a *10%* to *20%* growth rate is between *28.57%* and *71.42%.* The microfluidic device presented in this thesis would be able to target oxygen concentrations well within these ranges. Although, further testing on the accuracy of the method employed in this device would shed more light in its aptitude for these biofilm formation applications.

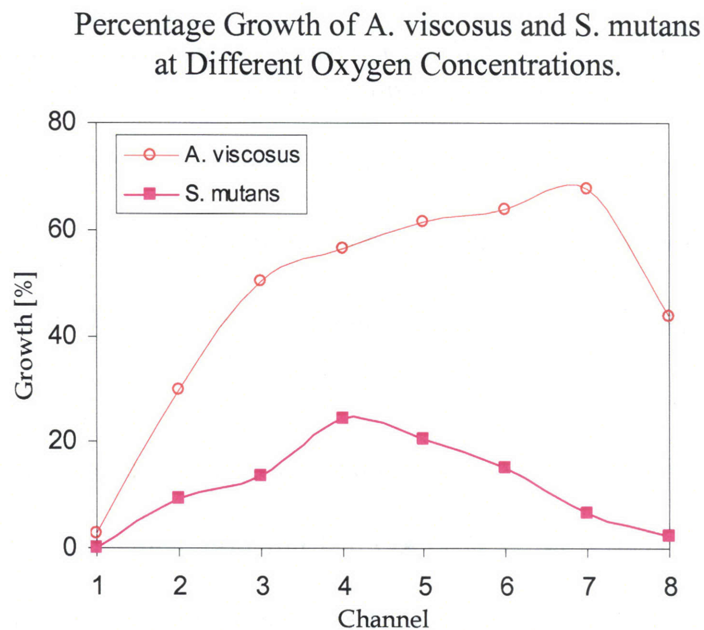


Figure 11: *Raymond Lam's uniculture experiment results. Numbering of channels in the x-axis correspond to (0, 1/7, 2/7, 3/7, 4/7, 5/7, 6/7, and 7/7) oxygen concentrations. The y-axis represents the % growth observed in both bacteria under these oxygen environments. Percentage growth was obtained by bacteria counting.*

Results are ambiguous as to if the device contains enough accuracy to tackle the biofilm formation feat to reveal useful results. An approach to improve the oxygen testing to further test the accuracy of the device would be to use phase detection instead of luminescent intensity to measure the oxygen concentration. The phase base approach of

luminescent lifetime is beneficial given that the lifetime is an intrinsic property of the PtOEPK molecule. This would eliminate the possibility of error associated with the intensity of incident light or differences in dye thickness or distribution. [5]. The luminescent intensity approach is susceptible to significant measurement errors when small changes in alignment of the sensor pad occur.

Concluding Remarks

This device design could allow for the regulation of oxygen levels in bacteria growth media flowing through the lower level channels, dynamically altering the conditions over time in the testing area. But the experimental results presented are ambiguous as to if the device contains enough accuracy to generate useful and accurate results. The current chip design in the Lam uniculture experiments provides an array of constant oxygen concentrations to individual testing areas. Further testing of the method presented for dynamic oxygen concentration control in-vitro is valuable given the potential benefits of using this method for the Lam dental plaque investigation, but also for a range of Lab-On-A-Chip applications and experimentation of cell behavior.

Appendix A

Variable Channel Lengths, Corresponding Resistances, and Expected Oxygen Concentrations [Chip C]

Oxy. Conc.	Oxy Length	Oxy Res.	Nit. Length	Nit. Res.
83.33%	960*10^-5	107.60*10^11	220*10^-5	21.285*10^11
71.40%	1942*10^-5	217.67*10^11	910*10^-5	88.044*10^11
66.67%	610*10^-5	68.372*10^11	350*10^-5	33.863*10^11
62.50%	810*10^-5	90.79*10^11	560*10^-5	54.181*10^11
41.17%	700*10^-5	78.460*10^11	1150*10^-5	111.26*10^11
44.44%	500*10^-5	56.046*10^11	730*10^-5	70.629*10^11
50.00%	1130*10^-5	126.66*10^11	1300*10^-5	125.78*10^11

Channel Valve Configuration [Chip C]

Oxygen

Flow Channels	Combinations for Open Channel	
1	3+1	44.44%
2	4+2	66.67%
3	1+2	41.17%
4	1+4	62.5%
5	3+4	83.33%
6	2+5	50%
7	X	X
8	3+2	71.4%

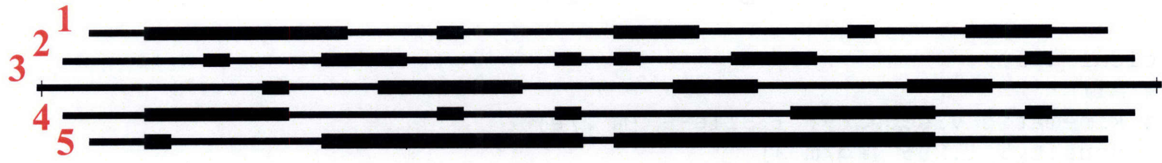
- No flow combination (5+4 and 5+1) (for 0% oxygen)

Nitrogen

Flow Channels	Combinations for Open Channel	
1		
2	2+5 and (5+4)	50%
3	1+2	41.17%
4	2+3	71.4%
5	1+3	44.44%%
6	1+4	62.5%
7	4+2	66.67%
8	3+4	83. 33%

- No flow combination (5+1) (for 0% nitrogen)

Valve System Diagram



Appendix B: Matlab Sript for Resistance Calculations

Resistance Calculation for Oxygen and Nitrogen

```
clear all;
% Air
% kinematic viscosity: 1.511e-5 [m^2/s]
% density: 1.205 [kg/m^3]
% viscosity: 1.73e-5 [Ns/m^2]

% Water
% kinematic viscosity: 1e-6 [m^2/s]
% density: 1000 [kg/m^3]
% viscosity: 1.0 x 10-3 [Ns/m^2]

% Nitrogen
% viscosity: 1.770029*10^-5

% Oxygen
% viscosity: 2.05056*10^-5

y = 2.05056e-5;

l = 350e-5;
w = 50e-6;
h = 20e-6;

r = 0;
for i = 0:1000,
    r = r + tanh((2*i+1)*pi*w/2/h)/(2*i+1)^5;
end
r = r*192*h/pi^5/w;
R = 12*y*l/w/h^3/(1-r)
```

Appendix C: ATCC Product Information Sheets for Bacteria

ATCC

Product Information Sheet for ATCC® 25175™

COLLECTION OF BACTERIA

ATCC NUMBER: 25175™

ORGANISM: *Streptococcus mutans*
Type strain. **Purified genomic DNA of this strain is available as ATCC® 25175D™.**

CITATION OF STRAIN:
If use of this strain results in a scientific publication it should be cited in that manuscript in the following manner: *Streptococcus mutans* ATCC® 25175™.

ATCC MEDIA:
#44 Broth: Brain Heart Infusion (BD 237500)
#44 Agar: Brain Heart Infusion Agar (BD 241830) or,
#260 Agar: Tryptic Soy Agar (BD 236950) with
5% Defibrinated Sheep Blood

CONDITIONS:
Temperature: 37°C
Atmosphere: Aerobic

BIOSAFETY LEVEL: 1
Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the following publication: *Biosafety in Microbiological and Biomedical Laboratories*, 4th ed. HHS Publication No. (CDC) 93-8395. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Washington DC: U.S. Government Printing Office; 1999. The entire text is available online at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm.

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Please see the enclosed Material Transfer Agreement (MTA) for further details regarding the use of this product. The MTA is also available on our Web site at www.atcc.org.

PROPAGATION PROCEDURE:
1. Open vial according to enclosed instructions.
2. Using a single tube of #44 broth (5 to 6 ml), withdraw approximately 0.5 to 1.0 ml with a Pasteur or 1.0 ml pipette. Rehydrate the entire pellet.
3. Aseptically transfer this aliquot back into the broth tube. Mix well.
4. Use several drops of the suspension to inoculate an agar slant and/or plate.
5. Incubate all tubes and plates at 37°C for 24-48 hours.

NOTES:
On #44 plates, two colony types may be seen: a) small, rough, irregular, white; and b) small, smooth, translucent.
Additional information on this culture is available on the ATCC web site at www.atcc.org.

REFERENCES:

1. Coykendall AL. Proposal to elevate the subspecies of *Streptococcus mutans* to species status, based on their molecular composition. Int. J. Syst. Bacteriol. 27:26-30, 1977.
2. Skerman VB, McGowan V, Sneath PH. Approved lists of bacterial names. Int. J. Syst. Bacteriol. 30:225-420, 1980.

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COLLECTION OF BACTERIA

ATCC NUMBER: 19246™

ORGANISM: *Actinomyces viscosus*

CITATION OF STRAIN:

If use of this strain results in a scientific publication it should be cited in that manuscript in the following manner: *Actinomyces viscosus* ATCC® 19246™.

ATCC MEDIA:

#44 Broth: Brain Heart Infusion (BD 237500)
#44 Agar: Brain Heart Infusion Agar (BD 241830)

CONDITIONS:

Temperature: 37°C
Atmosphere: 5% CO_2

BIOSAFETY LEVEL: 2

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the following publication: *Biosafety in Microbiological and Biomedical Laboratories*, 4th ed. HHS Publication No. (CDC) 93-8395. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Washington DC: U.S. Government Printing Office; 1999. The entire text is available online at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm.

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PROPAGATION PROCEDURE:

1. Open vial according to enclosed instructions.
2. Using a single tube of #44 broth (5 to 6 ml), withdraw approximately 0.5 to 1.0 ml with a Pasteur or 1.0 ml pipette. Rehydrate the entire pellet.
3. Aseptically transfer this aliquot back into the broth tube. Mix well.
4. Use several drops of the suspension to inoculate a #44 agar slant and/or plate.
5. Incubate the tubes and plate in an atmosphere of 5% CO_2 at 37°C for 48 to 72 hours.

NOTES:

Mature colonies are circular, convex, entire, off white, and opaque. This strain also grows well under anaerobic conditions.

Additional information on this culture is available on the ATCC web site at www.atcc.org.

REFERENCES:

1. Mycopathologia 1:81-87, 1938.
2. Gerencser MA, Slack JM. Identification of human strains of *Actinomyces viscosus*. Appl. Microbiol. 18:80-87, 1969. PubMed: 4896106
3. Georg LK, Pine L, Gerencser MA. *Actinomyces viscosus* comb. nov. A catalase positive, facultative member of the genus *Actinomyces*. Int. J. Syst. Bacteriol. 19:291-293, 1969.
4. Z. Augenheilkd. 79:477-510, 1932.

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Product Information Sheet for ATCC® 33238™

COLLECTION OF BACTERIA

ATCC NUMBER: 33238™

ORGANISM: *Campylobacter rectus*
Type strain. Deposited as *Wolinella recta.*

CITATION OF STRAIN:
If use of this strain results in a scientific publication it should be cited in that manuscript in the following manner: *Campylobacter rectus* ATCC® 33238™.

ATCC MEDIA:
#1539 Broth: Modified Chopped Meat Medium with Formate and Fumarate (*see below*)
#260 Agar: Tryptic Soy Agar (BD 236950) with 5% Defibrinated Sheep Blood

Ground beef (fat-free), 500.0 g
Distilled water, 1.0 L
1N NaOH, 25.0 ml
Mix meat, water and NaOH and bring to a boil with stirring. Cool to room temperature, skim fat from surface and filter, retaining both meat particles and filtrate. To filtrate, add sufficient distilled water to restore volume to 1.0 L. To this filtrate, add:

Trypticase (BD 211921), 30.0 g
Yeast extract, 5.0 g
K_2HPO_4, 5.0 g
0.025% Resazurin, 4.0 ml
Agar (*if desired*), 20.0 g
Boil and cool medium under 80% N_2-10% H_2-10% CO_2, and add:

Cysteine $HCl \cdot H_2O$, 0.5 g
Hemin Solution (see below), 10.0 ml
Vitamin K_1 Solution (see below), 0.2 ml
Adjust medium for final pH 7.0. Anaerobically dispense 7 ml medium into tubes containing meat particles (1 part meat to 5 parts fluid) under the same gas phase.

Vitamin K_1 Solution:

Vitamin K_1, 0.15 ml
95% Ethanol, 30.0
Store solution in brown bottle under refrigeration. Discard after one month.

Hemin Solution:

Hemin, 50.0 mg
1N NaOH, 1.0 ml
Distilled water to 100.0 ml
Autoclave at 121°C for 15 minutes.

[*Prepare a solution containing sodium formate and fumaric acid at a concentration of 6% each, adjust the pH to 7.0 and filter-sterilize. Prior to inoculation add 0.25 ml to a tube containing approximately 5 ml of the medium described above.*]

CONDITIONS:
Temperature: 37°C
Atmosphere: Anaerobic gas mixture, 80% N_2-10% CO_2-10% H_2

BIOSAFETY LEVEL: 1
Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the following publication: *Biosafety in Microbiological and Biomedical Laboratories*, 4th ed. HHS Publication No. (CDC) 93-8395. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Washington DC: U.S. Government Printing Office; 1999. The entire text is available online at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm.

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Please see the enclosed Material Transfer Agreement (MTA) for further details regarding the use of this product. The MTA is also available on our Web site at www.atcc.org.

PROPAGATION PROCEDURE:

1. Supplement medium to be used with formate and fumarate solution.

2. Open vial according to enclosed instructions.

3. Under anaerobic conditions, withdraw 0.5 ml of #1539

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broth from a single test tube (5 to 6 ml) and rehydrate the entire vial contents.

4. Aseptically transfer this aliquot back into the broth tube. Additional broth can be inoculated using 0.2 ml of this cell suspension per tube. A slant and a pre-reduced blood plate may also be inoculated with 0.1 ml each of the cell suspension. An aerobic blood plate may also be streaked to check for purity.

5. Incubate tubes and plate under anaerobic conditions at 37°C. Incubate second #260 plate aerobically at 37°C.

6. Within three to seven days, growth should be evident by slight turbidity and some sediment in the broth. Pinpoint translucent colonies will be formed on the anaerobic #260 plate. Some spreading occurs with additional incubation.

NOTES:

Three colony types may be seen: a) 1 mm in diameter convex variant; b) agar-pitting or corroding variant up to 5 mm in diameter; and c) spreading variant up to 5 mm in diameter. Any or all types may be recovered on primary isolation. No aerobic growth should be seen.

To observe growth, examine a wet mount of the broth under phase microscopy. The organism is a small, slender, straight sided rod with rounded ends and with a rapid darting motility. Motility is best observed in young cultures.

The cells do not Gram stain well using traditional procedures. To obtain the best results, use a basic fuchsin counterstain in place of the safranin.

Storage at liquid nitrogen temperatures, with 10% glycerol as the cryoprotectant, is recommended for long-tern preservation.

ANAEROBIC CONDITIONS:

Anaerobic conditions for transfer may be obtained by either of the following:

- Use of an anaerobic gas chamber, or
- Placement of test tubes under a gassing cannula system hooked to anaerobic gas.

Anaerobic conditions for incubation may be obtained by any of the following:

- Loose screw caps on test tubes in anaerobic chamber,
- Loose screw caps on test tubes in an activated anaerobic gas pack jar, or
- Use of sterile butyl rubber stoppers on test tubes so that an anaerobic gas headspace is retained.

Always use freshly prepared pre-reduced media or pre-reduced media that has been previously prepared but stored under anaerobic conditions. Resazurin in the media is a color indicator for anaerobic conditions. Observance of pink color in medium before use or during incubation shows anaerobic conditions have not been met and oxidation has occurred. Medium should be discarded.

Additional information on this culture is available on the ATCC web site at www.atcc.org.

REFERENCES:

1. Vandamme P, Falsen E, Rossau R, Hoste B, Segers P, Tytgat R, De Ley J. Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov.. Int. J. Syst. Bacteriol. 41:88-103, 1991. PubMed: 1704793
2. Tanner AC, Badger S, Lai CH, Listgarten MA, Visconti RA, Socransky SS. Wolinella gen. nov., *Wolinella succinogenes* (*Vibrio succinogenes* Wolin et al.) comb. nov., and description of *Bacteroides gracilis* sp. nov., *Wolinella recta* sp. nov., *Campylobacter concisus* sp. nov., and *Eikenella corrodens* from humans with periodontal disease. Int. J. Syst. Bacteriol. 31:432-445, 1981.
3. Badger SJ, Tanner AC. Serological studies of *Bacteroides gracilis*, *Campylobacter concisus*, *Wolinella recta*, and *Eikenella corrodens*, all from humans with periodontal disease. Int. J. Syst. Bacteriol. 31:446-451, 1981.
4. Lai CH, Listgarten MA, Tanner AC, Socransky SS. Ultrastructures of *Bacteroides gracilis*, *Campylobacter concisus*, *Wolinella recta*, and *Eikenella corrodens*, all from humans with periodontal disease. Int. J. Syst. Bacteriol. 31:465-475, 1981.
5. Guillot E, Mouton C. PCR-DNA probe assays for identification and detection of *Prevotella intermedia* sensu stricto and *Prevotella nigrescens*. J. Clin. Microbiol. 35:1876-1882, 1997. PubMed: 9196214
6. Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Wade WG. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl. Environ. Microbiol. 64:795-799, 1998. PubMed: 9464425
7. Wang B, Kraig E, Kolodrubetz D. A new member of the S-layer protein family: characterization of the crs gene from *Campylobacter rectus*. Infect. Immun. 66:1521-1526, 1998. PubMed: 9529076

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COLLECTION OF BACTERIA

ATCC NUMBER: 10953™

ORGANISM: *Fusobacterium nucleatum* subsp. *polymorphum*
Type strain. Deposited as *Fusobacterium polymorphum.*

CITATION OF STRAIN:
If use of this strain results in a scientific publication it should be cited in that manuscript in the following manner: *Fusobacterium nucleatum* subsp. *polymorphum* ATCC® 10953™.

ATCC MEDIUM:
#1490 Broth: Modified Chopped Meat Medium (*see below*)
For solid medium, add 2% agar.

Ground beef (fat-free), 500.0 g
Distilled water, 1.0 L
1N NaOH, 25.0 ml
Mix meat, water and NaOH and bring to a boil with stirring. Cool to room temperature, skim fat from surface and filter, retaining both meat particles and filtrate. To filtrate, add sufficient distilled water to restore volume to 1.0 L. To this filtrate, add:

Trypticase (BD 211921), 30.0 g
Yeast extract, 5.0 g
K_2HPO_4, 5.0 g
0.025% Resazurin, 4.0 ml
Agar (if desired), 20.0 g
Boil and cool medium under 80% N_2-10% H_2-10% CO_2, and add:

Cysteine HCl·H_2O, 0.5 g
Hemin Solution (*see below*), 10.0 ml
Vitamin K_1 Solution (*see below*), 0.2 ml
Adjust medium for final pH 7.0. Anaerobically dispense 7 ml medium into tubes containing meat particles (1 part meat to 5 parts fluid) under the same gas phase.

Vitamin K_1 Solution:

Vitamin K_1, 0.15 ml
95% Ethanol, 30.0
Store solution in brown bottle under refrigeration. Discard after one month.

Hemin Solution:

Hemin, 50.0 mg
1N NaOH, 1.0 ml
Distilled water to 100.0 ml
Autoclave at 121°C for 15 minutes.

CONDITIONS:
Temperature: 37°C
Atmosphere: Anaerobic gas mixture, 80% N_2-10% CO_2-10% H_2

BIOSAFETY LEVEL: 1
Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the following publication: *Biosafety in Microbiological and Biomedical Laboratories*, 4th ed. HHS Publication No. (CDC) 93-8395. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Washington DC: U.S. Government Printing Office; 1999. The entire text is available online at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm.

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Please see the enclosed Material Transfer Agreement (MTA) for further details regarding the use of this product. The MTA is also available on our Web site at www.atcc.org.

PROPAGATION PROCEDURE:
1. Open vial according to enclosed instructions.
2. Under anaerobic conditions, withdraw 0.5 ml of recommended broth from a single test tube (5 to 6 ml) and rehydrate the entire vial contents.
3. Aseptically transfer this aliquot back into the broth tube. A slant and a pre-reduced blood plate may also be inoculated with 0.1 ml each of the cell suspension. An

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aerobic blood plate may also be streaked to check for purity.

4. Incubate tubes and plate under anaerobic conditions at 37°C. Incubate blood plate aerobically at 37°C.

5. In 24 to 48 hours, growth is evident by turbidity in the broth. On agar surfaces, colonies are circular, with an entire margin, convex elevation, smooth surface and mottled appearance. No hemolysis occurs on blood agar. No growth should occur on the blood agar plate incubated aerobically. Cells are long thin rods with pointed ends.

ANAEROBIC CONDITIONS:

Anaerobic conditions for transfer may be obtained by either of the following:

- Use of an anaerobic gas chamber, or
- Placement of test tubes under a gassing cannula system hooked to anaerobic gas.

Anaerobic conditions for incubation may be obtained by any of the following:

- Loose screw caps on test tubes in anaerobic chamber,
- Loose screw caps on test tubes in an activated anaerobic gas pack jar, or
- Use of sterile butyl rubber stoppers on test tubes so that an anaerobic gas headspace is retained.

NOTES:

Always use freshly prepared pre-reduced media or pre-reduced media that has been previously prepared but stored under anaerobic conditions. Resazurin in the media is a color indicator for anaerobic conditions. Observance of pink color in medium before use or during incubation shows anaerobic conditions have not been met and oxidation has occurred. Medium should be discarded.

Additional information on this culture is available on the ATCC web site at www.atcc.org.

REFERENCES:

1. Ohio J. Sci. 53:141, 1955.
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COLLECTION OF BACTERIA

ATCC NUMBER: 25611™

ORGANISM: *Prevotella intermedia*
Type strain. Subgroup 4197; group A. Deposited as *Bacteroides melaninogenicus* subsp. *intermedius*.

CITATION OF STRAIN:
If use of this strain results in a scientific publication it should be cited in that manuscript in the following manner: *Prevotella intermedia* ATCC® 25611™.

ATCC MEDIUM:
#1490 Broth: Modified Chopped Meat Medium
For solid medium, add 2% agar to formula below.

Ground beef (fat-free), 500.0 g
Distilled water, 1.0 L
1N NaOH, 25.0 ml
Mix meat, water and NaOH and bring to a boil with stirring. Cool to room temperature, skim fat from surface and filter, retaining both meat particles and filtrate. To filtrate, add sufficient distilled water to restore volume to 1.0 L. To this filtrate, add:

Trypticase (BD 211921), 30.0 g
Yeast extract, 5.0 g
K_2HPO_4, 5.0 g
0.025% Resazurin, 4.0 ml
Agar (*if desired*), 20.0 g
Boil and cool medium under 80% N_2, 10% H_2, 10% CO_2, and add:

Cysteine HCl·H_2O, 0.5 g
Hemin Solution (*see below*), 10.0 ml
Vitamin K_1 Solution (*see below*), 0.2 ml
Adjust medium for final pH 7.0. Anaerobically dispense 7 ml into tubes containing meat particles (1 part meat to 5 parts fluid) under the same gas phase.

Vitamin K_1 Solution:

Vitamin K_1, 0.15 ml
95% Ethanol, 30.0 ml
Store solution in brown bottle under refrigeration. Discard after one month.

Hemin Solution:

Hemin, 50.0 mg
N NaOH, 1.0 ml
Distilled water to 100.0 ml
Autoclave at 121°C for 15 minutes.

CONDITIONS:
Temperature: 37°C
Atmosphere: Anaerobic gas mixture
80% N_2-10% CO_2-10% H_2

BIOSAFETY LEVEL: 2
Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the following publication: *Biosafety in Microbiological and Biomedical Laboratories*, 4th ed. HHS Publication No. (CDC) 93-8395. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Washington DC: U.S. Government Printing Office; 1999. The entire text is available online at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm.

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Please see the enclosed Material Transfer Agreement (MTA) for further details regarding the use of this product. The MTA is also available on our Web site at www.atcc.org.

PROPAGATION PROCEDURE:
1. Open vial according to enclosed instructions.
2. Under anaerobic conditions, withdraw 0.5 ml of the recommended broth from a single test tube (5 to 6 ml) and rehydrate the entire vial contents.
3. Aseptically transfer this aliquot back into the broth. Additional tubes may be inoculated with 0.5 ml each from the suspension. Also, 0.1 ml may be inoculated onto a slant. Streak several blood plates to check for colonial morphology and purity.

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4. Incubate tubes under an anaerobic atmosphere at 37°C. Incubate one agar plate anaerobically for colony formation, and one aerobically for aerobic contamination check.

5. Within 24-48 hours, growth should be evident by turbidity in the broth. On agar surfaces, colonies are pinpoint, circular, entire, convex, and black pigmented, no hemolysis. No growth should occur on the blood agar plate incubated aerobically.

ANAEROBIC CONDITIONS:

Anaerobic conditions for transfer may be obtained by either of the following:

- Use of an anaerobic gas chamber, or
- Placement of test tubes under a gassing cannula system hooked to anaerobic gas.

Anaerobic conditions for incubation may be obtained by any of the following:

- Loose screw caps on test tubes in anaerobic chamber,
- Loose screw caps on test tubes in an activated anaerobic gas pack jar, or
- Use of sterile butyl rubber stoppers on test tubes so that an anaerobic gas headspace is retained.

NOTES:

This organism requires the addition of Vitamin K_1 and Hemin to the media to obtain growth. See media section for details.

Always use freshly prepared pre-reduced media or pre-reduced media that has been previously prepared but stored under anaerobic conditions. Resazurin in the media is a color indicator for anaerobic conditions. Observance of pink color in medium before use or during incubation shows anaerobic conditions have not been met and oxidation has occurred. Medium should be discarded.

Additional information on this culture is available on the ATCC web site at www.atcc.org.

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ATCC **Product Information Sheet for ATCC® 33277™**

COLLECTION OF BACTERIA

ATCC NUMBER: 33277™

ORGANISM: *Porphyromonas gingivalis*

Type strain. Deposited as *Bacteroides gingivalis*. Quality control strain for bioMerieux Vitek products. **Purified genomic DNA of this strain is available as ATCC® 33277D™.**

CITATION OF STRAIN:

If use of this strain results in a scientific publication it should be cited in that manuscript in the following manner: *Porphyromonas gingivalis* ATCC® 33277™.

ATCC MEDIUM:

#1490 Broth: Modified Chopped Meat Medium (*see below*)
For solid medium, add 2% agar to formula below.

Ground beef (fat-free), 500.0 g
Distilled water, 1.0 L
1N NaOH, 25.0 ml
Mix meat, water and NaOH and bring to a boil with stirring. Cool to room temperature, skim fat from surface and filter, retaining both meat particles and filtrate. To filtrate, add sufficient distilled water to restore volume to 1.0 L. To this filtrate, add:

Trypticase (BD 211921), 30.0 g
Yeast extract, 5.0 g
K_2HPO_4, 5.0 g
0.025% Resazurin, 4.0 ml
Agar (*if desired*), 20.0 g
Boil and cool medium under 80% N_2-10% H_2-10% CO_2, and add:

Cysteine HCl H_2O, 0.5 g
Hemin Solution (*see below*), 10.0 ml
Vitamin K_1 Solution (*see below*), 0.2 ml
Adjust medium for final pH 7.0. Anaerobically dispense 7 ml medium into tubes containing meat particles (1 part meat to 5 parts fluid) under the same gas phase.

Vitamin K_1 Solution:

Vitamin K_1, 0.15 ml
95% Ethanol, 30.0
Store solution in brown bottle under refrigeration. Discard after one month.

Hemin Solution:

Hemin, 50.0 mg
1N NaOH, 1.0 ml
Distilled water to 100.0 ml
Autoclave at 121°C for 15 minutes.

CONDITIONS:
Temperature: 37°C
Atmosphere: Anaerobic gas mixture,
80% N_2-10%CO_2-10%H_2

BIOSAFETY LEVEL: 2

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the following publication: *Biosafety in Microbiological and Biomedical Laboratories*, 4th ed. HHS Publication No. (CDC) 93-8395. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Washington DC: U.S. Government Printing Office; 1999. The entire text is available online at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm.

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Please see the enclosed Material Transfer Agreement (MTA) for further details regarding the use of this product. The MTA is also available on our Web site at www.atcc.org.

PROPAGATION PROCEDURE:

1. Open vial according to enclosed instructions.
2. Under anaerobic conditions, withdraw 0.5 ml of recommended broth from a single test tube (5 to 6 ml) and rehydrate the entire vial contents.
3. Aseptically transfer this aliquot back into the broth tube. A slant and a pre-reduced blood plate may also be inoculated with 0.1 ml each of the cell suspension. An

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aerobic blood plate may also be streaked to check for purity.

4. Incubate tubes and plate under anaerobic conditions at 37°C. Incubate blood plate aerobically at 37°C.

5. Within 48 hours, growth should be evident by turbidity in the broth.

ANAEROBIC CONDITIONS:

Anaerobic conditions for transfer may be obtained by either of the following:

- Use of an anaerobic gas chamber, or
- Placement of test tubes under a gassing cannula system hooked to anaerobic gas.

Anaerobic conditions for incubation may be obtained by any of the following:

- Loose screw caps on test tubes in anaerobic chamber,
- Loose screw caps on test tubes in an activated anaerobic gas pack jar, or
- Use of sterile butyl rubber stoppers on test tubes so that an anaerobic gas headspace is retained.

NOTES:

Always use freshly prepared pre-reduced media or pre-reduced media that has been previously prepared but stored under anaerobic conditions. Resazurin in the media is a color indicator for anaerobic conditions. Observance of pink color in medium before use or during incubation shows anaerobic conditions have not been met and oxidation has occurred. Medium should be discarded.

On agar surfaces, colonies are pinpoint, circular, entire, and convex with no hemolysis. No growth should occur on the blood agar plate incubated aerobically.

Additional information on this culture is available on the ATCC web site at www.atcc.org.

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