# An oxygen-controlled *in vitro* model of the gastrointestinal human-microbiome interface

by

Steven John Holcomb

S.B., Mechanical Engineering, Massachusetts Institute of Technology (2016)
S.B., History, Massachusetts Institute of Technology (2016)

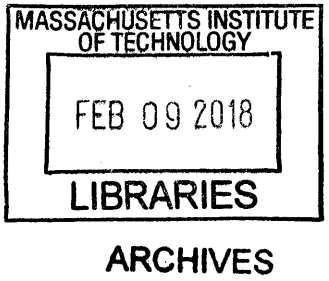
Submitted to the Department of Mechanical Engineering
in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Mechanical Engineering

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

February 2018



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## Abstract

The gastrointestinal system plays a vital role in the functioning of the human body, processing food into useable energy, controlling homeostasis, and serving as the front line of the immune system. The intestines are aided in their many functions by the gut microbiome, a collection of 100 trillion anaerobic bacteria cells that live inside the GI tract. Although they play an essential part in the organ system, they remain little-represented in *in vitro* gastrointestinal models because of the difficulty of replicating the anaerobic conditions of the intestines.

We constructed an *in vitro* model capable of growing aerobic epithelial intestinal cells along with anaerobic microbes in the same bioreactor. A device called the apical flow module seals a 12-well transwell and provides an inlet and outlet port into the apical chamber. Media is deoxygenated using nitrogen bubbles before it is pumped using a nitrogen-actuated pneumatic pump block. Microbes are injected into the anaerobic fluid through a rubber septum injection port before the fluid flows into the sealed transwell. Effluent is collected in sterile tubes at a controlled height so as to regulate the apical side pressure. Oxygen is provided to the basolateral human epithelial cells through basolateral circulation achieved using a pneumatic circulation plate.

Preliminary testing confirms our ability to control the oxygen in all parts of the system and to grow cocultures of human and bacteria cells. Epithelial cells grown in our bioreactor show signs of behaving more similarly to cells *in vivo* when exposed to the conditions present in our system, providing researchers with an oxygen-controlled gastrointestinal *in vitro* model.

Thesis Supervisor: David L. Trumper

Title: Professor of Mechanical Engineering

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## Acknowledgements

Throughout the course of working on this thesis, I was given a great deal of help from many people, to all of whom I am extremely grateful. First of all, David Trumper and Linda Griffith took me onto their project in the first place. Professor Trumper taught me about purposeful engineering design and carefully monitored my progress throughout my time on this project, providing useful feedback, asking focused questions, and instilling a rigorous approach to engineering that I hope to take with me. His passion for teaching is apparent through the time and effort that he devotes to his students, and I am a better engineer having worked under him.

Professor Griffith too has been incredibly supportive throughout the work, advising useful, targeted approaches to our questions and helping to understand the data that we acquired. Her determined approach to scientific research will help to ensure that valuable data can emerge from the work put in on this project.

Furthermore, none of the work described here would have been possible without the enormous efforts put in by Yu-Ja Huang. He devoted vast amounts of time and energy to advancing this project, running constant experiments even with the bulky, cumbersome hardware that we often had on hand, and his dedication to his work is inspiring. I will miss working closely with him, but I have no doubt that he will continue to be successful in his efforts to advance our understanding of the human-gut microbiome interface.

I would also like to thank Timothy Kassis, Dan Rathbone, Luis Soenksen, Brij Bhushan, and Collin Edington for their willingness and availability to help me throughout my time at MIT. I was fortunate to be able to work with people who made my time both productive and enjoyable.

The research was funded by grants sponsored by DARPA, NIH, and Boehringer Ingelheim.

## Contents

## List of Figures

## List of Tables

## Chapter 1: Gut Physiology and Literature Review

Laboratory research has the potential to cure some of humanity's most pervasive medical ailments, and it is progressing at a faster rate today than ever before. Scientists in university and private industry laboratories are prolific in investigating the human body and developing treatments for the many diseases that humanity faces. Research on human organ systems takes many forms, including animal models and *in vitro* testing using specialized organ models. *In vitro* models are particularly useful for researchers as they provide a great deal of flexibility in performing studies. Since these systems are not reliant on animal growth or human volunteers, scientists can grow cells taken directly from human bodies and subject them to wide-ranging stimuli to gather information about disease spread, drug efficacy, and the functions of different organs. Despite its presence in, quite literally, every human, one organ that still requires a great deal of exploration and attention from scientists today is the gastrointestinal system.

The human intestinal system is a vital part of the human body, processing food that we eat into useable energy and controlling homeostasis that ensures the body can continue to function even as external conditions change. A unique aspect of the intestines is that they contain the "human microbiome," a collection of approximately $3.8 * 10^{13}$ single-celled bacteria that live within the GI tract.[1] In fact, there is a roughly 1-1 parity of human cells that make up the body to bacteria living within it, and about 1 pound of a typical human's body weight comes from this vast legion of bacteria. Not merely passengers riding along with their human host, however, the bacteria of the microbiome actually serve numerous vital roles to their

host's body, aiding with the breakdown of food into energy and assisting the immune system of the human host.[2] Despite their presence and importance in the intestines, only recently have scientists been able to examine the microbiome closely. Research indicates a close relationship between the bacteria of the human microbiome and the functioning of organs as diverse as the liver and the nervous system, demonstrating that the trillions of bacteria living inside every human are indispensable to the survival of that human.

In spite of the importance of these bacteria, however, they continue to go unrepresented in most *in vitro* organ models that laboratories use to study the intestinal cells. It is difficult to work with the bacteria because many of them are anaerobic, meaning they either require a low oxygen environment to survive, or in the case of strict anaerobes, that they cannot survive except at very low oxygen levels, bordering on anoxia. These particular oxygen stipulations stand in contrast to every human cell which, being aerobic, require oxygen to function. *In vivo*, the body adapts to supply the human cells with oxygen via the bloodstream while depriving anaerobic bacteria of that same oxygen by forming a gradient across the GI tract walls. However, in a laboratory environment, the logistical hurdle of supplying human cells with oxygen while keeping anaerobic bacteria strictly deprived of it has proven challenging while researching the gut.

Our research seeks to provide scientists with an accurate *in vitro* model of the gastrointestinal system, capable of hosting anaerobic bacteria cells along with aerobic human cells, by creating hardware that controls both oxygen levels and flow rate across the cells under investigation.

## 1.1 Gut Physiology

The gastrointestinal (GI) tract is comprised of the organs dedicated to extracting energy from consumed food.[3] The tract is divided into two sections: the upper GI system contains the mouth, esophagus, and stomach, the organs that facilitate initial food consumption, while the lower GI, the small and large intestines, performs the majority of digestion.[4] Our gut model serves to replicate the conditions of the lower GI, which also hosts the gut microbiome.

### 1.1.1 Lower GI Anatomy

The intestines are a long, winding tube that extends throughout the body, connecting the upper GI to the anus. The bulk of the length of tubing is made up of the small intestine, also called the gut, which is the longest organ in the body and performs the majority of digestion.[4] Ranging from approximately 2-3 cm in diameter, the small intestine stretches for 3 m. As the stomach passes chyme, partially-digested food, into the intestine, it utilizes a process called peristalsis, waves of radially symmetric muscle contractions, to move the chyme downstream.[5] Bile alkalizes the acidic chyme as enzymes degrade the material into its constituent, usable macromolecules. These macromolecules are then absorbed through the surface of the small intestine. The surface is covered in thousands of finger-like structures called villi, which increase the surface area of the intestines, as well as a layer of mucus that serves as the front line of the immune system.[6]

The large intestine, also called the colon, stretches 2.5 m with a diameter of approximately 6 cm and facilitates water absorption. The mucus layer coating the large intestine is thicker than that in the small intestine, growing thicker still toward the distal colon.[4] Chyme may remain in the large intestine for as long as several days before passing out of the body.[7]

### 1.1.2 Gut Cell Types

Several different types of cells compose the environment of the intestines. Epithelial cells called enterocytes form the villi of the intestinal wall and facilitate absorption of nutrients.[7] Also making up the surface of villi are mucus-producing goblet cells. Beneath the surface of the villi, in a region called the lamina propria, cells including macrophages and dendritic cells support the gut's immune system. Figure 1-1 below provides a cross-section of the cell types forming a villus structure in the small intestine. Each of these human cell types will have a role in our *in vitro* model of the intestinal system.

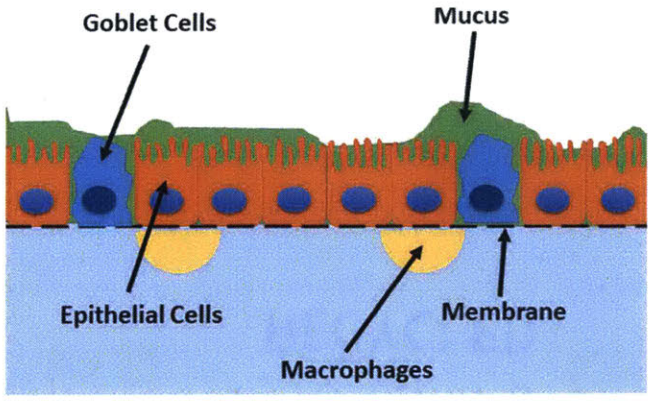


**Figure 1-1:** Cross-section of cell types comprising villus structure. The walls of the intestines are made up of enterocytes and goblet cells, which grow above immune cells including macrophages.

### 1.1.3 Gut Oxygen Gradient

An important and unique feature of the gut is the gradient in oxygen levels throughout the cells of the intestines. Cells receive their required oxygen from blood vessels throughout the lamina propria. This oxygen is consumed by the various submucosal cell types until the environment at the lumen is almost entirely anoxic at the midpoint of the lumen.[8],[9] Oxygen levels within the intestine range from 5 kPa partial pressure of oxygen at the pyloric sphincter down to essentially anoxic at the large intestine. The level of oxygen in the intestines facilitates the growth of anaerobic bacteria that comprise the gut microbiome, and control of oxygen levels is an essential part of our *in vitro* model. Figure 1-2 compares the oxygen gradients throughout the intestines. [10]

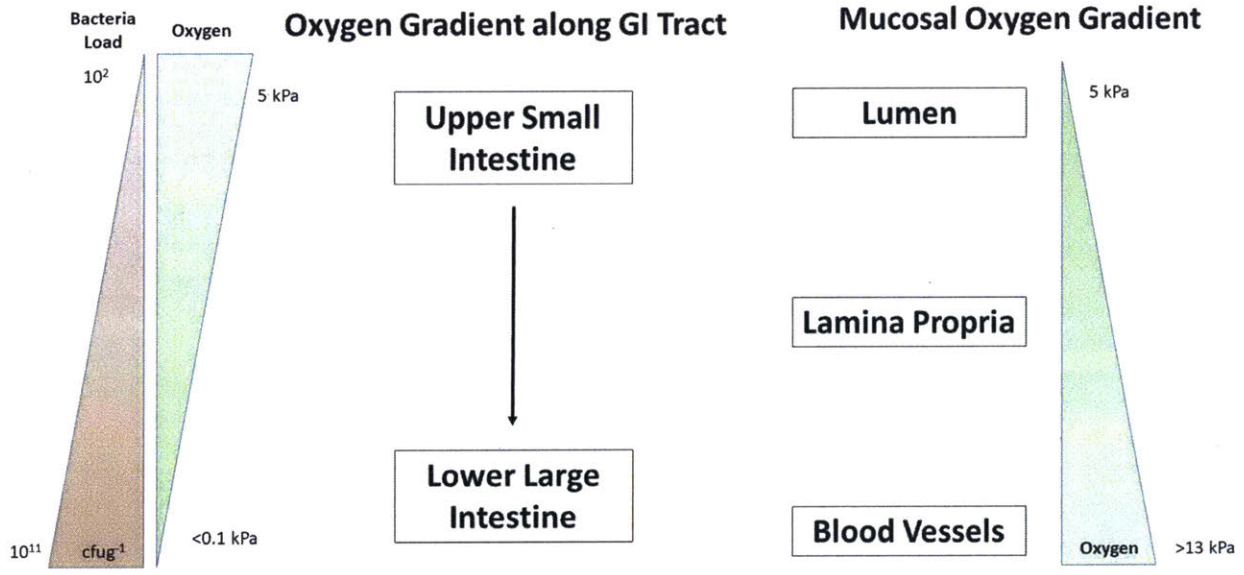


**Figure 1-2:** Oxygen and bacteria levels throughout the intestines. Note that the bacteria load is inversely proportional to the amount of oxygen present at any point in the intestines.

### 1.1.4 Gut Microbiome

Living within the confines of the lower GI tract are trillions of bacteria cells, thousands of different species, making up the gut microbiome.[1],[2],[3],[10] These bacteria cells aid the gastrointestinal system in performing its duties, and they may even be involved in the functioning of other organ systems throughout the body. Despite their vital role to the body, however, they are largely unrepresented in *in vitro* gastrointestinal organ models due to the difficulty in growing them along with human cells. The anaerobic conditions of the intestines, explained in section 1.1.2, mean that any bacteria growing within the GI tract must be anaerobic. Some of these anaerobic bacteria are facultative anaerobes, which can grow in aerobic or anaerobic conditions, but change their behavior based on which environment in which they exist. Others are strict anaerobes, which cannot survive if they are exposed to oxygen. In contrast, human cells are aerobic and require oxygen to survive. Whereas *in vivo* human cells can acquire their oxygen from the bloodstream in the lamina propria and create a gradient to prevent oxygenation of the lumen, for *in vitro* models no system exists that is fully able to create such a microenvironment. There are, however, numerous systems that exist to model other aspects of the intestines, as discussed below.

## 1.2 Literature Review

Various *in vitro* gastrointestinal models exist and helped to guide the development of our model. We will examine five different models, comparing their various strengths and

weaknesses in simulating the intestines and explaining the roles they played in informing the requirements of our own model:

1. Static Monoculture Model
2. Flow Monoculture Model
3. Flow Coculture Model without Mechanical Strain
4. Flow Coculture Model with Mechanical Strain
5. Oxygen-Controlled Monoculture Model

Finally, we examine the apical flow module, a device designed to be compatible with a standard transwell used in static monocultures to provide apical flow control, which will serve as a foundation for the oxygen-controlled *in vitro* model that we will present:

### 1.2.1 Static Monoculture Model

By far the most common conventional intestinal organ models use epithelial cells in transwells under static conditions. A monolayer of epithelial cells grown on a transwell membrane can be subjected to various tests, for example introducing drugs or other stimuli to measure cytotoxicity of the gut cells.[11] While simple to acquire materials and operate, especially using the ubiquitous transwell format, the model proves insufficient to provide a comprehensive gut model, failing to represent flow in the intestines, oxygen gradient across cell types, and the gut microbiome.

### 1.2.2 Flow Monoculture Model

To correct the drawbacks of the static monoculture model, some laboratories improve the intestinal mode by introducing flow, and therefore shear, to the system. One such model cultures cells in contained wells that are exposed to flow on one side.[12] Cells growing on that side of the channel experience wall shear stress, subjecting them to an environment more physiologically similar to that of cells *in vivo*. However, there is no method of inoculating bacteria in the system, nor is it oxygen controlled.

### 1.2.3 Flow Coculture Models

The importance of the gut microbiome in the functioning of the intestines is rapidly becoming more apparent to scientists, and some laboratories introduce bacteria into coculture models with human cells. One such model, displayed in figure 1-3, grows epithelial cells and microbes in separate chambers connected through a porous membrane.[13] The model exposes both cell types to fluid flow and allows exchange of nutrients between the membranes. However, the model lacks oxygen control, preventing growth of anaerobic microbes. Furthermore, the porous membrane separating the cell types prevents microbes from behaving as they would *in vivo*, adhering to mucus, which is absent from this model.

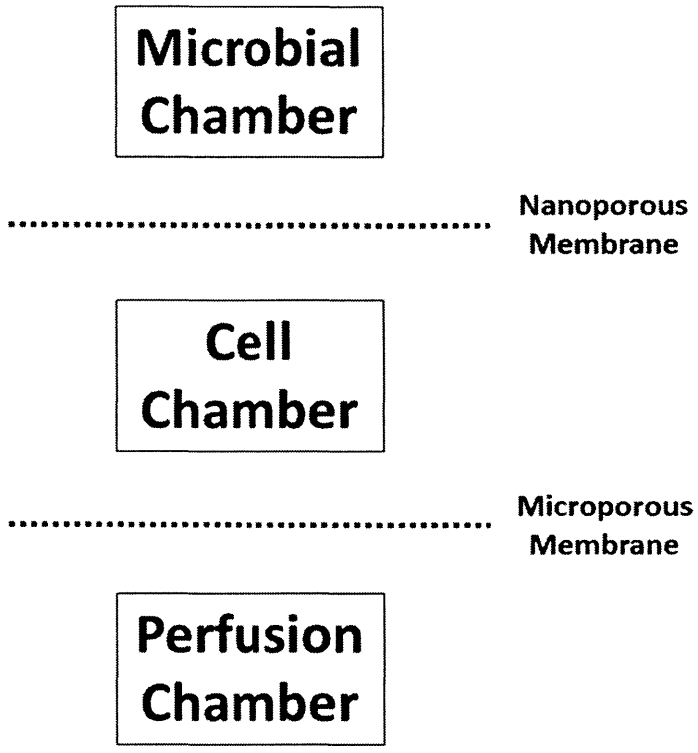


**Figure 1-3:** Coculture model to grow epithelial cells along with microbes. Porous membranes separate chambers that grow the different cell types.

Other *in vitro* models seek different approaches to replicate the conditions in the gut. Another model introduces peristalsis-like motion to mimic the movement of the gut.[14] This PDMS microfluidic chip uses two pneumatic channels surrounding a fluidic channel, as shown schematically in figure 1-4. The pneumatic channels are alternately subjected to vacuum, causing mechanical strain in the fluidic channel between them. Epithelial cells and microbes are seeded on one layer of a porous membrane, while media flows on both sides of the membrane. The model is designed to simulate multiple aspects of the intestinal system, however drawbacks still exist that prevent it from being fully representative. In particular, the model does not seal oxygen, so anaerobic microbes found in the intestines *in vivo* cannot

be grown. Furthermore, PDMS is highly absorptive, and therefore prevents drug studies from being possible to conduct onboard the platform.[15]

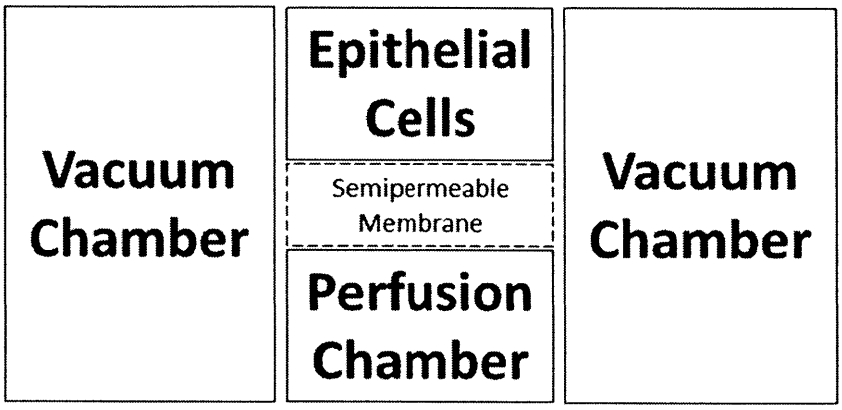


**Figure 1-4:** Flow coculture model featuring flow and mechanical strain subjected on both human and bacteria cells.

### 1.2.4 Oxygen-Controlled Flow Monoculture Model

Researchers recognize the need for oxygen control in creating accurate simulations of organ systems, and attempts have been made at creating such a system. One such model, shown in figure 1-5 below, flows oxygen-controlled media through a PDMS microfluidic chip, with normoxic media on one side and anoxic media on the other, thereby creating an oxygen gradient through cells grown between the channels.[16] Oxygen is removed from a portion of the media by bubbling inert gas through the media before flow, and a syringe pump draws the anoxic media through oxygen-impermeable tubing. Despite these steps, however, the model proved insufficient to entirely control oxygen, which leaked through the highly-permeable PDMS. Therefore, it cannot support anaerobic bacteria cells, nor can the PDMS chip permit drug studies to be performed on the device.

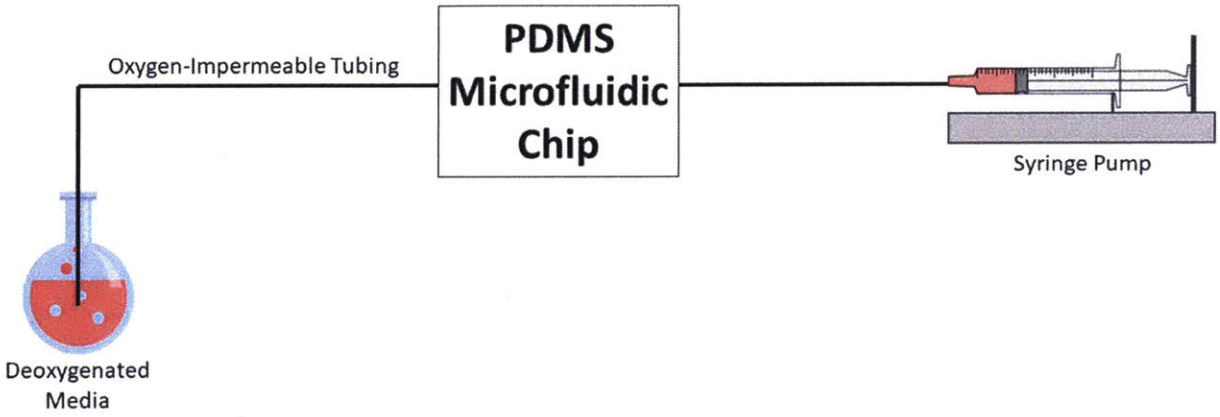


**Figure 1-5:** Oxygen-controlled microfluidic monoculture model. Oxygenated and deoxygenated media are flown on either side of a membrane, creating an oxygen gradient through the membrane.

We examined the abovementioned systems, analyzing the aspects about each that allows them to better simulate aspects of the gastrointestinal system. Our system draws from information presented by similar previous work, but innovates on solutions to some of the pressing challenges left unresolved by the prior art, in particular the ability to control oxygen and grow all of the necessary cell types to create a comprehensive *in vitro* gastrointestinal model.

## 1.3 The Apical Flow Module

A final existing model to examine, and the one that serves as a foundation for our oxygen-controlled system, is the apical flow module. Originally designed by Transon Nguyen in 2015 as part of his Master's thesis research, the apical flow module is a device that seals into a standard 12-well transwell (#3460, Corning Life Sciences, Tewksbury, MA) and

controls the fluid and oxygen to which cells growing in the transwell are exposed.[5] Figure 1-6 below displays the apical flow module as it fits into a transwell.

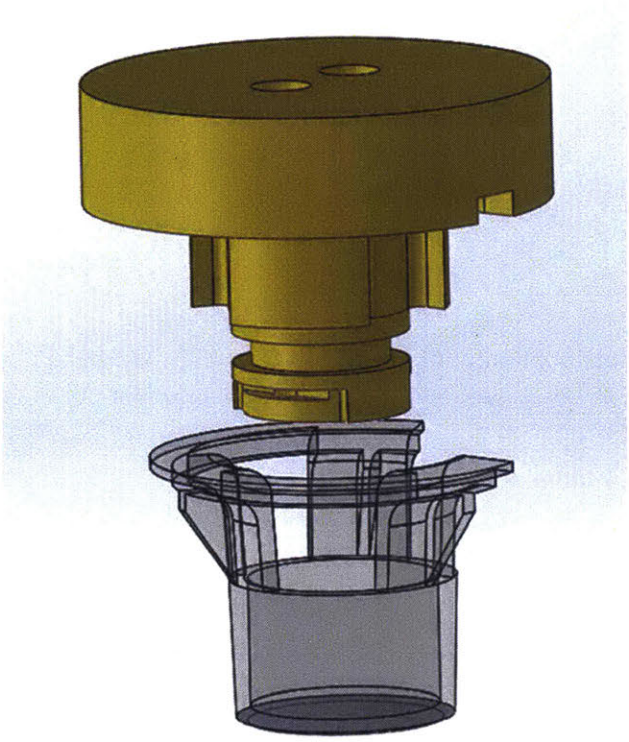

**Figure 1-6:** Apical flow module and 12-well transwell. The apical flow module seals over the top of the transwell and controls the fluid that enters and exits the transwell.

The apical flow module contains an inlet and outlet port leading into the apical chamber. An O-ring forms a radial seal in the transwell to seal fluid and gas exchange around the apical flow module. Using the module, the fluid to which cells are exposed can be controlled through the inlet and outlet ports, providing flow over a certain rate to simulate different parts of the intestines. Furthermore, the O-ring seal prevents oxygen exchange around the apical flow module, so oxygen-controlled fluid can flow through the module to create an anaerobic environment appropriate for a certain part of the intestines.

The module has been successfully applied to control flow over cells, but not to control oxygen. In our system, we utilize the apical flow module and construct a system around it capable of controlling flow and oxygen and introducing anaerobic microbes to recapitulate the microenvironment of the gastrointestinal system.

## Chapter 2: System Design and Configuration

The complex physiology of the gut requires a similarly comprehensive set of hardware in order to replicate it *in vitro*. To accomplish such a replication, we construct a system composed of several components that combine to provide functions, including fluid flow, oxygen control, and sampling, which allow us to create an accurate *in vitro* simulation of the gut. In the following sections, we analyze each of the individual components of the overall system, document their use, and discuss the design considerations that affect their creation.

We set out to design a device with the following functional requirements, each of them necessary to create a comprehensive oxygen-controlled *in vitro* gastrointestinal model:

- Oxygen control on apical and basolateral sides
- Flow control, including:
    - Programmable flow rates
    - Uniform flow over cells
- Introduction of anaerobic microbes
- Sterile assembly and operation

Figure 2-1 displays the overall configuration of the apical flow path, from pumping to effluent collection. The design and testing of the system was conducted in collaboration with Yu-Ja Huang.

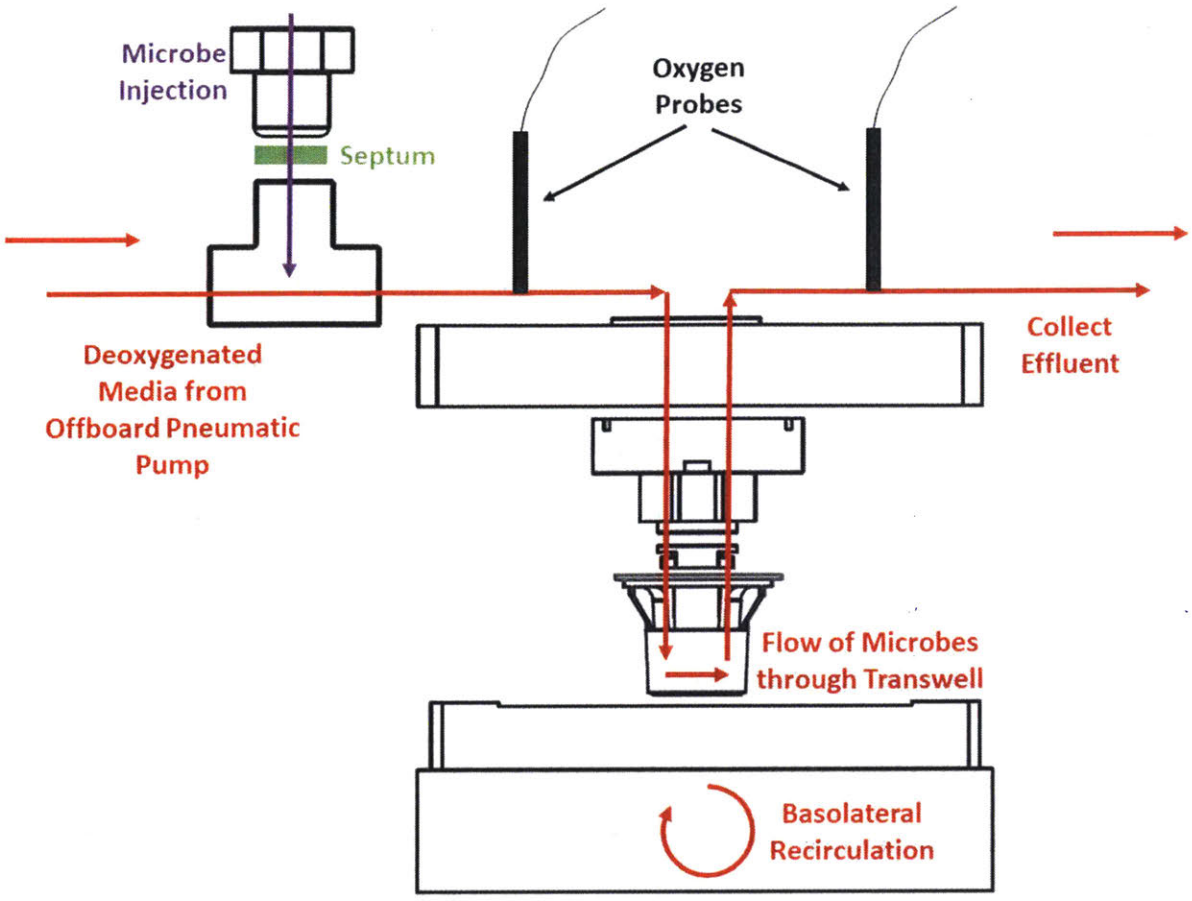


**Figure 2-1:** Schematic of system configuration showing fluid flow path through the apical module from pump to effluent collection.

## 2.1 The Gut Apical Flow Module

Central to our gut model is the apical flow module, a device that seals the bioreactor and facilitates the control of fluid and oxygen to which the cells are exposed, creating a microenvironment that is able to closely recapitulate the hypoxic domain in the lumen of the intestines. The device fits into a standard 12-well transwell (#3460, Corning Life Sciences, Tewksbury, MA) and seals above the membrane on the interior of the transwell. The first generation device was designed as a Master's thesis project by Transon Nguyen in 2015. [5]

### 2.1.1 Apical Flow Module Concept

As described in section 1.1.2, the intestines involve numerous types of cells, human and bacteria, working in tandem. An accurate in vitro model must also include a diverse array of cells, each of which require unique conditions to function properly. The *in vitro* gut model constructed in our lab uses a coculture of enterocytes and mucus-producing goblet cells grown along with bacteria from the gut microbiome, including the anaerobic *E. coli*, *B. fragilis*, and *B. theta*.[17] In the future, the basolateral side of the transwell will be seeded with macrophages from the immune system. In this manner, the apical side of the transwell will contain the cell types required to simulate the luminal environment of the intestines, and the basolateral side can simulate the circulatory system. As we design the hardware necessary to create such a biological environment *in vitro*, we keep the roles of the two sides of the transwell in mind. Figure 2-2 on the following page demonstrates the environment required for the *in vitro* gut model, including the various cell types and the flow of fluid on each side.

In order to create the microenvironment required for an accurate gut model within a 12-well transwell, we adapted an existing piece of hardware, the apical flow module, to seal the bioreactor.[5] Originally designed by Transon Nguyen in 2015, our apical flow module consists of a cylindrical polysulfone boss that extends into the transwell with a cap that mates with the top of the transwell to set the height of the boss above the transwell membrane. An ISO 3601 Standard size 012 Viton O-ring creates a radial seal between the inside of the transwell and the apical flow module, sealing the inner chamber of the transwell from the environment. Internal channels machined through the boss of the apical flow module provide

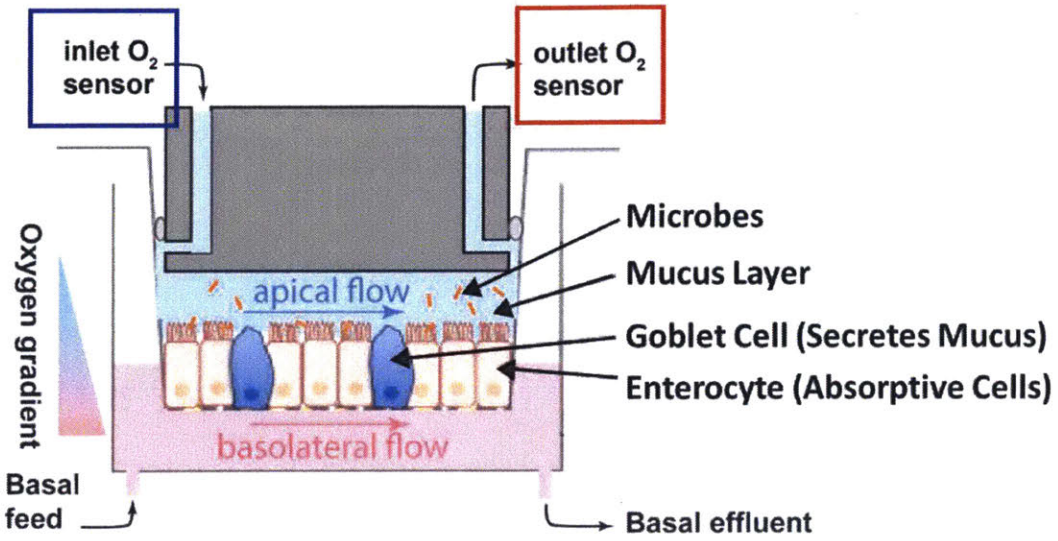


**Figure 2-2:** Physiology of *in vitro* gut model. Human cell types, including goblet cells and enterocytes, sit on the transwell, while bacteria cells grow in the apical media and in the mucus later on top of the human cells. Oxygen-controlled media is flowed over the apical side, while fully oxygenated media circulates on the basolateral side. Unpublished graphic reproduced with permission from Griffith lab.

an inlet and outlet for fluid to be provided to and removed from the sealed compartment. These channels are accessed through two 10-32 fittings machined into the top cap of the module, as shown in figure 2-3.

The apical flow module was initially conceived with two purposes in mind: 1) to provide flow, and 2) to control oxygen to which cells growing in a transwell are exposed. Through the inlet and outlet ports and oxygen-impermeable material and sealing capabilities, the module accomplishes both purposes, and provides a useful piece of equipment to aid the development of our *in vitro* epithelial human-gut microbiome model.

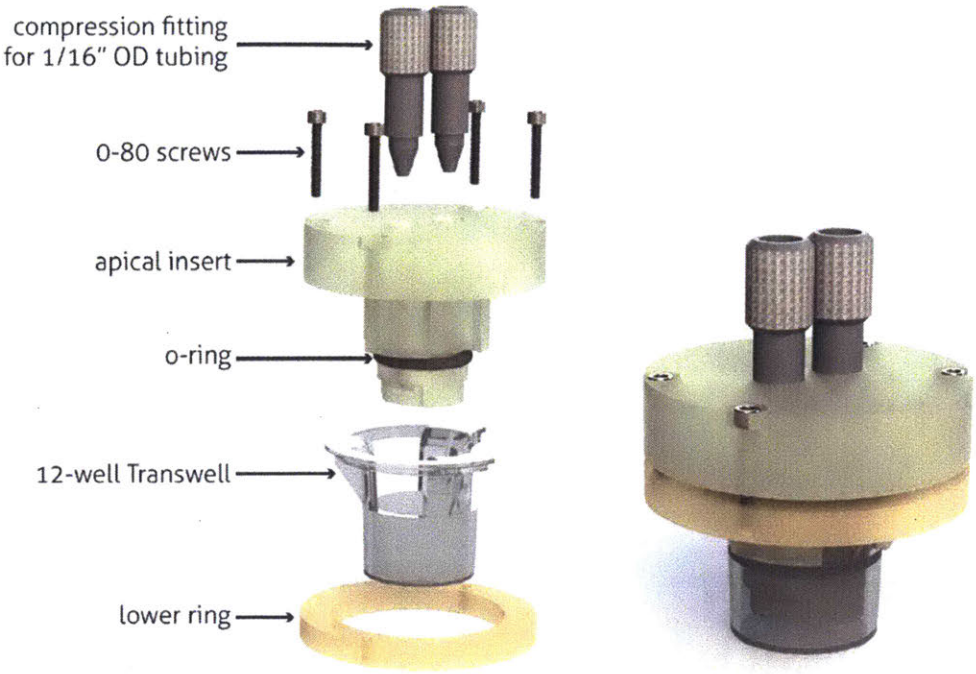


**Figure 2-3:** Components of the first generation apical flow module. Reproduced from [5] with permission from MIT.

### 2.1.2 Sealing

The first generation apical flow module uses an O-ring seal with an 11 mm x 1 mm silicone O-ring. However, the seal at times proved ineffective, and could bind on or shear onto interior features of the transwell and compromise sterility, so any future use of the device required a seal redesign. Figure 2-4 displays a cross-section of the current method of sealing, a 012 Viton O-ring seal.

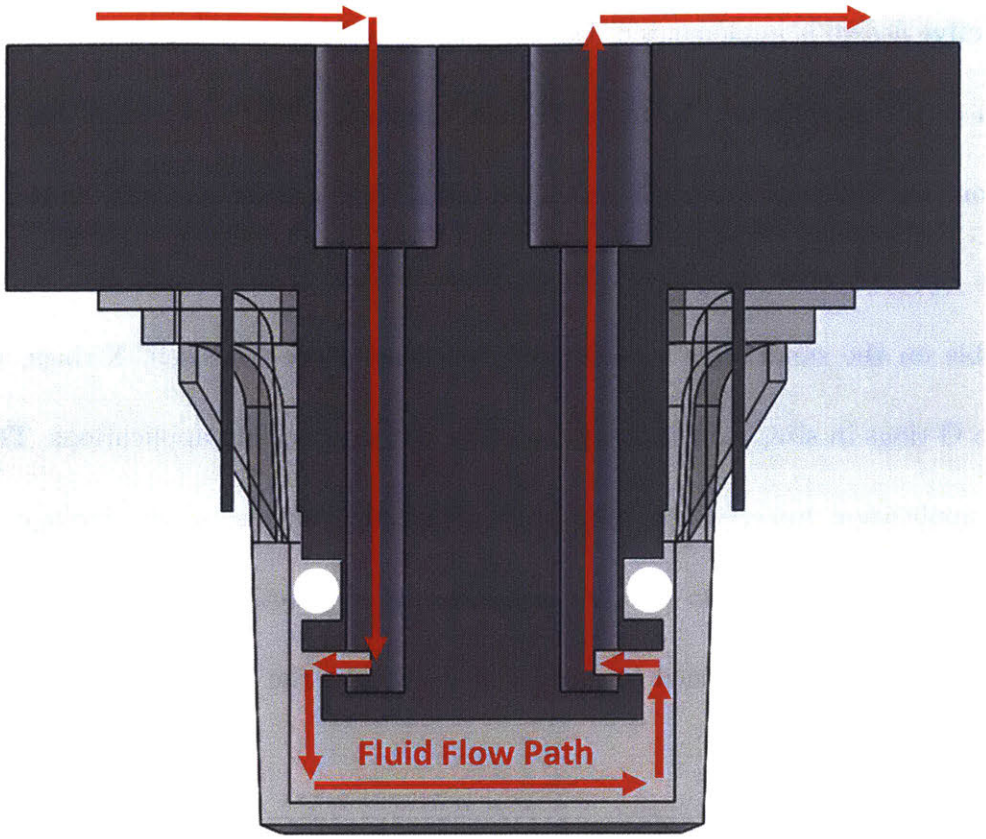


**Figure 2-4:** Cross-section of current O-ring seal. A 012 Viton O-ring seal applies a radial seal to the apical side of the transwell.

Seal Types

The apical flow module utilizes a radial seal, provided by an O-ring, to seal the inner well of the transwell, as seen in figure 2-4 above. An O-ring seal provides numerous benefits to the design: it is readily available, easy to assemble, and provides a reliable static seal to prevent liquid and gas from leaking into or out of the transwell.

However, an O-ring is not the sole type of seal that may work with the apical flow module, and alternate seal types are investigated as part of our design process to ensure the

most effective design is implemented. Designs such as a lip seal, spring-loaded face seal, or X-ring each earn consideration, however all have drawbacks that discourage their use in our application. Lip seals and face seals provide a radial sealing force necessary to connect to the transwell, but both have significantly larger cross sections than O-rings, and are impractical to assemble on the small scale mandated by the size of the transwell. X-rings, while more similar to O-rings in size, are primarily useful for dynamic sealing applications. They require a larger application force, which may have unintended effects on the biology within the transwell, and are worse than our O-rings in terms of practicality of assembly or sealing ability. Therefore, we determined to redesign and improve the O-ring seal.

### 2.1.3 O-ring Redesign

The first generation apical flow module seals with an 11 mm ID x 1 mm CS silicone O-ring that sits in an O-ring groove 0.925 mm wide and 0.561 mm deep. However, there are two drawbacks to this design. First, the material choice of silicone, while soft and compressible, and therefore easy to seal, is also highly permeable to drug absorption. Continuing with silicone as an O-ring material would limit the applications of the *in vitro* model, as drug studies could be skewed by absorption of the drug into the O-ring, rather than into the cells. Second, the O-ring sits far too shallow in the groove. A 1 mm cross section already being relatively small, the groove is required to be shallow so that the O-ring could contact both surfaces, the bottom of the groove and the inside of the transwell. However, the shallow groove proves insufficient to contain the O-ring, which has a tendency to shear along

the inner surface of the transwell and out of the groove upon assembly of the module. Such shearing compromises the sterility and fluidic control of the system, introducing unnecessary risks to any experiment conducted using the apical flow module. Furthermore, repeated shearing of the O-ring causes cuts to form in the silicone, further compromising sealing.

As shown in figure 2-5, we redesigned the O-ring groove to fit an ISO 3601 Standard size 012 Viton O-ring. Viton is a chemical-resistant fluoropolymer elastomer ideal for our applications due to both its resistance to drug absorption as well as its characteristic softness, measuring at a durometer of 60A, "soft" on the ASTM D2240 hardness scale.[18] The 012 O-ring, with an OD of 12.81 mm and CS of 1.78 mm, is significantly larger than the previous 11 mm ID x 1 mm CS O-ring. The redesigned O-ring groove is likewise larger, measuring 2 mm wide and 1.2875 mm deep, which provides enough space for the Viton O-ring to compress while remaining firmly within its groove.

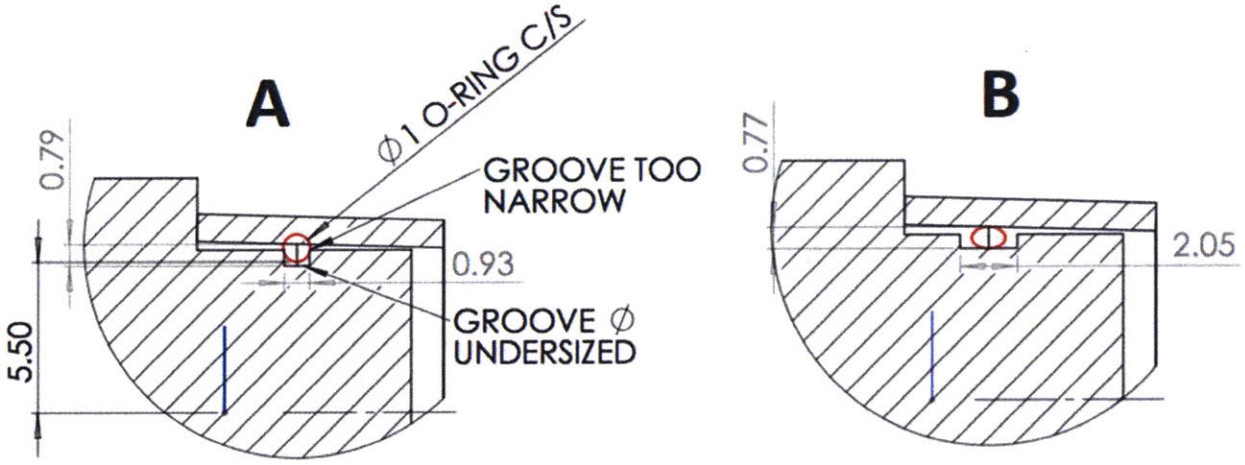


**Figure 2-5:** O-ring redesign. While the original O-ring sits in a groove too shallow and too narrow (**A**), a resized groove can effectively seat a different size O-ring (**B**).

The O-ring sizes are compared in an O-ring calculator provided by Apple Rubber Products Inc. (Lancaster, NY), which compares the gland size with the expected compression of the rubber O-ring. For the initial 11 mm x 1 mm O-ring design, the calculator predicts that the groove width provided is insufficient to fit the O-ring, and that the O-ring will not be able to stretch the required amount to properly seal the device. The redesigned O-ring, in contrast, is within the acceptable bounds recommended by the calculator. Figure 2-6 compares the first-generation O-ring design (**A**) with the redesigned O-ring (**B**). Note that the silicone O-ring shears out of its O-ring groove and along the wall of the transwell, whereas the Viton O-ring makes a consistent radial seal against the transwell.

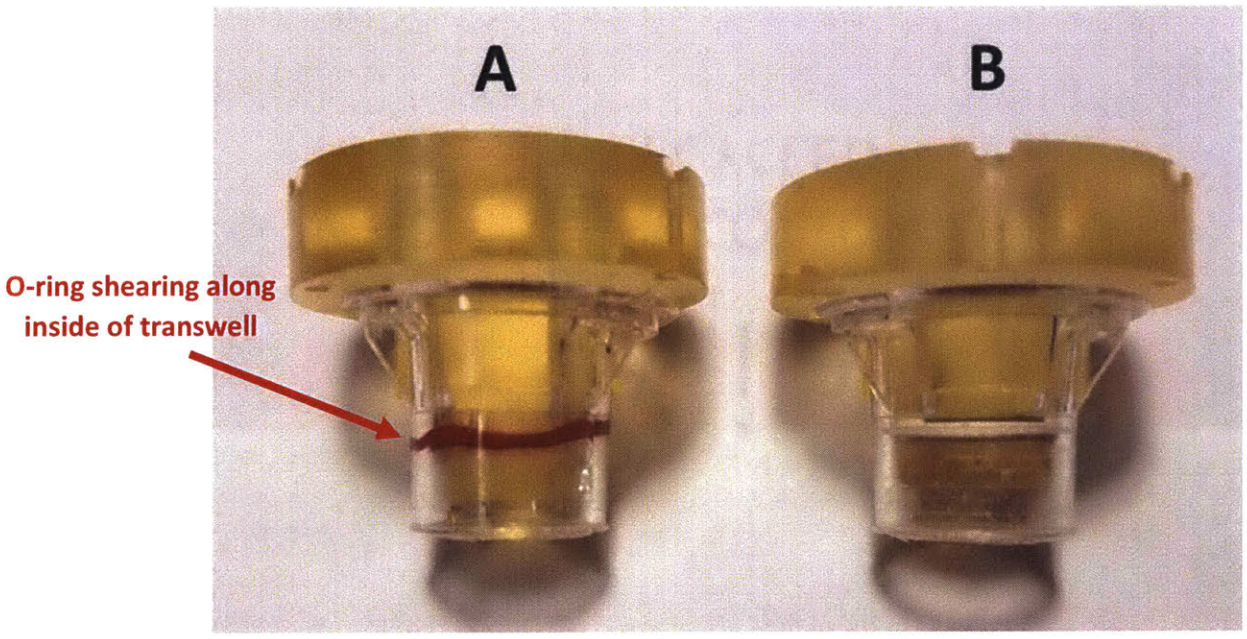


**Figure 2-6:** Comparison of first generation silicone O-ring (**A**) and redesigned Viton O-ring (**B**).[5] Note the shearing of the silicone O-ring out of the groove and along the side of the transwell, compromising sealing efficacy. The deeper-seated, thicker Viton O-ring makes a clean radial seal.

The devices containing redesigned O-ring grooves are tested under various flow conditions to determine the quality of the seal. The apical flow modules are inserted into a transwell with a sealed membrane, so all liquid enters and exits through the inlet and outlet ports on the module. The standard expected pumping rate of 10 μL/min is tested, along with rates far outside of the expected range, 0.1 μL/min and 100 μL/min. In all three cases, no leaking is observed, so the seal is determined to be reliable.

### 2.1.4 Material Choice

For any device to be used in biological applications, it is necessary to consider numerous aspects of the materials used; biocompatibility, gas control, and ability to be machined all earn attention as we proceed with designing the oxygen-controlled microfluidic system.

#### Biological Considerations

The apical flow module is machined out of polysulfone, a thermoplastic polymer that remains stable even at elevated temperatures required for autoclaving. An important requirement for each part of the system is that it be able to be sterilized to prevent growth of contaminants such as unwanted bacteria, yeast, or fungi. The preferred method of sterilization in biological applications is autoclaving, a process that elevates temperature and pressure inside of a pressure chamber to sterilize experimental components. Polysulfone is able

to withstand the high temperatures that autoclaving involves. Other materials considered, including polypropylene and acrylic, are not able to be autoclaved, so are much less desirable as materials for the module as long as it must be reusable.

An additional biological consideration involves the intended purpose for our system for drug testing. *In vitro* organ models can be applied to test preclinical drugs on human cells without risking adverse effects on human volunteers. The most common material for laboratory microfluidic systems, PDMS, is highly permeable to many types of drugs, so cannot be utilized in our device.[15] PDMS also allows oxygen to permeate through it, as indicated in the device examined in section 1.2.4. Polysulfone is not significantly drug absorptive or oxygen permeable. Other materials including stainless steel are able to be autoclaved and are resistant to drug and oxygen absorption, but are not as easily machined and are optically opaque, as outlined in the following section.

Machining Considerations

Besides biological considerations, the other major consideration made in selecting a material for the apical flow module is machinability. Polysulfone is a hard enough plastic that it does not easily crack when machined. A relatively small number of apical flow modules are required for our initial testing, on the order of 12-24 at a time. Because of this, the most expedient manufacturing option is machining each individual module, rather than a larger-scale manufacturing option such as injection molding or casting. Should the use of apical flow

modules become more widespread, we recommend transitioning the manufacturing from individual machining to molding or some other large-scale industrial manufacturing method.

### 2.1.5 24-well Scale-Down

In addition to the redesigned apical flow module compatible with a 12-well transwell, we also created a modified apical flow module to fit into a smaller 24-well transwell. The apical flow module is designed to seal into a standard 12-well transwell. With a surface area of approximately 1.12 $cm^2$, the transwell requires about $10^7$ cells to entirely fill the membrane, a full membrane being necessary so that the cell layer seals the fluid on the apical side of the transwell. While this transwell size is common in biological experiments, it can be difficult to acquire enough cells to entirely fill many transwells, especially when working with primary cells taken directly from human donors. At times it can be beneficial to divide the cells being used in an experiment into multiple smaller transwells, creating a larger number of replicates.

We therefore designed a scaled-down apical flow module compatible with a standard 24-well transwell, which has a surface area of 0.33 $cm^2$ and requires only 29% of the number of cells in a 12-well transwell to entirely fill and seal the membrane, allowing scarce primary cells to be separated into more testing replicates. The new design is similar to that of the 12-well apical flow module, with an extruded boss extending into the transwell at a height set by a cap contacting the top of the transwell. Inlet and outlet ports extend into the module,

allowing tubing to be connected using ferrules and fittings. A cross-section of this module along with the path of fluid flow through the device is shown in figure 2-7.

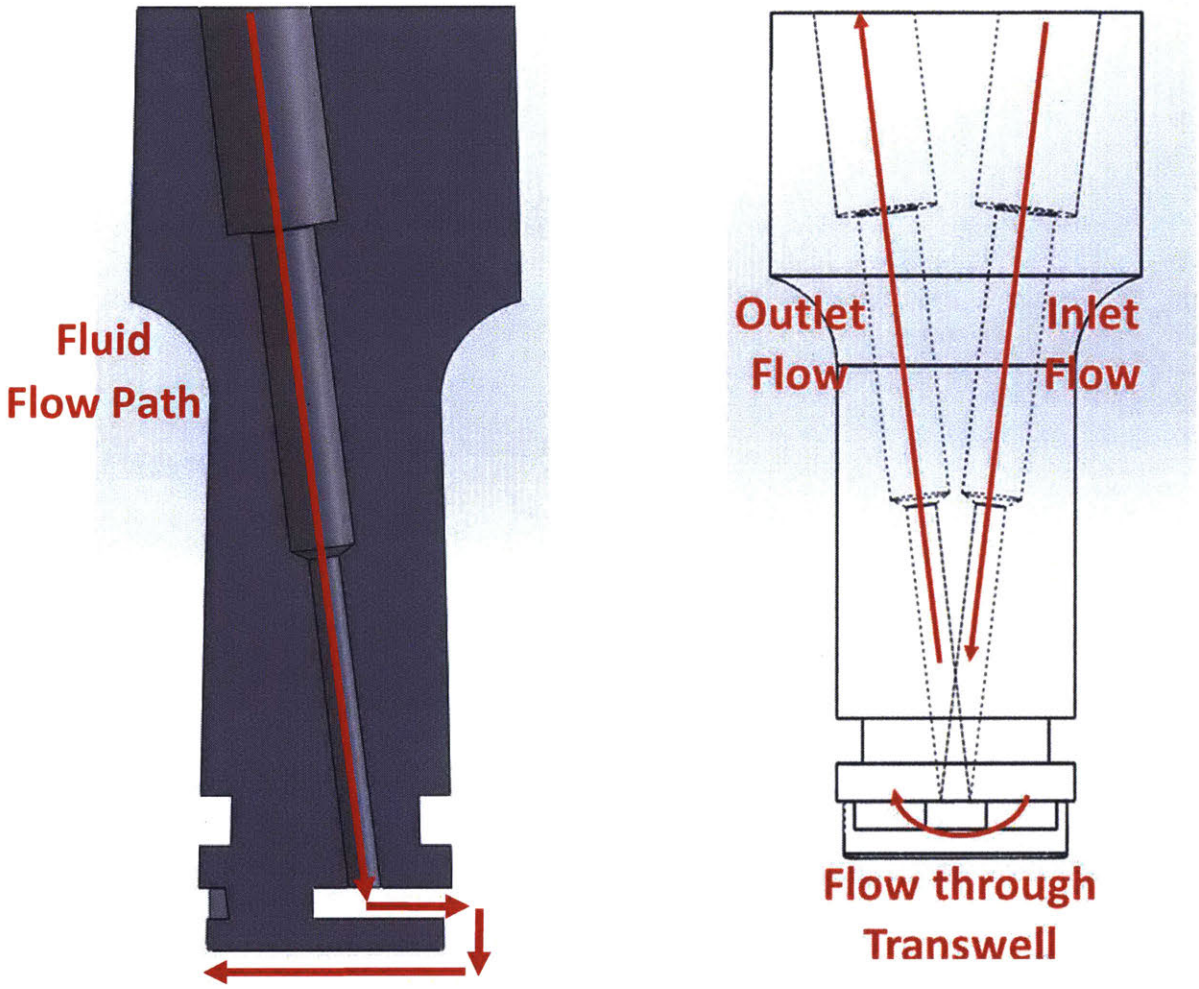


**Figure 2-7:** Cross-section of scaled-down 24-well transwell apical flow module and the path of fluid flow through the device. The fitting, threading, and bore are drilled at a 30 degree angle to allow room for two fittings to be placed on the top cap of the module. Fluid flows through the angled bores and along the surface of the transwell in the same manner as it does in the 12-well apical flow module.

The scale of the updated design presents unique challenges for fitting all necessary components onto the very small space available. The design calls for an O-ring groove to seal the transwell, bores extending from the top of the plug into the well, and space for fittings to

seal tubing into the device. The inner diameter of the transwell, 6.5 mm, is the maximum space available on a device inserted into the well. However, the smallest fittings compatible with 1/16 inch OD tubing, 6-32 size, have an outer diameter of 3.8 mm each, requiring more space than the transwell's size allows if they are to be placed in the same manner as the fittings on the 12-well apical flow module. To overcome this hurdle, the bores are drilled at a 30 degree angle, as shown in figure 2-7, which permits both fittings to be placed on the lid without contacting each other.

An O-ring seal is likewise implemented in a similar manner to that in the 12-well apical flow module. A 5 mm ID x 1 mm CS Viton O-ring sits in an O-ring groove 1.2 mm wide and 0.51 mm deep. The same Apple Rubber O-ring calculator places these measurements within the acceptable compression range. Sealing testing conducted on the smaller apical flow modules mimics that performed on the larger modules, with three flow rates, 0.1 μL/min, 10 μL/min, and 100 μL/min, all being used on the modules to test the outer bounds of their sealing capabilities. In all three cases, no leaking is observed. Similar to the 12-well apical flow modules, the 24-well modules are machined out of polysulfone.

## 2.2 Oxygen Sensing

In order to be able to grow anaerobic cells in our system, it is necessary to control and monitor the oxygen levels of the media in various parts of the system. To this end, we utilize

optical oxygen probes in the source media and inline in the fluidic path, both to validate and test our system as we design it, and to report oxygen levels during experiments.[19]

### 2.2.1 Optical Oxygen Probes

We monitor oxygen levels using optical oxygen probes designed by Lucid Scientific (Atlanta, GA). The probes function by using rapid optical pulses from an LED to illuminate a ruthenium complex through an optical fiber. By measuring the rate of decay of the resulting ruthenium fluorescence, the probes can detect the amount of oxygen present. The sensors have a resolution of 0.05 kPa at 20 kPa oxygen.

The probes are mounted at the tip of a 1 mm ID stainless steel needle casing. An optical cable connects the probe with a controller box, which can host up to four probes at a time. Figure 2-8 shows the probe setup along with a closer look at the probe tip.

We place one probe directly in the source media container to ensure that our media is fully deoxygenated before we begin flow through the system. We also have probes mounted inline with the tubing at the inlet and outlet of the apical flow module. The probes are connected with the flow path through stainless steel T-junctions that hold the tip of the probes directly in the flow path.

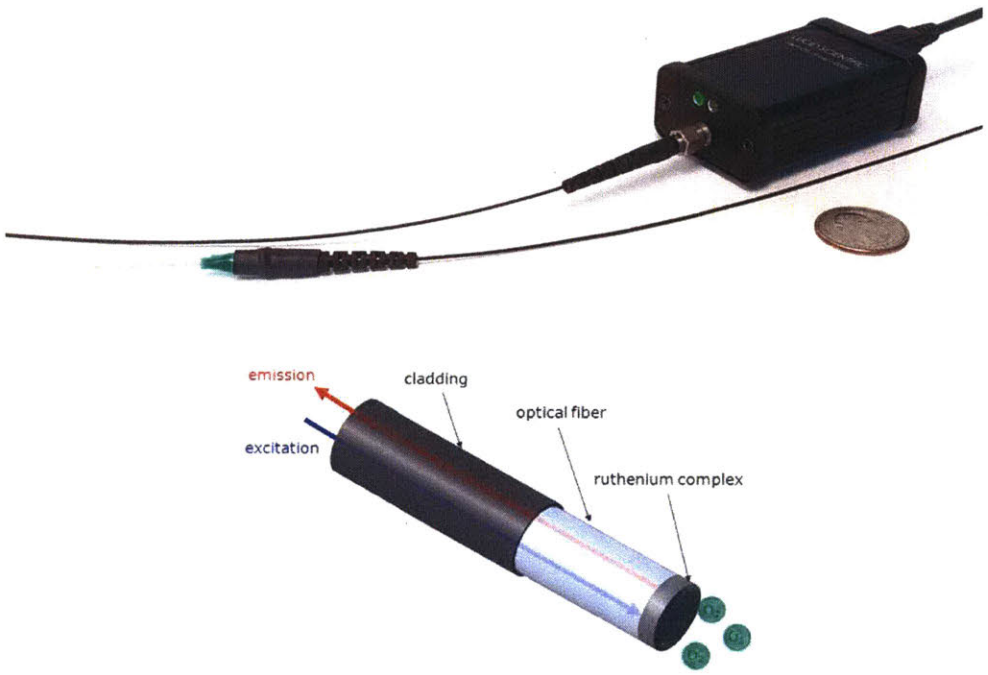


**Figure 2-8:** Lucid Scientific (Atlanta, GA) optical oxygen probes. The decay of the florescence of a ruthenium complex in the probe tip is measured as an LED flashes past. Reproduced with permission from Lucid Scientific.

Initially, polypropylene (PP) junctions were implemented to hold the oxygen probes in line with the apical flow path. However, the junctions proved insufficient to seal oxygen in the fluid. Figure 2-9 on the following page displays the results of testing conducted on the PP junctions connected with stainless steel tubing to a syringe pump. The deoxygenated source media is compared to the oxygen measured by the probe in a PP junction over a 9 hour stretch of time. The probe in the PP junction measures an oxygen level almost 1.5 kPa higher than the source media, and the measured oxygen continues to rise over time, indicating that oxygen is able to leak through the PP junction. Therefore, in our design only stainless steel junctions are used to hold the oxygen probes in line with the apical flow path. The use of stainless steel tubing is further discussed in section 2.3.4.

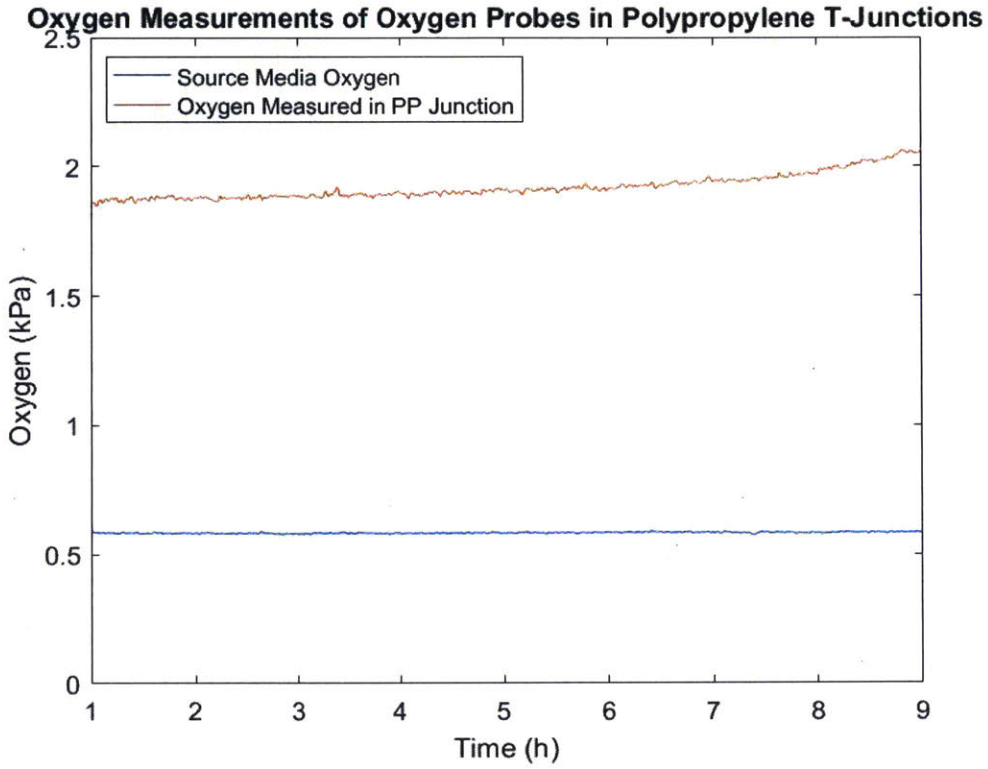


**Figure 2-9:** Polypropylene (PP) T-junction oxygen measurement. Deoxygenated source media at a consistent 0.58 kPa oxygen level flows through stainless steel tubing to be measured by an oxygen probe connected through a PP T-junction. The oxygen probe detects almost 1.5 kPa higher oxygen reading than the source media, and the detected oxygen continues to rise over time.

Alternate probe placements were also investigated. It is essential that the tip of the probes remain fully immersed in fluid in order for them to function properly over the course of the testing, so locations other than junctions in the tubing may be possible to host probes. One possibility includes placing an oxygen probe directly into the apical flow module, to measure the oxygen level on the apical side of the transwell itself. However, we note that the probe operates by continuously emitting short pulses of blue light. Prolonged exposure to bright light may have unintended effects on cells growing in the transwell, so that possible probe placement location is ruled out so as not to subject cells to this illumination. Another

option is to place inlet and outlet probes directly into the channels on the apical flow module. However, the geometry of the apical flow module is too crowded to permit the addition of more items on the cap of the module. Fittings needed to seal the probes in the channel would compete for space with fittings necessary to connect the tubing to the internal channels. Furthermore, the polysulfone material out of which the modules are machined is translucent and allows light to pass through, which introduces the same possibility of light affecting the cells in the transwell. For these reasons, T-junctions upsteam and downstream of the module are the best locations for the oxygen probes.

Probes are calibrated to their control boxes before the beginning of any test. The probes are placed in a solution of 250 mg sodium sulfite in 125 mL PBS or water. Sodium sulfite binds with oxygen in solution and is used to set a baseline zero oxygen level to calibrate all of the probes. The solution is then left overnight in an environment matching the temperature of the experiment, either ambient or in an incubator, until the solution becomes fully saturated with oxygen once again. Then, all of the probes being tested can be set to the same level of zero and ambient oxygen, so that any variation in readings due to differences in ruthenium levels are mitigated by their calibration to the same endpoints.

## 2.3 Pumping

Our *in vitro* intestinal system operates by circulating fluid from an oxygen-controlled source media container, past a bacterial injection port, through the apical side of a transwell

containing human intestinal cells, and into a collection tube. Oxygen probes monitor the oxygen levels of the source media inlet fluid before flowing through the bioreactor, and also monitor outlet fluid from the bioreactor. The fluid is circulated by a pneumatic pump block actuated with compressed nitrogen gas, as described in more detail below. Stainless steel tubing connects the various parts of the system and prevents oxygen diffusion into the deoxygenated fluid. In this section we will examine the design of the pumping system and choice in materials and equipment that enable the system to operate.

### 2.3.1 Pneumatic Pump Block

The fluid media throughout the model is circulated using a bi-directional pneumatic diaphragm micropump following a design by Walker Inman,[20] a cross-section of which is shown in figure 2-10 on the following page. The pneumatic pump has a number of advantages that suit it to our application. The design is compact, easily sterilizable, and the pumping is precise even at low flow rates. We were also able to draw from the experiences of lab members who were involved in the pump design or who have extensive experience with using the pumps. Furthermore, we are able to maintain control of oxygen tension by controlling the compressed gas used for actuation, as elaborated in section 2.3.3.

The pump consists of two blocks: a pneumatic block machined from acrylic, and a fluidic block machined from polysulfone, with a flexible polyurethane membrane separating them. Torque-controlled screws sandwich the membrane between the two blocks. In each

channel, three sets of valves in both the matching pneumatic and fluidic sides alternate between open and closed states to push fluid through the channel. A valve opens when vacuum is applied through the pneumatic side, pulling the flexible membrane to fill the pneumatic side and open the fluidic valve to be filled with fluid. The valve closes when compressed gas forces the membrane to fill the fluidic side, and when the succeeding valve simultaneously opens, fluid is forced in the flow direction. At least one valve is closed at all times to prevent backflow. V-cuts in the polysulfone pneumatic plate prevent the membrane from blocking the fluidic pathway by decoupling the valve and pump actuation, so pressure on the pump cannot reach and close the valves when not intended to. Figure 2-10 demonstrates the pneumatic pumping sequence.

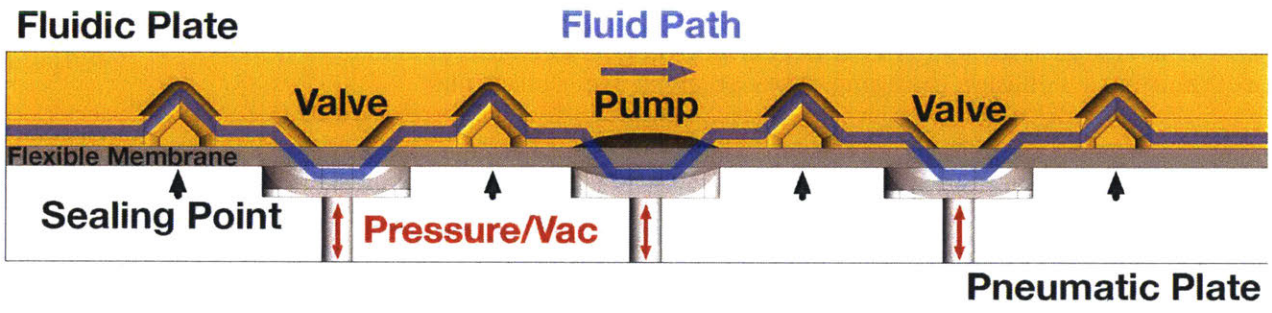


**Figure 2-10:** Cross-section of pneumatic pump valves, with the microfluidic polysulfone plate (top) and the acrylic pneumatic plate (bottom) sealing the flexible polyurethane membrane between. As the pneumatic lines alternate between applying pressure and vacuum, the membrane changes direction to open or close the valves on the fluidic side, forcing liquid through the path. Unpublished graphic reproduced with permission from Griffith lab.

With a stroke volume of 1 μL, the pump can alter its actuation frequency to set precise pumping rates. Furthermore, the stroke volume relative to the pressure requirement results in a high compression ratio, allowing the pump to self-prime and also tolerate bubbles without

hindering pump performance. It is important to note that the pump delivers a pulsatile flow, however it is diffused before it reaches the bioreactor by the multiple curves in the flow path from the pump to the bioreactor. The apical flow path contains five curves from the point fluid exits the final valve in the pump to the point where it flows over cells in the transwell. The pump expels 1 μL of fluid in intervals set by the programmed flow rate. At every point that the fluid reaches a corner in the apical module, the pulse of fluid is diffused so that it resembles laminar flow more closely. The high number of curves separating the pump from the apical chamber mean that we can model the flow in the apical chamber as laminar despite the pulsatility of the pump block.

The pump is controlled using software developed in conjunction with Continuum Innovation (Boston, MA). The software controls the actuation of the pump, and can be programmed to change pumping rates at given time intervals.

We tested the pumps to determine whether the pumping rate displayed on the program matches the amount of liquid collected at the outlet of our system. We sealed the porous membrane of the transwell to prevent liquid from leaking through, then operated the pump at a rate of 1 μL/min. After allowing the pump to operate for 24 hours, we measured the amount of liquid collected. The 1.44 mL collected matches the expected amount of fluid moved by the pump, therefore we consider the pump reliable, on the basis of this and other experiments with many pumping systems in our research group.

### 2.3.2 Alternative Pump Considerations

Use of the pneumatic pump provides numerous benefits to the system, however other pumps were also considered before arriving at the present pump. The first generation apical flow module was designed with the intention of using the device with a syringe pump. The many benefits of a syringe pump include continuous, non-pulsatile flow and a great degree of control over the flow rate, including the ability to program different flow rates over different time intervals. However, the syringe pump is impractical for long term applications for several reasons. First, there is no way to continuously deoxygenate media in a syringe before it flows through the system. Most syringe materials are permeable to oxygen, and even those that are not, such as stainless steel, cannot be relied on to seal oxygen over the course of a multi-day experiment. Because of these oxygen concerns, a syringe pump cannot be used to apply positive pressure to push fluid through the system. While it would be possible to place the syringe downstream of the bioreactor, applying negative pressure to such fluid from a continuously deoxygenated source media container, such application of negative pressure to the bioreactor could upset the pressure balance on the transwell. Furthermore, syringes have more limited volume capacities than other liquid storage containers, so over the course of a multi-day experiment it would be necessary to change out the syringes on the pump. Due to these impracticalities, a syringe pump is less viable than the pneumatic pump applied in the system.

Another alternative pump considered is a peristaltic pump. Peristaltic pumps function by rotating multiple rollers over an arc of flexible tubing. As the rollers move, they compress

the tubing and force the fluid between rollers forward, thus applying positive pressure to the fluid occupying the tubing. One major benefit to using a peristaltic pump is that it would be possible to use it to mimic the physiological process of peristalsis, the manner of pumping in the gastrointestinal tract where smooth muscle contracts in waves to propel fluid down the tract. Despite the physiological similarity, however, the pump proves impractical for our application. The pump necessitates the use of flexible tubing for the rollers to compress and through which to move fluid. However, flexible tubing is also permeable to oxygen, a perennial problem in material choice in our system. Because of its inability to guarantee oxygen control, a peristaltic pump is unfeasible for our oxygen-controlled model.

A final contender to drive pumping in our system is a gravity feed. By setting the height of the source media container above the height of the effluent outlet port, we can ensure that the force of gravity drives fluid continuously through the system. Such a pumping scheme appears simple and reliable to implement, however it too proves unworkable. The pressure exerted by the static media is given by the Bernoulli equation:

$$\Delta P = \rho * g * \Delta h \tag{1}$$

As fluid drains out of the source media container, the height of the container will decrease, decreasing the pressure exerted by the fluid. As the pressure decreases, the flow through the system will also decrease. Inconsistent flow rates may cause unintended consequences in any experiments conducted with our system by introducing additional variables. Furthermore, the lack of ability to program different flow rates at different times

also makes a gravity feed less appealing a pump option than others. Therefore, despite its simplicity in implementation, a gravity feed lacks necessary features for our system.

Though many possible pumping options exit, after investigating each of them in turn, it becomes apparent than a pneumatic pump is best-suited to our system, so it is implemented for all future testing.

### 2.3.3 Nitrogen Pump Actuation

To prevent oxygen from leaking into the system through the pneumatic pump, the pump is actuated using compressed nitrogen on the pneumatic side. After deciding on using a pneumatic pump to drive the fluid in the system, it becomes necessary to reconcile the drawbacks of the pump. In particular, it quickly becomes apparent that the pump has the potential to introduce oxygen into the system through the permeable polyurethane membrane in a manner illustrated in figure 2-11.

The valves are closed by flowing compressed gas through the pneumatic line, forcing the polyurethane membrane to expand and fill the fluidic valve. In most uses, the pump is actuated using compressed air at a standard atmospheric composition of 78% nitrogen, 21% oxygen, and 1% trace gasses.[21] Pumping at this composition, oxygen can diffuse into the system through the polyurethane membrane. In order to prevent such leaking, 100% compressed nitrogen is used exclusively to actuate the pump. This manner of pump actuation

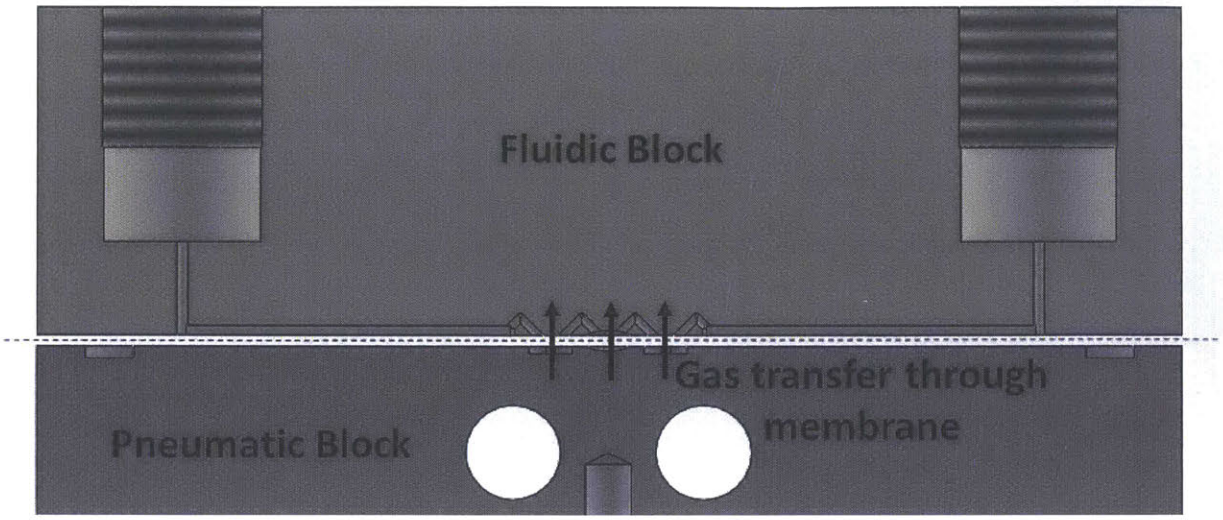


**Figure 2-11:** Transfer of gas through polyurethane membrane of pump block. Gas flows through the pneumatic side of the block to actuate the valves, however the porous polyurethane may allow gas to transfer from the pneumatic side into the fluid on the fluidic side, thereby changing the gas content in the apical side.

requires a relatively large amount of nitrogen, as much as 15 lbs per 48 hour experiment, to continuously actuate the pump. More efficient methods of actuating the pump will be discussed in section 4.1.

Figure 2-12 demonstrates the result of such nitrogen actuation. In this experiment, we deoxygenate water using nitrogen bubbles, then flow it past oxygen probes using a pneumatic pump block actuated with compressed nitrogen. While the pump takes some time, about 1.5 hours, to flush any oxygen bubbles that may have been in the system at the start of the flow, it is able to maintain an extremely low oxygen level the longer it runs. We found that the oxygen level of the outlet fluid differs from the inlet fluid by only 0.2 kPa after 10 hours of flow.

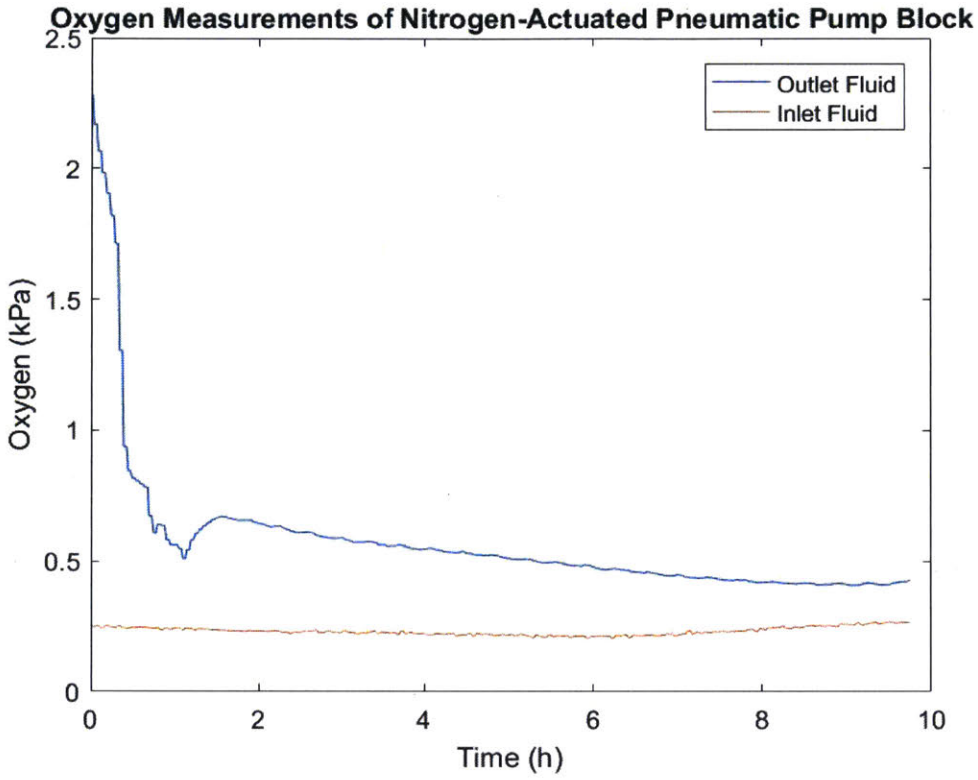


**Figure 2-12:** Measurements of fluid before and after flow through nitrogen-actuated pneumatic pump. While the pump and tubing require approximately one hour to be purged of oxygen entirely, after being fully purged the nitrogen-actuated pump keeps oxygen levels below 0.5 kPa, an acceptable level to grow most anaerobic bacteria.

### 2.3.4 Stainless Steel Tubing

Throughout the system, stainless steel tubing is used to connect all of the elements involved in apical fluid circulation in order to prevent oxygen leaking into the fluid. As many of the design decisions described above make clear, controlling the oxygen content of the apical fluid is an essential part of the system, and the primary element of our system that separates it from many *in vitro* gastrointestinal models that exist currently. Because the apical fluid channel contains so many different pieces of hardware, including the pump block, microbial injection port, oxygen probes, and the bioreactor itself, there is enormous risk of

oxygen leaking through either the tubing or the connectors that combine the various parts. After testing several materials, as explained below, stainless steel was found to be the best material for preventing oxygen leaking into the system.

Although stainless steel is known to be impermeable to oxygen, it is rigid and thus can be difficult to work with. All tubing lengths must be measured and cut ahead of time, as the rigidity of the tubing allows for limited flexibility in placing it. In order to examine alternate tubing options, we tested several types of tubing common to microfluidic applications. Table 2-1 gives the oxygen permeability of several materials often used to make microfluidic tubing.

**Table 2-1:** Gas permeability for different gasses of several polymers commonly used in microfluidic tubing. Table from IDEX Health and Science (Philadelphia, PA).

**POLYMER TUBING GAS PERMEABILITY:**

(Permeability is listed as $cm^3/100\ in^2 \cdot 24\ h \cdot atm/mil$ @ 25°C)

| | | | |
|---|---|---|---|
| **Halar ECTFE polymer** | | **PFA fluoropolymer** | |
| Carbon Dioxide | 330 | Carbon Dioxide | 2,260 |
| Nitrogen | 10 | Nitrogen | 291 |
| Oxygen | 25 | Oxygen | 881 |
| **PEEK polymer** | | **Tefzel fluoropolymer** | |
| Oxygen | 14 | Carbon Dioxide | 250 |
| **Radel R polymer** | | Nitrogen | 30 |
| Carbon Dioxide | 830 | Oxygen | 100 |
| Nitrogen | 36 | **Ultem PEI polymer** | |
| Oxygen | 195 | Oxygen | 37 |
| **FEP fluoropolymer** | | | |
| Carbon Dioxide | 1,670 | | |
| Hydrogen | 2,200 | | |
| Nitrogen | 323 | | |
| Oxygen | 748 | | |

For our application we tested polytetrafluoroethylene (PTFE), polyether ether ketone (PEEK), and C-Flex ULTRA to determine their oxygen permeability levels. PTFE, also called

Teflon, is a synthetic fluoropolymer that makes up a flexible tubing that is often used in microfluidic systems. PEEK is similarly useful for its flexibility and water absorption properties, so it easily integrates into microfluidic systems. C-Flex ULTRA is a flexible thermoplastic touted for its low permeability.[22] As a final comparison, we also examined stainless steel. We flowed deoxygenated fluid through each type of tubing and measured its oxygen level before and after flow. Figure 2-13 displays the difference in oxygen levels for each type of tubing.

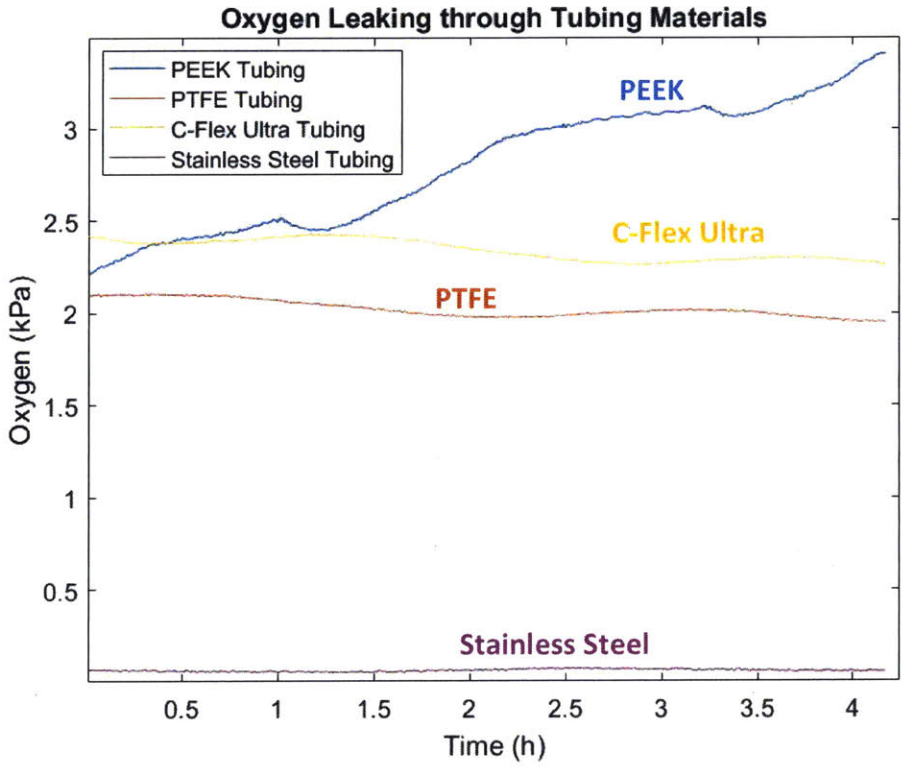


**Figure 2-13:** Comparison of oxygen leaking through each type of tubing tested. A 4 hour block of time is selected from 4 different flow tests. Each type of tubing received the same deoxygenated flow at a rate of 10 μL/min. The outlet oxygen measurements after flow through a 1 m length of tubing are given here. PEEK tubing sees an average of 2.9 kPa of oxygen leaking. PTFE sees an average of 2.1 kPa of oxygen absorbed. C-Flex Ultra allows an average of 2.3 kPa to enter the fluid stream. Stainless Steel, in contrast, allows only a negligible amount of oxygen to enter the tubing, demonstrating its ability to seal oxygen effectively.

Each of the three polymer tubing materials demonstrate some oxygen diffusion into the media. Over the course of flowing deoxygenated fluid at a rate of 10 μL/min through a 1 m length of tubing, some oxygen is able to diffuse through permeable materials. As the pump continues to flow deoxygenated media through, a balance between the rate of deoxygenated media flow and the rate of oxygen diffusion into the media is established, and the oxygen concentration levels out in some materials. PEEK tubing resulted in 2.9 kPa of oxygen to diffuse into the fluid over the course of 1 m of flow. PTFE sees an average of 2.1 kPa diffusing in, and C-Flex Ultra allows 2.3 kPa to enter the stream in the same 1 m. In contrast, stainless steel allows only a negligible amount of oxygen to enter the fluid stream. One of the major biological requirements of our *in vitro* system is for it to have the ability to grow all types of bacteria in the human microbiome, including strict anaerobes that cannot be exposed to any oxygen. Even a relatively small amount of oxygen leaking is unacceptable. Therefore, from flow testing through the tubing we concluded that stainless steel is the best tubing material to ensure a deoxygenated system under all flow conditions.

## 2.4 Bacteria Injection

Bacteria are injected into our system through a septum injection port built into a T-junction inline with the flow path. The system requires a method of adding bacteria to the bioreactor once flow is underway. Bacteria cannot be seeded on the transwell along with the human epithelial cells because of the anaerobic nature of many of the bacteria of the microbiome. Although the media in the system is deoxygenated prior to commencing flow,

our oxygen measurements indicate that it can take as many as 10 hours for all parts of the system to fully reach anaerobic levels of oxygen. Because of this, it becomes necessary to incorporate an injection port to add bacteria once flow through the system is underway.

### 2.4.1 Injection Port Design

Our injection port uses a stainless steel T-junction with a rubber septum sealed with a stainless steel fitting. The through channel in the junction matches the inner diameter of the stainless steel tubing connecting to it. A second channel of the same diameter and 1/4 inch long connects orthogonally to the through channel and opens to a flat surface 3/8 inch wide. A 3/8 inch diameter, 1/16 inch thick Buna rubber septum is then placed over the opening and compressed when a 7/16-14 fitting is fully tightened. A chamfer at the base of the fitting screw, seen in the sloped base of the screw visible in figure 2-14, ensures that the base is flat when it compresses the rubber septum. This fitting also has a 3/8 in long bore through its center, which connects with the bore in the T-junction. The compressed Buna rubber septum is sufficient to seal both liquid and gas even after being pierced with an injection needle. When not in use, the injection port is capped with a rubber cap to maintain sterility. All parts of the injection port are autoclavable. Figure 2-14 displays all of the parts of the septum injection port.

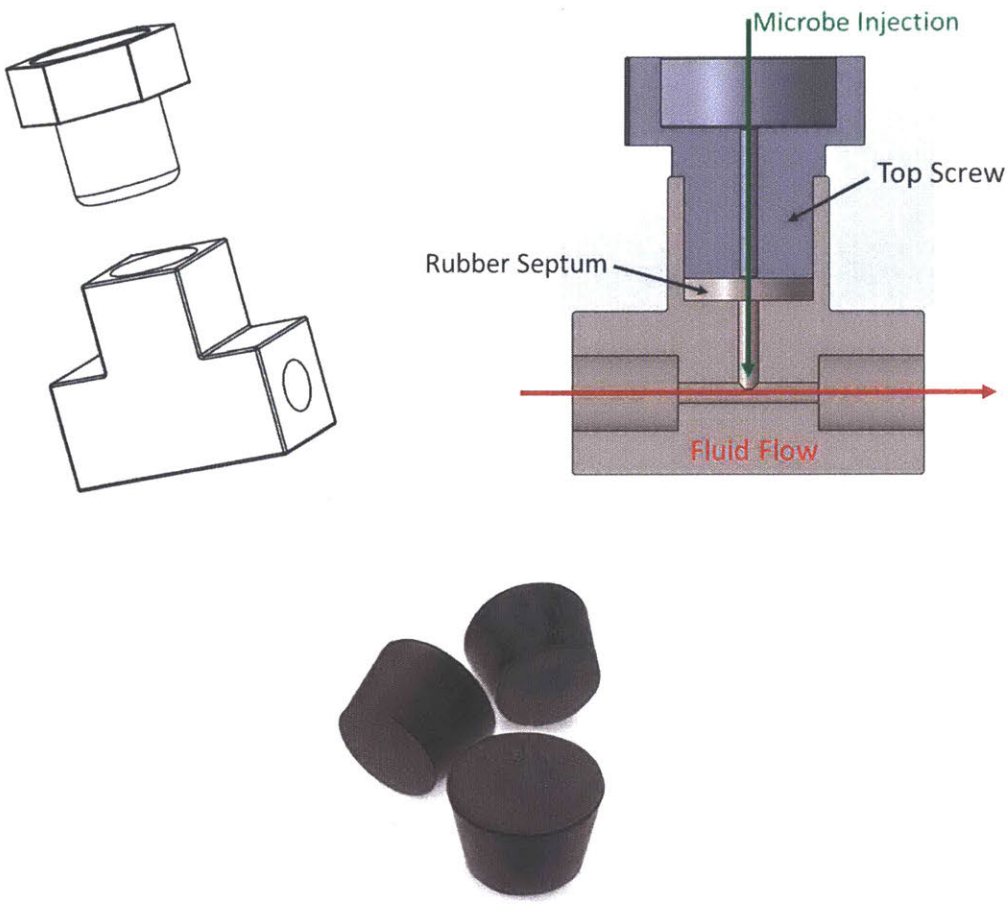


**Figure 2-14:** Septum injection port exploded view and cross-section. A 7/16-14 fitting seals a Buna rubber septum over a channel connecting to the fluid path in a T-junction. A needle pierces the septum to inject microbes directly into the flow path. A Fisher Scientific (Pittsburgh, PA) rubber stopper cap is used to seal the injection port when it is not in use.

### 2.4.2 Injection Port Testing

When designing the microbial injection port we investigated alternate hardware designs to the septum. While effective at sealing fluid and oxygen, the septum has several drawbacks. Handling sharp needles in a laboratory environment is always a risk for injury or contamination, so a design that does not involve needles would be preferable. Furthermore, sterility of the septum can be difficult to maintain, especially when piercing the rubber. The

entire system must be transported from an incubator to a sterile hood in order to perform the injection in an appropriately sterile environment. Thus, alternate microbe injection methods are investigated.

The primary alternate injection design that we investigated is the use of a Luer lock injection port. Not requiring any needle, the Luer lock is compatible with a standard syringe, and features a check valve imbedded into the device to prevent back flow. It also has a sterile cap that is able to be swabbed with alcohol, ensuring sterility before and after each injection. Figure 2-15 shows an example of the needleless Luer lock injection valve.

**Figure 2-15:** Needleless Luer lock injection valve. Part acquired from Qosina (Ronkonkoma, NY).

We designed a stainless steel T-junction with a Luer lock attachment, so the needleless injection port could be attached to the flow path. We then conducted tests on the needleless injection port and the septum. In both tests, deoxygenated fluid was flowed through the port, with oxygen levels measured before and after flow past the injection ports. The flow was

stopped for 20 hours, to simulate the steps of injecting the microbes and giving them time to adhere to the mucus and cells in the transwell. Flow was then resumed, and the oxygen level measured at the outlet port indicates how much oxygen was able to permeate through the injection port while flow was stopped. Figure 2-16 shows the results of the test for the needleless injection port.

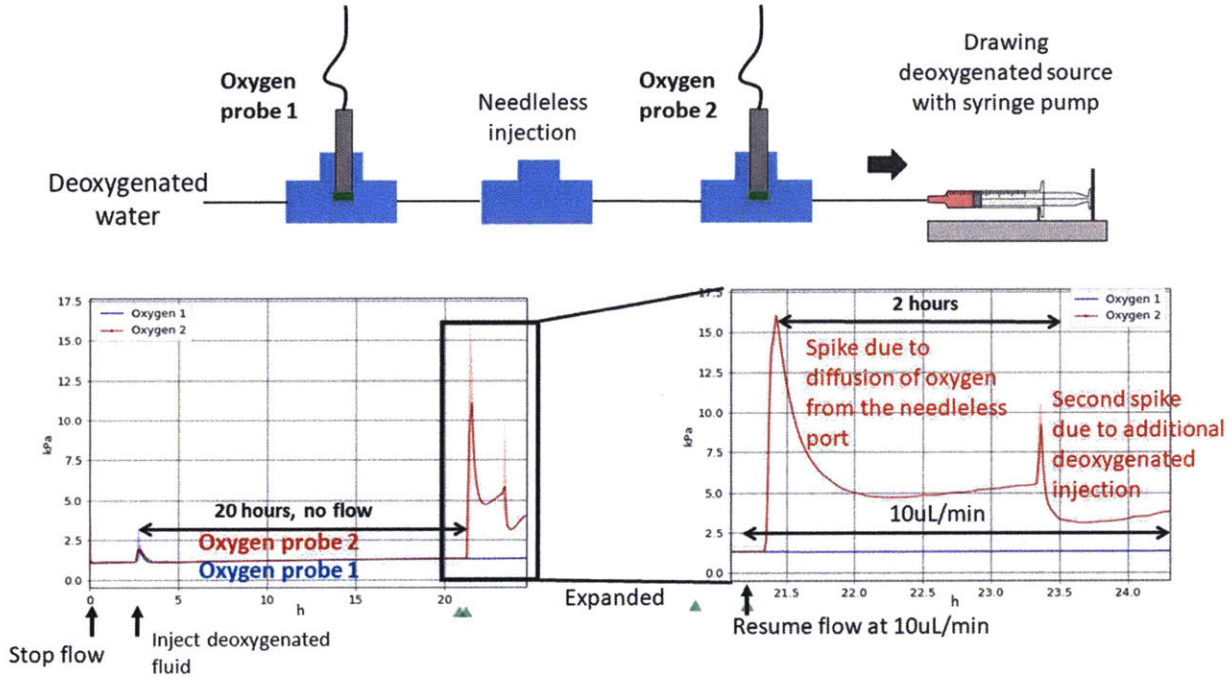


**Figure 2-16:** Test setup and results for oxygen leaking through needleless Luer lock injection port. Once flow is resumed at 20 hours, the oxygen levels of the outlet port spikes past 15 kPa of oxygen. Even after flow resumes for 2 hours, the oxygen level of the outlet tubing never returns back to the deoxygenated state of the inlet. This demonstrates that oxygen that leaked into the injection port cannot be flushed out of the system, compromising the oxygen sealing of the system.

After flow resumed past the needleless injection port, the oxygen level of the outlet tubing rose past 15 kPa of oxygen, indicating that oxygen was able to leak through the port while the flow was stopped. While this leaking on its own may not compromise the oxygen

sealing of the system, even after the flow resumed for an additional 2 hours, the oxygen level of the outlet tubing still did not drop to the deoxygenated level of the inlet tubing. This indicates that enough oxygen leaked into the injection port that it could not be flushed out even with fully deoxygenated flow. Thus the needleless injection port is untenable for our system.

The stainless steel septum was also tested under the same conditions, with the results displayed in figure 2-17.

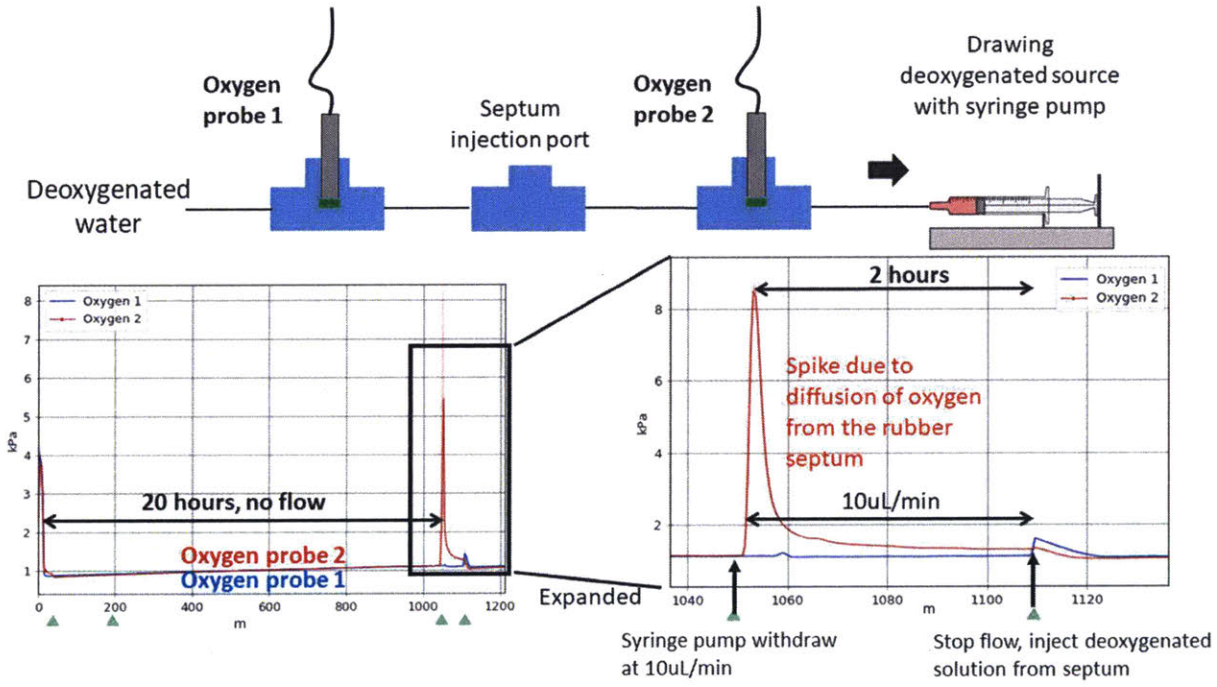


**Figure 2-17:** Test setup and results for oxygen leaking through septum injection port. Once flow is resumed at 20 hours, the oxygen level of the outlet port spikes past 8 kPa of oxygen. However, after flow resumes for an additional 2 hours the oxygen level of the outlet tubing drops back to the deoxygenated level of the inlet tubing, demonstrating that oxygen can be successfully flushed out of the port.

After flow resumed past the rubber septum injection port, the oxygen level of the outlet tubing rose past 8 kPa of oxygen, indicating that oxygen was able to diffuse into the port during the 20 hours when the flow was stopped. Once flow resumed for an additional 2 hours, the oxygen level of the outlet tubing dropped to the deoxygenated level of the inlet tubing. This indicates that oxygen can be flushed out of the system with deoxygenated flow. Therefore, the septum injection port is a more reliable design to ensure that our system maintains its low oxygen level necessary to grow anaerobic bacteria cells throughout the duration of a multi-day experiment. We also plan to seal the port with a rubber cap, shown in figure 2-14, when it is not in use to maintain sterility and further hinder oxygen diffusion.

### 2.4.3 Microbe Injecting Procedure

When injecting microbes through the septum, flow is stopped and the system is moved from the incubator into a sterile hood to ensure sterility. A mixture of microbes in media is transferred in a syringe from its broth, whether growing in an anaerobic chamber in the case of anaerobes or growing in atmospheric conditions for other bacteria. A 23 gauge 1/2 inch long needle is used to puncture the septum without making contact with the bottom of the through channel. Before microbes are injected, all three valves on the pump block are closed to prevent backflow into the source media. Such back flow cannot be permitted, as bacteria cells would grow in the source media. With the only opening in the sealed flow path being at the outlet past the apical flow module, all of the bacteria solution should move in the direction of the flow path, which remains open at the outlet. A volume of bacteria solution greater than

the volume of the tubing leading up to the bioreactor is injected to entirely fill the length of tubing leading up to the transwell. After injection, the device is transferred back to the incubator and flow is resumed for a short time until the volume of the apical side of the transwell is entirely full of microbe solution, then flow is stopped again. The microbes are given time to adhere to the human epithelial cells and mucus later before flow is resumed; typically, flow is stopped for 30 minutes. Through this device, microbes are able to be introduced to the deoxygenated system without hindering its ability to seal fluid and oxygen.

## 2.5 Effluent Collection Tube Stand

After flowing through the bioreactor, effluent is collected in 50 mL sterile test tubes. The tubes are connected to the flow path and held up by a stand that uses threading matching that of the tubes. The Bernoulli equation (1) dictates that height difference affects pressure changes at two different points in the system. In our case, the points of the system that open to atmosphere are the tubing outlet and the basolateral fluid free surface. In order to determine what height difference is needed to maintain equal pressure we must also take into account the pressure drop throughout the tubing. $\Delta P$ through tubing is given by the Hagen-Poiseuille equation (2) below:

$$\Delta P = \frac{8 * \mu * L * Q}{\pi * r^4} \tag{2}$$

In our system, the dynamic viscosity $\mu = 7.8 * 10^{-4}$ Pa*s for the cell medium used in the system, Advanced DMEM.[23] Pipe length L = 1.542 m. Volumetric flow rate Q = 10

$\mu L/min = 1.67 \times 10^{-10}$ $m^3/s$. Pipe radius $r = 3.97 * 10^{-4}$ m. Therefore, the pressure drop due to pipe resistance throughout our system $\Delta P = 20.5$ Pa. For a density of DMEM $\rho = 10^3$ $kg/m^3$ and acceleration of gravity $g = 9.8$ m/s, the Bernoulli equation (1) gives that a pressure drop of 20.5 Pa would require a height difference of 0.002 m to account for, a negligible difference in a system of our scale. Therefore, the Bernoulli principle dictates that the height of the outlet port match the height of the fluid on the basolateral side of the transwell, shown in figure 2-18.

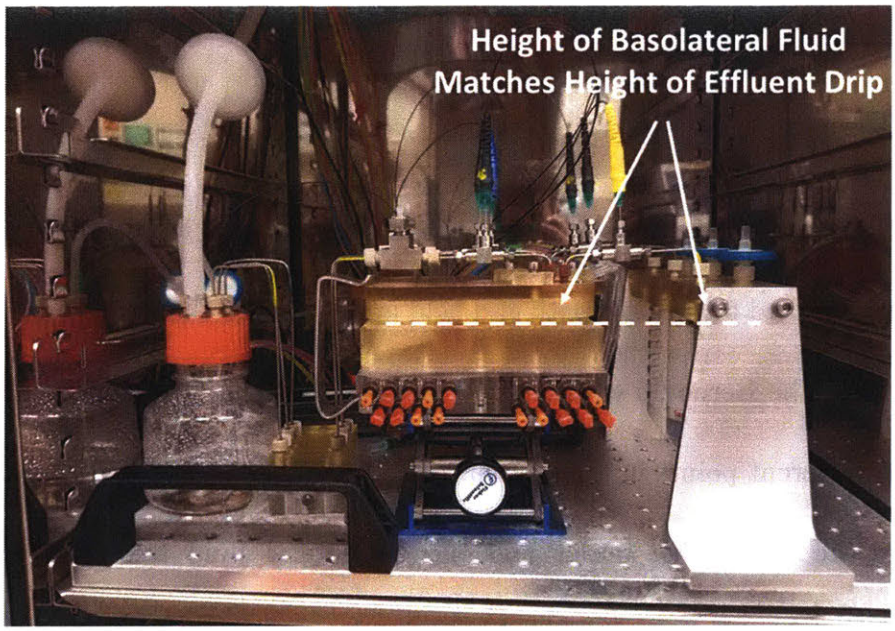


**Figure 2-18:** Overall system setup in incubator. The scissors jack is used to raise the height of the basolateral fluid free surface in the 3xGL well to the same height (132 mm) as the drip out of the effluent tube stand, ensuring equal hydrostatic pressure at both points.

The polysulfone stand features three sets of internal threading matching the 50 mL sterile test tubes used for collecting effluent. Each threaded port has two ports opposite the test tube where the tubing and a HEPA filter vent can be attached. Using this vent prevents outside contamination of the effluent. Two aluminum legs support the stand with the outlet

132 mm above the base of the stand. However new legs can be switched out to set a different height. The polysulfone stand can be autoclaved.

## 2.6 Basolateral Oxygenation

In order to ensure that the human gastrointestinal cells receive the oxygen they need to function properly, it is necessary to keep the media on the basolateral side of the transwell saturated with oxygen. The apical side must remain deoxygenated so anaerobic bacteria cells can survive, however the human cells can consume all of their required oxygen from the basolateral side alone if the media there maintains an appropriate level of oxygen. The basolateral media is continuously circulated using a series of pneumatic pumps on a device, which we refer to as the 3x Gut-Liver (3xGL) interaction device. Figure 2-19 displays the 3xGL in use with apical flow modules.

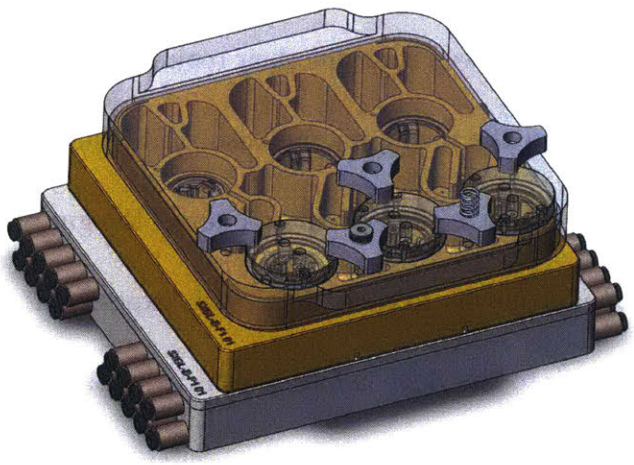

**Figure 2-19:** Basolateral circulation plate with sealed apical flow modules.

### 2.6.1 3xGL Basolateral Circulation

Up to this point all parts of the system described support the apical side microenvironment on the transwell. In this case, the primary purpose of these elements of the system is to circulate deoxygenated fluid for the bacteria on the apical side. However, the human cells and their oxygen requirements cannot be neglected, so the basolateral side of the transwell comes into play. In a static experiment, media on the basolateral side of the transwell is replaced approximately every 48 hours to prevent the media from being depleted of nutrients. Instead of replacing the basolateral media, which would require access to the basolateral side of the transwell and the intervention of a researcher, we plan to accomplish the necessary media oxygen replenishment through circulation.

The sealed transwells in the apical flow module are placed in the well of our lab's 3x Gut-Liver interaction (3xGL) device, which circulates media through not only the well sized to the 12-well transwells incorporated in our bioreactor, but also other wells with large surface areas. The human cells in the transwell consume their required oxygen through the transwell's semipermeable membrane from the media on the basolateral side, reducing the oxygen level in that media. The depleted media is then circulated out of the gut well and into neighboring wells. As the depleted media moves from well to well, exposing it to atmospheric oxygen levels, oxygen diffuses back into the fluid before it returns to the gut well to again be taken up by the human cells. A cross-section of the 3xGL circulation pathway is shown in figure 2-20 between the mixer well (**A**), the liver well (**B**), and the gut well (**C**), which holds the transwell.

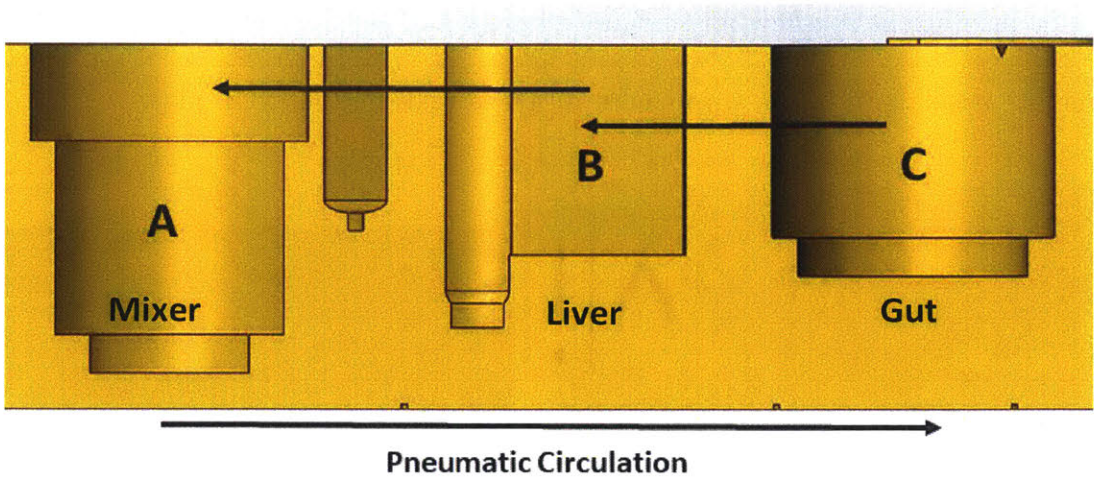


**Figure 2-20:** Cross-section of basolateral circulation in 3xGL plate. Well (**A**) is the mixer, well (B) is the liver, and well (**C**) is the gut, which holds the transwell. Pneumatic pumps move fluid from the gut to the mixer, and the fluid height spills over from mixer to liver and from liver to gut.

We placed an oxygen probe on the basolateral side of the apical flow module to examine the oxygen level of the basolateral media under three different conditions: full circulation throughout all three wells, recirculation within the gut well only, and no circulation. On the following page figure 2-21 (**1**) demonstrates the routes of circulated media in these experiments, figure 2-21 (**2**) demonstrates how the probe monitored oxygen through the apical flow module, and figure 2-21 (**3**) displays the oxygen measurements on the basolateral side.

When fully circulating, the basolateral media remains saturated with oxygen at about 19 kPa. Merely recirculating the media within one well drops the oxygen level down to below 15 kPa. Turning off circulation entirely causes the oxygen level to continue to fall. While it is unclear what threshold of basolateral oxygen is necessary to support human cell growth, the trend indicates that less than full circulation drops oxygen levels below atmospheric levels

and may be insufficient to allow for human cell growth. Therefore, we conclude that we will fully circulate basolateral media throughout all three wells on the 3xGL plate.

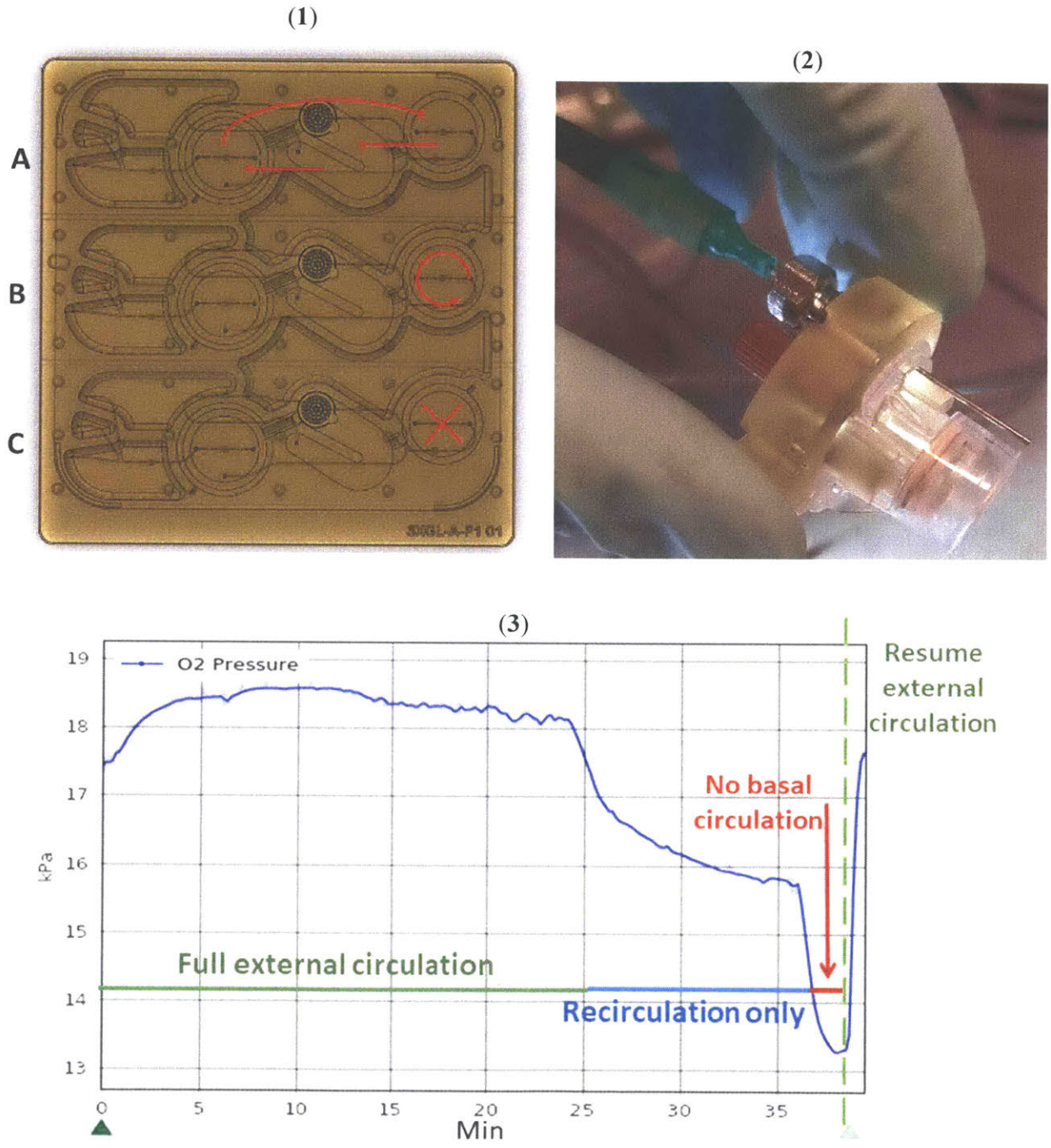


**Figure 2-21:** (**1**) The methods of circulating basolateral media included full circulation between all three wells (**A**), recirculation within the gut well only (**B**), and no circulation (**C**). (**2**) Basolateral oxygen is measured using a probe through the apical flow module at a height matching the membrane of the transwell. (**3**) The data indicate that only with full circulation does the basolateral media remain close to full atmospheric oxygen saturation at 19 kPa.

### 2.6.2 3xGL Function

The 3xGL plate was originally designed to study interactions between *in vitro* gut and liver model interactions in three replicates. Figure 2-22 displays the top view of the chip. Pneumatic pumps, functioning identically to those described in section 2.3.1, circulate fluid from one well to another. When the fluid height of one well grows due to fluid addition, it can spill over into neighboring wells via a spillway, thus regulating fluid height between wells.

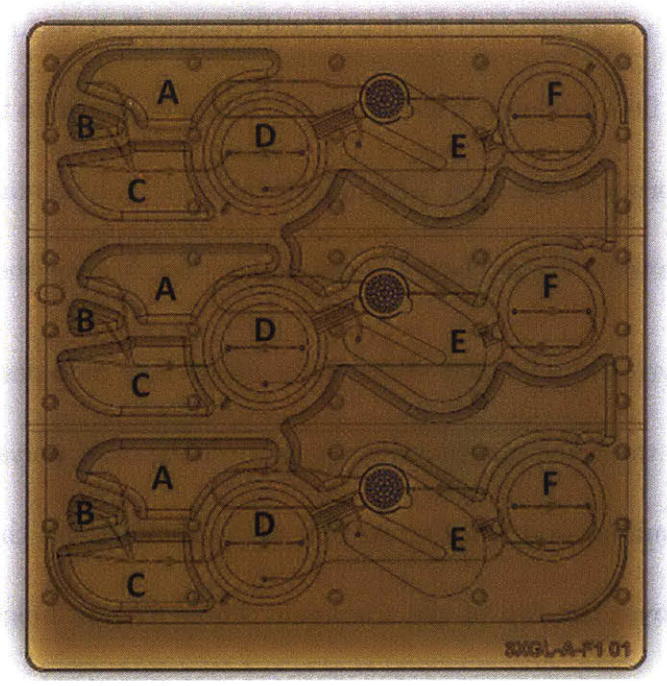


**Figure 2-22:** 3x Gut-Liver Interaction Plate Wells **A**, **B**, and **C** are used for drug and/or media storage, and can pump drugs into the other well at programmed intervals. Well **D** is a mixer that connects all of the wells. Well **E** features the liver model including a liver scaffold necessary to simulate the filtering function of the liver *in vivo*. Well **F** hosts the gut, grown on a 12-well transwell.

While the 3xGL plate was not conceived with a gastrointestinal human-gut microbiome model in mind, it is apparent that the device is successful at achieving a necessary part of our

system, basolateral oxygenation, so it was put to use. With other minor modifications, the 3xGL plate becomes compatible with the apical flow modules, as described below.

### 2.6.3 3xGL-Apical Flow Module Lid

We developed a lid sized to fit over the 3xGL plate that also incorporates apical flow modules, thereby closing the top of the plate and protecting the wells from contamination. While the 3xGL plate has a well sized to fit a 12-well transwell, it was not originally designed to be used with apical flow modules, whose caps add 9 mm of height to the top of a transwell. The 3xGL plate is normally operated with an off-the-shelf polystyrene lid that covers the plate and leaves its contents visible, however the lid does not fit over the apical flow modules.

We instead developed a lid that allows three apical flow modules to be sealed over the 3xGL plate. Sized to the same specifications as the off-the-shelf polystyrene lid, our lid is machined out of polysulfone and is taller, so that its height matches the height of the apical flow modules, 9 mm over the top of the transwell. Three 32 mm holes equal in size to the diameter of the apical flow module cap are machined in the lid to fit the modules. The modules are sealed by ISO 3601 Standard size 026 O-rings sitting in grooves 1.39 mm deep and 2.76 mm wide. Once in place within the O-rings, the caps are locked in place by five spring-loaded toggles that keep the modules firmly pressed flat into the 3xGL well. The lid is then clamped to the base of the 3xGL pneumatic plate with two stainless steel clamps. All parts of the 3xGL lid are autoclavable. The lid allows the apical flow modules to be used to the 3xGL

plate while maintaining the sterility of the basolateral fluid. Figure 2-23 gives a top view of the 3xGL-Apical Flow Module lid.

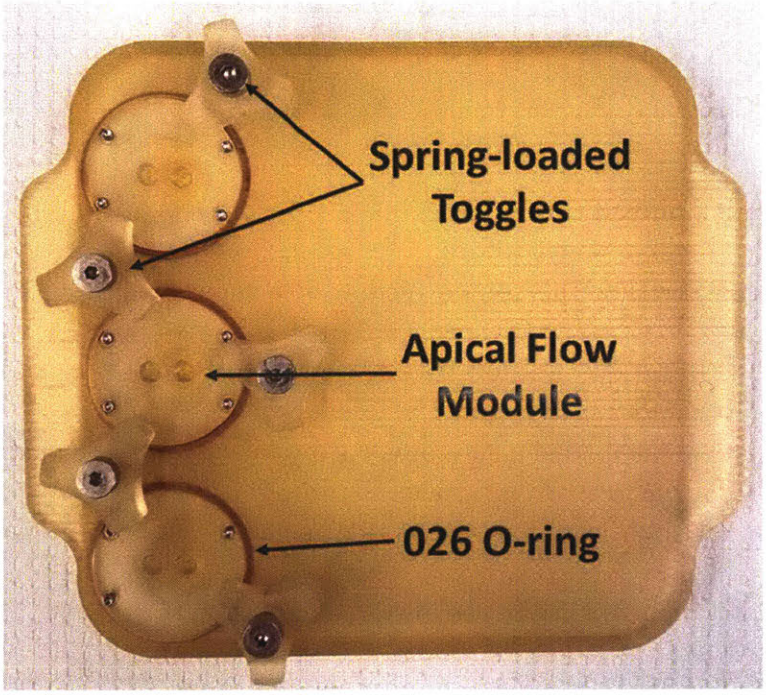


**Figure 2-23:** Top-view of 3xGL-Apical Flow Module lid. The lid allows for assembly of modular apical flow modules while maintaining sterility of the entire system.

During assembly, each length of stainless steel is pre-cut to lengths measured ahead of time. Stainless steel tubing is extremely rigid, and once measured and bent at the necessary places, the tubing holds its own shape and is strong enough to hold the injection port and oxygen probe T-junctions in place. The height of the apical flow modules, which we can alter based on the height of the tubing we cut, is dictated by the height of the outlet port on the effluent stand. The 3xGL and custom lid are assembled and placed on a scissors jack, which is raised into place until the apical flow modules sit snug in the O-rings in the lid. Once in place, the lid toggles are closed and the lid is clamped to the 3xGL plate. Through modification

to the 3xGL lid, the basolateral fluid can be oxygenated while deoxygenated apical fluid is pumped through the bioreactor.

## 2.7 Apical Oxygen Control

Before media is pumped through the apical side of the transwell, it is deoxygenated by bubbling nitrogen gas through the source media. The media is stored in a container large enough to support the duration and flow rate of the planned experiment. Pumped directly to the base of the container, the nitrogen gas convects away oxygen in the media solution through diffusion. A ventilation tube leads outside of the incubator, allowing displaced oxygen and excess nitrogen to be removed from the media without upsetting the gas balance in the incubator where the experiment takes place. A HEPA filter prevents back contamination through the ventilation line. A long oxygen probe extends into the media container and continuously monitors the oxygen level of the source media, ensuring that it remains at the proper level called for by the experiment. A custom cap with six ports seals into a standard GL45 cap, allowing all of the different probes, filters, and tubing necessary to maintain the gas content of the media to enter the container. Figure 2-24 displays the hardware needed to deoxygenate the source media.

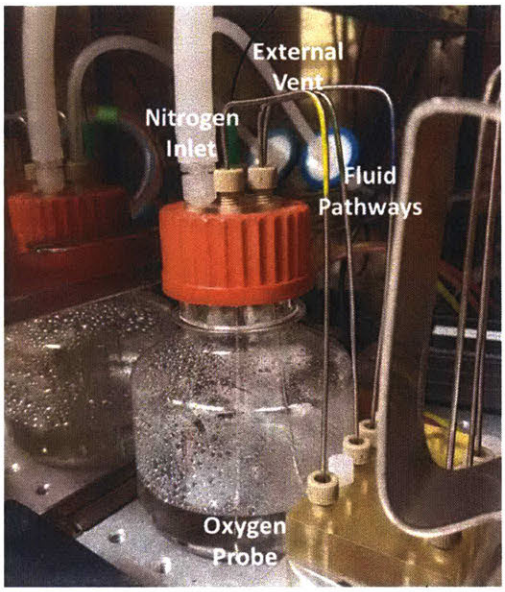


**Figure 2-24:** Source media container, including ports for nitrogen inlet, external ventilation, an oxygen probe into the media, and three stainless steel fluid pathways.

Nitrogen is continuously bubbled into the source media throughout the entire experiment to ensure continuous deoxygenation of the media. While it is necessary for the media to remain anoxic, excessive bubbling can be wasteful, as it continuously pumps in nitrogen even once the media has reached 0 kPa of oxygen. A 48 hour experiment can use as much as 25 lbs of nitrogen gas. Continuous bubbling of nitrogen also causes evaporation of media in the container. Possible solutions to these ongoing problems are discussed in section 4.1.

## Chapter 3: Biological Testing

The purpose behind designing and building the oxygen-controlled *in vitro* model described in the previous section is to provide researchers with a viable platform to grow human intestinal cells and microbes together in an environment that mimics the conditions of the intestines *in vivo*. In the course of building the hardware, we performed numerous validation tests to ensure that the pumping rate and oxygen control are consistent with what we programmed. In both cases, we proved that the pump rate and the control of oxygen throughout the system perform as we expect them to (refer to sections 2.3.1 and 2.3.4, respectively).

Once the system could be reasonably relied on to supply the cells with the environment necessary for their survival, we next endeavored to perform preliminary tests on the biology which the system will be used to study. We perform three distinct phases of biological tests to ensure that the system can support human and bacteria cells appropriately:

1. Oxygen gradient validation, to ensure the human cells are exposed to oxygen levels matching physiological conditions
2. Anaerobic monoculture, to ensure that the human cells receive appropriate oxygen levels from basolateral circulation alone
3. Anaerobic coculture, to ensure that both the aerobic human and anaerobic bacteria cells can survive and grow together

The testing was conducted along with Yu-Ja Huang, a postdoctoral fellow in the lab.

## 3.1 Oxygen Gradient Validation

*In vivo* human gastrointestinal cells create an oxygen gradient from the lamina propria to the lumen as successive cell types consume oxygen supplied through the bloodstream, to the point where the interior of the intestines is entirely devoid of oxygen.[2] The oxygen gradient is an essential part of both how the human cells function and also how they create the environment necessary for bacteria of the gut microbiome to exist and serve their function.[10],[24]

### 3.1.1 Cell Line Oxygen Gradient Testing

To examine the behavior of cells on our platform, we first cultured Caco-2 HT29 cell lines, which consist of a 9:1 ratio of enterocytes to goblet cells, on transwells. Fast-growing and durable, immortalized cell lines such as Caco-2 cells are particularly useful to test on our platform because of their ease to acquire, grow, and maintain. Testing cell lines on our platform gives us a clear indication of whether or not conditions are survivable for human cells. While robust and useful for preliminary experimentation, however, they are not substantive subjects for comprehensive human tests. As cancer cells, they multiply much more quickly and consume resources, including oxygen and nutrients, at higher rates than primary cells taken directly from human donors do.[25] Therefore, they serve us in a useful preliminary role, but are not relied upon to deliver data representative of the human cells that make up most of the body.

A monolayer of Caco-2 cells is grown on transwells and subjected to apical flow at a rate of 10 μL/min at 5 kPa oxygen saturation, about the level present in the upper small intestine, while basolateral oxygen is fully circulated. Oxygen probes at the inlet and outlet of the apical flow module monitor the oxygen consumption of the cells on the transwell. Figure 3-1 gives the oxygen data recorded during the 48 hour experiment.

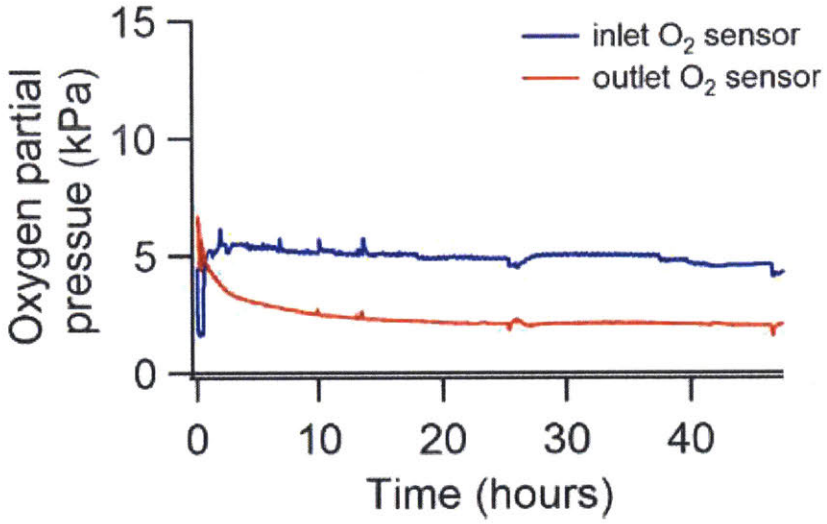


**Figure 3-1:** Inlet and outlet oxygen measurements of Caco-2 HT29 cells in transwell. The approximately 2.5 kPa difference in oxygen levels suggests that the cells consume oxygen and create an oxygen gradient across the monolayer.

The Caco-2 cells survive on the transwell under low-oxygen, though not entirely anoxic, apical conditions. Furthermore, the difference in oxygen levels of the inlet and outlet tubing suggests that the cells consume apical oxygen and create a gradient across the cell monolayer. This test provides us with confidence that the conditions to which we subject our cells will not kill them outright, giving us leave to move forward to test with more substantive, though also more fragile, cell types.

### 3.1.2 Primary Cell Oxygen Gradient Testing

Primary epithelial cells are intestinal cells taken directly from human donors. We acquired and grew primary cells in transwells then subjected them to the same conditions to which we subjected the Caco-2 cells in 3.1.1. Figure 3-2 gives the oxygen data from our primary cell testing.

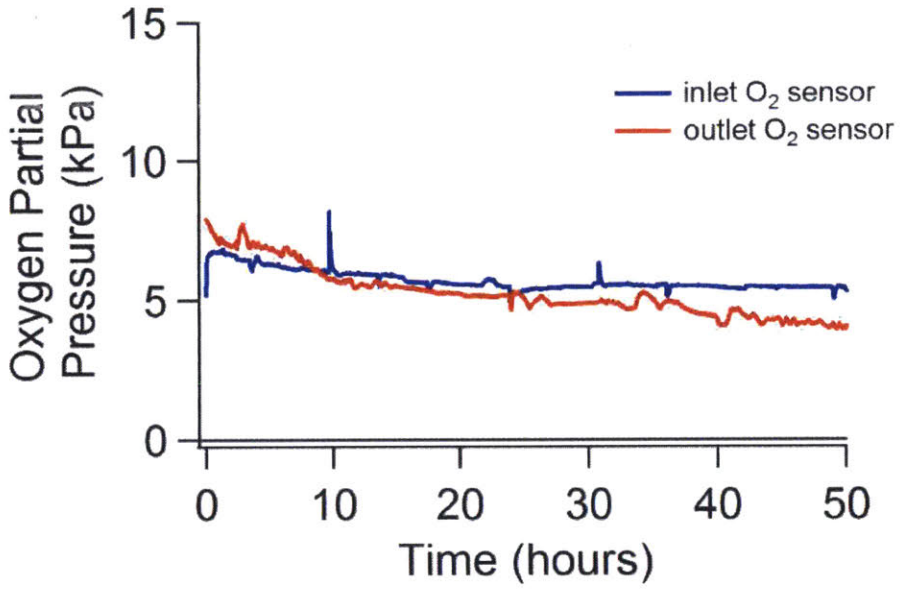


**Figure 3-2:** Inlet and outlet oxygen measurements of primary epithelial cells in transwell. The difference in oxygen levels varies over time, but the difference in oxygen levels and calculated oxygen consumption rate suggests that the cells create an appropriate oxygen gradient across the monolayer.

As expected, the primary epithelial cells consume oxygen at a significantly lower rate than the cancerous Caco-2 cells.[26] Furthermore, the amount of oxygen they consume changes over time, which also matches the expected physiological conditions more closely. The test proves that both cell lines and primary cells can survive in the system and also create an

oxygen gradient necessary to support microbes. The successful tests pave the way for the next set of testing, anaerobic apical flow.

## 3.2 Anaerobic Apical Flow Monoculture

Once it is apparent that our system creates an oxygen gradient capable of supporting human and bacteria cells together, the next step is to entirely deprive the apical side of oxygen in an anaerobic monoculture. In this set of testing, the human cells on the transwells must uptake all of the oxygen necessary for them to survive from the basolateral side of the transwell, while the apical side remains fully anoxic. A monolayer of Caco-2 HT29 cells is seeded on transwells. Deoxygenated apical media flows at a rate of 10 μL/min, while basolateral oxygen is fully circulated to maintain normoxic conditions, as examined in section 2.6.1.

After 48 hours, the Caco-2 cells not only survive the anaerobic apical flow, they also begin to exhibit unique feature formations. Figure 3-3 shows fluorescent-stained confocal images of 3-dimensional dome-like structures that are observed to form in the initially-flat monolayer of cells.

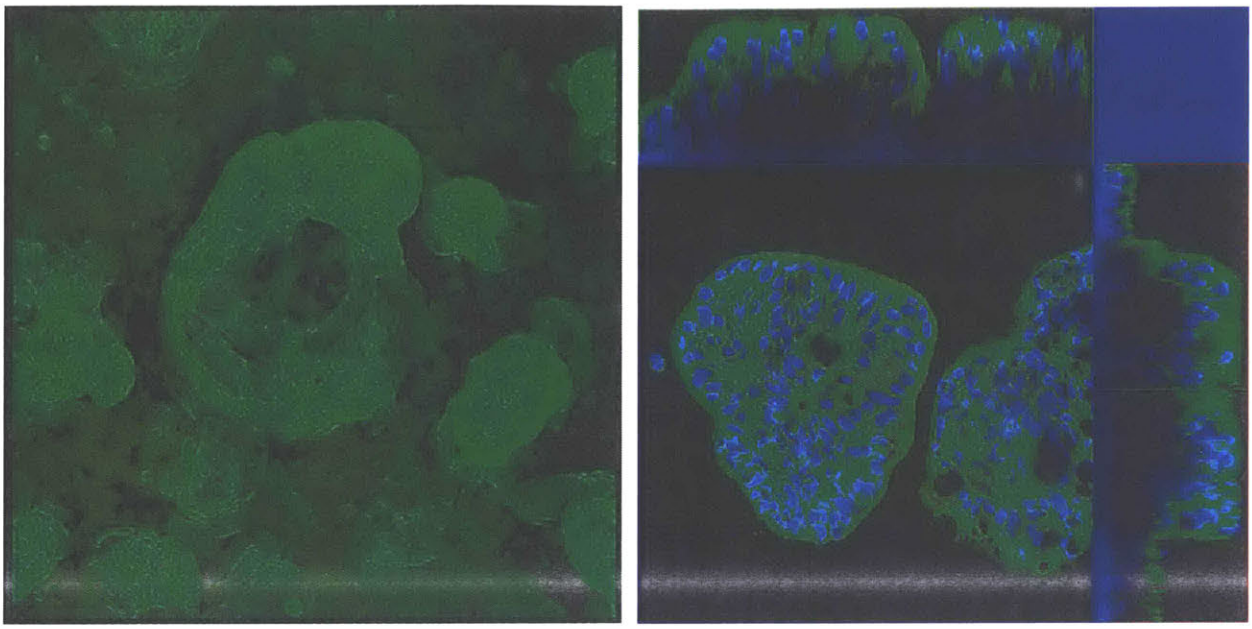

**Figure 3-3:** Confocal images of 3D dome-like structures present on the Caco-2 monolayer after 48 hours of anaerobic apical flow. The data are collected with Yu-Ja Huang.

The dome-like structures observed demonstrate not only that the human cells can survive 48 hours of anaerobic apical flow, gaining their necessary oxygen from basolateral media alone, but also that the cells behave differently when under anaerobic flow conditions. The observed dome formations mimic the villi structures found in the intestines *in vivo*, and it is possible that they indicate that the cells in our system behave similarly to how they do in the body when experiencing similar physiological conditions.

## 3.3 Anaerobic Apical Flow Coculture

The final validation test required to confirm that our system operates properly, and can be used as a worthwhile tool for researchers, is to test an anaerobic apical flow coculture.

A monolayer of Caco-2 HT29 cells is grown on the apical side of transwells. Deoxygenated apical media flows through the system for 12 hours, until all of the oxygen is reliably flushed from the tubing and ports. Then, an injection of $1.0 * 10^6$ *B. theta* anaerobic bacteria, roughly a 1-1 parity of human to bacteria cells, is added through the septum injection port. After stopping flow for 1 hour to allow the microbes to settle on the mucus layer of the human cells, flow at 10 μL/min is resumed for 48 hours.

The Caco-2 cells survive and show signs of even more widespread feature formation than was present in the anaerobic monoculture alone. Figure 3-4 shows the features on the transwell, along with a live-dead stain, with live cells displayed in blue while dead cells are red. The live-dead stain proves that the majority of the human cells observed on the transwell

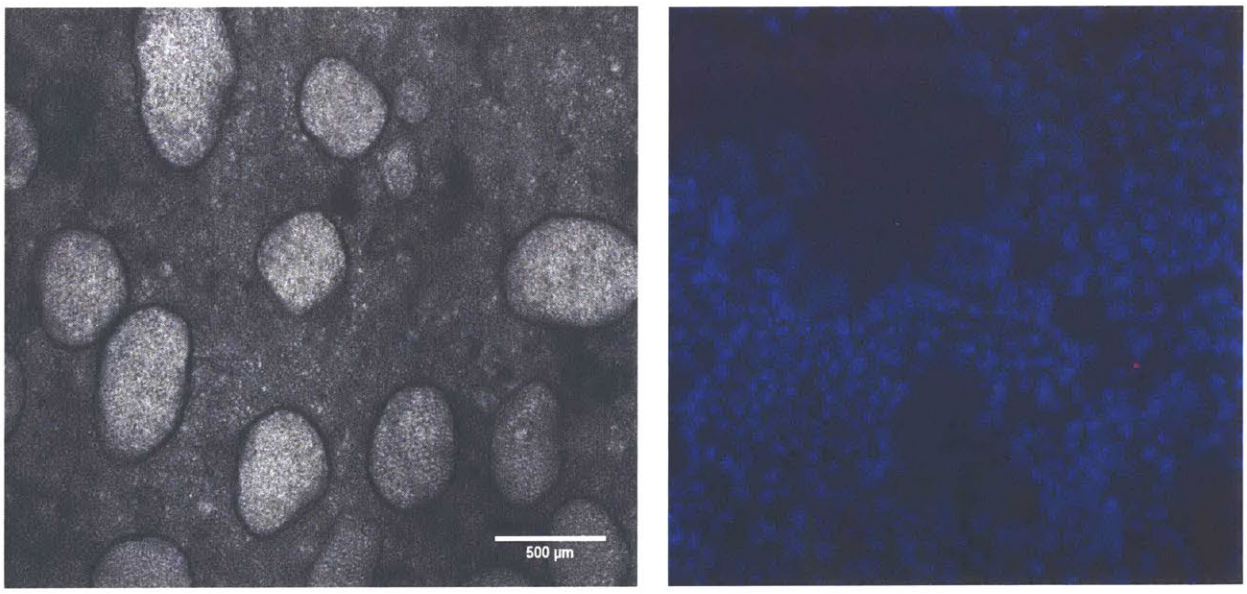


**Figure 3-4:** Feature formation and live-dead stain of anaerobic apical flow coculture experiment. Dome-like features forming among the Caco-2 cells are clearly apparent after the cells are grown in anaerobic apical conditions along with *B. theta* microbes. Furthermore, the live-dead stain proves that most of the human cells remain alive (blue) throughout the experiment. The data are collected with Yu-Ja Huang.

are alive, therefore do not interact with the microbes competitively for nutrients supplied by the media solutions. Furthermore, the widespread nature of the dome-like features formed in the anaerobic coculture demonstrates the changed behavior of the human cells when grown along with bacteria in conditions more similar to those of the intestines.

The anaerobic apical flow coculture is compared to a control consisting of an anaerobic apical flow monoculture that did not receive an injection of *B. theta* microbes. The monoculture received the same anaerobic apical media and featured the same basolateral circulation, but did not feature an injection port. The results of the control are displayed in figure 3-5, revealing a much less featured Caco-2 monolayer. Not entirely devoid of features, the monolayer does display some signs of dome formation, similar to that observed in the

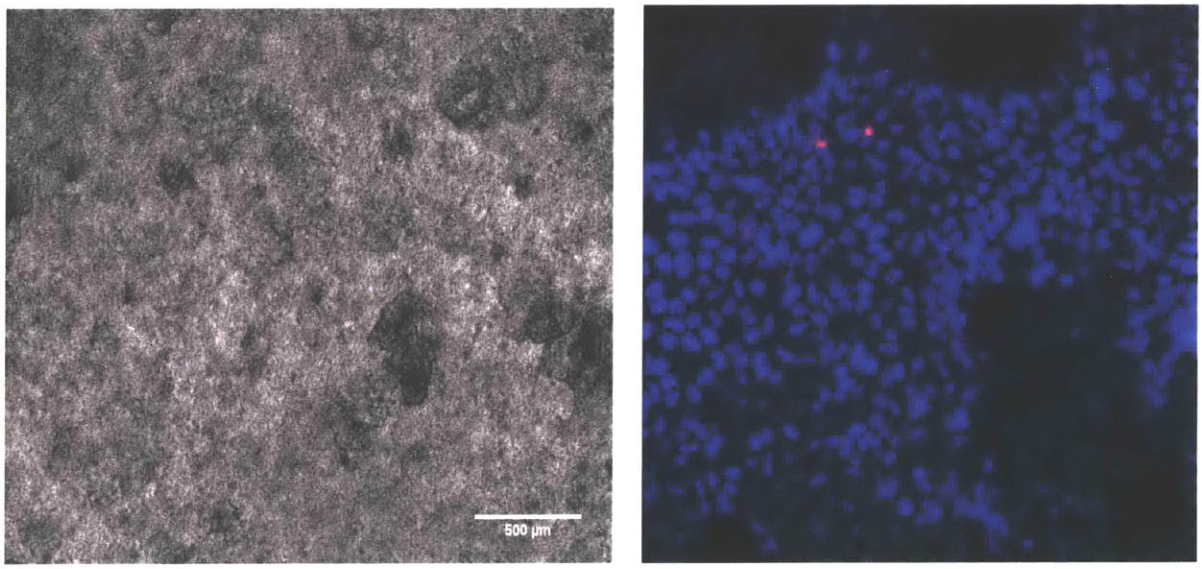


**Figure 3-5:** Feature formation and live-dead stain of anaerobic apical flow monoculture experiment. Dome formation can be observed in the Caco-2 cells, seen in the darker spots of the figure, however the feature formation is much less widespread than that of the anaerobic coculture of figure 3-4. The live-dead stain of Caco-2 cells reveals that the majority of the Caco-2 cells remain alive (blue) after the experiment. The data are collected with Yu-Ja Huang.

experiment documented in section 3.2. However, the dome features are not nearly as apparent or widespread as those seen in the anaerobic coculture in figure 3-4. The live-dead stain of the anaerobic monoculture reveals a ratio of live to dead Caco-2 cells comparable to that seen in the anaerobic coculture, revealing that the observations of feature formation are not attributed to any difference in the Caco-2 cells on the different transwells.

While the observations noted in the anaerobic apical flow coculture test are so far insufficient to draw conclusions about the microenvironment of the gut, they do demonstrate the viability of the system that we created as a tool for researchers to grow aerobic human and anaerobic bacteria cells together in the same *in vitro* model. Furthermore, the initial data are promising in suggesting that the coculture is an important method of understanding the functioning of the intestines, and that human cells behave differently, potentially more similar to *in vivo* cells, when they are grown in environments that more closely mimic physiological conditions. Using our system, scientists will be better armed to understand the workings of the gastrointestinal system and all aspects, including the elusive gut microbiome, that contribute to it.

# Chapter 4: Future Work and Conclusions

## 4.1 Future Work

Our oxygen-controlled *in vitro* gastrointestinal human-microbiome model, while functional and able to support cells in a physiologically accurate environment, is not a completed system ready to be deployed to laboratories around the world. The system is made up of more than 200 individual components that must each be autoclaved and assembled before use in an experiment. Furthermore, a significant quantity of nitrogen is used in each experiment to purge oxygen from the system. In both the complexity of the system and the use of nitrogen, it is possible to increase the efficiency of operation and ease of use of the model.

### 4.1.1 Source Media Oxygen Control Loop

One particular inefficiency in our current system is the use of nitrogen in purging oxygen from the system. Nitrogen is continuously pumped through the source media during experiments to ensure that the media that flows through the apical path remains deoxygenated at all times. The oxygen level of the source media is continuously monitored by an oxygen probe. However, the probe is simply used as a Boolean check to confirm that the oxygen level of the source media remains low. The compressed nitrogen line leading into the media remains open throughout the duration of the test.

There are two main concerns with the present method of source media deoxygenation. First, the continuous bubbling of nitrogen for 48 hours or longer is wasteful, and possibly unnecessary, as oxygen does not diffuse back into the deoxygenated system instantaneously, so the bubbling does not need to continue uninterrupted. Furthermore, continuous pumping of gas into the media container causes evaporation of media in the container, decreasing the amount of liquid media available for the system to flow through the apical channel, and also wasting media.

A future plan to improve the system is to build an oxygen control loop into the source media. The probe that monitors the media can be used to manage an On/Off control system. Once the oxygen level of the source media reaches 0 kPa of oxygen, the nitrogen line can be closed. A threshold value of allowable oxygen in the source media can be set as an upper limit for the On/Off control. Once enough oxygen diffuses into the source media that the oxygen level reaches the upper threshold, the control system will reopen the nitrogen line and return the media to anoxic conditions. Such a control system will prevent waste of nitrogen and media, and can prolong the length of experiments without researcher intervention.

### 4.1.2 Closed Loop Nitrogen Pump Actuation

Another inefficiency in the current system is the method of actuating the pneumatic pump block. In order to prevent oxygen leaking through the compressed gas mixture actuating the pump, compressed nitrogen is used to apply pressure to the pumping sequence (refer to

section 2.3.3). Similar to the continuous nitrogen bubble in the source media, continuous nitrogen actuation of the pump block uses more nitrogen than is necessary, as nitrogen is vented out of the pump block when the actuation sequence changes to vacuum.

A future addition to the system to prevent nitrogen waste is to scavenge nitrogen used to actuate the pump block. A closed loop of nitrogen in each pneumatic line can have pressure and vacuum applied without requiring continuous pumping of nitrogen from stored compressed gas. This closed loop will allow the system to apply pressure to each valve using the same nitrogen gas throughout the entire duration of the system, preventing unnecessary use of nitrogen. Figure 4-1 presents a diagram of one possible closed loop configuration, where a scavenging pump separates the nitrogen actuating the pump as it alternates between applying pressure and vacuum so nitrogen can be scavenged and re-used to actuate the pump.

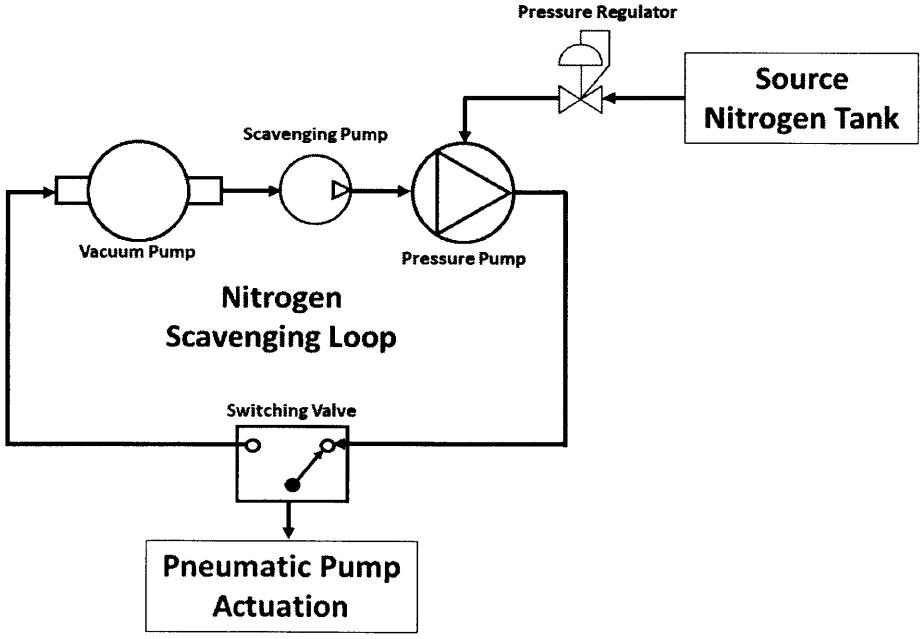


**Figure 4-1:** Diagram showing possible configuration of closed loop nitrogen pump actuation. The same nitrogen gas within the closed loop can be used to actuate the pneumatic pump continuously. A diaphragm pump can keep the nitrogen gas and pressure or vacuum pumps separate.

### 4.1.3 Increased Throughput Redesign

Two major weaknesses of the current system are: 1) limited throughput due to only three replicates, and 2) the complexity of system setup. The current model allows only three transwells to be tested at a time. This replicate number is driven by the number of channels in the 3xGL plate, but it is also influenced by the size and complexity of the system, the other major drawback. With over 200 individual components, the system takes several hours to assemble and disassemble, and each distinct part brings increased risk of contamination, oxygen leaking, or failure in some other way. While the system in its current form is functional, it is not yet sufficiently convenient to use.

To make the system more appealing as a tool to researchers studying the gastrointestinal system, it must be reduced in complexity and allow a higher throughput of replicates per experiment. In pursuit of both of these goals, a partnership has been established with Continuum Innovations (Boston, MA) to build the varied components of the system onto a single platform. This platform will consist of two layers of pumping, onboard apical pumping and basolateral circulation.

The current apical flow path consists of, sequentially, an initial source media container, a pneumatic pump block, a microbe injection port, an inlet oxygen probe, the apical flow module, an outlet oxygen probe, and an effluent collection container. Between each of these components stainless steel tubing, measured and cut to predetermined lengths, connects and seals fluid and oxygen. The integrated design will negate the need for stainless steel tubing by combining all of these separate components onto one plate. An acrylic lid will contain the

pneumatic channels to actuate the apical pump. Separated by a polyurethane membrane, a polysulfone plate will contain the apical fluid path, including a septum injection port, apical flow module fitting into a 12-well transwell, and outlet oxygen probe. Six replicates will be placed on a single lid. Removing the need for a separate pump block and tubing segments will allow the design to be much more compact as well as easier to assemble and operate for longer times. Table 4-1 provides the functional requirements of this integrated system:

**Table 4-1:** Functional requirements of integrated 6-way apical flow device.

| **Functional Requirement** | **Notes** |
|---|---|
| 6 apical flow modules | Higher throughput |
| Onboard apical pumping | Eliminates the need for stainless steel tubing |
| Outlet oxygen sensing | Only outlet needs to be measured as source media is also measured |
| Microbial injection | Onboard injection ports |
| Oxygen control of apical side | All parts of the integrated system must remain deoxygenated |
| Basolateral circulation | Oxygenate basolateral fluid |

A specialized basolateral flow plate will contain six wells containing 12-well transwells, along with six connecting circulation wells with large surface areas, to properly oxygenate the basolateral fluid. The redesigned basolateral circulation plate will use space more efficiently than the current 3xGL basolateral circulation plate by eliminating the excess wells of the 3xGL that are not currently used for oxygenation. All of the drug reservoirs and the mixer

well currently taking up space on the 3xGL plate but not used for oxygenating basolateral fluid will be removed, so the basolateral oxygenation can occur on a much smaller plate.

The new, integrated design will consist of fewer parts, will take up a smaller volume, and will contain more replicates than the current system. It will be possible to run several integrated systems inside of the same incubator, greatly increasing the number of replicates studied per experiment and giving researchers more opportunity to gain valuable information about the gastrointestinal human-microbiome interface.

## 4.2 Conclusions

The gastrointestinal human-microbiome interface holds a wealth of information about the functioning of the human body, the spread of disease, and the relationship between humans and our environment. The difficulties with replicating the interface *in vitro* have prevented researchers from understanding more key details of this important system. As it becomes more apparent how vital the gut microbiome is to the human body, the need for an accurate and easy to use *in vitro* model becomes all the more apparent.

Our present model functions in a manner that no existing *in vitro* model does by allowing the culturing of strict anaerobic bacteria cells alongside aerobic human cells. However, its drawbacks, especially its complexity and low throughput, prevent it from being put to wide use. Once the redesigns outlined above are put into place, the system will be a much more viable tool to allow research on the gut microbiome to proceed more rapidly.

Immune studies, drug testing, and even targeted disease therapies are among the applications for a comprehensive *in vitro* gastrointestinal model such as ours, and the system can prove to be a powerful tool for tackling the greatest medical challenges of our day.

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