Microfluidic Approaches for Algae Culture in Controlled Oxygen Environments

BY

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THESIS

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This thesis is dedicated to my parents, with thanks for supporting every decision I've ever made, and for instilling in me the sense of humor required to deal with the ramifications of those decisions (ha). I love you both.
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<tbody>
<tr>
<td>PDMS</td>
<td>poly(dimethylsulfoxide)</td>
</tr>
<tr>
<td>CO2</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>O2</td>
<td>Oxygen</td>
</tr>
<tr>
<td>N2</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>HIF</td>
<td>hypoxia inducible factor</td>
</tr>
<tr>
<td>H2</td>
<td>Hydrogen</td>
</tr>
<tr>
<td>MEMS</td>
<td>micro electro-mechanical systems</td>
</tr>
<tr>
<td>PtOEPK</td>
<td>platinum(II) octaethylporphine ketone</td>
</tr>
<tr>
<td>H2O2</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>smFRET</td>
<td>small molecule fluorescence energy transfer</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>PMMA</td>
<td>poly(methyl methacrylate)</td>
</tr>
<tr>
<td>UV</td>
<td>ultra-violet</td>
</tr>
<tr>
<td>PS</td>
<td>polystyrene</td>
</tr>
<tr>
<td>TAP</td>
<td>tris-acetate-phosphate</td>
</tr>
<tr>
<td>CoSO4</td>
<td>cobalt(II) sulfate</td>
</tr>
<tr>
<td>PC</td>
<td>polycarbonate</td>
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SUMMARY

Microfluidic platforms are excellent tools for studying the effects of precisely controlled oxygen and other gas environments on various biological systems, from mammalian cell culture to bacteria to algal species. Here, microfluidic approaches for exploring the effect of gas environments on members of the Volvocine algae are presented. A multilayer microfluidic device incorporating gas and hydration channels for the successful culture of *Chlamydomonas reinhardtii* under carefully controlled gas conditions was built and characterized. A device for generating and on-chip storage of droplets of *Chlamydomonas reinhardtii* in oil was developed for observation of the microalgae’s proliferation over several days. Additionally, it was demonstrated that *Volvox carteri* grows successfully in a microfluidic device.
1: INTRODUCTION

1.1 Research purpose

The purpose of this research is to develop microfluidic devices that allow for easy manipulation of algae samples and the precise control of the gas environments of volvocine algae so that high-throughput experiments examining algae’s behavior in these precisely controlled gas environments can take place. In addition, the purpose of this research is to build an understanding of algae’s behavior in microfluidic devices, so that a suite of these devices for efficient, high-throughput control of algae culture might be developed.

Microfluidics provide excellent platforms for precise control of the gas microenvironment. Under anoxic conditions, *Chlamydomonas reinhardtii* produces H\(_2\) (Grossman 2011, Melis 2000), which is desired as a fuel source. Using microfluidics to precisely control the oxygen concentration and light intensity in which chlamydomonas is grown can help algae biologists to better study the mechanism causing this increased H\(_2\) production, as this mechanism is not yet fully understood (Banti 2013). In addition to H\(_2\) production, microalgae such as *Chlamydomonas reinhardtii* are a source of biodiesel, as their oil reserves can be extracted to produce this alternative fuel source (Wijffels 2010). We explore here *Chlamydomonas reinhardtii*’s response in proliferation to various gas environments, as greater proliferation could yield more fatty reserves that can be tapped for biodiesel production.

1.2 Microfluidic devices

An introduction to the construction of microfluidic devices using photolithography and soft lithography is followed by a description of the properties of poly(dimethylsiloxane) (hereafter PDMS) and an explanation of the advantages of working at the microscale, including properties of diffusion.
1.2.1 Photolithography

Masters for microfluidic devices are often made using photolithography. “Master” is the term for the silicon wafer topped with patterned structures from which PDMS molds are cast, which is discussed in a later section. Photolithography is the process by which these raised structures are generated on the silicon wafer; a thin layer of photoresist—a chemical that will react with light—is deposited onto the silicon wafer and a patterned mask placed over the wafer, and then the photoresist is exposed to light through the patterned mask. A positive photoresist, when exposed to light of a designated wavelength and intensity, becomes soluble, whereas a negative photoresist exposed to light of a designated wavelength and intensity becomes insoluble. In the following experiments, negative photoresists are used, which means that unexposed regions of the photoresist are washed away during development. (Vladimirsky 1998).

Photolithographic technology was originally developed for use by the microelectronics industry to build integrated circuits (Whitesides 2001). Micro electro-mechanical systems (hereafter MEMS) was developed to enable the transition from observing the microscopic world to acting and measuring in the microscopic world (Tabeling 2006). Patterning for MEMS technologies also includes wet etching and dry etching. Chemical etching involves exposure of a substrate to a chemical through a mask resulting in a pattern etched into the substrate. Dry etching includes physical bombardment to create a pattern as well as chemical reactions that involve attracting a reactive chemical species to the surface of the substrate via electric fields (Tabeling 2006). When dry etching via physical bombardment is done at sufficiently high energies, ions are implanted in the material. This technology is applied in the doping of semiconductors (Tabeling 2006).

MEMS processes were leveraged to fabricate microfluidic channels, as no technological hurdles existed to impede making this transition (Tabeling 2006). Biologists recognized the potential in microfluidics to conduct high-throughput, small-volume experiments (Fodor 1993, Chee 1996). Also
clear was the possibility for process integration by developing “lab-on-a-chip” technologies, which is described in greater detail in a later section.

1.2.2 Soft lithography

Soft lithography was developed as a response to some of the limitations of photolithography, however using soft lithographic molds often still requires that some photolithographic techniques be employed in the early stages of device development. In the following experiments, for example, photolithography is used to build photoresist-patterned masters from which soft lithography molds are formed. Some of the limitations of photolithography include a limited ability to make surfaces compatible with cells and proteins, limited control over surface properties, expense, and a technology setup that is not readily available to most biological labs (Whitesides 2001).

As it is used in the following studies, soft lithography requires making an elastomeric stamp by cast molding it from the master made using photolithographic techniques (Haverkorn van Rijsewijk 1982). Uncured PDMS is poured over the master and cured on a hot plate, resulting in cross-linking of the PDMS (Clarson 1993). Subsequently, the cured PDMS mold can be easily peeled from the master, and is now patterned with the negative of the master’s pattern (Xia 1998). Pieces of PDMS or pieces of PDMS and glass can be bonded together using oxygen plasma treatment in order to construct complete devices. A detailed explanation of this process is provided in the Materials and Methods chapter.

1.2.2.1 PDMS

PDMS is an elastomer commonly used in soft lithographic applications, and is the elastomer used in the studies reported here. It has many highly desirable properties (Xia 1998), including optical clarity; straightforward surface modification; high permeability to gas; and mostly reversible reactions with surface treatments (Clarson 1993). Oxygen permeability in PDMS is superior to other
thermoplastics (Ochs 2014), and the gas permeability of microfluidic devices is very important for the success of biological experiments, as biological systems rely on efficient gas exchange and precise gas conditions.

As described earlier, uncured PDMS is poured over a master and then cured on a hot plate. The very low glass transition temperature \( T_g \) allows PDMS components (base and curing agent) to be liquid at room temperature (Xia 1998). The elasticity of the polymer allows for easily peeling of the PDMS, which in turn means that masters can be used many times before they need to be replaced—up to fifty times or more (Xia 1998).

PDMS does not swell in the presence of water (Clarson 1993), which is important for biological experiments that involve the use of liquid media. It is inherently hydrophobic, but can be rendered hydrophilic through oxygen plasma treatment (Xia 1998).

1.2.3 Advantages of the microscale

Many of the benefits of using microfluidic devices are due to the inherent advantages of operating at the microscale. These advantages are discussed in the subsequent sections, and include laminar flow, diffusion, a high surface area to volume ratio, and process integration.

1.2.3.1 Laminar flow

Microfluidic devices are characterized by low Reynolds numbers (Re), which are calculated according to the equation

\[ \text{Re} = \frac{v \cdot l \cdot \rho}{\mu}, \]  

(Equation 1)

where \( v \) is the velocity of the liquid moving in the channel, \( l \) is the width of the channel, \( \rho \) is the density of the liquid, and \( \mu \) its viscosity. When \( \text{Re} < 2000 \), fluid flow is laminar, which necessitates that all
transfer within the microfluidic device is a result of diffusion, as turbulent flow (and thus mixing) is not present (Whitesides 2001).

1.2.3.2 Diffusion in microfluidic systems

Because microfluidic systems are characterized by low Reynolds numbers, no turbulent flow is present to enhance mixing within a microfluidic system, and so simple diffusion adequately describes the transport of diffusive species within a microchannel.

Simple—or Fickian—diffusion is described by

\[ J = -D \frac{\partial C}{\partial x}, \] (2)

where \( J \) is the diffusive flux, \( D \) the coefficient of diffusion for a chemical species in a given medium, and \( C \) the concentration of the chemical species. For the purposes of examining diffusion in one direction, this equation can be expressed as

\[ J = -D \frac{\Delta C}{\Delta x}, \] (3)

where \( \Delta x \) may represent, for instance, a microfluidic channel width and \( \Delta C \) the difference in concentration from one side of the channel to the other. The relationship

\[ x^2 = 2Dt, \] (4)

describes the mean-square displacement of a particle in relation to time lapsed in the system (Tabeling 2006). Because time depends on the square of displacement, diffusion on the microscale is much faster than diffusion on the macroscale. In order to illustrate the effect of scale on diffusion time, consider two observers in a room with a jar of sulfur. Observer one is positioned 10 micrometers away from the jar, while observer two is positioned one million times farther away at 10 meters from the jar. At \( t = 0 \), the jar is opened and at \( t_1 \) the sulfur molecules (and odor) reach observer one at 10 micrometers. At \( t_2 \) the sulfur molecules reach observer two at 10m. Considering only Fickian diffusion as a method of transport and making use of Equation 4, it would take \( 10^{12} \) times longer for the molecules to reach observer two at
10m as it would observer one at 10 μm. Of course in reality, an observer on the macroscale would be able to smell the sulfur rather quickly, but that is due to the presence of turbulent flow, thermal gradients which facilitate transport in the air. While relying on diffusion for an experiment at the macroscale would be an either costly or impossible time commitment, diffusion can be a readily leveraged mode of transport at the microscale and within microfluidic devices.

PDMS, as was previously stated, is a commonly used polymer in microfluidics due to its many desirable qualities. For this discussion, though, of interest is its high permeability to gas. Microfluidic experiments for oxygen control frequently involve gas diffusion from a channel through a thin PDMS layer (or membrane) into another area of the device—perhaps another channel or a reservoir. It is important to note the differences in the way gas—specifically oxygen for the purposes of this discussion—behaves in PDMS versus water. Under identical conditions, oxygen gas is 1.7 times more diffusive in PDMS than in water, and six times more soluble in PDMS than in water (Polinkovsky 2009).

### 1.2.3.3 Surface to volume ratio

As all three-dimensional geometries shrink, the surface area to volume ratio of the geometry in question increases. For instance, a channel (rectangular prism) that is 25mm long, 1 mm high and 1 mm wide has a surface area to volume ratio of 4.4:1, whereas a channel that is 25mm long, 50μm high and 50μm wide has a surface area to volume ratio of 80.1:1. The latter dimensions of this rectangular prism are typical of the dimensions of a straight microfluidic channel.

The surface area to volume ratio of a microfluidic system is of importance when the surfaces of the microfluidic network are modified with an agent that is to interact in some way with the medium flowing through the device. For instance, experiments involving capture of rare cells, such as those that aim to capture circulating tumor cells in blood, require treating the surface of the device with antibodies
that will interact with and trap specific cells (Stott 2010, Riethdorf 2007). Because of the high surface
area to volume ratio of the channel, there is a greater chance that the cells of interest will interact with
the antibodies on the modified microfluidic surface in will in turn be trapped.

**1.2.3.4 Process integration**

Microscale systems allow for process integration, which is one of the reasons that microfluidic
systems are such a popular area of research. Process integration, as the name suggests, allows for the
combination of several laboratory processes onto a single chip—thus another name for microfluidics,
“lab-on-a-chip” (Tabeling 2006). The advantages of process integration include saving time and
reagents. Microfluidics launched as a field from MEMS in the early 1990s, and in that same decade
chips were developed that performed multiple processes on single chips, including a chip that performed
chemical reactions and capillary electrophoresis (Jacobsen 1994) and another that fully analyzed DNA
samples on a single chip (Burns 1998).

**1.3 The importance of the oxygen microenvironment in biological systems**

The oxygen microenvironment is studied extensively in mammalian systems, and many efforts
have been made to improve *in vitro* control of the O₂ microenvironment. Cellular function and behavior
are affected by the partial pressure of O₂, or oxygen tension, in the microenvironment. Oxygen tension
impacts a variety of vital biological processes including but not limited to embryonic development,
metabolism, and angiogenesis. Despite this fact, oxygen tension is an often-overlooked aspect of *in vitro*
systems aiming to reconstruct physiologically realistic microenvironments. Even in standard cell culture,
physiologic oxygen tension is largely disregarded. Incubators maintain cells at 37°C in 5% CO₂ (to
buffer the pH of cell culture media) and balanced air. The balanced air consists of approximately 21%
oxygen. However, the use of an ambient level of 21% oxygen in cell culture does not reflect the range of
oxygen tensions found normally in the cells and tissues of the human body. Normoxia, the term for the normal level of oxygen, is 21% in the earth’s atmosphere; we typically breathe a 21% oxygen gas mixture. In physiological contexts, normoxia in cells and tissues (Table 1) is well below ambient atmospheric oxygen tension, typically falling between 2-9% (Mohyeldin et al. 2010). Even in the most oxygenated parts of the body—the arteries, lungs, and liver—normoxia is only between 10-13% (Hung et al. 2011) so 21% oxygen is actually hyperoxic or above physiological oxygen concentrations. Other tissue-specific oxygen levels include 5% in venous blood (Barnikol and Pötzschke 2012), 1-7% in bone marrow (D'Ipollito et al. 2006), 0.5-7% in the brain (Ivanovic 2009), and 1% in cartilage (D'Ipollito et al. 2006).

The level of oxygenation is important, as it is a balance of oxygen availability and oxygen consumption that is necessary to maintain normoxia. Variations in oxygen tension—from above physiological oxygen tension (hyperoxia) to below physiological levels (hypoxia) or even complete absence of oxygen (anoxia)—trigger potent biological responses. For instance, hypoxia has been shown to support the maintenance and promote proliferation of regenerative stem and progenitor cells (Mohyeldin 2010, Hung 2011, Basciano 2011, Suda 2011). Paradoxically, hypoxia also contributes to the development of pathological conditions including systemic inflammatory response, tumorigenesis, and cardiovascular disease, such as ischemic heart disease and pulmonary hypertension.

Research has shown that a reduction in oxygen tension results in extensive alterations in gene expression. A vast array of hypoxia-related cell signaling pathways can be activated, including those for angiogenesis, metabolism, migration, proliferation, differentiation, and apoptosis. Virtually all hypoxia-related alterations in gene expression rely on the transcriptional activity of hypoxia inducible factors (HIF), a family of transcriptional factors (Ke 2006). The HIF family of transcription factors includes three known isoforms of α subunits: HIF-1α, HIF-2α, and HIF-3α. Of these isoforms, HIF-1α regulation has been best characterized (Semenza 2001). The HIF-1α subunit is continuously synthesized and
degraded under normoxic conditions. Following exposure to hypoxic conditions, HIF-1α accumulates rapidly. HIF-1α is stabilized and enters the nucleus where it heterodimerizes with the non-regulated subunit HIF-1β and initiates downstream transcription.

Both *Chlamydomonas reinhardtii* and higher order plants have mechanisms to accommodate changes in oxygen availability, specifically by activating fermentation pathways under hypoxic or anoxic conditions. Although *Chlamydomonas*’s relation to higher order plants means that well-understood pathways in *Chlamydomonas* can aid understanding of pathways in higher order plants and *vice versa*, the fermentation pathways in *Chlamydomonas* evolved independently of those of higher order plants (Mustroph 2010). As such, that the specifics of some of these pathways in higher order plants are well-understood has not translated to a clear understanding of these mechanisms in *Chlamydomonas*.

Oxygen control in plant and algal systems is of interest for a variety of reasons. Changing environmental conditions, such as flooding, can expose algae and plants to transient periods of hypoxia. Better understanding of plant responses to hypoxia can lead to the development of mutant strains of crops that will better tolerate hypoxic conditions, thus modulating the often-drastic effects of climate change (and thus flooding) on crop yields (Banti 2013). Some proteins encoded in the *Chlamydomonas* genome that are involved in anaerobic metabolism are also present in other anaerobic eukaryotes (Banti 2013), so understanding how these proteins respond to anoxic conditions can provide information about other organisms. Anaerobic metabolism in *Chlamydomonas* results in pathways that produce, among other things, H₂, which is being explored as a possible alternative fuel source. H₂ is only produced in *Chlamydomonas* under anoxic (or nearly anoxic) conditions (Melis 2000). Culturing *Chlamydomonas* under precisely controlled anoxic conditions using microfluidic devices could help to elucidate this pathway and also provide a means for understanding the H₂ production pathway enough such that
mutants increasing H₂ production can be developed, or for the development of strains that are more tolerant of anoxic environments (Banti 2013).

While not specifically related to oxygen, this discussion can be extended by including control of the CO₂ environment. *Chlamydomonas reinhardtii* has a mechanism for concentrating CO₂ under low-CO₂ conditions, and this pathway is well understood. Because of the divergence of *Chlamydomonas reinhardtii* and *Volvox carteri* from a common ancestor, there is reason to believe that an analogous mechanism may be present in volvox. Indeed, Yamano et al. demonstrated that volvox has a higher affinity for inorganic carbon (Ci) under low CO₂ conditions than high CO₂ conditions, and it is believed that the mechanism for concentration CO₂ is composed of inorganic carbon transport systems (Yamano 2011). As will be explained in subsequent sections, the applications of microfluidic control of the oxygen microenvironment to mammalian and bacterial cell culture systems are extensive. Considering the roles of oxygen and carbon dioxide in algal systems, it is worthwhile to explore how microfluidic platforms might also be used to better study these algae under controlled gas environments.

### 1.4 Control of the oxygen microenvironment

Controlling the oxygen microenvironment *in vitro* is important in many aspects of biology. This section explains how oxygen tension control is approached using conventional methods, as well as some of the limitations to these systems. Then, microfluidic approaches to O₂ tension control are explored.

#### 1.4.1 Using traditional methods

To study cellular behavior in low concentrations of oxygen, hypoxic chambers, workstations, and perfusion chambers have been the most widely used tools to create hypoxic environments. Unfortunately, these tools offer a single choice of a hypoxic level at a time. The homogenous oxygen levels provided also do not replicate oxygen gradients found *in vivo* which form from radial and axial
diffusion of oxygen from the microvasculature and metabolic consumption of oxygen by surrounding cells. Ultimately, the single oxygen level macroenvironments in such culture systems do not establish oxygen gradients that are physiological.

Hypoxic chambers remain as the tool of choice for imposing variable oxygen conditions because they are small enough to be housed inside a standard incubator, do not require specialized equipment for operation, and have the added advantage of being inexpensive (~$500) as compared to hypoxic workstations (~$50,000). Their price has made hypoxic chambers an attractive tool for labs interested in studying cells in low oxygen tension but not necessarily specializing in hypoxia. The hypoxic chamber consists of a vessel in which to place cell culture plates and dishes that can then be purged with a gas mixture of interest, sealed, and placed in an incubator. However, hypoxic chambers have several limitation: they are prone to leaks, are inherently low throughput, require considerable incubator space, cannot replicate anoxic conditions even when purged with nitrogen, equilibrate slowly (on the order of several hours), and are not compatible with microscopic analysis. Additionally, the oxygen level within a hypoxic chamber is imprecise. The oxygen concentration is not stably the same concentration as the infused gas throughout the chamber as transport limitations create a discrepancy between the gas concentration within the infused headspace and the gas concentration at the bottom of the culture dish.

The hypoxic workstation is a relatively large, sealed biosafety cabinet purged with a gas of interest, monitored with oxygen sensors, and equipped with its own incubator in one corner of the cabinet. Due to its cost, the hypoxic workstation is generally only found in labs specializing in hypoxia research. A workstation is attractive because, as compared to the hypoxic chamber, it is equipped with a small, gas-modulated bench top to perform conventional biological assays such as western blot and PCR preparations. The workspace is useful because hypoxic factors, like the HIF family of transcription factors, degrade rapidly upon re-equilibrating with atmospheric oxygen. Therefore, performing such assays in a sealed, hypoxic environment is ideal to achieve the best results. Atmospheric equilibration is
a concern when using hypoxic chambers, rather than hypoxic workstations, as they must be opened to retrieve cell culture contents or even to change media, forcing equilibration with ambient surroundings and an unintended intermittent hypoxia exposure which has been found to alter cell fate and function (Bhaskara 2012).

Though the workstation offers a precisely controlled, homogenous oxygen environment and space to perform biological assays, the setup is cumbersome. Small, delicate manipulations must be done from outside the cabinet while wearing bulky, integrated rubber gloves. Additionally, like the hypoxic chamber, the workstation cannot be easily coupled to live-cell microscopy unless a microscope is housed within the incubator. Overall, the hypoxic workstation is expensive and leaves too large of a footprint to be readily accessible to a wide population of researchers.

1.4.2 Using microfluidics

Microfluidics platforms have been used to control the oxygen microenvironment and to measure the effect of oxygen concentration on biological materials in a variety of ways, including exposing biological specimens to various constant concentrations of oxygen, discrete regions of different oxygen concentrations, and oxygen gradients, as is evidenced in the following sections. Several variations of microfluidic platforms have been used to generate the desired oxygen environment for these experiments, including devices that rely on diffusion from liquid or gas, utilizing on-or-off-chip mixers, leveraging cellular oxygen uptake to deplete the oxygen, relying on chemical reactions in channels to generate oxygen gradients in a device, and electrolytic reactions to produce oxygen directly on chip.

1.4.2.1 Early model

An early demonstration of oxygen modulation in a microfluidic device is a bioreactor system composed of four microfluidic cell culturing regions stacked one on top of the other, with a media
perfusion channel providing flow to the cell culture chamber. Inserted into the middle of the bioreactor (flanked above and below by two cell culturing regions) is what the authors call an “oxygen chamber,” which is connected via channels to the environment outside of the bioreactor. This serves as a way to allow gas from the outside environment to easily modulate the oxygen conditions within the culturing chamber. After culturing, this oxygen chamber design resulted in a 5-fold increase in cell growth compared to a 2-layer bioreactor and 8-fold increase compared to a 4-layer bioreactor without the oxygen chamber. Additionally, albumin production was monitored from cultured hepatocytes and only the cells grown in the 4-layer bioreactor with the oxygen chamber showed increased albumin production over a 12-day experiment (Leclerc 2004). Although no gas was perfused through the device, it represents a conceptual prototype for the more intricate and precise devices to follow.

### 1.4.2.2 Diffusion from gas

The most popular method for oxygen control within microfluidic channels is by diffusion of oxygen from gas channels across a thin, gas-permeable PDMS membrane and into liquid-filled cell culture channels. Oxygen control is demonstrated in a variety of ways, including discrete control (obtaining multiple, uniform oxygen concentrations), spatial control (binary oxygen concentrations, and spatial gradients of oxygen concentrations), and temporal control (switching between oxygen concentrations at set time intervals or maintaining a constant oxygen concentration over time). These different methods for oxygen control have been applied to different biological systems, and serve purposes beyond simply controlling oxygen concentration, ranging from improving the quality of an experiment, to creating a more physiologically realistic environment in which to grow cells, to studying the mechanisms of different diseases.

For example, a device was developed to address photobleaching problems in smFRET (single molecule fluorescence resonance energy transfer). Pathways for photobleaching in smFRET can be
mediated by oxygen. The device, which loads samples for smFRET, incorporates channels through which nitrogen gas is perfused, thus resulting in oxygen diffusion out of the adjacent channels without the use of chemical additives (a more common solution to the photobleaching problem) that can interfere with the smFRET signaling. This decreases the oxygen concentration around the area of the sample, and hence reduces photobleaching (Lemke, 2009). This example demonstrates that oxygen control has applications beyond cellular response to oxygen levels and demonstrates how microfluidics can enhance experimental data pertaining to an improved unveiling of biological phenomena.

### 1.4.2.2.1 Discrete control of oxygen levels

A number of devices exist for exercising discrete control of oxygen levels on a microfluidic platform. These devices may be designed such that one device contains several isolated regions of discrete oxygen concentrations, or such that they maintain constant oxygen concentrations over a period of time. Vollmer et al. presented a system for dynamic delivery and sensing of oxygen in perfusing medium when oxygen is delivered via a gas channel. PtOEPK oxygen sensors are placed in etched wells of a glass slide at the inlets and outlets of a microfluidic network to monitor oxygen levels. A custom excitation/collection module was created to house the device and monitor oxygen levels from the sensors. Again, oxygen diffuses from a gas channel across a PDMS membrane and into liquid channels. While this system is not applied to biological studies, it is an early example of the use of *in situ* PtOEPK sensors for oxygen characterization, and is a major advance in the development of microfluidic platforms for oxygen control of the microenvironment (Vollmer 2005).

Polinkovsky et al. present two devices in which individual growth chambers within two microfluidic devices take on discrete values ranging linearly between 0 and 100% oxygen concentration over nine chambers in one device, and exponentially from 0 to 21% oxygen concentration over nine channels in the second device. In these experiments, Ruthenium dye was used to characterize the oxygen
levels. For each device, the oxygen concentrations in the gas channels are achieved by flowing two gases through a three-step on-chip mixing channel network, culminating in nine separate channels of discrete concentration that flow over the growth chambers, which contain media. The growth chambers can be used to culture yeast, bacteria or mammalian cells, and for the purposes of this experiment *E. coli* division rates as a function of oxygen concentration were determined (Polinkovsky 2008). This device is interesting and useful because it presents a way to use one device to deliver many oxygen concentrations, and this in conjunction with multiple growth chambers means that high-throughput oxygen-controlled experiments can be conducted.

A microfluidic platform for culturing aerobic and anaerobic bacteria and mammalian cells is achieved using an on-chip mixer that creates a series of discrete oxygen concentrations by mixing oxygen and nitrogen that range linearly from 0 to 42%, and cells were cultured in channels at these oxygen concentrations. Incorporated into the device is a valve multiplexer, which was used to replace media in each of the eight wells at regular intervals. A custom excitation module was designed using LEDs as the excitation source and paired with a custom infrared detection module. PtOEPK polystyrene sensors were embedded in wet-etched wells of a glass slide. The sensors were calibrated using water with different concentrations of oxygenated water and correlated with the Stern-Volmer relation. Cell density and growth rates were studied with *E. Coli*, *A. Viscous*, *F. Nucleatum*, and embryonic fibroblast cells (Lam 2009). This device couples oxygen and valve control, and demonstrates that techniques necessary for cell culture (media replacement, in this case) can be incorporated into these experiments.

In addition to microfluidic culture chambers of custom microfluidic devices, Oppegard et al. developed a microfluidic insert for a standard 6-well plate was developed, which can be used to modulate oxygen concentration in cell culture in lieu of a hypoxic chamber, and with better oxygen control than a hypoxic chamber. The device’s oxygen concentration is characterized using a ruthenium coated substrate, and was further validated by monitoring HIF-1alpha expression in cells, to ensure that
it agreed with expression levels from traditional methods. The device is an insert for a standard six-well plate with a designated gap from the bottom of the plate, and has a microfluidic channel that runs through the mold, allowing gas to diffuse through the bottom of the mold to oxygenate or deoxygenate the multiwell plate. The oxygen concentration within the device rapidly changes when the input gas is changed, and can maintain a steady oxygen concentration (10%) over five days (Oppegard 2009). The main innovation of this work was adapting microfluidic oxygen control to the 6-well plate which is a standard workhorse of biomedical research. In a follow up paper they expanded on this theme to develop an insert for Boyden chambers which also nest into a multiwell plate for cell migration studies. The device’s ability to maintain an oxygen concentration in conjunction with its ability to adjust to a new oxygen concentration quickly when a new gas is flowed through the device make it very useful for both constant oxygen concentration studies as well as intermittent hypoxia studies. Hypoxia studies were conducted using an invasive breast cancer cell line (Oppegard 2010) and it was found that intermittent hypoxia resulted in different migration than constant hypoxia.

Abaci et al. present a microbioreactor for consistent, long-term oxygen control of the microenvironment with live computer monitoring of oxygen concentration in the device. The format of the device is a top gas channel which is used to diffuse oxygen into a lower, closed culture channel via a PDMS membrane. Both channels are etched into polymethylmethacrylate (PMMA). Fluorescent sensors were used to monitor the device’s dissolved oxygen level. The system is composed of sensor patches (flat 3 mm discs), fiber-optic guides, and a 4-channel transmitter device which interfaces with a computer. Media was constantly perfused at a relatively slow flow rate of 0.02 ml/h. The temporal responses of the oxygen tension in the channel in static conditions in which the gas channel was supplied with discrete oxygen levels (21%, 5%, and 1%) was compared to dynamic conditions which utilized the same discrete oxygen levels in the gas channel but also media perfusion in the culture channel at 0.5 ml/h were compared, and resulted in similar oxygen profiles. The shear stress introduced
by perfusion was negligible compared to shear stress levels reported to affect cell behavior. To demonstrate the bioreactor’s abilities, fibrosarcoma cells were cultured and cell viability, cell density and circularity tests were performed at 1% and 21% dissolved oxygen concentrations (Abaci, 2012).

Another example of a microfluidic device used to study the behavior of cancer cells in hypoxic and normoxic environments is presented by Funamoto et al., who designed a PDMS microfluidic device with an integrated 3D gel for cell culture flanked by media channels. Each media channel was separated from a gas channel by a 150µm diffusion gap. Oxygen diffusion between the incubator environment and the PDMS device was inhibited by a polycarbonate (PC) film bonded above the channels. The device was validated using a ruthenium-coated glass cover slip as an oxygen sensor. The device’s utility was demonstrated by studying the migration of human breast cancer cells (MDA-MB-231) in hypoxia. Time-lapse live-cell 3D confocal imaging was acquired to determine the cancer cell migration within the gel extracellular matrix (ECM). Cells in the gel showed increased net displacement, total path length, and their ratio (persistence) increased under hypoxia as compared to normoxia. Despite demonstrating that the device design lends itself to establish a gradient across the gel, cells were not studied in a gradient. Increased migration corresponds with increased invasive behavior of breast cancer cells reported in other studies (Funamoto 2012).

In another example, oxygen was used to control the polymerization of HbS blood as a model of a vasoocclusive crisis in sickle cell disease. Again, gas diffuses from a gas channel, across a PDMS membrane, and into the blood perfusion network. Occlusive and relaxation events due to HbS polymerization and depolymerization, respectively, are measured as a function of oxygen concentration by monitoring blood flow velocity in their microfluidic device. The device is presented as a tool to study sickle cell disease and possible future clinically useful agents to block HbS polymerization. CO binding is used to demonstrate that HbS polymerization can be blocked even in cases of extreme deoxygenation.
(Higgins 2007). This device represents the first model of the dynamic sickling process without influences of endothelial cells.

For some experiments, a binary oxygen concentration profile is useful to elicit a biological response as a result of exposure to two distinct oxygen regions. In the previously-mentioned paper, Oppegard et al. show the oxygen profile of a dual-condition microchannel, which can maintain a stable binary oxygen profile over fourteen days. Additionally, the author presents an interdigitated microfluidic channel network that generates a cyclic oxygen profile (Oppegard 2009). Mauleon et al. modified an existing brain slice chamber with a PDMS membrane and microfluidic channel layer. This device allows different areas of a 350 um thick brain slice to be exposed to different oxygen concentrations independently. Oxygen levels and calcium sensitive dyes were used to validate delivery of oxygen to discrete regions of brain slice anatomy (Mauleon 2011).

1.4.2.2 Oxygen gradients in the microenvironment

Diffusion from gas in microfluidic devices can also be used to generate gradients of oxygen concentration within the device. As a continuation of the work previously described by Polinkovsky et al., Adler et al. modified this device by controlling input to nine gas channels with computer-actuated three-way solenoid valves, which produce different mixtures to feed to the channels. Additionally, channel wall thicknesses are decreased so different gas concentrations generate a gradient rather than discrete oxygen concentrations. Again, Ruthenium dye is used to characterize the oxygen gradient. The authors propose that this device could be used to study unicellular organisms’ responses to chemotactic gradients, and noted that the device design would allow a user to modify the gradient intensity of specific regions of interest within the device (Adler 2009).

Most microfluidic oxygen control devices are limited to oxygen control within microfluidic channels, but a demonstration by Lo et al. presents gas channels buried within a gas permeable substrate
of a larger open well for two different microfluidic networks. One design relies on the diffusion between parallel flow gas channels, and the second design operates via direct mixing of gas in network channels. The oxygen profile generated via the parallel channel device is more linear compared to the mixing network device, from which the profile is strongly sigmoidal. The devices were characterized using a ruthenium substrate placed directly against the PDMS diffusion membrane. This device was used to determining the ROS response of cells exposed to 0-100% oxygen gradients. The results indicated ROS response is modulated by oxygen microgradient profiles as expected in hypoxia and hyperoxia (Lo 2010).

Microfluidic systems for controlling oxygen at the microscale have been applied to address a variety of physiologically relevant questions, for examining the behavior of cells in different and tightly-controlled oxygen environments, and they have been applied to studying specific pathologies including cancer, stroke, and sickle cell disease.

### 1.4.2.3 Diffusion of oxygen from liquids

Exposing cells and tissues to different oxygen levels can also be accomplished by oxygen diffusion from liquids. In many cases, media is equilibrated with the appropriate gas before introducing it to cultures. For instance, a commonly used method to mimic the induction of hypoxia is to place cell cultures in medium that has been bubbled with nitrogen (Holleyman 2001). The equilibration of media with nitrogen is frequently done in addition to housing the cultures in a hypoxic chamber or gas-controlled incubator (Wyld 1998) to reach even lower levels of oxygen. In microfluidic systems, cell culture media is also frequently equilibrated with appropriate gas mixtures to control oxygen content. In these systems, constant perfusion of the equilibrated media is usually necessary to maintain the desired oxygen level. Without an ideal, closed system, the oxygen concentration of media will re-equilibrate with ambient surroundings (i.e. the atmosphere) over time.
One example is the work by Grist et al. where diffusion from liquids was used to establish oxygen gradients across a central channel (Grist 2012). To create the on-chip oxygen gradient, off-chip gas bubblers produced deoxygenated and oxygenated water, which was fed via oxygen-impermeable tubing into designated control water channels. A deoxygenated control water channel flanked one side of a media perfusion chamber containing cells while an oxygenated water channel flanked the other side. Only a thin, PDMS barrier (~100 µm) separated the control water channels from the cell chamber. The high gas permeability of PDMS and the difference in the oxygen level in the control water channels allowed for the spatial gradient generation in the cell chamber. The perfusion rate of the oxygenated or deoxygenated water (100 µL/min) was maintained by syringe pumps which withdrew media, creating negative pressure to pull the liquid through the device. The media perfusion rate in the cell chamber was purposely maintained at a value several magnitudes lower (0.3 µL/min) than the control water channels to permit the formation of a stable oxygen gradient in the cell chamber.

Integrated PtOEPK sensors monitored the oxygen gradient and COMSOL® simulation modeling was undertaken to predict the oxygen gradient profile. However, the model did not fit the acquired data. Namely, the range of oxygen concentrations created experimentally was not as large as that predicted in the simulation. It is suggested that this discrepancy was due to the gas-permeable nature of PDMS and the rapid re-equilibration of the device’s channels with the ambient partial pressure of oxygen. The researchers expect that coating the outside of the PDMS block with an oxygen-impermeable coating could improve the available oxygen control.

Adjustment of the oxygen concentration in liquid is not limited to being performed prior to being introduced into microfluidic systems. On-chip hydration layers can be used as a means to allow diffusion from liquids. The hydration layer reduces the introduction of undesired bubbles created from direct diffusion of gas to a liquid in the experimental portion within a microfluidic device. A work in which a microfluidic device was designed to assess vaso-occlusive risk in sickle cell disease
demonstrated the utility of a PBS layer in preventing dehydration of blood samples and allowing gas transport to the blood (Wood 2012). In cross section, the design stacked a gas channel on top of a PBS channel on top of the blood sample channel. Oxygen concentration in the gas channel of the chip was modulated with solenoid valves from N2 and air sources, and oxygen levels were monitored with a fiber optic O2 sensor at the outlet of the gas channel. Deoxygenation of blood from sickle cell patients resulted in a reduction of flow velocity and blood conductance under the same pressure drop across the device. The reduction of flow velocity is due to the sickle shape adopted by the red blood cells (RBCs) as they became deoxygenated. The molecular basis for the shape change is a variant hemoglobin molecule, HbS, which is the result of a mutation in the gene coding the β-globin protein. Deoxygenation of these RBCs causes HbS to polymerize into long chains, which stiffens the cell and leads to the shape change. The morphological change then causes changes in flow by increasing the apparent viscosity of blood, resulting in differences in the rates of change in blood conductance (defined as the velocity per unit pressure drop). The rate of change in blood conductance was leveraged to measure disease severity.

Diffusion from gas to liquid within a microfluidic device can cause undesired bubbles in the experimental portion of the microfluidic device. In this case, a hydration layer may be added to a device, between the gas channel and the channel that will contain the sample. The gas bubbles are then introduced to the hydration layer rather than the experimental region of the device, and the constant flow of the liquid through the hydration layer washes bubbles away. Wood et al. added a hydration layer in the sickle cell device described above and used it to characterize the severity of sickle cell disease by creating an in vitro model of in vivo sickling, with sickling events induced by sudden deoxygenation of blood. The purpose of this experiment was to demonstrate a method for assessing disease severity in sickle cell patients, and the authors were able to do so by correlating the rate of change of blood rheological properties to disease severity (Wood 2012). This paper is especially significant to this thesis as it gave us the idea to include a hydration layer to prevent bubbles in our device.
1.4.2.4 In-channel reactions to generate gradients

Skolimowski et al. (2010) was the first to use an oxygen scavenger to create a gradient in a microfluidic cell culture device. A biofilm of *P. aeruginosa* was cultured on a thin PDMS membrane below which a serpentine channel carried the scavenger (10% sodium sulfite with 0.1 mM CoSO4 as a catalyst) which irreversibly consumed oxygen from the culture above. A glass slide with a PtOEPK sensor formed the top of the culture chamber and was used to characterize the effect of media flow on the gradient and to monitor oxygen levels of the culture. A media flow rate was chosen to apply a gradient that reduced oxygen saturation at the end of the device by 60% from ambient. Attachment of *P. aeruginosa* was shown to gradually decrease along the length of the decreasing oxygen gradient (Skolimowski 2010).

Chen et al. took this idea one step further by developing a device that generated and scavenged oxygen on chip using a pair of chemical reactions. The device consisted of a central cell culture channel that is flanked on either side with a chemical reaction channel. An oxygen gradient was formed across the central channel by an oxygen-generating reaction, H$_2$O$_2$ and NaOCl, and an oxygen-scavenging reaction, pyrogallol and NaOH. Each chemical species entered the chip through a dedicated channel, and both respective reactions were initiated on chip by a serpentine mixer just before meeting the cell culture channel. With proper throttling of flow in each channel, a steady, linear gradient could be formed across the cell culture and was characterized with a liquid ruthenium-based dye. Carcinomic human alveolar basal epithelial cells were cultured in the device under an oxygen gradient and with or without Tirapazamine, an anti-cancer drug that is activated to a toxic radical at low oxygen levels, to verify the oxygen sensitive effects on cancer cells (Chen 2011). This paper is of particular importance to this thesis, because it served as the motivation to use pyrogallol as a calibrating agent for determining the oxygen environment in our microfluidic devices.
1.5 **Measuring oxygen in microfluidic devices**

Measuring oxygen concentration in the microenvironment or within cell cultures presents specific challenges. The geometry and sample sizes are small. Many times, experiments require real time measurement as well as high spatial resolution of oxygen tension. Several tools have been adapted for measuring oxygen tension in the microenvironment, including employing either Clark-style electrodes or luminescent optical sensors.

For microfluidic systems, optical oxygen sensors are the tools of choice. They have several advantages over Clark-style electrodes. They do not consume oxygen, so they can be used in low or no flow environments and remain stable for long-term studies. Where Clark electrodes require an electrical connection to each position to be measured, optical sensors allow measurement over the whole area of the sensor, and at any number of discrete points. These sensors take advantage of oxygen-indicating fluorophores that are quenched in the presence of oxygen. The degree of quenching is determined by the oxygen partial pressure. The relationship between intensity and oxygen partial pressure is described by the Stern-Volmer equation,

\[
\frac{\tau_0/\tau}{I_0/I} = 1 + K_q[O_2] \quad (5),
\]

where \(I_0\) and \(\tau_0\) are the intensity and excited state lifetime in the absence of oxygen, respectively, \([O_2]\) is oxygen concentration, and \(K_q\) is the quenching constant. A Stern-Volmer calibration curve must be made for each sensor and application. When a sensor is calibrated, a corresponding Stern-Volmer curve is created by measuring the intensity at two known oxygen partial pressures. The intensity data collected is fitted to the Stern-Volmer equation to elicit corresponding oxygen partial pressure. Typically, a basic fluorescent microscopy setup is sufficient to monitor a fluorescent sensor-equipped device, although custom excitation/detector modules can also be created for portability, miniaturization, or placement in
Fluorophores are sensitive to photobleaching (attenuated intensity after long-term, constant excitation), but short periodic exposures are typically used to avoid photobleaching.

There are two main types of oxygen indicating fluorophores: ruthenium-based and metalloporphyrin-based. Oxygen-indicating fluorophore dyes such as these can be incorporated into sensors and probes to fit many applications. The simplest method may be directly flowing a suspension of 1 mg/ml of Ruthenium tris(2,2’-dipyridyl) dichloride hexahydrate (RTDP) through the device channels. This allows measurement of oxygen tension throughout the entire fluidic network (Sud 2006).

However, ruthenium is toxic, and to avoid adding toxic dye into culture media, the dyes can be permanently incorporated into a plastic matrix and embedded into channel walls or features. This PS/PtOEPK sensor can be cut and installed in regions where oxygen tension measurements are to be taken (Sinkala 2010, Vollmer 2005). The method for fabricating these sensors is described in the Materials and Methods section.

Oxygen indicating dyes can also be incorporated into a fiber-optic probe. The fluorophore is housed in an oxygen permeable medium at the tip of a fiber optic probe. With a proper optical setup, excitation and detection can now be performed through the fiber. Oxygen can be monitored by placing the fluorophore-doped tip in the sample or network. The advantage of fiber optic oxygen sensors is that they can be moved within the sample during measurements and be reused.

Both of these fluorophores are employed as oxygen indicators in various microfluidic studies. For the experiments conducted as a part of this research, platinum(II) octaethylporphine ketone (PtOEPK) was the sensor of choice.

1.6 The volvocine algae

The volvocine algae include unicellular *Chlamydomonas reinhardtii*, colonial *Gonium pectorale*, colonial *Pandorina morum*, colonial *Eudorina elegans*, and multicellular spheroid *Volvox carteri*, which
has several large centrally located gonidial cells and thousands of terminally differentiated, exteriorly located somatic cells (Starr 1969). The three colonial species are listed in increasing order of cell count and likeness in appearance to *Volvox carteri* (Kirk 1998). Because *Volvox carteri* and *Chlamydomonas reinhardtii* diverged from a common ancestor only fifty to seventy-five million years ago (Rausch 1989), they are used as model organisms for studying the evolutionary development of multicellularity and formation of terminally differentiated cells with designated functions.

Each of these volvocine algae is composed of cells that are very much like *Chlamydomonas reinhardtii*, and each contains $2^n$ of these cells, where $n$ varies from species to species (larger algae have higher $n$ values). Interestingly, when *Chlamydomonas reinhardtii* is dividing, a single cell enlarges $2^n$ fold and then undergoes multiple fissions in order to produce $n$ cells, and these cells then break out of the mother cell’s wall. Before the cells break out of the mother cell’s wall, they look quite a bit like a closely packed group of colonial cells (Kirk 1998).

*Chlamydomonas* proliferating inside of and then breaking out of the mother cell is analogous to what happens during volvox proliferation. The volvox life cycle lasts approximately 48 hours (Kirk 1998). The gonidial cells on the interior of the volvox undergo embryogenesis, which involves condensing of the gonidia followed by cleavage. Ultimately, the many-celled embryo inverts, and the volvox individual now appears to have several miniature volvox individuals inside of it. Eventually, these offspring hatch out of the mother volvox, and these newly-hatched individuals repeat the process. *Volvox* undergoing embryogenesis can be seen in Figure 1. *Volvox* approaching hatching with mostly mature individuals inside can be seen in Figure 2.
Figure 1. Volvox individuals with inverted embryos.
1.6.1 *Chlamydomonas reinhardtii*

The algal species for which most of the microfluidic devices in these experiments were designed is *chlamydomonas reinhardtii*, which is a member of the genus *Chlamydomonas*. *Chlamydomonas reinhardtii* (hereafter referred to as simply chlamydomonas) is a unicellular, photosynthetic green alga and, as mentioned in the previous section, a member of the volvocine algae. It is the only unicellular member of the volvocine algae, is bi-flagellated, and is typically approximately 10μm in diameter, though its size ranges from 5-30μm as the cell size varies throughout the cell division cycle (Harris...
Members of the genus *Chlamydomonas* are found in climates with a wide range of temperatures and environments. For instance, snow algae include multiple species of *Chlamydomonas* and, as the name suggests, these algae are found in snow or ice (Harris 2009, Thomas 1972). Other species demonstrate tolerance for acidic environments and high saline environments (Harris 2009). Originally, chlamydomonas was isolated from a soil sample (Harris 2009).

In the laboratory, chlamydomonas cultures can be expanded on agar plates or in liquid medium (Harris 2009), with agar plates also allowing the opportunity for long-term storage. The cultures should be maintained in the range of 20-25 degrees Celsius with constant stirring in order to prevent shading in more dense cultures (Harris 2009), and a light-dark cycle should be initiated if synchronous cell growth is desired (Harris 2009). In light, exponential growth is exhibited by the algae (Harris 2009). When in liquid culture, chlamydomonas may form palmelloids, which are clusters of algae that are stuck together. In *Chlamydomonas reinhardtii* these tend to form due to incomplete hatching of daughter cells from the mother wall during mitosis, and complete cells in groups of four or eight are visible inside of a single cell wall, whereas other species of *Chlamydomonas* may form as a result of environmental stress (Harris 2009). Palmelloid formation may be the mechanism leading to the formation of colonial algae such as *Gonium pectorale* (Kirk 1998).

As was mentioned in a previous section, chlamydomonas receives much attention due to its potential as a source for biofuel. *Chlamydomonas* produces H₂ as a result of anaerobic metabolism, which occurs under anoxic conditions, but this pathway is not fully understood. H₂ is, of course, a viable alternative fuel source, and better understanding of this mechanism and how to more efficiently generate H₂ could be of great use. Proliferation of chlamydomonas under precisely controlled gas conditions could be a helpful tool for studying this mechanism.

In addition to its potential as a generator of H₂ as an alternative fuel source, chlamydomonas stores lipid and triglyceride bodies, and these oils can be extracted and converted to biodiesel (Wijffels
One of the factors hindering the use of microalgae as a viable alternative fuel source is production capacity (International Energy Agency 2010). If specific gas conditions would result in increased proliferation rates or in an increased amount of fatty bodies in each chlamydomonas, this could help the effort to produce greater amount of algae without an increased demand for space. A recent study showed that chlamydomonas lipids were affected by growth in dark, anoxic conditions, including degraded fatty acids but increased triacylglycerols (Hemschemeier 2013).
2: MATERIALS AND METHODS

2.1 Microfluidic device fabrication

All microfluidic devices were made using standard soft photolithographic procedures, which are explained in detail in the introduction. Briefly, negative photoresist is spun to the desired thickness on a silicon wafer and exposed to ultraviolet (UV) light under a mask so that some regions of the photoresist are exposed to the light and others protected from it. When the wafer is treated with developer solution, the regions that were exposed to the UV light remain intact on the wafer and those that were protected from the UV light wash away. The result is a photoresist pattern on the wafer matching the pattern from the mask, now called a master. When a PDMS mold is to be cast from the master, the photoresist pattern is the negative of the final desired pattern.

2.1.1 Multilayer algae culture chamber device

The multilayer algae culture chamber device was composed of five layers. From the bottom of the device to the top, the layers consisted of a glass slide, a gas channel layer, a membrane, a hydration channel layer with a built-in membrane, and finally a chamber layer, which contained the algae during experiments. The gas channel layer was used for the perfusion of gas, which diffused through the device and into the chamber layer which housed the algae. This allowed for culturing algae in precisely controlled oxygen conditions. A membrane layer was used between the gas and hydration layers for the purpose of separating the two layers, thus containing water to the hydration layer. Water was perfused through the hydration layer in a direction opposite to the flow of the gas through the gas channel in order to generate countercurrent flow. The hydration layer helped to prevent the formation of gas bubbles in the culture chambers, which appeared in the culture chambers when the chambers were separated from
the gas layer by only a membrane. By adding a hydration layer between the gas layer and culture chamber, gas bubbles that might form appear in the hydration layer, which is constantly moving and supplied with fresh water, and so the gas bubbles are washed away before reaching the algae culturing chambers. The membrane layer built into the hydration layer prevented mixing of water from the hydration layer with the contents of the culture chambers. The top layer contained three chambers for culturing *Chlamydomonas reinhardtii*.

### 2.1.1.1 Fabricating masters for individual layers

SU-8 masters were fabricated using standard photolithography techniques (as described in the introduction). From these SU-8 masters, PDMS molds used for construction of the devices were formed.

#### 2.1.1.1.1 Gas layer

The gas layer master was made by spinning SU-8 2100 photoresist (MicroChem) to a thickness of 100 microns on a silicon wafer that had been dehydrated by baking at 120 degrees Celsius for 15 minutes and allowed to cool to room temperature prior to use. SU-8 was first poured onto the wafer, and the wafer was then spun at 500 rpm for 10 seconds followed by 3000 rpm for 30 seconds. Spinning SU-8 onto a wafer results in the formation of an edge bead, which is a circle of finite thickness at the edge of the wafer with a higher photoresist profile than the rest of the wafer. The edge bead was removed by using a razor blade to scrape it from the wafer. The wafer was then baked on a hot plate at 65 degrees Celsius for 5 minutes to prevent thermal shock and then at 95 degrees Celsius for 25 minutes. The wafer was covered with an inverted petri dish while on the hot plate to prevent bubble formation.

After this initial bake (called a soft bake), a photomask patterned with the gas channel design was placed on top of the wafer, and a clear, glass weight on top of the photomask. The purpose of the weight was to ensure that the photomask was flush against the photoresist. The wafer was then exposed
to UV light for a time such that the photoresist was exposed to at least 240mJ/cm$^2$ of UV energy. The time for this exposure varied depending on the intensity of the UV bulb being used. This time was then doubled to account for the presence of the glass weight.

Following UV exposure the wafer was placed on a hot plate to bake a second time (called a post-exposure bake), first at 65 degrees Celsius for 5 minutes and then a 95 degrees Celsius for 12 minutes. Again the wafer was covered with an inverted petri dish to prevent bubble formation. When the wafer finished baking, it was left to cool to room temperature on the hot plate before proceeding.

The cooled wafer was placed in a petri dish and covered with SU-8 developer and placed on an orbital shaker in a fume hood in order to clean the unexposed SU-8 from the wafer. When visual inspection showed that most of the unexposed SU-8 had been washed from the wafer, the developer was removed from the dish, fresh developer was added, and the wafer was again left to soak in the developer on the orbital shaker to finish the development process. When the wafer finished developing, it was removed from the developer solution, rinsed with isopropanol, and dried with compressed N$_2$.

### 2.1.1.2 Hydration layer

The hydration layer master was made by spinning SU-8 2050 (MicroChem) to a thickness of 50 microns on a silicon wafer that had been dehydrated by baking at 120 degrees Celsius for 15 minutes and then cooled to room temperature prior to use. SU-8 2050 was poured onto the wafer and first spun at 500 rpm for 10 seconds and then at 3250 rpm for 30 seconds. After spinning, the edge bead was removed in the same fashion as for the gas layer.

The wafer was baked for 5 minutes at 65 degrees Celsius and then for 9 minutes at 95 degrees Celsius under an inverted petri dish to prevent bubble formation. It was then covered with the photomask patterned with the design for the hydration layer, which was weighed down with a clear, glass weight to ensure that the photomask was flush against the photoresist. The photoresist-coated
wafer was then exposed to UV light through the photomask for a length of time such that the wafer was exposed to at least 160mJ/cm$^2$.

After UV exposure, the wafer was baked again, first for 5 minutes at 65 degrees Celsius and then for 7 minutes at 95 degrees Celsius. When this post-exposure bake was complete, the wafer was allowed to cool to room temperature on the hot plate before being developed. The hydration layer wafer was developed in the same fashion as the gas layer wafer.

### 2.1.1.1.3 Algae chamber layer

The algae chamber layer master was made by spinning SU-8 2150 (MicroChem) to a thickness of 200 microns on a silicon wafer that has been dehydrated by baking at 120 degrees Celsius for 15 minutes and then cooled to room temperature prior to use. SU-8 2150 was poured onto the wafer and spun at 500 rpm for 10 seconds and then 2750 rpm for 30 seconds. Edge bead removal was again performed.

The wafer was baked at 65 degrees Celsius for 5 minutes and then at 95 degrees Celsius for 40 minutes under and inverted petri dish. It was then covered with the photomask patterned with the algae chamber design and topped with a clear glass plate to ensure that the mask was flush against the wafer. It was exposed to UV light for a length of time such that the wafer was exposed to at least 115mJ/cm$^2$.

The wafer was post-exposure baked for 5 minutes at 65 degrees Celsius and 15 minutes at 95 degrees Celsius and then allowed to cool to room temperature on the hot plate. It was developed in the same manner as described for the gas layer. Several iterations of the design for this chamber layer of the device were auditioned.

For experiments with *Volvox carteri*, the procedure for making this layer was changed. In order to achieve a master with an SU-8 depth of 500 microns, SU-8 2150 was spun to a thickness of 250 microns by spinning at 500 rpm for 10 seconds and then 30 seconds at 2250 rpm. Edge bead removal
was followed by a soft bake for 7 minutes at 65 degrees Celsius and 60 minutes at 95 degrees Celsius; the spinning and edge bead removal steps were repeated (thus depositing a second layer), and the wafer was soft baked at 65 degrees Celsius for 10 minutes and then 90 minutes at 95 degrees Celsius. The wafer was exposed to 600 mJ/cm² of energy through the loading layer mask, and the post-exposure bake was done for 5 minutes at 65 degrees Celsius and 30 minutes at 95 degrees Celsius. After these changes, the wafer was processed as the wafer for the 200 micron layer.

2.1.1.2 Treatment with aminosilane

Completed masters were treated with Trichloro(1H,1H,2H,2H-perfluorooctyl)silane (Sigma-Aldrich, 448931), which helps to prevent PDMS from sticking inside of small SU-8 structures and delamination of long, narrow structures. To treat the masters, the silicon wafers with SU-8 structures were placed in a vacuum chamber. A glass slide with a 30µL drop of Trichloro(1H,1H,2H,2H-perfluorooctyl)silane was also placed in the vacuum chamber. The vacuum was turned on and pulled air out of the chamber continuously for thirty minutes, and then the chamber was sealed so that a vacuum could be maintained. The vacuum was maintained for two hours before releasing and using the wafers.

2.1.1.3 PDMS molds for individual layers

For the gas and chamber layers of the multilayer algae culture chamber device, PDMS was mixed in a ratio of 10:1 base to curing agent, degased, and poured onto the silicon wafer patterned with SU-8, which was placed face-up inside of a Petri dish. The wafer and PDMS were degased to ensure that bubbles were not present; this was especially important for the gas and hydration layers, which contained many small holes as part of a structure to generate posts. After all bubbles were removed, the PDMS-covered wafer was baked on a hot plate at 85 degrees Celsius for one hour in order to cure the
PDMS. Cured PDMS was then peeled from the SU-8 master, yielding a layer of PDMS with a pattern on one side and smooth PDMS on the other.

The PDMS hydration layer was made by pouring degased 10:1 base to curing agent PDMS onto the hydration layer master and spinning it for 10 seconds at 500 rpm and 30 seconds at 600 rpm. This generated a PDMS hydration layer with a thin membrane built in. Similarly, the second membrane layer was made by spinning PDMS onto a blank silicon wafer for 10 seconds at 500rpm and 30 seconds at 800 rpm, which resulted in a membrane 100 microns thick. After spinning, wafers were baked at 85 degrees Celsius for 30 minutes, until the PDMS cured.

2.1.1.4 Assembling the device

The individual PDMS layers of the multilayer algae culture chamber device were irreversibly bonded together using oxygen plasma treatment. A stainless steel dispensing needle with edges that were beveled was used in each layer of the device that required inlet/outlet ports, and gave holes of diameter 2.4mm (McMaster Carr, 6710A17). First, holes were punched for each of the inlet/outlet ports in the algae chamber layer. Next, the patterned side of the PDMS chamber layer and the PDMS hydration layer membrane (which had not yet been peeled from the wafer) were treated with oxygen plasma. This was done using a corona treater (Electro-Technic, model BD-20A). While running, the corona treater is hovered over each region of PDMS that needs to be deposited with reactive O$_2$ species. The surfaces that have been treated (in this case, the patterned side of the PDMS chamber layer and the membrane side of the combined hydration-membrane layer) were pressed together and then placed on a hot plate at 120 degrees Celsius under a weight. They were baked for 20 minutes before proceeding.

The combined chamber and hydration-membrane layers described above were cooled to room temperature. A knife was used to slice through the PDMS membrane to the silicon wafer all the way around the combined layers, and the chamber layer plus membrane-hydration layer was gently peeled
away from the silicon wafer to reveal the patterned side of the membrane-hydration layer. The hydration layer side (patterned side) of the hydration-membrane layer was treated with the corona treater along with the plain membrane (still attached to the silicon wafer) in the same manner as described above. These treated surfaces were then bonded together and placed on a hot plate for 20 minutes at 120 degree Celsius and weighted down. The order of the layers on the hot plate from bottom to top were membrane layer on top of silicon wafer, hydration layer with built-in membrane layer, and chamber layer.

After baking and cooling to room temperature, a knife was used to cut through the PDMS membrane to the silicon wafer around the combined layers, and the membrane was peeled (along with the attached layers) from the silicon wafer. The newly revealed side of the membrane layer was treated with oxygen plasma along with the patterned side of the gas layer, and these layers were pressed together to bond irreversibly. The gas through chamber layer was placed on a hot plate at 120 degrees Celsius under a weight and baked for 20 minutes.

After cooling to room temperature, holes were punched through the entire device at the gas channel inlet and outlet ports as well as the hydration channel inlet and outlet ports. For this step, it was very important to wait until the device was completely cool or else crack would form around the ports when punching holes, which would lead to a poor seal between the PDMS and an inlet/outlet tube and ultimately leaking.

Finally, the flat side of the gas channel PDMS layer and a glass slide were treated with oxygen plasma and bonded together, then again placed on a 120 degree Celsius hot plate and baked under a weight for 20 minutes. After this step, the device was fully constructed. A picture of the device with different dyes highlighting the loading layer, gas layer, and hydration layer is depicted in Figure 3. The loading layer went through multiple iterations, of which this was one. Ultimately, the loading channels were shortened such that they were located over the gas and hydration layers. This prevented algae from clustering in the long channels away from the imposed gas conditions.
Figure 3. The multilayer algae culturing device. The gas channel is yellow, the hydration channel is red; the overlap between these two looks orange. The loading layer is green.

2.1.2 Droplet generating device

A droplet generating device was designed to form droplets of chlamydomonas suspended in TAP medium—which is a typical medium used for chlamydomonas culture (described in greater detail in a later section)—inside of an oil layer. The device was also designed to transport the droplets from the site of their formation to a storage chamber in a style similar to an existing algae droplet generator (Pan 2011), but with all of these components contained on the same chip rather than a two-chip system.

2.1.2.1 Fabricating masters for droplet generating device

The droplet generator and housing chamber master was made by spinning SU-8 2050 (MicroChem) to a thickness of 50 microns on a silicon wafer that had been dehydrated by baking at 120 degrees Celsius for 15 minutes and then cooled to room temperature prior to use. SU-8 2050 was poured
onto the wafer and first spun at 500 rpm for 10 seconds and then at 3250 rpm for 30 seconds. After spinning, the edge bead was removed in the same fashion as described in 2.1.1.1.

The wafer was baked for 5 minutes at 65 degrees Celsius and then for 9 minutes at 95 degrees Celsius under an inverted petri dish to prevent bubble formation. It was then covered with the photomask patterned with the design for the droplet generator, which was weighed down with a clear, glass weight to ensure that the photomask was flush against the photoresist. The photoresist-coated wafer was then exposed to UV light through the photomask for a length of time such that the wafer was exposed to at least 160mJ/cm².

After UV exposure, the wafer was baked again, first for 5 minutes at 65 degrees Celsius and then for 7 minutes at 95 degrees Celsius. When this post-exposure bake was complete, the wafer was allowed to cool to room temperature on the hot plate before being developed. The droplet generator layer wafer was developed in the same fashion as described in 2.1.1.1. Finally, the completed master was treated with Trichloro(1H,1H,2H,2H-perfluorooctyl)silane as described in 2.1.1.2.

2.1.2.2 Assembling the device

A PDMS mold was made for the droplet generator and housing chamber in the same manner as for the multilayer algae culture device. The cured mold was peeled off, and holes were punched for the oil and media inlets and the outlet, using the same size (2.4mm diameter) as for the multilayer algae culture device. The corners were cut off of a two inch by three inch glass slide. One side of the glass slide and the side of the PDMS mold with the droplet generator pattern were treated with oxygen plasma using the corona treater in the same fashion as described in 2.1.1.4. The treated PDMS and glass slide were pressed together and placed on a 120 degree Celsius hot plate under a weight and left to bake for 20 minutes.
After cooling to room temperature, the droplet generator and housing chamber device was filled with Novec 1720 (3M) such that the device was fully coated in order to render the generator channels and housing chamber hydrophobic. The Novec 1720 was left to evaporate, and the device was baked at 130 degrees Celsius for 15 minutes. Next, uncured PDMS in a 10:1 ratio was poured into the bottom of a three inch diameter petri dish. The device was placed on top of the PDMS (slide side down), and placed on a hot plate under a weight to cure. This ensured that the droplet generator and chamber device was affixed to the bottom of the petri dish.

### 2.2 Oxygen characterization

For these experiments, all oxygen characterization incorporated platinum(II) octaethylporphine (Frontier Scientific) ketone (hereafter PtOEPK) sensors into the microfluidic device. The fluorescent intensity of the PtOEPK sensor was monitored in response to changing oxygen concentrations, and from this the oxygen concentration inside the device was determined.

#### 2.2.1 PtOEPK sensor fabrication

PtOEPK solution is composed of PtOEPK powder and a solution of PS dissolved in toluene. First, a 35% w/w solution of PS/toluene is made. PS and toluene were mixed together in a glass jar, covered, and left to mix on a shaker for 24 hours. PtOEPK powder was added to this PS/toluene solution at 1mg/mL and left to shake for another 24 hours. The resulting solution was poured onto a glass slide and spun at 2000rpm. The slide was placed under a hood and protected from light by covering with aluminum foil and left overnight so that the toluene could evaporate. The result was a sensor with PtOEPK embedded in a thin PS film.
2.2.2 PtOEPK sensor incorporation into multilayer algae culture device

PtOEPK sensors were incorporated into the multilayer algae culturing device in two ways. For the purpose of measuring oxygen at the bottom of the culturing well, a PtOEPK sensor was placed on top of the membrane built into the hydration layer just before its PDMS mold finished curing. This ensured that the sensor was stuck at the top of the membrane, and then when this layer was plasma bonded to the chamber layer, the sensor was positioned at the bottom of the chamber well. In order to place a sensor at the top of the culture chamber well, a cue tip was barely dipped in uncured PDMS and then the cue tip was dabbed onto the top of a different well in the culture chamber layer. After the culture chamber layer and hydration-membrane layer were bonded together, there was one well with a sensor at the bottom of the well and another well with a sensor at the top of the well.

2.2.3 Characterization using compressed air and nitrogen as calibrating agents

In order to determine the oxygen environment achieved in the chamber layer of the device when flowing gas through the gas layer and water through the hydration layer, the PtOEPK sensor inside the device first needs to be exposed to oxygen standard concentrations for calibrations. The fluorescent intensity of the PtOEPK sensor, which is quenched in the presence of oxygen, when exposed to these standards is then compared to the fluorescent intensity of the PtOEPK under experimental conditions, and by employing the Stern-Volmer equation (Equation 5), the oxygen concentration in the culture chamber (where the PtOEPK sensor is located) under experimental conditions can be calculated.

First, the culture chambers were filled with water. To measure the fluorescent intensity of the sensors in response to gas environments, compressed air was flowed through the hydration layer and the fluorescent intensity of the sensor measured, and then N₂ was flowed through the hydration layer and the corresponding fluorescent intensity of the PtOEPK sensor measured. These PtOEPK sensor fluorescent
intensity values in response to air (~21% O\textsubscript{2}) and N\textsubscript{2} (0% O\textsubscript{2}) served as calibration values when using the Stern-Volmer equation.

After calibration was complete, the experimental measurement was performed. Water was flowed through the hydration layer at a rate of 0.5mL/hour and gas was flowed through the gas layer. First, compressed air was flowed through the gas channel for five minutes, and then the gas was switched to N\textsubscript{2} and the measurement continued until the fluorescent intensity values of the PtOEPK sensor reached a stable number. For the bottom of the well, the complete compressed air to N\textsubscript{2} measurement took 45 minutes; this same experiment took 60 minutes for the top of the well. This was performed three times, and then the intensity values were plugged into a MATLAB program that calculated the oxygen concentrations by using the Stern-Volmer equation.

Measurements were taken in the same fashion but with a chamber filled with medium containing \textit{chlamydomonas reinhardtii} in order to demonstrate that the same oxygen concentrations achieved without algae can be achieved with algae present. Additionally, 24-hour measurements with algae present were conducted in the same fashion in order to test whether a 0% oxygen environment (formed via N\textsubscript{2} gas) could be maintained over 24 hours.

\textbf{2.2.4 Characterization using pyrogallol as calibrating agent}

The multilayer algae culture device oxygen environment was also characterized by using the oxygen scavenger pyrogallol and water as a calibrating agents rather than compressed air and N\textsubscript{2} as calibrating agents. For this experiment, bottom to top order of the construction of the device, from the gas layer to the algae chamber layer, was reversed. This was because pyrogallol changes from being a clear to a very dark brown liquid as it scavenges oxygen, and switching the order of the layers of the device prevented a dark solution from being present between the fluorescent sensor and the fluorescence
detector—and thus affecting accurate detection of fluorescence—without changing the geometry of the device.

2.2.4.1 Making pyrogallol solution

The oxygen-scavenging pyrogallol solution was made by making a solution of pyrogallol in water at a concentration of 100mg/mL and a 1M NaOH solution and then combining these two solutions at a ratio of 1:1. Because pyrogallol becomes an oxygen scavenger in basic conditions, the two solutions (pyrogallol in water and 1M NaOH) had to be combined under inert conditions to maintain the scavenging potential of the pyrogallol. This was achieved by placing the two solutions in a glove bag that was flushed with N₂ gas and working inside the glove bag to combine the two solutions in a 1:1 ratio. The new solution was pulled into a syringe that would be used to pump the solution into the microfluidic device while still in the N₂ glove bag.

2.2.4.2 Using pyrogallol in oxygen characterization

The oxygen environment in the multilayer algae culture device was also measured by using pyrogallol, an oxygen quencher, to calibrate the device. A PtOEPK sensor was present in the bottom of one of the wells of the culture chamber layer, and again the fluorescent intensity of the sensor as a function of oxygen concentration was measured. Again, the chamber well was filled with water. First, water was flowed through the hydration layer at a rate of 0.5mL/hour, and the fluorescent intensity of the PtOEPK sensor with water on the other side of the membrane was measured. Then the water was switched to pyrogallol at the same flow rate, and the fluorescent intensity of the PtOEPK sensor was recorded as pyrogallol scavenged oxygen, and this was continued until the fluorescent intensity value reached a stable number.
After calibrating with pyrogallol, the hydration layer was flushed with water until the fluorescent intensity of the PtOEPK sensor returned to a value typical of ambient conditions. The oxygen environment characterization was then performed under experimental conditions, with water pumped through the hydration channel at a rate of 0.5mL/hour and gas flowed through the gas channel in the direction opposite to the water. Compressed air was injected into the gas channel for the first five minutes, followed by N\textsubscript{2}. This procedure was performed three times. Fluorescent intensity values were plugged into the MATLAB Stern-Volmer program to calculate oxygen concentration values as with experiments performed used compressed air and N\textsubscript{2} as calibrating agents.

2.3 Chlamydomonas culture

*Chlamydomonas reinhardtii* were cultured at room temperature. The cultures were grown in 200mL Erlenmeyer flasks which were topped with aluminum foil and placed on an orbital shaker. Each Erlenmeyer flask was filled with 50mL TAP medium, and was inoculated with enough chlamydomonas such that the desired cell concentration was achieved. Cell counting was done with either a hemacytometer or an automated cell counter (BioRad TC-20 Automated Cell Counter).

2.3.1 TAP medium

TAP medium was made according to a standard recipe. Three solutions are used: TAP salts, a phosphates solution, and Hutner’s trace elements. The TAP salts are made by adding 15.0g of NH\textsubscript{4}Cl, 4.0g MgSO\textsubscript{4}·7H\textsubscript{2}O, and 2.0g CaCl\textsubscript{2}·2H\textsubscript{2}O to a flask and adding water to a volume of 1L. The phosphate solution is made by adding 28.8 g K\textsubscript{2}HPO\textsubscript{4} and 14.4 g KH\textsubscript{2}PO\textsubscript{4} to a flask and adding water to a volume of 100mL. Hutner’s trace elements were purchased. The final TAP solution used for chlamydomonas culture was made by combining 2.43g Tris, 25mL of the TAP salts solution, 0.375mL of the phosphates
solution, 1.0mL of Hutner’s trace elements, 1.0mL of glacial acetic acid, and adding water to 1L. This final solution is then autoclaved.

### 2.3.2 Growth curve

The chlamydomonas growth curve was determined in order to demonstrate that chlamydomonas growth was indeed following a typical S-curve shape. First, chlamydomonas were inoculated at approximately $0.5 \times 10^5$ cells/mL, and a cell concentration measurement taken to determine the exact concentration. Beginning 24 hours after inoculation, cell concentration measurements were taken approximately every eight hours until the end of the exponential growth phase was achieved. Measurements were taken using an automated cell counter (BioRad, TC-20).

### 2.3.3 Algae diameter measurement

Determining the diameter of chlamydomonas cells was achieved using the automated cell counter in conjunction with the TC-20 Data Amalyzer, software that measures the diameter of the cells from the sample that was counted.

### 2.4 Multilayer algae culture device experiment

The following sections describe the execution of two different approaches to measuring chlamydomonas proliferation when housed in the multilayer culture device and exposed to varying gas environments. The first approach is for counting chlamydomonas off-chip, and the second is for monitoring chlamydomonas proliferation on-chip.
2.4.1 Multilayer algae culture device for counting chlamydomonas off-chip

Each of the three wells of the algae culture chamber was filled with chlamydomonas. This was done by first fitting a syringe with a female luer barb (Cole Parmer Female Luer Barb, part number 45501-01) and then filling the syringe with chlamydomonas culture. Chlamydomonas was injected into wells by inserting the luer barb into the port for the well and then pushing the syringe plunger until chlamydomonas emerged from the opposite port. Each well was filled in this manner, and the inlet and outlet ports for each well were plugged. The plugs were made by melting the larger side of a reducing connector in the flame of a Bunsen burner (Cole Parmer Reducing Connector, Polypropylene, part number EW-40621-95). The un-melted end of the reducing connector was then filled with uncured PDMS. The PDMS was then cured, and the plug ready for use. The small (un-melted, PDMS-filled) end of the reducing connector was plugged into the port. Two of these plugs were needed for each well of the device.

After filling the wells with chlamydomonas, the hydration layer was filled, and water was continuously pumped through the hydration layer at a flow rate of 0.5 mL/hour from a 30 mL syringe using a syringe pump (New Era Pump Systems, NE-300). The syringe was fitted with a 21 gauge needle with a blunted end. The syringe was connected to the hydration layer inlet port via Tygon tubing with an outer diameter of 3/32” (Cole Parmer Tygon lab Tubing, EW-06407-70). The tubing was inserted directly into the port. At the outlet port of the hydration layer, a piece of the same tubing was connected and led into a container to collect water.

Gas was connected to the gas inlet channel via polypropylene tubing connected to a rotameter, which was in turn connected to a regulator on the gas tank. The gas flow was controlled by the rotameter (Omega, FL-5311G), which was maintained at 14 CCM. Tygon tubing was connected to the outlet for the gas channel in the same fashion as described for the hydration channel, and the end of the tube was
placed in a flask containing water to check for bubbles to ensure that the gas was indeed flowing through the channel.

After the algae were loaded into the device and the hydration and gas channels hooked up to their respective sources, the device was left for 24 hours. In order to count cells, each well was flushed and the algae collected into a microcentrifuge tube. Tygon tubing was connected to the inlet and outlet of each well. First, air was flowed through the tubing to push the algae out. Then 50 microliters of TAP medium was flushed through the well via the tubing to collect remaining algae. The tubing was then flushed with air again to push the liquid into the microcentrifuge tube. Counts were done using either a hemacytometer or an automated cell counter (BioRad, TC-20).

### 2.4.2 Multilayer algae culturing device for monitoring chlamydomonas on-chip

The procedure for 2.4.1 was repeated, except that the device was placed on a microscope stage and photos were taken at the beginning of the experiment and the end of the experiment, or continually over 24 hours. The device was not flushed but instead the algae were counted by observation inside 200 micron diameter chambers.

### 2.5 Droplet Formation

The droplet-generating device in its Petri dish was placed on the stage of an inverted microscope. Tygon tubing was connected to the outlet of the droplet-generating device and also to the oil inlet. The droplet-generating device was filled with oil containing surfactant (Dolomite PicoSurf 2% in FC-40, 3200202). Oil and algae each were pumped through syringes with flow rates controlled by syringe pumps. The syringes were connected to Tygon tubing with an outer diameter of 3/32” (Cole Parmer Tygon lab Tubing, EW-06407-70) via needles with blunted ends. After the device was filled with oil, the oil was flowed through the device at a rate of 100 microliters per hour, and then the algae was
connected via Tygon tubing and flowed at a rate of 200 microliters per hour. The flow rates were left for twenty minutes, until droplets formed. After droplets formed at this rate, the flow rate for the oil was changed to 1000 microliters per hour, and droplets formed again after twenty minutes. A picture of the device is presented in Figure 4.

After the chamber region filled with droplets, the exit tube was clamped off and the syringe pumps shut off. The Petri dish in which the device was nestled was filled with water and refilled daily. Droplet diameter was measured by using Metamorph software to draw lines across droplets and find the length of the lines.

Figure 4. The droplet generating device filled with blue dye. The left-most port is the oil inlet; the middle of the three ports is the chlamydomonas inlet; the right-most port is the exit port. Droplets form where the oil and algae channels intersect, and the large, hexagonal region is where the droplets are stored.
3: RESULTS

3.1 Chlamydomonas growth curve results

Chlamydomonas growth in its characteristic S-curve is shown in Figure 5. The lag phase lasted for 36 hours, and the exponential phase for 60 hours. The stationary phase began after 106 hours. At 192 hours, a drop-off in chlamydomonas concentration was observed.

Figure 5. Chlamydomonas growth curve shows the concentration of the stock chlamydomonas culture as a function of time.

3.2 Oxygen characterization results

Oxygen concentration achieved in the well of the multilayer culture chamber device was determined using PtOEPK sensors. Two methods were employed to calibrate the sensor, and these results are shown below.
3.2.1 Calibrating with gas

Oxygen characterization was done for the multilayer algae culture device, and Figures 6 and 7 show the oxygen profile at the bottom and top of the well, respectively, using compressed air and N₂ gas as calibrating agents. Oxygen environment at the bottom of the well begins at 19.1% and goes down to 0.0%, and at the top of the well begins at 17.5% and goes down to 0.1%. Figure 8 shows and overlay of the plots for the oxygen environments at the top and bottom of the well.

![Graph of Oxygen Concentration Over Time](image)

Figure 6. The oxygen concentration at the bottom of the multilayer algae culturing device was determined using a PtOEPK sensor, and calibrated by perfusing compressed air and then N₂ through the hydration layer.
Figure 7. The oxygen concentration at the top of the multilayer algae culturing device was determined using a PtOEPK sensor, and calibrated by perfusing compressed air and then N\textsubscript{2} through the hydration layer.

Figure 8. Overlay of the curves of oxygen characterization at the bottom and top of the well.
The oxygen environment was also characterized with algae present in the well. Figure 9 shows the oxygen profile of the well with chlamydomonas present, changing the gas environment from compressed air to N\textsubscript{2}. The oxygen environment begins at 16.6\% and goes down to -1.5\%. Figure 10 shows the oxygen profile in the device with chlamydomonas present over 24 hours. The oxygen environment begins at 17.8\% and goes down to -2.7\%.

![Graph showing oxygen profile](image)

Figure 9. The same oxygen characterization was performed with chlamydomonas inside of the well.
Figure 10. The oxygen characterization was repeated and continued for 24 hours with chlamydomonas inside of the well.

### 3.2.2 Calibrating with pyrogallol

Oxygen characterization of the device using pyrogallol was performed, and the results are presented in Figure 11. After calibrating with ambient water and pyrogallol, as described in the Materials and Methods section, the measured oxygen environment began at 21.6% and went down to -0.2%.
Figure 11. The oxygen concentration in the well of the multilayer culture chamber device is displayed, first when compressed air is perfused through the gas channel and then N₂. Water and pyrogallol were used as the calibrating agents in the hydration layer.

3.3 Multilayer culture chamber device results

The results of algae proliferation measurements are presented for cases of algae being counted off-chip. The results of experiments for which algae proliferation was monitored on-chip are described.

3.3.1 With off-chip counting

Results for an n=3 study of chlamydomonas proliferation in the multilayer algae culture device is presented in Figure 12. The results show the fold increase in chlamydomonas concentration over the
initial stock concentration. Results for the individual trials of these experiments are presented in Figure 13.

Figure 12 (above). Chlamydomonas proliferation is presented for stock conditions as well as chlamydomonas in the multilayer microfluidic device, with air, N\textsubscript{2}, or 5% CO\textsubscript{2} balanced with air perfused through the gas channel. Relative proliferation as the fold increase over initial stock culture conditions is presented.
Figure 13 (above). Chlamydomonas proliferation is presented for stock conditions as well as chlamydomonas in the multilayer microfluidic device, with air, N\textsubscript{2}, or 5% CO\textsubscript{2} balanced with air perfused through the gas channel. Results for n=1, n=2 and n=3 are presented as separate graphs.

For all trials, greater proliferation of chlamydomonas was observed in the microfluidic device than for the stock solution under any tested gas environment. All experiments were initiated at the late stationary/early logarithmic phase of growth, between numbers 4 and 5 on the growth curve, as in Figure 14.

Figure 14. Time points for algae growth curve measurements presented as numbers for easy reference.

All proliferation experiments were initiated between points 4 and 5 on the curve.
3.3.2 With on-chip counting

For this experiment, a paralyzed strain of chlamydomonas was used with the goal of counting the algae in each central chamber region at 0 hours and at 24 hours to see if there was a clear difference in proliferation based as a function of gas environment. Time lapse video results showed that the algae did not stay in place and that this method would not work to determine algae proliferation as a function of gas environment.

3.3.3 Algae diameter measurements

Algae diameter measurements were acquired for the algae that were grown in the multilayer algae culture device for which off-chip counting was performed. Figure 15 shows the average algae diameter for each gas across three trials.

![Graph showing average algae diameter across three trials.](image)

Figure 15. The average measured diameters for each culturing condition (including the diameter of the chlamydomonas in stock solution before the algae were injected into the microfluidic device) across three trials.
3.4 Droplet generator results

The droplet generator experiments resulted in successful generation of droplets containing chlamydomonas, and droplets were formed at a rate of 375 droplets/second. Droplet diameter was stable over five days (shown in Figure 16). Two strains of chlamydomonas—one paralyzed and one with functioning flagella (Arg2 and C125, respectfully)—were tested in these droplets, and each strain proliferated successfully. Figure 17 shows the Arg2 strain on day one Figure 18 on day five, with Figure 19 offering a close-up view of these droplets on day five. Figure 20 shows C125 droplets on day one, and Figure 21 shows a large algal structure on day two.

![Droplet diameter chart]

Figure 16. Droplet diameter was monitored over the course of five days.
Figure 17. Arg2 algae droplets on day one. Small dark spots inside of the droplets are chlamydomonas cells.

Figure 18. Arg2 algae droplets on day 5.
Figure 19. Close-up view of Arg2 algae droplets on day 5. Clusters of up to 18 cells are visible.

Figure 20. C125 chlamydomonas strain in droplets on day 1. Small dark spots are individual chlamydomonas cells inside of the droplets.
Figure 21. Large C125 cellular body inside of a droplet on day 2.

3.5 Volvox growth in multilayer culture chamber

*Volvox carteri* embryos were placed in the multilayer algae culture device in order to demonstrate that they can indeed grow successfully inside of a microfluidic device. These results are shown in Figure 22. Additionally, an adult individual was placed in a microfluidic device and left to demonstrate that an individual would complete its growth cycle and successfully hatch. This result is shown in Figure 23.
Figure 22 (above). Volvox embryos that grew into adults inside of the wells of the multilayer culture chamber device.
Figure 23. A single volvox individual (left) successfully grew and hatched new volvox individuals inside of a microfluidic chamber.
4: DISCUSSION

4.1 Chlamydomonas growth curve

The chlamydomonas growth curve showed the characteristic sigmoidal shape, including the logarithmic growth phase promised as a function of growing the algae in light. Performing this measurement not only demonstrated that the algae grew appropriately in our lab conditions, but provided a basis of comparison for growth rates between the stock conditions and microfluidic conditions. This is important as we were learning algae culture, which had not previously been done in our lab.

4.2 Oxygen characterization

Initially, calibrations for oxygen characterization in the device were done by flowing nitrogen gas and then compressed air through the hydration layer, which was separated from the PtOEPK sensor by a thin PDMS membrane (~100 microns). This approach yielded measured starting oxygen concentrations much lower than 21%. This could be attributed to PtOEPK’s sensitivity to pressure and temperature (switching from calibration with compressed gas to experimentally flowing water through the hydration layer would have affected this) or due to differences in light intensity detected by the microscope as a result of switching from a gas-filled hydration layer to a water-filled hydration layer.

When we switched to calibrating with water and pyrogallol, the resulting oxygen profile had more sensible values. Calibrating with water and pyrogallol resulted in constant pressure and temperature from the calibration to the experiment, and also the presence of water in the hydration layer during calibration meant that there was no change in the amount of liquid that light passed through from calibration to the experimental procedure.
4.3 Multilayer algae culture device

As the results for the multilayer algae chamber device show, this method for growing chlamydomonas for the sake of determining relative proliferation among different gases did not yield consistent results. Over the course of the experiments, no one gas condition emerged as yielding the most or the least proliferation. While it could simply be that the algae are not consistently responding to different gas environments, we believe that inconsistencies in these result stem not from an inherent lack of response by the chlamydomonas, but rather from the way the algae are removed from the device. If, for instance, after flushing the algae from the wells, some small amount of media is left behind in the well or tubing and does not enter the collection vial, this vial could yield a much higher concentration of algae than a vial for which all of the media is collected in the vial.

The issue with unloading the device and achieving consistent results among gas environments aside, using this style of device did demonstrate consistently that culture inside of the multilayer microfluidic device yielded much higher final algae concentrations than culture in stock conditions. We believe that this is due to less shading of the algae inside of the microfluidic chamber (and thus greater light exposure), and also due to improved diffusion of gas into the culture chamber over conventional culture in a flask. This device, or one like it, could be used to culture chlamydomonas under specific gas environments for the purpose of examining the algae’s genetic response to these conditions, where relative proliferation would not be the end goal.

Comparative studies of the diameter of chlamydomonas after exposure to each of these gases also did not yield results that convinced us that any great effect on the diameter of the cells took place. If a certain gas resulted in much larger diameter values, this might have indicated that palmelloids were formed in response to the stress of that particular gas environment.

As was mentioned briefly in the results section, monitoring paralyzed strains of chlamydomonas on-chip in search of a significant difference in proliferation rates in response to different gases proved
fruitless. A 24-hour time lapse of the region of interest (ROI) showed that cells frequently moved in and out of the ROI, and so any results suggesting greater proliferation under certain gas conditions could easily have been due to cells floating into the ROI. We believe that this movement of cells was due to small pressure changes within the device, possibly due to the hydration layer below the culture chamber.

Culture of *Volvox carteri* in this device did prove useful as a means of demonstrating that volvox cultures can be successfully grown and expanded in microfluidic devices. Due to the lack of success of these experiments, we decided to move to microfluidic droplet culture as algae can be more easily confined within droplets.

### 4.4 Droplet generating device

The droplet generator successfully produced droplets of algae suspended in medium encapsulated by oil. Droplets were produced at a rate of 375 droplets/second, and were transported to the housing chamber that held the droplets. Chlamydomonas proliferated inside of the droplets. For strain C125, some large chlamydomonas bodies were observed, such as that which is pictured in the results section. These large bodies are either large cells getting ready to undergo fission (as is described in the introduction), or they are palmelloid bodies. Unfortunately our imaging quality makes it difficult to see the body clearly, but it appears as though multiple cell outlines are visible, so it is quite possible that this is a palmelloid body.

The size of the housing chamber made it difficult to pack the chamber fully enough that no movement of the droplets took place. As a result, imaging specific droplets over several days was not possible. For future experiments, this can be fixed by transporting the droplets to multiple, smaller chambers that are the size of the visual field. This way, even if the droplets take time to settle into a final
position, the number of droplets with, for instance, one cell on day one and the number of droplets with, for instance, 12 cells on day three can be determined.

Simple modifications following the pattern set by the multilayer algae culturing device could be made to add hydration and gas layers to the droplet generator. These combined with separated, smaller housing chambers would make for individual areas exposed to different gas environments. Exposure to three different gas conditions could be done in parallel on the same device, and algae proliferation within droplets monitored. This would eliminate the problems associated with the multilayer algae culture device; specifically, the algae would not leave the droplet unless the droplet burst, and there would be no need to extract the chlamydomonas from the device for the sake of counting, as this could be done on-chip. Formation of palmelloid bodies in response to gas environment could also be monitored using this format. These droplets can also be used for live imaging of chlamydomonas division.
5: FUTURE DIRECTIONS

Because successful growth and monitoring of chlamydomonas culture has now been demonstrated in microfluidic devices, the next step is to integrate gas and hydration channels into the existing chlamydomonas droplet-forming device. Integrating these channels can be done by converting the single-layer droplet generating device into a device with multiple layers of PDMS membranes and channels, much like the multilayer culture chamber device. Chlamydomonas proliferation as a function of varying levels of oxygen and carbon dioxide will be determined. Additionally, using the multilayer culture chamber device (or perhaps a similar device with valves in place for easier flushing of the device), chlamydomonas can be cultured in gas environments, and the resulting samples can be analyzed, for instance for increased H$_2$ production and expression of various genes.

Further microfluidic tools that may be developed include a volvox c-section device, which would automate the cumbersome process of isolating a young volvox individual from a contaminated culture, as well as a valve array for studying chlamydomonas in multiple gas and nutrient environments simultaneously. Additionally, studies that target O$_2$ and CO$_2$ to specific regions of a developing volvox embryo could be achieved by combining a trap (Tan 2006) with PDMS membranes and gas channels in a multilayered device.
6: CONCLUSIONS

Microfluidic modalities for precise control of oxygen concentration were combined in an architecture that allowed for precise control of the gas environment of chlamydomonas culture. Existing technology for generating chlamydomonas droplets was consolidated into a single-chip format, and combining this droplet generator with the algae culturing device with integrated gas and hydration channels will allow for the monitoring of chlamydomonas proliferation under precisely controlled gas environments. These technologies can be further used to explore genetic changes in chlamydomonas as a response to these gas environments. Insights into algae proliferation and genetic changes as a function of gas environment could be useful for studies in the evolution of multicellularity, or for research into chlamydomonas use as a biofuel. Additionally, it was demonstrated that *Volvox carteri* grows successfully in microfluidic devices, so further studies of volvox in precisely controlled conditions may be explored, as well as the development of a suite of microfluidic tools for use in volvox culture.
CITED LITERATURE


Yamano, T., Fujita, A., Fukuzawa, H.: Photosynthesis characteristics of a multicellular green alga Volvox carteri in response to external CO_{2} levels possibly regulated by CCM1/CIA5 ortholog.
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PUBLICATIONS


CONFERENCES


Hadron Collider Physics Symposium 2006, Duke University, Poster Presentation – Bayesian Learning of Neural Networks for Signal/Background Discrimination in Particle Physics.

Argonne Undergraduate Research Symposium 2006, Argonne National Laboratory, PowerPoint Presentation – summarizing the results of Summer 2006 research described above.
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