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Development of Microfluidic Devices to Study Algal Chemotaxis and Long-Term Growth Dynamics

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DEVELOPMENT OF MICROFLUIDIC DEVICES TO STUDY ALGAL CHEMOTAXIS AND LONG-TERM GROWTH DYNAMICS

A Thesis
Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science in

The Cain Department of Chemical Engineering

by
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B.S., Mississippi State University, 2014
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ABSTRACT

Harmful algal blooms pose a threat to human health and the environment. Many complex factors influence their formation and development, and much is still unknown. One major influencing factor that is well-known yet poorly studied is algal chemotaxis. Many studies have examined chemotaxis in other organisms, but very little about chemotaxis in algae has been studied, largely because existing technology and assay techniques are inadequate. Microfluidics offers many possibilities for cell biology, and has been applied to the study of chemotaxis in other organisms already. These techniques can be applied to the study of algal chemotaxis as well.

We present in this study a flow-free microfluidic gradient generator for the study of algal chemotaxis. The device consists of a bottom layer of agarose hydrogel and a top layer of PDMS imprinted with thee parallel microchannels. A gradient is formed by flowing media containing a chemoeffector in one of the outer channels and a buffer solution in the other, providing two constant-concentration boundary conditions. The center channel is left flow-free to allow cells to migrate freely in response to the gradient. We demonstrate the device operation using Chlamydomonas reinhardtii as our model organism, exposing the cells to a gradient of nitrogen in the form of ammonium.

We also further demonstrate the device’s utility by modifying it for use in long-term culturing of algal cells, with the ultimate goal of studying allelopathy by culturing two species side-by-side. The modified device contains a second cell culture channel, enabling us to culture two different cell populations that are chemically connected via diffusion but kept physically separate. As a proof of concept, we successfully culture C. reinhardtii first in a 3-channel device, then in both channels of a 4-channel device.
CHAPTER 1. INTRODUCTION

1.1 Harmful Algal Blooms

In the last few decades, harmful algal blooms (HABs) have become an increasing threat in coastal waters all over the world. HABs are characterized by the accumulation and sometimes dominance of a single algal species, which can lead to a range of negative effects in the immediate environment. Many HAB species produce toxic compounds that can harm fish and other marine species, as well as causing negative health effects in humans either by consuming contaminated seafood or ingesting contaminated water. Some HAB toxins, such as brevetoxin produced by the diatom *Kernea brevis*, can also become aerosolized in the spray produced by rough surf, where they can then be inhaled and cause illness even far from the shore. Some blooms can generate substantial amounts of biomass, which undergoes aerobic decay by bacteria as the bloom begins to die off, consuming large amounts of dissolved oxygen in the water column and leading to hypoxia or anoxia, which in turn leads to widespread fish kills. Blooms can also become so dense that they block sunlight to the seabed beneath them, killing off local marine vegetation. In addition to their impact on human health and the environment, these events can also cause wide-reaching economic problems. Fish kills severely impact the fishing industry, which for many coastal communities can account for a large portion of the local economy. Tourism also suffers, as the potential health effects make tourism undesirable or outright dangerous. Thus, there is a need to better understand the environment factors which lead to the initiation and propagation of an HAB.

There are many environmental factors that affect the growth and accumulation of microalgae and influence the proliferation of blooms (Figure 1.1). Moreover, recent studies have hypothesized that different algae species have different requirements to initiate the formation of a bloom. The most well established factor in the literature is increased nutrient content in the water column, especially dissolved nitrogen (N) and phosphorus (P). A well-known trigger for the formation of algal blooms is eutrophication, the increase in nutrient content of coastal waters due to fertilizer or sewage runoff. A dramatic shift in the natural ratio of nitrogen and phosphorus in the water column can create conditions that greatly favor a single species, which, when combined with other factors, can lead to a bloom. One specific example is that of cyanobacteria (blue-green algae) in Lake Pontchartrain. Some species of cyanobacteria, such as *Anabaena* spp., are capable of fixing atmospheric nitrogen, allowing them to thrive when the concentration of dissolved nitrogen is relatively low, while others, such as *Microcystis* spp., can quickly take over when dissolved nitrogen concentrations are high. This has raised concerns regarding eutrophication of the lake when the Bonnet Carré Spillway is opened during flood events, rapidly discharging nitrogen-rich Mississippi River water into the lake.

Temperature and light are also major considerations for bloom formation. Most algal blooms occur in the spring, when the days are longer resulting in optimal light exposure and increased water temperatures. Additionally, nutrients that could not be utilized during the winter due to light limitations are in great abundance. Once these dissolved nutrients are used up, the algae begin to die off, leaving low populations during the summer months. There is typically a smaller bloom in the fall when nutrients have again been allowed to accumulate and light is still temporarily adequate, followed by another low population state during the winter as both light and temperature become too low.
Algae can bloom in response to increased nutrient concentrations, as well as changes in temperature, salinity, and light conditions. Most algal species prefer warmer temperatures, and all algae require light for photosynthesis. Some species also produce toxins for various reasons, such as defense from predators or to inhibit other species that compete for nutrients.

Water salinity also has a significant effect on the formation of a bloom. Discharge of fresh water from a river can create a region of low salinity near the shore, which can create favorable conditions for algal species that prefer lower salt content to grow. River discharge is usually greater in the spring when the winter snows begin to melt. This change in salinity coupled with fertilization and subsequent runoff from agriculture during the spring planting season can cause highly favorable conditions for algal blooms to occur. As demonstrated in the literature, this is a well-known problem in the Gulf of Mexico, where the Mississippi River discharges large amounts of fresh water containing agricultural runoff, leading to blooms of primarily *Pseudo-nitzschia* each spring.

There have been significant efforts by NOAA and independent researchers to develop metrics to predict the formation of HABs; however, there is still a lack in the fundamental understanding of what is occurring at the biological level in algae in a bloom. For example, recent work identified that the increase in biomass in an algal bloom does not correlate with the doubling time of many bloom-causing species. This suggests that the accumulation of algae in a bloom event may be partially due to their migration from bulk water to the coast, where algal blooms tend to occur. However, little is currently known about the migratory behavior of algae during the initiation and propagation of a bloom.
1.2 Chemotaxis

Cell migration is critical to the development of any cellular system. In multicellular organisms, many types of cells migrate during early development, only becoming stationary once fully differentiated. Others, such as cells involved in immune response, remain migratory throughout the life of the organism. Unicellular organisms typically migrate in order to find more favorable living conditions. There are several types of directed migration, termed “taxis”, that cells can undergo in response to different stimuli. Many photosynthetic cells, such as algae and cyanobacteria, undergo phototaxis in response to gradients of light in order to optimize light collection for photosynthesis. Mammalian immune cells can perform haptotaxis and durotaxis in response to gradients of adhesive ligands and mechanical properties in the extracellular matrix, respectively. Many types of cells also migrate in response to temperature (thermotaxis) and electric fields (electrotaxis). But the most commonly encountered and best understood method of directed cell migration is in response to gradients of attractive or repulsive chemicals, termed chemotaxis.

Chemotaxis has been studied at length in several model organisms including the slime mold *Dictyostelium discoideum*, mammalian neutrophils, and mammalian fibroblasts. *(Table 1)* *D. discoideum* is a multicellular “social amoeba” that lives on forest floors and in soil, feeding on decaying plant matter and was one of the first organisms whose chemotactic response was studied. When food becomes scarce, these cells begin to release waves of cyclic adenosine monophosphate (cAMP) which is used to attract other cells. This creates waves of motion in which *D. discoideum* cells migrate towards each other, allowing the entire organism to relocate itself to a location where food is more abundant.

<table>
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<th>Table 1. Summary of research on model chemotactic cells.</th>
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<tr>
<td>Neutrophils / <em>Dictyostelium discoideum</em></td>
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<tr>
<td>Receptor</td>
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<td>Chemoattractant (s)</td>
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<tr>
<td>Class of PI3K</td>
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Neutrophils are a type of immune cell found in mammals that aid in wound healing. When the skin is broken, endothelial cells release gradients of chemokines and adhesive ligands, which attract neutrophils and other immune cells to remove cellular and extracellular debris and engulf invasive bacteria to prevent infection. This is accomplished via chemotaxis in response to foreign peptides that are secreted by invading cells. Some of the many chemoattractants toward which neutrophils migrate include N-formylmethionyl-leucyl-phenylalanine (fMLP), a peptide released by bacteria that stimulates neutrophils to attack; interleukin-8 (IL-8), a chemokine released by endothelial cells to attract immune cells to points of infection; and complement component 5a (C5a), a protein fragment that draws neutrophils to the site of infection as well as triggering the expression of adherent molecules on the cells’ surface.
There are many similarities between the chemotactic response of *D. discoideum* cells and neutrophils. Both types of cells are amoeboid, meaning their method of migration follows the same pattern: a) the cell extends part of its plasma membrane, called a pseudopod, towards the source of the chemoattractant, b) the pseudopod forms adhesive contacts with the surface, c) the bulk of the cell is pulled to the new site, and d) the old adhesive contacts on the rear of the cell are detached. Due to their similarities of movement, these cells are also capable of moving at similar speeds, around 20 µm·min⁻¹. Surprisingly, despite their disparate nature, both cells use G protein-coupled receptors to sense chemoattractant gradients and induce migration towards it via outside-in signaling.¹⁰ Both cell types also respond to similarly shaped gradients with steepnesses on the order of 1 to 4% intensity change over the length of the cell.

The third well-studied cell type is the fibroblast. Fibroblasts are a connective tissue cell, the most common connective cell in mammals, that are involved in synthesis of extracellular matrix proteins and play a prominent role in wound healing. When the skin is broken, platelettes in the blood begin to form a clot, then release platelette derived growth factor (PDGF), which stimulates fibroblasts in the surrounding tissue to migrate towards the wound and begin repairing it, proliferating rapidly and regenerating the extracellular matrix to form new tissue.¹⁰ The mechanism of motion for fibroblasts is superficially similar to that of the amoeboid neutrophils, extending a portion of its cytoplasm forwards and then pulling the rest of the cell in the same direction; but in fibroblasts the extension, the lamellipodium, is supported by networks of F-actin, rather than the cytoskeleton. These actin networks take several minutes to construct and break down, causing the cell to move slower by at least one order of magnitude, about 1 µm/min.

Additionally, fibroblasts possess much stronger adhesion to the extracellular matrix, owing to the fact that their adhesion is mediated by integrins, further slowing its movement. The receptor that detects PDGF is a tyrosine kinase, further differentiating fibroblast chemotaxis from that of neutrophils. Finally, fibroblasts require much steeper gradients, in excess of 10% intensity change over the cell’s length.

Despite the extensive research into these model cell types, there remains virtually no research into chemotaxis in algae. The current model for algal migration follows two different types of tactic movement. Algae will migrate vertically upward during the day in response to light via phototaxis, while they will migrate vertically downward at night in response to chemical stimuli via chemotaxis (Figure 1.2). This behavior is referred to as Diel Vertical Migration (DVM). Additionally, recent studies have identified another metric by which algae migrate called gravitaxis or geotaxis.¹¹ This method requires the algae to modulate the lipid content resulting in cells that are denser or less dense to facilitate their vertical movement in the water column. This suggests that the migratory behavior of algae is highly complex and, thus, requires further study. Understanding these processes, especially the types of nutrients and intensity and steepness of the gradients that induce chemotaxis, is vital to deeper understanding of the dynamics within a bloom. However, as free-swimming organisms, microalgae do not follow the same patterns of movement as the adherent or matrix-dwelling cells that have been studied thus far, nor do they respond to the same types of chemical stimuli. Thus, a different approach must be applied when studying the tactic movement of these freely swimming algae.

Unfortunately, although many traditional methods to study the chemotactic response of cells have been developed none of them are particularly well-suited to studying algal migration. One of the earliest, and most popular, methods for studying chemotaxis was developed by Boyden in 1962, dubbed the Boyden chamber or the transwell assay.¹² In this technique, cells are placed in a well with a microporous membrane as the bottom, then the well is placed in a second well
containing a chemoattractant, creating a gradient of the attractant across the membrane. Cells then burrow through the membrane and the chemotactic response is quantified by counting the number of cells in the lower well at the end of the experiment. This technique is quite useful for determining which chemicals will elicit a chemotactic response for selected types of cells. Additionally, its ease of use and lack of specialized equipment have made it the dominant method to study chemotaxis for the last five decades. Unfortunately, it does have the serious limitation that one cannot directly observe cellular chemotaxis in a time-dependent manner to determine their migration path or mode of locomotion. Additionally, transwell assays are limited to only one type of gradient that can be established between the source and sink chamber. To overcome this, various improvements to the technique have been developed, starting with the Zigmond chamber in 1977, in which cells and chemoattractant are placed on a glass cover slip so as to be viewable with light microscopy and separated by a narrow microchannel through which the cells migrate. Despite this and other improvements over the subsequent decades, the transwell assay still possesses limitations that make it unsuitable for testing various aspects of algal chemotaxis.

There have been very few studies that actually explore the chemotactic response of algae. Original methods have attempted to use bulk culture flasks with a camera mounted on the side to observe the swimming behavior of algae. Recently, an assay developed by Leick and Helle\cite{leick1993} for studying bacterial chemotaxis using a capillary loaded with a known chemoattractant has been applied to algae. In this technique, the capillary tube is loaded with a fixed concentration of nitrogen and dipped into a petri dish of algae in suspension. The algae are then allowed to swim into the capillary for a fixed period of time, removed, and then counted at the end of the assay to determine how many cells ‘responded’ to the gradient. This method is more suitable to the study of algal migration as it allows the cells to swim freely rather than forcing them to burrow through a membrane or constricting them to a narrow channel barely wider than the cell itself. However, this method has several limitations including the lack of any direct observation of cells, unsteady
gradients from the capillary, and the high probability for false positives of randomly migrating cells or cells that are drawn into the capillary by capillary action. Thus, it is imperative to development a new method to study algae chemotaxis that overcomes these limitations.

1.3 Microfluidics

Microfluidics is the field of study involving the manipulation of fluids at sub-millimeter length scales. Microfluidic devices offer a number of useful traits that make them very useful for analysis of single cells and have increased its popularity immensely over the past decade. Microfluidic devices can be constructed and assembled using equipment and techniques that were already well established in the semiconductor industry. Early microfluidic devices were mostly interested in electrophoretic phenomena, that is, the movement of particles suspended in liquid within microchannels under the influence of an electric field. This, combined with the convenience of preexisting technology, made silicon and glass the obvious choice for materials of construction. As the potential for use in cellular biology became more apparent, however, it became clear that these materials were not ideal for most applications. One of the biggest issues was that silicon is opaque, making it unsuitable for visible microscopy. It is also relatively expensive, and methods for fabricating devices from it are difficult and costly. Alternative materials and methods were needed.

The first soft lithography techniques were developed by Bell Labs in the 1970s and first applied to microfluidics in the 1980s. Soft lithography is the process of creating a master mold of one or several microfluidic devices on a silicon wafer, then using this mold to cast the devices in elastomeric materials. A typical soft lithographic fabrication sequence is shown in Figure 1.3. A photoreactive polymer (e.g., SU-8) is applied to a silicon wafer by spin coating, and then exposed to ultraviolet light, using a photomask to ensure only certain parts of the polymer become crosslinked. The un-crosslinked polymer is removed with a specialized solvent and the remainder baked to fully crosslink the remaining resin and more firmly affix it to the wafer. Once the master mold is prepared, a liquid elastomer is poured over the mold and allowed to set, then peeled off, forming the microfluidic device. Holes must be punched into the device to allow liquid to be injected and removed, and in many applications the device is permanently bonded to glass to seal the microchannels and provide rigidity.

![Figure 1.3. Soft lithography](image-url)
The preferred material for most modern microfluidic devices is polydimethylsiloxane (PDMS), first used by Whitesides in 1998. PDMS is optically transparent, making it very useful for light microscopy. It is flexible and tough, providing a marked improvement over the brittleness of glass and allowing it to be easily removed from the fragile silicon mold. The flexibility of the material has also allowed for the development of several types of microfluidic valves, enabling very fine control of fluids within the devices. If rigidity is desired, PDMS can be easily bonded, either reversibly or irreversibly, to glass, owing to its silicon-based chemistry. This same chemistry allows for surface treatments to make the material more hydrophobic or hydrophilic, depending on the desired application. PDMS is also fairly cheap and easy to work with, allowing for rapid prototyping and quick preparation for development and testing of devices. Furthermore, PDMS is gas-permeable and biologically inert, making it ideal for biological applications.

Microfluidic devices also allow for precise control over the dynamics of fluid flow within the channels. Whether a fluid stream is laminar or turbulent can be determined from a dimensionless quantity known as the Reynolds number, Re. This number is a ratio between the inertial and viscous forces acting within a fluid, and is proportional to both the fluid velocity and length scale of the system (i.e. the diameter of a pipe or channel through which the fluid flows), and inversely proportional to the fluid’s viscosity. In a high-Reynolds number system (Re > ~2100), fluid flow is turbulent and induces large amounts of mixing. In a low-Re system, however, fluid flow becomes laminar, following smooth streamlines with almost no mixing except by diffusion. Due to the small length scales involved, flow in a microfluidic device is almost always laminar, allowing very precise control of the flow, as well as pressure and concentration profiles within the device. In addition, interfacial tension and capillary action have much larger effects on such small scales, allowing novel applications such as formation of stable droplets for cell encapsulation and sorting; conversely, gravitational forces are almost negligible on such scales, preventing suspended particles from settling or allowing fluids to move through microchannels via capillary action even against gravity.

Due to its many unique qualities, microfluidics offers a host of entirely new techniques for studying chemotaxis. Due to the length-scale diffusive mixing phenomena that occur in laminar flows, it is quite easy to create smooth gradients of chemicals within microfluidic channels. The earliest microfluidic gradient generators relied on this principle, the first of which being the three-inlet parallel-flow microchannel, also called the “T-sensor”, developed by Mao et al. for the study of bacterial chemotaxis (Figure 1.4). In this device, two microchannels containing a chemoeffector and buffer solution, respectively, converge into a single channel and flow in parallel. Due to the laminar nature of fluid flow in microfluidics, the two streams do not mix, but instead slowly diffuse into one another creating a smooth gradient across the channel. Bacteria are injected down the centerline of the channel, where they are exposed to the gradient and allowed to migrate chemotactically. The end of the channel splits into twenty-two outlet channels spaced evenly.
across the width of the channel, and the chemotactic response is quantified by counting the number of cells in each outlet. Mao et al. used the device to test the response of E. coli to L-aspartate and found a strong response at a concentration of only 3.2 nM, nearly three orders of magnitude below the detection limit of the capillary assay at roughly 1 µM, demonstrating the utility of the device. Unfortunately, this and other flow devices have one major limitation: the gradient is inherently unstable. The cells are injected at the point where the chemoeffector and buffer streams meet which is the point where the gradient is extremely steep (almost a step change in concentration). As the cells move down the channel, the two streams have more time to diffuse into one another, creating an increasingly shallow gradient. The initial steepness can skew the response for the rest of the channel, and cells may change their response based on the slope of the gradient.

An improvement of the device was introduced by Lanning et al., using only two channels with bacteria suspended in both, providing an initially uniform cell distribution and exposing the cells to the gradient more gradually as the two streams diffuse into each other. Lanning et al. also performed an important test by stopping the flow and allowing the gradient to diffuse evenly down the length of the channel, which allowed the cells to migrate in the absence of flow. Lanning found that flows up to 1 mm·s⁻¹ did not affect the chemotactic response of E. coli. This remains the only test for determining the effect of flow on chemotactic migration in microfluidics. Unfortunately, this device still has the severe limitation that the gradient it produces changes down the length of the channel.

Further improvements to the design by Mao et al. have created gradients that are increasingly linear and stable; however, they all have one major limitation: they require flow to operate. This allows quantification of chemotactic response at the endpoint, but makes it very difficult to study the motion of cells during migration. To that end, many flow-free devices have been developed. Many of these devices use “stopped flow”, that is, the device is initially set up using flow, then the flow is stopped to allow the gradient to develop by diffusion alone. One such device is the nutrient-pulse injector developed by Stocker et al. This device uses two flow inlets in-line with one another, one for cells and one for the chemoeffector. The cell inlet divides into two channels, which then rejoin to form a single wider channel (Figure 1.5). The chemoeffector is injected in a narrow stream at the point where the two cell channels meet, creating a thin stripe of chemoeffector between the two cell streams. Flow is then stopped and the gradient allowed to develop through diffusion alone, and the cells allowed to respond chemotactically. This device was designed to mimic conditions found in the ocean, where bacteria are subjected to pulses of dissolved organic matter excreted by larger organisms. While such systems match real-world scenarios and are thus very useful to study, gradients generated in such a way are highly transient. For many situations, including studying algal chemotaxis, it is

Figure 1.5. Nutrient pulse injector developed by Stocker et al.
necessary to create a steady, linear gradient in the absence of direct flow.

The first microfluidic gradient generator capable of producing steady gradients with no flow or shear was developed by Diao et al.\textsuperscript{17,19} (Figure 1.6). This device consisted of a nitrocellulose membrane from which three parallel microchannels were carved using a laser. The membrane was then sandwiched between a glass slide and a Plexiglas manifold, with reservoirs carved out over the ends of each channel. One of the outer channels (the “source” channel) was filled with a chemoeffector, while the other (the “sink” channel) was filled with a buffer solution with no chemoeffector. The center channel (the “test” channel) was loaded with cells. The chemoeffector would then diffuse through the nitrocellulose membrane, creating a gradient from the source channel to the sink channel. One important feature of this device over previous designs was that the source and sink channels provided constant concentration boundary conditions, rather than the no-flux boundary conditions found in flow-based devices, providing a steady gradient with constant slope after some initial setup time. The separation of the channels also protected the cells from the direct flow in the outer channels.

Figure 1.6. The nitrocellulose device developed by Diao et al.\textsuperscript{19} A) Device dimensions. B) Schematic illustrating the construction of the Plexiglas manifold.

The device generated by Diao et al. provided the groundwork for future flow-free microfluidic gradient generators, and many subsequent designs used adaptations of the original geometry. This device did, however, have two major drawbacks. First, due to the method of fabrication and setup, Diao et al. could not eliminate residual flow in the test channel for channels thicker than 140 µm, thus negating one of the device’s most attractive features. And secondly, because the channels were embedded within the membrane, the gradient in the test channel would be disrupted as the cells were loaded and subsequently reform by diffusing across the channel, subjecting the cells to a transient gradient for several minutes. Future devices worked to eliminate these problems.

The first improvement came from Cheng et al., who replaced the nitrocellulose membrane with an agarose hydrogel\textsuperscript{17,20}. The fluidic channels were cast into the agarose hydrogel using soft lithography, instead of laser etching, making fabrication much easier. Casting the channels and
support as a single piece also prevented deformation of the channel walls, eliminating residual flow. This device has been used in several studies on chemotaxis including Kim et al.\textsuperscript{21}, Zhuang et al.\textsuperscript{22}, and Chang et al.\textsuperscript{23} A modified version of this three-channel device was developed by Ahmed et al. who combined the agarose hydrogel with a PDMS top layer, imprinting the channels in either the PDMS or the agarose in three different arrangements: 1) all three channels in agarose as with Cheng et al., 2) all three channels in PDMS, or 3) source and sink channels in agarose and test channel in PDMS\textsuperscript{17, 24} (Figure 1.7). Ahmed et al. found that by changing the manner in which the channels were imprinted into the agarose or PDMS resulted in different shaped gradients. They also found that the third arrangement is optimal for studying bacterial chemotaxis.

![Figure 1.7. The three channel configurations tested by Ahmed et al.\textsuperscript{24}](image)

Understanding algal chemotaxis is vital to the deeper understanding of HABs. A significant amount of work has gone into the development of microfluidic devices for studying chemotaxis in various types of cells, but almost none of this has been focused on the chemotactic response of algae. Free-swimming cells require a flow-free environment in order to observe their migration, and a steady gradient to fully quantify their response. Such platforms have been developed to study the chemotaxis of bacteria, but a stronger focus on algae is required.
CHAPTER 2. A MICROFLUIDIC GRADIENT GENERATOR FOR THE STUDY OF ALGAL CHEMOTAXIS

Many of the principles developed for the studying chemotaxis in bacteria can also be applied to the study of algal chemotaxis. In this section we will detail the design, fabrication, characterization, and optimization of a three channel ‘flow-free’ microfluidic device capable of creating linear gradients. We will describe the challenges we encountered in creating the device and the solutions identified to create a truly flow free center channel. Then, we will provide initial data showing a chemotactic response in *C. reinhardtii* using the device.

2.1 Device Design and Fabrication

The microfluidic device designed for this study is similar to those presented above. It consists of two layers: a bottom layer of 3 wt% agarose to facilitate chemical diffusion and a top PDMS layer into which three parallel fluidic channels are imprinted (Figure 2.1). We have

![Figure 2.1. Device overview. A) Top view of device showing the layout of the microchannels. The gradient generation area, where the channels are closest together, is 10 mm long. Each channel is 600 µm wide, and the spacing between the channels is 450 µm. B) Side view of device showing the PDMS and agarose layers as well as the Plexiglas housing. C) Photograph of the assembled device.](image-url)
designed and fabricated several devices with different geometries modulating the width of the center channel or the spacing between the outside fluidic channels and the center ‘flow-free’ channel. Additionally, we altered the thickness of the agarose and PDMS layers as well as the height of the channels. Fluid flow was induced in the channels using positive pressure flow via syringe pump so we designed a Plexiglas chamber to contain the device, prevent leakage, and keep it fluid tight. Previous devices used negative pressure to induce flow in the top and bottom channels; however, we were unable to replicate this type of flow without causing significant leakages in the fluidic channels.

The microfluidic devices were fabricated using a combination of soft lithography and PDMS replication. Device geometries and dimensions were drawn up using design software (AutoCAD) which allowed for the production of transparency masks (CAD/Art) to transfer the design to the silicon master. The silicon master wafers were fabricated using 3” silicon wafers (University Wafer). SU-8 photoresist (MicroChem) was spin-coated onto the wafers to create a positive relief of the fluidic channels on the surface of the wafer, then baked on a hot plate at 95°C for 30 minutes. To achieve a channel height of 150 µm, we had to perform a two-step fabrication where each layer of SU-8 developed on the wafer was 75 µm. The SU-8 layer on top of the wafer was crosslinked via UV light for 45-165 seconds. The UV exposure time was determined based on documentation provided by MicroChem regarding SU-8 photoresist crosslinking which depended on the thickness of the photoresist layer. The exposed wafer was then baked again at 95°C for 30 minutes to two hours, again depending on thickness of the photoresist, then developed using MicroChem’s SU-8 Developer solution. Developed wafers were then hard-baked at 150°C for 30 minutes to an hour. Finished wafers were treated with a silane [(tridecafluoro-1,1,2,2-tetrahydrooctyl) trichlorosilane] via physical vapor deposition in order to reduce sticking when removing devices from the mold. The design of the photomask allowed for the generation of four microfluidic devices per wafer.

Microfluidic devices were generated using established PDMS replication techniques using Sylgard 184 Silicone Elastomer Kit (Ellsworth Adhesives). Liquid PDMS was prepared using a ratio of either 10:1 or 5:1 by weight of base to curing agent. The components were mixed thoroughly by hand in plastic weigh boats then placed in a vacuum chamber for 20-60 minutes to fully degas the mixture prior to pouring to prevent bubble formation in the devices. Once fully degassed, the liquid PDMS was poured over the silicon master wafer in a petri dish and placed on a hot plate at 65°C for a minimum of 8 hours but normally overnight. Once fully cured, the PDMS replicas were removed from the silicon master, excised with an X-acto knife to separate the individual devices, and punched at the inlet and outlet ports using a blunted 18-gauge needle (PrecisionGlide; Becton, Dickinson and Company). The devices were then placed in a clean petri dish for storage. For all experiments, devices were plumbed using 0.022”ID x 0.042OD Microbore PTFE tubing (Cole Parmer).

Agarose was prepared by pipetting 31 mL of liquid 3% agarose into a standard 100 mm petri dish at least one hour prior to device assembly. The agarose was then cut into approximately 20 by 30 mm rectangular pieces using a razor blade for use in the device. Devices were assembled by hand in a horizontal clean bench to minimize any debris trapped in the device.

Cell Culture & Reagents

The algal species chosen for our experiments was *Chlamydomonas reinhartii*, a freshwater species. This species was chosen because of its use as a model organism for studying many
aspects of cell biology, primarily due to the ease with which it can be grown and its easy to manipulate genome. All stock cell cultures were kindly provided by Dr. Naohiro Kato (LSU, Biological Sciences). Cells were cultured in 500mL flasks using 1X TAP (tris-acetate phosphate) media. 1X TAP was prepared by diluting a 10X stock using deionized water. 10X TAP was prepared by combining 10 mL each of 2M tris-acetate stock, phosphate buffer solution, a nutrient stock containing ammonium chloride, calcium chloride, and magnesium sulfate, and Hutner’s trace metals, then bringing the mixture to 1 L with deionized water. The culture flasks were maintained on an orbital shaker under a fluorescent light bank controlled by a timer to provide the cells with a 14h:10h light-dark cycle. Cells were resuspended once a week by transferring 1 mL of algae culture into 300 mL fresh 1X TAP.

Cells were counted manually every two days using a hemocytometer. For each experiment, 10 mL of cells were centrifuged at 4000 rcf for 5 minutes, the supernatant drawn off, and the pellet resuspended to a concentration of $7 \times 10^6$ cells·mL$^{-1}$ in nitrogen-free TAP.

Microscope

Two microscopes were used during development of the device. Preliminary data was gathered using a Zeiss PrimoVert phase contrast microscope equipped with an Axiocam 105 color digital camera. The majority of later experiments were performed using a Leica DMi8 microscope equipped with a Hamamatsu ORCA-Flash 4.0 digital camera. The Leica microscope’s electronically controlled shutter allowed us to keep the algae in the dark for the majority of an experiment, only exposing them to light for a few milliseconds at a time for image capture.

2.2 Identifying and Overcoming Challenges in the Development of a Flow-Free Device

Published studies by Ahmed et al. and Chang et al. demonstrated the utility of the devices; however, they did not complete describe certain aspects of the assembly nor significant challenges found in the device. Here, we will detail challenges we encountered during the characterization of the device. One significant challenge we encountered was the presence of endogeneous flow in the flow free channel. Below we will describe our hypothesis for the cause of this flow and our solutions to eliminate it in the center channel. The initial height of the fluidic channels was 75 μm and the initial PDMS recipe used a 10:1 ratio with 15 g base and 1.5 g curing agent, resulting in a thickness of 2-3 mm. In order to secure the inlet and outlet tubing, PFTE tubing was precut (~2 inches in length), inserted into the device and then liquid PDMS was dabbed around the base of the tubing and allowed to cure, sealing the tubing to the inlet and outlet ports. For operation, longer pieces of tubing were connected to the device using metal connectors made from blunted 23 gauge needles (PrecisionGlide; Becton, Dickinson and Company). Initially, agarose thickness was not controlled, with liquid agarose poured by hand into a petri dish with no measurement. Additionally, in keeping with prior research from other groups, the device was planned to work with negative pressure, pulling media through the device from a reservoir using a syringe pump. It was expected that this negative pressure would suction the PDMS to the agarose, eliminating the need for additional support to hold the device together. Unfortunately, there were numerous problems with this setup.

Negative Pressure and Structural Integrity

The first problem encountered was the inability to generate fluid tight channels with negative pressure-driven flow. Due to the pliable nature of both PDMS and agarose, the channels had a
tendency to collapse when negative pressure was applied, blocking flow. We attempted to decrease the flow rate to increase the pressure inside the channels; however, this resulted in the seal between the PDMS and agarose weakening enough for the layers to detach. It was ultimately decided that some sort of frame would be needed to hold the layers together and that the device would need to use positive pressure-driven flow. To overcome this limitation, we designed an external chamber made out of Plexiglas.

The chamber consisted of two Plexiglas plates held together by small screws. The bottom plate was featureless except for the screw holes at each corner. The top plate had, in addition to its screw holes, two slots to allow the tubing from the device to pass through (visible in Figure 2.1 C). Our initial design attempted to create threaded screw holes; however, Plexiglas is too soft and malleable to hold threading for more than one or two uses, so it was decided to use small nuts to secure the screws instead. The original Plexiglas used for the chamber was 3mm thick. However, this became a problem as the focal length of our microscope (Zeiss) was not long enough to resolve the channels beyond 4x magnification due to the thickness of the device. New plates were fabricated from 1.5mm Plexiglas to compensate.

Dehydration

Another significant challenge we encountered in the operation of the device was the dehydration of the center culture channel. Many initial experiments failed due to the center channel drying out which resulted in either algal death or removal from the experimental window due to the advance of an air pocket (Figure 2.2) We attempted to refill the channel with new algal suspension, but it was quickly discovered that once the channel began to dry the damage was already done.

![Figure 2.2. Cells forced out of the channel by an advancing air pocket. Images are 10 seconds apart.](image)

Our initial hypothesis was that this observation was due to evaporation from the inlet and outlet ports. To address this, we attempted different methods to inject the algae into the center channel. First, the inlet and outlet ports of the center channel were plumbed with short pieces of PFTE tubing for ease of loading cells. Cells were loaded using syringes equipped with 23 gauge needles, which were inserted into the tubing. Once the center channel was filled, the liquid in the tubing was expected to provide a reservoir to keep the channel filled; however, we observed that this volume of buffer would deplete during the course of the experiment. Once this issue was discovered, we switched to plugging the ends of the tubing plumbed into the devices using closed-off, blunted 23 gauge needles to provide an airtight seal. This approach also did not work, so we next decided to remove the PFTE tubing from the inlet and outlet ports in the center channel and instead cover the inlet and outlet ports with small PDMS blocks after loading the
cells. Covering the port, whether with metal plugs or PDMS blocks, did prevent evaporation and help with the drying issue, but did not completely resolve it.

After further analysis of the devices, we discovered that the presence of air in the center channel itself occurred in the middle and not near the ports. Thus, we hypothesized that the presence of gas in the channels was due to the agarose itself losing water through evaporation from the exposed sides of the device. The first attempt to solve this problem was to increase the water content of the agarose. The original agarose recipe was 3% by weight, prepared using 1X TAP media or nitrogen-free 1X TAP (N-free). This was changed to 1%, to increase the water content in the hydrogel. Additionally, we attempted to decrease evaporation by decreasing the surface area:volume ratio of the agarose slab by increasing the thickness. As mentioned above, the thickness of the agarose was not originally controlled very carefully, and was typically 2-3 mm in thickness. It was decided to increase this thickness to 5 mm and to more precisely regulate the thickness by pipetting a fixed volume of liquid agarose into a petri dish. For the desired thickness, this volume was calculated to be 31 mL using standard 100 mm petri dishes. These changes did help to eliminate channel drying which resulted in the use of the 1% agarose bottom layer; however, it was later discovered that these modifications resulted in new challenges.

1% agarose is much softer and more fragile than 3%, so great care had to be taken when handling it during device assembly. This resulted in several failed experiments or devices that could not be used. We also ran into problems when assembling the device and tightening the screws causing the agarose to infiltrate the fluidic channels and block flow or hinder the motion of algae in the center channel. Extreme cases resulted in the deformation or splitting of the agarose. Great lengths were taken to attempt to prevent overtightening of the screws and deformation of the agarose. We measured the thickness of the agarose before assembling every device using an electronic caliper and determined the spacing between the top and bottom Plexiglas plates to ensure the proper tightness. We also tried to use plastic spacers around the screws to prevent overtightening; however, this was ineffective due to slight changes in agarose thickness after pouring. We attempted to have available interchangeable spacers with multiple lengths or some method to adjust the size of the spacers as needed, but this method was ultimately deemed too cumbersome and abandoned. Finally, the pliable nature of the agarose also contributed to the residual flow in the center channel, as discussed in the next section.

Ultimately, the 1% agarose was abandoned and the 3% was reinstated. This required that we explore alternative approaches in device assembly to continue to prevent channel drying. One potential solution to this was to create a humidified environment so that evaporation from the agarose could not occur. This included wrapping the exposed sides of the device in parafilm; however, this was rejected as being too awkward to handle, as well as causing stress on the device as the parafilm was pulled tight. We considered creating a chamber to house the device during experiments, in which would be placed one or more small water reservoirs whose evaporation would humidify the chamber, but this was rejected because the construction of such a chamber to both fit on our microscope (Leica) and allow the tubing to enter and exit was deemed too complicated. Thus, the humidified environment approach was abandoned in favor of a more manageable solution, decreasing the flow rate of the syringes. The hypothesis was that increasing the residence time of the liquid in the top and bottom channels (by decreased the volumetric flow rate through them) would result in a more saturated hydrogel. The initial flow rate selected for the top and bottom channels was 15μL·min⁻¹. Decreasing the flow rate in the top and bottoms channels to 5μL·min⁻¹ resulted in the agarose being able to retain its moisture.
The issue was still not fully resolved, however. While the agarose was no longer a problem, it was frequently observed that bubbles would form in the channel and grow slowly over the course of the experiment, often to the point of filling the entire channel. It was determined that this was not due to drying, but to the evolution of dissolved oxygen from the PDMS. As noted earlier, PDMS is gas permeable\textsuperscript{25}, which is an advantage for biological systems as it allows for gas exchange. However, as we discovered, freshly cured PDMS often contains absorbed atmospheric gases that can diffuse out of the polymer when exposed to areas of low concentration of the gas, such as cell media in a microchannel. This was easily remedied by degassing the PDMS in a vacuum chamber for 30 minutes prior to device assembly. The result was a two-layer microfluidic device capable of maintaining a flow-free fluidic environment in a center channel with no direct flow.

**Residual Flow**

While we were able to address many of the design challenges with the microfluidic device, one issue remained throughout most of the testing. This was the presence of residual flow in the ‘flow-free’ center channel. One of our earliest hypotheses was that the flow was caused by capillary forces or gravity pushing liquid into the channel from the tubing used to plumb the channel. It was hoped that the metal plugs used to prevent evaporation would also prevent the liquid from being pulled down the tubes due to gravity, but the effect was not sufficient. Eventually, plumbing of the center channel was eliminated altogether in order to prevent this. The flow was mitigated somewhat, but not eliminated.

It was next hypothesized that the residual flow in the center channel was caused by a pressure differential introduced during device assembly. One potential cause was uneven agarose. The first attempt to combat this was to fabricate a large, rectangular “boat” with walls of even height and a flat bottom. The idea was that the agarose could be poured up to the rim of the wall, providing an even and replicable thickness across a large amount of agarose. There were several issues with this approach, however. The first attempt failed due to the material chosen for the construction of the boat deforming when heated. A second agarose boat was fabricated using a more thermally resistant material, but it soon became apparent that the boat could not practically be used as intended. The amount of precision required to pour liquid to exactly the level of a retaining wall is not possible without carefully measuring the liquid volume beforehand. Therefore, all agarose poured into this boat possessed either a concave or convex meniscus, not the perfectly flat surface it was meant to achieve. Furthermore, pouring such a large amount of agarose all at once turned out to be imprudent, as only a few pieces could be used before the agarose dried out or became contaminated and could no longer be used, generating a large amount of waste. It was decided that it was more practical to simply pipette a known volume of liquid agarose into a petri dish, placing the dish on a level surface and leaving it undisturbed until fully set.

Unfortunately, this did not completely resolve the issue of residual flow in the center channel, although it made device assembly easier. PDMS and agarose are both very flexible and deform under pressure, thus it was suggested that the pressure exerted by the fluid flowing through the source and sink channels, however small, could cause enough deformation to induce a pressure gradient throughout the device. Thus, we initiated several changes to reduce the effect of this deformation. First, new channel geometries were selected with heights of either 100 or 150 µm to decrease the surface area:volume ratio of the channels, thereby decreasing the relative amount of area for the pressure to work on to affect the channel volume. Secondly, the ratio of
base to curing agent when preparing liquid PDMS was decreased from 10:1 to 5:1, increasing the Young’s modulus of the PDMS from 2.66 MPa to 3.59 MPa and reducing the amount of deformation. Also the thickness of the PDMS replicas was increased by switching from a total mass of 16.5 g to a total mass of 330 g (base + curing agent). This nearly doubled the thickness of the PDMS replica to approximately 5mm and provided more mass in which to distribute the deforming forces. These changes reduced the flow substantially, and allowed us to begin gathering preliminary chemotactic data. These new devices were tested with 1% agarose; however, it was determined experimentally that the 3% agarose offered superior performance and reduced flow in the center channel.

Surprisingly, one source of induced pressure came from the PDMS used to plumb and seal the tubing to the inlet and outlet ports. Because the drops were added on top of the PDMS, they created raised areas around the base of the tubing. These lumps were wider than the slots in the Plexiglas plate through which the tubing was fed, so when the device was assembled the ends of the PDMS slab under the ports would be pushed down, creating a higher pressure at either end of the device than in the middle. Sealing the tubing had originally been done because the thinness of the PDMS slab meant that the inner walls of the ports did not provide enough grip to hold the tubing in place on their own, making it easy for tubing to fall out. Fortunately, with the adoption of the new thicker PDMS this problem was eliminated and the sealing was no longer necessary, so it was abandoned. This also had the effect of saving considerable time in fabricating devices as plumbed devices would need to bake for several hours in order to cure the sealant.

Although the pressure gradient inside the device was significantly reduced at this point, there was still enough flow within the channel to bias the tactic movement of the algae and reduce our confidence in a solely chemotactic response. Thus, one more change was necessary to finally create a truly flow-free environment. Since the pressure gradient could not be fully eliminated due to the need to maintain some pressure between the agarose and PDMS to maintain fluid tightness, it was decided to find a way to allow the pressure inside the test channel to equalize before beginning each experiment. This was deemed impossible using the rigid PDMS blocks to close off the ports, so a new method had to be found. Our first attempt was to leave the inlet and outlet ports open and add a few drops of excess media over the ports. By paying attention to the direction of flow and adding more media to the downstream side we were able to stop the flow for a short period of time (~2-3 min), but the media overlaid at the input and output ports would evaporate over the course of the experiment, allowing the channel to dry up. Thus, something less volatile was required. Both liquid PDMS and mineral oil were tested to cover the inlet and outlet ports, but the weight of the substances allowed them to force their way into the channel and displace the media. Finally, liquid agarose was tested to cover the inlet and outlet ports and was discovered to work exceedingly well (Figure 2.3). As a liquid the agarose is able to move with the liquid at the ports, allowing pressure to equalize; it then quickly solidifies to seal the ports and prevent evaporation and further pressure fluctuations. Initial fears that the heat of liquid agarose might harm the cells seem to be unfounded, as the cells seem completely unaffected.

Final Device Overview

The current device has changed significantly from the original design (Figure X). The PDMS thickness and stiffness have both been increased and the microchannels have been raised to 150µm. The agarose layer has been increased to approximately 5mm in thickness and is always prepared on a level surface to ensure evenness. Tubing is no longer sealed into the ports of the
device, instead simply inserted into the ports and held in place by friction, and each length of tubing is now a single piece. The flowrates of the source and sink channels have been decreased to 5 µL·min⁻¹. Cells are now directly injected into the port of the test channel, and the ports subsequently sealed with liquid agarose. This device is now truly flow-free and ready for use in studying algal chemotaxis.

2.3 Experimental Methods and Results

A total of ten device geometries were designed in order to create different types of chemical gradients within the flow free test channel (Table 2). We designed microfluidic devices with two widths for the center channel (600 µm and 1200 µm) and five different spacing lengths between the flow-free center channel and the top (source) and bottom (sink) channels: 200, 450, 800, 1200, and 1600 µm. To date, we have tested six of these devices at various points during development; however, the majority of the studies presented here used the the 600 µm wide channel with 450 µm spacing between the three channels (device B).

Based on what has been published about the diel vertical migration of algae, algal cells are more strongly phototactic during the day in order to capture sunlight for photosynthesis, and more strongly chemotactic at night in order to forage for nutrients (Figure 1.2). As such, we set-up the continuous culture of *C. reinhardtii* so that the night cycle would fall during the daytime hours to allow for experimentation during day for us but at night for the algae to increase the strength of the cellular response. Initial tests of the algal chemotactic response included a nitrogen starvation period using N-free TAP prior to experimentation to create a baseline migratory response in cells. This approach, termed serum-starving, is common in chemotactic studies for model cell lines like fibroblasts and neutrophils. Initial experiments were performed
Table 2. Device geometries

<table>
<thead>
<tr>
<th>Device</th>
<th>Center Channel Width (µm)</th>
<th>Channel Spacing (µm)</th>
<th>Tested</th>
</tr>
</thead>
<tbody>
<tr>
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<td>600</td>
<td>200</td>
<td>Yes</td>
</tr>
<tr>
<td>B</td>
<td>600</td>
<td>450</td>
<td>Yes</td>
</tr>
<tr>
<td>C</td>
<td>600</td>
<td>800</td>
<td>Yes</td>
</tr>
<tr>
<td>D</td>
<td>600</td>
<td>1200</td>
<td>Yes</td>
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<tr>
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<tr>
<td>F</td>
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<td>Yes</td>
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<tr>
<td>G</td>
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<td>450</td>
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<td>1200</td>
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<td>J</td>
<td>1200</td>
<td>1600</td>
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</tr>
</tbody>
</table>

under ambient light conditions in the lab for the sake of convenience; however, we switched to conducting the experiments in the dark to eliminate any unwanted light bias in the migratory response.

To induce a chemotactic response of *C. reinhardtii*, we used the chemoattractant ammonium (NH$_4^+$), a natural source of nitrogen for many algal species and a well-known chemoattractant for *C. reinhardtii*. As the primary nitrogen source for this species, ammonium is present in 1X TAP media, so standard 1X TAP was used in the source channel of the device, while nitrogen-free 1X TAP (N-free) was used in the sink channel. 1X TAP was originally used to prepare agarose for the device, but it was realized early that preexisting ammonium in the agarose could bias the gradient and the cellular response, so we switched to making the agarose with N-free 1X TAP. Furthermore, it was determined that 1X TAP did not contain enough ammonium (~0.75mM) to generate a tactic response in *C. reinhardtii*, so a nitrogen-spiked 1X TAP media was prepared by adding excess ammonium. Initially, the spiked TAP was prepared by weighing out solid ammonium chloride and dissolving it in TAP; however, this resulted in poorly reproducible media recipes. Instead, a stock N-spiked 1X TAP solution with very high nitrogen content (approximately 12mM) was prepared. Media with the desired concentration was then prepared by combining this nitrogen-spiked stock solution with N-free TAP.

Gradient Characterization

Prior to studying the algal chemotactic response, it was necessary to confirm the stability of the chemical gradient developed in the center channel using fluorescent microscopy (Figure 2.4). Gradient characterization was performed using device B and chemical diffusion was observed by flowing 10µM FAM (5,6-carboxyfluorescein) in the source channel and deionized water in the sink channel for a duration of 6 hours. Images were taken every three minutes for the duration of the time course. A duplicate experiment was performed using 10µM rhodamine 6G with similar results (data not shown). The data clearly demonstrates that the device is capable of generating a steady linear gradient, with a gradient forming within one hour and achieving steady state after approximately 5 hours.
Figure 2.4. Fluorescent gradient characterization of the flow-free microfluidic gradient generator. A) Fluorescent image of gradient. B) Quantified data showing development of gradient over time. A gradient was formed in 30-60 minutes, and the gradient reached steady state after 5 hours.

Collecting Preliminary Chemotactic Data

The algal chemotactic response was evaluated during the development of the microfluidic device; however, the bulk of the data collected during this period is biased by residual flow and, thus, unusable. We have, however, observed a potent chemotactic response in C. reinhardtii using the microfluidic device (Figure 2.5).

Figure 2.5. Montage of early chemotactic response.
For this experiment, the cells were nitrogen starved for one hour in the dark. The device was assembled using 1% agarose. N-spiked TAP was prepared with approximately 7mg of ammonium chloride dissolved in 40mL 1x TAP, for a concentration of 3.4mM. As this experiment was meant to gather preliminary data, the gradient was allowed to develop for 40 minutes to ensure a gradient was present rather than allowing it to develop to steady state. N-spiked TAP was prepared with approximately 7mg of ammonium chloride dissolved in 40mL 1x TAP, for a concentration of 3.4mM. The cells were loaded into the device and recorded for two hours with the lights on. Cells began visibly migrating within 30 minutes and were fully accumulated on the source side of the channel by the end of the experiment. While preliminary, these studies do confirm the ability of C. reinhardtii to migrate preferentially up a nitrogen gradient in the microfluidic device. Unfortunately, we were unable to replicate this experiment due to the aforementioned challenges in the device. This has led to our recent efforts with the newly optimized device discussed below.

Determining Optimal Gradient Conditions to Induce a Chemotactic Response

Once the microfluidic device performance was optimized, we then set out to quantify and characterize the algal chemotactic response. To accomplish this, we performed a series of experiments were using device B, varying the nitrogen concentration in the source channel from 1 mM to 11 mM at 2 mM intervals. All these experiments were performed in the dark, and the gradient was allowed to develop in the device for 3 h prior to injecting cells. For this range of concentrations, no chemotactic response was observed in the cells. However, we did observe an interesting phenomenon in the inlet and outlet ports after the experiment was concluded. When the ports were examined at the end of the experiment it was discovered that most of the cells in the port had accumulated on the source side of the port. As shown previously (Figure 2.1 A), the ends of the source and sink channels flare outward to a final distance of approximately 5 mm from the center channel, so the gradient at the ends of the channel is much shallower than that in the center of the device where the channels are closest. Thus, we hypothesized that the gradient in the middle of the center channel was simply too steep for the cells to detect. As describe above, the steepness of the chemical gradient does influence the ability of a cell to perform tactic movement. Thus, it is probable that too steep of gradient cannot be sensed by algae and that they require shallower gradients similar to D. discoideum and neutrophils. To explore this, we set out performing additional experiments using devices with 800, 1200, and 1600 µm channel spacing.

To further support the hypothesis that gradient steepness influences the algal chemotactic response, we performed a second set of experiments using device B. However, the device was only allowed one hour of gradient development time before seeding the center channel with cells to provide a less intense gradient. For this set of experiments, we started out using 2 mM ammonium in the source channel and increased it to 10 mM at 2 mM increment increases for each experiment. Similar to above, no chemotactic response was observed in the center channel, but a strong bias towards the source side of the channel was observed in the inlet and outlet ports with an ammonium concentration of 6 mM and 10mM. This strengthened our suspicion that the gradient in this device was too steep.

To explore the possibility of gradient steepness on the chemotactic response of algae, we performed another set of experiments using device G in order to increase the distance between source and sink and decrease the gradient steepness. Ammonium concentrations tested in the device ranged from 2 mM to 8 mM at increments of 2 mM. We decided to neglect the 10 mM ammonium concentration as no response was observed from either the 6 mM or 8 mM
ammonium concentration. We did observe a slight bias in the swimming cells towards the source side of the channel for 4 mM, but no accumulation. In response to this promising evidence, we performed three more experiments using the same geometry and nitrogen concentrations of 3 mM, 4 mM, and 5 mM in the source channel. Unfortunately, no responses were observed during the experiments; however, when the lights were turned back on at the end of the 4 mM experiment a response was observed within 15-20 minutes. (Figure 2.6)

Previous studies by Ermilova et al. concluded that the chemotactic response of C. reinhardtii was highly dependent upon the sexual cycle of the algae, which was highly dependent upon the light and nutrient exposure in the cells. This published work supports a hypothesis that the presence or absence of light influences the tactic response of algae, which coincides with our previous observations of algae accumulation in the ports after the light in the microscope room was turned back on. In order to investigate possible light-dependence on chemotactic response, the three experiments were repeated in the light, again using device G and nitrogen concentrations of 3mM, 4mM, and 5mM. The 3mM experiment failed due to bubble formation in the channel because the PDMS had not been properly degassed, but responses were observed for both the 4mM and 5mM experiments (Figure 2.7).

In effort to quantify the type of chemotactic response observed in the microfluidic device, we sought out to count the number of cells in the device and their respective location – closer to the source or the sink channels. The chemotactic response for both successful experiments was quantified by dividing the channel into four equal horizontal segments and counting the number of cells in each segment at five time points. Data is presented in Figure 2.7. The data shows a clear increase in the number of cells near the source side of the channel over the course of the experiment, with approximately 50% of all cells accumulated at the source after 90 minutes for 4mM and 60 minutes for 5mM. This response indicates that C. reinhardtii can detect gradients of ammonium between 1.9 and 2.4 mM-m⁻¹ and at intensities between 4 and 5 mM.

2.4 Conclusions

We have developed a truly flow-free microfluidic gradient generator for the study of algal chemotaxis and have demonstrated its utility. Our device is capable of generating steady, linear gradients without exposing the cells to any flow. Our experiments with C. reinhardtii have shown that the gradients generated by our device can induce a chemotactic response in algae, as well as test the effects of gradient intensity and steepness. We intend to continue testing these effects using the other device geometries we have developed. We also plan to further explore the effects of light exposure on the tactic response.
Figure 2.7. Quantified chemotactic response by C. reinhardtii. A) Cell channel at 60 minutes during 4mM experiment, showing channel divisions for quantification. B) Quantified data for 4mM experiment. A clear accumulation of cells is observed in Region 1, nearest to the source channel. C) Cell channel at 60 minutes during 5mM experiment. D) Quantified data for 5mM experiment.
Allelopathy is the process by which chemicals excreted by an organism inhibit, kill, or sometimes stimulate the growth of another organism. Many species of phytoplankton, including those known to form HABs, secrete allelopathic chemicals in order to fend off predators or inhibit other algae that compete for resources. One example is the cyanobacteria *Microcystis* spp. which is known to produce the hepatotoxin microcystin during HABs. This particular species naturally occurs in Lake Pontchartrain in Louisiana and is suspected to have allelopathic interactions with centric diatom species such as *Skeletonema* spp. This precise interplay between the two species has implications in the initiation and propagation of HABs of the different species. One challenge in studying phytoplankton allelopathy is the need for occurrence. Researchers have to wait until a bloom occurs to study the growth of select species and to obtain water samples to identify potential allelochemicals.

Additionally, current laboratory-based methods for studying allelopathic interactions among algae are extremely limited. The most common technique is to culture two species separately, then filter the cells out of the media of one culture and introduce the filtrate into the media of the other culture to see how any chemicals excreted by the first species affects the second. Unfortunately, this method of study excludes any chemicals that would be produced only in the presence of the second species, and also any chemicals the second species might produce in response to the first and what affect they would have. This technique also limits the ability to study the allelopathic response of cells at the individual level, only looking at population-wide effects. Thus, a new method to directly assess allelopathic interactions is needed. Here we detail preliminary work performed using the three channel microfluidic device to culture a single algal species for extended time periods (up to 12 days). This novel microfluidic approach allows for the direct, daily counts of cells which leads to a more accurate assessment of algal growth. While preliminary, the work presented in this section has the potential to be used to study the allelopathic interactions between multiple HAB-causing species.

### 3.1 Device Design

The three channel microfluidic gradient generator can be easily deployed to allow for the long-term culturing of algal species. The features that make the device capable of studying algal chemotaxis can also be harnessed to allow for sustain growth of algae. The only difference is instead of supplying the device with a source and sink, the device is supplemented with growth media in both the top and bottom fluidic channels. Thus, the microfluidic device behaves like a chemostat where the nutrient concentrations in the center culture channel ultimately reach a steady state. Moreover, we can easily modify the geometry of the center channel to include a second ‘flow-free’ channel to allow for the simultaneous co-culture of two different algal species. The addition of a second test channel allows the two species to be cultured alongside one another, while remaining physically separated for observation. Allelochemicals are allowed to diffuse from one channel to the other, enabling chemical cross-talk between the two cell populations (Figure 3.1).

This long-term culture device again consists of a bottom agarose layer and a top PDMS layer in which the microchannels are imprinted via soft lithography. The channels are all 600 µm wide, the spacing between the inner and outer channels is 450 µm, and the space between the two inner channels is 200 µm (Figure 3.1, D). Due to the semi-permanent nature of the device,
Figure 3.1. Overview for the 4-channel co-culture device. A) Allelopathy is the process by which an organism produces chemicals that influence the growth, survival, or reproduction of another. B) and C) Examples of positive and negative allelopathy, respectively, between two species cultured in the 4-channel device. D) Overview of the device design. The channels are all 600 µm wide, spacing between the outer and inner channels is 450 µm, and spacing between the inner channels is 200 µm. Nutrient media is supplied through both flow channels, while two species of cells are cultured in the two inner channels.

PDMS blocks or agarose drops were not sufficient to fully seal the culture channels, so tubing with metal plugs was required. Because the purpose of this device is culturing cells rather than directing their migration, a gradient is not needed, so both outer channels are supplied with fresh culture media in order to continuously provide the cells with nutrients. The gradient was characterized in this device using the same technique as for the gradient generator in order to demonstrate the diffusion of culture media into the growth channels.

As a proof of concept, we successfully cultured the model unicellular algal species *C. reinhardtii* in both the three-channel and four-channel devices for ~10 days. For both devices, the top and bottom fluidic channels were constantly supplied with 1X TAP. The device was placed in the algal culture station beneath a bank of fluorescent lights controlled by a timer to provide the cells with a 14:10 light-dark cycle. The 1X TAP media was fed through the device using two 10mL syringes (Becton, Dickinson and Company) placed in a syringe pump at a flowrate of 15µL·min⁻¹, which could last for 12 hours before needing to be changed. Because of the long-term nature of the experiment, constant observation was not required, so the device was left alone until time to change out its feed syringes, which was performed twice a day (9 am/9 pm) for the duration of the experiment. Cell growth was assessed by collected daily images of the entire culture channel at the 9 am syringe swap. Below we detail some of the challenges that occurred in the development of this microfluidic long term growth approach in addition to the preliminary data obtained for the growth of *C. reinhardtii* in both the three channel and four channel devices.
3.2 Identifying and Overcoming Challenges in the Development of a Long-Term Culturing Device

As with the development of the microfluidic device to study algal chemotaxis, there were some challenges in the development of this device to perform long-term growth. Below we outline the specific challenges and the solutions we devised to perform long-term growth in a microfluidic device.

Dehydration

Initially, long term algal culturing in the microfluidic device experienced significant trouble with dehydration, exacerbated by its much longer run time. In addition to drawing water out of the culture channel, the evaporation of the agarose would also cause it to shrink, loosening the seal between the two layers and resulting in leakage. We initially attempted to address this issue by increasing the water content of the agarose by changing it from 3 wt% to 1 wt% and increasing the total thickness of the slab to 5mm. This proved to be more successful for the long-term culturing than for the chemotactic studies as residual flow was not as great a concern; however, it did not completely eliminate the problem.

Unlike with the chemotaxis experiments described above, it was relatively easy to create a humidified environment for the device. Because the device spent the majority of its time sitting on a table, there was no need for complex construction. Our first attempt was to use an overturned crystalizing dish, supported on scrap pieces of PDMS to provide a gap under the rim of the dish wide enough for the tubing to pass through. Two small petri dishes filled with water were placed next to the device to provide humidity. This arrangement worked quite well for a time, until a completely unforeseen problem arose. The gap between the table and the the edge of the dish proved to be too large to keep out contaminants, specifically fruit flies. During multiple syringe swaps, one or more fruit flies was observed to be crawling on the side of the device, attracted by the agarose and the ethanol used to sterilize the outside of the device. Without exception, we would observe bacterial contamination in the device within a day or two after the fruit fly encounter which ultimately ruined the experiment.

To overcome this limitation, a new chamber was built from Plexiglas to replace the crystalizing dish. This new chamber had notches cut into the bottom edge of two of its sides to allow the tubing to pass under the walls without being crushed, while also allowing the box to sit directly on the table to keep out any insects or other pests (Figure 3.2). This chamber did have some minor flaws, though. If the tubing inside the box did not lay right it would put torque on the device and cause it to flip over. However, experience and care proved to be the easiest solution to this challenge and after switching to this chamber we eliminated the problems related to dehydration.

Figure 3.2. The long-term culturing device inside the Plexiglas humidity chamber.
Contamination

By far the biggest challenge we encountered with this experiment was the ease with which it can become contaminated with bacteria. Because the device was designed to grow cells, it also enabled the growth of unwanted cells which could then interfere with the algae. The result was that many early experiments were terminated due to bacterial contamination in the culture channel (Figure 3.3).

One of the first sources of contamination encountered was contaminated media. Early on, 1X TAP was aliquoted in batches of 50mL at a time, which was far more than enough to fill two 10mL syringes used to infuse the device with growth media. Excess 1X TAP was placed in the refrigerator to be used for the next syringe swap. Unfortunately, the syringes used at the time were not sterile, nor was the environment in which the swap was performed, so the 1X TAP frequently became contaminated. The bacteria present in the 1X TAP were then drawn into the syringes and injected into the device, where they began to proliferate. One particularly bad infestation almost completely blocked one of the flow channels before the device was terminated. Following this incident, we adjusted our experimental protocol with increased emphasis on aseptic technique to minimize contamination.

The next most common source of contamination was the ambient laboratory environment. Ideally, the device would be kept in a sterile environment such as a biosafety cabinet, but such an environment was not available for this project. As a result, bacteria would often adhere to the exposed sides of the agarose slab, where it could then proliferate due to the abundance of nutrients. Many of these colonies would burrow between the PDMS and agarose interface and eventually grow to the point where they are able to enter one of the feed channels or the culture channel. In addition to obscuring visibility of the device, these bacterial cells can disrupt the growth and activity of the algal cells in the channel by consuming the nutrients in the growth media.

The only way found to combat this type of contamination was prevention, leading to the development of a strict sanitation protocol for assembling and handling the device. First, prior to the assembly of the device, anything that could be autoclaved was sterilized (e.g., tubing, media, agarose). Anything that could not be autoclaved was cleaned thoroughly with 70% ethanol (e.g., PDMS). Device assembly was performed inside a HEPA-filtered environment using a horizontal clean bench, which would also be wiped down with ethanol prior to assembling the device. Lastly, immediately after assembly and after any time the device was handled, the outside of the device would be swabbed with an ethanol-soaked Kimwipe, paying special attention to the interface between the agarose and PDMS. These steps, when properly followed, have completely eliminated the hazard of bacterial contamination.
Leakage

The long-term culturing experiments in the microfluidic device also experienced leakage, but for different and more difficult to treat reasons. First, the choice of 1 wt% agarose as the bottom hydrogel layer caused issues. As stated previously, 1% agarose is quite soft and deformable, and it also shrinks as water evaporates from it. Because of this, it was quite common to find the device sitting in a puddle of TAP as the agarose would form to the shape of the PDMS, then relax or shrink, pulling away from the PDMS and breaking the seal. This usually did not harm the algae, so standard procedure was to simply wipe up excess liquid and adjust the screws to compensate during every syringe swap. It was not until after acquiring the humidity chamber that it became feasible to use 3 wt% agarose rather than 1 wt%, eliminating this source of device leakage.

The second source of leakage was deformation of the PDMS itself. As with the gradient generator, sealing the tubing into the ports using liquid PDMS was halted once it was discovered that the sealing PDMS caused uneven pressure across the device. It was soon discovered, however, that sealing the device for long term culturing was necessary. Over long enough time scales, PDMS will permanently deform the same as agarose. Because the tubing was slightly bigger than the hole punched for the port, the PDMS around the port gradually stretched and became loose enough that media began leaking out around the tubing. In order to fix both problems, the Plexiglas plate was placed on the device before the sealing PDMS was applied, allowing the Plexiglas to lie flat while still providing a good seal for the tubing. Because of this, it became necessary to plumb the device with only short pieces of tubing and connect longer pieces using metal connectors. This new protocol ultimately eliminated any channel leakage and allowed for the successful culturing of algae in the microfluidic device.

3.3 Quantification of Cell Growth Within the Long-Term Culturing Device

Once all of the issues had been worked out, long-term culturing of algae in the microfluidic device proved quite simple. The device was assembled as per the sanitary protocol outlined above. Cells were loaded at a concentration of $5 \times 10^4$ cells·mL$^{-1}$ and the channel was imaged. The device was then placed under the fluorescent lights within the humidity chamber. Syringes were changed every 12 hours, at 9am and 9pm, and images were taken every 24 hours immediately following the 9am syringe swap.

Algal growth dynamics were quantified by using the cell counter plugin for ImageJ (NIH) to count the total number of cells within the device (Figure 3.4). Cells were counted in three parts of the channel and their average density used to estimate the total number of cells in the entire channel. This number was double-checked by comparing it to a total cell count of the entire channel for a given day and found to be in good agreement, differing by less than 3%. Unfortunately, we were unable to quantify algal growth beyond day seven due to the fact that the cells started to grow so well that they became too dense to accurately count. However, quantification of algal growth over the first seven days provided adequate information regarding the cells’ growth dynamics within the device. For the first 4 days, the cells remained in their lag phase, with the population not changing much at all. After day 4, however, the cells entered the exponential growth phase. Fitting the data to a trendline of the form $C = C_0 e^{kt}$, where $C_0$ is the initial concentration and $k$ is the doubling time in days, we found that the doubling time is 1.0195 days, matching the doubling time of 22-26 hours given by Pocock and Falk.28
A second experiment was performed using the 4-channel culturing device. The 4-channel device is designed to provide a culturing environment for two separate species in order to study their allelopathic interactions while keeping them physically separate. The diffusive properties of the device were characterized using fluorescent microscopy. An overview of the device is shown in Figure 3.5. In order to demonstrate the capability of the device to culture algae cells, we chose to culture only *C. reinhardtii* in both channels as a proof of concept. As this experiment was just recently completed, we are still in the process of quantifying the algal growth dynamics in both center channels, but a cursory analysis suggests that the cells follow the same pattern of four days of lag phase, followed by exponential growth with doubling time of close to one day. Images are provided in Figure 3.6.

In summary, we have shown that our 3-channel flow-free microfluidic gradient generator can be easily adapted for long-term culturing of cells. We have optimized the conditions for growing algal cells and successfully performed multiple culturing experiments. The addition of a second cell channel allows for the culturing of two separate species at once. This can be of great use in the study of allelopathy, as it allows the separate cultures to interact chemically while remaining physically separate for observation. We plan to begin culturing other species in the future in order to continue development of this device.
Figure 3.5. Overview of the 4-channel co-culture device. A) Device geometry, as shown in Figure X. B) Brightfield image of the device showing the four channels. C) Close-up image of the culture channels with algae. D), E), F), G) gradient data using FAM (D, E) and rhodamine (F, G).

Figure 3.6. 8-day montage of *C. reinhardtii* growth in the co-culture device.
CHAPTER 4. CONCLUSIONS

Harmful algal blooms are a major hazard to human health as well as the environment. The factors that trigger and influence HABs are numerous and complex, and we must understand them in order to prevent and mitigate HABs and their effects. One important facet is the role of cell migration, most notably, chemotaxis. Chemotaxis is a well-known phenomenon occurring in virtually all biological systems, but it is poorly studied in algae. Traditional chemotaxis assays are not suited to the study of algal chemotaxis, so a new method is needed. Microfluidics provides many opportunities for study in biological sciences, including the study of chemotaxis. Microfluidic platforms have already been developed for the study of bacterial chemotaxis. Many of the principles developed in this field can also be applied to study chemotaxis in algae.

We set out to develop a microfluidic platform specifically for the study of algal chemotaxis. Building on past developments with bacterial chemotaxis, our device is a 3-channel flow-free diffusive gradient generator composed of two layers: a PDMS top layer into which the fluidic channels are printed, and a 3 wt% agarose bottom layer to allow the generation of gradients through diffusion. While the design is simple, several challenges had to be overcome in order to optimize the device. However, once these obstacles had been dealt with we were successful in inducing a chemotactic response and have begun to characterize the ideal gradient conditions required for chemotaxis. In the future we plan to further study the effects of gradient steepness and intensity, as well as the effects of light exposure on chemotaxis. We are also examining possible alternative materials for the construction of the device in collaboration with Dr. John Pojman (LSU, Chemistry).

We also examined our device for use in long-term culturing of algal cells, with the ultimate goal of producing a platform for studying allelopathy. With the addition of a second cell channel, two species may be cultured in the same device, allowed to allelopathically influence one another while being kept separate. Modification of the microfluidic device for long-term culturing presented a different set of challenges, but ultimately they were overcome and the culturing of cells in the 3-channel device was successful. Applying what we learned, we were also able to culture cells in the 4-channel device. We hope to continue this work by acquiring a second species in order to demonstrate the co-culturing of multiple species within a single device.
REFERENCES


VITA

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