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THE HYDRODYNAMIC MODEL OF GIARDIA LAMBLIA ATTACHMENT

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ABSTRACT

*Giardia lamblia* is a flagellated, intestinal parasite that resists peristalsis by attaching to the intestinal wall. Despite investigation over the past 40 years, the mechanism of attachment remains controversial. Here we provide experimental and computational evidence in support of an active hydrodynamic attachment mechanism. Using spinning disk confocal microscopy, we show that fluorescent quantum dots move in a directed manner under the ventral surface of the parasite: entering under the anterior end of the ventral disk through a small opening created by the overlap of the disk’s spiral array of microtubules before exiting under the flexible posterior zone of the disk and through the ventral groove. On average, the quantum dots traveled with a speed of ~5 μm/s—as predicted by our computational model—which translates into a negative pressure differential (with respect to atmospheric pressure) sufficient to provide the previously measured force of attachment. Additionally, our model predicts that *Giardia* should be capable of attaching to uneven and porous substrates (akin to intestinal cell microvilli), and we provide evidence that *Giardia* can attach with roughly equivalent forces to low porosity polyacrylamide and glass surfaces under flow, but increasing porosity compromises the attachment strength. We computationally verified these data using a morphologically accurate 2-D finite element model of the parasite’s ventral surface and found that the ventral flagella’s waveform and beat frequency are sufficient to generate the observed flow. The experimentally measured fluid flow at *Giardia*’s ventral surface, the attachment of trophozoites to porous surfaces, and the
computationally predicted flagellar-driven fluid flow in *Giardia’s* ventral surface geometry provide a clearer understanding of the role that fluid dynamics play in *Giardia lamblia’s* attachment mechanism and support our proposed hydrodynamic model.
In loving memory of my parents, Theodore J. Picou Jr. and Deborah S. Picou. The research and writing of this dissertation is dedicated to my family, friends, and advisors who were instrumental in making sure I stayed course and finished my graduate degree.

*Pro aris et focis*

Many thanks,
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Chapter 3 is a variation of my first—and main—paper on Giardia lamblia attachment adapted for this dissertation. As such, many people contributed to the work presented. The text for the introduction, the simulation of fluid flow at the ventral surface, and the theory behind to attachment to porous surfaces sections were written in large part by Dr. Heidi Elmendorf and Dr. Jeff Urbach with additions and edits done by myself. While I was responsible for the experimental work, computational analyses of results, particle/cell tracking, and some of the simulation computations, the base COMSOL model was produced by Jamie Polackwich with the help of Ryan McAllister. Theoretical work was a joint effort of Dr. Tom Powers and Dr. Jeff Urbach.
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Chapter 1: Biology introduction

1.1 Introduction

*Giardia lamblia* (syn. *Giardia intestinalis, Giardia duodenalis*) is a flagellated, parasitic protozoan that causes the diarrheal disease giardiasis. *Giardia lamblia* is a waterborne pathogen with the most common infections occurring due to ingestion of infectious cysts via drinking water contaminated with fecal matter. *Giardia* has been found to infect a wide range of organisms including humans, domestic animals like dogs, cats, and ferrets, as well as livestock such as cattle, goats, and pigs. *Giardia* infection occurs worldwide; there are ~280 million cases of symptomatic disease and ~500,000 new cases are reported annually. The most pronounced infections occur, however, in elderly people and children: up to 6-8% of children in developed countries become infected\(^2\). In humans, giardiasis is the most common form of intestinal parasitic disease in the United States\(^3\) and has affected nearly 33% of people in developing countries\(^2\). Giardiasis can either be asymptomatic or symptomatic\(^4\) and symptoms include diarrhea, cramps, and abdominal pain.

1.2 Giardia biology

*Life cycle*

*Giardia lamblia* has a two-stage life cycle comprising infectious, non-motile cyst and motile trophozoite stages. Humans or animals ingest waterborne cysts that undergo excystation upon passage through the stomach. Excystation occurs when the tetra-nucleated cysts encounter host stomach acids and various digestive enzymes\(^5\). Following excystation in the proximal small intestine, *G. lamblia* transitions into the motile, bi-nucleated trophozoite stage. Because each cyst
has four nuclei, it undergoes duplicate rounds of cytokinesis that results in four trophozoites. *Giardia lamblia* trophozoites are binucleated, tetraploid organisms. Both nuclei are transcriptionally active, contain copies of five chromosomes, and are identical. Trophozoites replicate in and colonize the small intestine where they can be primarily found in the lower duodenum and jejunum. As a noninvasive parasite, *G. lamblia* trophozoites do not physically penetrate host tissue or enter host cells. Instead, they reside in the mucus layers where they attach to the intestinal wall. At some point during colonization a subset of the trophozoites either migrate or are brought further down the small intestine via peristalsis and encyst back into the non-motile cyst stage near or in the colon. After encystation, the cyst is passed through the body and returned to the environment via fecal matter.

**Basic biology**

*Giardia lamblia* cysts are elliptical in shape and are roughly 5 µm wide and 7 µm long. The *Giardia lamblia* trophozoite, however, has defining and unique morphological characteristics that are important for motility and attachment. Overall, the parasite has bilateral symmetry and the shape resembles half of a teardrop where the curvature lies at the dorsal surface. This is an airfoil-like morphology, which is an optimal aerodynamic shape\(^6,7\) and is 10-15 µm long and 5-9 µm wide\(^5\). The trophozoite has eight flagella consisting of ventral, caudal, anterior, and posterior lateral pairs that all emanate from basal bodies located above the ventral disk and between the nuclei. The anterior half of the ventral surface features a ventral disk composed of a concave spiral array of microtubules and a ventrolateral flange, which is a protrusion of cell membrane that hangs over the edges of the ventral disk. The ventral disk is ~8
µm in diameter and has a height of ~2 µm⁹. The spiral array of microtubules (~50 in total⁹ with each being ~20 µm in length⁹) is highly organized and is linked to microribbons that extend to the ventral disk’s overlap zone (denoted by “anterior opening” in Chapter 2) which is where the spiral array of microtubules overlaps⁹. The ventral surface’s posterior half contains a concave ventral groove that runs in an anterior-to-posterior fashion, is open on the bottom, and houses the ventral flagella⁵,¹⁰.

Motility

One of the interesting features of trophozoites is that their flagella serve different purposes depending on locomotive state: free swimming and planar swimming. Free swimming resembles a corkscrew motion and is caused by rotation along the trophozoite longitudinal axis and an undulation of the cell body’s posterior end (the caudal region). The asynchronous beating of the anterior flagella along with lateral bending of the caudal region assist in trophozoite turning as well⁸. During this state, the intercellular portion of the caudal flagella flex the trophozoite’s caudal region resulting in forward propulsion. One can think of this as analogous to a dolphin’s kicking motion. Free swimming trophozoites can rapidly modulate their speed and exhibit speeds ranging from 12-40 µm/s ⁶,⁸. As trophozoites near the attachment surface they transition to planar swimming with speeds ranging from 1-15 µm/s. In this state the trophozoites exhibit neither longitudinal rotation nor caudal flexion and instead exhibit stable motion parallel to the attachment plane. Unlike free swimming trophozoites, where propulsion is primarily due to caudal flexion, planar swimming propulsion is generated by different flagella pairs. The ventral
flagella pair are primarily responsible for forward thrust (82%). The anterior flagella are also responsible for forward thrust (18%), albeit in a minor way.

1.3 Why we should study attachment

*Giardia lamblia* attachment plays a vital role in the organism’s pathogenesis and attachment to the host epithelial wall must occur in order for the parasite to reproduce and maintain infection. There are three primary reasons why studying *Giardia* attachment is important: public health, biophysics, and pathogenesis. The first reason, public health, includes current treatment methods. The second reason, biophysics, involves the future potential of understanding such a complex way for a microorganism to attach and move in an environment like the small intestine. The third reason, pathogenesis, is that we largely do not understand *Giardia’s* attachment mechanism and a full understanding of the organism’s pathogenesis cannot occur until we learn more about how it maintains infections.

Most *Giardia lamblia* infections are self-clearing\(^\text{11}\). That being said, current prevention and treatment methods exist but are either lacking in efficacy or safety, as they are contraindicated for pregnant women. The primary method of prevention is the maintenance and accessibility of clean drinking water. While simple on the surface, the capability of clean sewage and water treatment is out of reach for poor and isolated communities. If infection does occur, there are a few drugs that are currently used. The first is the 5-nitroimidazole compound class which includes metronidazole, tinidazole, ornidazole, and secnidazole. Out of these compounds, metronidazole and tinidazole are the most effective *in vitro*. The second class of drugs is antihelmintics that naturally fail to provide *Giardia* specificity. Because most drug treatments
fail in ~20% of cases, it is imperative that *Giardia* specific drugs with high efficacy are found. Due to the unique nature of *Giardia* attachment (described in detail in this dissertation), *Giardia’s* attachment mechanism and the molecular underpinnings of its attachment mechanism potentially serve as an ideal drug target. Indeed, experiments done in our lab have shown that a drug causing trophozoite flagellar paralysis cause detachment (Walls-unpublished) and, should it cause paralysis *in vivo*, would assist the body in clearing infection.

The second reason for studying *Giardia* attachment is that it is useful from a biophysics perspective. Currently, the fluid dynamics surrounding biological systems in a mucosal environment are not well understood. In addition to gaining an understanding of how organisms move and attach in complex environments such as mucus, there are industrial applications that will benefit from understanding *Giardia’s* attachment mechanisms. One of the main benefits involves swimming microrobotics and targeted drug delivery\textsuperscript{12–14}. A primary obstacle to developing these microrobots is a lack of understanding when it comes to how organisms move and attach in environments characterized by low Reynolds numbers\textsuperscript{15}, where viscous forces dominate inertial forces. Because *Giardia* operates in the low Reynolds number regime, understanding how it is able to attach and detach to host epithelial walls will provide insights regarding the engineering of robotics that move in similar environments.

Lastly, in order to thoroughly understand *Giardia lamblia* pathogenesis we must study how the organism attaches to the intestinal epithelium and maintains infection. Although there have been many investigations into how the parasite attaches, the exact mechanism by which it does so is largely unknown. *Giardia* trophozoites do not invade host cells and reside in and under the intestinal mucus layers. By better understanding how trophozoites attach in a mucus environment
it will provide insight as to how other organisms that do not invade host cells are able to maintain attachment in these adverse conditions. Should *Giardia* possess a unique attachment mechanism among parasitic protozoans, understanding how the organism attaches will result in a new discovery as to how organisms can attach to surfaces. Finding out how *Giardia* attaches is the focus of this dissertation and will be explained in the coming chapters. Before we get to that, however, it is necessary to understand the environment that *Giardia* trophozoites maintain infection and attach to their host.

1.4 Attachment environment

**Host response**

Although this dissertation does not address the host immune response in response to infection, it has been shown that it influences both susceptibility and disease clinical presentation. As is the case with many pathogenic organisms, both susceptibility to *Giardia lamblia* infection\(^1\) and disease severity\(^1\)\(^7\),\(^1\)\(^8\) is increased in immunocompromised hosts. Additionally, evidence suggests that that *Giardia* infections elicit a pro-inflammatory response in the intestine\(^1\)\(^9\). Parasite clearance occurs both through diarrhea and via a T cell response due to the activation and differentiation of B cells through the generation of anti-*Giardia* antibodies and also through B cell independent mechanisms\(^2\)\(^0\).

**Mucus layers**

The gastrointestinal mucus layers are the first line of defense against infection and must be crossed if a pathogen hopes to survive. Mucus is composed of two distinct layers—loosely
adherent bordering the lumen and firmly adherent bordering the epithelium—and comprise enzymes, immunoglobulins, salts, microbiota, proteins, and glycoproteins (mucins). The loosely adherent layer can be traversed by microorganisms and macro/micro-molecules. The firmly adherent layer, however, is generally impermeable to microorganisms. Gastric mucus and intestinal mucus is secreted by goblet cells found in their respective epithelium. Mucins have at least one PTS (proline, threonine, serine) domain that is O-glycosylated to form glycopeptides and are either gel-forming or trans-membrane. In the small intestine, MUC2 is the gel-forming mucin and MUC3, MUC12, MUC13, and MUC17 are the trans-membrane mucins (MUC1, MUC4, and MUC16 are also trans-membrane mucins but are not intestinal mucins). Gel-forming mucins either form a web of linear-polymers or a network of N-terminal-trimer polymers and trans-membrane mucins form a brush layer at the epithelial surface. The mucus layers have varying thicknesses depending on their locations. The loosely adherent layer in the duodenum is on the order of 100 µm thick and the firmly adherent layer, which is often thinner than the loosely adherent layer, is only ~16 µm thick. Many factors govern the thickness, composition, and rheological properties of the mucus layers such as mucin synthesis and secretion, mucin degradation, pH levels, and mechanical shear forces (Reviewed in). Although mucus has very different micro- and macro-rheological properties, the total composition of mucin types form a viscoelastic gel. As a viscoelastic substance, mucus has frequency-dependent shear moduli comprising elastic (G') and viscous (G'') components. The elastic component can be thought of as a gel's ability to return back to its original form after a shear stress is applied. The viscous component can be thought of as the loss component such that a fluid loses some ability to return back to its original form after a shear stress is applied. These
moduli are important in determining the mechanical properties of mucin that microorganisms and molecules encounter when attempting to cross the mucus layers. Of particular importance is the ratio of $G'$ to $G''$ found in the firmly adherent mucus layer that cannot generally be penetrated by microorganisms. The firmly adherent layer is a viscoelastic gel, where $G' > G''$, as opposed to a viscoelastic fluid. In contrast, the loosely adherent layer is a viscoelastic fluid, where $G'' > G'$.

**Navigation to the attachment surface**

In order for *G. lamblia* trophozoites to establish infection by attachment to the epithelial wall, they must first travel through the both the small intestine lumen and a meshwork of mucus ~170 µm (loosely adherent + firmly adherent) thick. The antimicrobial nature inherent with respect to both the antimicrobial molecules and the biophysical properties of the mucus layers make their transversal an interesting aspect of pathogenesis. Because of this, parasitic protozoans as well as bacteria interact with the mucus layer through many different specific mechanisms that can be broadly defined as mucus alteration and/or locomotion. The intestinal murine nematode *Trichuris muris* elicits different mucosal changes during acute and chronic infections. In acute infection, GABA-α3 (a receptor in mucosal epithelium) expression is increased in response to elevated IL-13 levels which leads to goblet cell hyperplasia and increased glycoprotein secretion and may assist in parasite expulsion. In chronic infection, the mucus barrier is depleted and contains lowly charged mucins which may affect mucus composition. *Entamoeba histolytica* takes a more direct approach in that it actively dissolves the mucus—*E. histolytica* secretes mucinases that cleave MUC2. One of the few organisms that can directly penetrate the firmly
adherent mucus layer (specifically that of the stomach) is *Helicobacter pylori* and it does so through a combination of locomotion and mucus alteration. The enzyme urease facilities *H. pylori*'s traversal through the firmly adherent mucus layer\textsuperscript{27} by increasing local pH which causes the mucus to act more like a viscoelastic fluid than a viscoelastic gel\textsuperscript{28,29}.

While it is possible that *Giardia lamblia* trophozoites exhibit some form of mucus alteration, the interaction between *Giardia lamblia* and mucus is largely unknown as few interactions between *G. lamblia* and mucus have been investigated. Two studies found that trophozoite attachment increases in the presence of mucus from the duodenum\textsuperscript{30} and that duodenal mucus supports trophozoite growth in serumfree medium\textsuperscript{31}. Using 2-photon microscopy to image trophozoite interactions with sections of mouse duodenum, it was found that many trophozoites directly attach to the duodenal villi (our lab unpublished). Other trophozoites in this study were seen either freely swimming above and around the villi or were attached within ~2 µm of the villus surface, indicating that trophozoites have the ability to penetrate and reside in the firmly adherent mucin layer. These findings are not unique, due to transmission and scanning electron micrographs indicating that trophozoites attach to intestinal villi’s microvilli\textsuperscript{32}. Mucosal defenses against *G. lamblia* are also not well understood but include IgA antibodies and B cell-independent mechanisms (reviewed in\textsuperscript{17}).

1.5 Attachment

Attachment to host organisms and host cells is an important factor in pathogen virulence and is often a drug development target. For intracellular pathogens, attachment is a temporary necessity that they must undertake before entering a host cell. Extracellular pathogens, however,
must maintain attachment while combatting host defenses and external forces when attempting to replicate and survive. Similar to motility through the mucus layers, parasitic protozoans often employ attachment mechanisms specific to their pathogenesis. Extracellular parasitic protozoans like *Entamoeba histolytica* employ lectin-mediated binding\(^{33}\) or adhesins, as is the case with *Trichomonas vaginalis*\(^{34}\).

Attachment plays an integral part in *Giardia lamblia*’s lifecycle due to the trophozoite’s habitation of the small intestine where it is constantly acted upon by peristaltic forces. Despite the importance of trophozoite attachment, we neither have a complete understanding *Giardia*’s attachment mechanism nor of how it maintains attachment in small intestine’s mucus environment. Although the specifics of *Giardia*’s attachment are unknown, current hypotheses fit into three main categories: lectin/adhesin-mediated binding, ventral disk clutching, and hydrodynamic suctioning.

**Lectin/adhesin-mediated binding**

Lectin or adhesin-mediated binding is a highly specific form of attachment where lectins on the *Giardia* trophozoite surface would bind to receptors on intestinal epithelial cells. Due to this attachment mechanism’s prevalence in the biological world, multiple studies have been conducted to investigate specific binding in *Giardia lamblia* and there are a few general lines of evidence that support this theory: *Giardia*’s surface is negatively charged due to the presence of N-acetyl-D-glucosamine residues\(^{35}–^{37}\); possible glucose-mannose and mannosyl binding via *Giardia*’s glucose-mannose-specific lectin and a mannose-6-phosphate binding lectin\(^{36}–^{38}\); and *Giardia* attachment to small intestine epithelial cells as opposed to colonic cells\(^{39}\).
There are many arguments against lectin/adhesin-mediated binding as Giardia’s primary attachment mechanism. Although trophozoites display preferential attachment to small intestine epithelial cells as opposed to colonic cells, lectin- and adhesin-mediated binding (specific sticking) is improbable as anything more than an ancillary attachment mechanism since trophozoites attach do, in fact, attach to both types of epithelial cells. In 2006, Hansen et al investigated trophozoite attachment to differentially treated surfaces. Using a centrifuge-based detachment assay, the researchers attached trophozoites to uncoated (plain) glass, poly-L-lysine, Teflon-like (tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane, and polyethylene glycol (PEG) coated glass slides. The experimental concept behind each of these treatments are as follows: if trophozoites exhibited nonspecific electrostatic binding then they would preferentially attach to poly-L-lysine coated surfaces; if van der Waals force played a significant role in attachment they would preferentially attach to glass as opposed to the Teflon-like surface; and if they had specific binding then a polyethylene glycol surface would inhibit trophozoite binding due to its inhibition of protein absorption and screening of surface charges. Interestingly, they found that trophozoites attached equally well to all surface treatments tested with 50% detachment occurring at normal forces of $1.51 \pm 0.20 \text{ nN}$ and 90% detachment occurring at $2.43 \pm 0.33 \text{ nN}$.

**Ventral disk clutching**

Due to the unique nature and orientation of Giardia lamblia’s ventral disk, it has long been thought to play a central role in the parasite’s attachment mechanism. These hypotheses revolve around either contractile-based clutching or modulating the concavity of the disk such that it grasps the attachment surface. This hypothesis is supported by scanning electron micrographs...
of imprints left by attached *Giardia* and transmission electron micrographs of attached *Giardia*'s ventral disk embedded in epithelial microvilli \(^{32,40,41}\).

There are two biological/cytoskeletal arguments against ventral disk clutching. These hypotheses revolve around the ventral disk changing conformation in a significant manner during attachment or grasping inherent topographical features of the attachment surface. Firstly, large conformational changes of the ventral disk have not been seen *in vitro*\(^{42}\). Secondly, the ventral disk’s dorsal ribbons are linked by filaments that cannot withstand constant conformational changes\(^{43}\). Another argument against the clutching theory is with respect to behavior. Experiments in our lab have shown that attached trophozoites can pivot and slide while remaining attached to glass surfaces under fluid flow (unpublished). A static, clutching attachment mechanism does not allow for this type of attached motion. Additionally, an argument against ventral disk clutching is experimental in nature. Most attachment studies are done on glass slides or glass coverslips, which are very smooth and lack the surface features necessary for the ventral disk to grasp onto.

**Negative pressure-based attachment models**

There are two different negative pressure-based attachment models: a hydrodynamic model driven by fluid flow between the ventral surface and the parasite’s attachment substrate and a passive model where the ventral disk serves as a suction cup. In the active model proposed by Holberton in 1973, *Giardia*’s ventral disk and groove act as a semi-closed system where ventral flagella-driven fluid flow creates a negative pressure differential (with respect to ambient pressure) underneath the organism\(^{44}\). In contrast, the passive model comprises a suction cup.
mechanism where ventral disk contraction expels fluid from under the ventral disk creating a seal and generating negative pressure under the ventral disk.\(^{45,46}\)

Both the active and passive hydrodynamic suction models have evidence suggesting they are not the primary attachment mechanism. Holberton’s work was theoretical in nature, did not include experiment, and was morphologically inaccurate which led to unrealistic attachment forces (and pressures under the ventral disk\(^{8}\)). The Holberton model has the ventral flagella emanating from under the ventral disk. Due to modern technology, imaging, and further biological study we know that the basal bodies responsible for the flagella are above—not below—the ventral disk and that the ventral flagella enter the ventral groove at the disk’s posterior end. Holberton’s active model also has the ventral surface fluid flow occurring in the two grooves surrounding the ventral disk (between the ventral disk and the ventral flange). While there does exist space for fluid flow there, these reservoirs do not empty into the ventral groove which means that ventral flagella beating is not responsible for that fluid motion.

The passive suction model is improbable due to both experimental evidence and the \textit{in vivo} nature of trophozoite attachment. Although it has recently been suggested that \textit{Giardia} remains attached in the absence of ventral flagella beating\(^{45}\), the data presented do not support the authors’ conclusions. In these experiments the authors used TIRF to look at surface contact during attachment, disrupted flagellar motion using a morpholino-based knockdown of PF16, and overexpressed a dominant negative form of alpha2-annexin which caused irregular flagellar waveforms. The first issue found in this study is the use of TIRF microscopy to indicate surface contact. Indeed, TIRF can be used to indicate surface proximity of a sample, but due to its inherent z-resolution limit of \~100\text{nm}\(^{47}\) it cannot definitively determine surface contact.
Secondly, the overexpression of dominant negative alpha2-annexin has a population penetration of ~82% according to the authors—meaning 18% of the parasites were not affected. Thirdly, neither the PF16 morpholinos nor the alpha2-annexin dominant negative parasites exhibited flagellar paralysis. The organisms simple had decreased flagellar function (irregular beating and waveforms). Lastly, and most importantly, the authors’ data indicate that the organisms did experience decreased attachment in the various attachment assays. In the shear force attachment assays there was ~20% decreased detachment in the PF16 morpholino trophozoites and ~30% less attachment for the alpha2-annexin knockdown. In the centrifugal assays, PF16 morpholino attachment decreased by ~40% and the alpha2-annexin knockdown attachment decreased by ~60%.45.

In vivo the parasite attaches in the transmembrane mucin layer of host epithelial cells which is a porous environment. Similarly, it attaches to host epithelial cells, which are, by definition, semi-permeable. This has also been shown in vitro during the experiments showing preference for small intestinal epithelial cells. As such, a tight seal around the ventral disk’s periphery is unlikely due to the porous nature of host cells.

Concluding thoughts on attachment hypotheses found in literature

In addition to the evidence against each current hypothesis regarding the primary mechanism of Giardia attachment, the limits and robustness of Giardia’s attachment mechanism are also shown through changes in environmental conditions. One of the earliest investigations into trophozoite attachment determined that there was an important physiological factor that defined general attachment ability: temperature. It was found that reducing temperature to 24°C severely
limited Giardia’s attachment ability and attachment did not occur at 12º C or below\textsuperscript{48}. While those data do not provide evidence for a particular attachment mechanism, they indicate that attachment is heavily reliant on metabolic processes and energy generation. In 2008, Hansen and Fletcher compared trophozoite attachment in differing aqueous conditions to determine what environmental factors might cause Giardia detachment\textsuperscript{49}. These tests were conducted by modulating media osmolality, tonicity, and pH as a proxy for environmental changes attached trophozoites may experience in the small intestine. While the researchers did not measure detachment force, they conducted experiments under a steady flow rate of 0.3 mL/min and compared detachment profiles. They found that cells detach in a tonicity-dependent manner and that neither changes in pH nor osmolality affected cell attachment. These data provide further evidence that Giardia trophozoites are able to attach with equal rigor under variable environmental conditions.

1.6 A new model of Giardia attachment

It is possible that each hypothesized attachment mechanism plays a part in Giardia lamblia attachment \textit{in vivo}. However, one of the defining characteristics of Giardia lamblia trophozoite attachment is its robust nature—trophozoites can attach to a wide range of substrates under varying conditions. Because of this, all of the current hypotheses only explain a subset of Giardia attachment scenarios and fail to provide a mechanism that encompasses the wide range of attachment conditions.

My research, and the data presented in coming chapters, seeks to address these concerns by presenting a new, refined model of Giardia lamblia attachment based on ventral flagellar-driven
fluid flow at the ventral surface. Evidence for ventral fluid flow originated with TIRF experiments that show fluorescent microspheres entering and exiting the area under the ventral disk and ventral groove during attachment. To date, there have been no experimental studies of the fluid dynamics involved in Giardia’s attachment mechanism. Although many have researched different aspects of Giardia’s attachment and proposed various models in attempts to explain their findings, the only investigations of fluid at the ventral surface were theoretical in nature and done by David Holberton in 1973 and 1974. The field has largely dismissed the his hydrodynamic hypothesis due to its inconsistencies with experiment.

We hypothesize that ventral flagella-driven fluid flow at the ventral surface of Giardia lamblia generates a negative pressure differential (relative to ambient pressure) sufficient to account for the force attachment (originally proposed Holberton but refined by our lab). There are three conditions which must be met in order for a flagella-driven attachment model—and consequently our hypothesis—to hold: i) The ventral flagella create directional fluid flow at the ventral surface; ii) The fluid flow is capable of generating a negative pressure differential sufficient to account for the force of attachment; iii) The negative pressure differential—and therefore partial attachment—can be actively generated on a porous surface despite fluid influx at the ventral surface. We will test these three conditions using a combination of biophysical assays and computational models.
Chapter 2: Physics introduction

2.1 Introduction

Most studies investigating Giardia lamblia’s attachment mechanism have used strictly biological approaches. While the resulting attachment theories explain certain aspects of Giardia’s attachment behavior, they fail to describe and predict the parasite’s robust attachment capabilities (described in Chapter 1). It is clear, then, that classical biological approaches alone are incapable of discerning Giardia’s primary attachment mechanism and an interdisciplinary approach is necessary. Our hypothesized attachment mechanism is hydrodynamic in nature: ventral flagella-driven fluid flow between ventral surface of Giardia lamblia and the attachment substrate generates a negative pressure differential (relative to ambient pressure) sufficient to account for the force attachment.

Because the hydrodynamic model sits at the intersection of biology and physics, a brief explanation of the underlying cellular mechanical and physical principles that serve as the model’s foundation is in order.

2.2 Eukaryotic and ventral flagella motion

Eukaryotic flagella are comprised of a centralized bundle of microtubules known as the axoneme that are arranged in a 9+2 array of microtubule doublets (a single doublet in the center encircled by nine doublets). These doublets are connected by dynein motor proteins that slide each doublet relative to a single neighboring doublet and they do so in a unidirectional manner—the proteins “walk” from the positive end (where the microtubules originate from) towards the negative end (where the microtubules lead). As the motoring (sliding) activity propagates along
the length of the flagellum—and because each doublet slides relative to a single neighboring doublet—it creates a bending motion where the moving doublets bend towards the stationary doublets. The canonical sinusoidal waveform of eukaryotic flagella is thus created as the sliding doublets change along the flagellum’s length\textsuperscript{50,51}. 

*Giardia lamblia*’s ventral flagella exhibit standard eukaryotic flagella motion but do so in a unique and interesting way. As the wave propagates along the length of the flagella, the bends occur where the flagella and sides of the ventral groove meet. Due to the trapezoidal shape of the ventral groove, we can model the ventral flagella as a travelling wave with a linearly increasing amplitude modeled by

\[
y(x,t) = h \ast s(x) \ast \sin(kx - \omega t)
\]  

(2.1)

where \(x\) is the linear distance along the center of the ventral groove, \(y\) is the perpendicular displacement of the flagellum, \(\omega\) is the flagellar wave’s angular frequency, \(t\) is time, \(s(x)\) is the line for the ventral groove’s edge, \(k = 2\pi/\lambda\) (\(\lambda\) is wavelength), and \(h\) is the ratio of flagellum amplitude to the ventral groove height (\(h = 1\) for healthy parasites, Figure 3).

2.3 *Navier-Stokes*

The Navier-Stokes equations are the equations of motion for viscous fluids and result from applying Newton’s 2\textsuperscript{nd} law to fluid motion. Specifically, Navier-Stokes is the partial differential equation dealing with the conservation of momentum for Newtonian fluids. When dealing with incompressible fluids, these equations take the form
\[
\rho \left( \frac{\partial \vec{v}}{\partial t} + (\vec{v} \cdot \nabla) \vec{v} \right) = -\nabla p + \eta \nabla^2 \vec{v} \quad (2.4)
\]

where \( \rho \) is density, \( \vec{v} \) is flow velocity, \( t \) is time, \( p \) is pressure, and \( \eta \) is dynamic viscosity. In the low Reynolds number regime where inertial forces are negligible, Navier-Stokes can be linearized and simplified because the nonlinear inertial terms \( (\rho \vec{v} \cdot \nabla \vec{v}) \) go to zero\(^5\). The simplified form of Navier-Stokes (together with the continuity equation \( \nabla \cdot \vec{v} = 0 \)) becomes the Stokes equations for creeping flow

\[
\nabla^2 \vec{v} = \frac{1}{\eta} \nabla p \quad (2.5)
\]

As a result, the linearized equations for fluid motion are linear in velocity and pressure that serves as an integral part of our hydrodynamic model as well as our computational simulations.

2.4 Low Reynolds number regime

Because our proposed *Giardia lamblia* attachment model is hydrodynamic—based on fluid flow—an understanding of how fluid behaves at the micron scale is essential. Generally when one thinks of an object moving in fluid one pictures something on the scale of a human or marine animal swimming in a large body of water where the fluid is characterized by turbulent flow and inertia. On small scales, however, different properties dominate and its motion is governed largely by viscous—instead of inertial—forces and is laminar in nature. In laminar flow, the fluid layers are parallel and do not undergo mixing like they do in turbulent flow. The
Reynolds number is a dimensionless quantity that helps differentiate between these two regimes, helps predict the transition from laminar to turbulent flow, and is the ratio of inertial to viscous forces within a fluid (2.2).

\[ Re = \frac{\text{inertial forces}}{\text{viscous forces}} \]  

(2.2)

Inertial and viscous forces can be expressed specifically as

\[ Re = \frac{\rho VL}{\mu} \]  

(2.3)

where \( \rho \) is fluid density, \( V \) is the fluid velocity scale, \( L \) is length scale, and \( \mu \) is fluid viscosity. Mathematically, the Reynolds number comes from the non-dimensionalization of the Navier-Stokes momentum equation where length, flow velocity, time, and pressure are substituted with dimensionless variables. The resulting non-dimensionalized Navier-Stokes equation yields \( (1/Re) \) as the parameter leading the viscous term, which allows one to predict behaviors of particular fluid regimes depending on the dominance of viscous or inertial forces.

In the low Reynolds number regime, where \( Re \ll 1 \) and is the case for my research, viscous forces dominate due to low fluid velocities and small characteristic length scales. The low Reynolds number regime is important for my research not only because it describes the qualitative nature of flow conditions (laminar flow) but also because it allows for the simplifications of the Navier-Stokes equations for fluid motion.
2.5 The hydrodynamic model

The hydrodynamic model of *Giardia lamblia* attachment proposed here is an active hydrodynamic model (a comparison of active vs passive models is discussed in Chapter 1) centered on the ventral flagella acting as a fluid pump that continuously draws fluid under the ventral surface. In order to understand our model it is best to think of *Giardia’s* ventral surface (the surface where attachment occurs) as a semi-closed system consisting of two chambers (Figure 2.1): a chamber created by the ventral disk and a chamber created by the ventral groove which houses the ventral flagellum. The system is semi-closed in that the first chamber (created by the ventral disk) has a small opening at the anterior end and opens to the second chamber (created by the ventral groove) which then leads out from under the parasite at its posterior end. These two chambers are also connected and, for simplicity’s sake, assumed to be open to each other—meaning there is an opening between the two chambers and they are not separated by a barrier. Because of the semi-closed nature, there is a clear path for fluid to flow directionally at the ventral surface (Figure 2.2): fluid enters the anterior opening of the ventral disk, travels through the first chamber, enters the ventral groove, and then exits the posterior end of the ventral surface.

In order for there to be a pressure drop across the ventral surface, fluid must flow across each of the system’s openings. We can gain insight into how this occurs by looking at a simpler geometry where the small anterior opening (designated “ao” in Figures 2.1 and 2.2) can be modelled as a small hole in an infinite, thin screen. In 1958 Hasimoto investigated the relationship between viscous flow through a small slit and pressure drop and found the flow conductance (the ratio of the total flow to the pressure drop) to be
\[
\frac{Q}{\Delta p} = \frac{\pi a^2}{8\eta} \tag{2.2}
\]

where \( Q \) is the total flux, \( \Delta p \) is the pressure drop, \( 2a \) is slit width, and \( \eta \) is viscosity (Hasimoto 1958). Thus, the pressure drop as a function of fluid flux is

\[
\Delta p = \frac{Q8\eta}{\pi a^2} \tag{2.3}
\]

Because the ventral disk is a large round channel, the pressure drop across the ventral disk will be much smaller than that associated with (2.3), resulting in uniform low pressure throughout the chamber. As there is a second opening between the ventral disk chamber and that of the ventral groove, there is another (albeit small and insignificant compared to that across the anterior opening) pressure drop as fluid flows between the two chambers.

In our model the ventral flagella act as the fluid pump. Due to the ventral flagella being bounded by the ventral groove (the wave spans the width of the groove and the height of the groove is largely offset because of the ventral flagella’s specialized fins along the bottom edge), this acts as a positive displacement pump where the flagella trap fluid in the groove and displace it as the flagella beat. Since the fluid is incompressible, the fluid displaced by the flagella must be balanced by the fluid entering the disk through the small anterior opening—making it an active model. To determine the amount of fluid discharged by the ventral flagella, one has to
integrate Equation 2.1 over the length of the ventral groove. However, we can approximate the fluid flux generated by the ventral flagella as

$$Q \approx f\lambda \bar{s} \quad (2.4)$$

where $Q$ is fluid flux, $f$ is flagella frequency, $\lambda$ is wavelength, and $\bar{s}$ is the average height of the ventral groove found from the term $s(x)$ in Equation 2.1 (the line representing the ventral groove edge) assuming the ventral flagella span the entire groove width (Figure 2.3). As the flagella beat, they draw fluid through the small anterior opening of the ventral disk which results in drastic pressure drop at the anterior opening, the low pressure is maintained throughout the ventral disk chamber, and then the pressure gradient returns to atmospheric pressure over the length of the ventral groove as it opens at the posterior end. A computational simulation of *Giardia*’s ventral surface serves as a visual representation and is found in Figure 2.4. We see that the minimum pressure (maximum pressure drop) of -6.5 Pa is found at the ventral disk’s anterior opening and is largely maintained throughout the area under the ventral disk and the beginning of the ventral groove. As the ventral groove opens towards atmospheric pressure at the posterior end, we see a gradual increase in pressure until it reaches atmospheric equilibrium (Figure 2.4).

We can approximate trophozoite force of attachment (suction force) by the equation

$$F_{suction} \sim \Delta p A_{parasite} \quad (2.5)$$
where $\Delta p$ is the pressure drop found across the anterior opening (2.3) and $A_{\text{parasite}}$ is the parasite area.

2.6 The hydrodynamic model with respect to porous surfaces

One of the hydrodynamic model's conditions is that the negative pressure differential—and therefore partial attachment—can be actively generated on a porous surface despite fluid influx at the ventral surface. In our simplified model, *Giardia*’s ventral surface is a semi-closed system where the largest pressure drop occurs at the ventral disk’s anterior opening (equation 2.3, simulated in Figure 2.4)—which also happens to be the only place fluid enters the system. When attached to porous substrates, however, the pressure differential at the ventral surface will drive fluid through the substrate and into the system. Because the ventral flagella act as a positive displacement pump, the total flux at the ventral surface is flagella-driven, which is to say that the flux is independent of the pressure differential. Therefore, flow through the substrate will cause a reduction in flow through the anterior opening of the ventral disk and will result in a decrease in the pressure drop at the anterior opening.

The relationship between the pressure differential and fluid flux on porous surfaces can be modeled as two parallel impedances

$$Q_{\text{total}} = \delta P \left( \frac{1}{k_s} + \frac{1}{k_o} \right) \quad (2.6)$$

where $Q_{\text{total}}$ is the total flux, $\delta P$ is the pressure differential, $k_s$ is the substrate impedance, and $k_o$ is the anterior opening impedance. Thus, we can predict that substrate porosity will reduce
attachment force when the substrate impedance approaches the impedance of the anterior opening which results in the flux through the substrate becoming a significant fraction of the flux through the anterior opening of the ventral disk.

2.7 Rheometer-based attachment assay

In the coming chapters we test parasite attachment using a rheometer-based attachment assay. The assays are conducted using a stress-controlled rheometer (Anton Paar MCR-301) with a parallel-plate tool geometry (Figure 2.5) where the rheometer controls the rotational rate of the tool. Due to the low Reynolds Number regime, the fluid flow is laminar and is described as Couette flow (flow between two parallel plates where one plate is moving and the other is kept stationary). The fluid flow is generated by the viscous drag exerted on the fluid—via the top moving plate—and the shear rate is defined by

\[ \dot{\gamma} = \frac{v}{h} \]  

(2.7)

where \( \dot{\gamma} \) is shear rate, \( v \) is the velocity of the moving plate, and \( h \) is the distance between the two parallel plates. This shear-driven flow is a case of simple shear, which is a type of laminar flow where a single component of the velocity vectors has a non-zero value and the velocity gradient is constant and perpendicular to the fluid flow.

For our purposes, we can approximate the shear force exerted on the attached parasites as

\[ F_{\text{shear}} = \dot{\gamma} \eta A_{\text{parasite}} \]  

(2.8)
where $F$ is the shear force, $\gamma$ is the shear rate, and $A_{\text{parasite}}$ is the area of the parasite. It is important to note that these assays do not directly test our pressure-based attachment mechanism. Instead, the assays exert shear force, which is perpendicular to the normal force. Using the relationship between an object moving due to shear force and static friction, we can relate the experimentally tested shears forces to the normal force. For two solid non-moving objects relative to each other, maximum force of static friction force is given by

$$F_{s}^{\text{max}} = \mu_s N \quad (2.9)$$

where $\mu_s$ is the coefficient of static friction and $N$ is the normal force. When the maximum static friction force is exceeded, the object starts to slide. By combining equations 2.5 and 2.9

$$F_{s}^{\text{max}} = \mu_s (\Delta p A_{\text{parasite}}) \quad (2.10)$$

we can see what shear force is needed to exceed the maximum static friction force and dislodge—sliding in our simplistic model—attached Giardia trophozoites.
Figure 2.1. Two-dimensional model of *Giardia lamblia’s* ventral surface. The circular chamber is the ventral disk (vd) with an anterior opening (ao) on the bottom left and the bare zone (bz) denoted by a black filled circle. The ventral groove (vg) emanates from the posterior end of the ventral disk and contains the ventral flagella (vf – blue line).
Figure 2.2. Two-dimensional model of *Giardia lamblia*’s ventral surface with the hydrodynamic model’s proposed fluid flow. The circular chamber is the ventral disk (vd) with an anterior opening (ao) on the bottom left and the bare zone (bz) denoted by a black filled circle. The ventral groove (vg) emanates from the posterior end of the ventral disk and contains the ventral flagella (vf – red line). A potential path for fluid flow at the ventral surface is denoted by blue arrows. The fluid enters the ventral disk at the anterior opening, travels around the bare zone, and is pumped out through the ventral groove by the ventral flagella.
Figure 2.3. Two-dimensional model of *Giardia lamblia*’s ventral flagella amplitude with respect to the ventral groove height. The ventral flagella (blue line) modeled as a travelling wave with a linearly increasing amplitude as it approaches the height of the ventral groove (black lines). The ratio of the amplitude to the height of the ventral groove (h) approaches 1 as the ventral flagella span the ventral groove’s height.
Figure 2.4. A morphologically accurate two-dimensional finite element model of the pressure field under *Giardia lamblia’s* ventral surface during one phase of the ventral flagella oscillation. The circular chamber is the ventral disk (vd) with an anterior opening (ao) on the bottom left and the bare zone (bz) denoted by a white circle in the middle of the ventral disk. The ventral groove (vg) emanates from the posterior end of the ventral disk and contains the ventral flagella (vf – black line). The pressure field is measured in Pascals where black indicates the minimum pressure and light grey indicates maximum pressure (atmospheric). The yellow streamlines denote fluid flow lines and the red arrows denote fluid velocity vectors. The average pressure differential under the ventral surface is -1.4 Pa.
Figure 2.5. Schematic of a parallel plate rheometer geometry. The fluid flow is generated by the viscous drag exerted on the fluid by the moving plate which creates a velocity gradient (red arrows). The edge shear rate ($\dot{\gamma} = \frac{v}{h}$) is determined by the velocity of the rotating plate’s edge divided by the height, which is the distance between the two plates. Our experiments are conducted at 2/3 the distance to the edge thus making the local shear rate approximately $(2/3) \times$ edge shear rate.
Chapter 3: Novel flagellar pump mediates attachment of \textit{Giardia lamblia}

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3.1 Introduction

Many microbes exist in environments characterized by fluid flow; this is true of both pathogens subjected to physiological fluid movement in animal hosts and environmental microbes subjected to water flow in aqueous milieu. The ability of microbes to attach to surfaces despite the shear forces exerted by fluid flow is a prerequisite for their proliferation and survival. Most microbes are quite specific in their attachment, relying on specific ligand-receptor interactions to attach in a suitable niche.

\textit{Giardia lamblia} is an intestinal pathogen of humans and other mammals that is endemic
in much of the world\textsuperscript{2}, with estimated incidence rates of 20-30\% in developing nations\textsuperscript{54} and 0.5-1\% in the U.S.\textsuperscript{55,56}. Symptomatic individuals suffer from diarrhea and other upper gastrointestinal complaints, and both symptomatic and asymptomatic individuals with chronic infections suffer growth effects from long-term malabsorption\textsuperscript{57-59}.

The parasite survives in water supplies in an environmentally-resistant form known as a cyst. Following ingestion, exposure to acids and proteases in the stomach trigger excystation and the emergence of the trophozoite form of the parasite that attaches with its ventral surface against the microvilliated surface of epithelial cells in the small intestine. The trophozoite is a teardrop-shaped cell with an elaborate cytoskeleton. The ventral surface of the trophozoite is dominated at the round anterior end by a large domed structure (the ‘ventral disk’) formed by a spiral array of microtubules and in the tapering posterior half by a chamber (the ‘ventral groove’) in which the ventral pair of flagella beat continuously in a semi-sinusoidal waveform.

Previous research over the past 40 years into the mechanism by which \textit{Giardia lamblia} attach to the small intestine has resulted in evidence to support three different models that we refer to here as the ‘sticking’ (Lectin/Adhesin-Mediated Binding), ‘clutching’ (Ventral Disk Clutching), and ‘sucking’ (Negative pressure-based attachment models) models of attachment (Discussed in Chapter 1). The ‘sticking’ model – the traditional microbial attachment model – predicts that adhesion between surface molecules on \textit{Giardia} and intestinal cells is primarily responsible for attachment. While there is evidence that pre-treatment of either the parasite or host cells with lectins reduces parasite attachment\textsuperscript{36}, other data clearly demonstrate that \textit{Giardia} attaches with equal force to surfaces with a wide variety of charges, including hydrophobic surfaces, arguing against ‘sticking’ as the dominant force of attachment\textsuperscript{1}. The ‘clutching’ model
predicts that the parasite contracts the edges of the ventral disk to allow the parasite to cling to the surface of intestinal cells. Numerous transmission electron micrographs show cross-sections of the parasite attached to intestinal cells with the edges of the disk dug deeply into the cell surface\textsuperscript{44,60}, and scanning electron micrographs of intestinal cells from which \textit{Giardia} have recently detached show clear footprints of the edges of the ventral disk left behind on the surface of the intestinal cells\textsuperscript{60}. Although there is little to directly argue against the ‘clutching’ model, there are two primary lines of evidence: there is no evidence of contractile proteins at the edges of the ventral disk – indeed, \textit{Giardia} has been found to not contain any myosin homologs\textsuperscript{61} – and thus a cellular mechanistic explanation is still lacking for this model; and trophozoites are able to attach to surfaces lacking clutchable topographical features such as glass and plastic. The ‘sucking’ model, first described by Holberton in the 1970’s\textsuperscript{43,44} proposes that \textit{Giardia} attaches using a hydrodynamic mechanism in which the beating of the ventral pair of flagella moves fluid under the parasite to generate negative pressure. Although this model has attracted a great deal of interest over the years, there has been no direct evidence to support a hydrodynamic model of attachment.

Here, we provide evidence to support the sufficiency of the hydrodynamic model to promote attachment of \textit{Giardia} to its substrate. The Holberton model makes two important predictions, both of which we test here with confirmatory results. First, a hydrodynamic force of attachment relies on a pressure differential between the ventral and dorsal surfaces of the parasite: the pressure in the region between the ventral surface of the parasite and substrate must be lower than the pressure above the dorsal surface of the parasite. This pressure differential will push the parasite onto the surface. We identify the source of this pressure differential by
documenting for the first time the existence of a directional fluid flow between the parasite and substrate. Unexpectedly, and in contrast to Holberton’s initial model, we find that on a solid glass substrate, fluid moves under the ventral disk itself through a small gap created by the spiral nature of the disk at its anterior end and out the flexible posterior portion of the disk into the ventral groove. Second, a hydrodynamic force of attachment should allow the parasite to attach to a porous surface: the continual generation of the pressure differential would permit attachment – albeit at perhaps reduced forces – to virtually any surface since the mechanism does not rely on the creation of a tight seal between the substrate and ventral disk. This latter point seems especially important since the normal intestinal milieu of Giardia requires the parasite to attach to the surface of mucus-coated, microvilliated intestinal epithelial cells – a highly irregular substrate that clearly would be nearly impossible to attach to with a passive suction cup – a role more commonly ascribed to the ventral disk in the Giardia literature. In support of this second prediction, we find that Giardia attaches with roughly similar force profiles to both solid glass and porous polyacrylamide substrates.

3.2 Characterizing fluid flow at the ventral surface

Our negative pressure-based active hydrodynamic model of Giardia lamblia attachment depends upon the parasite’s ability to induce flow from a small, previously unremarked-upon opening in the anterior end of the ventral disk, under the ventral surface, out an opening in the posterior end of the ventral disk, and through the ventral groove before leaving the posterior end of the parasite’s ventral surface. Generally, eukaryotic organisms use the propagating wave motion of their flagella to generate propulsion, enabling them to swim. While this is true for
Giardia’s ventral flagella during planar swimming, the unique geometry of the organism’s ventral surface and the continuous beating of the ventral flagella during attachment (Figure 3.1) lead us to hypothesize that the system creates a positive displacement pump responsible for maintaining directed fluid flow and a pressure gradient necessary to facilitate attachment.

We tested our anterior to posterior fluid flow hypothesis by using a solution of 20nm diameter fluorescent quantum dots, 10% Ficoll, 4% bovine serum albumin, and particle tracking velocimetry. We chose 10% Ficoll because it yielded the highest viscosity for which Giardia’s flagella behaved ‘normally’, beat with a mean frequency near that of 0% Ficoll controls (Chapter 5, Figure 5.1), and because it decreased quantum dot Brownian motion. Bovine serum albumin, when present in high levels, binds nonspecifically and prevents the quantum dots from sticking to the glass coverslip—Giardia’s plane of attachment. Together, these properties allowed us to use the quantum dots as stand-ins for fluid tracers in this low Reynolds number regime.

To measure the fluid dynamics at the ventral surface of attached trophozoites, we captured video of attached cells in the quantum dot solutions using fluorescence at 60x with a temporal resolution of 50 ms. As a product of the quantum dots’ size and the small size of the anterior opening of the ventral disk, we were only able to track particles at the ventral surface of ten attached trophozoites (Aggregated in Figures 3.3 and 3.4). Of these trophozoites, one cell was captured having three separate quantum dots enter the anterior opening of the ventral disk (Figure 3.2). Surprisingly, the three dots took nearly the exact same route through the cell (Figure 3.2G, overlay). The dots entered the small anterior opening of the ventral disk, traveled around the bare zone by following the disk’s spiral array of microtubules, left the disk’s posterior opening, travelled through the ventral groove, and then out from under the parasite’s posterior
Although the quantum dots for the other nine trophozoites did not all enter the same respective anterior opening (possibly due to ventrolateral flange morphology or uneven proximity of the ventral disk to the attachment surface), the quantum dots displayed similar trajectories: entered the disk, traveled around the bare zone while under the disk, exited the disk’s posterior opening, and travelled through the ventral groove (Figure 3.3).

We determined the trajectories of the quantum dots using an in-house PTV program adopted from John Crocker of University of Pennsylvania (Crocker 1996) (Figure 3.2A,C,E, Figure 3.3, Figure 3.4) by measuring the frame-by-frame instantaneous velocities of each quantum dot. Before looking at all the particle trajectories in aggregate, we can get an idea of fluid flow at the ventral surface by analyzing the motion of the three quantum dots that travelled under a single parasite. The first quantum dot (Figure 3.2A) had an average speed of $4.72 \pm 3.7 \, \mu m/s$ and a median speed of $3.41 \, \mu m/s$ over a total of 97 frames. However, MATLAB did not catch the last part of the trajectory when the dot makes two rapid revolutions, apparently caught in the flagellar motion, as it was leaving the ventral groove. The second quantum dot (Figure 3.2C) had an average speed of $2.04 \pm 2.92 \, \mu m/s$ and a median speed of $0.62 \, \mu m/s$ over a total of 313 frames. Although this is much lower than the first, the quantum dot got stuck at the posterior outlet of the ventral disk before leaving. We believe the flow still continued but the physical size of the quantum dot caused it to stop. Excluding the recorded speeds when the particle was stuck (<1.5 \, \mu m/s), the average speed was $5.23 \pm 3.18 \, \mu m/s$ with a median speed of $4.69 \, \mu m/s$. Like the first quantum dot, MATLAB could not track the particle leaving the posterior end of the ventral groove and reported an error stating the parameters were too extreme—meaning the dot moving too quickly—as it made a similar revolution. The third quantum dot (Figure 3.2E) had an
The quantum dot was very faint so the image sequence’s contrast was enhanced 0.4%, and MATLAB picked up the entire trajectory—even during the exit revolution experienced by the previous two quantum dots.

To get a rough, general idea of the fluid flow field at the ventral surface we aggregated the quantum dot tracks from each attached parasite (Figure 3.3) and then divided the ventral surface into zones (Figure 3.4) for granular analysis. As described above, the particles followed similar trajectories under each parasite. They entered the ventral surface of the attached trophozoites under the anterior end of the ventral disk, travelled around the bare zone to the disk’s posterior, entered the ventral groove, and then exited the parasite at the ventral groove’s open posterior end. Before entering the ventral surface, particle motion was consistent with that of Brownian motion (data not shown). Ventral surface zones are shown in schematic form (Figure 3.4) and defined as the following: Left Anterior (Figure 3.4 Box A, outlined in Figure 3.3), Proximal Bare (Figure 3.4 Box B, outlined in Figure 3.3), Right Anterior (Figure 3.4 Box C, outlined in Figure 3.3), Left Posterior (Figure 3.4 Box D, outlined in Figure 3.3), Posterior Exit (Figure 3.4 Box E, outlined in Figure 3.3), and Ventral Groove (Figure 3.4 Box F, outlined in Figure 3.3). Similar to the single parasite with three quantum dot captures, the aggregate flow field suggests pronounced flow under the ventral disk in the Left Anterior and Left Posterior zones (Figure 3.4A mean 9 ± 6 μm/s; median 7 μm/s; Figure 3.4D mean 9 ± 8 μm/s; median 8 μm/s). That being said, there was significant flow in the Proximal Bare and Right Anterior zones as well (Figure 3.4B mean 6 ± 4 μm/s; median 5 μm/s; Figure 3.4C mean 5 ± 5 μm/s; median 4 μm/s) which is indicative of persistent fluid flow under the ventral disk. In all instances the
fastest flow occurred in the Ventral Groove zone (Figure 3.4F mean 18 ± 13 μm/s: median 17 μm/s) where the ventral flagella are housed and serves as the flagellar pump.

Contrary to previously published data\textsuperscript{45}, these data indicate directed anterior-to-posterior fluid flow at the ventral surface of attached \textit{Giardia lamblia} trophozoites and lay the foundation of our active hydrodynamic attachment model.
Figure 3.1. *Giardia lamblia* trophozoites continuously beat the ventral flagella during attachment. A-D: Each image is of the same trophozoite attached to a glass coverslip and is separated from the previous image by 5 frames. Images were taken at 32º C in PBS under 60x DIC with a temporal resolution of 10 ms for the whole stack. Because of this, each image shown is ~50ms apart.
Figure 3.2. A single attached trophozoite demonstrates consistent, directed fluid flow at its ventral surface as demonstrated by three separate quantum dot particle tracks. Trajectories of three quantum dots that went through the compartment composed of the ventral disk and ventral groove. The cell’s “shadow” can be seen at the center of the image. (A,C,E) Each color corresponds to a different dot, and each dot went through the cell at a different time. (B,D,F) Max intensity fluorescent overlay of the dots at each time point. (A,B) MATLAB code does not catch the last part of the trajectory. Average speed: 4.72 ± 3.7 μm/s. Median speed: 3.41 μm/s. Total frames used: 97 (50 ms apart). (C,D) MATLAB code does not catch the last part of the trajectory because the dot was moving too fast (error reported when the parameters were too extreme). Average speed: 5.23 ± 3.18 μm/s (excluding speeds <1.5 μm/s when dot got caught where the ventral disk opens to the ventral groove). Corrected median speed: 4.69 μm/s. Total frames used: 313 (50 ms apart). (E,F) MATLAB code catches the dot’s entire trajectory. Average speed: 7.04 ± 4.73 μm/s. Median speed: 5.79 μm/s. Total frames used: 66 (50 ms apart). Contrast was enhanced to allow 0.4% of pixels to be saturated. (G) Overlay of the three trajectories in A, C, E. Images were taken under fluorescence at 60x using Qtracker 655 non-targeted quantum dots (Invitrogen).
Figure 3.3. Aggregate particle tracks indicate directed anterior-to-posterior fluid flow at the ventral surface of attached *Giardia lamblia* trophozoites. Each color represents the frame-by-frame instantaneous velocity vectors of separate quantum dots under individual trophozoites. All tracks were rotated and normalized to an average sized trophozoite ventral disk. Traced boxes are the ventral surface zones (left-to-right, top-to-bottom): Left Anterior, Proximal Bare, Right Anterior, Left Posterior, Posterior Exit, and Ventral Groove. Large red vectors are the average velocity vector for each zone.
Schematic of ventral surface zones

Box A Total Speed
mean = 9.6 µm/s, median = 7.2 µm/s

Counts
speed (µm/s)

Box B Total Speed
mean = 6.4 µm/s, median = 5.2 µm/s

Counts
speed (µm/s)
Figure 3.4. Speeds of instantaneous particle tracks display persistent fluid flow at the ventral surface of attached *Giardia lamblia* trophozoites. Schematic of ventral zones boxes A-F (left-to-right, top-to-bottom): Left Anterior, Proximal Bare, Right Anterior, Left Posterior, Posterior Exit, and Ventral Groove. Histograms of each zone show the instantaneous speeds of each track where the x-axis is speed (μm/s) and the y-axis is instantaneous particle count.
3.3 Trophozoite attachment to a variety of substrates under shear flow

It has been experimentally shown that *Giardia* can attach to a variety of non-porous substrates \(^1,45,49\) which fits well with the passive suction model. However, the active hydrodynamic model we propose dictates that trophozoites are able to maintain attachment to porous substrates both under fluid flow and no-flow conditions—an attachment feature that is biologically relevant, has never been shown before, and is unexplainable by current attachment theory. To demonstrate that fluid flow found at the ventral surface is capable of generating a negative pressure differential sufficient to account for the force of attachment on both non-porous and porous substrates, we subjected parasites to shear stress using a microscope-rheometer that lets us both control the amount of shear generated and visualize the results instantaneously.

While these methods do not directly test pressure-mediated suction, they indirectly test our model by a) subjecting attached trophozoites to the range of forces our model predicts they can withstand, and, b) the controlled influx of fluid flow via porous substrates disrupts our model in a predictable fashion. For non-porous substrates, we used uncoated glass coverslips. For porous substrates, we created 50 µm thin polyacrylamide gels with consistent rheological properties (Supplemental Figure 3.2) by varying acrylamide percentages (5%, 6%, and 7% acrylamide by volume) while maintaining a 6% cross-link density. We chose a cross-link density of 6% because it offers a wide hydrodynamic permeability range that is well defined\(^62\) for the percentage of acrylamide chosen.

To test that flagella-driven fluid flow is capable of generating a sufficient negative pressure to account for the force of attachment on non-porous substrates we allowed Syto16-
labeled trophozoites to attach to non-porous glass coverslips and then subjected them to a step-wise linear increase in shear rates ranging from 100-8000 s\(^{-1}\) over a period of 120 seconds (Figure 3.5). Parasite attachment was computed by imaging once per second (in correspondence with the step-wise linear ramp of shear rate) and tracking the persistence of individual parasites throughout attachment assay’s length. Originally, we attempted to count present cells but quickly experienced inflated cell counts due to cells entering the frame and fluorescent streaks created by out-of-focus detached cells. In order to overcome these issues, we differentiated between false positives by comparing them to cells that maintained attachment and persisted in sequential frames. We found that cells attached to glass coverslips exhibit shear-dependent attachment profiles—mainly they detach as shear rate increases in an expected fashion. Fifty percent detachment occurred at 879.56 ± 126.99 s\(^{-1}\). Interestingly and in accordance with previously data gathered in our lab (data unpublished), a characteristic of glass attachment is that attached parasites exhibit sliding behavior that is distinguishable from detached parasites due to their speed and proximity to the attachment plane (data not shown).

Although there have been studies that investigated *Giardia* attachment to inherently porous epithelial cell lines\(^{39,41}\), they did not investigate the effect of porosity on attachment. Using the proposed attachment model, we can estimate the effect of substrate porosity on attachment force. The pressure differential will drive fluid will flow through the substrate and into the space ventral beneath the ventral disk, thereby reducing the flow through the small anterior opening and the resultant pressure drop. We can model this situation as two parallel impedances, so that the relationship between the pressure differential and the flux is

\[
Q_{\text{total}} = \delta P \left(\frac{1}{k_s} + \frac{1}{k_o}\right) \quad \text{(Chapter 2 Equation 2.6)}
\]

where \(k_s\) is the impedance of the substrate and \(k_o\) is the
impedance of the anterior opening. We expect that the total flux is approximately independent of the pressure differential (i.e. that the flagellar pump runs like a ‘positive displacement’ pump, as indicated by our simulations with different values of AO), and predict that the force of attachment will be reduced by substrate porosity only when the flux through the substrate becomes a significant fraction of the total flagellar-driven fluid flux (or equivalently, when the impedance of the substrate approaches the impedance of the anterior opening).

We used 5%, 6%, and 7% polyacrylamide gels (with permeability values of $k_{5\%} = 9.64 \times 10^{-18}$ m$^2$, $k_{6\%} = 5.24 \times 10^{-18}$ m$^2$, and $k_{7\%} = 3.13 \times 10^{-18}$ m$^2$, computed from experimentally measured permeability as a function of cross-link density [62]) to cover the porosity that should negatively affect our attachment model; with 5% being the most porous and having the most negative effect on attachment and 7% being porous enough to affect attachment but where the parasites should still be able to maintain attachment under the subjected shear. Cells attached to 5% gels detached so rapidly that a 50% detachment shear rate cannot reasonably be determined (Figure 3.6). Cells attached to 6% polyacrylamide gels fared better in that they were able to maintain attachment on the low end of subjected shear rates but reached 50% attachment at 266.19 ± 48.22 s$^{-1}$ (Figure 3.7). Cells attached to 7% polyacrylamide gels, on the other hand, attached with high proficiency—much better than cells attached to 5% and 6% (Figure 3.9)—and reached 50% attachment at 2217.18 ± 316.65 s$^{-1}$ (Figure 3.8). While it was fully expected that cells should have been able to maintain strong attachment to 7% gels, the level with which they did so was a surprise. We predicted that cells should be able to withstand ~3x the forces of those attached to 6% as opposed to the 8-fold difference seen here.
Despite the discrepancy between attachment to 6% and 7% polyacrylamide gels, our hydrodynamic model is the only Giardia attachment model that can explain these data and cellular attachment to porous surfaces. Specifically, the passive suction model dictates that trophozoites cannot attach to any porous surfaces due to the impossibility of a complete seal being formed at the ventral disk periphery.
Figure 3.5. *Giardia lamblia* trophozoites exhibit shear rate-dependent detachment when attached to glass. The percentage of originally attached cells vs the local shear rate [1/s] at 2/3 the distance from tool center to the outer edge. The x-axis is the local shear rate at 2/3 the distance from tool center to the outer edge; the y-axis is the percentage of original cells remaining. The orange line (012616_Glass_Coverslip1_TrialA) represents the first trial with the initial frame set to timepoint 3; the grey line (012616_Glass_Coverslip2_TrialA) represents the second trial with the initial frame set to timepoint 6; the blue line (012416_Glass_Coverslip2_TrialA) represents third trial with the initial frame set to timepoint 7.
Figure 3.6. *Giardia lamblia* trophozoites rapidly detach from 5% polyacrylamide substrates under shear flow. The percentage of attached cells to a 5% polyacrylamide substrate vs the local shear rate [1/s] at 2/3 the distance from tool center to the outer edge. The x-axis is the local shear rate at 2/3 the distance from tool center to the outer edge and is adjusted for polyacrylamide substrate thickness; the y-axis is the percentage of original cells remaining. The orange line (08252016_5PA) represents the first trial with the reference frame set to timepoint 0. The grey line (09232016_PA) represents the second trial with the reference frame set to timepoint 0. The blue line (08022016_5PA) represents the third trial with the reference frame set to timepoint 0.
Figure 3.7. *Giardia lamblia* trophozoites exhibit intermediate shear rate-dependent attachment to 6% polyacrylamide substrates. The percentage of attached cells to a 6% polyacrylamide substrate vs the local shear rate [1/s] at 2/3 the distance from tool center to the outer edge. The x-axis is the local shear rate at 2/3 the distance from tool center to the outer edge and is adjusted for polyacrylamide substrate thickness; the y-axis is the percentage of original cells remaining. The blue line (10282016_6PA) represents the first trial with the reference frame set to timepoint 0. The orange line (11032016_6PA) represents the second trial with the reference frame set to timepoint 0. The grey line (11102016_6PA) represents the third trial with the reference frame set to timepoint 0.
Figure 3.8. *Giardia lamblia* trophozoites exhibit strong shear rate-dependent attachment to 7% polyacrylamide substrates. The percentage of attached cells to a 7% polyacrylamide substrate vs the local shear rate [1/s] at 2/3 the distance from tool center to the outer edge. The x-axis is the local shear rate at 2/3 the distance from tool center to the outer edge and is adjusted for polyacrylamide substrate thickness; the y-axis is the percentage of original cells remaining. The blue line (05232016_7PA) represents the first trial with the reference frame set to timepoint 0. The orange line (06162016_7PA) represents the second trial with the reference frame set to timepoint 0. The grey line (06222016_7PA) represents the third trial with the reference frame set to timepoint 0.
Figure 3.9. *Giardia lamblia* trophozoite detachment profiles are substrate porosity-dependent. The average percentage of attached cells to a 7%, 6%, and 5% polyacrylamide substrates vs the local shear rate [1/s] at 2/3 the distance from tool center to the outer edge. The x-axis is the local shear rate at 2/3 the distance from tool center to the outer edge and is adjusted for polyacrylamide substrate thickness; the y-axis is the percentage of original cells remaining. The solid colored lines (red, blue, black) represent the average trophozoite attachment to each substrate and the shaded regions (light red, light blue, grey) represent the standard deviation between trials for each respective substrate. The red line represents the average attachment of trophozoites to 7% polyacrylamide with the reference frames set to timepoint 0. The blue line represents the average attachment of trophozoites to 6% polyacrylamide with the reference frames set to timepoint 0. The black line represents the average attachment of trophozoites to 5% polyacrylamide with the reference frames set to timepoint 0.
3.4 Simulating the effects of ventral flagella in the ventral surface geometry of attached Giardia lamblia trophozoites

The experimentally observed fluid flow suggests the following ‘flagellar pump’ model for Giardia attachment to smooth surfaces: The beating of the flagella in the ventral groove continually draws fluid from underneath the ventral disk. The fluid is incompressible, so the fluid exiting the disk must be instantaneously balanced by a flux of fluid into the disk through the small anterior opening at the overlap of the microtubule spiral array\textsuperscript{44,60,9}. The small opening represents a high impedance for fluid flow, and therefore requires a large highly localized pressure gradient. As a result, the pressure in the ventral disk will be significantly below the external pressure. The ventral disk, by contrast, provides a relatively wide channel for fluid flow, so there will be an approximately uniform low pressure present throughout the area under the disk. Thus, the purpose of the disk is not to generate low pressure, but to distribute the flow-generated low pressure over a large area.

We have quantitatively tested this model by numerically solving the equations of motion for fluid flow in a two-dimensional model of Giardia with beating flagella. The model geometry, shown in Chapter 2 Figure 2.1 and Figure 3.10A (this chapter), and associated dimensions, are derived from experimental observations of attached parasites\textsuperscript{44,60,9}. Specifically, the ventral disk (VD) is modeled as a circular region of diameter 6 microns, with small anterior opening (AO) of width $a$, and an excluded area with diameter 1.6 microns due to the protrusion of the cell membrane through the bare zone (BZ), centered in the VD. The wedge shaped ventral groove (VG) is 1.4 microns wide at the posterior opening (PO), and extends 4.0 microns, expanding to a final width of 2.6 microns. The beating of the flagellum is modeled as a traveling wave with a
linearly increasing amplitude, \( y(x,t) = h s(x) \sin(kx - \omega t) \), where \( x \) is the linear distance along the centerline of the parasite starting at the PO, \( y \) is the perpendicular displacement of the flagellum, \( \omega \) is the angular frequency of the flagellar wave, in this case \( \omega = 2\pi \times (10 \text{ µm/s}) / 2.66\text{µm} \), which equals about 23.62(s\(^{-1}\)), and \( k = 2\pi/\lambda \), with \( \lambda = 2.66 \text{ µm} \), and \( s(x) = 0.15x + 0.7 \) is the line matching the edge of the ventral groove, so that when \( h = 1 \) the peaks of the flagellar wave just make contact with the walls of the VG at every point in the beating cycle.

The fluid flow and pressure changes generated by the flagellar motion are calculated by solving the viscous flow equations with no-slip boundary conditions on all surfaces using a commercial finite element package (see Methods). A snapshot of a typical solution is shown in Figure 3.10B, for \( a = 125 \text{ nm} \) and \( h = 0.9 \). The arrows representing the instantaneous velocity of the fluid show that the velocity is high and a rapidly changing function of position close to the flagellum, while the flow under the ventral disk is small but relatively uniform, consistent with the experimental observations (Figure 3.3). As expected, the low pressure generated at the base of the flagellar pump is spread over the entire area of the VD and the anterior pressure gradient is confined to a very small region around the AO.

The relationship between the pressure drop generated by the pump and the flux through the anterior opening can be estimated from the equation for a small aperture in an infinite straight barrier (Chapter 2, Equation 2.3). Figure 3.11 summarizes the results of model calculations for values of the AO (\( r \)) of 86 nm, 116 nm, and 190 nm and wave amplitude/channel height ratios of 0.1, 0.5, and 0.9 (flux as a function of wave/amplitude/channel height is shown in Figure 3.12A). The expected linear relationship between \( \delta P \) and \( Q/r^2 \) is satisfied in all cases.
The general features of the flow field do not change with the phase of the flagellar wave, but fluid flux through the parasite, area-averaged pressure underneath the ventral disk, \( P_{\text{ave}}(t) = \frac{1}{A} \int_{\text{parasite}} P(x, y, t) \) (where \( A \) is the total area of the parasite), and the average force on \textit{Giardia} do show some variation with time (Figure 3.13, Table 3.1, Figure 3.12B respectively).

Assuming that the pressure outside of the parasite is approximately uniform and constant, the parasite will experience an average force of attachment equal to \( F_{\text{ave}} = \frac{A}{T} \int_{\text{cycle}} (P_0 - P_{\text{ave}}(t)) \), where \( P_0 \) is the external (atmospheric) pressure. For the conditions shown in Fig. 3.10C, \( F_{\text{ave}} = 50 \ pN \).
Figure 3.10. Morphologically accurate two-dimensional finite element models of the fluid velocity field and pressure field under *Giardia lamblia*’s ventral surface during one phase of the ventral flagella wave cycle. (A) Schematic of *Giardia*’s ventral surface geometry. Inlet represents the ventral disk’s anterior opening and has a diameter of 0.125 µm. The ventral disk is modeled having a radius of 3 µm. The bare zone is modeled as a circle in the middle of the ventral disk and has impermeable boundaries such that fluid cannot enter that area. The ventral flagella are modeled as a single wave with a total length of 4 µm and includes 1.5 wavelengths (wavelength of 2.66 µm). The ventral groove outlet represents the posterior opening of the ventral groove and has a diameter of 2.67 µm. (B) COMSOL simulation of representing one ventral flagella phase of the fluid velocity field under *Giardia*’s ventral surface. Red indicates maximum velocity and deep blue indicates minimum velocity. Red arrows indicate fluid velocity vectors and the yellow streamlines indicate fluid flow lines. XY-axis denote distance. Velocity field is in meters per second [m/s]. Structures and shapes are the shown in (A). The ventral flagella are simulated by a wave with increasing amplitude propagating from left to right (anterior to posterior). The minimum velocity is 0 and the maximum velocity is 5.4e-5 m/s. (B) COMSOL simulation of representing one ventral flagella phase of the pressure field under *Giardia*’s ventral surface. Very light grey indicates maximum pressure and black indicates minimum pressure. Red arrows indicate fluid velocity vectors and the yellow streamlines indicate fluid flow lines. XY-axis denote distance. Surface pressure is in Pascals [Pa]. Structures and shapes are the shown in (A). The ventral flagella are simulated by a wave with increasing amplitude propagating from left to right (anterior to posterior). The maximum pressure differential for the phase shown is 1.62 Pa.
Figure 3.11. The pressure drop at *Giardia lamblia*’s ventral disk anterior opening linearly increases with fluid flux through the anterior opening. Scatterplot showing the linear relationship of the pressure drop at *Giardia lamblia*’s ventral disk anterior opening as a function of fluid flux over the ventral disk anterior opening. Pressure was computed for three values of $W$ where $W$ is the ratio $\frac{A_{\text{max}}}{\text{Channel Height}}$ ($A_{\text{max}}$ is the maximum wave position in the channel and Channel Height is the ventral groove edge): $W = 0.1$ (blue dots), $W = 0.5$ (orange dots), $W = 0.9$ (grey dots). Trendline is represented by a dashed yellow line. The x-axis is log(pressure) and the y-axis is log(flux/r$^2$) where r is the anterior opening size.
Figure 3.12. Both the average flux through the ventral groove outlet and the average force on Giardia increases as the ventral flagella amplitude approaches the ventral groove height. (A) COMSOL simulation results of the average fluid flux through the ventral groove outlet over one complete ventral flagella wave cycle as a function of $\frac{A_{\text{max}}}{\text{Channel Height}}$ ($A_{\text{max}}$ is the maximum wave position in the channel and Channel Height is the ventral groove edge). The x-axis is the ratio of $A_{\text{max}}$ to Channel Height. The y-axis is flux through the ventral groove outlet [$m^2/s$]. (B) COMSOL simulation results of the average force on Giardia over one complete ventral flagella wave cycle as a function of $\frac{A_{\text{max}}}{\text{Channel Height}}$ ($A_{\text{max}}$ is the maximum wave position in the channel and Channel Height is the ventral groove edge). The x-axis is the ratio of $A_{\text{max}}$ to Channel Height. The y-axis is average force on Giardia in newtons [N].
Figure 3.13. Flux through *Giardia* trophozoite’s ventral surface varies with ventral flagellar wave phase. COMSOL simulation results of the average fluid flux through the ventral groove outlet over one complete ventral flagella wave cycle broken up into 12 phases (where n = phase = 0-11). The x-axis is phase number (n) and the y-axis is fluid flux through the ventral groove outlet ($m^2/s$).
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Table 3.1. **Area-averaged pressure under *Giardia* trophozoite’s ventral surface varies with ventral flagellar wave phase.** COMSOL simulation results of the average fluid flux through the ventral groove outlet over one complete ventral flagella wave cycle broken up into 12 phases (where Frame (n) = phase = 0-11).
Supplemental Figure 3.1. Delaunay triangulation enables one to distinguish between attached parasites and bright streaks created by moving, detached parasites. A representative image of a Delaunay triangulation (red lines) and the corresponding dual graph of the Voronoi diagram (blue lines) of a set of potential attached parasites (centroids denoted by bright spots).
Supplemental Figure 3.2. 5%, 6%, and 7% polyacrylamide substrates exhibit distinguishable storage moduli. $G'$ [Pa] vs Polyacrylamide percentage gels with 5% crosslinker density by volume. The y-axis is the measured storage modulus, $G'$, in Pascals; the x-axis is the percent acrylamide contained in the polyacrylamide substrates with 6% crosslinker density by volume. The red dots are 5% acrylamide, the orange dots are 6% acrylamide, and the grey dots are 7% acrylamide.
3.5 Methods

Cell culture

*Giardia lamblia* trophozoites were maintained anaerobically in borosilicate glass tubes or polystyrene flasks. Parasites were grown at 37°C in modified TYI-S-33 media (Keister, 1983), where 0.024 M sodium bicarbonate replaced the traditional phosphate buffer solution. For all experiments, *Giardia* cultures were grown to mid-log phase (~ 80% confluency) and then were placed on ice for 20 minutes to allow cellular detachment.

Preparation of viscous fluids

Ficoll (GE Healthcare Life Sciences) was added to 1X PBS at concentrations of 0% (control), 5%, 10%, 15%, and 19%. A UBBELOHDE Viscometer (Cannon Instrument Company, 0.002795 mm²/s² constant, 0.156% expanded uncertainty) was used to determine the viscosities of the various solutions. Due to temperature stability issues, 32°C was used, as it was the most stable temperature over the extended time frames necessary for viscosity measurement by hand. The viscosities were as follows: 1.08 ± 0.03 cP (centipoise; mPa•s) for 0%, 2.23 ± 0.01 cP for 5%, 4.77 ± 0.05 cP for 10%, 9.65 ± 0.08 cP for 15%, and 14.66 ± 0.40 cP for 19% Ficoll in 1x PBS.

Determination of ventral flagella oscillatory frequency

*Giardia lamblia* cultures were iced for 20 minutes to allow cellular detachment. 25 μL of cells were pipetted on pre-cleaned Superfrost MicroscopeSlides (Fisherbrand) and covered with 22x50 Microscope Cover Glass (Fisherbrand). Using a heated objective, slides (with cells
present) were brought to an equilibrium temperature of 32°C to allow for attachment and to ensure that fluid viscosity remained at calculated values. After this 5-minute equilibrium period, attached cells were imaged in 1X PBS at 60x DIC using a Nikon Eclipse Ti-U microscope with a 1200hs camera manufactured by PCO AG. Video was captured with a temporal resolution of 10 ms. To ensure cellular fitness, imaging occurred for a period of 30 minutes or less. Data sets were analyzed using the free software package ImageJ (NIH) by manually recording the recurrence of peak-to-peak amplitudes of the ventral flagella.

Quantum dot solution preparation and cell imaging

Cells were imaged in solution composed of 1X PBS, 10% Ficoll, 4% bovine serum albumin, and a 1:25 dilution of Qtracker 655 non-targeted quantum dots (Invitrogen). The 10% Ficoll was chosen because it has the highest viscosity (4.77 ± 0.05 cP) for which *Giardia* maintained steady flagellar beating while reducing the quantum dots’ Brownian motion, and bovine serum albumin was used as a general non-sticking aid to prevent the quantum dots from sticking to the glass microscope coverslip. Video was captured at 60x fluorescence using a Nikon Eclipse TE2000-U spinning disk confocal microscope with an Andor iXon DV887 camera and a temporal resolution of 50 ms using SlideBook (Intelligent Imaging Innovations) microscope imaging software.

Particle tracking

Particle tracking was accomplished using a MATLAB adaptation by Daniel Blair and Eric Dufresne of the software developed by David Grier, John Crocker, and Eric Weeks (Crocker
The algorithm works as follows: grayscale images are converted to black and white images by applying a bandpass filter to subtract the background and thresholding--this allows for the location of the bright spots’ centroids to be determined with sub-pixel accuracy. Then, the instantaneous trajectory and corresponding speed of each particle was found by linking the position of bright spots in one image to the closest bright spot in the following image.

Coverslip preparation

Hydrophilic bottom coverslips were prepared using 25 mm diameter circular coverslips. The coverslips were passed over the inner blue flame of a Bunsen burner (with the side to be activated facing down) to remove any particles and ready the coverslips for activation. A small amount of 0.1 N NaOH was pipetted on the activated sides and spread evenly using a Kimwipe to leave little NaOH on the surface and air dried for approximately 10 minutes. 50 µL of Aminopropyl-trimethoxysilane (APTMS, Sigma Aldrich) was pipetted on the center of the coverslips and spread evenly over their surfaces using the pipette tip. After waiting 5 minutes to react, the coverslips were placed in dH$_2$O for approximately 10 minutes to allow complete removal of the excess APTMS, to prevent adverse reaction with the subsequent glutaraldehyde activation, resulting in an orange residue. Following the dH$_2$O wash, coverslips were air dried and then placed in a fume hood. The coverslips were completely covered with 0.5% glutaraldehyde (Sigma Aldrich) and incubated for 30 minutes. Excess glutaraldehyde was removed, and the coverslips were rinsed in dH$_2$O for 10 minutes before allowing to air-dry.
**Fluorescent cell labeling**

Cells were centrifuged at 2500g in a Beckman GPR centrifuge for 10 minutes. The supernatant was removed and the cell pellet was resuspended in 497 µL sterile 1X DPBS in a sterile flat-top 2.0 mL plastic microcentrifuge tube. A new 10 µL aliquot of Syto16 was thawed and centrifuged at ~2000g 15 seconds to pellet clumped dye then 3 µL of the supernatant was added to the cell sample. The sample was then wrapped in aluminum foil and placed in the end-over-end tube roller at 4˚C for 30 minutes, then centrifuged at 2500 RCF (g) in a Beckman Microfuge R at 4˚C for 10 minutes. After supernatant removal, the cells were resuspended in 130 µL sterile 1X DPBS, wrapped in aluminum foil, and placed on ice until use.

**MicroRheo attachment assay - glass**

A glass coverslip was prepared by washing in this sequence: ethanol, deionized H2O, ethanol, deionized H2O and dried using a Kimwipe. The clean coverslip was then placed in the MicroRheo cup and the rheometer was zeroed with a PP25 tool. After zeroing, the tool was raised to 100 mm. Forty-nine µL of cells was then placed on the coverslip and the tool was lowered to 100 µm. The cell sample was incubated for 10 minutes (with the water circulator running at 37˚C) to allow for complete cell attachment. After the cell attachment phase, the rheometer was set to take measurements every second (rotation template; fixed point measurement duration of 1 second) for 120 seconds in a step-wise linear ramp from 100 s⁻¹ to 8000 s⁻¹.
**MicroRheo attachment assay - polyacrylamide**

The hydrophilic coverslips were placed in the MicroRheo cup and then solutions of the respective polyacrylamide mixtures were pipetted onto the coverslip. The PP25 tool was then lowered to a height of 50 µm after zeroing for the 30 minute polymerization process. 49 µL of cells were then placed on the coverslips and the tool was lowered to 150 µm for experimentation. The cell samples were incubated for 10 minutes (with the water circulator running at 37˚ C) to allow for complete cell attachment. After the cell attachment phase, the rheometer was set to take measurements every second (rotation template; fixed point measurement duration of 1 second) for 120 seconds in a step-wise linear ramp from 100 s⁻¹ to 8000 s⁻¹.

**Imaging**

Images were captured with a 50 ms exposure time and 950 ms delay between images, under fluorescence, every second from ~10 seconds before the rheometer started running until it finished.

**Cell counting**

Potential parasites were first found via a raw count script adapted from our in-house PTV software. During this step, the images were subjected to band-pass filtering for isolation of bright areas corresponding to parasite size. Then centroid location, brightness, and eccentricity were recorded for each potential cell and for each image. We then took a computational geometry-based approach (Supplemental Figure 3.1) to filter out the false positives generated by general noise and fluorescent streaks. During the analyses we found that parasites would often enter and
exit the frame due to sliding. To remedy this, we recorded the position and brightness of cells in a user-defined reference image (generally the first image before the start of fluid flow) and then would compare the position of that cell to the reference frame throughout the attachment assay. Because cells sometimes come into and out of focus temporarily, we allowed for a two frame grace period where cells could not show up for two frames and then reappear the following frame. Circles with radii of 2x cell radii were drawn around each reference cell and the reference cells were allowed to move within this circle while still being counted as attached. If the cells disappeared for more than two frames or moved outside of the allowable circle then they were considered detached and were not counted from that point forward.
Chapter 4: Investigating the hydrodynamic model at individual parasite resolutions

4.1 Introduction

We examined the ventral flagella oscillatory frequencies of attached *Giardia* trophozoites in order to investigate the source of attachment force variation seen in our previous experiments (Chapter 3) and in the literature\textsuperscript{45,46,49}. What has not been documented in the literature, however, is there exists large variation in ventral flagella frequency of attached trophozoites. Instead, the ventral flagella frequencies of attaches trophozoites has been reported in a range of 8-11 Hz (average of 10 Hz)\textsuperscript{63} for *Giardia lamblia* and 18 Hz in *Giardia muris*\textsuperscript{43,60}. In Chapter 3, we showed that there exists continual directed fluid flow at the ventral surface of attached *Giardia* trophozoites and we expect that this flow is generated by the beating of *Giardia*’s ventral flagella—an expectation that is supported by the computational model (Chapter 3, Figure 3.10) of *Giardia*’s ventral surface and the propagation of a wave with increasing amplitude bounded by the ventral groove. Due to the ventral surface geometry of attached trophozoites, our active hydrodynamic model predicts that the flagellar pump runs like a positive displacement pump and there is a linear relationship between the change in pressure (and resulting attachment force) at the ventral surface and fluid flux. Thus, the wide range of ventral flagella frequencies of attached trophozoites is predicted to correspond to a wide range of ventral flagellar-generated fluid flux and attachment force.
4.2 Attached trophozoites exhibit a wide range of ventral flagella frequencies in no-flow conditions

We first measured baseline flagellar frequencies of attached trophozoites in no-flow conditions to determine if there exists sufficient variation in attached trophozoite ventral flagellar frequencies to account for differences in attachment forces. To do this, we compared trophozoite ventral oscillatory frequencies when attached to glass (control), 7% polyacrylamide substrates, 6% polyacrylamide substrates, and 5% polyacrylamide substrates. Initially we only measured ventral flagella frequencies when parasites were attached to glass (Figure 4.1), while subsequent experiments were done in pairs with separate glass controls for each set of polyacrylamide experiments: glass + 7% PA (Figure 4.2), glass + 6% PA (Figure 4.3), and glass + 5% PA (Figure 4.4).

We originally measured the ventral flagella frequencies of parasites attached to glass and, contrary to previously published literature\textsuperscript{43,60,63}, the ventral flagella of attached trophozoites vary from ~1-24 Hz (Figure 4.1) with a mean frequency of 7.5 Hz and a median frequency of 6.5 Hz (n = 508 trophozoites). Not only is there wide distribution of ventral flagella frequencies within a population, but there is also extreme variation between populations measured on different days (left column in Figures 4.2,4.3,4.4). Because of the variation both within and between populations of cells, we cannot compare ventral flagella frequencies of trophozoites attached to glass and polyacrylamide as a whole and instead compare them on a day-to-day basis.

There is also variation in ventral flagella oscillatory frequencies of trophozoites attached to 7% polyacrylamide substrates in no-flow conditions (Figure 4.2, right column). These experiments were done on five different days and ventral flagella frequencies were recorded for
parasites attached to glass coverslips (control) and 7% polyacrylamide substrates. Because of the variation within populations, we used the two-sample Kolmogorov-Smirnov test to compare the ventral flagella frequency distributions. The two-sample Kolmogorov-Smirnov test is nonparametric and tests whether two distributions differ. The null hypothesis is that both samples come from the same parent distribution. When comparing the oscillatory frequencies of the paired glass and 7% PA experiments there was no discernible difference—Day 1-5 p-values are 0.21, 0.78, 0.26, 0.83, and 0.70 (Table 4.1). The trophozoites maintain attachment and do not detach. Therefore the lack of ventral flagella frequency difference is in accordance to our theoretical model because the influx of fluid at the ventral surface due to the porosity of 7% polyacrylamide is not a significant fraction of the total flagellar-driven flux.

Similar to trophozoites attached to 7% polyacrylamide, there is a wide range of ventral flagella frequencies when attached to 6% polyacrylamide substrates (Figure 4.3, Right Column). These experiments were done on three different days and ventral flagella frequencies were recorded for parasites attached to glass coverslips (control) and 6% polyacrylamide substrates. The difference between ventral flagella frequencies of trophozoites attached to 6% polyacrylamide substrates and the paired control datasets (Table 4.1) varies from day-to-day: on Day 1 there was a significant difference between the two datasets (p = 0.009), on Day 2 there was not a significant difference (p = 0.29), and on Day 3 there was not a significant difference (p = 0.08). Although there was not a significant statistical difference over all three days, Days 1 and 3 indicate a biological difference in ventral flagella beat frequencies and it is possible that Day 2 was an outlier—something that can potentially be remedied with more experimental trials.
There is also a wide range of ventral flagella frequencies when trophozoites are attached to 5% polyacrylamide substrates (Figure 4.4, right column). Like the pairs of 6% polyacrylamide experiments, these were done on three different days and ventral flagella frequencies were recorded for parasites attached to glass coverslips (control) and 5% polyacrylamide substrates. Also like the 6% polyacrylamide datasets, the ventral flagella frequencies of trophozoites attached to 5% polyacrylamide substrates varies on a day-to-day basis. However, there was a significant difference of ventral flagella frequencies on 6% polyacrylamide when compared to paired controls for each day (Table 4.1): Day 1- p = 0.0004, Day 2- p = 1.2e-7, Day 3- p = 0.3. Although these experiments were done under no-flow conditions, I hypothesize that the organisms respond to an increase in fluid flux at the ventral surface by increasing their ventral flagella frequencies in an attempt to maintain a negative pressure differential sufficient to account for their attachment.
Figure 4.1. *Giardia* trophozoites attached to glass in no-flow conditions exhibit a wide range of ventral flagellar frequencies. Histogram of trophozoite ventral flagella frequencies under no-flow conditions while attached to glass coverslips (n = 508 trophozoites, mean = 7.5 Hz, median = 6.5 Hz). Frequency [Hz] is on the x-axis and probability density is on the y-axis.
Figure 4.2. There is no discernible difference between ventral flagella frequencies under no-flow conditions on paired glass coverslip and 7% polyacrylamide gel experiments. Left column: Histograms of trophozoite ventral flagella frequencies under no-flow conditions while attached to glass coverslips for Days 1-5. Frequency [Hz] is on the x-axis and probability density is on the y-axis. Right column: Histogram of trophozoite ventral flagella frequencies under no-flow conditions while attached to 7% polyacrylamide gels for Days 1-5. Frequency [Hz] is on the x-axis and probability density is on the y-axis. That a randomly chosen sample from the experiment will fall within that range.
Figure 4.3. There is a slight difference (depending on day) between ventral flagella frequencies under no-flow conditions on paired glass coverslip and 6% polyacrylamide gel experiments. Left column: Histograms of trophozoite ventral flagella frequencies under no-flow conditions while attached to glass coverslips for Days 1-3. Frequency [Hz] is on the x-axis and probability density is on the y-axis. Right column: Histogram of trophozoite ventral flagella frequencies under no-flow conditions while attached to 6% polyacrylamide gels for Days 1-3. Frequency [Hz] is on the x-axis and probability density is on the y-axis.
Figure 4.4. There is an increase in trophozoite ventral flagella frequencies attached to 5% polyacrylamide gel experiments under no-flow conditions when compared to trophozoites attached to glass coverslips. Left column: Histograms of trophozoite ventral flagella frequencies under no-flow conditions while attached to glass coverslips for Days 1-3. Frequency [Hz] is on the x-axis and probability density is on the y-axis. Right column: Histogram of trophozoite ventral flagella frequencies under no-flow conditions while attached to 5% polyacrylamide gels for Days 1-3. Frequency [Hz] is on the x-axis and probability density is on the y-axis.
<table>
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<th># parasites on polyacrylamide</th>
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Table 4.1. Table of Kolmogorov-Smirnov comparison results for ventral flagella frequencies of trophozoites attached to glass coverslips (control) and 5%, 6%, 7% polyacrylamide substrates under no-flow conditions.
4.3 Simulating the effect of ventral flagellar frequency change on negative pressure generation

Our active hydrodynamic model predicts that an increase of ventral flagella-driven fluid flux at the ventral surface will correspond to an increased pressure drop and attachment force. To simulate this, we made morphologically accurate two-dimensional finite element models of the pressure field under Giardia lamblia's ventral surface and modulated the ventral flagellar wave speed. The first model is our “base” simulation with a wave speed of $1e^{-5}$ m/s (Figure 4.5A). For this simulation we find an average pressure differential of 1.43 Pa which corresponds to an attachment force of $5e^{-11}$ N (Table 4.2). Next, we increased the wave speed by a factor of 1.5 (Figure 4.5B). In this case, the linear relationship between pressure and fluid flux (Figure 4.6) predicts that we should see a 50% increase in the pressure differential when compared to our base case and this is exactly what we see. An increased wave speed of $1.5e^{-5}$ m/s leads to an average pressure differential of 2.13 Pa which corresponds to an attachment force of $8e^{-11}$ N (Table 4.2). Lastly, we decreased the wave speed by a factor of 0.5 (Figure 4.5C). As predicted by the linear relationship between pressure and fluid flux, we see an average pressure differential of 0.72 Pa which corresponds to an attachment force of $2.5e^{-11}$ N (Table 4.2).

An interesting aspect of the predicted pressure differential at the ventral surface is its periodic nature (Figure 4.6). The simulations take place over one complete cycle of the ventral flagellar wave and the complete cycle is broken up into 12 phases (the snapshots shown in Figures 4.5A, 4.5B, and 4.5C correspond to the first phase—frame 0—of each simulation). By graphing pressure differential vs frame, we can see that the pressure differential peaks during the phases representing the points in time when the ventral flagella’s anterior end comes into closest proximity with the ventral groove edges. During these phases, the position of the ventral flagella
cause them to pull the maximum amount of fluid from the ventral disk’s anterior opening which results in the greatest drop in pressure. Because attachment force is a function of pressure differential, we see that the attachment force also varies with phase and peaks in concert with the generated pressure differentials (shown in Table 4.2 for each simulation).
Figure 4.5. A morphologically accurate two-dimensional finite element model of the pressure field under *Giardia lamblia*’s ventral surface changing with wave speed during one phase of the ventral flagella oscillation. [A-C] The circular chamber is the ventral disk with an anterior opening on the bottom left and the bare zone denoted by a white circle in the middle of the ventral disk. The ventral groove emanates from the posterior end of the ventral disk and contains the ventral flagella (black sinusoidal wave), which are modeled as a travelling wave with a linearly increasing amplitude. The pressure field is measured in Pascals where black indicates the minimum pressure and light grey indicates maximum pressure (atmospheric). The yellow streamlines denote fluid flow lines and the red arrows denote fluid velocity vectors. [A] The pressure field under *Giardia*’s ventral surface when the ventral flagella wave speed is $1 \times 10^{-5}$ m/s. The average pressure differential under the ventral surface is $-1.4$ Pa. [B] The pressure field under *Giardia*’s ventral surface when the ventral flagella wave speed is $1.5 \times 10^{-5}$ m/s. The average pressure differential under the ventral surface is $-2.1$ Pa. [C] The pressure field under *Giardia*’s ventral surface when the ventral flagella wave speed is $1 \times 10^{-6}$ m/s. The average pressure differential under the ventral surface is $-0.7$ Pa.
Figure 4.6. The periodicity of the pressure differential is a result of ventral flagellar phase. Pressure Differential (Pa) vs Frame of Simulation. The y-axis is the pressure differential (Pa) between outside of the cell and under the ventral disk and the x-axis is frame number. The simulation occurs over one complete cycle of the ventral flagella and is broken up into twelve points in time (frame number corresponds to each point in time, 0-11). Pressure differential was simulated for the base case (control simulation) with a wave speed of 1e-5 m/s (orange dots), 1.5x base case with a wave speed of 1.5e-5 m/s (blue dots), and 0.5x base case with a wave speed of 5e-6 m/s (grey dots). W corresponds to the ratio of ventral flagella wave height to the ventral groove edge. Normally the ventral flagella fill the groove which would result in W = 1. However, there is a boundary limit in the simulation at W = 1 so W = 0.9 was used.
Table 4.2. Table of Frame number, Force (N) and Pressure Differential (Pa) for the simulations of *Giardia lamblia*’s ventral surface with wave speeds (left to right) of 1e-5 m/s, 1.5e-5 m/s, and 5e-6 m/s. Max P, Min P, and Avg P correspond to the maximum, minimum, and average pressure differentials found over one complete wave cycle.
4.4 Methods

Cell culture

*Giardia lamblia* trophozoites were maintained anaerobically in borosilicate glass tubes or polystyrene flasks. Parasites were grown at 37°C in modified TYI-S-33 media (Keister, 1983), where 0.024 M sodium bicarbonate replaced the traditional phosphate buffer solution. For all experiments, *Giardia* cultures were grown to mid-log phase (~ 80% confluency) and then were placed on ice for 20 minutes to allow cellular detachment.

Preparation of glass coverslips for polyacrylamide gel substrates

Hydrophobic top slides were prepared using plain glass microscope slides. After cleaning off the surface with mild detergent, slides were thoroughly rinsed with dH2O and then dried using Kimwipes. 500 µl of Sigmacote (Sigma-Aldrich) was then allowed to sit on the clean surface for 2 minutes. Upon removal of the solution, coverslips were rinsed with dH2O to remove any excess siliconized articles and allowed to air dry before use.

Hydrophilic bottom coverslips (upon which the gels were polymerized) were prepared using 25 mm x 25 mm square glass coverslips. The coverslips were passed over the inner blue flame of a Bunsen burner (with the side to be activated facing down) to remove any particles and ready the coverslips for activation. A small amount of 0.1 N NaOH was pipetted on the activated sides and spread evenly using a clean coverslip (to leave little NaOH on the surface) and were allowed to air dry for approximately 20 minutes. 50 µL of Aminopropyl-trimethoxysilane (APTMS, Aldrich) was pipetted on the center of the coverslips and spread evenly over their surfaces using the pipette tip. After waiting 5 minutes to react, the coverslips were placed in
dH$_2$O for approximately 10 minutes to allow complete removal of the excess APTMS, otherwise it will adversely react with the subsequent glutaraldehyde activation leaving an orange residue. Following the dH$_2$O wash, coverslips were allowed to air dry and then placed in a fume hood. The coverslips were completely covered with 0.5% glutaraldehyde that we prepared from a stock solution and allowed to sit for 30 minutes. Excess glutaraldehyde was removed, and the coverslips were rinsed in dH$_2$O for 10 minutes before allowing to air-dry.

**Preparation of polyacrylamide gel substrates**

Polyacrylamide gels were prepared by diluting the water-based stock solutions of 40% Acrylamide (Sigma), 2% Methylenebisacrylamide (Sigma), and 1X Dulbecco’s phosphate-buffered saline. The amount of Acrylamide varied by volume (5%, 6%, and 7%) depending on experiment and Methylenebisacrylamide was held to 6% by volume to achieve a 6% cross-link density. For polymerization, 10 µL of 10% ammonium persulfate (Sigma) and 3 µL TEMED (Sigma) solution was added to the Acryl/Bis, mixed well, and 32 µL of the total solution was pipetted onto the activated coverslips in order to achieve a gel thickness of 50 µm. The siliconized top slides were placed on top, and the coverslips were then inverted so the slide’s weight would not negatively affect gel preparation. Gels were allowed to polymerize for 30 minutes and the hydrophilic coverslips (and attached gel) were then removed with tweezers. 1X DPBS was placed on top of the gels for hydration while awaiting experiment.
**Cellular imaging**

Cells were centrifuged at 2480 RPM in a Beckman GPR knee-high centrifuge for 10 minutes. The supernatant was removed and the cell pellet was resuspended in 200 µL sterile 1X DPBS in a sterile flat-top 2.0 mL plastic microcentrifuge tube. 40 µL of cells were then pipetted onto clean glass microscope slide and the polyacrylamide gel-containing coverslip was placed on top. The sample was then inverted, placed on a Nikon Eclipse TE2000-U spinning disk confocal microscope, and brought to 37º C to allow for attachment. Video was captured at 60x DIC using by a Andor iXon DV887 camera with a temporal resolution of 11 ms using SlideBook (Intelligent Imaging Innovations) microscope imaging software. To ensure cellular fitness, imaging occurred for a period of 15 minutes or less. Data sets were analyzed using the free software package ImageJ (NIH) by manually recording the recurrence of peak-to-peak amplitudes of the ventral flagella.

**Statistical tests and histogram compilation**

Histograms of the ventral flagella frequencies were created using the hist() function included in the R-statistical programming package and Kolmogorov-Smirnov tests were conducted using the ks.test() function in R. Probability density function was overlaid onto all histograms and when integrated over a particular range will give the probability that a randomly chosen sample from the experiment will fall within that range.
Chapter 5: Miscellaneous experiments that led to the hydrodynamic model

5.1 Introduction

Throughout my graduate career there have been many experiments that were preliminary studies, proof-of-concept experiments, or ultimately did not yield usable results. Nevertheless, these experiments guided my future work and will serve as the foundation for experiments done in the future. In this chapter I will discuss the three experiments that I consider the most illuminating when it comes to understanding our hydrodynamic model and the reasons we chose to conduct them—even if two were ultimately deemed a “failure.”

The first experiment I discuss can be considered foundational due its necessity for measuring the fluid flow at Giardia's ventral surface (Chapter 3 and here in Chapter 5). When it comes to testing fluid flow at the ventral surface (for our hydrodynamic model), the parasite must be able to beat the ventral flagella in fluid of varying viscosity with consistent waveforms. This is important for two reasons: trophozoites inhabit the small intestine where the environmental fluid does not possess the viscosity of water; and the Brownian motion of small fluid tracers in water-like fluid is substantial so decreasing that motion while not negatively affecting flagellar behavior is necessary so we can track them in our quantum dot experiments (Chapter 3).

The second and third experiments discussed here sought to address two aspects of Chapter 3: fluid flow at the ventral surface of attached trophozoites and the attachment force of attached trophozoites resulting from the hydrodynamic model. In the second experiment, we attempted to trace the intensity shifts of fluorescent dextran as it moved under the ventral surface of attached trophozoites using fluorescence correlation spectroscopy. In our hydrodynamic
model, trophozoite attachment is active in that the ventral flagella pull fluid through the anterior end of the ventral disk, move it under the ventral disk, through the ventral groove, and then out the posterior end of the parasite. Along with this fluid flow is a decrease in pressure with respect to the ambient pressure surrounding the parasite and thus the parasite is pushed down onto the attachment surface. By using a solution containing fluorescent dextran, we sought to determine the presence of fluid at the ventral surface and to measure its motion. In the third experiment, we attempted to use traction force microscopy to measure the attachment forces responsible for deformations in the attachment surface. Measuring the traction forces cells exert on their environment is not a new concept as it has been used to study everything from cell-induced traction and contractile forces in human airway smooth muscle cells\textsuperscript{64,65} to the effects of substrate elasticity on endothelial cell network formation and the effect of matrix density on capillary morphogenesis\textsuperscript{66}. By examining the traction forces exerted on flexible polyacrylamide substrates under the ventral disk, we could compare them to the attachment forces predicted by our hydrodynamic model as well as those measured in experiment\textsuperscript{1,45}.

In all, these experiments were either a fundamental part of future experiments or provided a basis and direction for experimentation going forward.
5.2 Flagella oscillatory frequency in viscous fluid

Although the theoretical basis of our hydrodynamic model applies regardless of fluid viscosity (Equations found in Chapter 2), it is necessary to test the ventral flagella mechanics of attached trophozoites in solutions with various viscosities. This is important for two reasons. The first is biological: *Giardia lamblia* experiences dynamic fluctuations in fluid viscosity while inhabiting the small intestine. The second is increasing fluid viscosity will decrease the Brownian motion of our 20nm quantum dots during our particle tracking experiments (Chapter 3). We prepared solutions using 1X PBS and various percentages of Ficoll, a hydrophilic polysaccharide, and measured their viscosities using a viscometer. Ficoll was used because we were able to get significant changed in viscosity without altering the osmolarity of the solutions; this is important because *Giardia*’s natural habitat, the small intestine, stays ~300mOsm. The viscosities ranged from roughly that of water or 1.08 cP, to 2.23 cP, 4.77 cP, 9.65 cP, and 14.66 cP. These ranges were chosen because 6% mucin solutions (the percentage of MUC2 mucin found in the small intestine) have a viscosity of ~7 cP.

To test the flagellar mechanics we captured video of attached parasites (n = >100 parasites at each viscosity) at 37ºC under 60x DIC with a temporal resolution of 10 ms. As a control, 1X PBS with a viscosity of 1.08 cP was used. Under these conditions the ventral flagella had an oscillatory frequency of 7.93 ± 3.86 Hz (Figure 5.1). After doubling the viscosity to 2.23 cP, the frequency also doubled to 14.39 ± 6.88 Hz. At 4.77 cP, flagella roughly returned to control frequencies of 7.2 ± 5.56 Hz on average. At 9.65 cP, flagellar frequency lowered to 3.17 ± 2.89 Hz. At the highest tested viscosity, 14.66 cP, flagellar frequency approached zero (data not shown).
Similar to the experiments discussed in Chapter 4, there were a wide range of ventral flagella frequencies at each viscosity tested (Figure 5.2). Because of this, doing statistical analyses on frequency means is suboptimal. We reconciled this by conducting two sample Kolmogorov-Smirnov tests to compare frequency distributions for each experimental condition (5%, 10%, and 15% Ficoll) with the frequency distribution of the 0% Ficoll control condition. When compared with the control condition, 5%, 10%, and 15% Ficoll experiments displayed significant statistical differences. For 0% vs 5% Ficoll, \( p = 1.7 \times 10^{-15} \). For 0% vs 10% Ficoll, \( p = 0.002 \). And lastly, for 0% vs 15% Ficoll \( p < 2.2 \times 10^{-16} \).

At all tested viscosities except for 14.66 cP, the ventral flagella maintained a steady beat frequency, a semi-sinusoidal waveform, and were in phase. At 14.66 cP, however, the ventral flagella did not maintain a steady beat frequency (or did not beat at all), proper waveform, and frequently began beating out of phase (data not shown). It is important to note that attachment forces were not measured for any of these experiments since they were done under no-flow conditions. At viscosities less than 14.66 cP parasites were considered attached if they were stationary during the data collection period.
Figure 5.1. Attached trophozoites exhibit canonical ventral flagella oscillatory frequencies in fluid viscosities four times that of PBS. A traditional box and whiskers plot showing the oscillatory frequency of the ventral flagella in solution of varying viscosities. The vertical axis is beat frequency in Hz of attached parasites (n > 100 trophozoites) and the horizontal axis is percent Ficoll in PBS along with the corresponding dynamic viscosity expressed in cP (1 centipoise = 1 millipascal-second). Here, the whiskers show the maximum and minimum values of each data set, the yellow box is the first quartile, the blue box is the third quartile, and the division between the two boxes is the median. Percent Ficoll from right to left: 0% Ficoll in PBS, 5% Ficoll in PBS, 10% Ficoll in PBS, 15% Ficoll in PBS. Dynamic viscosity ($\mu$) from right to left: 1.08 cP, 2.23 cP, 4.77 cP, 9.65 cP as measured by viscometer.
Figure 5.2. *Giardia lamblia* trophozoites exhibit a wide range of flagellar frequencies in solutions containing 0%, 5%, 10%, and 15% Ficoll. Top Left: Histogram of ventral flagella frequency for parasites attached to glass coverslips in a 1X PBS solution containing 0% Ficoll (Dynamic viscosity of 1.08 cP). Top Right: Histogram of ventral flagella frequency for parasites attached to glass coverslips in a 1X PBS solution containing 5% Ficoll (Dynamic viscosity of 2.23 cP). Bottom Left: Histogram of ventral flagella frequency for parasites attached to glass coverslips in a 1X PBS solution containing 10% Ficoll (Dynamic viscosity of 4.77 cP). Bottom Right: Histogram of ventral flagella frequency for parasites attached to glass coverslips in a 1X PBS solution containing 15% Ficoll (Dynamic viscosity of 9.65 cP). Frequency [Hz] is on the x-axis and probability density is on the y-axis. Probability density function for each experiment is overlaid on the histogram, and when integrated over a particular range will give the probability that a randomly chosen sample from the experiment will fall within that range.
5.3 Investigating fluid flow at the ventral surface using fluorescent dextran

Before we used 20nm quantum dots for fluid tracers to investigate fluid flow at the ventral surface of attached *Giardia* trophozoites, we attempted to use fluorescent dextran and a technique known as fluorescence correlation spectroscopy (FCS). The reason for choosing a bulk fluid tracer like fluorescent dextran is because it would demonstrate what all of the fluid is doing instead of using quantum dots which tell us what the fluid around that particular particle is doing. FCS allows one to analyze the fluctuations of fluorescent particles based on their intensity and concentration. In the correlative analytical approach, one can determine the average number of fluorescent particles and their motion as fluorescent intensity fluctuates throughout the image. FCS has been used to investigate membrane protein dynamics, kinase multimerization in synaptic processes, and various other membrane composition and cellular trafficking processes.

For the purpose of our experiments we sought to trace the motion of patches of fluorescence intensity as they moved under the ventral surface of attached *Giardia* trophozoites. To accomplish this, we suspended trophozoites in a solution containing 10% Ficoll, 5% glucose, and 10 mg/mL fluorescent dextran (3000 MW, Alexa Fluor 488 conjugate, ThermoFisher) and allowed them to attach to clean glass coverslips. We then imaged the trophozoite ventral surfaces with a temporal resolution of 36 ms and attempted to measure shifts in fluorescent intensity (representative image in Figure 5.3). Although the experiments did not yield usable data for correlative analysis, they did provide qualitative evidence of continuous fluid presence under the ventral surface of attached parasites. Contrary to the passive hydrodynamic model’s predictions, these data indicate that significant fluid flow exists throughout the ventral disk and ventral
groove (Figure 5.3A) as is shown by the presence of fluorescent solution at the ventral surface. What is also interesting about these findings is the definitive lack of fluorescent signal under the bare zone. These data support our hypothesis that fluid enters the anterior end of the ventral disk, persists around the bare zone, exits the area under the ventral disk and into the ventral groove, and enters the surrounding solution at the ventral groove’s posterior end. Despite these experiments not providing quantitative data regarding fluid motion, they did provide evidence that the cavities formed by *Giardia*’s ventral surface are not closed systems.
Figure 5.3. Solution containing fluorescent dextran suggests the presence of fluid at the ventral surface of attached *Giardia lamblia* trophozoites. [A]: Fluorescent image of trophozoite ventral surfaces while attached to glass in a solution containing fluorescent dextran. The bright portions of the image are a result of the fluorescent dextran and the dark portions of the image are parts of the trophozoite ventral surface that are in contact (or are in close proximity to) the glass surface. [B]: Bright field image of the same trophozoites found in [A] taken immediately following the fluorescent image. Both images were taken using a laser scanning confocal microscope with a temporal resolution of 36 ms and a 63x objective; and are images taken out of an image sequence containing 500 images in total.
Using traction force microscopy to investigate pressure changes under the ventral disk

The interactions between cells and their environments affect everything from cell proliferation and cell death to cell movement and attachment. Traction Force Microscopy (TFM) is an important technique that gives us the ability to characterize the cellular forces exerted on attachment surfaces and better understand the roles traction and stress play on both the cell and its environment. In order to measure these forces, one must find the deformation of a flexible substrate and then infer the associated traction field from the known substrate displacement. This technique utilizes fluorescent polystyrene microspheres with diameters of ~0.2 μm that are evenly dispersed in a flexible polyacrylamide substrate. The substrate’s stiffness, or Young’s Modulus, is a function of the acrylamide to bis-acrylamide ratio used while polymerizing the gel and is essential in determining the forces necessary to deform the substrate while under stress. Increasing either the acrylamide percentage or the bis-acrylamide cross-linker percentage lead to stiffer gels and it is important to choose ratio where the gel is stiff enough to prevent tearing but soft enough to allow for measuring beat microsphere displacement.

To obtain a general understanding of how Giardia lamblia trophozoites attached to flexible polyacrylamide substrates behave and what effects these substrates have on their morphology we created polyacrylamide gels with various acrylamide to bis-acrylamide ratios and observed trophozoite attachment behavior. In order to record attached trophozoite behavior, we labeled cell membranes with Alexa Fluor 488 (Thermofisher) conjugated wheat germ agglutinin (WGA)—which binds to N-acetyl-D-glucosamine found on the cell membrane—and then took Z-stacks from the dorsal surface of attached parasites to ~6 μm below the attachment plane using a 0.2 μm vertical step-size (Figure 5.4). The first substrates we created were “soft”
substrates with stiffness of ~80-150 Pa and were comprised of 4% acrylamide and 0.04% bis-acrylamide. Upon attaching to the soft substrates we made two unique observations that, to this day, are absent from *Giardia* literature. The first observation is that attachment of trophozoite groups create fissures in the substrate and that these fissures grow in size over time as more trophozoites congregate to the area (data not shown). The second and most surprising observation is that in some cases the trophozoites take on an entirely new morphology. The canonical shape of a *Giardia* trophozoite is one of a half teardrop (discussed in Chapter 1) with a convex dorsal surface and a flat ventral surface. When attached to “soft” polyacrylamide gels, however, the inverse sometimes occurs: the parasites exhibit a flat dorsal surface and a convex ventral surface (Figure 5.4A)—a shape analogous to an umbrella flipping inside out due to wind. What is even more interesting than the shape itself is that these trophozoites were viable (upon visual inspection) and the ventral flagella continued to beat normally. Because of the unusual behavior found on “soft” polyacrylamide gels, the next gels we created had 5% acrylamide, 0.03% bis-acrylamide, and over twice the stiffness (~310 Pa). When attached to these “stiff” gels the parasites exhibited both normal behavior and their standard teardrop morphology (Figure 5.4B).

The next step in our experimentation was to measure the forces attached trophozoites exerted on the polyacrylamide gels and see how they compare to the forces predicted in our computational model. The concept behind this idea was to measure the traction forces exerted on the substrate by analyzing microsphere displacement at the ventral surface. Because the area under the ventral disk acts as a negative pressure conduit, we expected to see measurable microsphere displacement at the anterior end of the ventral disk where fluid is pulled through by
the ventral flagella. Ultimately these experiments were inconclusive due to noisy traction field results and very little motion that could be seen under the ventral disk (due to a z-resolution barrier). However, we were able to measure the deformation and traction fields in the ventral groove (Figure 5.5). In a representative timepoint of our experimentations, we found that the substrate deformed in an anterior-to-posterior direction between 0.1-0.15 μm under and surrounding the ventral groove (Figure 5.5C) with a maximum stress of 5.98 Pa (Figure 5.5D). These deformation and traction fields dynamically changed during experimentation but follow a similar pattern (data not shown). While we are unsure of the exact reasons for substrate deformation, it is likely a result of ventral flagellar contact with the substrate surface as well as cell body motion caused by parasite shift during attachment.
**Figure 5.4.** *Giardia lamblia* trophozoites exhibit substrate tensile stiffness-dependent morphology when attached to polyacrylamide gels. [A-B]: 3-dimensional visualizations of fluorescent (WGA labeled; green) *Giardia* trophozoites attached to polyacrylamide substrates embedded with fluorescent microspheres (red). [A]: “Inverted” trophozoite (WGA shown in green) attached to a polyacrylamide gel (microspheres shown in red) with a Young’s modulus of ~80 Pa. [B]: Trophozoite (WGA shown in green) displaying typical morphology when attached to a polyacrylamide gel (microspheres shown in red) with a Young’s modulus of ~310 Pa.
Figure 5.5. *Giardia* flagella deform polyacrylamide substrates as they beat. [A]: Composite image of a *Giardia lamblia* trophozoite attached to a polyacrylamide gel embedded with fluorescent microspheres. Image was formed by taking a fluorescent image (the white dots are the fluorescent microspheres) with the bright field channel open concurrently so the un-labeled trophozoite could be seen on top of the gel. Image was taken on a spinning disk confocal microscope using a 60x objective. Red box denotes the area under the trophozoite that was analyzed for the deformation and traction fields. [B]: Fluorescent image of displaced microspheres embedded in a polyacrylamide gel that was analyzed. [C]: Deformation field with displacement vectors at timepoint 28 (key denotes amplitude in µm). [D]: Traction field with displacement vectors at timepoint 28 (key denotes shear stress in Pa). Max shear stress = 5.98 Pa, Sum force applied = 41.3 pN, Net force applied = 31.3 pN.
5.5 Discussion

The experiments discussed in this chapter play an important role in my journey to understand *Giardia lamblia*’s attachment mechanism and provided the groundwork for discovering our hydrodynamic model. When compared to the experiments of Chapter 3 and Chapter 4, one can see the general philosophy I developed to determine how an organism attaches to a surface and then investigate different aspects of the same question: What happens at the parasite-surface interface? At the most basic level, the first experiment where I measured ventral flagella frequencies in fluid of varying viscosity investigated the cellular mechanics aspect of this question—mechanically speaking it investigates how the parasite acts. The second experiment continues this philosophy by investigating the fluid dynamics at the boundary separating the parasite and the surface it attaches to. In *Giardia*’s case it leads one to determine whether or not the parasite’s actions cause fluid flow during attachment. Lastly, the third experiment comes at the parasite-surface interface from the opposite direction by investigating how the attachment surface response. Because the parasite is physically attaching to a surface, one has to ask if the surface responds in a way that can shed light on the mechanism of interaction between it and the parasite.

Although the first experiment was ultimately used to find the optimal balance between flagellar function and increased viscosity for the purpose of tracking fluid tracers, it did not start out that way. *Giardia* attaches in a mucosal environment where the fluid viscosity is most likely much greater than water. Because of this, it is important to determine how the organism’s flagella move in fluids with increased viscosity. There have been other experiments (not discussed in this dissertation) along the way that attempted to investigate flagella motion in
mucus as well as an investigation into the rheological properties of reconstituted mucus. In nearly all cases, we have found that the ventral flagella behave “normally” in fluids with different viscosities and different properties. As a result, an active attachment mechanism such as our hydrodynamic model is plausible both in vitro and in environments similar to those found in vivo.

A central tenant of our hydrodynamic model is that directed fluid flow is actively generated at *Giardia lamblia*’s ventral surface. In the second experiment discussed in this chapter we attempted to measure intensity shifts in fluid containing fluorescent dextran in order to learn whether or not fluid existed at the ventral surface and to determine its motion. Generally speaking, fluorescence correlation spectroscopy works best in defined volumes where the magnitude of fluorescence fluctuations differs greatly. In our experiments the final data was too noisy to generate any usable results, yet the experiments were illuminating in that they showed both the presence of fluid at the ventral surface and that the ventral surface is not made up of closed cavities. Instead, the area under the ventral disk and the ventral groove are semi-closed in nature. The experiments indicated from a qualitative standpoint that fluid does, in fact, exist throughout the ventral surface of attached trophozoites and exists in a manner that is consistent with what we would expect from an active hydrodynamic attachment mechanism.

Lastly, we attempted to determine the traction forces exerted on a flexible polyacrylamide substrate by attached trophozoites. Similar to the fluorescent dextran experiments, these studies did not yield usable data. Although these experiments showed that there was deformation where the cell body comes into contact with the surface itself, they did not offer clues as to how the parasite was attaching in the first place. However, they indicated that
we were incorrectly thinking about how to measure negative pressure generation. Although one might naturally assume that investigating surface deformation would be a good indication of this type of attachment force (and might be in other systems), in Giardia’s case a change in parasite morphology could potentially serve a similar purpose. Specifically, the bare zone is a protrusion of the cell body under the ventral disk that gets pulled down and comes into contact with the attachment surface upon attachment. A future experiment, discussed more in Chapter 6, might shed light on what sort of suction force is necessary in order to create the bare zone.
5.6 Methods

Cell culture

*Giardia lamblia* trophozoites were maintained anaerobically in borosilicate glass tubes or polystyrene flasks. Parasites were grown at 37°C in modified TYI-S-33 media (Keister, 1983), where 0.024 M sodium bicarbonate replaced the traditional phosphate buffer solution. For all experiments, *Giardia* cultures were grown to mid-log phase (~ 80% confluency) and then were placed on ice for 20 minutes to allow cellular detachment.

Preparation of viscous fluids

Ficoll (GE Healthcare Life Sciences) was added to 1X PBS at concentrations of 0% (control), 5%, 10%, 15%, and 19%. A UBBELOHDE Viscometer (Cannon Instrument Company, 0.002795 mm²/s² constant, 0.156% expanded uncertainty) was used to determine the viscosities of the various solutions. Due to temperature stability issues, 32°C was used, as it was the most stable temperature over the extended time frames necessary for viscosity measurement by hand. The viscosities were as follows: 1.08 ± 0.03 cP (centipoise; mPa•s) for 0%, 2.23 ± 0.01 cP for 5%, 4.77 ± 0.05 cP for 10%, 9.65 ± 0.08 cP for 15%, and 14.66 ± 0.40 cP for 19% Ficoll in 1x PBS.

Determination of ventral flagella oscillatory frequency

*Giardia lamblia* cultures were iced for 20 minutes to allow cellular detachment. 25 µL of cells were pipetted on pre-cleaned Superfrost Microscope Slides (Fisherbrand) and covered with 22x50 Microscope Cover Glass (Fisherbrand). Using a heated objective, slides (with cells
present) were brought to an equilibrium temperature of 32°C to allow for attachment and to ensure that fluid viscosity remained at what was previously calculated. After this 5-minute equilibrium period, attached cells were imaged in 1X PBS at 60x DIC using a Nikon Eclipse Ti-U microscope with a 1200hs camera manufactured by PCO AG. Video was captured with a temporal resolution of 10 ms. To ensure cellular fitness, imaging occurred for a period of 30 minutes or less. Data sets were analyzed using the free software package ImageJ (NIH) by manually recording the recurrence of peak-to-peak amplitudes of the ventral flagella.

*Fluorescent dextran preparation and fluorescent imaging*

*Giardia* trophozoites were resuspended in a solution that contained 1X DPBS, 10% Ficoll, 5% glucose, and 10mg/mL fluorescent dextran (3000 MW, Alexa Fluor 488 conjugate, ThermoFisher). Cells were then placed on a clean glass coverslip in a laser scanning confocal microscope (Leica SP5 confocal microscope) and allowed to attach for 10 minutes. After attachment, images were collected at the ventral surface with a 63x objective under alternating fluorescence and bright field at an exposure time of 36 ms.

*Preparation of glass surfaces for polyacrylamide gel substrates*

Siliconized top coverslips were prepared using 18 mm diameter circular coverslips. After cleaning off the surface with compressed air, 500 μl Gel Slick Solution (Cambrex Bio Science) was allowed to sit on the surface for 2 minutes. Upon removal of the solution, coverslips were rinsed with dH2O to remove any excess siliconized articles and allowed to air dry before use.
Hydrophilic bottom coverslips (upon which the gels were polymerized) were prepared using 25 mm diameter circular coverslips. The coverslips were passed over the inner blue flame of a Bunsen burner (with the side to be activated facing down) to remove any particles and ready the coverslips for activation. A small amount of 0.1 N NaOH was pipetted on the activated sides and spread evenly using a Kimwipe to leave little NaOH on the surface and were allowed to air dry for approximately 10 minutes. 50 μL of aminopropyl-trimethoxysilane (APTMS, Aldrich) was pipetted on the center of the coverslips and spread evenly over their surfaces using the pipette tip. After waiting 5 minutes to react, the coverslips were placed in dH₂O for approximately 10 minutes to allow complete removal of the excess APTMS, otherwise it will adversely react with the subsequent glutaraldehyde activation leaving an orange residue. Following the dH₂O wash, coverslips were allowed to air dry and then placed in a fume hood. The coverslips were completely covered with 0.5% glutaraldehyde (Fluka) and allowed to sit for 30 minutes. Excess glutaraldehyde was removed, and the coverslips were rinsed in dH₂O for 10 minutes before allowing to air-dry.

Preparation of polyacrylamide gel substrates

Polyacrylamide gels were prepared by diluting the water-based stock solutions of 40% acrylamide (Sigma) and 2% methylenebisacrylamide (Sigma), plus 100 μL of fluospheres carboxylate-modified microspheres with 0.2 μm diameter (Invitrogen) to dH₂O to bring the solution up to 1 mL at 5% acrylamide:0.03% bis-acrylamide. The solution was allowed to degas in a dessicator for 15 minutes to remove any unwanted air bubbles before the polymerization process was started. For polymerization, 10 μL of 10% ammonium persulfate (Sigma) and 3 μL
TEMED (Sigma) solution was added to the acrylamide/bis-acrylamide/microsphere mixture, mixed well, and 20 μL of the total solution was pipetted onto the activated coverslips. The siliconized top coverslips were placed on top, and the coverslips were then inverted so the microspheres would settle at the top of the polymerized gel. Gels were allowed to polymerize for 45 minutes and were then placed in dH₂O for 10 minutes to loosen the top coverslip from the gels. To ready the Polyacrylamide gels for microscopy, the glass bottoms of 35mm Glass Bottom Dishes (MatTek) were removed, and the dishes’ bottoms were lightly covered with vacuum grease. The gel-bearing coverslips were then placed where the original glass bottoms had been, ensuring that the Polyacrylamide gels were unharmed and in the center of the dishes’ holes. 1X PBS was placed on top of the gels for hydration and placed at 4°C for storage. Young’s modulus of the gels was measured by an Anton-Paar MCR-301 rheometer and found to be ~310 Pa on average.

Cell imaging and determination of traction forces

Images were captured at 60x fluorescence using a Nikon Eclipse TE2000-U spinning disk confocal microscope with an Andor iXon DV887 camera using SlideBook (Intelligent Imaging Innovations) microscope imaging software. 3-dimensional visualization was achieved by taking Z-stacks from the dorsal surface of attached parasites to ~6 μm below the attachment plane using a 0.2 μm vertical step-size. Files were converted into 8-bit tiff using Imaris scientific 3D/4D image processing and analysis software (Bitplane). They were then imported into MATLAB for calculation of the traction stress field. The algorithm is Fourier transform based and determines
the traction stress field of the polyacrylamide gels from the deformation field using the Boussinesq Green’s function by Sabass, et al (Sabass, et al, 2008).

Statistical analysis

Statistical analysis of ventral flagella frequencies was conducted using the Kolmogorov-Smirnov test (ks.test()) function found in the R statistical software.
Chapter 6: Summary and concluding thoughts

6.1 Summary

Attachment plays an integral part in *Giardia lamblia*'s lifecycle due to the trophozoite’s habitation of the small intestine where it must attach to the epithelial wall in order to reproduce and survive. Despite the vital role attachment plays in *Giardia* pathogenesis, the details surrounding *Giardia*’s attachment mechanism—in fact, the attachment mechanism as a whole—have remained unknown. Until now, hypotheses regarding *Giardia*’s attachment mechanism have fallen into three categories: lectin/adhesin-mediated binding, ventral disk clutching, and hydrodynamic suctioning. In Chapter 1, I discuss the positives and negatives of these hypotheses when it comes to explaining the robust nature of *Giardia* attachment. Trophozoites can attach in many conditions and to a variety of substrates. When combined with the complexity of *Giardia*’s *in vivo* environment, the previous hypotheses for attachment are found wanting.

The research and data presented in this dissertation support a new, refined model of *Giardia lamblia* attachment—the hydrodynamic model. We hypothesize that ventral flagella-driven fluid flow at the ventral surface of *Giardia lamblia* generates a negative pressure differential (relative to ambient pressure) sufficient to account for the force of attachment. While a hydrodynamic hypothesis was originally proposed by Holberton44, it has since been refined by our lab. There are three conditions which must be met in order for a flagella-driven attachment model—and consequently our hypothesis—to hold: i) The ventral flagella create directional fluid flow at the ventral surface; ii) The fluid flow is capable of generating a negative pressure differential sufficient to account for the force of attachment; iii) The negative pressure
differential—and therefore partial attachment—can be actively generated on a porous surface despite fluid influx at the ventral surface.

For the first time, we show that there is, in fact, directed fluid flow at the ventral surface of attached *Giardia* trophozoites (Chapter 3) and that the ventral disk does not create a closed, sealed system as is previously thought\(^ {45} \). Through morphologically accurate computational modelling of the ventral surface, we provide evidence this directed fluid flow is generated by the continuous beating of the ventral flagella (Chapter 3). Using our computational model and a unique flow-based attachment assay that we created, we have shown that this directed fluid flow can generate a negative pressure differential (with respect to ambient pressure) sufficient to account for the parasite’s force of attachment (Chapter 3). Similarly, we show that partial attachment can be actively generated on porous surfaces despite additional fluid influx at the ventral surface during attachment (Chapter 3).

Additionally, the research presented in this dissertation indicates that the hydrodynamic model not only describes bulk parasite behavior (attachment at the population level) but also accounts for the ventral flagella behavior of individual parasites during attachment (Chapter 4). In these experiments, we show that parasites exhibit different ventral flagella beat distributions depending on what substrate they are attached to in no-flow conditions (porous vs nonporous and on surfaces with varying porosity). Also, for the first time, the data presented in Chapter 4 shows that there is a wide range of ventral flagella beat frequencies of attached trophozoites and this range follows a similar distribution to that found in trophozoite attachment force. Lastly, Chapter 4 presents the ongoing experiments where I investigate ventral flagella motion of individual parasites in flow conditions.
Lastly, the data and experimentation shown in Chapter 5 displays the general framework I followed to figure out the primary attachment mechanism of *Giardia lamblia*. We show that *Giardia* is able to maintain canonical ventral flagella beating in fluids of varying viscosity (Chapter 5) which is ultimately unsurprising considering *Giardia's in vivo* attachment environment. Also discussed in Chapter 5 are ways in which I attempted to investigate *Giardia's* attachment mechanism that did not produce usable data but guided the experiments shown in Chapter 3.

In total, the experiments and data presented in this dissertation provide evidence that *Giardia lamblia* utilizes an active hydrodynamic attachment mechanism. In essence, attached trophozoites are micron-sized fluid pumps. The ventral surface geometry (in combination with the ventral flagella) serves as a positive displacement pump that generates a drop in pressure along the anterior opening of the ventral disk, maintained throughout the area under the ventral disk, and inside the ventral groove which results in attachment via actively generated suction force.

6.2 Future directions

Although the research presented in this dissertation provides a thorough foundation for *Giardia lamblia's* attachment mechanism, there are aspects of *Giardia* attachment that need to be addressed and experiments that should be done before our understanding is complete. While I am confident that the hydrodynamic model proposed in the previous chapters serves as the basal mechanism for trophozoite attachment, *Giardia* attaches to host cells in a very complex fashion as dictated by the complexities of its environment. Due to the *in vitro* nature of my research, it is
imperative that future work build upon our system, increase complexity, and investigate the following aspects of *Giardia* attachment: directly assess pressure generation at the ventral surface; attachment in viscoelastic fluids; attachment to host cells; and detachment.

Directly assessing suction force exerted on the attachment substrate by attached trophozoites is the first step researchers should take going forward. In Chapter 5 I discussed our attempts at using traction force microscopy to measure substrate deformation at the attachment surface. Even though those experiments ultimately failed, they inform us that this question should be examined from a different perspective—we should look at *Giardia*’s morphological changes. A defining factor of trophozoite attachment is the formation of the bare zone which is a protrusion of the cell body emanating from the center of the ventral disk’s spiral array of microtubules and culminating at the attachment surface. What makes this morphological feature so fascinating is that it only presents itself upon attachment. We hypothesize that the pressure gradient at *Giardia*’s ventral surface caused by the hydrodynamic model pulls the cell body downwards towards the attachment substrate. Because of this, bare zone formation may be used as a proxy for suction force and have already begun the requisite preliminary experimentation. Using a cell membrane stain (CellMask, Invitrogen) and confocal microscopy, we can measure the bare zone’s volume in attached cells. Along with knowing membrane elasticity, the bare zone volume will allow us to determine the pressure change necessary to pull that volume of cell mass down into the ventral disk cavity. These experiments should give us a direct way of measuring the pressure changes that result from the hydrodynamic model.

In the future, the next set of experiments should build upon the complex nature of *Giardia* attachment by making increasing the experimental system’s similarity to the parasite’s
in vivo environment. I envision increasing similarity in two ways: investigating attachment in viscoelastic fluids and to intestinal epithelial cells. As discussed in Chapter 1, *Giardia lamblia* colonization occurs in a mucosal environment and the trophozoites attach in mucus—a viscoelastic fluid. Unlike PBS or growth media (the two media in which I conducted attachment assays), mucus has both viscous and elastic properties. While we expect the hydrodynamic model to apply in viscoelastic fluids and explain *Giardia* attachment, testing the model’s validity in more in vivo-like fluid is necessary to gain a greater understanding of its attachment mechanism. The second way researchers can increase complexity is by investigating attachment to intestinal epithelial cells. Specifically, attachment tests involving LS174T cells (human colonic epithelial cells) would serve this purpose. LS174T cells have a few advantages as a host cell substrate: they are mucus producing and form microvilli upon monolayer formation. For these experiments one could grow a monolayer of LS174T cells in a flow-cell, allow trophozoites to attach to the monolayer, and then measure attachment force by flowing media or LS174T-generated mucus over the attached trophozoites. LS174T cells also present the ability for researchers to determine the fluid flow field at *Giardia*’s ventral surface when parasites are attached to cells containing microvilli. Like the fluid flow experiments done in Chapter 2, one could use quantum dots as fluid tracers and use particle tracking velocimetry to discern a greater understanding of how fluid behaves at the parasite’s ventral surface.

Lastly, a complete understanding of *Giardia lamblia* attachment cannot be obtained without studying trophozoite detachment. Until this point in time, my experiments have attempted to answer the question, “How does *Giardia lamblia* attach?” What these experiments do not acknowledge, however, is that *Giardia* attachment is a process involving both attachment
to a surface and detachment from that surface. Indeed, one of the defining features of *Giardia* attachment is that the parasite can rapidly attach and detach from various surfaces in no-flow and flow conditions. It will be interesting to see how the fluid flow field differs during parasite detachment as a result of morphological or flagellar-based changes. While we have not ignored the importance of understanding detachment, the experiments necessary to understand detachment are largely limited by technology. The temporally rapid attachment and detachment of *Giardia* combined with the parasite’s micron scale make detachment-focused experiments a challenge. Hopefully future researchers will have the knowledge and technology present to investigate the minute changes that occur to attached trophozoites right before they detach and reposition.

6.3 Impact and concluding thoughts

The research findings presented in this dissertation are important not only because they provide a further understanding of *Giardia lamblia*’s attachment mechanism but also because they result in a paradigm shift surrounding cellular attachment in general. The attachment mechanisms of extracellular parasites are often defined by the specific nature of their pathogenesis. In the case of hookworms, the organisms physically latch onto host cells using structural features analogous to teeth. In the case of *Entamoeba histolytica* and *Trichomonas vaginalis*, the organisms chemically attach via lectin- or adhesin-mediated binding. My research has provided evidence for a third category of cellular attachment—a hydrodynamic mechanism of attachment—that is neither based on physical latching or chemical binding. There is no reason to suspect that only *Giardia* utilizes this form of hydrodynamic attachment. It has been shown
that *Vorticella* creates suction forces during feeding\textsuperscript{71,72} which is particularly interesting because the suction is cilia-mediated. Thus, I propose that organisms containing ventral disk-like structures or ventral channels and flagella/cilia located inside or around the ventral surface possess all of the components necessary for a hydrodynamic attachment mechanism—they have a fluid reservoir (ventral disk) and a fluid pump (flagella/cilia) that actively create suction and pull themselves onto the underlying cells.
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