Characterization of Vibrio cholerae Motility Using High-Throughput 3D Tracking

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Characterization of \textit{Vibrio cholerae} Motility Using High-Throughput 3D Tracking

A thesis presented by

Anisha Mittal

to

the Faculty of the

Harvard John A. Paulson School of Engineering and Applied Sciences

in partial fulfillment of the requirements for

the Bachelor of Arts degree with honors in

Biomedical Engineering

Adviser: Katja Taute

Harvard University

Cambridge, MA

April 3, 2020
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In submitting this thesis to the Harvard John A. Paulson School of Engineering and Applied Sciences in partial fulfillment of the requirements for the degree with honors of Bachelor of Arts, I affirm my awareness of the standards of the Harvard College Honor Code.

Name: Anisha Mittal

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Acknowledgements

Writing this thesis in the midst of the recent COVID-19 pandemic has shown me just how little we know about the spread of infectious diseases and how important it is to study the mechanisms behind pandemic diseases (like cholera). This thesis is dedicated to furthering the study of an infectious disease that has been around for centuries, but still disproportionately affects low-resource communities.

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Abstract

Motility is known to contribute to pathogenicity in many bacterial species, but the underlying mechanisms are mostly unknown. We strive to characterize motility behavior in *Vibrio cholerae*, the causative agent of cholera, and identify adaptations relevant to pathogenicity. Here we present a quantitative characterization of *V. cholerae* motility in liquids using a recent high-throughput 3D tracking method, enabling a more comprehensive analysis of motility behavior. We typically acquired more than 5,000 individual 3D trajectories in 10 minutes. After an experimental protocol was developed that yielded a motile fraction greater than 90% and population-averaged swimming speeds greater than 90 μm/s and analysis program optimized with a false positive and negative detection rate of only 3-4%, we demonstrated for the first time that *V. cholerae* exhibits run-reverse-flick motility in liquids, and quantified population distributions of turning angles, swimming speed, and run durations. We also characterized another novel behavioral feature: deceleration events during runs. Our findings provide a baseline for studies of *V. cholerae* motility in complex environments more closely mimicking the host as well as chemotaxis assays. An understanding of *V. cholerae* navigation strategies could provide insight into the early stages of infection.
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I. Introduction

Bacteria play an important role in a myriad of biological and chemical processes, influencing the environment, agriculture, and human health. Microbial motility is a dynamic feature that underlies many of these processes. The specific method of motility can influence how bacteria navigate and interact with complex physical and chemical environments. For many pathogenic bacteria, the ability to adapt motility to specific environmental conditions and chemical stimuli influences their virulence. The Taute Lab at the Rowland Institute strives to characterize motility behavior for a variety of relevant bacteria to better understand this dynamic microbial feature and gain insight into the underlying mechanisms that drive different species’ specific motility patterns.

For the past year, I have been working with the Taute Lab to characterize the motility behavior of *Vibrio cholerae*, the causative agent of cholera, both in liquid environments as well as in complex ones more closely mimicking the human host. *V. cholerae*’s motility is a property crucial to successful cholera infection and disease spread, however, its exact motility strategy has never before been characterized. *V. cholerae* swims with a single polar flagellum and many other species with this flagellar architecture have been shown to exhibit a specific motility called “run-reverse-flick.” Standard 2D microscopy methods have so far been unable to determine whether *V. cholerae* also displays this motility pattern. 3D bacterial tracking, however, yields long trajectories with multiple turning events, enabling a more comprehensive and accurate analysis of motility behavior.

This thesis applies a novel high-throughput 3D tracking method recently developed by the Taute Lab to understanding and quantifying *V. cholerae* motility in liquids. First we will
investigate growth and motility conditions to optimize *V. cholerae* motility for further statistical analysis. We will then modify various steps in the high throughput 3D tracking method to ensure adequate application to studying *V. cholerae* motility. We will then use this tailored technique to demonstrate for the first time that *V. cholerae* exhibits run-reverse-flick motility in liquids, and to quantify population distributions of the associated motility parameters. The application of this method also reveals a novel behavioral feature of *V. cholerae* motility: deceleration events during runs. Preliminary studies of more clinically relevant behavior, such as *V. cholerae* chemotaxis and motility in more complex media are also presented. This thesis thus provides a foundation for understanding *V. cholerae* motility and its role in environmental adaptations as well as impacts on human health.
II. Background and Literature Review

In this section, I detail the motivations behind studying motility of *V. cholerae* and what is already known about this bacterium. Additionally, I describe the techniques typically used for studying motility and how the high-throughput 3D tracking method used in this thesis improves upon the limitations of previous bacterial tracking methods.

**Bacterial Motility Patterns**

Motility is a quality crucial to a diverse set of microbial functions such as responses to environmental stimuli, interactions with other microorganisms, and even pathogenicity. Bacteria have developed a variety of active mechanisms to navigate through different media, including swarming across a surface, twitching using extending pili, or gliding involving focal-adhesion complexes [1]. Swimming, the type of motion explored in this thesis, is movement of individual bacteria in liquids and is typically mediated by a flagellar system. This system has three major components [2]. First is the flagellum, a rotating semi-rigid helical proteinaceous filament extending from the cell’s surface. The second, the basal body, anchors the flagellum to the cell envelope and is engaged in rotation of the filament by the use of a complex rotational nano-motor. The third major component, referred to as the hook, serves as a universal joint that connects the filament to the basal body. Flagellar motors mentioned in this thesis work bi-directionally allowing alternation between clockwise and counterclockwise rotation. The flagellar motors are driven by ion gradients across the cell membrane which cause the flagella to rotate. Thus far, two coupling ions, H+ and Na+, have been described as energy sources. Often a specific bacteria’s motility strategy is determined by its flagellar architecture.
*Escherichia coli*, for example, has four to eight flagella emerging from its cell body and displays what is known as a “run-tumble” motility pattern. This pattern has been detailed in *E. coli* since the 1970’s and arises from two alternating flagellar motor states [3]. When all the motors spin counter-clockwise (as seen from behind), the flagella forms a bundle supporting linear swimming segments or “runs” at speeds of ~10-30 µm/s. When one or more of the motors spin clockwise, the bundle is destabilized, causing a cell reorientation, called a “tumble” (Figure 1 a). *E. coli* always maintains its flagella in a “pushing” state, where the bacterium swims forwards with the flagella pushing the cell body through the fluid from the rear. This alternating running and tumbling behavior is common among other peritrichous bacteria (those with multiple flagella over the cell body), including *Salmonella enterica* and *Bacillus subtilis* [4]. Bacterial motility patterns for other species with different flagellar architectures are only starting to be explored and understood [5], [6]. The “run-reverse-flick” motility pattern that will be of interest in this thesis was only discovered less than 10 years ago, although the polarly flagellated species that perform it, including the majority of marine bacteria, have been studied for decades [7].

Many marine bacteria are monotrichous species, meaning they have a single polar flagellum on the cell body and thus cannot perform the same motility mechanism as *E. coli*. Unlike *E. coli*, these bacteria can switch between forwards and backwards motion by reversing the direction of rotation of their single flagellar motor. When the flagellum is pushing the cell head, the bacteria swims forwards, and when the flagellum is pulling the cell head, the bacteria can swim backwards, essentially reversing along the same path. Observations, however, of the marine bacterium *Vibrio alginolyticus* changing swimming direction away from this “back-and-forth” path without multiple flagella led to the discovery of a new motility strategy – the run-reverse-flick [7]. This motility pattern is a three-step cyclic motion (Figure 1 b-c). Motor reversal after a
forwards run results in a $180^\circ$ reorientation (reversal). Motor reversal after a backwards run can be followed by a flick of the flagellum which results in a reorientation of swimming direction with a smaller angle (on average $90^\circ$) and wider distribution.

The flicking behavior is mechanically driven by a buckling instability of the hook, or the connection between the base of the flagellum filament and its intracellular motor [4]. The drag force on the cell head and the thrust from the flagellar propulsion compress the hook causing it to

Figure 1. Different bacterial species with different flagellations display different motility patterns in liquid. Panel a, b courtesy of K. M. Taute & M. Grognot. (a) Peritrichous *E. coli* displays “run-tumble” motility driven by two distinct flagellar motor states: counter-clockwise rotation causing (forward, black) runs and clockwise rotation causing tumbles. (b) Monotrichous *V. alginolyticus* “run-reverse-flick” motility. Motor reversal after a forward run (black) induces a “$180^\circ$” reorientation. Motor reversal after a backward run (red) is followed by a flick (circle), a reorientation event of wider distribution and smaller angle. (c) Diagram of a “flick” adapted from Ref. 4. Flicking events are caused by a buckling instability of the hook after a backwards run, reorienting the bacterium in a forward direction.
“buckle” and the flagella to deform, reorienting the bacteria in a different direction (Figure 1c). The two distinct turning events (reversals and flicks) are alternating most of the time, but the buckling leading to a flick can fail to happen, therefore a bacterium can display multiple reverses in a row, but not two flicks in succession. The run-reverse-flick motility behavior along with a higher average swimming speed seems well-suited to marine bacteria: *V. alginolyticus* has been demonstrated to climb a chemoattractant gradient faster than *E. coli* and to accumulate more tightly around its source, in what is probably an adaptation to the marine environment where encounters with food are rare and transient (as opposed to the enteric environment of *E. coli*) [7], [8]. Much is still unknown about the motility mechanisms of various bacteria with similar flagellar architectures and the motility strategies of many clinically relevant pathogens, such as *Vibrio cholerae*, have yet to be characterized.

**Vibrio cholerae**

*Vibrio cholerae* is the causative agent of cholera, a severe diarrheal disease still endemic in many countries with an estimated 2.9 million cases annually and 95,000 deaths per year between 2008 and 2012 [9]. To this date, there have been seven cholera pandemics and while transmission of cholera is rare in high-income countries, the lack of access to safe water and sanitation facilities in low-income countries allows cholera to still afflict millions of people. *V. cholerae* is a gram-negative bacterium with a single polar flagellum that often resides in marine environments, such as rivers, estuaries, and oceans. This marine bacterium is often ingested and the disease transmitted through contaminated water sources. *V. cholerae* can be found and ingested as individual free living cells, microcolonies, or biofilms [10].
Once ingested, the bacteria colonize the small intestine in human hosts. Motility has been shown to be a crucial feature for pathogenicity, putatively in allowing the bacteria to navigate towards the epithelium and penetrate the mucus layer covering the intestinal epithelium [10]. Non-motile mutants of *V. cholerae* strains were shown to have a marked reduction in virulence compared to motile strains, suggesting that motility may increase the association of the bacteria with intestinal mucosa, described as a single loose and unattached 150 µm layer [11], [12]. After reaching the intestinal epithelium layer, it is hypothesized that a variety of nonspecific adhesins including the flagellum allow the bacteria to determine whether it has reached the appropriate infection niche through reversible attachment [10], [13]. After attachment to the intestinal epithelium, the bacteria multiply and form microcolonies mediated by toxin-coregulated pilus (TCP) [10]. TCP enables the virulence cascade including secretion of cholera toxin (CT), which triggers the full progression of the disease symptoms of “rice water” stool containing extremely
motile bacteria. This stool contaminates the environment and contributes to disease spread during epidemics.

There are multiple strains and mutants of *V. cholerae* that are studied, each isolated from the different pandemics. The two main biotypes studied are the “classical”, mainly dominant before the 1960’s, and “El Tor”, which is the cause of the 1961 pandemic [14]. This thesis utilizes the classical *V. cholerae* strain O395 wildtype (wt) and toxin deleted (ΔctxAB), generously provided by Edward T. Ryan at MGH, which has also been extensively used for molecular analysis of virulence factors [15].

Some preliminary studies on surface motility of *V. cholerae* and its flagellar motor have been conducted [16], [17] as well as a set of studies in the 90’s on motility in liquid or agar [17], [18]. These studies examined *V. cholerae* motility using growth conditions in LB with various concentrations of NaCl salts, more rarely in VPG. Once the bacteria are grown to a desired concentration, they can be diluted in a so-called motility medium whose role is to drastically slow growth while maintaining environmental conditions that support motility. Several teams used TMN, a media often used for marine bacteria, as a motility medium also with varying salt concentrations. Overall, as it was shown that the flagellar motor of *V. cholerae* is driven by Na+, Na+ is often used at or above 100 mM in growth and motility media [17]. These literature protocols yielded high non-motile fractions and were either not focused on swimming motility, or measuring only average speeds on a small number of (short) trajectories. In order to study *V. cholerae*’s motility, this thesis optimizes growth conditions for motility starting with these previously defined protocols, aiming at increasing the obtained swimming speeds and reducing the non-motile fraction of bacteria. While it has been hypothesized that *V. cholerae* should exhibit run-reverse-
flick motility [19], the exact motility strategy of the bacteria has never before been characterized comprehensively, in part due to limitations in visualization and tracking methods.

**Previous Tracking Methods**

Studies of bacterial motion are often limited by the method of imaging. For bacteria, which are very small (on the order of microns), microscopes have a very shallow field of view at higher resolutions, meaning bacteria can swim out of focus in less than a second. Additionally, if placed into a thin, quasi-2D sample container, the bacteria’s motility is altered due to surface interactions. Consequently, most motility studies are performed in bulk fluid to avoid surface interactions.

Conventional light microscopy only allows for 2D imaging, where bacterial trajectories can be captured within the microscope’s limited focal plane or by projecting into 3D [7], [20]. Projections can introduce ambiguities to the trajectories, making it hard to accurately determine turning angles or the motion of bacteria perpendicular to the plane [21]. 2D slicing, or tracking within a restricted focal plane, has reduced errors in trajectory parameters but introduces bias into the run durations of trajectories captured as well as limits the number of trajectories captured as a right turn (such as a flick event) will take the bacteria out of the plane [21]. A proposed “solution” for increasing trajectory time has been the 2D tracking of cells along a surface, but this induces potential biases as surface effects can alter swimming behaviors (e.g. circling) and lead to strong experimental biases [22]–[24]. Thus, 2D methods of tracking bacteria do not provide a fully accurate or comprehensive analysis of motility behavior, despite having contributed important insights to the bacterial motility field (such as first revealing the run-reverse-flick motility).
3D tracking improves upon limitations of 2D imaging, but few techniques have mastered high performance, high-throughput, and technical simplicity. The current gold standard of 3D tracking involves automatically moving the sample to keep a bacterium in focus at the center of the field of the microscope and using the necessary displacements to determine the bacterium’s trajectory [25]. This method requires a specialized technical set-up and is restricted to characterizing an individual cell’s motility at a time, limiting its ability to efficiently characterize behavior of a whole population. Digital holographic microscopy is another method of gaining accurate 3D trajectory information by reconstructing 3D object images from a digitally magnified hologram [26]. This provides more information than traditional microscopy and can image multiple bacteria at high speed, but requires a customized technically elaborate set-up to image smaller organisms such as bacteria [27]. Off-focus imaging can also gather 3D information of multiple bacteria by using their diffraction ring intensity patterns as a measure of the distance of the object (the z position) from the imaging focal plane [28]. While off-focus imaging provides significant technical simplicity, current methods use just the diameter of the outermost ring, foregoing part of the information contained in the bacteria image. Additionally, the need for fluorescent tagging constrains spatiotemporal resolution due to photon limitations and other coupled methods such as darkfield demonstrate decreased ranges of imaging in the z-direction [21], [29], [30]. This thesis utilizes a novel high-throughput 3D tracking method developed by Taute et al. in 2015 that address the limitations of previous 2D and 3D methods as it yields accurate turning angle measurements and long trajectories with multiple turning events using a technically accessible set-up, allowing for more comprehensive characterizations of population and individual motility patterns.
III. Materials and Methods

The general approach of the high-throughput 3D tracking method utilized in this thesis and its specific workflow are presented here. This thesis presents modifications to various steps of the workflow in order to tailor the method to analyzing *V. cholerae* motility.

**High-Throughput 3D Tracking Method Principles**

This thesis utilizes a novel method that allows for bacteria tracking in 3D using a standard phase contrast microscope, developed by Taute et al. in 2015, whose lab at the Rowland Institute I currently work in [21]. This method maximizes correlation between the bacteria’s diffraction rings and a reference library, determining the bacteria’s 3D position over time, with a precision of $<0.5\ \mu m$ in x,y and $<1\ \mu m$ in z. The reference library was created by aligning and combining z stacks of 1 µm silica beads to show how diffraction patterns in phase contrast microscopy are related to an object’s z position. In order to mitigate ambiguity in the sign of the z position, spherical aberrations were introduced into the optical path, inducing asymmetry of intensity patterns about the focal plane [31]. These aberrations enable an unambiguous and re-usable library, mapping diffraction ring pattern to z position (*Figure 3* a-b).

This technique improves on the limitations in performance, technical simplicity, and high-throughput of the previous methods. By utilizing the full complex diffraction patterns produced by the bacteria at a particular z position along the optical axis, (unlike previous defocused phase contrast microscopy which utilized just the outer diffraction ring), we can increase the z-range of tracking to 200 µm. The temporal rate of the tracking is also improved, as it is only limited by the detector readout speed. With just the use of a phase contrast microscope and a camera, the method can typically acquire more than 5,000 trajectories in just 10 minutes. With such a precise,
technically simple 3D tracking method, accurate turning angle measurements can be made and trajectories significantly longer than typical run lengths can be acquired. Additionally, any assay compatible with phase contrast microscopy can be combined with this tracking method, allowing comprehensive characterization of bacteria motility behavior in simple chambers, microfluidic systems, and even complex 3D environments. Any micron-sized bacteria can be tracked and their diverse motility trajectories obtained using this broadly applicable method.

![Diagram of bacterial tracking method](image)

**Figure 3. Reconstructed 3D trajectory from correlation to reference library.** Panels a, b, c, and d reproduced from Ref. 21. (a) Vertical slice from reference library combining 73 image stacks obtained for 1 µm silica beads. (b) Horizontal slices of reference library. (c) Images of *E. coli* bacterium at corresponding marked z positions. (d) Reconstructed 3D trajectory of bacterium. (e) Example image of swimming *V. cholerae* bacteria through the phase contrast microscope during a typical experiment.

**High-Throughput 3D Tracking Method Workflow**

For each bacterial species tracked using the high-throughput 3D tracking method, a specific workflow is followed from preparing the bacteria, to data acquisition, and analysis of trajectories to fully quantify associated motility parameters. A part of my work was to define and develop the adequate parameters for *V. cholerae* in various steps of this workflow (delineated in blue).

**Bacterial Sample Preparation.** A typical bacterial sample preparation goes through three steps: striking an agar plate from glycerol stocks (bacteria stored at -80°C) and allowing colonies
to grow, picking one of them (to ensure monoclonal population) and growing it to saturation in liquid medium (overnight culture), then diluting a small volume into fresh medium and growing it (day culture) to a specific bacterial density, as measured indirectly by optical density (OD) at 600 nm. In the case of *V. cholerae*, previous work suggested certain growth conditions (page 12); my work was to test these growth conditions or propose new ones in order to obtain the optimal swimming conditions (high speeds, low non-motile fraction, stable over experimental time). The final determined growth conditions for *V. cholerae* are presented in Results. The general growth conditions were colony growth on a 1.5% agar LB plate at room temperature, followed by an overnight culture of 2 mL at 30°C, then a 10-mL day culture grown at 30°C, shaken at 200 rpm. All media contained 100 µg/mL kanamycin when working with *V. cholerae* strain O395 ΔctxAB, to maintain the toxin deletion and ensure safety. Bacteria were harvested from the day cultures in the mid-exponential phase, determined by measuring optical density at 600 nm (OD) of the bacterial sample. A range of OD’s were tested to determine the OD at which the bacteria display optimal motility. Custom sample acquisition chambers were created before the experiment by placing an 18 x 18 mm coverslip on top of a microscopy slide with three layers of parafilm as a spacer between them creating a chamber of a height of ~300 µm. The harvested bacteria were diluted to a target OD between 0.002-0.006 at 600 nm in a motility medium I experimentally determined to be optimal and injected slowly into the chamber. The ends of the camber were then sealed with hot wax and the sample immediately observed under the microscope.

**Microscopy and Data Acquisition.** The sample chamber was then placed on an inverted Nikon TE2000-U microscope with a x 40 phase contrast lens. The objective lens’s correction collar was set to 1.2 mm to introduce spherical aberrations, which introduce asymmetry about the focal plane in the point spread function to avoid localization problems near the focus. Recording
acquisitions were taken \( \sim 134 \mu m \) above the bottom surface of the chamber so as to capture bacteria in the bulk of the liquid. The illumination was adjusted to \( \sim 20,000 \) counts per pixel. We recorded movies of 1,000 to 4,000 frames given the experiment, at 30 fps to fully capture the motility of fast swimming *V. cholerae*. The data is then saved as a series of 16-bit tiff files.

The next steps allow for data preparation and then tracking of bacterial trajectories using a set of custom-written MATLAB programs routinely used in the laboratory.

**Image Down Sampling.** The obtained recordings are down sampled by a factor of two as previous works in the lab determined that the tracking resolution was not affected by this loss of data while it allowed for substantial space saving.

**Background Correction.** A median-based background correction technique was used to remove unwanted background features and to obtain the best contrast possible from the diffraction rings (median pixel value determined on a sliding window of 101 frames). This removes all stationary objects and creates a flat background.

**Image Cross-Correlation.** The bacteria position in the z direction was localized by computing the normalized image cross-correlation between the bacterial image and the reference library images. The reference image yielding the highest correlation value gives the corresponding z position of the bacteria. The \((x, y)\) position is identified as the lateral shift between the two images that maximizes the cross-correlation value. This cross-correlation is repeated for each frame in the recording to assemble bacteria trajectories (*Figure 3* c-d).

**Tracking Algorithm.** Once a bacterium is identified, it is tracked forwards and backwards in time. The last known position of the bacteria is used to constrain the search radius against the reference library in all three dimensions. The reference image with the maximum cross-correlation
value is used to extract the z position, based on the maximal displacement possible at an estimated maximum possible bacterial swimming speed (~200 µm/s for *V. cholerae*). The trajectory ends if the cross-correlation value is below a certain threshold. New bacteria entering the frame are identified if the cross-correlation value is above a certain threshold and its position does not lie within a joining distance of a known bacterium (typically 10 pixels in x and y and 30 reference library slices with a spacing of 134 nm in z). The new bacterium is then tracked backwards in time and if its position at a past frame lies within the joining distance of the end of another trajectory, the two trajectories are joined. This algorithm yields the 3D trajectories of bacteria over time in the chamber during a specific recording.

**ADMM Filtering.** After trajectories are obtained, the positions were smoothed using 2nd order ADMM-based trend-filtering [24], [32] with a regularization parameter $\lambda = 0.3$ for *V. cholerae*.

**Analysis.** The specific threshold parameters to analyze turning and run events from bacterial trajectories obtained depend on the bacteria in question. Velocity vectors are computed as forward differences in position, and direction changes are computed as the angles between subsequent velocity vectors. A turning event is determined by an angular threshold ($\alpha_c$), where a turn is identified if it is above said threshold. $\alpha_c$ is determined relative to the median angular change ($\Delta\psi_{med}$) displayed during runs of a particular trajectory: $\alpha_c = A\Delta\psi_{med}$ where $A$ is a constant determined through visual inspection of trajectories so as to minimize the number of false positive and false negative detected turns. While analysis methods have been developed in the lab for *V. alginolyticus*, this thesis required modification of the analysis method to first ensure adequacy of the method to *V. cholerae* and second, determine the optimal parameters tailored to *V. cholerae* trajectory analysis. The process of determining the specific angular threshold and
verifying the analysis method in obtaining turning events and backwards and forwards runs for *V. cholerae* is described in Results.

This thesis applies the high-throughput 3D tracking method to quantify the motility of *V. cholerae*. After modifying the workflow to tailor it to analyzing *V. cholerae*, characterization of its motility in liquids is presented for the first time.
IV. Results

Determining Growth and Motility Conditions

To explore *V. cholerae* motility behavior, and later navigation strategies, a protocol ensuring vigorous and sustained motility over time had to be developed. Previously published protocols using LB and TMN with various salt concentrations yielded high non-motile bacteria fractions (up to 70%) and decreased swimming velocities (ranging from 44-99 µm/s) [16], [17]. In developing growth and motility conditions for optimal *V. cholerae* motility, these previous conditions were initially tested and then new conditions were proposed.

*V. cholerae* bacterial colonies were first grown on 1.5% agar LB plates at room temperature. Overnight cultures of individual colonies were then grown in 2 mL LB at 30°C, shaken at 250 rpm. Day cultures were made with 50 µL of the saturated bacteria in 10 mL of different growth media at 30°C, shaken at 200 rpm (Table 1). Bacteria were grown to OD 0.3 and at time zero, the culture was diluted 50-100x in fresh growth media and injected into a sample motility optical chamber and imaged for measurement of initial motility conditions in growth media. The bacterial culture was then diluted in corresponding motility medium and at subsequent controlled increments of delay time, injected into the optical chamber and imaged for 3D tracking, to determine whether motility could be sustained over typical experimental time (up to an hour). The average bacterial swimming speed and percentage of non-motile bacteria in the population (taken to be bacteria with average swimming speeds less than 25 µm/s) over time were compared across the various growth and corresponding motility media to determine which conditions yielded the optimal environment for studying *V. cholerae* motility.
Table 1. Pairs of growth and motility media. Bacteria were diluted into these pairs of growth and motility media. LB5, LB10 and the TMN motility media of corresponding salt concentration were conditions previously tested with V. cholerae. The M9GM/M9MM were theorized to yield enhanced motility. Media compositions are provided in Appendix.

<table>
<thead>
<tr>
<th>Growth Medium (GM)</th>
<th>GM Ingredients</th>
<th>Motility Medium (MM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M9GM</td>
<td>M9 salts w/ 0.4% pyruvate</td>
<td>M9MM</td>
</tr>
<tr>
<td>LB5</td>
<td>LB w/ 5 g/L NaCl</td>
<td>TMN</td>
</tr>
<tr>
<td>LB10</td>
<td>LB w/ 10 g/L NaCl</td>
<td>TMN</td>
</tr>
</tbody>
</table>

Figure 4. Comparison of motility parameters in various media. Bacteria were grown to OD 0.3 in different growth media (LB5, LB10, or M9GM). Bacterial cultures were then diluted into respective motility media (TMN of corresponding salt concentration, or M9MM), and after a controlled delay time, injected into a sample chamber and imaged for 3D tracking. At time zero, one dilution was performed in clean growth medium, for measurement of initial conditions in growth media. We measure and compare the average bacterial speed (a) and population non-motile fraction (b) as a function of time, for the pairs of growth and motility media listed in Table 1.
As seen in Figure 4, the conventional, rich growth media such as LB5 and LB10 fail to provide conditions of high initial average velocities (at t = 0). Other media mentioned in the literature (VC, 10% LB, or VPG) were also tested but yielded so little motile fraction that we did not subsequently track bacteria. TMN, a motility medium often used for marine bacteria, does not then provide an environment to sustain the initial motility over time, with noticeable drops in average swimming speed to \(~40 \mu m/s\). The poorer medium M9GM yields initial motility of \(~80 \mu m/s\), and its corresponding M9 motility media allows vigorous and sustained velocity greater than 95 \mu m/s for over an hour. Additionally, while LB5 and LB10 show non-motile fractions of 20-60\%, the M9 media display non-motile fractions of only 5-10\%. Based on these comparisons, it was determined that M9GM and M9MM yield the most optimal growth and motility conditions for future V. cholerae experiments.

After determining the growth and motility media, we then had to define a specific OD at which to harvest bacteria that would lead to optimal motility, again determined by balancing swimming velocity and non-motile fraction. The bacteria were harvested during mid-exponential phase at various ODs and injected into motility chambers and imaged for 3D tracking. The mean velocities of the motile population and fraction of non-motile bacteria were compared. It was determined that an OD between 0.35 and 0.4 maximized the average swimming velocities while balancing a non-motile fraction between 5-10\% (Table 2).
In addition to OD, the time between dilution of the bacterial culture and injection and subsequent imaging is an important factor to consider as bacterial motility can change over time as the bacteria acclimate to the motility medium. Conducting a time-series experiment would show approximately the delay time between dilution and data acquisition at which the motility is

<table>
<thead>
<tr>
<th>OD</th>
<th>Delay Time (min)</th>
<th>Mean Motile Velocity (µm/s)</th>
<th>% Non-Motile</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>20</td>
<td>96.0</td>
<td>42.2</td>
</tr>
<tr>
<td>0.25</td>
<td>45</td>
<td>98.7</td>
<td>33.3</td>
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<tr>
<td>0.30</td>
<td>40</td>
<td>96.9</td>
<td>10.5</td>
</tr>
<tr>
<td>0.35</td>
<td>45</td>
<td>98.3</td>
<td>7.1</td>
</tr>
<tr>
<td>0.45</td>
<td>45</td>
<td>84.2</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Table 2. Comparison of motility parameters across various optical densities. Bacteria were harvested at the OD listed and then injected into imaging chambers after the corresponding delay time. Mean velocities calculated for bacteria with average swimming speeds greater than 25 µm/s. Non-motile fractions calculated by dividing the number of non-motile bacteria (speed less than 25 µm/s) by the total number of bacteria.

![Figure 5](image)

Figure 5. Comparison of motility parameters over time. Bacterial cultures were diluted into M9 motility media and injected into imaging chambers at various delay times. Average bacterial speed of the motile population (a) and the percentage of non-motile bacteria (b) are compared for different delay times.
optimized and has also stabilized. Bacteria were harvested at OD 0.35-0.4 from three different cultures (made from three independent *V. cholerae* colonies) and diluted into M9 motility media. Six distinct motility recording acquisitions were taken for each culture (1,000 frames each at 30 fps) over the course of 1.5 hours after dilution (*Figure 5*). Culture 1 (in blue) showed a decreased swimming velocity and larger non-motile fraction than the other two cultures, but this discrepancy can be attributed to drastic differences in growing time (culture 1 reached OD 0.4 much faster than the others) and a decreased density of bacteria (200 bacteria in culture 1 vs. ~2000). Variability between cultures in growing time, in particular, was observed for *V. cholerae*. The potential reasons governing this are unknown, but it is hypothesized that different overnight cultures can lead to different bacterial states depending on homogeneity of solution and growing conditions. An investigation into the underlying causes of these variations is out of the scope of the project, however, we mitigated this effect by growing multiple cultures at a time and harvesting bacteria from cultures that took longer to reach the optimal OD. Despite this, it can be seen that after ~45 min, the bacterial swimming velocities stabilize at a high speed and the non-motile fraction of bacteria also seem to start to stabilize. It is more difficult to see a consistent trend in non-motile fraction between cultures as non-motile fractions are noisy to measure because they are based on a very small number of individual bacteria that do not leave the field of view, whereas the motile fraction consists of a constant turnover of individuals in the field of view. Because there are minimal fluctuations in swimming speed after this delay time, we conclude that injections and recordings should be conducted ~45 minutes after dilution into motility medium.
The growth and motility conditions determined here were subsequently used for all _V. cholerae_ motility experiments presented in this thesis (Figure 6). These conditions allow for the exploration of _V. cholerae_ motility by ensuring optimal motility (stable high swimming speeds and high motile fractions), allowing for comprehensive analysis and quantification of motility behavior.

**Determining Analysis Program Parameters**

After trajectories are obtained for _V. cholerae_ motility over time, a custom-written MATLAB program used in the lab analyzes each trajectory to extract turning and run events. The program can output a 3D plot of each trajectory with runs and turning events identified (red represents a turn, and runs alternate between light and dark blue after each turn). The program also outputs an array of the point positions of the start and end of each run detected as well as the
associated speed information from each individual bacterium that is analyzed (determined by whether their trajectories were long and fast enough to be analyzed). This analysis program allows for further statistical characterization such as determining population distributions of motility associated parameters.

The analysis program depends on bacteria-specific thresholds on the rate of angular change that determine what is considered a turning event and what is considered a run (page 18). A turn is identified if the rate of angular change is above an angular threshold \( \alpha_c \), which is defined as

\[
\alpha_c = A \Delta \psi_{\text{med}} \quad \text{where } A \text{ is a constant and } \Delta \psi_{\text{med}} \text{ is the median angular rate of change displayed during runs of a particular trajectory. A run can be defined by a certain number of points, specified by the variable } c \text{ (if } c = 1, \text{ then 2 points below the threshold are required to begin a run). For example, for } V. \text{ alginolyticus, } A \text{ was set at 4 and } c = 2, \text{ meaning that a new run would start at time } t_i \text{ if } \Delta \psi_i, \Delta \psi_{i+1} < \alpha_c, \text{ requiring a minimum of 3 points to make a run} \ [21]. \text{ These threshold parameters are determined by visual comparison of original trajectories and their corresponding marked analysis output plot in order to balance the false positive and false negative turning events detected by the analysis program.}

This analysis step in the method workflow had to be tailored to analyze \( V. \text{ cholerae} \) trajectories. In order to determine the optimal parameters, a subset of 50 random trajectories were generated in MATLAB (with trajectory duration ranging from 3-6 seconds) from an experiment with \( ~9,000 \) trajectories. The analysis program was then run on this subset of trajectories with varying parameters for \( A \) and \( c \), and the number of various types of discrepancies between each original trajectory and its analysis identification were noted by visual inspection in addition to comments specific to each trajectory. After initial qualitative analysis of trajectories, we observed for the first time that \( V. \text{ cholerae} \) displays run-reverse-flick motility \( (\text{Figure 9 b-c}) \). Because of this
specific pattern, in addition to recording false positive and negative turns, we also had to ensure that the analysis would not attempt to mark two successive flicking events. The types of discrepancies thus recorded were false positive turns, false negative turns, and false negative runs. A false positive turn occurs when a turn is marked by the analysis program but does not exist in the trajectory (Figure 7 a). A false negative turn occurs when a turn visible in the trajectory is not marked by the analysis program (Figure 7 b). A false negative run occurs when the analysis seems to have marked two successive flicking events, but in reality presumably missed a reversal and a backwards run before the 2nd flick (as two flicks physically cannot occur in sequence) (Figure 7 c). In order to ensure the analysis did not miss backward runs, the variable c was reduced and again tested so fewer points would be required to mark a run.
Figure 7. Types of discrepancies recorded between original trajectory and analysis program output. Turns in the analysis output are marked in red and runs alternate between light and dark blue after turns. The discrepancies are delineated by open blue circles. (a) Example of false positive turn. (b) Example of false negative turn. (c) Example of false negative run – the analysis marks one turn suggesting that two flicks occur in succession, however, there is a missed reversal and backwards run before the second flick.
After the analysis was run with $A$ ranging from 5 to 7 at $c = 1$ and $c = 0$, the normalized percentages of the various discrepancies were plotted (Figure 8). At $c = 0$, the percentage of false negative runs and false negative turns are drastically reduced. $A = 6$ is the only value that balances minimal false positive and false negative turns. Thus, it was determined that $A = 6$ and $c = 0$ yield the greatest percentage of perfectly analyzed trajectories for *V. cholerae*, with a false negative turn rate of 4%, a false positive turn rate of 3%, and a false negative run rate of 3%. These analysis parameters were then used to provide comprehensive statistical characterization of *V. cholerae* motility for experiments with much larger sets of trajectories.

![Figure 8. Normalized percentages of discrepancies recorded for various A values. Discrepancies recorded by visual comparison between original trajectories and analysis program output with varying A and c parameters. Percentages calculated by dividing the number of each problem by the total number of turning events, and multiplying by 100.](image)

**Run-Reverse-Flick Characterization**

After growth conditions and analysis parameters were finalized, large-scale motility acquisitions were conducted on bacteria from three different cultures grown from three independent *V. cholerae* colonies. In total, 27,036 individual trajectories were acquired. We observe average population speeds of the motile bacteria to be ~100 µm/s (Figure 9 a).
Additionally, by qualitative analysis of trajectories, we present here for the first time that *V. cholerae* displays run-reverse-flick motility in liquids ([Figure 9] b-c). This motility pattern can be distinctly observed in these example trajectories as a cyclic, three-step motion, as described in the Background section (page 9).

![Figure 9](image)

**Figure 9.** *V. cholerae* displays a run-reverse-flick motility. (a) Normalized distribution of swimming speeds of bacteria from three independent experiments. In total, 27,036 individual trajectories were acquired. (b) Labelled analysis of *V. cholerae* trajectory, where cyclic, 3 step motion is observed. Flicks are denoted by large open circles and reversals are pointed out by arrows. The trajectory starts at the closed black circle. (c) Longer example *V. cholerae* trajectory displaying run-reverse-flick motility. Trajectories are selected as representative of a population of 100 µm/s average speed, grown by the previously defined protocol.

We then applied the analysis program to present the first statistical characterization of *V. cholerae* motility behavior. The analysis program is correctly able to identify turning and run
events for *V. cholerae* trajectories (Figure 10 a). The population distribution of turning angles present in the trajectories acquired is bimodal (Figure 10 b). This bimodal distribution is typically expected for run-reverse-flick motility patterns, as the two distinct turning events are distributed about different average turning angles (flicks display a wider distribution around a smaller angle, usually 90°, while reversals are distributed around 180°) [7]. From the population distribution of run duration, we observe that backward runs are significantly shorter in duration than forward runs (Figure 10 c).

![Image](image.png)

**Figure 10. V. cholerae displays a run-reverse-flick motility.** (a) Representative example *V. cholerae* trajectory where a run-reverse-flick motility can already be observed by eye, and its algorithmic analysis. Turning events are detected by thresholding the instantaneous angular change. We observe a cyclic, three-step motion typical of run-reverse-flick motility: forward, reverse, and flick. Motor reversal after a forward run induces a “180° reorientation. However, motor reversal after the backward run is followed by a flick of the flagellum, created by a buckling instability of the hook, resulting in a reorientation event of wider distribution and smaller angle. Our custom code accurately identifies each event. (b) Turning angle distribution, with 27,646 turning events. The bimodal distribution with peaks at ~90° (flicks) and 180° (reversals) is typical of run-reverse-flick motility. (c) Forward and backward run durations distribution, from respectively 7,224 and 4,261 algorithmically identified runs (out of 17,382 total runs). Backward runs are significantly shorter than forward runs (average 0.18 seconds versus 0.61 seconds respectively). (d) Bivariate histogram mapping the angle before each run against the angle after each run. Colors represent the frequency of observations.
We can additionally show that this motility pattern is indeed run-reverse-flick by examining the relationship between the angle before and after a run (Figure 10 d). By the relative densities, we can see that when the angle before a run is ~90° (a flick), the angle after the run is almost always ~180° (a reversal). This supports the fact that two flicks cannot occur in succession. Similarly, when the angle before the run is ~180° (a reversal), the angle after the run can either be another reverse, but is more likely to be a flick (~90°). In this way, we can see that *V. cholerae* motility displays linear segments, or runs, interrupted by alternating reversals and flicks, typical of run-reverse-flick motility. This run-reverse-flick motility is also observed in the *V. cholerae* O395 wildtype (wt) strain, as seen by visual inspection of trajectories obtained from motility acquisitions with the wt (Figure 11).

![Figure 11. Example V. cholerae wildtype trajectories. Run-reverse-flick motility can be observed even in the O395 wt strain. Trajectories are selected as representative of a population with 100 µm/s average speed.](image)

**A Novel Feature: Deceleration Events**

While determining analysis program parameters and visually inspecting *V. cholerae* trajectories, I noticed a novel feature that appeared in most trajectories – deceleration events during
runs. Bacteria usually appear to decelerate as they are turning, which can be seen when examining the instantaneous speed and angular change trace over the course of a trajectory (Figure 12 a-b). However, it was also observed that there were deceleration events during linear segments that were not accompanied by any significant angular change. Additionally, these decelerations were observed only during forward runs, however deceleration events during backward runs may easily be missed due to the backward runs’ significantly shorter duration. This feature is observed in numerous trajectories and even in the wt strain (Figure 12 c-d).

Figure 12. V. cholerae trajectories display deceleration events during runs. a) Typical bacterial trajectory and (b) its associated instantaneous speed and angular change trace. Deceleration events are denoted by black arrows. Abrupt deceleration events can be seen during the forward runs (dark blue). This feature is observed in numerous trajectories (c and Fig. 10a) and even in the O395 wt strain (d).
Preliminary Chemotaxis Studies

Chemotaxis, or the movement of bacteria along a gradient of increasing or decreasing concentration of a particular substance, enables bacteria to interact with their environment and respond to specific chemical stimuli. This unique navigation mechanism can have important implications such as promoting pathogenicity, establishing symbiotic interactions, and shaping geochemical fluxes [33]–[35]. A recently developed chemotaxis assay within our lab combines microfluidically created chemical gradients with the high-throughput 3D tracking method, allowing for characterization of bacterial chemotactic behavior on a population scale while also revealing individual chemotactic mechanisms [24]. This assay was applied to preliminary studies of *V. cholerae* chemotaxis in a gradient of 10 µM/mm of the non-metabolizable analog of the amino-acid aspartate, methyl aspartate (MeAsp), a well-known chemoattractant for *E. coli*.

The chemical gradient is created in a commercially available microfluidic device (IBIDI µ-slide Chemotaxis) with a 70 µm high, 1 mm long, and 2 mm wide central channel connecting two 65 µl reservoirs (Figure 13). One of these reservoirs is filled with a solution of bacteria with the chemoattractant and the other reservoir is filled with chemoattractant-free bacterial solution. This establishes a quasi-static, linear gradient field in about an hour, by simple diffusion of the chemoattractant. Recordings of the central channel are then conducted using the high-throughput 3D tracking method and 3D trajectories extracted. 50-100 bacteria are typically tracked simultaneously in 3D in the center channel of the microfluidic device. From the trajectories obtained, the chemotactic drift velocity can be determined as the population-averaged velocity along the direction of the gradient (x). In these preliminary studies, we performed several acquisitions in the chemotaxis chambers, but the results were difficult to reproduce between experiments. From these various acquisitions, we obtained a range of drift velocities for *V.
*cholerae* in a 10 μM/mm methyl aspartate (MeAsp) gradient (4.12 ± 0.61 μm/s, 2.50 ± 0.48 μm/s, 0.5 ± 0.56 μm/s, and 0.6 ± 0.46 μm/s) in order of increasing OD of the harvested bacteria recorded (within OD 0.35-0.4). The seeming decrease in drift velocity as OD increases led to the discovery that the drift is highly dependent on the OD of the culture. Further analysis into this behavior is being explored in order to find an OD at which we can obtain reproducible chemotaxis behavior (likely at an OD ~2.5).

**Figure 13. Schematic of the multiscale chemotaxis assay and its typical output illustrated with *E. coli*.** Figure and caption arranged from Ref. 24. (a) A quasi-static linear chemical gradient is established between two reservoirs containing a uniform concentration of bacteria. Bacteria are observed in the central portion of the linear gradient. (b) 5,045 individual trajectories containing 37,080 seconds of total trajectory time, obtained in 9 min of recording at 15 Hz in a typical experiment, allow for quantification of population-scale metrics such as the population average displacement up the gradient ($v_d$, drift velocity). (c) Two example trajectories (more than a minute long) demonstrating access to individual behavior.
V. Discussion

This thesis presents the first statistical characterization of run-reverse-flick motility of \textit{V. cholerae} in liquids in addition to quantifying population distributions of the associated motility parameters. A novel protocol for growth and motility conditions of \textit{V. cholerae} is described in Figure 6. Swimming speeds of greater than 90 $\mu$m/s and motile fractions greater than 90% are consistently sustained for over an hour when the bacteria are grown in M9GM, harvested at an OD 0.35-0.4, diluted in M9MM, and imaged ~45 minutes after dilution. These motility results greatly improve upon previously published protocols that demonstrate slower swimming velocities and lower motile fractions, although it is known that \textit{V. cholerae} can obtain speeds of up to 100 $\mu$m/s, consistent with our results [16], [17]. The M9 media was initially proposed after personal discussions at a conference (and recently mentioned in preprint [36]) indicating that a M9 growth media could yield higher swimming speeds for the \textit{V. cholerae} “El Tor” strain. The M9 media with 0.4% pyruvate may provide advantages in improving \textit{V. cholerae} motility as pyruvate is a metabolic intermediate of aerobic respiration. Since Vibrios possesses a respiration-driven Na$^+$ extrusion pump, we can hypothesize that by fueling the aerobic respiration, pyruvate allows for the maintenance of a Na$^+$ gradient across the inner membrane, which in turn fuels the flagellar motor pump, allowing motility [18]. This could be a partial explanation for the importance of M9 and pyruvate in obtaining motile cells.

With enhanced motility conditions achieved, statistical analysis was then performed on \textit{V. cholerae} trajectories obtained using the newly defined growth protocol. Modification of the analysis program was required, with final parameters of $A = 6$ and $c = 0$ being chosen. This indicates that an angle is defined if it surpasses six times the median angular change of the trajectory and a run can be defined by just one point. These parameters take into account the
observation that \( V. \text{cholerae} \) backward runs are very short and in order to be marked by the analysis, \( c \) must be reduced to zero.

This tailored analysis program allows for subsequent accurate statistical characterizations of \( V. \text{cholerae} \) motility in liquids. We demonstrate in Figure 10 that \( V. \text{cholerae} \) does indeed display run-reverse-flick motility in bulk liquid, the same pattern discovered in \( V. \text{alginolyticus} \) in 2011 [7]. Like \( V. \text{alginolyticus} \), \( V. \text{cholerae} \) is a marine bacterium with similar flagellar architecture, suggesting that it should display similar motility behavior, although its exact pattern had until now never been characterized. The higher swimming speeds and the run-reverse-flick motility pattern could allow the bacteria to more efficiently localize around nutrient sources, especially useful in environments where food is scarce, such as the ocean or rivers.

We additionally demonstrate a novel feature of \( V. \text{cholerae} \) motility in Figure 12. Trajectories seem to show abrupt and stark deceleration events during linear segments, or runs. The media the bacteria are immersed in are uniform so it is unlikely that these decelerations are in response to some local change in chemical concentration. Additionally, our initial qualitative association of these decelerations with forward runs is not a trend we were able to quantify on a population-scale, suggesting that perhaps the backward runs are so short that visual observation of these decelerations during backward runs is difficult. Future studies could perhaps include tethering experiments, which involve tethering the flagellum to a surface. This would allow visualization of motor rotation at high frame rates, potentially providing better measurements of the dynamics of the decelerations. Quantification of these decelerations could help elucidate the underlying mechanism and cause for these events.

While \( V. \text{cholerae} \) displays a run-reverse-flick motility like in other polarly flagellated species, giving insight into its abilities, the quantifications of backward and forward run durations
and decelerations show that it is also different from other species. The observation that \textit{V. cholerae} backward runs are so short compared to forward runs is a marked deviation from the run-reverse-flick motility of other marine bacteria like \textit{V. alginolyticus}, and is actually more similar to the motility behavior of \textit{Shewanella putrefaciens} [37]. This could suggest that \textit{V. cholerae} chemotaxis behavior and underlying mechanisms differ from standard run-reverse-flick chemotaxis. It is known that \textit{E. coli} extends forward runs when ascending a gradient to increase directional persistence, while many singly flagellated bacteria such as \textit{V. alginolyticus} extend \textit{both} forward and backward runs when ascending a gradient [24]. As the run duration distribution of \textit{V. cholerae} differs from other marine bacteria, it is unknown which underlying chemotactic mechanism \textit{V. cholerae} exhibits. These short backward runs and potentially different chemotaxis behavior ultimately could confer different advantages to \textit{V. cholerae} motility compared to other marine bacteria in specific environments.

Finally, we demonstrate that \textit{V. cholerae} can chemotact in a 10 \textmu M/mm MeAsp gradient. This presents preliminary studies of \textit{V. cholerae} chemotaxis behavior with a commonly used chemoattractant. However, our preliminary results were non-reproducible between cultures, suggesting that chemotactic drift velocity is related to OD and can differ drastically depending on the state of the culture. The variety in OD’s tested between 0.35-0.4 showed that somewhere along this range, there seems to be a sharp transition from a quantifiable chemotaxis response to a sudden absence of chemotactic response. This phenomenon may be linked with an ecologically relevant transition in \textit{V. cholerae}, and these preliminary results can provide a basis for further exploration of chemotaxis behavior.

The application of the high-throughput 3D tracking method towards studying \textit{V. cholerae} motility enabled comprehensive characterization of individual motility mechanisms as well as
population-based motility metrics. The high-throughput accessible with this method allows for the first ever statistical model of *V. cholerae* motility patterns and opens the door to future characterization of its motility in more complex media mimicking the host environment. A deeper understanding of *V. cholerae* motility could provide insight into the early stages of infection and adaptations to specific environmental conditions.
VI. Future Work

Bacteria have important impacts on the environment, agriculture, and human health, as well as a variety of other industries and sectors. Their motility has been shown to influence pathogenicity, yet little is known about specific motility mechanisms for many pathogens [11], [33]. Recent events emphasize how little we know about infectious disease and the importance of better understanding pandemic diseases (such as cholera). This thesis elucidates *V. cholerae* motility mechanisms in liquid, however, it is important that such a motility characterization be expanded to more complex, viscous media and 3D environments more closely mimicking the host.

*V. cholerae* specifically infects the intestinal epithelium in humans, requiring the bacteria to first penetrate a viscous mucus layer. Future studies of *V. cholerae* should thus explore its motility behavior in mucus-mimicking conditions. The simplest media would be agar, which primitively mimics the hydrogel properties of mucus. An example *V. cholerae* trajectory in 0.25% agar is presented in Figure 14, obtained using the high-throughput 3D tracking method. We can see that the bacteria can still reach high swimming speeds during certain segments, but then remains stuck or strongly decelerated for other segments. This behavior echoes previous results for *E. coli* motility in agar and porous environments [38], [39]. Further studies could help quantify this behavior for *V. cholerae* to better understand the distribution of decelerated segments and potential mechanisms behind this. In future studies, we could also test motility in viscous polymer solutions such as polyvinylpyrrolidone (PVP).
A more complex mucus-mimicking condition would be utilizing mucins directly. Mucins are a glycoprotein constituent of mucus, therefore, characterizing *V. cholerae* motility in mucins could provide direct clinical insights, however, commercially available purified mucins are unable to adequately mimic the rheological properties of human mucus [40]. Some preliminary studies of *V. cholerae* motility in 0.6% and 1.2% diluted purified mucin environments from human saliva (generously provided by the Ribbeck Lab at MIT) were performed using the high-throughput 3D tracking technique with more rigorous background correction required. Initial qualitative analysis shows *V. cholerae* retains in mucins its run-reverse-flick motility with shorter backward runs which expands the significance of our characterization in liquid (Figure 15). We additionally observe occasional strong decelerations and stops, however these segments occur much less than in 0.25% agar. Exploring *V. cholerae* motility further in mucin environments seems very promising in providing insights into the bacteria’s pathogenicity.
The data presented here is a culmination of a year-long project and this work has been presented in posters at the Boston Bacterial Meeting (2019) and the Gordon Research Conference on Sensory Transduction in Microorganisms (2020). The characterization presented in this thesis represents the first step towards a comprehensive understanding of *V. cholerae* navigation strategies. The high-throughput 3D tracking method provides novel insight into individual bacterial mechanisms while also resolving population based dynamics, resulting in the first statistical demonstration of run-reverse-flick motility in *V. cholerae*. Further studies exploring and more rigorously quantifying motility behavior including chemotaxis in aqueous and mucus-mimicking environments could reveal insights into virulence factors affecting the early stages of *V. cholerae* infection.

Figure 15. Example *V. cholerae* trajectories in mucin solution. Run-reverse-flick motility can be observed by visual inspection in trajectories acquired in mucin solution using the high-throughput 3D tracking method.
## VII. Appendix

### Media Compositions

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition</th>
</tr>
</thead>
</table>
| LB     | 1% Bacto Tryptone  
0.5% Bacto Yeast extract  
0.5% NaCl  
pH 7.0 |
| TMN    | 50 mM Tris-HCl  
300 mM NaCl  
5 mM MgCl₂  
5 mM glucose  
pH 7.5 |
| M9GM   | 5x stock M9 salts (Sigma)  
0.5% NaCl  
0.4% pyruvate  
2 mM MgSO₄  
1 mM CaCl₂  
pH 7.0 |
| M9MM   | M9 salts  
5 g/L NaCl  
2 mM MgSO₄  
1 mM CaCl₂  
5 mM glucose  
pH 7.0 |
**VIII. References**


