Investigation of Viscosity Effects on Bacteria Collective Motion using Particle Image Velocimetry

by

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B.Sc., Biological Science
Tsinghua University, Beijing, China, 2016

SUBMITTED TO THE DEPARTMENT OF
CIVIL AND ENVIRONMENTAL ENGINEERING
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF ENGINEERING IN ENVIRONMENTAL ENGINEERING
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

JUNE 2017

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Submitted to the Department of Civil and Environmental Engineering
on May 12, 2017, in Partial Fulfillment of
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ABSTRACT

Swimming bacteria are known to display collective motion resulting from their flagella propulsion. Various models of such collective behavior have been proposed, and different motion patterns are also well-documented. However, no previous research has investigated how contrasts in fluid viscosity would influence the collective motion of swimming bacteria. In this paper, Particle Image Velocimetry (PIV), a commonly-used method to measure non-intrusive, instantaneous and whole-field velocity, is applied to visualize the swimming patterns and behavior of *Escherichia coli* and *Vibrio alginolyticus*. Firstly, various PIV post-processing tools are examined; next, different PIV conditions are tested and post-processing methods are optimized to best visualize bacteria collective motion; finally, the influence of viscosity contrast on bacteria behavior is investigated, and the swimming patterns that result from injecting active suspensions of bacteria with fluids of different viscosity are investigated.

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ACKNOWLEDGEMENT

I would like to extend my gratefulness to my advisor, professor Ruben Juanes, and the doctoral candidate in our lab, Jane Chui. During the whole academic year, they are always ready to help when I reach out.

As a student majoring in biological science for my undergraduate, fluid mechanics was a completely new field for me, not to mention the visualization tools of flow. However, Jane taught me extremely patiently. From the basic principles of fluids, to bacteria collective behavior such as swarming, Jane taught me in such a progressive way that I was able to understand those knowledge by heart. Instead passing the knowledge directly, Jane encourage me to start with one simple piece of paper, and step by step, I'm now capable of understanding papers much faster than before. When it comes to the coding part, Jane also taught me how to solve problems with logical thinking, which will definitely benefit me in future. What's more, Jane is not only my tutor, but also my friend. When I told her my depression when rejected by one company, she encouraged me that I should never give up. Jane is the best tutor and lab mate I've ever met.

My advisor, professor Juanes, is also the best advisor. I took his class last semester, and his interesting teaching style and devotion to students left me a deep impression; after joining his lab, I'm also moved by his care for his students. Every time I meet problems during my research, he always encourages me and tells me "Don't worry", which gives me confidence for future work.

I'm also very grateful to my lab mates who have helped me during my research. Without their help, I might not finish my thesis smoothly.

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Chapter 1
Background

1.1 Collective motion

Collective motion (or collective behavior) is a common phenomenon in our daily life. From bird flocks to human gathering, and from colonies of army ants to swimming bacteria, collective behaviors take place all the time. For example, it is often noticed that the crowd will automatically line up in a queue when waiting for entry to someplace; a flock of birds always migrates together following a certain flying pattern; and even for objects as small as molecules such as proteins, the chemical bonds and molecular forces also generate the collective behavior of proteins, with which the proteins function to maintain the life of all the living things.

The definition of collective motion varies slightly in different researchres, but the essence is common to all of them. According to Tamás Vicsek et.al [1], collective behavior refers to the phenomenon that an individual unit's action is dominated by the influence of the ''others''. That is, if a unit behaves in a group, its behavior will be different from the behavior when the unit is alone. In general, a collective motion system is composed of the following elements. Firstly, the units of the system should be similar to each other. Take the flock of sheep as an example. If a wolf was counted in the system, the results would be absurd. Secondly, the units should be moving with a nearly a constant absolute velocity and are able to change their direction. This is easy to understand, as investigating a group with outliers would be misleading and meaningless. Finally, the units in the system should be able to interact with fellow units and respond to environmental change.

Investigation of bacteria collective motion started years ago. Back to the 1971, Keller and Segel noticed the collective behavior of *Escherichia coli* bands and developed a corresponding phenomenological theory [2]. In that paper, they found out that random motion and chemotaxis would lead to substrate consumption and change in the bacterial density, and they proposed partial differential equations to illustrate their theory. After that, many investigations have been conducted into the collective behavior of bacteria colonies [3] [4] [5], and many different patterns of bacteria colonies growing on wet agar surfaces have been found, including super-diffusing particles pattern, turbulent-like pattern and rotational pattern.

Combining simulations with experimental observations, Czirók et al. applied *Bacillus subtilis* to investigate the collective motion such as formation of rotating dense aggregates and migration of bacteria in clusters using control parameters like concentration of agar and peptone to control the nutrient level [6]. They discovered that under certain hostile environmental conditions, outstanding collective motion such as vortex occurred. In their paper, collective behavior of *Bacillus subtilis* colony is pictured, shaping a beautiful cyclic pattern.

1.2 Swimming and swarming of bacteria

There are many different patterns of bacteria movement. The most common movements are swimming, where rotating flagella push the cells forward in liquid, and swarming, where rotating helical flagella power multicellular movement. The extension and retraction of pili lead to twitching, while focal adhesion complexes lead to the active surface movement called gliding. Finally, sliding refers to the passive surface translocation powered by a surfactant [7]. Our research focuses on the visualization of bacteria collective motion in fluid, and to

understand this, swimming and swarming of *Escherichia coli* are essential. Therefore, both swimming and swarming are introduced and discussed.

Swimming

When it comes to swimming bacteria, first of all swimming is important for cellular organisms because swimming is essential to sustain the life of bacteria in search for food (chemotaxis), the reaction to light (phototaxis), and the orientation in the gravitation field (gravitaxis). Unicellular swimmers, such as bacteria like *Escherichia coli*, *spermatozoa*, and *Paramecia* are common micro-swimmers typically of a few to several tens of micrometers in size.

It is interesting to point out that the physics ruling the swimming in such micrometer scale is completely different from macro-world swimming rules. Reynolds number, a dimensionless quantity term used in the fluid mechanics field, is commonly applied to predict flow patterns when the flow situations are different. Generally, at low Reynolds numbers, laminar flow occurs and the characteristics of which is smooth, constant fluid motion; at high Reynolds numbers, on the other hand, turbulent flow occurs. Dominated by inertial forces, turbulent flow is characterized by chaotic eddies, vortices and other flow instabilities. Swimming at the micrometer scale is swimming at low Reynolds numbers [8], where viscous damping plays a dominant role, while in the macro-world high Reynolds number is the basic rule. In addition, bacteria gain propulsion from their flagella, which makes the motion of swimming bacteria even more complicated.

In our experiment, *Escherichia coli* and *Vibrio coralliilyticus* are used to visualize the bacteria collective motion. *Escherichia coli*, is a gram-negative, facultatively anaerobic, rod-shaped,

coliform bacterium of the genus Escherichia, while *Vibrio coralliilyticus* is also a kind of gram-negative, rod-shaped bacterium [9]. *Escherichia coli* is commonly found in the lower intestine of warm-blooded organisms [10]. Same to some other kinds of bacteria, *Escherichia coli* has flagella for their propulsion. Flagellated bacteria are classified into two categories: the first is monotrichous bacteria, which possess a single flagellum; the second is lophotrichous bacteria, which have flagella located at a particular spot on their surface; the third is amphitrichous bacteria, the single flagellum of which are located on each of the two opposite ends; and finally, the peritrichous bacteria, the surface of which are covered by multiple flagella [11]. *Escherichia coli* belongs to the peritrichous bacteria category [12]. In this research, both *Escherichia coli* and *Vibrio alginolyticus* are used.

According to previous studies, single *Escherichia coli* bacterium swims in a 'run-and-tumble' motion. In stage 1, the helical winding of all flagella is left-handed, rotating in the counterclockwise direction. In stage 2, flagella form a bundle and pushing the bacterium moving in a certain direction. Stage 1 and stage 2 are the old run phase. In stage 3, one flagellum of the bacterium changes its rotational direction to clockwise. At almost the same time, in stage 4 the helical direction of the flagella also change from left-handed to right-handed and a polymorphic transition occurs for flagella, making up the turmbling phase. In stage 5, the new run phase starts and the bacterium changes its swimming direction. In stage 6, all flagella start to rotate again in the same counterclockwise direction and the bundle is formed again. Finally, in stage 7 and 8, the bacterium returns to a directional motion and another round starts [13].

Swarming

When it comes to the collective behavior of bacteria, the patterns are very different from individual bacterium. When flagellated bacteria are moving in bulk liquid, where the locomotion of one individual doesn't affect the others, this movement is called swimming. However, at a moist surface or in a thin liquid film, flagellated bacteria will exert another pattern, which is called bacteria swarming [15]. Swarming, generally, refers to a rapid cellular bacterial surface movement powered by rotating flagella.

Swarming is a characteristic of *Escherichia coli* [16]. It is interesting to point out that *Escherichia coli* K12 cells do not secrete surfactants in the experiments. This is probably due to the fact that the bacteria absorb water from the agar below and therefore they are not sticking to the agar and the fellow bacteria [17]. Another interesting fact is that chemotaxis is not required for swarming in *Escherichia coli*. According to previous research, under the condition that flagellar motors uncoupled from the chemotaxis signaling pathway achieved by molecular methods, the cells are still able to form swarming pattern [18].

The typical characteristics of swarming bacteria is large-scale swirling and streaming motions. In one experiment, researchers made *Escherichia coli* move past a fixed micro-scope objective, videoed the whole process, and measured the expansion rate. They have found that cells of *Escherichia coli* will elongate, produce more flagella, and collective motion will show up on the surface of the agar [19]. In the edge area, the density of the bacteria is the lowest; in the peak area, the cell density is the highest, followed by fall-off, where the density drops, and plateau 1 and 2, where the density remains almost the same. In this paper, a monolayer appears at the edge of the swam. In the peak area, cells are very dense and active, moving towards different directions. In the fall-off area, density drops, and in the following plateau phases cells

are moving in different directions actively. Further experiments using smoke particles on the top surface near the swarm edge reveal that the surface of the swarm is stationary [19].

1.3 Visualization of bacteria collective motion

Many attempts have been made in the past decades to visualize the collective motion of bacteria. However, there are still challenges to face. One major challenge is that it is extremely difficult to follow and keep track of an individual bacterium among a huge number of bacteria. This difficulty results from the massive number of bacteria, the similarity between bacteria, and the fast speed of bacteria movement.

One method that is widely used in today's bacteria visualization is called "Particle Image Velocimetry" (PIV) [20]. This method is widely used to measure the velocity of a particle by recording the position of the particle in a flow field with multiple images and then analyzing the captured images. Unlike laser Doppler velocimetry, or hot-wire anemometry, which are also used to measure flows, the advantage of PIV is that this method is capable of producing two-dimensional or three-dimensional vector fields, together with the accuracy and resolution of single-point measurement technology and the ability to obtain the overall structure and instantaneous image [20].

The principle of PIV is relatively simple. First of all, a large number of particles, in our case, *Escherichia coli* suspension, for example, are put in a flow field. Next, using microscope with a digital camera, images of the suspension are taken during very short time intervals. In this way, the displacement of particles at each point is obtained by image analysis. The velocity vector at each point in the flow field can be obtained by the time interval between displacement,

and other motion parameters such as streamline and swirl can also be calculated. The components of a PIV system include seeding particles (*Escherichia coli* and *Vibrio coralliilyticus* in our experiment), imaging system and post-processing software.

Many studies have been conducted using PIV to visualize bacteria collective motion. Cisneros et al. (2007) evaluated the velocity field of thousands of swimming bacteria with PIV [21]. In their experiments, *Bacillus subtilis* is used to investigate the characteristics of the Zooming BioNematic (ZBN) phase, which refers to a collective phase where swimming *Bacillus subtilis* are densely populated. With PIV analysis, they found that *Bacillus subtilis* suspension will exhibit large-scale directional coherence. Significant spatial and temporal correlations of velocity and vorticity are also shown, which could be explained by local energy input by the swimmers [21].

Another study applied PIV into the particle dynamics investigation inside an evaporating droplet [22]. In this experiment, the effect of chemotaxis on evaporation-induced particle deposition patterns formation has been reached experimentally in drying droplets of bacterial suspensions. *Escherichia coli* bacteria were used as biological tracers, and droplet containing suspended microorganisms was visualized by LED light. The example of PIV analysis result of both live and dead bacteria is shown below. It has been found that the velocity vector pattern of live bacteria suspension droplet is different from the dead one, showing that chemotaxis can actually change the velocity fields and concentration patterns in swimming bacteria [22].

The influence of concentration change on the collective motion in an active suspension of *Escherichia coli* bacteria is also examined using PIV [23]. In this study, researchers found that if the concentration of bacteria suspension is increased from a dilute to a semi-dilute regime,

there will be a pattern change, from a dynamical cluster regime to a regime of 'bio-turbulence' convection patterns, which may exert significant influence on the macro-level. Large-scale collective motion can be seen from the PIV result, and velocity directions are also shown [23]. In summary, PIV is widely applied in particle researches and is also a powerful tool in the visualization of bacteria collective motion. Based on this situation, PIV is used in our experiment to further investigate bacteria collective motion under different viscosity.

Chapter 2
Motivation

Studies have been conducted concerning the influence of viscosity on bacteria mobility. For example, in 1977, researchers have found that generally, the average velocity will decrease when viscosity of the agar increase for flagellated bacteria such as *Pseudomonas aeruginosa*, *Spirillum serpens*, and *Escherichia coli* [24]. An average of 60 centipoise (cp) viscosity is needed to immobilize those bacteria, while 1,000 cp and higher viscosity is required to immobilize for *Spirillum gracile* and *Spirochaeta halophile* [24]. Another interesting study applied the semen of Japanese cattle to investigate the bovine sperm motion in environments of various viscosities [25]. They found that the motility of the sperm decreased with the increase of viscosity, and that the flagellum shape changed with the viscosity change, suggesting that bovine sperm has evolved to swim effectively in the highly-viscous oviduct [25]. The studies above investigated a lot about the mobility, but no collective motion of bacteria is mentioned in this paper.

Another paper researched the effect of viscosity on swimming by the lateral and polar flagella of *Vibrio alginolyticus* [26]. Using *Vibrio alginolyticus* as the model organism, they found out the functional difference between polar and lateral flagella in viscous environments [26]. However, this paper only goes into the flagella's role, while collective motion pattern of bacteria is still not mentioned.

When it comes to active bacteria suspensions, which exhibit singular macroscopic transport properties, researchers have found that at low shear, the viscosity is found to decrease with

concentration at values lower than the suspending fluid viscosity [27].

In addition to a viscosity vs shear rate characterization of an *Escherichia coli* suspension, it has also been found that in the dilute regime and at a low shear rate, the decrease of the apparent viscosity is linearly correlated with the decrease of bacteria concentration [28]. The strong viscosity reduction may play an important role when considering macroscopic transport and particle dispersion in porous systems or in capillary networks, which is the key to many applications including bacterial fluids [28]. The study on the rheology of bacteria fluids under viscosity change has been studied, but they still fail to consider the collective behavior of bacteria under conditions or especially varying viscosity remains unexplored.

In summary, previous studies either focus on the influence of viscosity on single bacterium and the motility of bacteria, or the rheology of bacteria under viscosity change, but no previous research has investigated how contrasts in fluid viscosity would influence the collective motion of swimming bacteria. Therefore, in this study, we apply PIV to visualize the collective motion of E. coli under viscosity contrasts. Firstly, PIV post-processing tools, specifically, "PIVlab" and "Mpiv" are examined; next, different PIV conditions are tested and post-processing methods are optimized to best visualize bacteria collective motion; finally, the influence of viscosity contrast on bacteria behavior is investigated. The swimming patterns that result from injecting active suspensions of bacteria with fluids of different viscosity are researched.

Chapter 3
Methods

3.1 Suspension culture of bacteria

In this paper, *Escherichia coli* (strain: ATCC® 9367™) and *Vibrio coralliilyticus* (strain: ATCC® BAA450™, medium marine broth 2216, kanamycin resistant) are used. Firstly, a 100 μl *Escherichia coli* or *Vibrio coralliilyticus* frozen culture is taken from the -80°C refrigerator. Next, the *Escherichia coli* or *Vibrio coralliilyticus* culture is transferred to a flask (with 50mg/mL kanamycin for *Vibrio coralliilyticus*), and LB stock solution is added to around 25 ml. The mouth of the flask is sealed with sterile cotton plugs and the cell culture is Incubate overnight at 20°C with continuous shaking at 240rpm. After that, the sample is centrifuged at 2300g for 10min, and the pellet is re-suspended to 0.35ml. Finally, the sample is examined with microscope to investigate the activity of bacteria.

3.2 Image-taking and PIV processing

When the bacteria sample is ready, one drop of sample solution is pipetted to a glass plate. The movement of bacteria are monitored with microscope and images are taken with Hamamatsu flash 3.0 microscope camera. Different image-taken conditions are tested, and two PIV analysis tools for image-processing are examined. The results and discussion are written in the following chapters.

Chapter 4
Examination of PIV analysis tools

There are several commonly-used PIV post-processing tools such as Mpiv, PIVlab and openPIV. Below, Mpiv and PIVlab are examined.

4.1 Mpiv

Mpiv is a relatively simple Matlab toolbox, consisting of two main programs and several external functions. Written on the basis of simple algorithms, the two main programs are one image processing and one post-processing program. A Graphic User Interface (GUI) can be used to run PIV analysis, or images can be directly analyzed by the Mpiv codes.

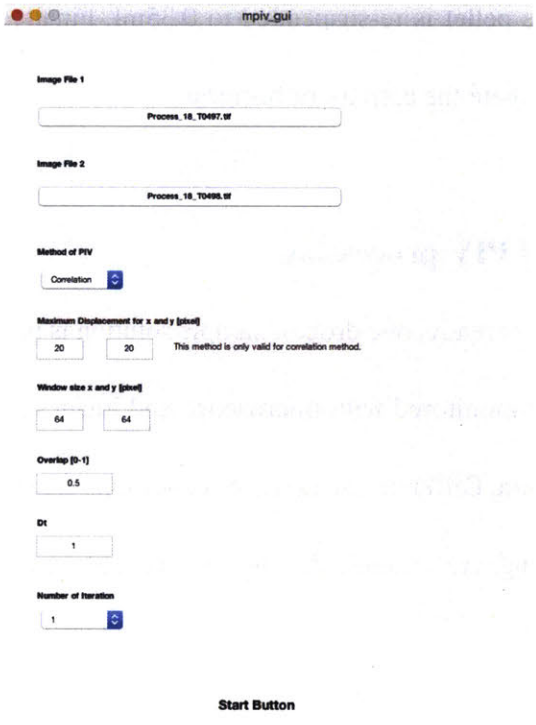

Figure 4.1: Illustration of Mpiv GUI.

The two basic principles are cross-correlation method and MQD (Minimum Quadratic Difference) method. The correlation algorithm is based on the conventional method, and the formula is listed as follows:

$$C(\Delta X, \Delta Y) = \frac{\sum_{i=1}^{N}\sum_{j=1}^{N}\left[f_1(X_i, Y_j) - \overline{f_1}\right]\left[f_2(X_i + \Delta X, Y_j + \Delta Y) - \overline{f_2}\right]}{\sqrt{\sum_{i=1}^{N}\sum_{j=1}^{N}\left[f_1(X_i, Y_j) - \overline{f_1}\right]^2}\sqrt{\sum_{i=1}^{N}\sum_{j=1}^{N}\left[f_2(X_i + \Delta X, Y_j + \Delta Y) - \overline{f_2}\right]^2}}$$

where f1 and f2 are the small windows from each image in the image pair, N is the window size and the overbar denotes mean quantity. The location of the maximum value (peak) in C is used as the mean particle displacement of this small area.

The Minimum Quadric Differences (MQD) algorithm functions through calculating the pixel value differences between the search windows. The formula is listed as follow:

$$C(\Delta X, \Delta Y) = \sum_{i=1}^{N}\sum_{j=1}^{N}\left|f_1(X_i, Y_j) - f_2(X_i + \Delta X, Y_j + \Delta Y)\right|$$

The location of the minimum value in C is used as the particle displacement.

The main component of Mpiv is mpiv.m and mpiv filter.m. The first one is the main route of Mpiv, and the latter one is to post-process the output from Mpiv, which includes two parts: spurious vectors elimination and missing vectors interpolation.

One thing needs to mention is that Mpiv does not have pre-processing methods, and DACE toolbox is needed to accompany Mpiv.

4.2 PIVlab

PIVlab is another popular Matlab toolbox for PIV post-analysis. A normal PIV analysis is made

up of three main steps: image pre-processing, image evaluation, and post-processing. The general work flow is illustrated in the picture below: firstly, the original pictures are inputted into PIVlab, and a Graphic User Interface (GUI) can be used to make the processing easier.

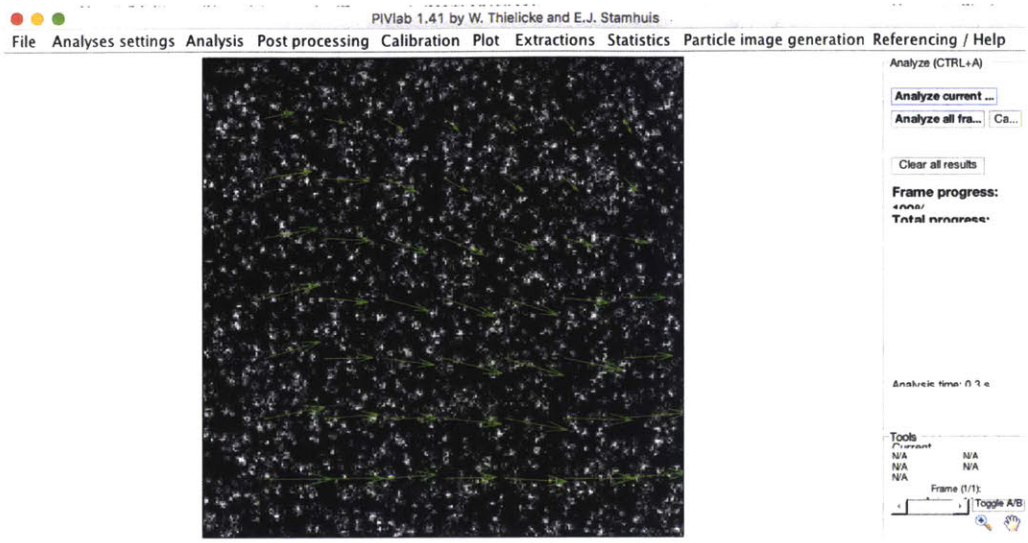

Figure 4.2: Illustration of PIVlab GUI.

Next, pre-processing methods could be chosen, and then the PIV settings are determined. After analysis, the speed of the bacteria in each interrogation window are calculated and vector validation is applied to remove errors. A movie of swimming bacteria can be directly exported from PIVlab.

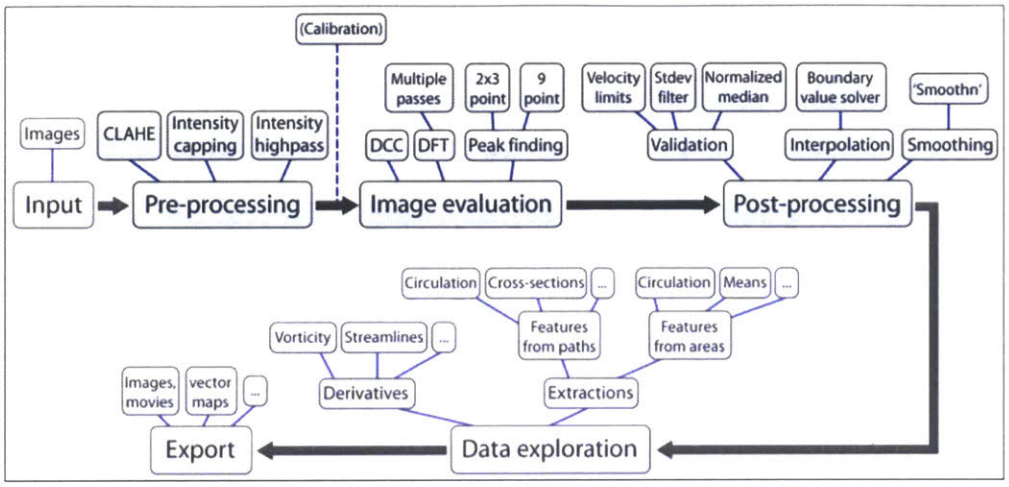

Figure 4.3: Illustration of general workflow of PIVlab.

In the analysis, the same two methods are used here: the direct cross correlation (DCC) and Minimum Quadric Differences (MQD). Normally we use DCC method for swimming bacteria images according to previous researches.

4.3 Test of Mpiv and PIVlab

Firstly, "image1.bmp" and "image2.bmp" are used for testing the two toolboxes.

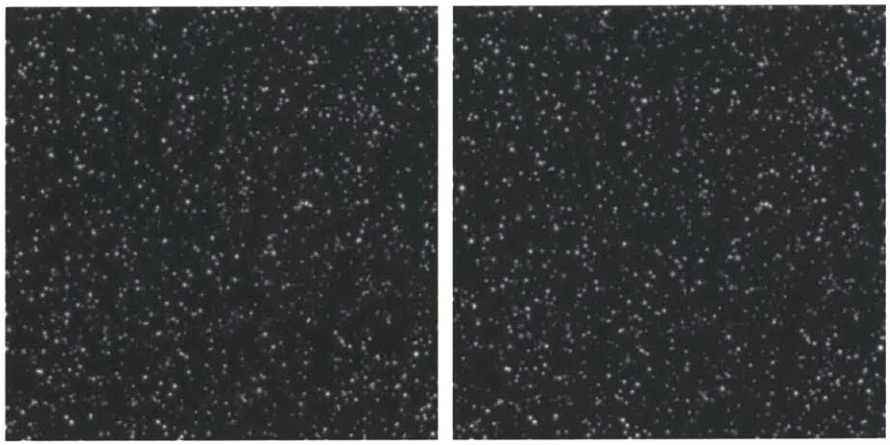

Figure 4.4: "image1.bmp" and "image2.bmp".

In Mpiv, choose DCC as the algorithm; the size of interrogation window is 64 pixels, overlap 50%. The result is shown as follows:

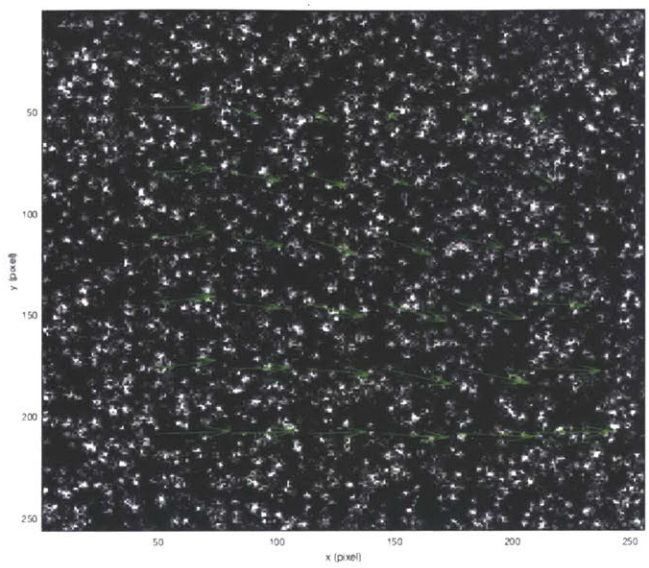

Figure 4.5: Mpiv result. Green arrows represent the swimming direction of the bacteria. The longer the arrow, the faster the swimming speed is.

In PIVlab, the DCC method is also chosen as the PIV algorithm; interrogation area is 64 pixels, and step 32 pixels (which is equal to overlap 50% in Mpiv); then the two pictures are analyzed. The result is shown as follows:

Figure 4.6: PIVlab result. Green arrows represent the swimming direction of the bacteria. The longer the arrow, the faster the swimming speed is.

As we can see from the two figures above, Mpiv and PIVlab generate similar results. The directions of swimming bacteria are the same for each interrogation window in both mpiv and PIVlab, which shows that both toolboxes work.

4.4 Analysis of pros and cons

As discussed above, both Mpiv and PIVlab can function well in PIV post-processing. Both of them have their pros and cons.

For Mpiv, since the code is very easy to read and modify, it is more convenient to adapt to different PIV images. However, additional pre-processing codes should be written, and the Mpiv is not able to process large pictures with many bacteria in one interrogation window.

For PIVlab, the GUI is easier to use. The advantages of PIVlab include:

1) Multiple images input: unlike Mpiv, where only 2 pictures can be imputed from the GUI. Additional loop is needed to input more pictures.
2) Pre-processing: PIVlab has its own pre-processing functions including contrast enhancement, removal of background signal, and the removal of very bright points.
3) Direct output: after processing, the results can be outputted directly from PIVlab into jpg, bmp, tif and avi (movie) as well.
4) Clear GUI: From left to right, the GUI includes pre-processing, analyzing, post-processing, calibration, and statistics. It is much easier for new comers to understand and get used to PIV analysis in a short time.

Based on the discussion above, PIVlab is chosen to do the result analysis in the following experiments.

Chapter 5
Optimization of image-taking conditions

Many factors affect the PIV visualization results. During the image-taking stage, magnitude of microscope and different microscopy techniques (phase-contrast and bright-field) will influence the quality of the images.

5.1 Testing of PIVlab

To test if PIVlab will be effective for our experimental setup, two swimming bacteria samples, a directional one and an un-directional one, are measured. The raw images used in this case are pictures of swimming bacteria. Phase-contrast technique is used, the magnitude of microscope is 20x, and images are taken at a frequency of 0.1 second/image. To make the analysis simpler and to avoid the interference from the surrounding, a 512*512 pixels square was cut out from the original picture as illustrated below.

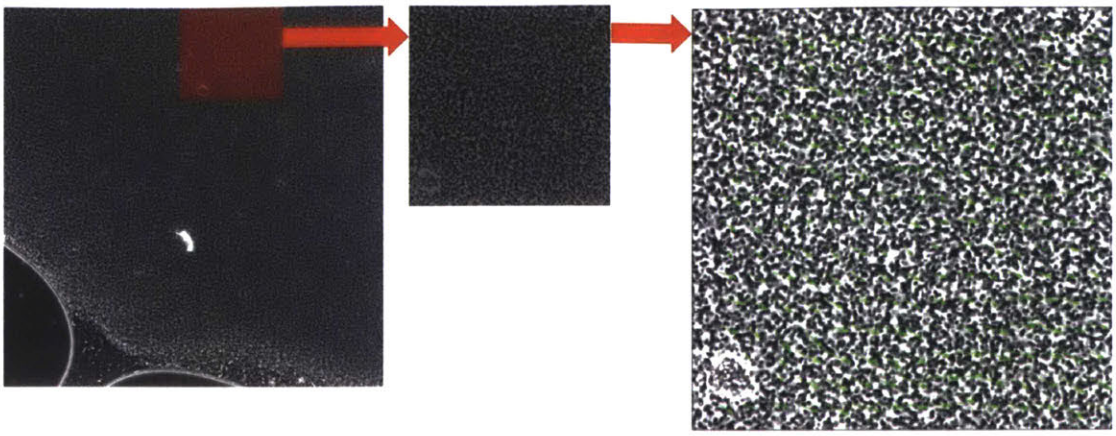

Figure 5.1: A 512*512 pixels square is chosen from the 2048*2048 pixels image, with phase-contrast technique, and then PIVlab is run to analyze the images, with interrogation window 64 pixels (1/16 of the whole image).

To remove the bright spots that will influence the analysis of PIVlab, MATLAB codes are written to change every pixel which is brighter than 30,000 into 30,000, and then run the PIVlab. The results are shown in the images below.

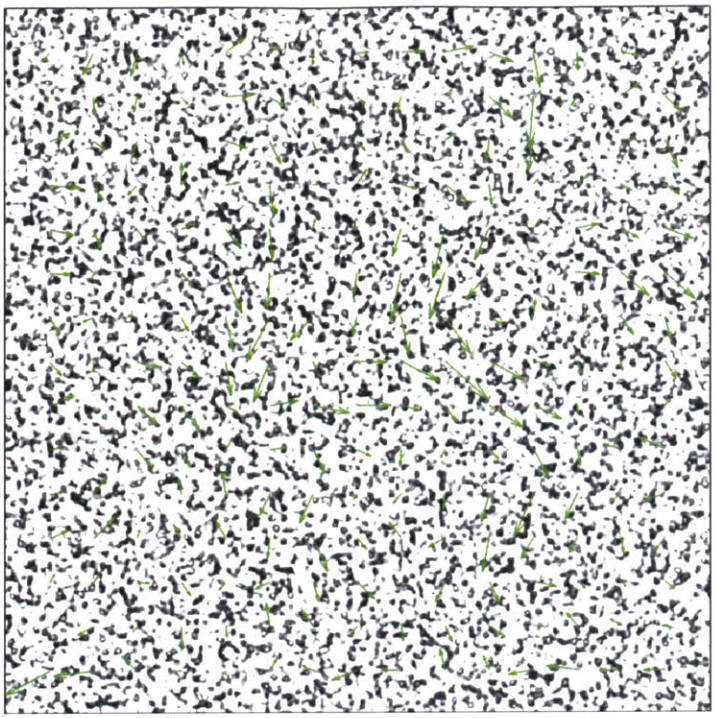

Figure 5.2: Image of bacteria swimming in random directions. Microscope magnitude 20x, 512*512 pixels from the 2048*2048 pixels image, phase-contrast technique, interrogation window 64 (1/16 of the whole image).

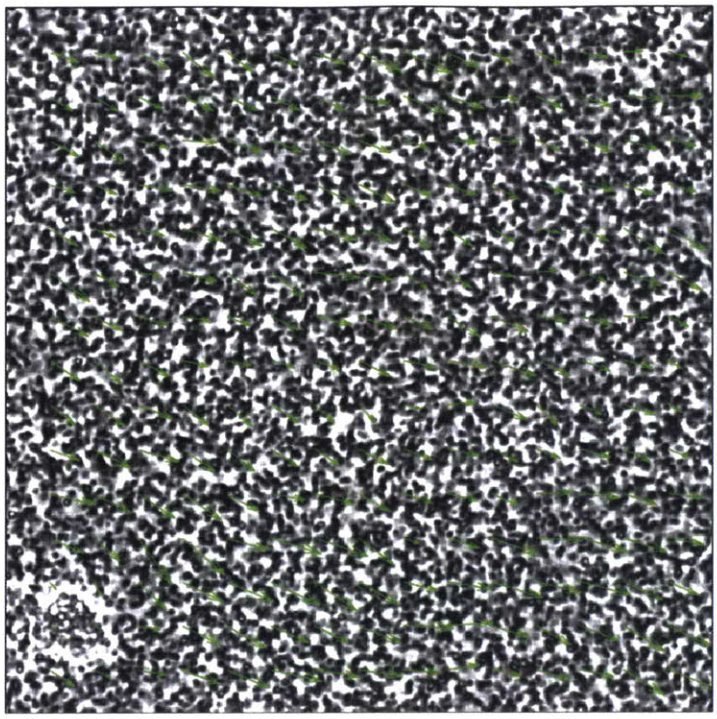

Figure 5.3: Image of bacteria swimming in the same direction. Microscope magnitude 20x, 512*512 pixels from the 2048*2048 pixels image, phase-contrast technique, interrogation window 64 (1/16 of the whole image).

From the two images above, we can see that in Figure 5.2, the arrows are in various directions, showing that bacteria are swimming randomly. In Figure 5.3, the arrows are pointing the right direction, showing that bacteria are swimming to the right. Since for the bacteria samples, one is directional and the other is un-directional, the PIVlab analysis shows the same results, proving that the PIVlab is capable of processing swimming bacteria samples and generating the correct flow direction. Moreover, since the bacteria swimming speed is 16-30μm/s, the swimming speed calculated by PIVlab all fall in this range, proving that the velocity calculation is also correct in this experiment.

Based on these observations, PIVlab is used in the following experiments.

5.2 The magnification of microscope

The magnification of microscope plays an important role in the visualization of bacteria collective motion. If the magnification is too large, then it could be difficult to obtain the whole picture of bacteria collective motion. If the magnification is too small, on the other hand, it would be difficult for PIV analyzing tools to recognize those swimming bacteria. In this chapter, 20X and 40X are tested to see which magnification is better for our experiment.

Based on the images above, 20x and 40x magnifications are compared. The following image shows the results of PIVlab in 40x:

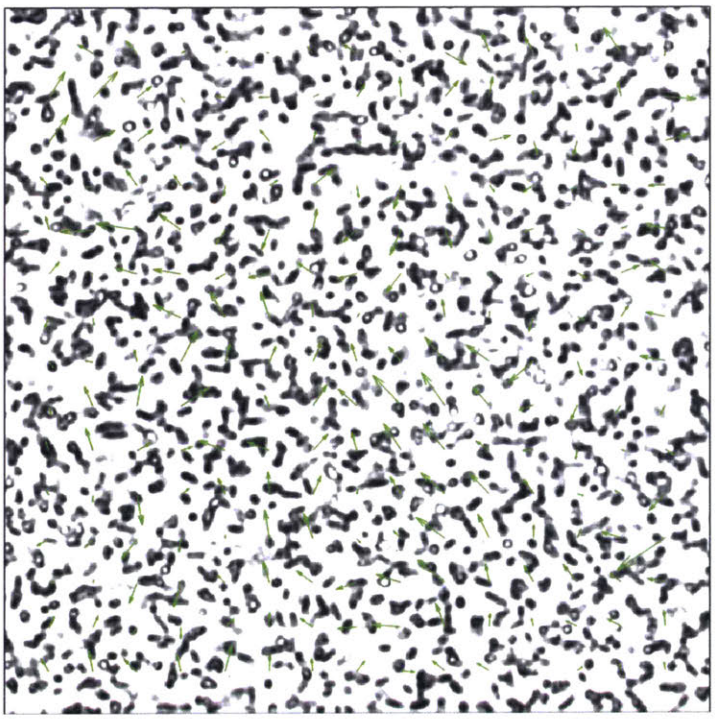

Figure 5.4: Image of bacteria swimming in the same direction. Microscope magnitude 40x, phase-contrast technique, 512*512 pixels from the 2048*2048 pixels image, interrogation window 64 (1/16 of the whole image).

As we can see from Figure 5.3 and Figure 5.4, in 20x image, a broader area is shown and it is easier to see bacteria collective motion. However, it should also be mentioned here that some swimming bacteria of both 20x and 40x are not detected. This may due to the fact that the pre-processing before PIVlab analysis is not good enough to remove noises; that there's not enough bacteria in each interrogation window; and that since some bacteria are covered with other bacteria, making the size and movement more difficult for PIVlab to catch. In these two images, the 20x one is better to get the whole picture of bacteria collective motion, but due to the fact that the two images are both a small part of the original images, it is better to apply the whole images rather than the parts.

Owing to the limitation of original images (the left bottom of the images includes air-water interface), a 1024*1024 pixels square were cut from the original 2048*2048 pixels images, as is illustrated in the figure below:

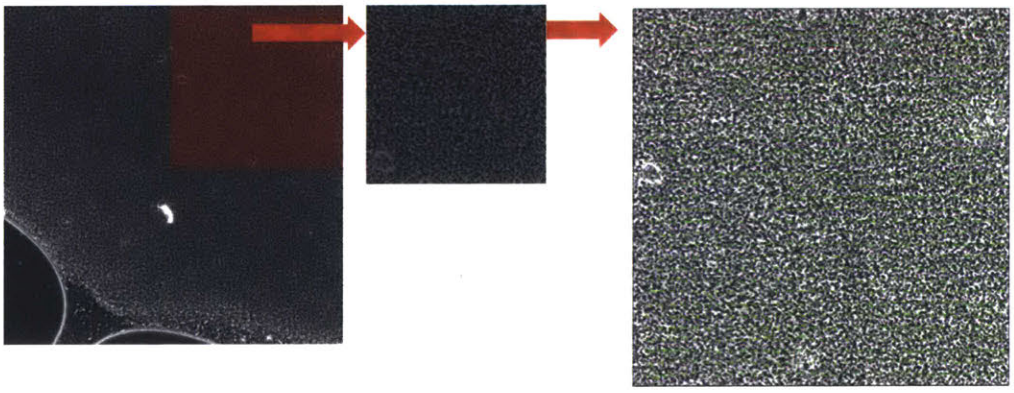

Figure 5.5: A 1024*1024 pixels square is chosen from the 2048*2048 pixels in image phase-contrast technique, and then PIVlab is run to analyze the images, with interrogation window 64 (1/16 of the whole image).

Based on the images above, 20x and 40x magnitudes are compared. The following images shows the results of PIVlab in both 20x and 40x:

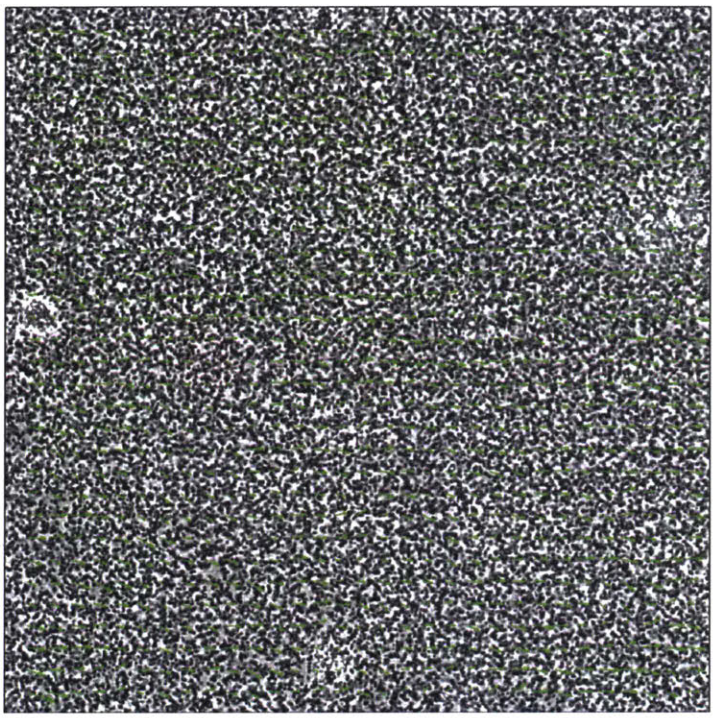

Figure 5.6: Image of bacteria swimming in the same direction. Microscope magnitude 20x, phase-contrast technique, 1024*1024 pixels from the 2048*2048 pixels image, interrogation window 64 (1/16 of the whole image).

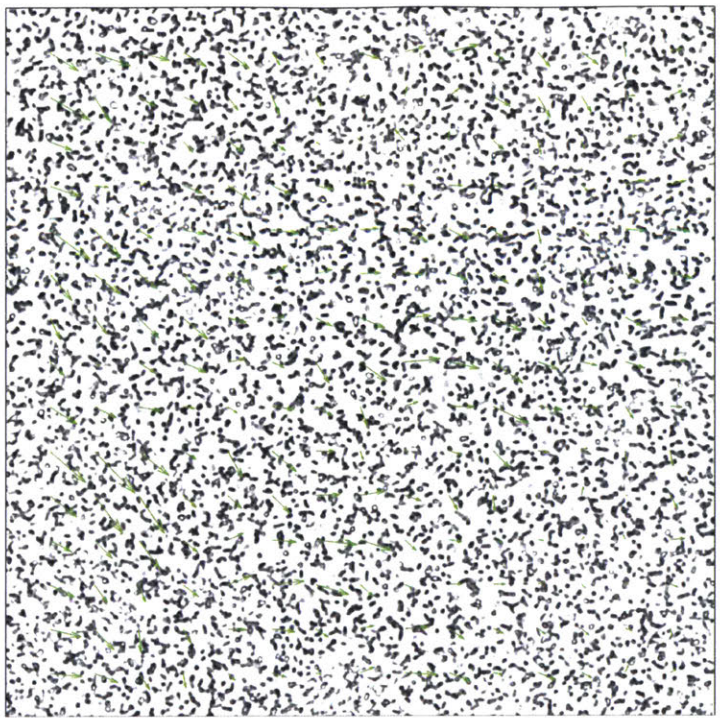

Figure 5.7: Image of bacteria swimming in the same direction. Microscope magnitude 40x, phase-contrast technique, 1024*1024 pixels from the 2048*2048 pixels image, interrogation window 64 (1/16 of the whole image).

From the two images above, the following conclusions are reached: Firstly, in both images, the bacteria are roughly swimming to the right direction; next, in the 40x case, some arrows are missing, meaning that it is difficult for PIVlab to detect the movement of all the bacteria. The explanation for the phenomenon is the same as the case above, but it's more likely due to the fact that there are not enough bacteria in each interrogation window.

Hence, to make sure that there are enough bacteria in each interrogation window, and that the whole picture of collective motion can be visualized, 20x is chosen in the following approach.

5.3 Phase-contrast and bright-field techniques

Phase-contrast refers to the microscopic technique which changes invisible phase shifts in light passing through a transparent specimen into variations in the brightness of pictures, where sample contrast comes from interference of different path lengths of light through the sample. The phase-contrast microscope functions by generating constructive interference or by reducing the amount of background light that reaches the image plane. Bright-field refers to the technique which applies transmitted white light to illuminate samples, and contrast in the sample is caused by attenuation of the transmitted light in dense areas since dense areas absorb more light. As the simplest illumination technique in light microscopes, bright-field microscopy is characterized by a dark sample on a bright background, hence getting the name. To select a better microscopic technique for PIV images, both phase-contrast and bright-field techniques are tested. Same as conducted in part one of this chapter, the magnitude of microscope is 20x, and images are taken at the frequency of 0.1 second/image. A 512*512 pixels square was cut out from the original picture. The phase-contrast result is shown before in Figure 5.7, and the bright-field result is shown below:

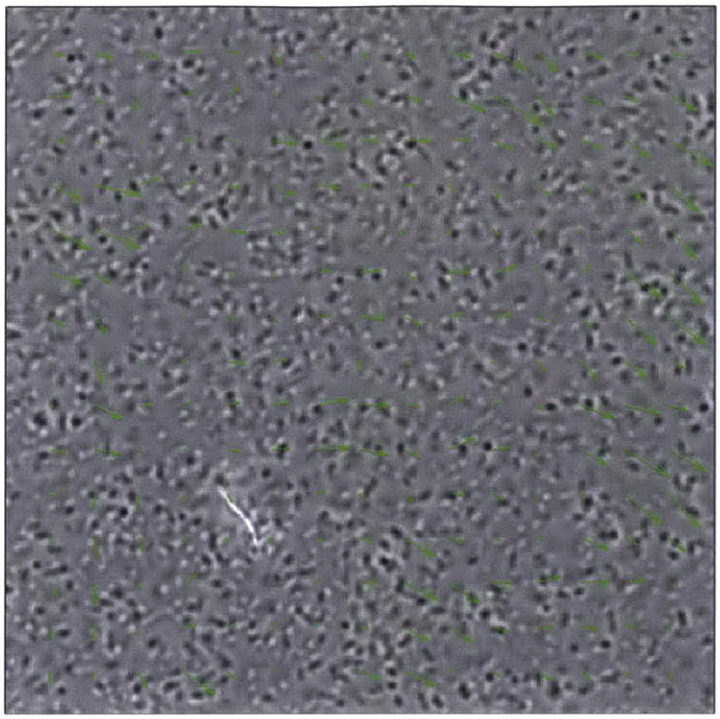

Figure 5.8: Image of bacteria swimming in the same direction. Microscope magnification 20x, 512*512 pixels from the 2048*2048 pixels image, bright-field technique, interrogation window 64 (1/16 of the whole image).

As we can see from Figure 5.7 and Figure 5.8, in both pictures bacteria are swimming to the right, though in the bright-field image it is not very uniform. This may due to the fact that since images were taken not at the same time, the uniform swimming direction is likely to change with the passage of time. Incorrect analysis of PIVlab is also a possible explanation. Next, for the bright-field image, PIVlab failed to detect many bacteria. As we can see from the bright-field image, the noise of the image is much higher than the phase-contrast one, making it difficult for pre-processing and for PIVlab to analyze. On the other hand, PIVlab analyzed the phase-contrast technique successfully, and a clear directional movement of bacteria is shown.

Based on the discussion above, phase-contrast is a better PIV microscope method and will be applied in the following experiments.

Chapter 6

Optimization of image-processing (PIVlab) methods

During the image-processing stage, the threshold control of the images, the size of interrogation windows, and different contrast enhancement methods, also affect the PIV analysis results. To obtain a clear PIV analysis of bacteria collective motion, the factors mentioned above are tested and optimized.

6.1 Contrast enhancement

As can be found in many sample images, the images taken from the microscope sometimes can be relatively dark and it is difficult to distinguish the swimming bacteria with the surrounding for PIVlab. One example is shown below. The original image is dark and difficult to distinguish swimming bacteria, and then after the process of PIVlab, though most of the swimming bacteria are detected, some patches are still missing.

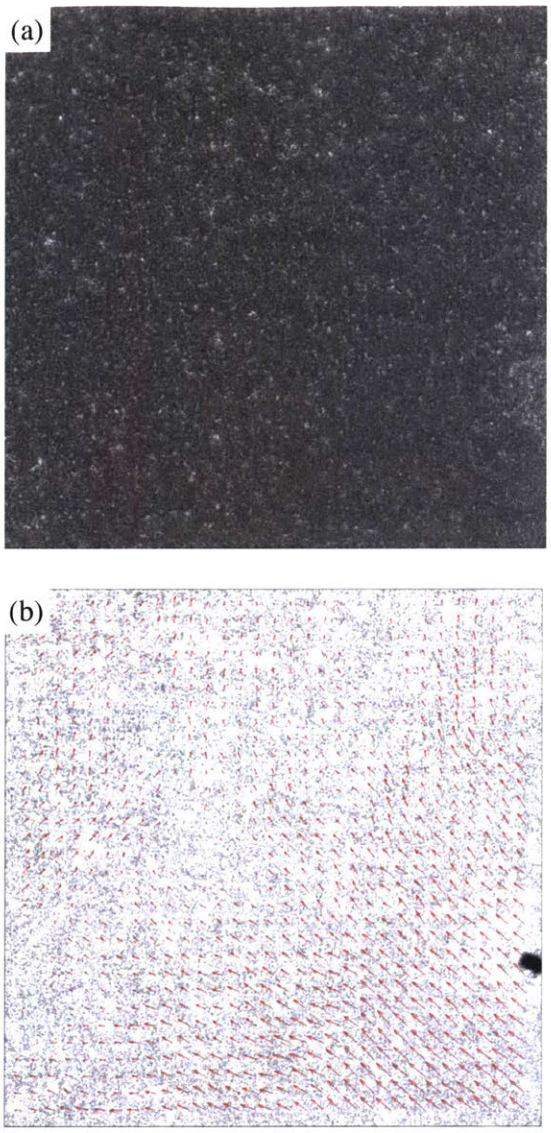

Figure 6.1: (a) The original image from one experiment. Image taken with 20x microscope using phase-contrast technique. (b) PIV analysis result. Some arrows are missing in the middle, showing that some bacteria are not detected.

To solve this problem, three functions to adjust contrast are tested in this experiment: "imadjust", "histeq" and "adapthisteq".

"imadjust" command enhances the contrast of the image through mapping the values of the input intensity image to new values. As a result, 1% of the data is saturated at low and high intensities of the input data by default. "histeq" functions by transforming the values in an

intensity image, making the histogram of the output image approximately obey uniform distribution by default, the process of which is also called histogram equalization. "adapthisteq", on the other hand, performs contrast-limited adaptive histogram equalization. The difference between "histeq" and "adapthisteq" is that instead of working on the entire image, "adapthisteq" operates on small data regions (tiles). Each tile's contrast is enhanced so that the histogram of each output region approximately matches the uniform distribution by default. In this way, contrast enhancement can be limited and therefore the possible background noise is not amplified too much to disturb the image. Based on theory, "adapthisteq" should be the best choice due to its precision and capability to avoid noise.

Images are taken at 20x microscope with phase-contrast technique; next, the three contrast enhancement methods are applied to the original images; then, a threshold of 30,000 is applied to remove the very bright spots; finally, the original images, the contrasted enhanced images, and the further modified images are examined in PIVlab to analyze the swimming pattern of bacteria. The images are shown below:

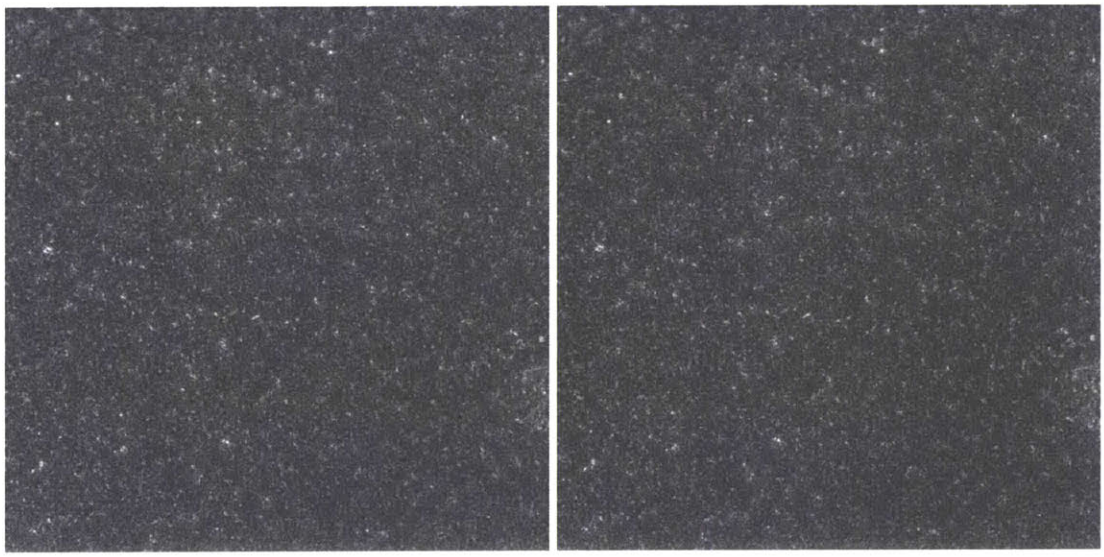

Figure 6.2: Original image pair: taken at 20x microscope with phase-contrast, and the time interval between the two pictures is 0.04 second.

PIV results of the unprocessed image pair (Figure 6.2), the three contrast enhanced ones are shown below:

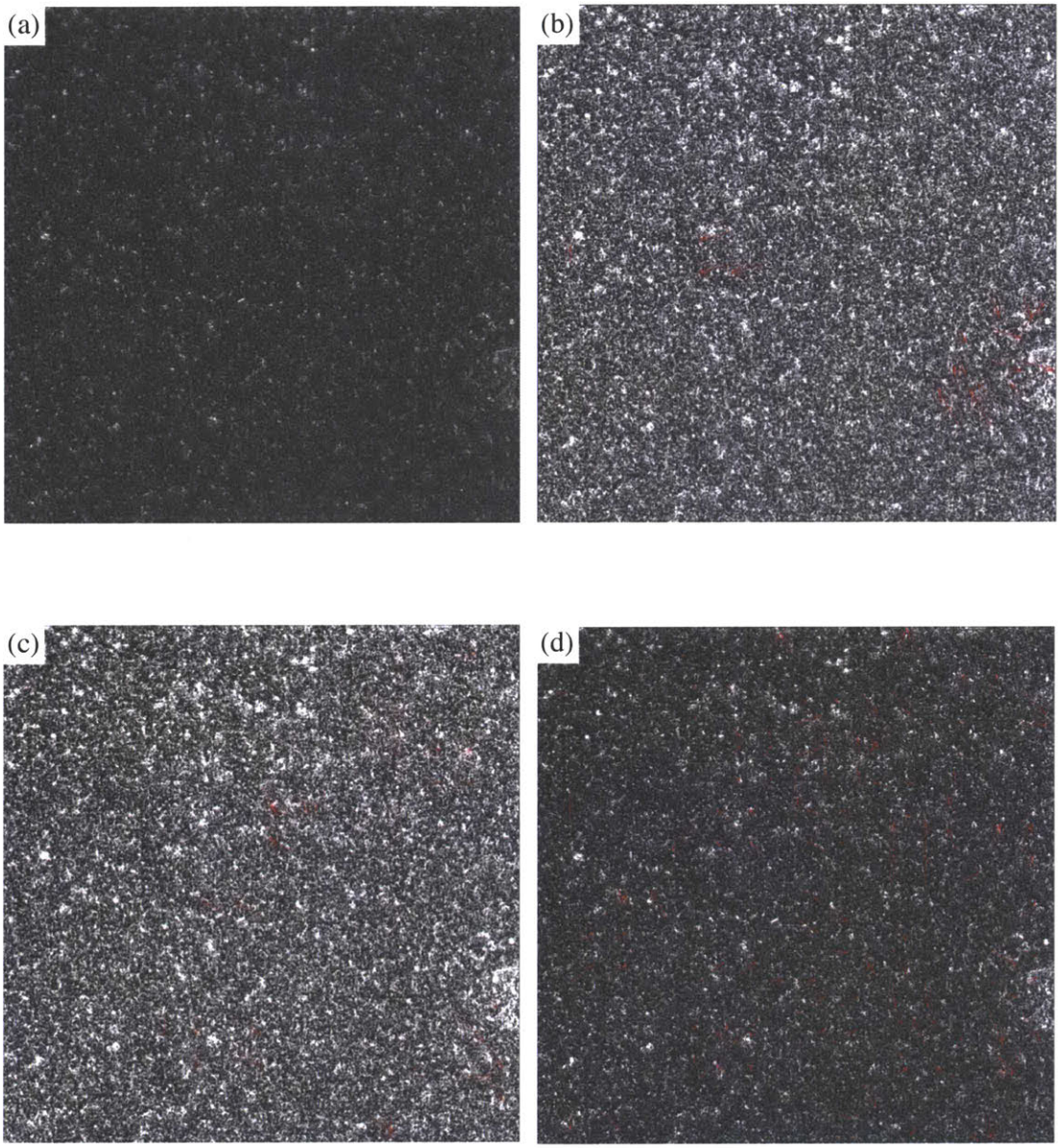

Figure 6.3: Examination of three contrast enhancement methods. (a) PIVlab analysis result of original image pair, interrogation window 256 pixels, with 50% overlapping. No arrows show up, so PIVlab fails to detect the swimming bacteria in this case. (b)contrast of the image pair is enhanced with "imadjust" command, (c) "histeq" command and (d) "adapthisteq" command. In (b) and (c), most of the bacteria are not detected, and in (d) around 40% is detected.

As can be seen from the images above, each contrast enhancement method makes the image easier to read. As predicted, "adapthisteq" is the best contrast enhancement method compared with the other two. However, the result is still disappointing since there's still a lot of bacteria missing, further processing such as removing the background noise should be applied. Like what has been conducted in the experiments before, all the bright spots are removed from the image by changing the pixels which are higher than 30,000 into 30,000. In this way, bright spots and other noise are removed. While bacteria are much darker (usually less than 10,000), this threshold will not influence the bacteria we want. PIV analysis results are shown below:

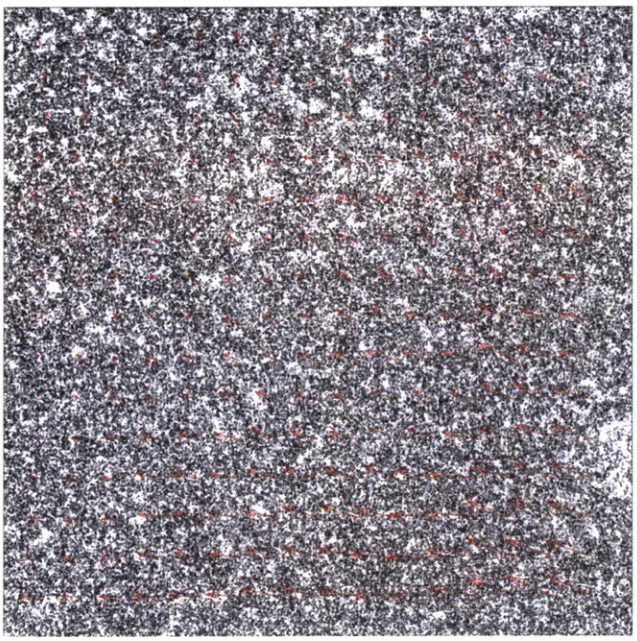

Figure 6.4: PIVlab analysis result of original image pair, interrogation window 256 pixels, with 50% overlapping. Before PIVlab running, the contrast of the images is firstly enhanced by "adapthisteq" command and the threshold are changed to 30,000.

As can be seen from the image above, almost all the swimming bacteria are detected and it is shown from the result that bacteria are swimming roughly to the left.

Since the total size of the image is 2048*2048 pixels, and the interrogation window size is 256 pixels with 50% overlapping, then if all the bacteria in each window is readable, then

(2048/(256*50%))-1=15 arrows should appear in each line or row, or 15*15=225 arrows should appear on the image in total. Building on this, the number of arrows can be counted to reflect the percentage of bacteria detected through each method. A table of the results are shown below:

Table 6.1: Efficiency of PIV processing different samples.

The percentage are calculated by the number of arrows divided by the total number of arrows that should appear in an image.

	Original	*"imadjust"*	*"histeq"*	*"adapthisteq"*	*"adapthisteq" plus background removal*
Detected bacteria	0%	9%	10%	40%	100%

As we can see from the table above, for the contrast enhancement method, the "adapthisteq" is the most efficient since it can enhance the contrast while at the same time avoid the enhancement of background noise. However, based on the results above, removing background directly by changing those brighter pixels into a certain number seems to be a more effective and economic way.

Therefore, for the contrast enhancement method, "adapthisteq" is the best choice, and the threshold of the background removal approach is further discussed in the next part.

6.2 Threshold of brightness

In the previous discussion, background noise is removed directly by changing those brighter pixels into a certain number. 30,000 is used as the threshold. To test whether 30,000 is the best

threshold, 30,000, 40,000 and 50,000 are tested to see which threshold is the best.

Based on the previous experiment, where images are taken at 20x microscope with phase-contrast technique, images are processed with "adapthisteq" contrast enhancement method and then different thresholds are applied to remove the very bright spots. The PIV analysis results are shown below:

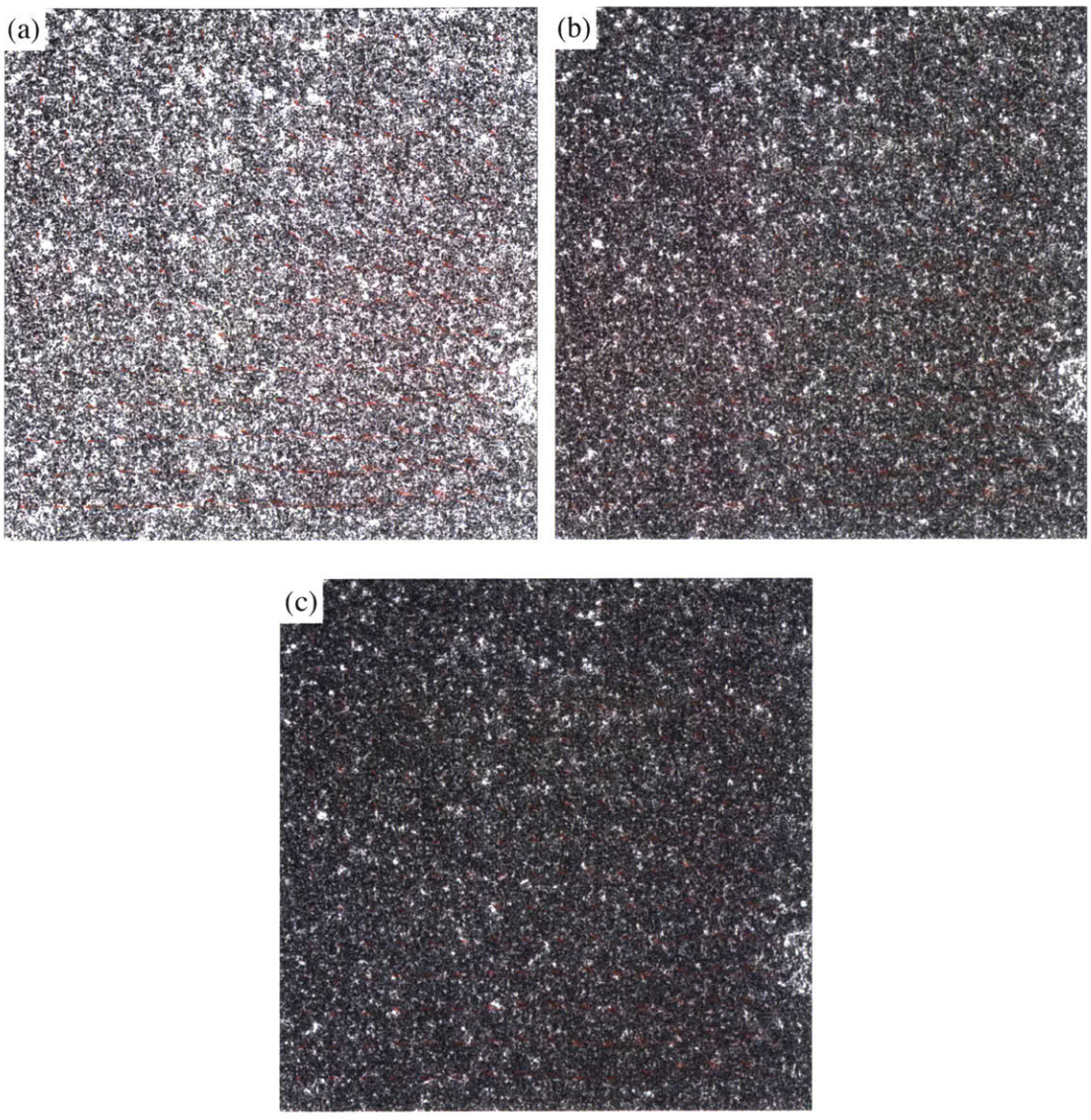

Figure 6.5: PIVlab analysis result of the same original image pair, with "adapthisteq" contrast enhancement method, interrogation window 256 pixels, with 50% overlapping. Brightness threshold of the images are (a)30,000, (b)40,000, (c)50,000.

As can be seem from the images above, the threshold of removing bright background noise doesn't influence the PIV results. Bacteria in each interrogation window are detected, and the directions of arrows in the three images are the same, proving that as long as the threshold falls in a certain range which functions through removing the background noise but doesn't influence the detection of swimming bacteria, then there's little difference in PIV analysis results.

Another round of experiment was run and new images are taken for further research. Images are taken at 60fps with 20x magnitude and phase-contrast technique. Since for these images the brightest (highest) is 255, while the lowest 0, 100, 150, and 200 are tested in this experiment. PIVlab results are shown below:

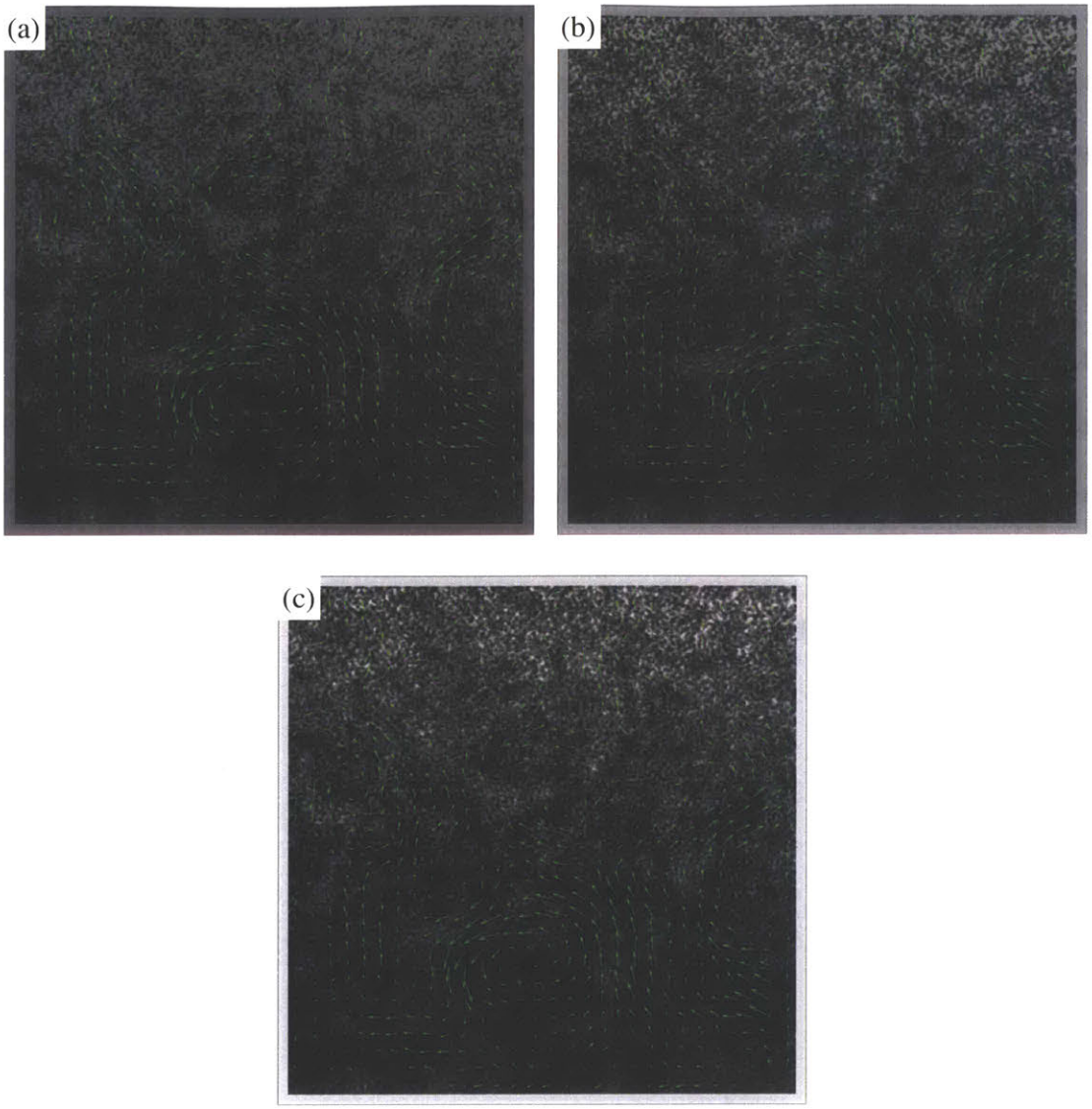

Figure 6.6: PIVlab analysis result of one image pair, interrogation window 32 pixels, with 50% overlapping. Brightness threshold of the images are (a)100, (b)150, (c)200.

These images show very clear bacteria collective motion. Also, even though the thresholds of background removal are different, the results are almost the same. The directions and length of arrows are mostly the same, but it is interesting to notice that for some spots where the velocities of bacteria are relatively slow, the three different thresholds give different results. The same spots of the three images above were investigated and the following images show the cut and enlarged parts:

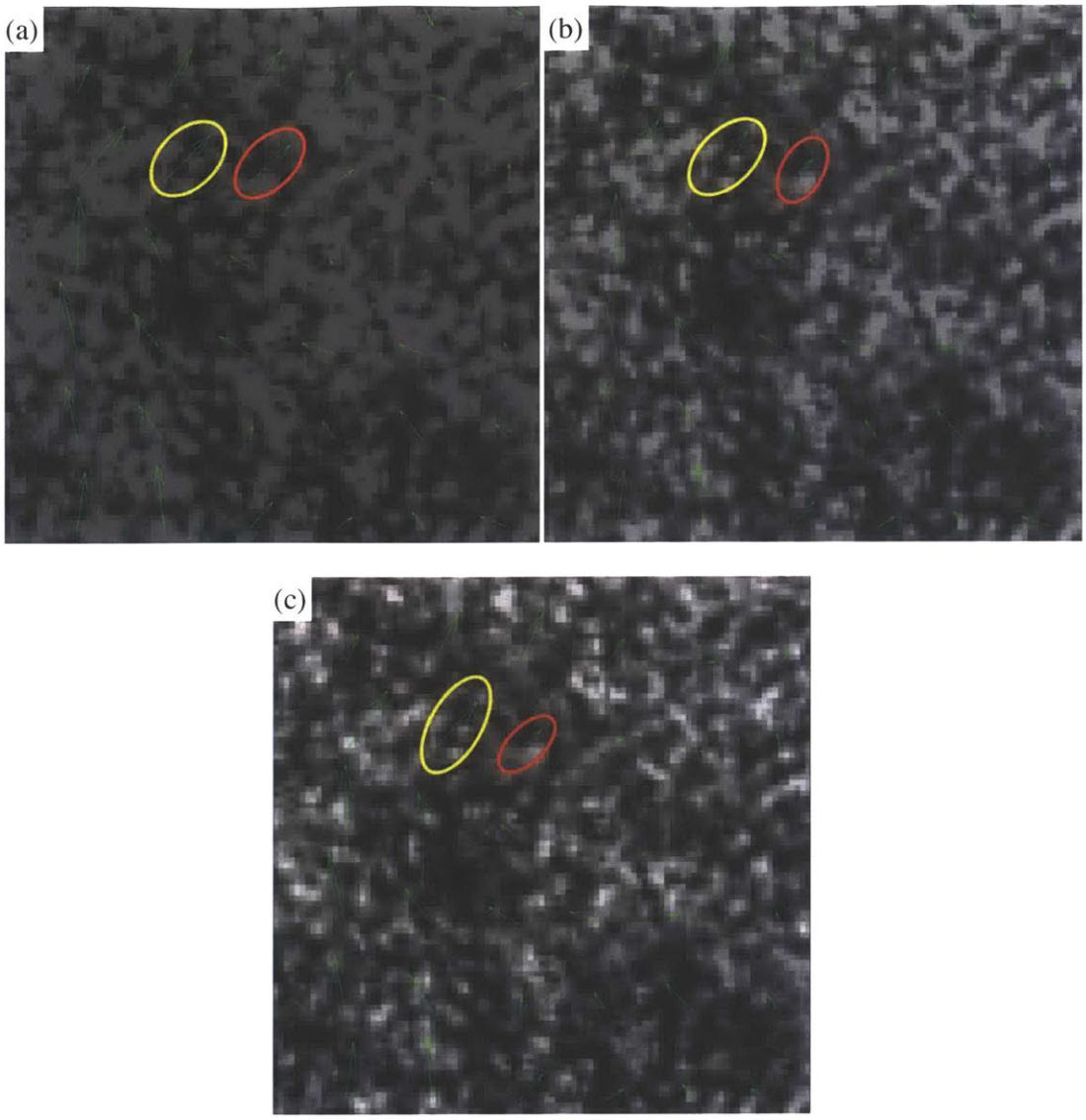

Figure 6.7: Images cut from Figure 6.6. Brightness threshold of the images are (a)100, (b)150, (c)200. Yellow circles contain arrows with the same velocity and direction, while red circles contain arrows with different velocity.

As can be seen from the figure above, in the yellow circles, the velocities of the arrows are all around u=0.8 px/frame, v=-0.8 px/frame; however, in the red circles, the velocities of the arrows are (a) u=0.69 px/frame, v=-0.65 px/frame; (b) u=0.37 px/frame, v=-0.35 px/frame; (c) u=0.36 px/frame, v=-0.35 px/frame. This is a big variance. Since the processed images are the same and the only difference is the threshold of background removal, the velocity should be

almost the same. One explanation to this phenomenon is probably that the 100 threshold is too low for PIVlab to distinguish, especially for those windows where bacteria are not dense (so the color is a bit lighter which may blend with the background if the threshold is too dark). Checking with the original images, it is found that the bacteria in the red circle seem to move slower than those in the yellow circle. Therefore, 150 might be a reasonable choice when setting the threshold of removing bright background noise.

Based on the two cases above, it is important to set threshold in a reasonable range. Higher number would be not that useful, while lower number would affect the detection of swimming bacteria. The best way to determine a threshold is to test several numbers in every new experiment and then determine a number.

6.3 Interrogation window

The size of interrogation window also influence the visualization of bacteria collective motion.

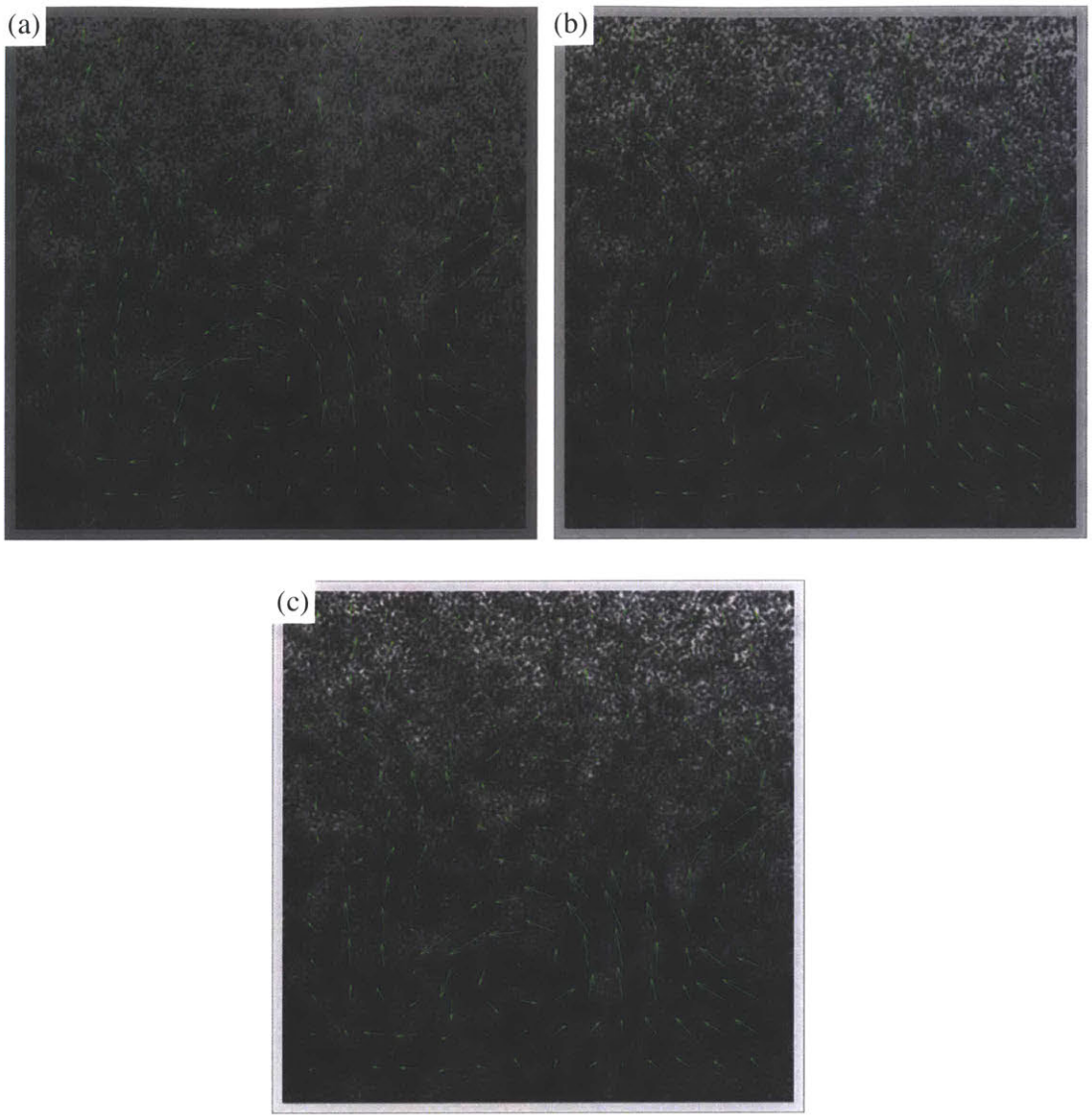

Figure 6.8: PIVlab analysis result of one image pair, interrogation window 64 pixels, with 50% overlapping. Brightness threshold of the images are (a)100, (b)150, (c)200.

As can be seen from Figure 6.6 & 6.7, 6.8, 64 pixels interrogation window is a better one to visualize the collective motion of bacteria more precisely. When it comes to the size of the interrogation window, it should be put in mind that for the two images for PIV analysis, there should be at least two-thirds of the bacteria in the interrogation window remain in the same window. Else the calculation will be imprecise.

6.4 Collective motion pattern

Previous research has found that there are when under flow, the direction of bacterial motion is independent of the width of the flow channel, while different collective motion patterns appear with various flow channel widths [29]. It has been found that the wider the channel, the more swirling patterns shown.

In our experiment, the width of image is around 0.67mm, which is much larger than the previous published paper, and more swirls are seen in our results. The difference may due to the fact that in their experiment the model organism is *B. subtilis*, while in this study *Escherichia coli* is applied. However, the results still prove that collective motion did happen in our experiment, and swirls are the characteristic of bacteria collective motion.

Chapter 7

Influence of viscosity on bacteria collective motion

7.1 Motion comparison between *Escherichia coli* and *Vibrio coralliilyticus*

In previous experiments, *Escherichia coli* is used as the model organism. Here, another type of bacteria, *Vibrio coralliilyticus* is also tested for PIV. *Vibrio coralliilyticus* is a kind of Gram-negative, rod-shaped bacterium [30]. The swimming speed of the two kinds are very different: the mobility of *Escherichia coli* is 16-30μm/s, while *Vibrio coralliilyticus* is around 200μm/s [31]. In the experiment, we did observe that *Vibrio coralliilyticus* did swim much faster than *Escherichia coli*.

However, compared with *Escherichia coli*, the collective motion is more difficult to observe from *Vibrio coralliilyticus*. In Figure 7.1, it is difficult to discern the collective behavior pattern with 64 pixels interrogation window size. If larger window size is applied, the details will be ignored, and what can be seen is just that the bacteria are moving towards left.

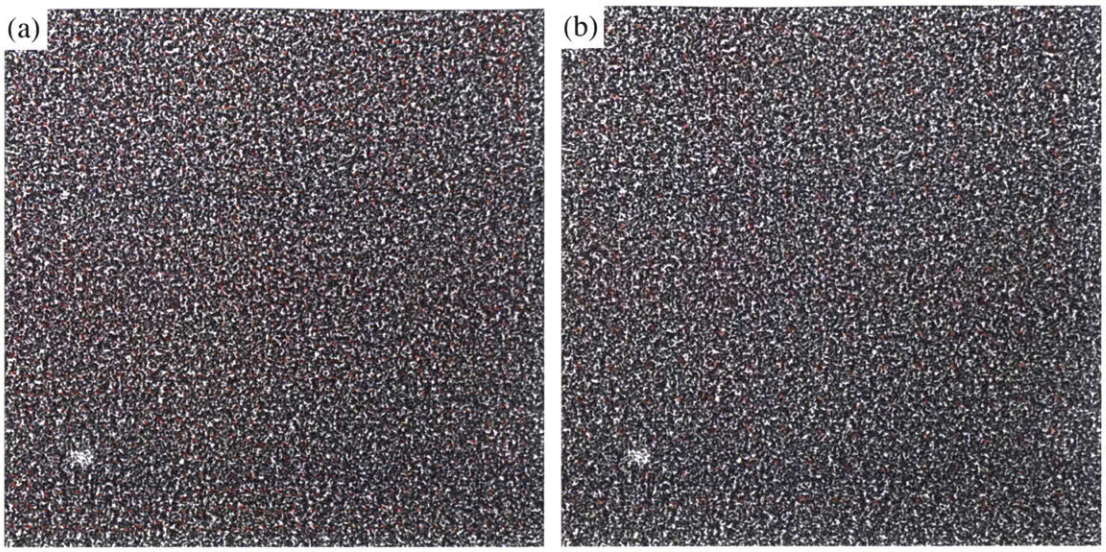

Figure 7.1: PIVlab results of Vibrio coralliilyticus using phase technique with 40x magnitude, (a) 64*64 pixels interrogation window and (b) 128*128 pixels interrogation window.

When it comes to the interrogation window size, in this experiment, 128*128 is a better choice since if we use 64 pixel square, not enough bacteria are included, and it's difficult to distinguish the arrows, too, with smaller window size.

Post processing of PIV data is generally required to obtain reliable results [32]. A basic method to filter outliers in PIVlab is to use vector validation. Velocity thresholds can also be determined by comparing each velocity component with a lower threshold and an upper threshold (t_{lower} and t_{upper}):

$$t_{lower} = u - n * \sigma u$$

$$t_{upper} = u + n * \sigma u$$

where u = mean velocity; σu = standard deviation of u. According to this calculation, the n will determine the actual range. In this experiment, 200 images are taken during 20s, so the time interval is 0.1s between consecutive images. Since the average swimming speed of Vibrio is around 200μm/s, so during each image pair, the longest distance a bacterium can travel is 20μm. Also, since the window size is 344μm for 2048 pixels, so between each frame pair, the average

distance a bacterium can travel should be around 120 pixels per image pair. Combined with the velocity data obtained from PIVlab, we can see that almost all the bacteria fall in the reasonable range.

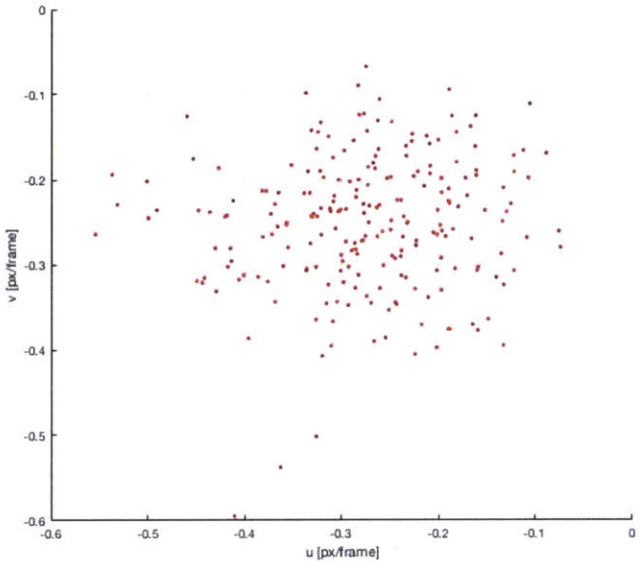

Figure 7.2: velocity of Vibrio coralliilyticus using phase technique with 40x magnitude calculated by PIVlab.

7.2 Fluorescent *Vibrio coralliilyticus* VS wildtype

Fluorescent *Vibrio coralliilyticus* cells are also used in this experiment to compare the phase technique and fluorescence technique. The *Vibrio coralliilyticus* strain in encoded with fluorescent gene (Texas Red) and fluorescent protein is expressed in those cells. Under microscope, fluorescent cells are observed.

However, after exported to TIF images, the contrast is so low that only pure dark image is observed. To solve this problem, MATLAB pre-processing is applied. After that, images are imported to PIVlab for further examination. The results are shown below:

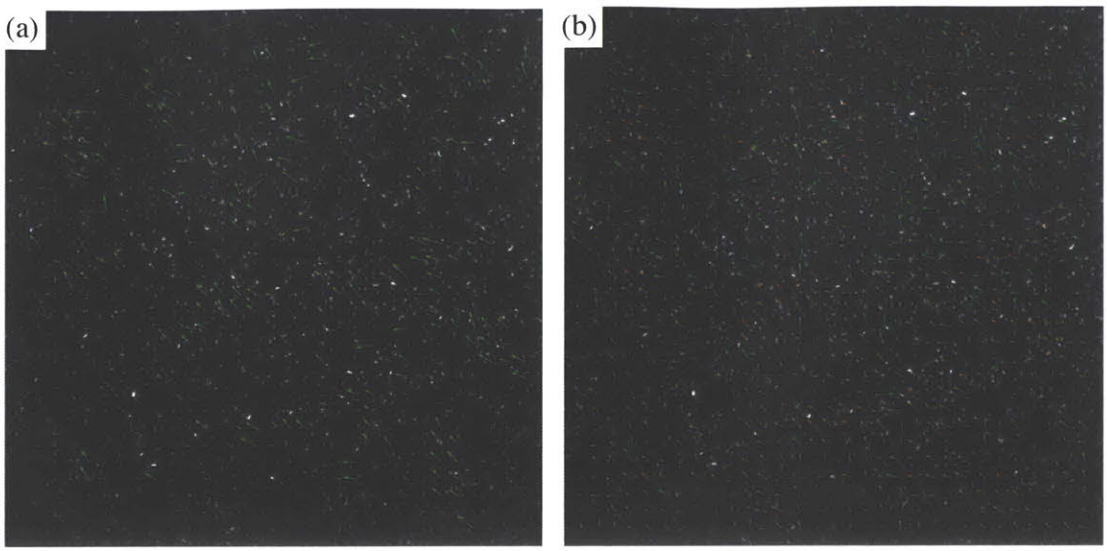

Figure 7.3: PIVlab results of Vibrio coralliilyticus (Texas Red) using fluorescent technique with 40x magnitude, 128*128 pixels interrogation window. (a) before vector validation and (b) after vector validation.

Comparing Figure 7.1 and 7.3, we can see that in Figure 7.1, there are more bacteria than in Figure 7.3. In theory, the number of bacteria should be roughly the same as all the other procedures are the same. One explanation could be that the expression of fluorescent protein is not abundant enough, leading to the missing of bacteria under fluorescent microscope. Another explanation could be that the fluorescent *Vibrio coralliilyticus* is not as lively as the wild-type. Therefore, part of fluorescent *Vibrio coralliilyticus* might be already dead when put under microscope. Human error could also be one possible reason, as the growth of bacteria is sometimes unpredictable; and the contrast enhancement process might also influence the results. Further tests into the contrast adjustment of the images is need for better results. However, if those lost cells are dead ones, then using this method might be a better one since the result will not be influenced by those dead cells which might be considered immobilized when using phase-contrast method.

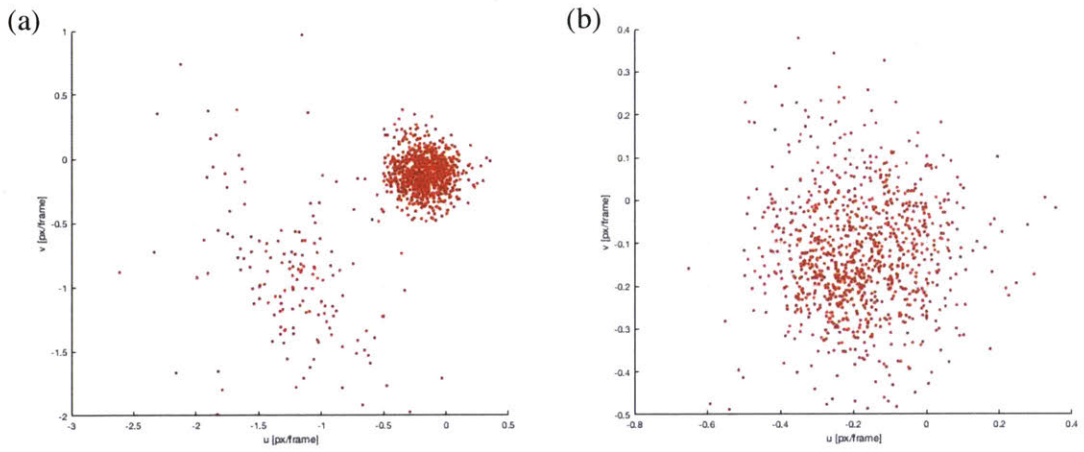

Figure 7.4: velocity of Vibrio coralliilyticus using phase technique with 40x magnitude calculated by PIVlab. (a) before vector validation and (b) after vector validation.

Another interesting phenomenon is the swimming speed difference. From Figure 7.2 we can see that the fastest swimming speed is 0.6 px/frame. However, in Figure 7.4, some bacteria even reach 3 px/frame velocity, which is 4 times as the normal ones (the time steps of images are taken into calculation). From 7.4 (a) we can see that a great number of bacteria swim between 0~0.5 px/frame, and the rest swim randomly in random directions. Those outliers could result from the failure of PIVlab to recognize bacteria correctly. After vector validation, the result is shown in Figure 7.4 (b).

Though not as clear as *Escherichia coli*, where swirls are observed easily, the *Vibrio coralliilyticus* sample also displays a few clues for collective motion.

7.3 Viscosity influence on *Vibrio coralliilyticus* collective motion

Using the same fluorescent *Vibrio coralliilyticus* cells, the impact of viscosity on the collective behavior bacteria is investigated. In this experiment, glycerol is used to make the solution more viscous. 40μl glycerol and water stock solution is mixed with 160 μl bacteria, reaching a final viscosity equals to 4 times of water. The figure below shows the PIVlab results before and after

vector validation.

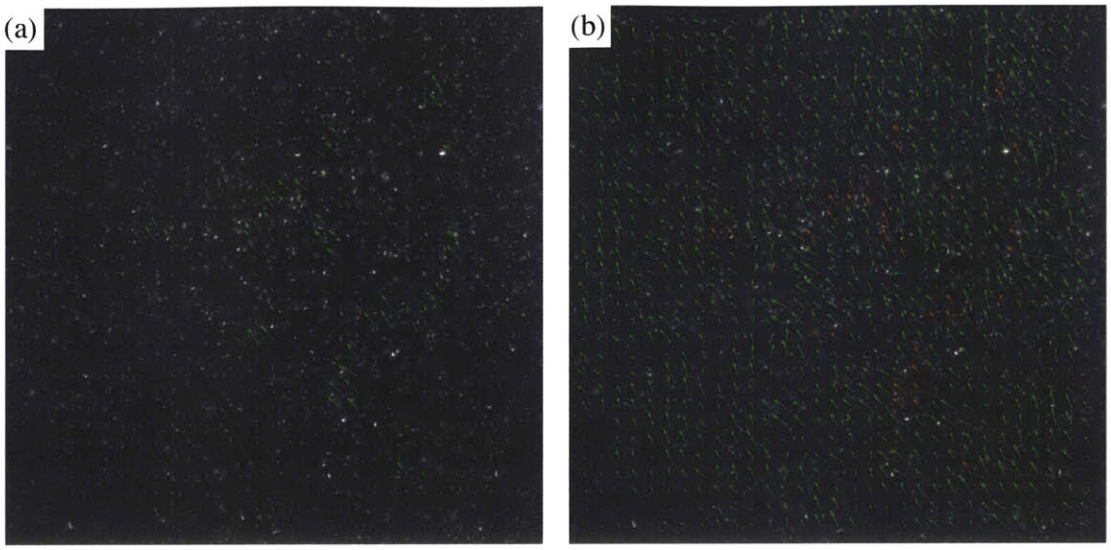

Figure 7.5: PIVlab results of Vibrio coralliilyticus (Texas Red) using fluorescent technique with 40x magnitude, 128*128 pixels interrogation window. (a) before vector validation and (b) after vector validation.

The same case as before, before vector validation, the velocity of bacteria varies so much that some are out of normal range. Vector validation is conducted to refine the u & v between 0~1 px/frame, as shown below.

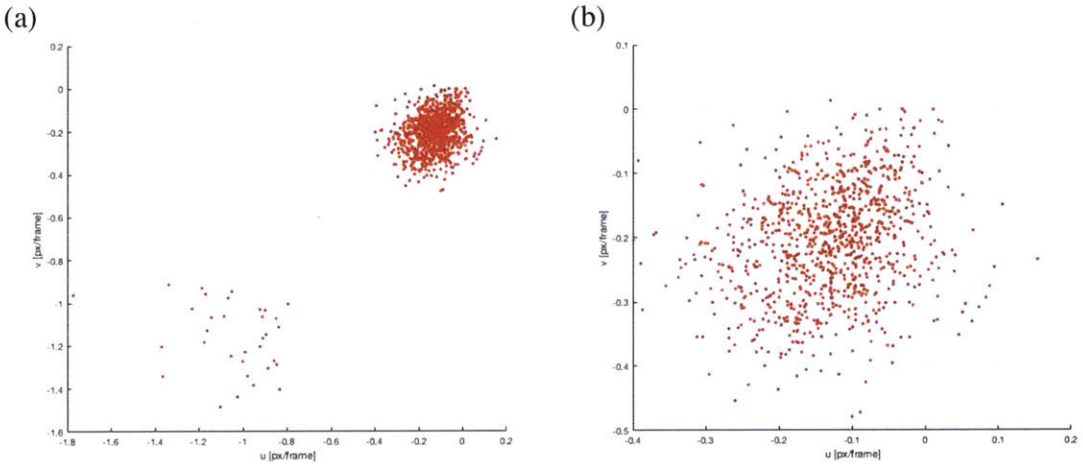

Figure 7.6: velocity of Vibrio coralliilyticus using fluorescent technique with 40x magnitude calculated by PIVlab. (a) before vector validation and (b) after vector validation.

The influence of viscosity can be viewed in two ways: the mobility and the collective motion pattern. For the sample with 4 times viscosity of water, the velocity range of the bacteria is

$$u = -0.4 \sim 0.2 \text{ px/frame}$$

$$v = -0.5 \sim 0 \text{ px/frame}$$

However, for the sample with water as the solution, the average velocity of the bacteria is

$$u = -0.8 \sim 0.4 \text{ px/frame}$$

$$v_{mean} = -0.5 \sim 0.4 \text{ px/frame}$$

We can tell from the data that the velocity range of low viscosity is smaller than the range of high viscosity one. Thus, higher viscosity will lower the mobility of bacteria. This result is in accordance with previous research, which shows that the average velocity will decrease when viscosity of the agar increase for flagellated bacteria [24].

When it comes to the influence of viscosity on bacteria collective motion, a detailed comparison between sample with 4 times viscosity of water and sample with water as the solution is shown below.

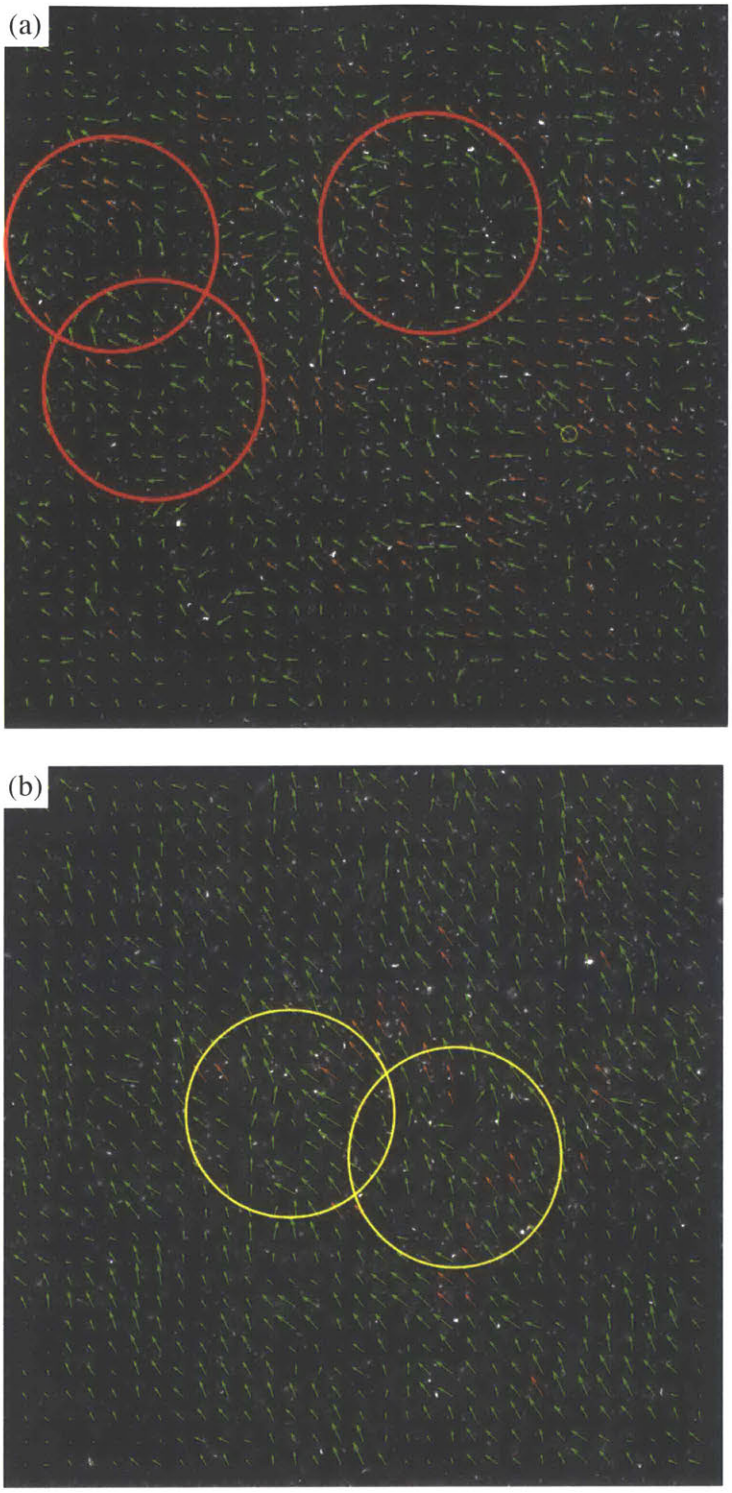

Figure 7.7: PIVlab results of Vibrio coralliilyticus (Texas Red) using fluorescent technique with 40x magnitude, 128*128 pixels interrogation window. (a) low viscosity and (b) high viscosity.

From Figure 7.7, we can see that though *Vibrio coralliilyticus* doesn't display collective motion

pattern as good as *Escherichia coli*, there's still clues for collective behavior, as pointed out in the red circle. Especially for the upper-left circle, a swirl-like pattern can be seen. However, for the high viscous sample, though in the yellow circle area the bacteria are not swimming in the same direction and might be the precursor of a swirl, no clear collective motion pattern can be observed. The non-uniform direction might result from the fact that the suspension is not smooth enough and impurities in the way of bacteria motion could also lead to such pattern.

Another simple observation is that in the low viscosity image, the swimming direction is diverse, while in the high viscosity one the bacteria swimming direction is more uniform, which implies that high viscosity will restrict the motion of bacteria, so high viscosity could also inhibit collective motion.

In summary, from current data it is reasonable to conclude that high viscosity will inhibit the formation of collective motion among *Vibrio coralliilyticus*.

Chapter 8

Conclusions

How contrasts in fluid viscosity would influence the collective motion of swimming bacteria is a problem that remains to be solved. In this paper, PIV is applied to visualize the swimming patterns and behavior of *Escherichia coli* and *Vibrio coralliilyticus*.

For the methodology part, it has been found that when visualizing *Escherichia coli*, using phase-contrast method at 40x magnitude is the optimum practice. When using PIVlab to analyze bacteria motion, a collective motion pattern has been observed which is similar to previous researches on bacteria suspension. It has been found that the function "adapthisteq" is a better contract enhancement method, though the threshold of brightness of each pixel exerts a more important role. The best interrogation window size and the threshold value largely depend on the images obtained from microscope camera.

The influence of viscosity contrast on bacteria behavior is also investigated. It has been found that increasing viscosity will result in the loss of mobility for swimming bacteria; and based on the *Vibrio coralliilyticus* results in this research, it could be concluded that high viscosity will inhibit the formation of collective motion among *Vibrio coralliilyticus*.

Though conclusion has been drawn from this research, there are still a lot of problems remaining to be solved. Firstly, more experiments using fluorescent *Vibrio coralliilyticus* should be conducted. Normally, three groups of data are needed to draw a conclusion, but due to the difficulty in cell cultivation and the fact that the microscope was broken for a while, only limited sets of data are obtained for analysis. In the future, more sets of parallel experiments should be conducted and more images should be taken to make the result more convincing.

Secondly, there is still room for image-preprocessing before PIVlab analysis. Currently, the threshold of brightness and contrast enhancement are tested to make the images of swimming

more readable for PIVlab to process. In the future, more different image processing methods should be tried, and if possible it would be more convenient to develop a new toolbox based on PIVlab for customized PIV experiments specially for our experiments.

Finally, due to limited time, the viscosity ladder was not tested. In the future, different viscosity solutions should be tested to see the influence of viscosity on bacteria collective motion. It would be interesting to see the bacteria collective motion pattern change when one man-made micro-swimmer (which is able to change speed and direction) is mixed with the bacteria suspension. The results would be useful to explain the underlying principles of bacteria collective behavior under specially varying fluid viscosity.

Appendix

MATLAB Code

1. Pre-processing of bacteria images from microscope camera

```
%This short program pre-processes the images obtained from camera for PIVlab analysis later%
clear all
close all
clc
% go to the place where pictures are stored
cd ('/Users/yinmenghu/Desktop/vibrio/_vcorr1dayphase_');

%read the pictures in matlab
srcfiles=dir('/Users/yinmenghu/Desktop/vibrio/_vcorr1dayphase_/vcorr1dayphase_T*.tif');

%create a loop to process all the pictures in this folder
for i=1:1:length(srcfiles);
   filename=strcat(srcfiles(i).name);
   I=imread(filename);
   %by putting all the values over 30000 into 30000
   %all those bright spots are eliminated
   I(find(I>30000))=30000;
   % change from uint 16 to uint 8 for best PIVlab post processing
   J=im2uint8(I);
   %go to another folder to store the processed images
   cd ('/Users/yinmenghu/Desktop/vibrio/Processed vcorr1dayphase');
   %create a new loop to name the images in 3 digits for more convenient
   %PIVlab analysis
    if i<10;
      name = ['00', num2str(i)];
   imwrite(J,name,'tif');
   elseif 10<=i & i<100;
      name = ['0', num2str(i)];
   imwrite(J,name,'tif');
   else
      imwrite(J,num2str(i),'tif');
   end
   close all
   %go back to the original folder
   cd ('/Users/yinmenghu/Desktop/vibrio/_vcorr1dayphase_');
end
```

2. Contrast and Threshold Test of Images

```
% This short program conducts Contrast and Threshold Test for PIVlab analysis later%
close all
clear all
clc
% read pictures, test 2 first
% go to the place where pictures are stored
cd ('/Users/yinmenghu/Desktop/Lab Project/0406/0406 original');

%read the pictures in matlab
srcfiles=dir('/Users/yinmenghu/Desktop/Lab Project/0406/0406 original/Process_18_*.tif');
%create a loop to treat all the pictures

for i=1:2;%pick 2 pictures
   filename=strcat(srcfiles(i).name);
   I=imread(filename);
   cd('/Users/yinmenghu/Desktop/Lab Project/0406/0406 threshold & contrast test');
   name = ['original', num2str(i)];
   imwrite (I, name, 'tif');

   % 3 process method
   Ia=imadjust(I);
   name = ['imadjust', num2str(i)];
   imwrite (Ia, name, 'tif');
   mm1=Ia;

   %threshold 30000, 40000, 50000
   m=[30000, 40000, 50000];
   for n=1:1:3;
   Ia(find(Ia>m(n)))=m(n);
   name = [num2str(m(n)), 'imadjust', num2str(i)];
   Ja=im2uint8(Ia);
   imwrite (Ja, name, 'tif');
   Ia=mm1;
   end

   %method 2
   Ib=histeq(I);
   name = ['histeq', num2str(i)];
   imwrite (Ib, name, 'tif');
   mm2=Ib;

    %threshold 30000, 40000, 50000
   m=[30000, 40000, 50000];
   for n=1:1:3;
   Ib(find(Ib>m(n)))=m(n);
   name = [num2str(m(n)), 'histeq', num2str(i)];
   Jb=im2uint8(Ib);
   imwrite (Jb, name, 'tif');
   Ib=mm2;
   end

    %method 3
```

```
    Ic=adapthisteq(I);
    name = ['adapthisteq', num2str(i)];
    imwrite (Ic, name, 'tif');
    mm3=Ic;

     %threshold 30000, 40000, 50000
    m=[30000, 40000, 50000];
    for n=1:1:3;
    Ic(find(Ic>m(n)))=m(n);
    name = [num2str(m(n)),'adapthisteq', num2str(i)];
    Jc=im2uint8(Ic);
    imwrite (Jc, name, 'tif');
    Ic=mm3;
    end

    close all
    cd ('/Users/yinmenghu/Desktop/Lab Project/0406/0406 original');
end
```

3. Pre-processing of fluorescent samples

```
%This short program pre-processes the images of flouruscent sample images obtained from camera
for PIVlab analysis later%
clear all
close all
clc
% go to the place where pictures are stored
cd ('/Users/yinmenghu/Desktop/vibrio/_vcorr1dayMay101to5M4_');

%read the pictures in matlab
srcfiles=dir('/Users/yinmenghu/Desktop/vibrio/_vcorr1dayMay101to5M4_/*.tif');

%create a loop to process all the pictures in this folder
for i=1:1:length(srcfiles);
   filename=strcat(srcfiles(i).name);
   I=imread(filename);
   % For the flourescent images, the contrast is so low that only black
   % color can be seen. This function magnititude the contrast by 200
   % times since the original threshold is 0~300 and the wanted should be
   % 0~60000
   J = imadjust(I,[0 0.005],[0 1]) ;
   % change from uint 16 to uint 8 for best PIVlab post processing
   J=im2uint8(J);
   %go to another folder to store the processed images
   cd ('/Users/yinmenghu/Desktop/vibrio/Processed _vcorr1dayMay101to5M4_');
   %create a new loop to name the images in 3 digits for more convenient
   %PIVlab analysis
    if i<10;
      name = ['00', num2str(i)];
   imwrite(J,name,'tif');
   elseif 10<=i & i<100;
      name = ['0', num2str(i)];
   imwrite(J,name,'tif');
   else
      imwrite(J,num2str(i),'tif');
   end
   close all
   %go back to the original folder
   cd ('/Users/yinmenghu/Desktop/vibrio/_vcorr1dayMay101to5M4_');
end
```

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