Analyzing twitching surface motility of *P.aeruginosa* bacteria by MATLAB tracking program and determining effects of surface conditions on rate of movement

*Phys*4002 Final report*

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Abstract

Pseudomonas aeruginosa is a highly versatile and adaptable bacteria becoming notorious for it's high resistance to host anti-microbials and antibiotics. As such it has caused chronic infections from biofilms forming on mucosal surfaces becoming a major medical problem. *P. aeruginosa* have been observed to undergo various surface motility modes such as swarming and twitching which are used to expand the colony outward. These modes of surface motility are highly cooperative in their behavior indicating a physiological change from planktonic to surface environment. Factors that affect their movement can be temperature, nutrition density, surface conditions, bacteria density as well as external defense proteins used to inhibit the growth of a biofilm. Effects of surface conditions are analyzed by spin coating thin agar films and behavioural changes in bacteria are observed. A method to analyze changes in behavior is presented here. A code written in MATLAB was developed and can be used to quantitatively identify and track trajectories of bacterial objects on a surface which has been used to determine the velocity and behavioural changes of individual bacteria. It promises to be a useful tool when quantitative measurements on bacteria movement are required.

Introduction

Opportunistic human pathogens such as *P. aeruginosa* bacteria can cause nosocomial pneumonia, and chronic infections such as urinary tract infections and sepsis in burn wound and immunocompromised patients.[1] In 2008 a paper was published by Hancock's group from the Centre for Microbial Diseases & Immunity Research, at the University of British Columbia, showing that cationic human host defense peptide LL-37

inhibits biofilm formation by stimulating twitching motility, as well as decreasing attachment and causing the down regulation of certain genes used in biofilm formation.[2] Twitching is a form of surface translocation used by a wide variety of bacteria that involve type IV pili. Pilus retraction can generate forces up to 140pN, which is approximately 10000 times the weight of a bacteria[3]. Important parameters such as temperature and moisture, as well as nutrition level and surface hardness may affect the surface motion. These parameters are to be investigated as well as a quantitative analysis technique is presented to measure the change in bacterial behaviour under varying conditions and in the future, in the presence of LL-37 protein. To do this a tracking program written in MATLAB was developed, allowing for the mean velocity to be calculated as well as a plot of the tracked positions, which can be used to better analyse individual motion.

Methods

Growing and imaging

Pseudomonas aeruginosa, strain PA01 wild type bacteria were grown on a standard LB agar plate from a frozen stock culture over night for 16 hours in an incubator at 37^{o}C. The standard LB agar plates contain salt, yeast extract and tryptone and are 1.5% (w/v). After growing the bacteria overnight, they are then transferred to a 1% (w/v) LB agar plate with the same ratio of nutrients, which is ideal for twitching to occur. After transferring them to the 1% plate, we incubated again for another 2 hours to allow time for the bacteria to transition into a surface associated state. Afterwards they were placed in a temperature-controlled cell, which maintained a 30^{o}C environment to

begin imaging. For imaging, a bright field microscope using 60X magnification and Image Pro Plus was used for the data acquisition.

Temperature and moisture control

From our observations of the standard method for producing twitching motion of bacteria on 1% agar surface, it was decided that temperature as well as moisture level during observation under the microscope should be controlled as this may have an affect on the rate of twitching motion. To do this we employed a simple design where a heating unit and thermal couple are attached to a custom designed stage mount to be used with the bright field microscope. Figure 1 shows a picture of the stage mount, where the glass slide sits in the middle of a heating unit. The slide holder is shown in figure 2. A water bath on one side of the holder is used to maintain a humid environment, with an opening that can be used to insert a probe to measure the vapour pressure of the surface of our sample. Measurements of these two variables hope to aid in the consistency of the twitching bacteria observations as well as provide a further understanding of the mechanism, if there is a strong dependence found.

Thin agar films

As an interesting experiment and as a way to control the nutrition level and hardness of the surface, we employed a new approach to produce the surface on which the bacteria would undergo their mode of translocation. We used a spin coater, rotating at 3000rpm to create thin films of agar, mixed with LB broth. This introduced some challenges since agar itself is a gel and so needed to be heated before as well as during the spin coating process to remain as a liquid long enough to fully cover the surface. Heating before was done using a hot plate with magnetic stir rod set to approximately

70^{o}C. A watch glass was placed on top of the beaker to keep the concentration constant. Heating lamps were used to try and keep the agar from cooling before spreading when placed on the glass slide in the spin coater and the temperature at the surface was approximately 40^{o}C. A further challenge when trying to create these films and keep them flat and uniform is that LB broth is composed of tryptone, yeast and electrolytes in the form of salt and are required to provide essential nutrients to the bacteria. The wide range of molecules makes uniform spreading as well as determining the uniformity of the film produced challenging. Although it was not done, due to a shortage of time, an idea to overcome this challenge is to perform the spin coating with just agar, and soak the thin films in liquid LB broth in hopes of re-introducing the nutrients as well as increasing the moisture content. To analyze the surface we used contact mode AFM.

Agar surface imaging

In order to compare the thin agar films to what is already known to be a suitable surface for bacterial twitching motion, AFM images of a 1% agar surface would be used to analyse the surface. Contact mode AFM could not be used since the gel surface was too soft to be registered, even with a low set point voltage. The AFM tip also easily damaged the surface. To overcome this, tapping mode AFM was used. Tapping mode AFM uses a vibrating cantilever and measures a change in phase of the natural frequency of oscillation in air this is then used to infer changes in height of the surface.

Quantitative analysis by MATLAB tracking

As a method to quantitatively determine differences in bacterial behaviour from either a change in temperature, moisture level or in the presence of LL-37, analysis using particle tracking provided a method to determine dispersion rates and plots of individual

bacterium movement. To do this an image processing program was developed that also produces a list of positions of the objects identified. This is done using several different MATLAB functions within the image toolbox. First the background is removed by first using “imopen” function which morphologically opens the image with a specified object to be ignored. I used an oval shape to form around the bacteria. A binary image is then the result after dividing the intensities to be normalized to 1, this is done by the “greythresh” function. Figure 3 shows a before and after sample processed image, figure 4 shows an example of the oval template used. The objects are then identified by “bwconncomp” function which looks for connected components in a binary image. It uses a connectivity of 8 (nearest and next nearest neighbours on a 2D lattice). Each object is given a Pixel Id list, which is a 1-by-NumObjects cell array where the kth element in the cell array is a vector containing the linear indices of the pixels in the kth object. The centroid positions of each object can be found by “regionprops” function, giving properties of the kth object. This image processing is then done for a stack of tiff images forming a position list for each time step, and the positions are connected in time by use of Grier and Crocker’s track algorithm originally written in IDL but converted to MATLAB by Blair and Dufresne. This algorithm is available to the public online and connects an unsorted list of positions to individual objects, essentially “connecting the dots” in time by assigning closest positions between time steps to one object. The track algorithm requires an approximate maximum displacement to limit the search to a local area around an object. It should be noted that it is is then best to use small time steps when imaging to allow for optimal tracking.

Results and discussion

Thin agar films and regular agar surface

As a way to produce a surface in which the nutrient content was not “infinite”, and the surface hardness was like that of a rigid structure such as medical equipment, table top or door handle, thin agar films were produced. Figure 5 shows a sample AFM image of the surface and as one can see there are clear dendritic-like structures formed. This is believed to be a result of the salt components and may suggest the agar dried out before gelation occurred. Figure 6 shows a sample AFM image of a regular agar surface, however unlike the thin agar films the surface could not be imaged using contact mode AFM and so tapping mode was used. This is a result of the surface being gel-like and was too soft to be registered when approaching the tip in contact mode, even with a low set point voltage. The surface was so soft in fact that even in tapping mode molecules were easily removed and adsorbed onto the tip of the cantilever, changing its vibration frequency and resulted in noisy imaging as the tip rastered across the surface. Another property that was found while imaging was that the surface is “sticky”. This was deduced from the AFM tip suddenly disengaging during imaging. This may be a result of as the AFM applies a voltage to keep the cantilever oscillating, a sudden release from adhesion to the surface resulted in an over compensated amplitude, disengaging the tip from the surface. These differences in surface properties and the very fact that the thin agar films were easily imaged using contact mode AFM shows how different the surfaces are and how soft the regular 1% surface is. Twitching was not observed on the thin agar films and from these findings it is believed that it is not simply a nutrition content effect but rather a result of the surface properties itself. It is expected that these bacteria do not move on hard surfaces but can only undergo twitching on soft, moist surfaces. These conditions

are often associated with biofilm formation and so this may provide further evidence that twitching motility mode is used to expand the colony in order to produce a biofilm.

Tracking

In order to quantitatively determine behavioural changes and to gain a more physical understanding of the twitching motility mode, a tracking program was designed and developed. Currently the program can be used to obtain the mean velocity of bacteria motion captured under a bright field microscope and produce a output plot of their resulted trajectories, figure 8 shows a sample trajectory plot of a sequence of 384 images separated by 0.5s containing approximately 783 bacterium per image. The mean velocity calculated by averaging over all instantaneous velocities is 0.447um/s, with a variance of 0.273um/s, see figure 9. This agrees with most literary sources where twitching occurs at approximately 0.5um/s [2]. The large variance is expected to be a result of the twitching motion itself. It is not a constant motion but jerky in its nature giving rise to varied speeds during the motion of this sort. Also, how frequently a bacterium moves is dependant on its position relative to other bacteria. From observations of the plotted trajectories, often bacteria that are isolated, i.e. has no neighbours outside of several body lengths, move to form “pods”, which can be described as small groups of bacteria that continue in a cooperative manner in the same direction. It has been suggested previously that isolated bacteria cannot move, however perhaps it is not that isolated bacteria cannot move but rather the bacteria that cannot move become isolated in time, since they will not move to form these cooperative areas. There are many potential behavioural observations that can be analyzed by the tracking program and it is designed to be flexible. A simple example

of how behavioural changes can be quantitatively analyzed is by comparing newly added bacteria to a surface with bacteria that which have been incubated on the surface for 2hrs. Newly added bacteria have a much lower mean velocity of 0.117um/s with variance 0.031um/s. Comparing this to the previous values for velocity show clear evidence that time is required for the bacteria to accommodate to a surface, undergoing a physiological change to allow for movement to occur and it is not instantaneous. Figure 10 shows the velocity distribution of the newly introduced bacteria. As was mentioned previously, twitching bacteria can be observed to form cooperative pods. Another quantitative analysis technique that can be implemented by the tracking program is to determine the identified objects orientation. This is still in early stages of coding however figure 11 shows a contour plot of calculated bacteria orientations, the different colours correspond to an angle relative to the x-axis. This could then be done for a stack of images and how the orientations change as a function of time could be analysed. What is expected to be observed is that the regions of similar orientation increase in size over time and may be a way to quantitatively determine stages of biofilm formation. This can be compared analogously to a non-organic system of liquid crystals and the orientation function of the bacteria can be correlated in a similar way to phase changes of the liquid crystal model. This is only in preliminary stages but is a promising direction for the project to develop towards. An interesting experiment would be to see whether non-motile bacterial strains still orientate and how the system may evolve with time as the colony grows in time.

Conclusions

As with any new project to a group, many challenges have been faced throughout. Some were overcome and others emerged as dead ends forcing the project to change as a result, however the overall goal remained the same: To characterize and understand the rate and behavior of the twitching surface motility mode of *P.aeruginosa* when colonizing a surface. This project certainly moved in the forward direction. A new method for developing a surface with variable rigidness and nutrition content was investigated by spin coating thin films of agar. Although this surface was not suitable for the PA01 to undergo twitching motility, new insight into surface conditions was found when both surfaces were imaged by AFM. The regular 1% agar surface is extremely soft and moist which are also important conditions for biofilm formation and only this type of surface produces twitching motion. The thin film surface was hard and evidence of drying rather than gelation was shown by the dendritic salt formations seen in the AFM image. The vast differences in the surface conditions and the change in bacteria behavior suggest that it is not just a nutrition content dependent motility mode. To better understand the twitching motility mode and its affect on biofilm formation it is best to analyze other external effects. In order to get a quantitative understanding of the bacteria motion, a tracking program was developed and produces promising results for future use towards understanding the twitching motility mode and its affect on biofilm formation. Currently the program can determine mean instantaneous velocities and output plotted trajectories for individual bacteria imaged under bright field microscopy. This showed evidence for an adaptation period by the bacteria since newly introduced bacteria to a 1% agar surface had a much lower mean velocity then compared to bacteria which were

incubated on the surface for 2hrs. There is however much more potential to the program other than velocities and distances but also the orientation of the bacteria motion can be correlated and analysed in time. Early stages of the technique have been developed and were presented showing promise to the idea. This may provide a method to quantitatively analyze biofilm formation where a dense colony should show correlated orientation. The program will also hope to quantitatively analyze the effect of LL-37 defense protein as well as others that may provide a promising effect to reduce biofilm formation and prevent nosocomial infections in the future.

Figures

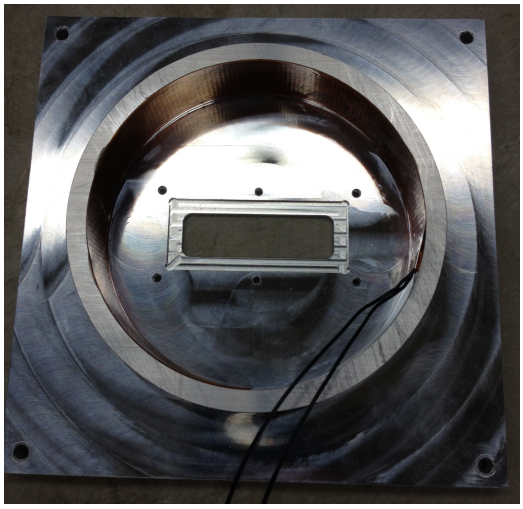

Figure 1: Showing custom-built stage mount with heating unit to be used with TIRF microscope

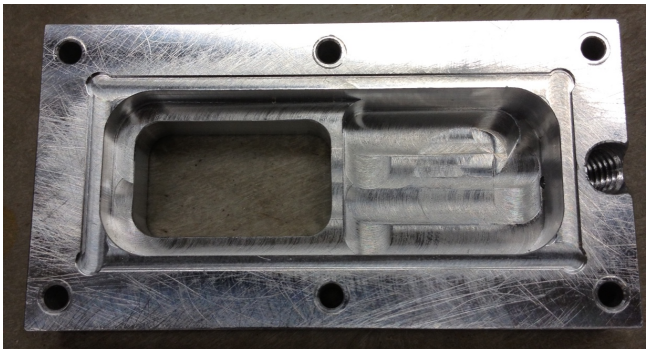

Figure 2: Showing custom-built slide holder with a site for a water bath to keep moisture levels high

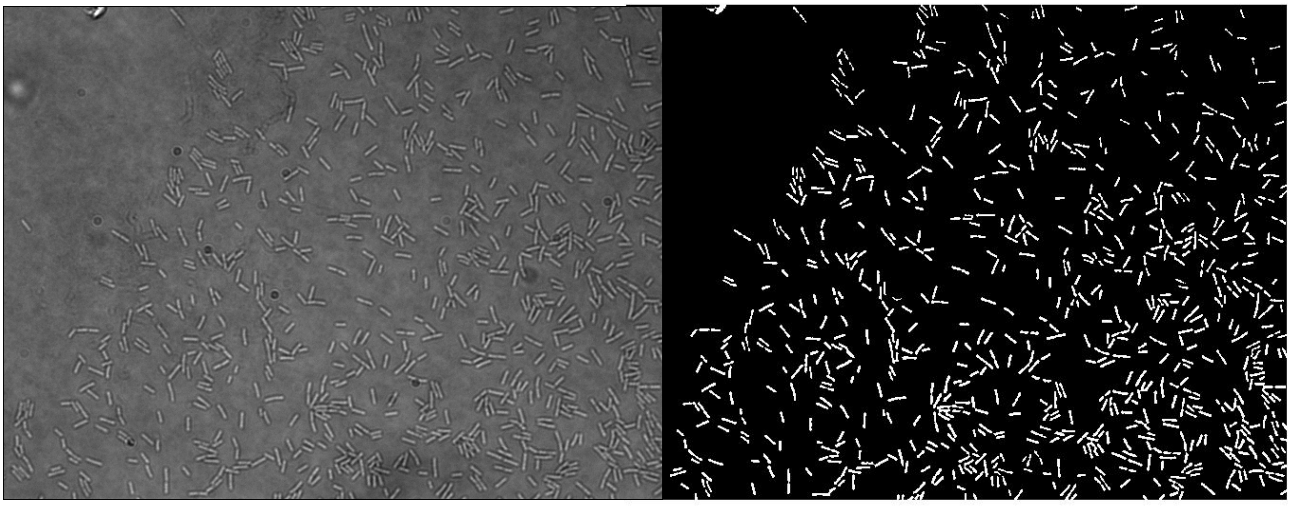

Figure 3: (left) An image taken of twitching *P.aeruginosa* at 60x from bright field microscope. (right) A processed image using MATLAB in which the background has been removed and individual bacteria are highlighted in white.

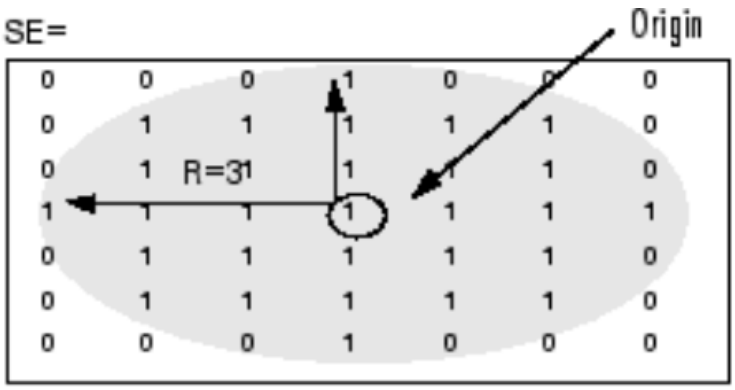

Figure 4: Diagram showing the binary object template and how the origin and major minor axis are calculated

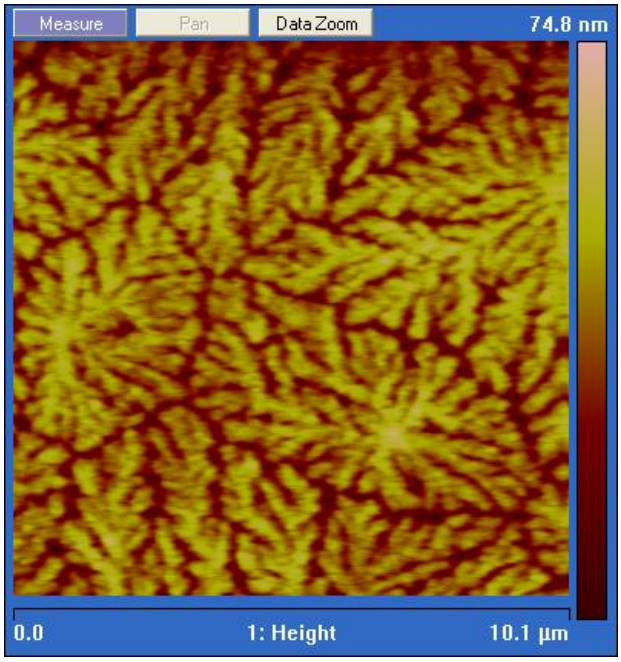

Figure 5: AFM contact mode image of thin agar film produced from spin coating

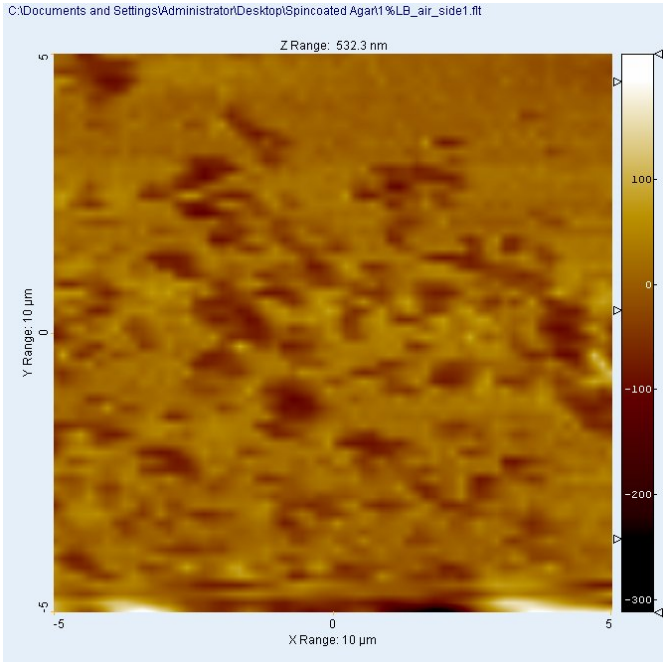

Figure 6: AFM tapping mode image of regular 1% agar surface. This is the agar-air interface surface

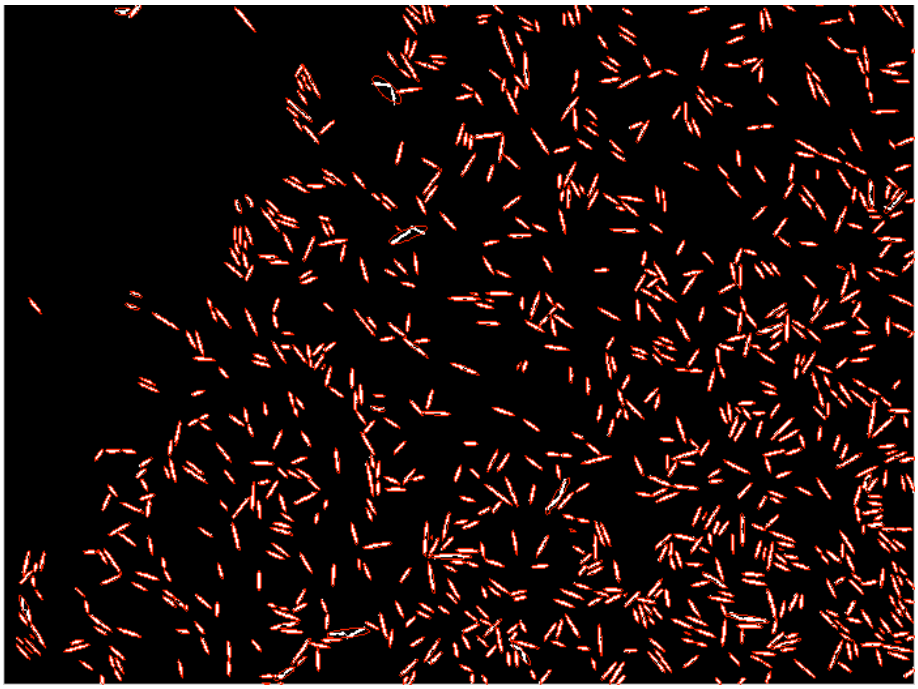

Figure 7: Showing the processed binary image representation of the original bright field microscopy image. The detected objects are outlined in red. Some can be seen to include more than one bacterium but for the majority only one is identified

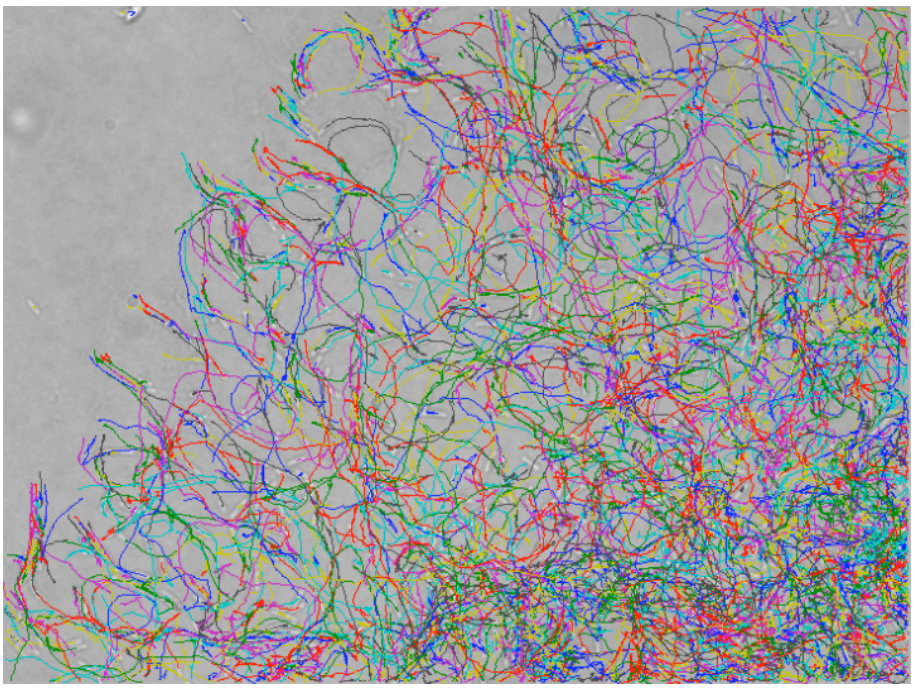

Figure 8: Plot of bacteria trajectories. Taken after 384 times steps separated by 0.5s. Each colour corresponds to a different trajectory and is over layered on top of the original bright field image.

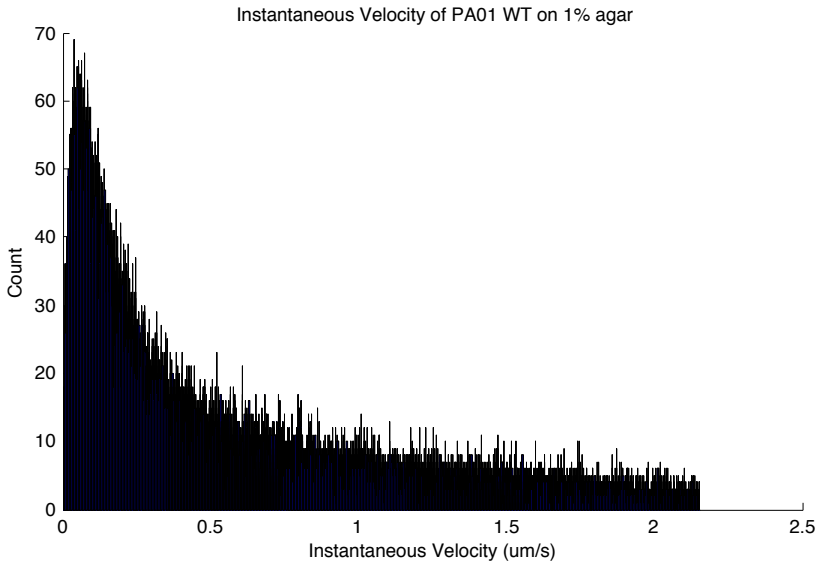

Figure 9: Plot of instantaneous bacteria velocities on 1% agar surface after 2hr incubation period before imaging. Mean velocity is 0.447um/s. The wide distribution can be attributed to the jerky nature of twitching motion as well as the surrounding conditions of each bacteria may affect their movement

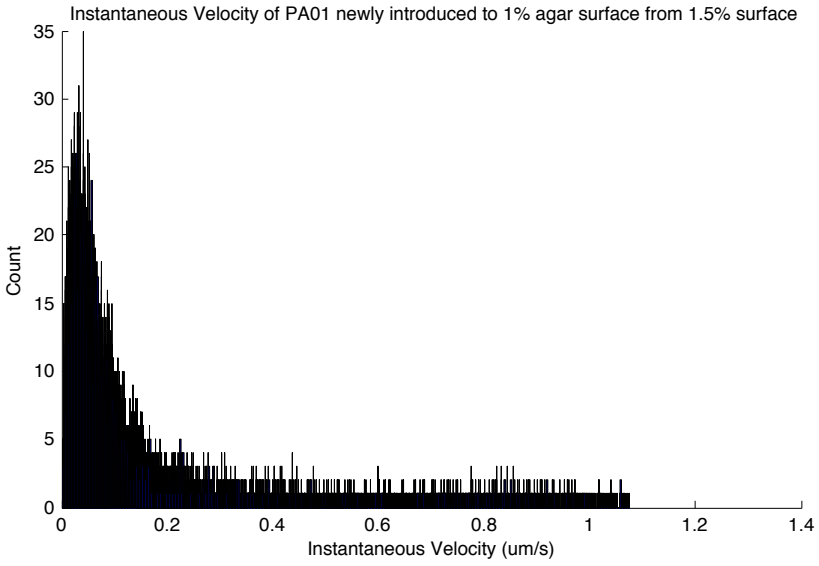

Figure 10: Plot of instantaneous bacteria velocities on 1% agar surface after no incubation period before imaging. Much narrower peak with mean velocity 0.117um/s showing evidence for an adaptation period required for bacteria to undergo physiological change before movement on surface can occur

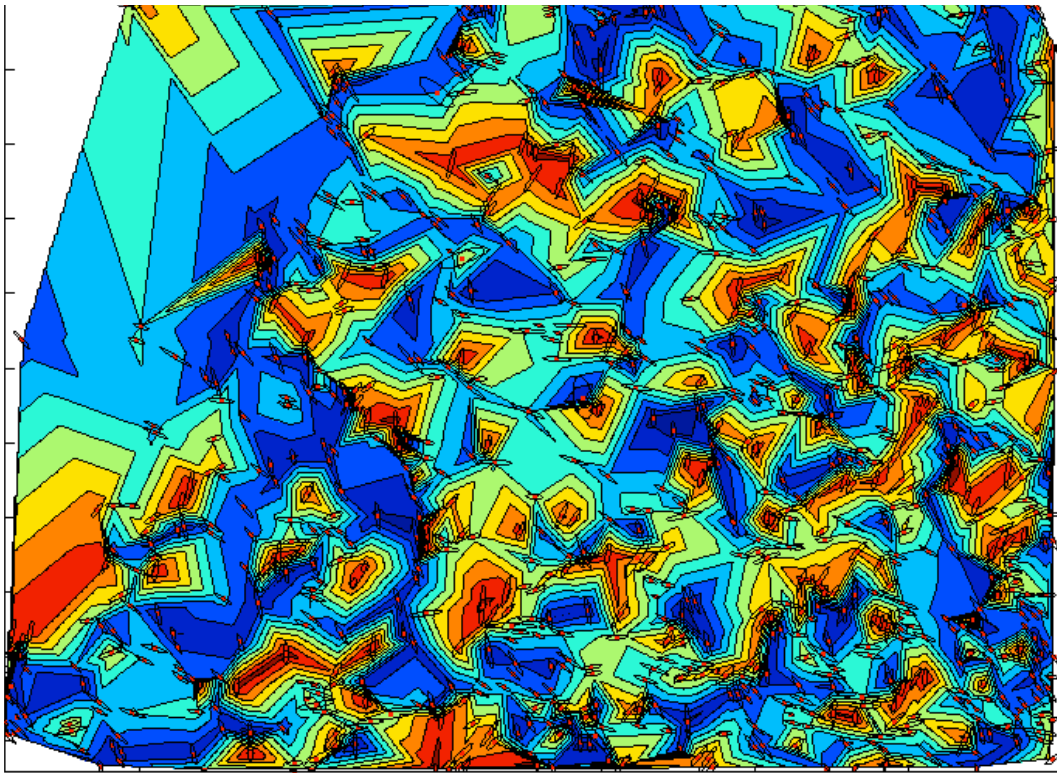

Figure 12: Contour plot showing areas of common orientation of bacteria. The colours indicate different orientations. Centroid locations and the outline of objects identified is also plotted to compare colours to orientation angles.

References

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