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Developing a method for imaging and analyzing live bacterial motion on various types of surfaces

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12 May 2015
Confidential-1

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Dear Madam:

This report, entitled "Developing a method for imaging and analyzing live bacterial motion on various types of surfaces", was prepared as my 2A Work Report for the University of Waterloo. This report is in fulfillment of the course WKRPT 200. The purpose of this report is to demonstrate the development process undergone in order to achieve a good method for live bacterial imaging and analysis, as well as present the results obtained on bacterial motion and settlement on various surfaces. It is a confidential-1 report.

The University of Toronto is one of the most renowned public research universities in the world, and the lab I worked in was part of its Materials Science and Engineering department. The department was designed with the establishment of four theme areas of specialization in mind, nanomaterials and nanotechnology being the most applicable.

The Hatton Lab Group at the University of Toronto is supervised by Dr. Benjamin Hatton, and specializes in micro and nanostructured surfaces and adaptive materials. A very common theme amongst the group members is designing and engineering antimicrobial surfaces. This report was written for my supervisor, Professor Benjamin Hatton.

I would like to thank Dr. Benjamin Hatton for providing me with this great opportunity to work in his lab, and obtain valuable research experience in the biomedical and nanotechnology fields. I would also like to thank Dalal Asker for helping me develop my project goals and methods, as well as helping me grow the bacteria necessary for my experiments. Finally, I would like to thank Nicolas Lavielle for providing me with a few samples of his surfaces to test. I hereby confirm that I have received no further help other than what is mentioned above in writing this report. I also confirm this report has not been previously submitted for academic credit at this or any other academic institution.

Sincerely,

Dhilan Bekah
ID 20512356

Contributions

Dr. Benjamin Hatton's Lab Group is part of the Materials Science and Engineering Department at the University of Toronto, and consisted of four postdoctoral research fellows, two graduate students, and one undergraduate student (me).

Professor Hatton returned to the University of Toronto after serving as a staff scientist in the Wyss Institute for Biologically Inspired Engineering at Harvard University, specializing in microstructured surfaces and adaptive materials. Most recently, he has been involved in patterning surfaces and surface structure to change wettability and adhesion properties for antimicrobial applications. As a result, the team's main project themes are bio-inspired nanomaterials design, self-assembly of nanocomposite and nanoporous structure, surface microstructure engineering, bacterial contamination of surfaces and biofouling, organic/inorganic templating, and adaptive surface structures.

As the undergraduate student, my tasks initially involved shadowing and helping the other group members with their projects, but after I completed all the necessary training, I was able to begin my own project. One of the more basic tasks required in the beginning was to get the dry lab correctly set up with the wireless network, as well as installing office and windows on all the computers. I then began to work with one of the graduate students on her colloidal ink jet printing project, which consisted of helping setup the XY-Stage for the print head, and the interactive Labview software associated with it. Then, once I completed the necessary biosafety training course at the University of Toronto, I was able to begin my project on analyzing live bacterial motion on various different material and structured surfaces.

This report focuses on my main project during my co-op experience with the Hatton Lab Group. During this time, I have gained a tremendous amount of knowledge, most of which is directly related to my academic experience as a nanotechnology engineer, but also as someone who is looking to enter into the field of biomedicine. The most valuable skill I acquired was working with a high-end microscope capable of a variety of different imaging techniques, but specifically fluorescence microscopy. It is rapidly expanding in many medical and biological research fields, and has been talked about several times in my own classes in regards to the applications of nanotechnology. In addition, being part of a team with group members that are far more knowledgeable and experienced has given me a lot of insight into what is most commonly used in nanotechnology and biomedical research, and what they are used for. As an example, one of the

graduate students was using Scanning Electron Microscopy (SEM) to image his nanoparticles that he synthesized, and I learned a lot about his whole general process from start to finish from our group meetings and presentations.

When Professor Hatton was working at the Wyss Institute at Harvard, he became interested with how bacteria move on different surfaces after observing them during one of his projects, but he was never able to follow through with it. One of the common themes of the group is antimicrobial surfaces, and so understanding how bacteria move and settle on surfaces would greatly help. Having me on the team with no prior project to pursue enabled him to start developing this idea of his. In addition, not only did I develop the imaging, but I also tested multiple analysis software for acquiring quantitative data. Writing this report has made me think more critically about my experience, and has made me realize that my project has in fact served as a tool for my group to use going forward to more efficiently acquire better results, rather than actually getting all the good results myself.

Summary

The purpose of the report is to describe the method that was developed for imaging live bacterial attachment on various surfaces and for qualitatively and quantitatively analyzing the acquired data. The report also, however, serves to demonstrate how the project carried a much greater importance in pioneering the testing with the new fluorescence microscope and imaging with the new software, while also developing a method to image in the future. Thus, the lab group can further pursue this project, as well as how it is related to the rest of the projects.

The report is structured in a way that first introduces the background knowledge to properly understand where the project problem comes from. Bacterial attachment and surface interactions are described, leading to the goal of the analysis (Section 1). The methodology is then described, elaborating on the process of learning the hardware and software necessary to run the experiments and analyze the resulting data. The experimental conditions are also explained, defining what is controlled and what is being changed (Section 2). The report then goes on to show all of the acquisition stages and surfaces used, and present examples of the acquired data for each. Simultaneously, the development of the imaging method is illustrated through the differences in the figures as the section progresses (Section 3). Finally, a more in depth analysis is presented on the software programs used, and what they can potentially do for extrapolation and more meaningful results in the future (Section 4).

The major conclusions in this report are that the process in developing a reliable and efficient imaging and image analysis method provided an equal or greater value to the results acquired from the project itself. In addition, the data presented reinforced the baseline research already done on cell attachment to modified surfaces, as linear patterns reduced attachment compared to flat surfaces. Furthermore, new data was presented that could be further analyzed to compare cell attachment and movement between specific material surfaces.

The major recommendations in this report are to further analyze the quantitative data obtained, as described in the software analysis (Section 4), and to continue refining the method by testing more surfaces and examining whether modifications need to be made. Due to the time limitations presented near the end of the co-op term, further data extrapolation was not possible, and so it is likely that the data presented will provide some very new insight as to how different materials affect surface attachment.

Conclusions

Based on the results of this report analysis, it was concluded that the process in developing a reliable and efficient imaging and image analysis method provided an equal or greater value to the results acquired from the project itself. The fluorescent microscope arrived during the work term and so this project was intended to familiarize oneself with its workings and capabilities. It enabled the pioneering of a new imaging method, as well as new analysis software.

The multichannel feature is commonly used with two colour filters for staining different parts of the organism. In this project, it was used with a combination of the common green fluorescent protein (GFP) filter, and either the brightfield (BF) filter or the Differential Interference Contrast (DIC) filter to acquire images, and it worked superbly. When using surfaces with defined structures, the patterns can be seen with either the BF or DIC filters, while the GFP filter overlays the stained bacterial cells on top, making qualitative analysis very easy.

Multiple software programs were also explored, with potential for further extrapolation in each. Based on the analysis in the report, CellC is ideal for quick enumeration of bacterial images, while the Matlab script is good for qualitatively tracking the motion of bacteria in videos / time-lapse videos.

Moreover, the results themselves reinforced the baseline research already done on cell attachment to modified surfaces, as linear patterns reduced attachment compared to flat surfaces. The PDMS microgrooved structures showed the same settlement patterns as expected, and coincided with previous lab group findings on the preferential growth patterns of bacteria on such surfaces.

Recommendations

Based on the analysis and conclusions drawn in this report, the main recommendations in this report are to further analyze the quantitative data obtained, as described in the software analysis (Section 4), and to continue refining the method by testing more surfaces and examining whether modifications need to be made. Moving forward, the key steps to be taken are:

1. Use the quantitative enumeration data from the CellC results to compare cell attachment densities on all the surfaces, and make correlations between what surfaces reduce cell attachment.
2. Use the qualitative data from the bactrack overlaid tracks to compare general movements between surfaces. Flat surface motion and microgroove surface motion would have very distinct tracks ideally.
3. Look further into the quantitative data from the bactrack generated statistics file to see how reliable the velocity numbers are by tracking positions over time and optimizing the Matlab script. Also examine how the velocities are affected from surface to surface, and see whether motion on the surface is altered.
4. Test more samples and surfaces to continue refining the imaging method by thinking of any new problems that could arise.
5. Test SLIP surfaces, as the time limitations of the work term prevented such trials, and they would be the next step in analyzing surface attachment. They are known to prevent settlement, but quantitative data using the aforementioned software programs would further reinforce this knowledge.

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1 Introduction

Bacteria exist in either planktonic (free in bulk solution) or sessile (as a unit attached to a surface or within a biofilm) populations ⁽²⁾. The formation of biofilms begins with the conditioning layer, which can be anything present in the bulk fluid that is able to settle on the substrate/surface ⁽²⁾. Then, a small number of bacterial cells that were transported from the bulk liquid to the now conditioned surface start to adhere. Phase contrast and confocal light microscopy have shown that planktonic cells are attracted to surfaces via motility (i.e. by force of its parts like the flagella) or by Brownian motion, and explore the area around them in species-specific behavior ⁽¹⁾⁽⁴⁾. Some species' cells roll across the surface before initiating their adhesion behavior, which leads to the formation of monolayers on the surface, as cells will roll away from already adherent cells. Cells of other species move very little before adhering, while others congregate in certain locations on the surface forming isolated microcolonies ⁽¹⁾. These observations show that bacterial cells can sense their proximity to surfaces, and react accordingly. At the Rowland institute at Harvard, the Berg group analyzed the motility of bacteria, and found two distinct motions by studying swimming E.coli. "Running" is when the cell moves steadily forward when pushed by a bundle of filaments, while "tumbling" is when the cell moves erratically in place with little net displacement when the bundle comes apart ⁽⁵⁾. While this report does not go into great depth in regards to individual cell motion, it does tend to the differences in settlement and overall motion of the bacteria.

1.1 Surface treatments and patterns

It is well known that bacterial adhesion to surfaces is mediated by the physical and chemical properties of the substrate, as well as the surface characteristics of the organism ⁽³⁾. In addition, topographical features that limit cell-surface interactions have been shown to inhibit surface colonization biofilm formation ⁽³⁾. In one study, patterned polydimethylsiloxane (PDMS) surfaces were used in static and microfluidic conditions, and cell attachment was observed. The results showed that the highest attachment density occurred on flat, un-patterned surfaces, and that linear patterned surfaces greatly reduced cell attachment ⁽³⁾. Moreover, surfaces with holes further reduced cell attachment ⁽³⁾. This project was intended to reinforce those observations, as well as introduce new results in regards to the material of the surfaces.

1.2 Goal of the Analysis

The initial objectives of the project were to image live bacterial attachment to various surfaces, use image analysis software to enumerate and track the bacteria, and to compare the effect of different surfaces on the bacterial motion. While all of them were met, this report serves to also demonstrate how the project carried a much greater importance in pioneering the testing with the new fluorescence microscope and imaging with the new software, while also developing a method to image in the future.

2 Methodology

In order to gather reliable data efficiently, a method for running experiments must be developed, especially when learning to work with new hardware and software. Modifications are inevitable and, more often than not, beneficial, but a baseline must be established with proper controlled, dependant, and independent variables.

2.1 Learning the hardware and software

The most essential part of the project was fluorescence microscopy, so learning how to operate the microscope and associated software was required. Before even working with the bacteria, however, 11- μm polystyrene colloidal particles were used to test out the image acquisition and analysis processes. The microscope used was the Olympus BX-63, and was capable of 5x – 100x magnification, with both air and water/oil immersion lenses. A few drops were placed in between a glass slide and a cover slip, and placed under the microscope lens. While the green fluorescent protein (GFP) filter was not used with these particles (as they could not be stained), these runs were meant to familiarize oneself with the basic focus and movement mechanics of the microscope. Furthermore, the Brightfield (BF) and Differential Interference Contrast (DIC) filters were used and explored, which proved to be extremely fruitful in the development of the imaging method, as described in subsequent parts of the report. Another benefit to running test acquisitions with the colloidal particles was learning the interface for the modular imaging software, cellSens, associated with the microscope. Getting a grasp on what features are useful and what is just noise saves valuable time, and allows for a greater number of more consistent results. Moreover, save and export settings were determined for easy future access and analysis. Finally, the analysis software was learned and tested with the colloidal particles to ensure that they were functioning correctly. One of the programs, entitled CellC, can be used to enumerate the number of cells in one or several images frames, following a few parameters such as thresholding, cluster division algorithm etc. CellC was run multiple times with acquired images of the particles, and an example of the total count figure generated is shown in figure 2-1.

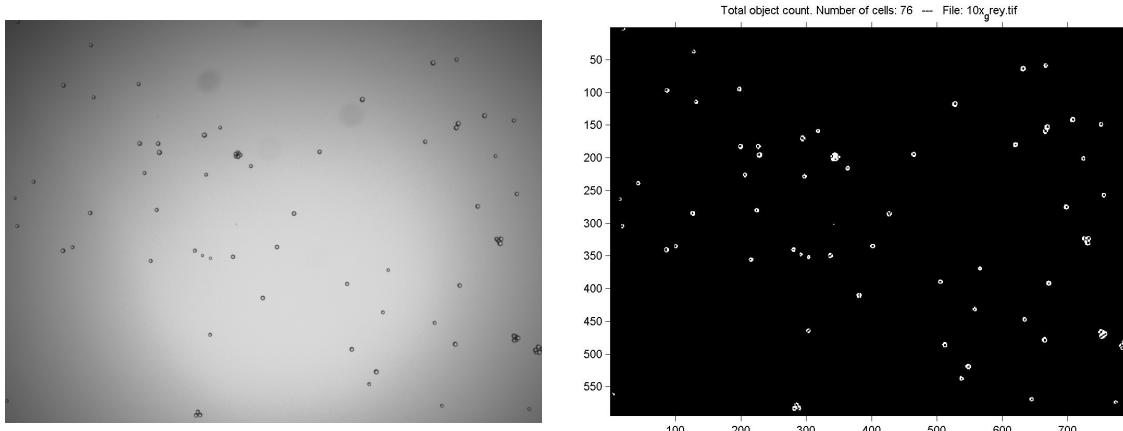


Figure 2-1. (a) 11- μm PS Colloidal Particles at 20x; (b) CellC enumeration of 11- μm PS Colloidal Particles at 20x

The other program tested with the particle data was a Matlab compiled script called backtrack, which analyzes a video file and generates a file containing information on the velocities of the particles. The second version of the script can also overlay the tracks it drew internally onto the original movie frames. The scripts are both open source code, and also required slight modification as the syntax was out of date. An example of the results, as well as a more in depth analysis on all of the software is done in section 4 of the report.

2.2 Experimental Conditions

One of the postdoctoral research fellows who also worked closely on the project had a strong background in microbiology, and had carried out several experiments that required similar conditions. As a result, *Escherichia Coli* was chosen as the bacteria strain, and the method of observation was fluorescence microscopy, so the Syto 9 Green Fluorescent Nucleic Acid Stain was used. The types of surfaces to be tested were smooth glass, smooth polyurethane, and smooth polydimethylsiloxane (PDMS), as well as microstructured polyurethane and PDMS. The process that was initially established went as follows:

- Cure desired surface onto a plain glass slide, and repeat for the number of trials required
- Put clear, double sided tape on 3 of the 4 surrounding sides of the surfaces
- Stain the bacterial culture in media with the Syto 9 dye and let sit for 10 minutes
- Make a 1/5 and a 1/10 dilution
- Place a cover slip on top of surface, with a little bit of the surface uncovered

- Deposit a small amount of the bacteria-containing liquid onto the uncovered surface (capillary action will draw the liquid under the cover slip)
- Examine using fluorescent microscope
- Acquire Data

From an engineering standpoint, processes must always be refined in order to maximize efficiency, and over the course of running the experiments, a few modifications were made. Firstly, since the bacteria were being stained in media, the image background appeared to also be stained under fluorescent light, resulting in worse image quality. This was due to the fact that the medium contained yeast extract, and since Syto 9 stains DNA, it stained parts of the medium as well. Therefore, the tubes were lightly centrifuged in order to harvest the cells from the culture medium, and then resuspend in buffer. In addition, any free dye was washed after staining to reduce the background even further, as can clearly be seen by comparing figure 2-2.

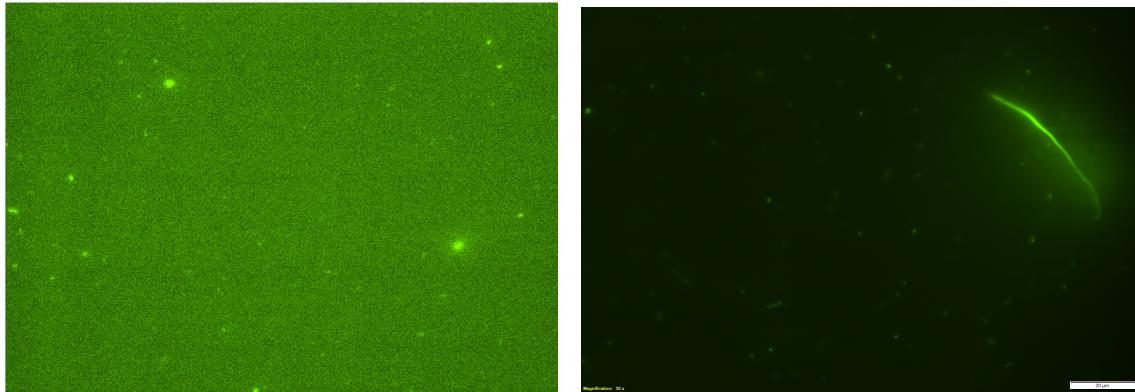


Figure 2-2. (a) Fluorescence image in medium; (b) Fluorescence image in buffer, after wash

The second modification arose from the fact that many of the surfaces were too elevated for the clear tape to provide a good boundary, as well as stick to the cover slip on top. This is because the process was originally designed for the smooth glass since that was the first surface to test. As a result, double-sided black tape (whose thickness could be easily adjusted to suit the surface thickness) was placed on the surrounding sides of the surface to be analyzed instead. The result of the change is shown in figure 2-3.



Figure 2-3. Simple microfluidic device for depositing liquid containing bacteria

3 Acquisition and Results

3.1 Smooth Glass

The microscope slides are made of glass themselves, and thus no surface curing was required. Initially, 3 concentrations for both 3-hour and 18-hour growth of the bacterial liquid were used to determine what would be most convenient and efficient for image analysis. As demonstrated by figure 3-1, the 1x dilution contained too much bacteria, and enumeration/tracking tasks would prove too difficult.

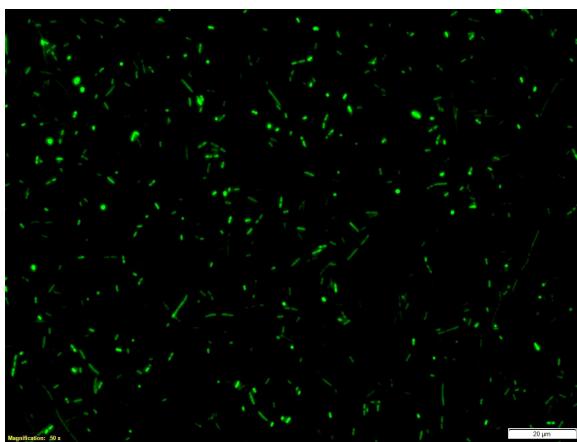


Figure 3-1. 1x dilution of 3-hour grown E.coli on smooth glass

In addition, there was practically no difference between the 3-hour and 18-hour culture growths (other than a bit more biofilm formation and chaining in the 18-hour, but it was not of great importance to the project). Therefore, going forward, only the 5x and 10x dilutions for the 3-hour growth cultures were used. Figures 3-2 and 3-3 demonstrate these conditions on smooth glass.

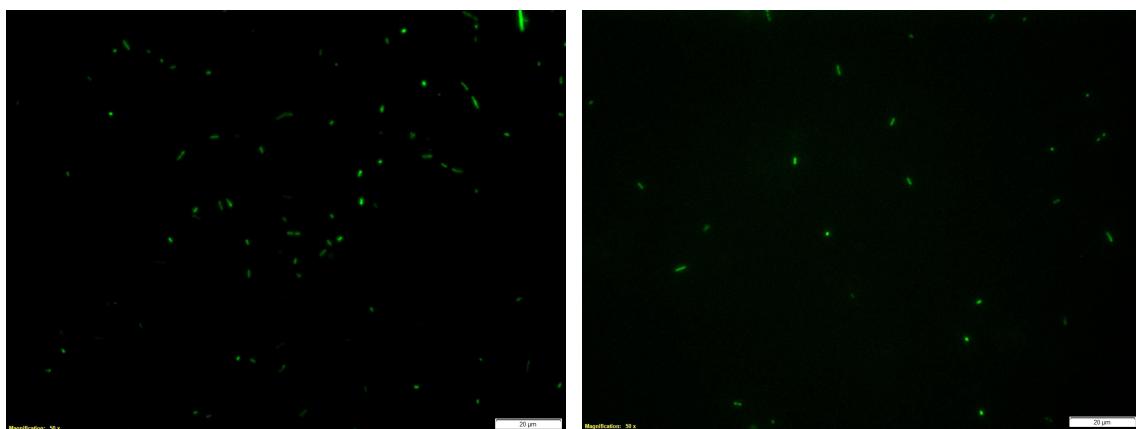


Figure 3-2. (a) 5x dilution of 3-hour grown E.coli on smooth glass (b) 10x dilution

3.2 Smooth Polyurethane

To create the smooth polyurethane surfaces, a few drops of polyurethane liquid are deposited onto a smooth PDMS mould, and then a glass slide is placed on top. The ensemble is then placed under the UV curing machine and, after about a minute, the glass slide is pulled off of the PDMS mould, leaving a thin smooth layer of polyurethane on the slide, as portrayed by figure 3-3.

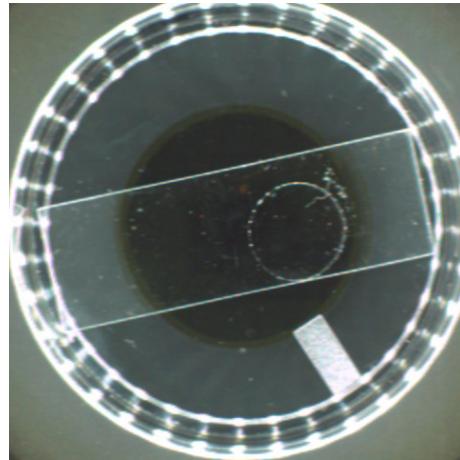


Figure 3-3. Smooth Polyurethane Surface

Several time-lapse images were taken at 15-20 frames every 5 seconds. A few of the best were compiled into video format for easy viewing, and can be accessed from the “Smooth Polyurethane” folder in the [Google Drive folder](#). Some of the results were analyzed with the software, as described in section 4.

3.3 Structured Polyurethane

Two different structures were tested using polyurethane as the surface material. One of them was less applicable as a microstructure since the pattern was too large, but still served as good acquisition practice, especially since it led to the development of a key part in the imaging method.

3.3.1 Waffle Patterned Structure

A few drops of the polyurethane liquid were deposited onto the waffle patterned PDMS mould, and cured onto a glass slide through the same process as before, as shown in figure 3-4.

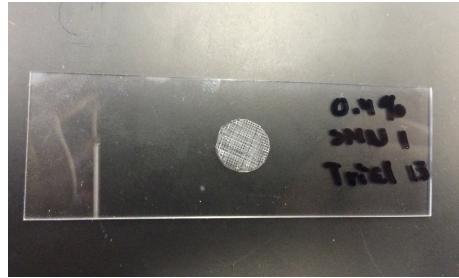


Figure 3-4. Waffle Patterned Polyurethane Surface

When the liquid containing the bacteria is deposited onto the surface, the small gap allows for bacteria to appear on different elevations (i.e. on the bottom and top surfaces, as well as flowing through the middle). The pattern, however, does not appear when using the fluorescence filter, and so the focus was adjusted using the BF filter, in order to make sure that the bacteria being observed are on the desired bottom surface. At this point in the process, the postdoctoral research fellow suggested trying a feature of the software called multichannel that allows you to image using two filters at the same time. Figure 3-5 illustrates the pattern from the BF filter, overlaid with the stained bacteria from the GFP filter.

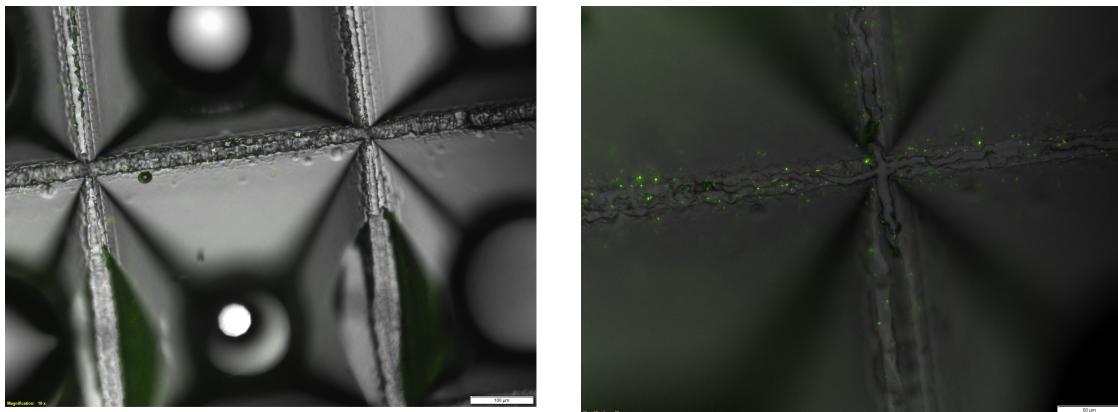


Figure 3-5. (a) Multichannel waffle-patterned structure at 10x; (b) At 20x

In addition, to ensure that the bacteria were not just settling on the ridges defining the pattern, videos were taken inside the holes (BF worked best in this case due to the light contrast inside the hole), and can be viewed in the “Structured Polyurethane 1” folder in the [Google Drive folder](#).

3.3.2 Sharklet Patterned Structure

Using the same curing method, sharklet pattern slides were created using the sharklet PDMS mould. Several videos and time-lapse images were taken with BF, GFP, and multichannel conditions, and can all be viewed in the “Structured Polyurethane 2” folder in the [Google Drive folder](#). The pattern is illustrated in figure 3-6, and figure 3-7 shows a multichannel snapshot of a time-lapse video capturing motion on the surface.

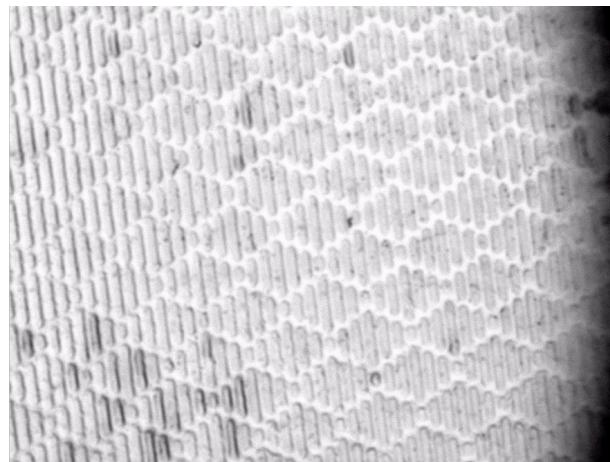


Figure 3-6. BF Sharklet-patterned structure at 50x



Figure 3-7. Multichannel Sharklet-patterned structure at 50x

3.4 PDMS

3.4.1 Microgrooves Structure 3

Ultimately, the project was intended to relate to and help the lab group with all the other projects, but more specifically with the anti-attachment and Slippery Liquid-Infused Porous (SLIP) surfaces. One of the postdoctoral research fellows working with microgrooved structures lent me some of his surfaces to test. No curing onto microscope slides was required, as he had already created these surfaces on PDMS in a clean room. This specific microgroove structure was his third, and had a groove height of 15 μm , a groove width of 10 μm , and a spread between the grooves of 30 μm .

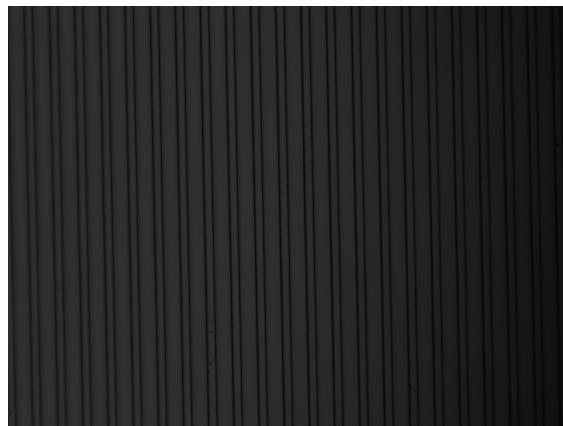


Figure 3-8. PDMS Microgroove structure 3

A snapshot at the beginning of the acquisition is shown in figure 3-9.

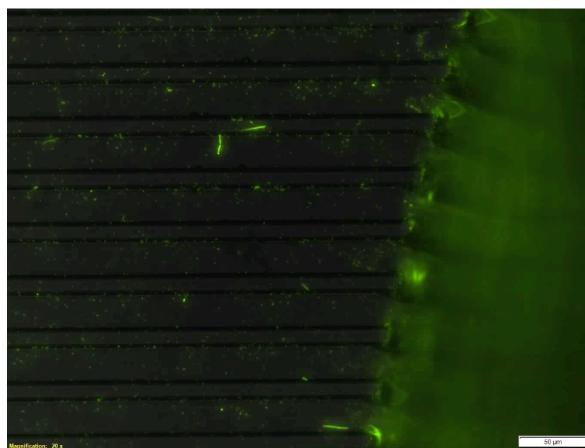


Figure 3-9. Multichannel PDMS Microgrooves 3 at the start

After about 15 minutes, the bacteria started to settle preferentially in between the grooves, as demonstrated by figure 3-10. The grooved structure can be depicted through the GFP filter only (no multichannel) due to the settlement pattern. This type of behaviour is exactly the same as when the bacteria are grown straight onto the surface, rather than just depositing them like this, as previous experiments tended to show preferential growth in between the grooves as well.

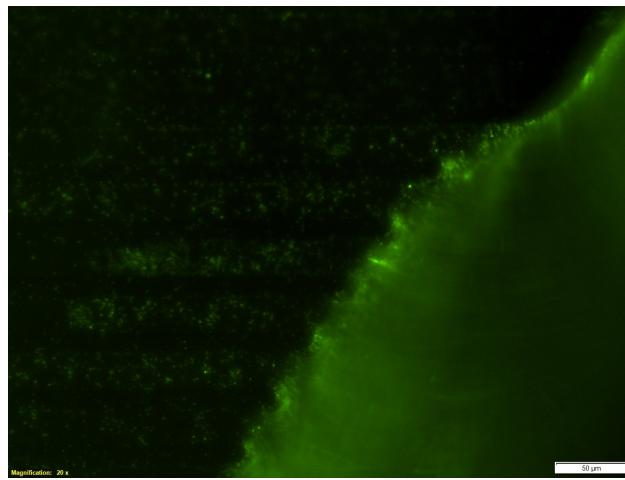


Figure 3-10. GFP PDMS Microgrooves 3 after ~15 minutes

Figure 3-11, taken around the same time as figure 3-10, illustrates a field of view that encompasses both smooth and grooved PDMS surface in order to be able to qualitatively compare the two.

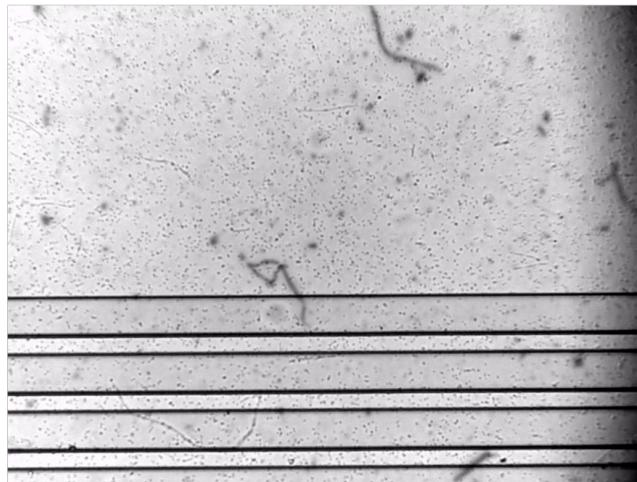


Figure 3-11. BF PDMS Microgrooves 3 and smooth PDMS after ~15 minutes

From the video, it can be seen that the walking/tumbling motion of the bacteria tends to be directionally oriented within the grooves, but is more sporadic on the smooth portion. Finally, figure 3-12 illustrates a snapshot of a DIC filter video after most of the bacteria had settled. This image filter provides a great sense of depth, and led to a new multichannel acquisition with the next sample, using DIC and GFP together.

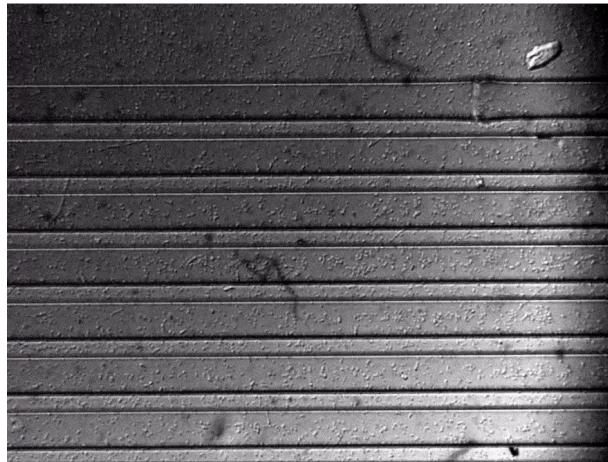


Figure 3-12. DIC PDMS Microgrooves 3 after ~30 minutes

All of the multichannel videos taken can be viewed in the “PDMS Microgrooves 3” folder in the [Google Drive folder](#).

3.4.2 Microgrooves Structure 8

This was the eighth microgroove structure, which had a groove height of 2 μm , a groove width of 10 μm , and a spread between the grooves of 20 μm . Figure 3-13 is a snapshot of a video capturing the initial fluidic motion traversing the surface after deposition, and shows the overall flow going across the grooves.

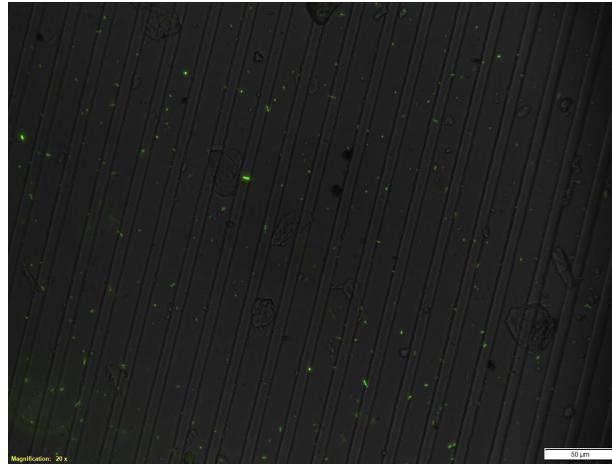


Figure 3-13. BF+GFP snapshot of PDMS Microgrooves 8 at the start

While it cannot be seen from viewing snapshots, the second video shows the traversing motion slow down, and by the third video (roughly 10 minutes after the start), the motion of the liquid and bacteria are more oriented in the direction of the grooves. A few more videos were taken, showing a decrease in the motion and velocity of the liquid as the bacteria began to settle. After about 20 minutes, the settlement pattern became very clear, as seen in figure 3-14.

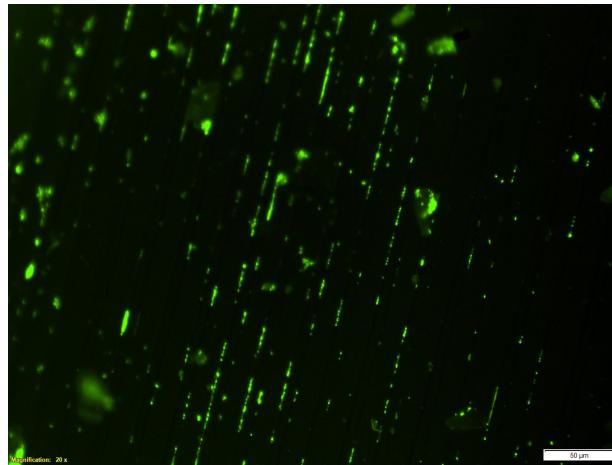


Figure 3-14. GFP snapshot of PDMS Microgrooves 8 after ~20 minutes

After the liquid began to dry out, the bacteria settled preferentially on the sides of the walls of the grooves protruding up. This coincided with the lab group's previous results with growing bacteria directly onto the surface, which showed that they first attached to the walls of the grooves, then populated the spread in between the grooves, and then finally filled the surface of the grooves

themselves after an extended period of time. More multichannel time-lapse videos were taken, and after about 25 minutes, this same development was observed, as demonstrated by figure 3-15.

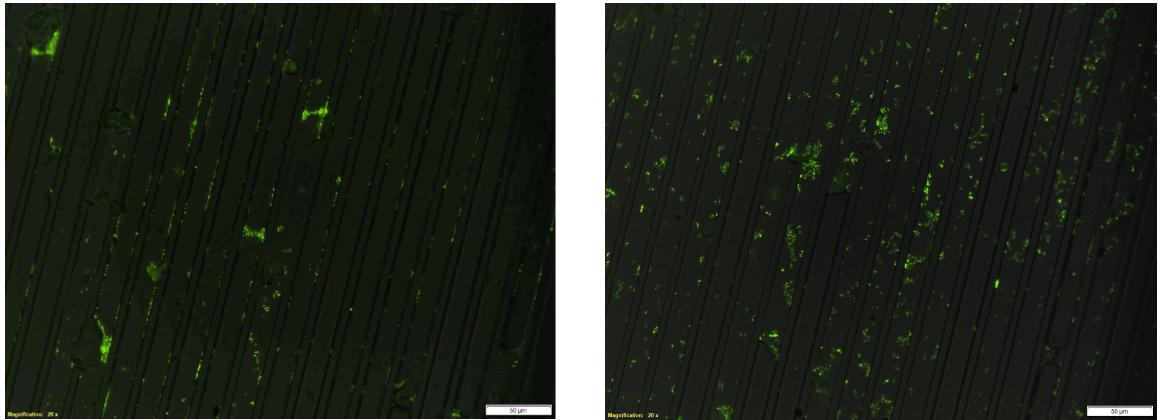


Figure 3-15. (a) BF+GFP PDMS Microgrooves 8 after ~25 minutes; (b) After ~30 mins

More and more of the surface began to show attachment and chaining in the spread between the grooves, and still no attachment on the surface of the grooves themselves. Shortly after, curiosity sparked an idea, which involved using DIC and GFP for the multichannel filters, rather than using BF and GFP as before. Exploring other areas of the surface led to the discovery of a very interesting pattern that had developed in the spread of the grooves, as depicted by figure 3-16.

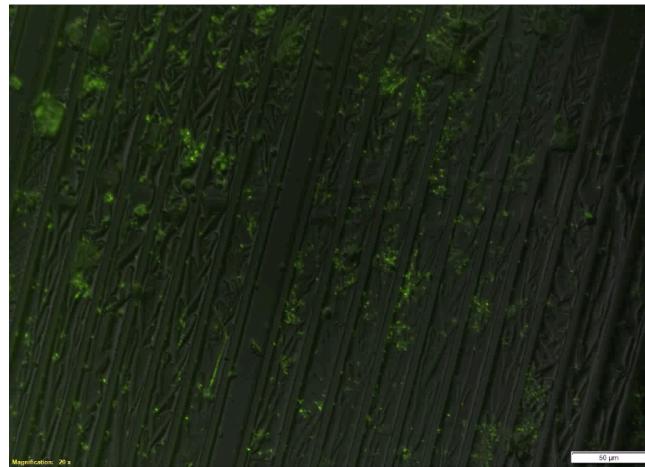


Figure 3-16. DIC+GFP of PDMS Microgrooves 8 after ~35 minutes

This pattern is not part of the original structure, and, at another location on the surface, figure 3-17 shows that the stained bacteria seem to be attached where the patterns appear. One possible speculation as to why that is could be that the buffer has created these patterns as it dried up.

Another possible speculation is that it is the bacteria themselves, forming biofilm, and that not all of the cells were stained.

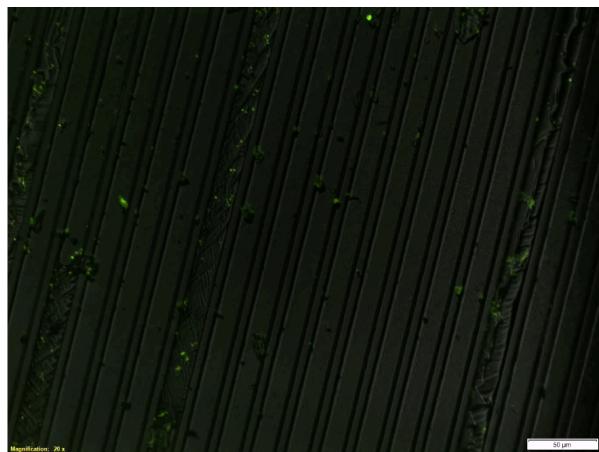


Figure 3-17. DIC+GFP of PDMS Microgrooves 8 after ~36 minutes

4 Software Analysis

After having established a reliable method to image bacterial motion on various surfaces, a few different software programs were used to obtain some quantitative data and achieve the original goal of enumerating and tracking the bacterial cells.

4.1 CellSens

The first software program that was used was the modular imaging software cellSens, as it was directly linked to the microscope. Aside from using it to change lenses, filters, acquisition and experiment settings (i.e. time-lapse length and frames), cellSens is very good for image adjustment. Most of the videos and time-lapse images acquired were rendered much clearer using the threshold adjustment depicted in figure 4-1. Using the GFP filter can cause the background to be a grainy green, which is less than favourable, but playing with the adjusters can increase the contrast so that the background appears blacker. All of the figures depicting acquisition images were modified in this manner.

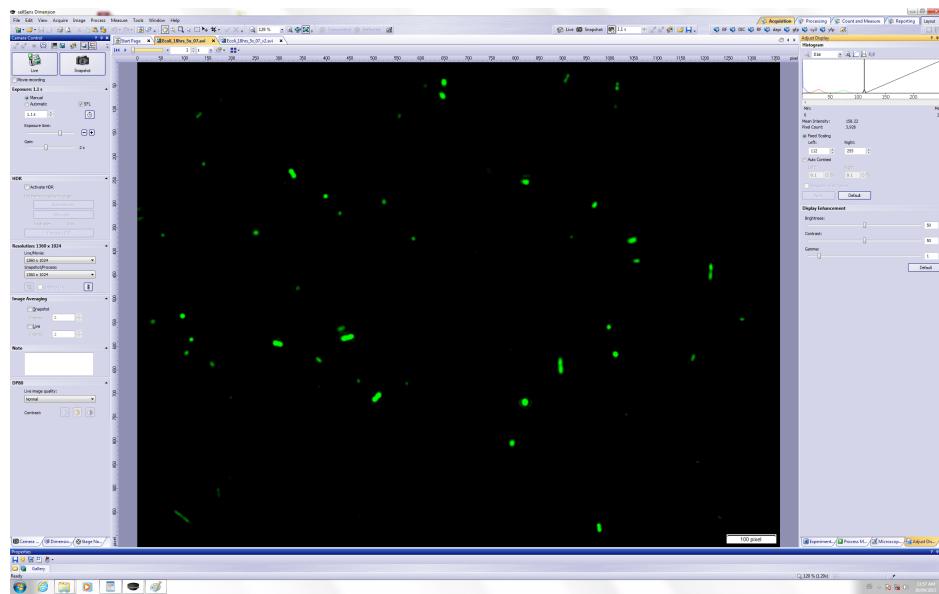


Figure 4-1. CellSens interface, with threshold adjusters in the upper right corner

The other essential part of this software is its ability to retain all of the original information in its native '.vsi' format, and save it into virtually any other format i.e. AVI, TIFF, JPEG, etc. This is important due to the specific input formats required of other software programs.

4.2 CellC

A lot of software programs capable of enumeration work on a single image basis. CellC on the other hand, is also capable of batch processing, which is very useful since time-lapse images are saved as a TIFF stack. Figure 4-2 shows the CellC interface.

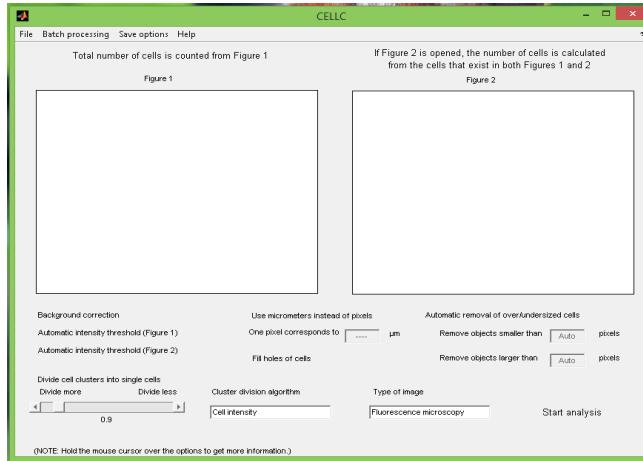


Figure 4-2. CellC interface

After converting the images to black and white (Image-J was used in this experiment) and loading them into CellC, there are a variety of parameters that can be controlled. These include, thresholding, cluster division algorithm (cell intensity or cell shape), degree of division, type of image (fluorescent or light), pixel-um interconversion, and cell size limits. With each image, a total count figure is generated (as seen in section 2.1), as well as a statistics file. An example of the results of that file are displayed in table 4-1.

Table 4-1. CellC Example Results File

Cell's serial number	Area of cell	Approximate volume	Length	Width	Intensity mean	Intensity maximum	Solidity	Compactness
1	20.7414	18.6043	3.2875	3.2875	114.2857	248	1	7.2401
2	14.8153	10.7664	2.7396	2.7396	109.1	255	1	7.447
3	80.0027	423.1626	9.3147	9.3147	179.3333	255	0.84375	3.4787
4	20.7414	18.6043	3.2875	3.2875	158.8571	220	1	7.2401
5	121.4856	797.6611	11.5064	11.5064	198	255	0.93182	3.4618
6	74.0766	236.344	7.6709	7.6709	184.92	255	0.96154	4.7494
7	23.7045	44.0992	4.3834	4.3834	139	249	1	4.6544
8	77.0397	352.7934	8.7668	8.7668	182.5769	255	0.89655	3.7817
9	44.446	114.6406	6.0272	6.0272	167.8	255	1	4.6159
Statistical means of columns								
0	53.0059	224.0751	6.3316	6.3316	159.3303	249.6667	0.9593	5.1854
Unit of measure: pixels								

Furthermore, a summary file is generated which compiles the mean information from each statistics file of each image analyzed. Aside from the fact that the enumeration process is very

easy to do with the batch processing feature, analyzing the numbers over time and comparing the trends between surfaces could lead to potential extrapolation on how surfaces affect bacterial attachment.

4.3 Bactrack

Backtrack is a Matlab compiled script that takes a tab-delimited queue file of designated tasks (Appendix A), analyzes the video file that it is directed to, and generates a text file containing the average speed, standard deviations of average speed, number of objects counted, number of objects considered moving, and percentage of objects moving. There is a second, movie version of the script that does all the same things as the first, but also overlays the tracks it drew internally onto the original movie frames. As an example, figure (a) of 4-3 is a snapshot of the original video taken with the microscope, while (after converting to black and white and running backtrack) (b) shows the end frame snapshot of the overlaid track. This program is useful for qualitative analysis between surfaces due to the motion being tracked, as well as quantitative analysis since velocities and number of moving cells are calculated.

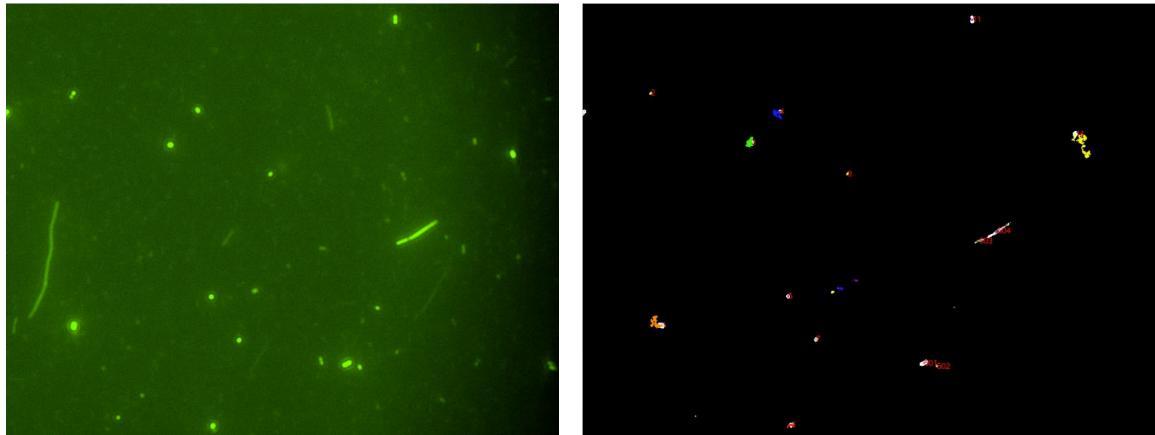


Figure 4-3. (a) Original video snapshot; (b) End frame snapshot with overlaid tracks

References

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Appendix A Bactrack Queue File Tasks

Queue File:

The queue file is a tab-delimited text file where each row is a separate analysis task and the columns are defined as follows (see "ExampleInput.txt" for an example):

Column 1:	Output directory (full path, with trailing \) (string)
Column 2:	Analysis task label (string)
Column 3:	Full path and file name of the movie input file (string)
Column 4:	Analysis task label 2 (should be duplicate of column 2) (string)
Column 5:	Threshold value (a decimal from 0.0 to 1.0) - this number scales onto the 256 possible gray levels and determines the thresholding cutoff for objects to include as the image is converted to a binary equivalent. 0.5 is generally OK.
Column 6:	First frame to analyze (w/r/t movie file in Col. 3) (integer)
Column 7:	Last frame to analyze (w/r/t movie file in Col. 3) (integer)
Column 8:	Frame rate of movie (frames / sec) (decimal or integer)
Column 9:	Scale of movie (pixels / unit length) (decimal)
Column 10:	Search radius (How many unit lengths to search from one frame to the next for the same objects. This can be a decimal. Small for slow objects, larger for fast objects.) (decimal)
Column 11:	Minimum number of contiguous frames that an object must be detected in to be considered "real." (integer)
Column 12:	Velocity threshold (in unit lengths / sec) that an object must be going to be considered "moving." (decimal)
Column 13:	Velocity thresholding state (0 for off, 1 for on) - sets whether velocity thresholding is performed. If it is "off," even stationary objects will be reported in the average speed. If it is on, only objects with velocity greater than that specified in Col. 12 will be included in average speed calculations.

There is no limit to the number of analysis lines possible in the queue file.