Proposed Methods to Optimize Growth, Imaging, and Analysis of *Bacteroides Thetaiotaomicron* Biofilms

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On my honor as a student, I have neither given nor received unauthorized aid on this assignment as define by the Honor Guidelines for Thesis-Related Assignments

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Proposed Methods to Optimize Growth, Imaging, and Analysis of Bacteroides Thetaiotaomicron Biofilms

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Abstract

Bacteroides thetaiotaomicron (*B. theta*), a human gut bacterium, holds significance in aiding human digestion and fostering human immune system development. When *B. theta* proliferates and adheres to surfaces, they can form biofilms, structured communities of bacteria enveloped in an extracellular matrix. Optical Density (OD) and Percent Coverage measure planktonic and adherent *B. theta* respectively. Understanding the correlation between these two metrics can help optimize *B. theta* growth measurements. This project proposes image analysis methods for obtaining percent area coverage from light microscopy (LM) and confocal laser scanning microscopy (CLSM) images. To validate these methods Crystal Violet (CV) and Viability (Live/Dead) staining were performed to measure the correlation between *B. theta* percent coverage and OD to optimize *B. theta* growth methods. To optimize biofilm formation methods, OD and coverage were measured for varying growth intervals and starting ODs. Image analysis methods proved promising but inadequately validated, as it was found that LM-imaged CV-stained biofilms grown for 72 hours exhibited a negative correlation (-0.825, p=0.113) between OD and percent coverage, while the CLSM-imaged viability-stained biofilms grown for 48 hours exhibited a positive correlation (0.825, p=0.18) between the two. While there was no significant difference between starting ODs in the measured coverage of CLSM-imaged viability-stained biofilms grown for 48 hours, the coverage of LM-imaged CV-stained biofilms grown for 72 hours was significantly higher (p=0.33) when begun at an OD of 0.05 than one of 0.1. Also, there was no significant difference at 48 or 72 hours in the change in OD between biofilm cultures begun at ODs of 0.05 and 0.1 but more research with increased replicates is recommended to confirm this outcome.

Keywords: B. Theta, Biofilm, Optical Density, Percent Coverage

Introduction

B. theta Biofilm Background

Bacteroides thetaiotaomicron (*B. theta*) is a predominant gut symbiont that contributes significantly to human digestion, including the breakdown of otherwise non-metabolizable polysaccharides[1]. *B. theta* plays an important role in the gut microbiome, which aids in the digestion of complex carbohydrates and fosters immune system development[2]. When *B. theta* proliferates and adheres to surfaces in colonies, it forms biofilms, which are structured communities of bacteria attached to surfaces and enveloped in an extracellular matrix[3]. *B. theta* biofilms adapt to gastrointestinal (GI) conditions by altering their metabolism[4] and colonization patterns[5].

Optimizing Biofilm Measurements

Understanding the formation of biofilms inside the body is an important aspect of modern medical research that is driving new strategies to treat infectious diseases and to understand the mechanisms by which bacteria colonize new niches[6]. For this to occur, there is sufficient room to optimize the methods of *B. theta* biofilm measurement through both percent coverage and Optical Density (OD) measurements. This can be done by comparing stained *B. theta* biofilm images with optical density measurements of *B. theta* planktonic growth. Previous research by O'Toole *et. al* has compared the two metrics using a crystal violet staining method, which functions by staining the *B. theta* cell wall, but does not stain the biofilm matrix specifically[7]. This report attempts to advance previous methods by incorporating additional viability staining to compare *B. theta* biofilm percent coverage and optical density, and by comparing optical density measurements of 48-hour and 72-hour *B. theta* biofilm samples. To determine the best methods for the growth and measurement of *B. theta* biofilm, the paper aims to (1) grow, stain, and image the biofilms of *B. theta* with varying initial ODs, measuring OD at every step, and (2) perform image analysis to determine the percent area coverage of each biofilm. It is hypothesized there should be a correlation between *B. theta* biofilm percent coverage and optical density, but the magnitude of this relationship can be measured in more detail in this report. It is also hypothesized that an initial OD of 0.05 will yield both a greater change in optical density throughout biofilm formation and a greater percent area coverage than an initial OD of 0.1.

Results

Coverage Quantification Validation

To validate the percent area coverage measurements that were extracted by image processing, coverage was plotted against the final OD of each biofilm dish and a linear regression was performed. While the relationship may not necessarily be linear between the OD of bacteria in a suspension and the coverage of bacteria in a biofilm of the same dish, the correlation between the two growth metrics provides a way to validate that the coverage measurements are accurate and precise enough to see a trend. Comparison against an already validated segmentation method, such as manual segmentation, would be needed to more confidently validate the coverage measurements.

Validation of Light Microscopy Analysis

Regression of LM-derived coverage against final OD (Figure 1) results in a non-significant (p=0.12) correlation of -0.825. This is an unexpected result and is addressed in the discussion section below.

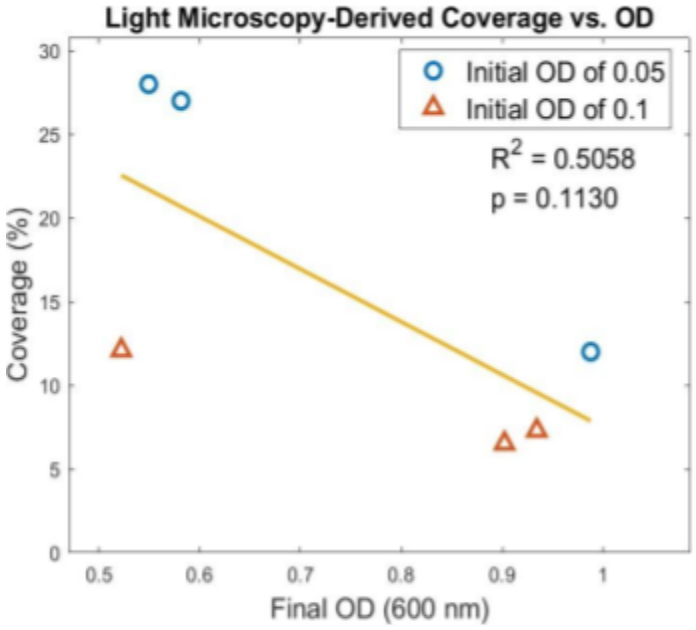

Figure 1: Light Microscopy-Derived Coverage vs. OD. Light microscopy imaged biofilms were grown for 72 hours and stained with crystal violet.

Validation of CLSM Analysis

Regression of CLSM-derived coverage against final OD (Figure 2) results in a non-significant (p=0.18) positive correlation of 0.825. One trial that yielded a coverage of approximately 0 at an OD of 0.38 seems to be an outlier. Visual inspection of the image confirms that there is little to no coverage compared to other samples, and a

regression that excludes this data point yields a non-significant (p=0.14) correlation of 0.976. (0.825, p=0.18)

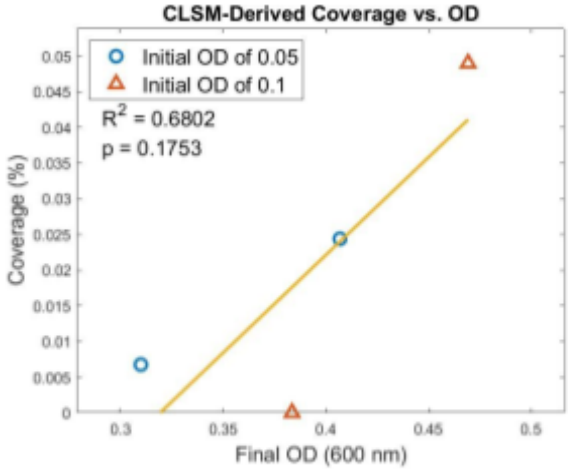

Figure 2: CLSM-Derived Coverage vs. OD. CLSM imaged biofilms were grown for 48 hours and stained with the live/dead stain.

Impact of Initial OD on Coverage

Trials run with the initial OD of the dish near 0.05 were compared to those run with an initial OD of 0.1 in order to evaluate the impact of initial OD on coverage and determine the condition that optimizes biofilm formation.

Confocal Imaging

A student's T-test on live/dead stained CLSM images of biofilms grown for 48 hours show no significant change in mean of percent area coverage in trials with an initial OD of 0.05 than the mean of trials with an initial OD of 0.1 (p=0.618, n=2) (Figure 3). Due to the small sample size, the suspected outlier could not be excluded.

Light Microscopy Imaging

A student's T-test on crystal violet stained LM images of biofilms grown for 72 hours shows a significantly higher mean of percent area coverage in trials with an initial OD of 0.05 than the mean of trials with an initial OD of 0.1 (p=0.033, n=3) (Figure 3).

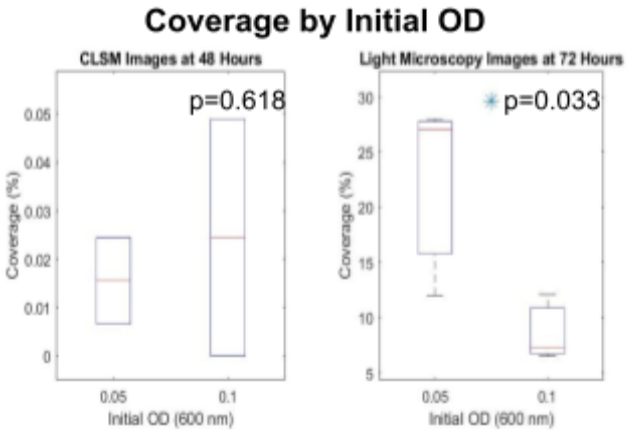

Figure 3: Coverage by Initial OD. Box plots of percent area coverage for initial ODs of 0.05 and 0.1 are shown for CLSM images of live/dead stained biofilms grown for 48 hours (left) and light microscopy images of crystal violet stained biofilms grown for 72 hours (right).

Impact of Initial OD on Change in OD

Trials run with the initial OD of the dish near 0.05 were compared to those run with an initial OD of 0.1 in order to evaluate the impact of initial OD on the change in OD over the duration of biofilm formation. This was done in order to understand growth in the suspension around the biofilm. A student's T-test on the difference after 48 hours between initial and final OD measurement of the cell suspension in which biofilms were grown results in no significant difference between the means of suspensions begun at an OD of 0.05 and those started at an OD of 0.1 (p=0.245, n=2). The same applied to the difference measured after 72 hours (p=0.484, n=3).

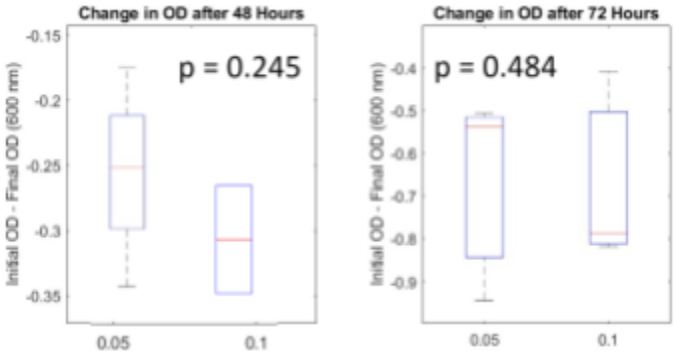

Figure 4: Change in OD by Initial OD. No significant difference was observed in dishes grown for 48 (left) and 72 (right) hours.

Discussion

Findings/Implications

Further modifications to the experimental setups will need to be performed to confirm the lack of significant differences encountered. For one, there needs to be a substantial increase in the number of replicates for both image sets, and also measurements of CV and CLSM data need to be performed at both 48 and 72 hour time points respectively to be able to measure the significant difference between the two.

CV Staining

Due to an error in the experimental design, biofilms were washed with 70% ethanol after CV staining and prior to imaging. This likely lysed many bacteria and severely damaged the biofilm. Because all LM images were of CV-stained biofilms, we conjecture that the LM derived percent area coverage is an underestimation of the true coverage. If true, this conjecture would invalidate both the following evaluation of our proposed LM image analysis methods and would also invalidate the significant difference found in coverage for CV-stained biofilms grown at varying initial ODs. We recommend that the experiments used here involving CV be repeated without the ethanol wash.

Validating Image Analysis Methods

The positive, although non-significant, correlation between coverage and final OD for CLSM images= viability of stained biofilms grown for 48 hours supports the use of the proposed CLSM analysis methods. Notably, however, there was a negative correlation measured between the percent coverage and final OD of LM-imaged CV-stained *B. theta* biofilms grown for 48 hours for samples with initial ODs of both 0.05 and 0.1. This could perhaps be due to decreased biofilm formation caused by increased competition for resources by the *B. theta* bacteria experienced at a higher OD as previous research by Chatdizaki-Livanis et. al has proposed the validity of this relationship but further research will be needed to experiment this in more detail[8]. This is supported by the later time point of biofilm imaging (72 hours instead of 48) and the higher final ODs observed (Figure 1). We recommend experiments measuring coverage under all conditions at both time points to further test the proposed image analysis methods.

Evaluating the Impact of Initial OD

No significant differences were observed in the change in optical density over the duration of biofilm formation for trials having an initial OD of 0.05 and trials having an initial OD of 0.1, but we recommend repeating the experiment with a greater sample size. For CLSM-imaged viability-stained biofilms grown for 48 hours, no significant difference was found between the coverage measured in biofilms cultured at an initial OD of 0.05 and 0.1, whereas for LM-imaged CV-stained biofilms an initial OD of 0.05 resulted in a significantly higher ($p=0.033$) mean coverage than an initial OD of 0.1. There were not enough successful trials for either viability-stained biofilms grown at 72 hours or CV-stained biofilms grown for 48 hours to perform statistics ($n=1$), so we recommend repeating the experiment with larger sample sizes both to confirm that an initial OD of 0.05 yields more coverage in CV stained biofilms at 48 hours and to test viability stained biofilms at both timepoints with more statistical confidence.

Future Work

More expanded work in the future could also focus on researching the impact of sphingolipids, which are a specialized group of lipids known to play a role in maintaining homeostasis and symbiosis in the gut microbiome. It has been proposed that the formation of sphingolipid membrane microdomains on *B. theta* may facilitate biofilm formation in the bacteria[9], but more extensive research can follow up on this. We hope that the growth conditions and coverage measurement methods proposed here will aid future experiments in accurately understanding the dynamics and mechanisms of *B. theta* biofilm formation.

Materials and Methods

Many of the following methods were performed in an anaerobic chamber. The chamber was maintained as a sterile environment at 37°C and under 42 ppm of oxygen at all times.

Plating and Growing Cultures of B. theta

Bacterial plates of *B. theta* cultured on agar (Supplement 1) were maintained in an anaerobic environment. From these plates, a 10 µL inoculum loop was used to transfer bacteria to new agar plates. Each newly streaked mother culture was covered and incubated for 48 hours at 37°C. After 48 hours bacteria was collected by a 10 µL inoculum loop and shaken into a tube with 3 mL of BHI media (Supplement 2).

Preparing Coverslips

The coverslips (Fisherfinest: premium cover glass, Cat#12-544-10, thickness No. 1)[10] that would be later used to measure biofilm growth were submerged in 70%

Ethanol in sterile(LabForce Cat#1188N82-LF)[11] 60 mm petri dishes for approximately 10 minutes. The ethanol was then removed from the petri dishes, and the dishes were placed inside of the anaerobic chamber for 24 hours to ensure that the coverslips could equilibrate in the anaerobic environment.

Adjusting Optical Density

After 24 hours of incubation, three 200 uL aliquot samples from one of the *B. theta*-BHI media complex tubes were placed in a row on a 96-well plate, and three 200 uL aliquot samples from another *B. theta*-BHI media complex tube were also placed in a row just below the initial samples on the well plate. Along with these, three 200 uL aliquot samples of BHI media were placed in a row just above the initial *B. theta*-BHI media samples. The 96-well plate was then placed inside of a Tecan Infinite 200 PRO plate reader[12] which was used to measure the OD of the *B. theta*-BHI media complex and the *B. theta* at a wavelength of 600 nanometers and then to export it to an excel sheet. The OD measurements were then placed in equation 1 below with the BHI to be added in terms of *B. theta*-BHI media sample volume (SV), the measured sample OD (SOD), and the desired OD (DOD). The equation then determined the remaining amount of BHI media needed to obtain an OD of 0.05 for one of the *B. theta*-BHI media complex samples and 0.1 for the other *B. theta*-BHI media complex sample. The two calculated amounts of BHI media were added respectively to the two *B. theta*-BHI media complex tubes that each of the samples came from, and the same sample measurement method was carried out to ensure that 0.05 and 0.1 OD could be measured for the two *B. theta*-BHI media samples respectively with a margin of error of ± 0.01. If the values were still off by more than 0.01, the equation below was reapplied, and the process was repeated until the targeted OD values were measured.

$$BHI\ to\ be\ added\ =\ SV((SOD/DOD)\ -\ 1) \quad (1)$$

Growing Biofilms

Once the accurate OD measurements for both of the *B. theta*-BHI media complex tubes was measured, each coverslip was removed from the 60 mm petri dishes they were incubated in and placed into another sterile 60 mm petri dish with a 70% Ethanol sterilized tweezer. Each of the petri dishes containing the coverslips were then submerged with 30 mL of either the 0.05 OD *B. theta*-BHI media complex tube or the 0.1 OD *B. theta*-BHI media complex tube which allowed for replicates of each condition. One set of the 0.05 OD and 0.1 OD petri dishes were then placed in the 37ºC incubator for 48 hours, and another set of the 0.05 OD and 0.1 OD petri dishes were placed in the 37ºC incubator for 72 hours.

Coverslip Staining

For both the 48 and 72 hours samples, the *B. theta*-BHI media suspensions were removed from the 0.05 OD and 0.1 OD petri dishes and transferred to waste after the allotted time had passed. Each of the petri dishes was then washed by 3 mL of sterile Phosphate-buffered saline (PBS).

Crystal Violet (CV) Assay

To perform the crystal violet staining for the 0.05 and 0.1 OD petri dish samples, CV was diluted in water to form a 0.1% solution. After cell suspension was aspirated, the biofilms underwent one wash with 3 mL of sterile PBS before 3 mL of 0.1% CV solution was placed into each petri dish and incubated for 15 minutes at room temperature. After incubation, the CV solutions were removed from each of the petri dishes and transferred to waste. The petri dishes were washed with 3 mL of sterile PBS again, and were then submerged in 3 mL of 70% ethanol for another 15 minutes at room temperature. After incubation, the 70% Ethanol was removed and transferred to waste.

Viability(Live/Dead) Staining

To perform the Live/dead (L13152 LIVE/DEAD BacLight Bacterial Viability Kit)[13] staining for the 0.05 and 0.1 OD petri dish samples, the coverslips were removed from the petri dishes they were present in and transferred to new, sterile 60 mm petri dishes with a sterile tweezer. The samples were then submerged with 3mL of sterile PBS. The staining solution was prepared by dissolving components A and components B in sterile PBS and placing the resulting reagent in an Eppendorf tube. After that, 15 µL of the solution was placed into each of the petri dish samples, and the petri dish samples were incubated for 15 minutes at room temperature under tin foil to protect the light-sensitive stains. After incubation, the petri dish samples were washed twice with 3mL sterile PBS.

Image Acquisition

Stained coverslips were mounted onto microscope slides using ethanol-sterilized tweezers by placing one drop of mounting oil on the slide and placing the coverslip with the side that was facing up during biofilm growth to be facing down on the slide, sandwiching the biofilm between the coverslip and slide.

Light Microscopy Imaging

Slides imaged via LM were imaged on an EVOS M7000[14]. The slide was set in focus at 10x, 20x, 40x, and 60x magnifications in sequence to set the focal plane, then images were acquired at the desired magnification. For 60x magnification oil immersion of the lens was used. For every biofilm the location on the coverslip that was imaged was chosen by the researcher on the basis of apparent biofilm formation. To reduce bias in choosing a location for imaging approximately 100 images were captured in order to increase the area analyzed.

Confocal Imaging

Slides imaged via CLSM were imaged on an ORCA-Fusion CMOS camera (Hamamatsu C14440-20UP)[15] with a 100x oil-immersion lens (SR HP Apo TIRF 100xAC Oil)[16] with an aperture of 1.49. 3 slices were taken 2 µm apart starting from the base of the biofilm which is at the top of the microscope slide. Pixel's were 0.065 µm in both dimensions and were collected with an exposure time of 0.1843s. For every biofilm the location on the coverslip that was imaged was chosen by the researcher on the basis of apparent biofilm formation.

Image Analysis

In order to perform both the LM and confocal image analysis, all of the software utilized was streamlined within the Moore's Lab Rivanna group to ensure adequate storage for the large quantity of acquired images. Rivanna is a UVA operated high performance cluster with large computational and memory capabilities[17].

Light Microscopy Image Analysis

The image analysis for the LM images was performed by CellProfiler[18], a free, open-source software used to measure phenotypical measurements from a streamline of images. For this to occur, all of the horizontal segment images were loaded into CellProfiler as TIFF files, and the intensity of the CV stains in each image was enhanced by selecting for holes within the range of 1 to 30 pixels to ensure that the biofilms could be adequately measured by the software. After that, the *B. theta* biofilms were identified using an adaptive otsu thresholding method with a threshold smoothing scale of 5 and a threshold smoothing factor of 0.7. For each image, the total image area, area occupied by the *B. theta* biofilms, and count of the biofilms was extracted to an excel spreadsheet. An example of a 2D visualization with this process is shown in figure 5.

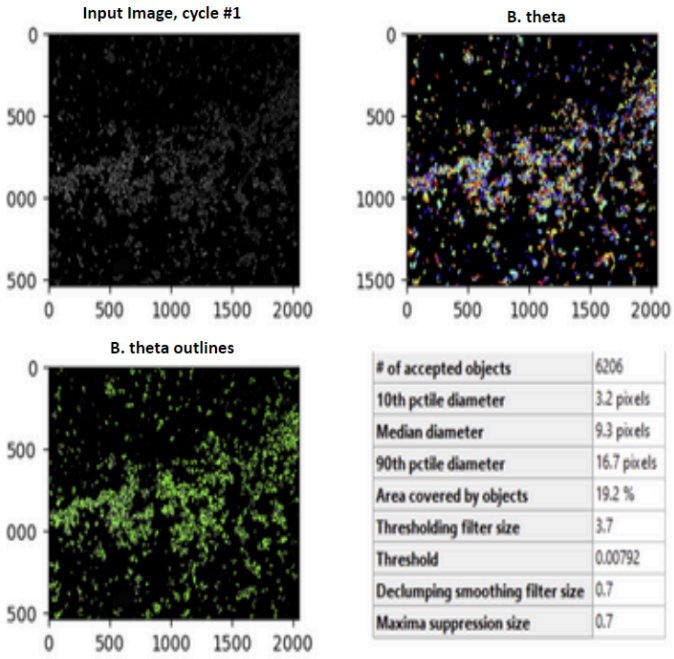

Figure 5: 2D *B. theta* Biofilm Visualization on Cell Profiler Example. The input image in the top left depicts the biofilm enhanced images being inputted into the program. The *B. theta* outlines image on the bottom left outlines all measured instances of *B. theta* biofilms. The *B. theta* image on the top right colors in all measured instances of *B. theta* biofilms. The data in the bottom right highlights the accepted B. theta biofilm objects, *B. theta* biofilm diameter measurements, *B. theta* biofilm percent coverage, and the thresholding information for each image.

Confocal Image Analysis

Analysis of CLSM images was performed on the BioVoxxel 3D Box extension[19] to the Fiji image processing software[20]. BioVoxxel 3D Box is not specially adapted to bacteria but is designed to perform general cell segmentation. The images were converted from the proprietary .nd2 format to TIFF by the Bioformats extension. TIFF stacks were then processed using BioVoxxel 3D Box using an adaptive Otsu thresholding method on the full histogram. Histogram holes were not filled. Then the maxima separation method was applied to a slight Gaussian blur of the image to 3D segment the image stack. For the maxima separation method, a spot sigma and detection radius of 0 were used. The image of labeled objects was converted to binary and each stack was overlaid to determine percent area coverage.

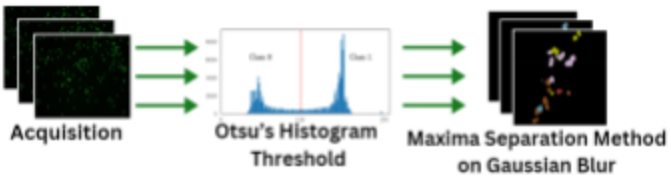

Figure 6: Overview of Processing on CLSM Images. Biofilms were live/dead stained. Otsu's method was used to reduce noise and the maxima method was used to prioritize the labelling of small objects.

Python scripts that processed and organized results were written to be callable by slurm scripts on the Rivanna cluster to efficiently process images and adjust resources by job needs. The developed slurm script called the Fiji and Anaconda linux modules.

Viable histogram threshold methods, object segmentation methods, and all adjustable parameters were optimized by applying a grid search method to rate options by visual inspection of output. In order to prioritize measurement of percent area coverage, the output was rated high if there was accurate identification of the biofilm area but was not optimized to reduce object bleeding or return accurate object counts. No image filtering was used to retain high contrast between bacteria's edges and background. No background subtraction was performed because it was not needed for segmentation and it negatively impacted the intensity of bacterial objects.

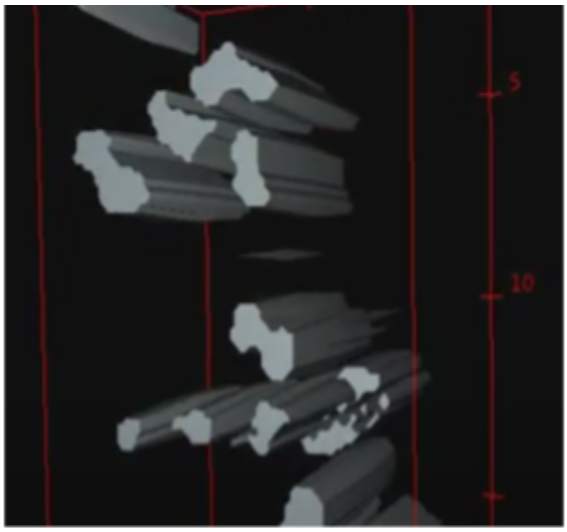

Figure 7: Visualization of 3D segmented biofilm. Shown is a small example of 3D biofilm segmentation. Scale bar is in μm.

End Matter

Author Contributions and Notes

N.S., L.M., A.V.A.S, and C.H. designed research. N.S. and L.M. performed research, N.S. and L.M. wrote software, N.S. and L.M. analyzed data; and N.S. and L.M wrote the paper.

The authors declare no conflict of interest.

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Supplementary Material

Supplement 1: Preparing BHI Agar Plates.

Materials: Brain Heart Infusion Broth (Sigma Aldrich), Becton Dickinson Microbiology Bacto Agar

1. Add 18.5 g of Brain Heart Infusion Broth and 7.5 g of Becton Dickinson Microbiology Bacto Agar to a 500 mL glass media bottle (adjust media amount as needed but maintain powder:water ratio)
2. Fill bottle with 300 mL of DI water, seal cap on bottle, and shake vigorously
3. Open bottle and wait for bubbles to mostly settle
4. Place stir bar and fill bottle to 500 mL mark
5. Loosely cap the bottle, place indicator cap, and autoclave on "liquid 2" cycle
6. After autoclaving, allow the bottle of agar to cool for about 5-10 mins while stirring to prevent it from solidifying
7. Add 1 mL per 100 mL agar (in this case 5 mL) of pre-sterilized vitamin k, L-cysteine, and hemin solutions each
8. Place plates in a laminar flow hood for pouring. Use a serological pipet to pour about 20-25 mL of agar onto each plate.
9. Allow the agar plates to solidify for 30 min before storing inverted at 4°C with parafilm.

Supplement 2: Preparing BHI Liquid Media.

Materials: Brain Heart Infusion Broth (Sigma Aldrich)

1. Combine the calculated amount of BHI media powder with the desired amount of water. The media should indicate the amount of powder to add to 1L of water.
2. Mix the bottle vigorously until somewhat dissolved. Autoclaving will further dissolve the media.
3. Loosen the cap on the bottle and place autoclave tape on the cap. Autoclave the bottle on the "Liquid 2" cycle.
4. After autoclaving, allow the bottle of agar to cool for about 5 mins.
5. Add 1 mL per 100 mL media of pre-sterilized vitamin k, cysteine, and hemin solutions each.
6. Tightly cap bottle and store at 4°C.