High-throughput analysis of single cell *Pseudomonas aeruginosa* growth

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Introduction

Cells within biofilms *(Figure 1)* are subjected to heterogeneous conditions *(Figure 2)*, leading to the development and necessity of bacterial subpopulations. *Pseudomonas aeruginosa* is a model biofilm, whose hibernation promotion factor gene and protein are implicated in the ability of its cells to successfully enter dormancy when stressed by starvation (*Figure 3*) . In the studies that follow, methods for analyzing the regrowth of *P. aeruginosa* in microfluidic drops are explored. Droplet microfluidics allows for the observation of single cell behavior. In this application, cells are isolated in monodisperse drops, and the regrowth is analyzed through confocal microscopy and high throughput fluorescence detection.

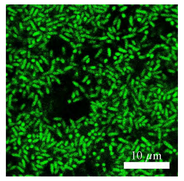


Figure 1: *Pseudomonas aeruginosa* biofilm

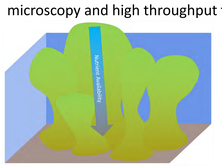


Figure 2: Nutrient distribution in a model biofilm

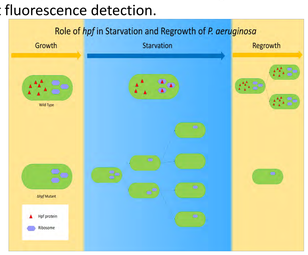


Figure 3: The role of the hibernation promotion factor in successful resuscitation after starvation periods

Bulk Answer

Both bulk regrowth tests and droplet regrowth tests indicate that *P. aeruginosa* cells possessing the *hpf* gene, the wild type, resuscitate better following starvation periods than Δ*hpf* mutants lacking the gene *(Figure 4.)* However, there are varying levels of regrowth even among cells of the same species. This heterogeneity of regrowth is of interest in gaining understanding of biofilms.

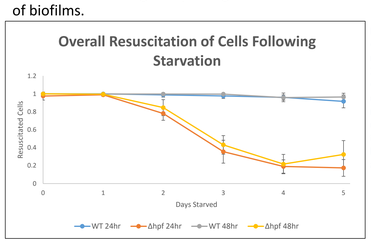
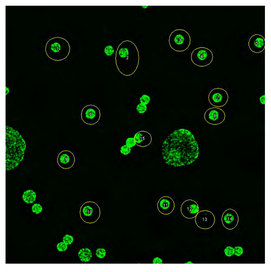


Figure 4: *Hpf* possessing PAO1 wild type cells and Δ*hpf* mutants were starved for 0, 1, 2, 3, 4, and 5 days. The cells were then diluted, and single cells were reintroduced to media within microfluidic drops. The cells were allowed to resuscitate for 24 and 48 hours. The PA01 wild type had a greater level of resuscitation.

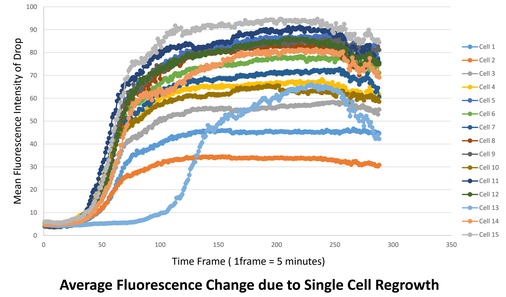
Single Cell Answer

Single *P. aeruginosa* cell growth has been analyzed through fluorescence output in confocal images. (*Figures 5,6.*) The cells exhibit different scale and rates of growth, made clear through visual analysis.

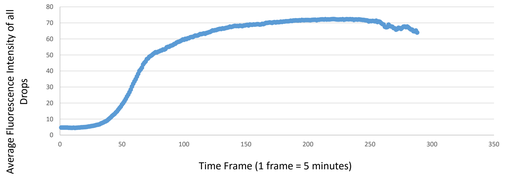
Figure 5: key to drops referenced in single cell regrowth fluorescence plots.
Figure 6: Plot of fluorescence change per drop over 24 regrowth period of *P. aeruginosa.*



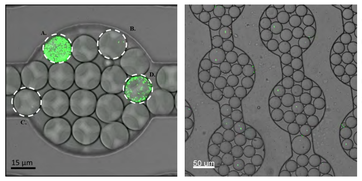
Varied Fluorescence Change Response in Drops due to Single Cell Regrowth



Average Fluorescence Change due to Single Cell Regrowth



Microfluidic Single Cell Analysis Methods



Visual: In order to gain data for analysis, the growth of cells in drops must be quantified. Growth has been defined with 3 categories: single cell, intermediate, and full (positive)(*Figure 7*) Growth classification can be done visually, with either continuous or end point confocal microscopy (*Figure 8.*) However, visual analysis takes a significant amount of time, and does not yield quantitative results.

Figure7 (left): Varieties of cell regrowth. Drops A and B are classified as single cell, drop D is intermediate, and drop A is a positive regrowth result.
Figure 8 (right): Image of single cells within drops immediately after encapsulation. This image was visually analyzed to confirm beginning concentration of cells to drops, a concentration that correlates to optical density.

High Throughput Detection: High-throughput detection is the desired form of analysis, as it takes a significantly shorter amount of time to process the data than visual methods, and yields a higher number of data points. In microfluidic high throughput detection, drops pass through a narrow channel on a microfluidic device at kilohertz rates (*Figure 9*). A laser is aligned so that it shines directly into the center of this channel, exciting any fluorescent material in the drops passing through the channel. The resulting fluorescence emission is directed into the optical train of the detection stand, where it is separated based upon wavelength (red, green, and blue) and reported to a computer interface as data that can be processed by the researcher – the intensity of the fluorescence response is conveyed as a peak. As a developing technology, a great deal of focus in this body of research is directed at optimizing high throughput detection for sensitivity and repeatability. Here, defining the single cell, intermediate, and full growth results using high throughput detection is the main objective, with the eventual goal of correlating specific cell numbers, at the early stages of growth, to detection results.

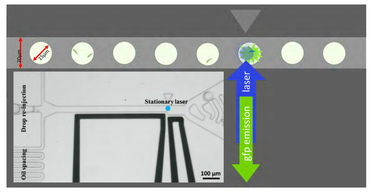


Figure 9: Schematic of detection device

Determining The Single Cell Threshold

One of the limitations of high throughput detection is in determining the threshold at which a detection peak indicates a single cell in a drop rather than noise. This capability is necessary to move forward in studying growth and regrowth heterogeneity in *P. aeruginosa* – living cells that are not replicating represent a little understood population in the post-starvation growth culture used to study the effect of *hpf* in cells. The method being used to determine this threshold is described *(Figure 10.)*

1. Dilution Preparation

A number of cell:drop dilutions are used to make sure the results are repeatable *(Figure 11).* Lower dilutions are used because they have the highest likelihood of always encapsulating just one cell when a cell is encapsulated – the cells fill drops based on the Poisson Distribution. 1mL of culture makes 5.65 x 10^8 drops. Cell/mL density is approximated through optical density.

Cell Dilutions for Threshold Study			
Trial	Cells/Drop	Cells/mL	OD
1	1/10	56500000	0.0565
2	1/20	28250000	0.02825
3	1/50	11300000	0.0113
4	1/100	5650000	0.00565

Figure 11: table of dilutions used in determining single cell threshold.

2. Drop Encapsulation

Bacteria are encapsulated in drops using a 15μm PDMS microfluidic drop making device *(Fig. 12).* Devices are fabricated using soft photolithography, plasma bonding, and functionalization with a hydrophilic treatment. The device operates based on flow focusing of water in oil *(Fig. 13).* The resulting drops are mono-disperse, meaning that they are all 15 μm, rather than a distribution of sizes. From the exit port, drops flow to a collection syringe.

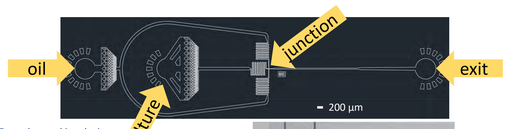


Figure 12: 15 um dropmaking device

Figure 13: Image of drops forming in a PDMS device due to coaxial flow at the junction. Image courtesy of W. Huck

3. Confocal Microscopy

Confocal Microscopy is used to verify the accuracy of the dilutions. Drops are put into a dropspot device[1] *(Figures 14, 15)* by very slow injection. The dropspot device acts as a well plate for the drops. The entire device is then imaged under a confocal microscope. In the resulting images, the number of drops (total), and cells/drop are counted *(Fig. 16).* The distribution of cells/drop will be compared to a Poisson[2] statistical prediction to determine if the dilution, determined by optical density, is correct.

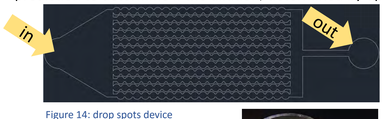


Figure 14: drop spots device

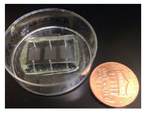


Figure 15: 3 drop spots devices in PDMS at scale

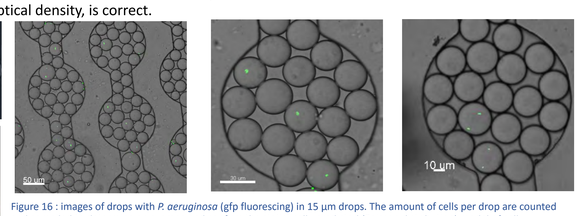
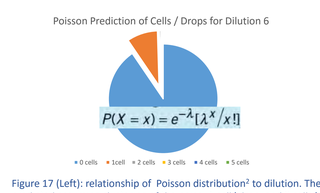


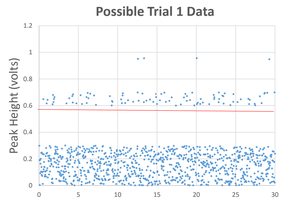
Figure 16 : images of drops with *P. aeruginosa* (gfp fluorescing) in 15 μm drops. The amount of cells per drop are counted and compiled to determine an average and confirm the statistically predicted (Poisson distribution) model of cell distribution.

4. High Throughput Detection

Drops are run through a detection chip at approximately 4kHz. The resulting data is analyzed, and ranges of peak values are assigned a corresponding number of cells/drop based upon segregations in the data set and the weight of those correlations compared to the predicted number of drops with 0, 1, or 2 cells based on the Poisson distribution[2] *(Figures 17,18,19).*

Trial	Cell/Drop	Cells/mL	OD
1	1/10	56500000	0.0565





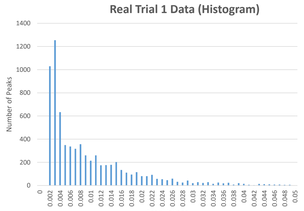


Figure 17 (Left): relationship of Poisson distribution[2] to dilution. The Poisson distribution predicts the likelihood that a drop will have 0, 1, etc. number of cells. Figure 18 (Center): possible dilution data, predicted assuming clustering of the noise, 1 cell/ drop, and 2 cells/drop. Note the predicted separation between signal for 1 cell and noise. Figure 19 (Right): Actual results of peak voltages in the form of a histogram. Note there was not distinct segregation between voltage populations.

Growth Curve Determination with High Throughput Detection

In order to begin defining detection results which indicate intermediate cell growth vs. full cell growth, normal growth must be defined in terms of high throughput detection results. To do this, cells in a 1cell:10 drops dilution were encapsulated in drops. A sample of the drops were imaged over 24 hours to gather fluorescence change over time data, using the confocal. Of the remaining drops, a sample was taken periodically through the first 12 hours of growth and detected. Preliminary results follow *(Figures 20, 21.)*

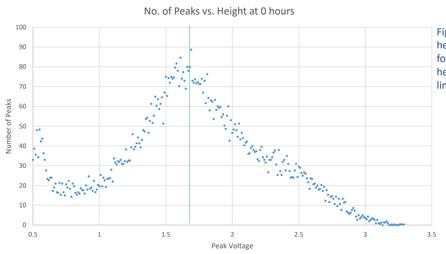


Figure 20: Histograms of peak height (volts) vs. number of peaks for all time points. The max peak height is designated by a green line.

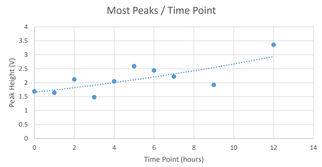
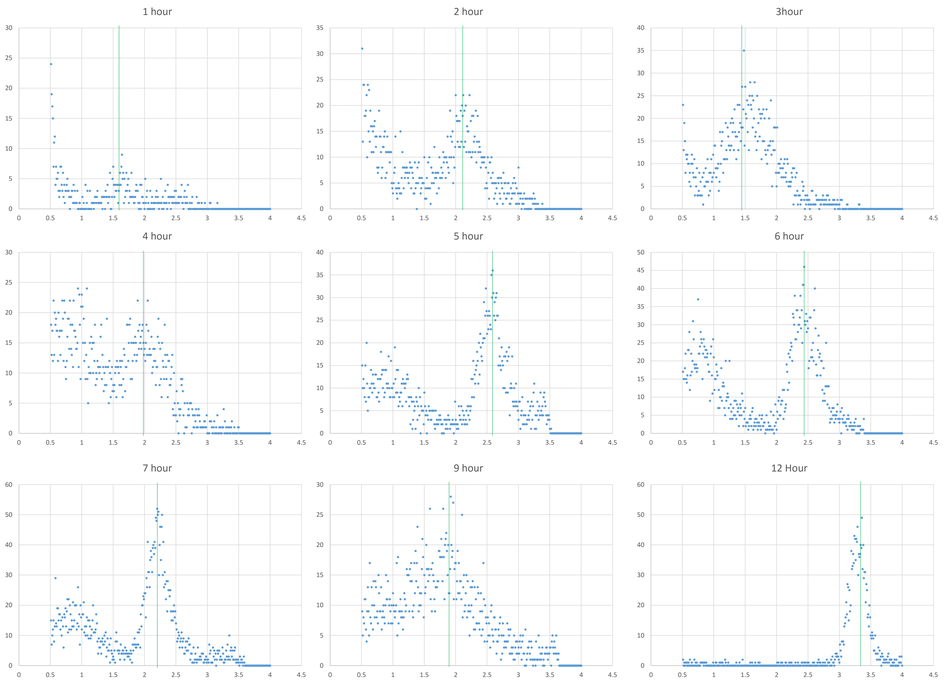


Figure 21: The change in max peak height of detection data over time. Preliminarily, results trend up. However, the experiment will be repeated to see if the trend holds. Optimal data is expected to correlate to the confocal fluorescence average growth curve, shown in figure 6.



Conclusion and Future Work

Methods are being developed, with preliminary success, for isolating and defining 3 subpopulations which categorize *P. aeruginosa* growth in drops: single cell, intermediate, and positive. Future work will involve further testing and optimization of these methods. The goal of these methods is to apply them to *P. aeruginosa* growth studies which gauge the effect of the *hpf* gene on the ability of cells to resuscitate after a starvation period. Upon defining the three key populations, we are hopeful that we will be able continue optimization of high throughput analysis in order to confidently assign detection results to cell numbers within drops.

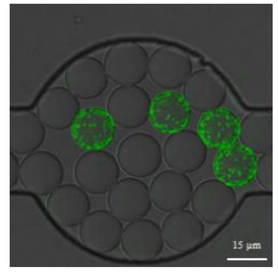


Image of *P. aeruginosa wt.* regrowth after starvation from HPF studies

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Chang Lab

References

1 “Dropspots: a picoliter array in a microfluidic device,” 2009, Schmitz *et.al*
2 “Single-cell analysis and sorting using droplet-based microfluidics,” 2013, Mazutis, et.al

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