

Quantitative Analysis of *Pseudomonas aeruginosa* Biofilm Images using Fractal Dimensions

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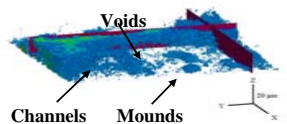
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1. Abstract

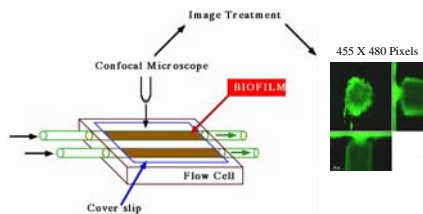
The natural mode of microbial growth is as organized biofilm communities on surfaces. It is now well established that it is very important to study bacteria such as *Pseudomonas aeruginosa* by growing it in the laboratory as a biofilm. The method is facilitated by the use of a confocal scanning laser microscopy (CSLM) that allows one to follow the development of the biofilm. The main difficulty faced is the quantitative and comparative analyses of the heterogeneous images. It was postulated that the bacterial biofilms have a fractal surface. An object is said to be fractal if its morphology does not change on different observation scales. If this conjecture is true then the mathematics of fractals imply that the two-dimensional cross-sections of the biofilms should have a fractal boundary. One way to establish fractal behavior is by studying the *Fractal Dimension* of the surface or that of the two-dimensional boundary along the cross section of the biofilm. In order to investigate the fractal behavior, we built a computer program called **Biofilm Image Processing (BIP)** to perform bulk processing of biofilm images produced by a CSLM. Since there is no consensus on a definition of the *Fractal Dimension* of a plane curve, our program computes it in eleven different ways. An analysis of the dependence of the fractal dimension on experimental parameters such as bacterial strains, nutrition concentration, and growth time was performed on a large number (over a thousand) of *P. aeruginosa* biofilm images. The merits and demerits of the different methods of computing the fractal dimension were studied. Finally, the potential limitations of biofilm image analyses (using fractal dimensions) have been investigated.

2. Introduction

The natural mode of microbial growth is as organized biofilm communities on surfaces.



P. aeruginosa can be grown in the laboratory as a biofilm. Biofilms can be inspected using a confocal scanning laser microscope (CSLM). By using a CSLM the 3-dimensional biofilm can be studied by acquiring cross-sectional images of the biofilm.

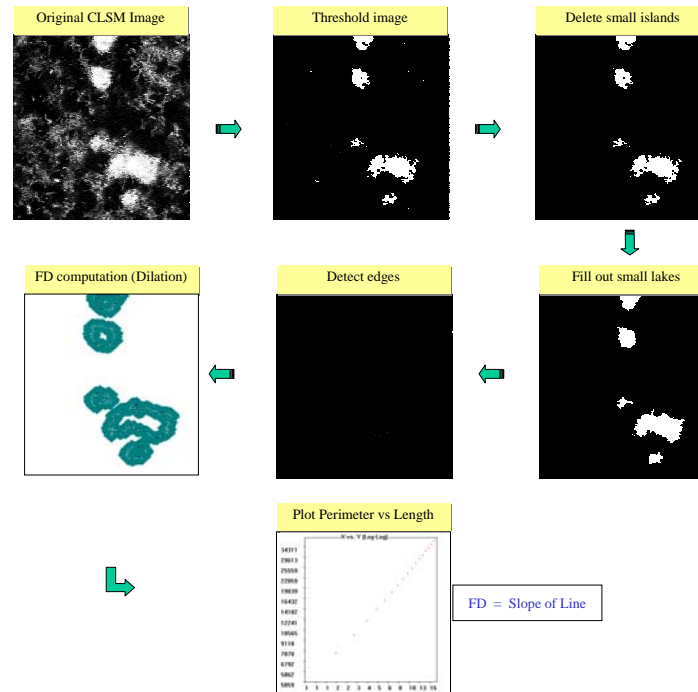


3. Quantitative Analysis of Biofilm Images

- There is a dire need for quantitative and comparative analyses of the images produced by the CSLM. The following quantitative measures (and their functions) of a biofilm have been studied by various researchers: biomass and biovolume, surface area, distribution of thickness, behavior of biofilm at the substratum, roughness coefficient, various diffusion parameters, and fractal dimension.
- It has been suggested that bacterial biofilms have a fractal surface. An object is said to be fractal if its morphology does not change on different observation scales. **Fractal Dimension (FD)** is a mathematical concept that helps to quantify fractal images.
- Reasons for quantitative analyses: to analyze temporal structural development in single- or multiple-species communities, comparison of different mutations, study the influence of nutrient or stress-inducing agents.

4. The Biofilm Image Processing (BIP) Software Package

- **Biofilm Image Processing (BIP)** is a software package used to investigate the potential fractal behavior of biofilm images.
- **BIP** includes several image processing tools, and 11 different methods to compute FD values.
- It has the ability to perform batch processing on a large number of images.
- It also performs statistical analysis to relate fractal behavior to experimental parameters such as bacterial strains, nutrition concentration, and growth time.



5. Computing Fractal Dimension

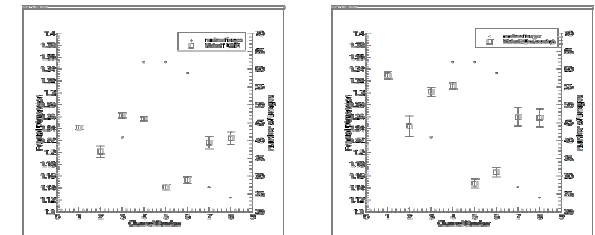
- There are no widely accepted definitions of FD. The software BIP computes it in 11 different ways. The methods include: **Euclidean Distance Map (EDM)**, **Minkowski Sausage Method (Dilation)**, **Box Counting Method**, **Corner Method (Counting and Perimeter)**, **Fast Method (Regular and Hybrid)**, **Parallel Lines Method**, **Cumulative Intersection Method**, and **Mass Radius (Short and Long)**.

6. Experiments and Results

- A large number of CSLM images were analyzed using **BIP**.
- The images were of 4 strains of *P. aeruginosa*. They include:

Channels 1 & 2	PAO1 wt	Normal wildtype strain
Channels 3 & 4	PAO1 rpoS	Cannot synthesize the stationary phase sigma factor RpoS
Channels 5 & 6	PAO1 <i>Apil(G-K)</i>	Unable to perform twitching motility
Channels 7 & 8	PAO1 <i>lasI</i>	Cannot synthesize the signal molecule OodHHL

[Strains PAO1 *Apil(G-K)* were provided by J. Kato and H. Otake, Hiroshima University, Japan.]
- FD values of images were computed; range of values for the middle layers of stacks were analyzed.



The ranges of the FD values for the four different strains are sufficiently distinct.

7. Conclusions and Discussions

Computing FD values

- On the whole, biofilm images are about as fractal as can be expected within the variabilities in nature.
- The most stable and reliable methods are: EDM, Minkowski Sausage Method, and Box Counting Method.
- The ranges of the FD values for the four different strains seem to be sufficiently distinct, supporting the view that the tendency and ability of a strain to form biofilm, as well as the quality of biofilm produced (quantified by the FD of the resulting biofilm) is a function of the genetic constitution of the bacterial strain.
- Wider ranges (for example, see channels 7 and 8) in the values of the FD indicate that the strain may have reduced ability to form a coherent biofilm.
- Fewer number of images (for example, see channels 7 and 8) indicate that an increased number of images could not be reasonably analyzed by **BIP**. Some images cannot be reasonably analyzed. The number of bad images seems to be correlated to the width of ranges of FD for that strain.
- No correlation was found between FD and other experimental parameters such as growth time.
- Considerable variation was observed for the FD values of adjacent layers of a *stack* of images.

Limitations of the data

Pixel sizes need to be much smaller for more accurate analysis. FD computations depend on it.

Limitations of the Experimental Setup

- Variability in readings – operator settings of voltages and offsets on the CSLM may vary.
- Variability in image quality and background noise – affects “thresholding” step.
- Variability in experimental conditions – these can never be perfectly simulated every time.

Other interesting parameters

Other researchers have identified the following: biomass & biovolume, surface area, distribution of thickness, behavior at substratum, roughness coefficient, diffusion parameters, and FD.

More sophisticated statistical analysis

Multivariate statistical analysis and improved error modeling are required for better analysis.