Identification of motility clusters per area in surfaces colonized by Pseudomonas aeruginosa

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Abstract. The biofilms are involved in pathogenesis and antibiotic persistence and resistance. The number of cells of a given species that will adhere to surfaces depends not only on the affinity of the cells but also on their number available for attachment. Therefore, the quick identification of the motile microorganism's area should be of great interest. The analysis of bacterial spatial patterns at the initial stage of biofilm formation is very important to know the success of the bacterial colonization. We propose a post processing method capable to distinguish motile microorganisms area of colonization in dynamic speckle images by applying Mathematical Morphologic techniques. The methodology would be effective for segmenting, detecting and describing patterns of colonization known not to be completely spatially random. The presented method is fast, reproducible, convenient, robust, and can be used to control the pattern, spacing, and orientation between colonies of different bacteria in order to prevent biofilm development.

1. Introduction

In natural environments, bacteria live mostly in biofilms [1], which are multicellular communities attached to surfaces. Motility gives access to surfaces suitable for biofilm formation, it is necessary for the recruitment of planktonic cells within the biofilm, and it is required for the spreading of biofilm on non-colonized surfaces [2]. Biofilm architecture was shown to be motility dependent in E. coli and in Pseudomonas aeruginosa [3,4]. These findings highlight the importance of discriminate clusters of motile bacteria in a sample. We recently developed a method which detects motile bacteria on a surface [5]. Analysis of bacterial spatial patterns at the initial stage of biofilm formation is very important to know the success of the bacterial colonization [6].
Chronic infections remain a major challenge for the medical profession and are of great economic relevance because traditional antibiotic therapy is usually not sufficient to eradicate these infections.

One major reason for persistence seems to be the capability of the bacteria to grow within biofilms that protects them from adverse environmental factors. In oral infections the number of cells of a given species that will adhere to oral surfaces depends not only on the affinity of the cells but also on their number available for attachment [7]. Therefore, the quick identification of motile microorganisms attached per area should be of great interest.

Pseudomonas aeruginosa is not only an important opportunistic pathogen and causative agent of emerging nosocomial infections but can also be considered a model organism for the study of diverse bacterial mechanisms that contribute to bacterial persistence [8]. In this context the elucidation of physiological characteristics, such as cell size (starved versus vegetative bacteria), and number of motile microorganisms per area, may influence their transport through porous media and should be considered. The identification of mechanisms responsible for the switch from planktonic growth to a biofilm phenotype and the pattern of motile bacteria aggregation per area in persistent disease should provide new insights in *P. aeruginosa* pathogenicity, and would contribute to a better clinical management of chronically infected patients.

The immediate goal of this work was to distinguish motile surface patterns per area of colonization by applying image processing techniques in particular Mathematical Morphology. The methodology would be effective for segmenting, detecting and describing patterns of colonization known not to be completely spatially random. The images of bacteria in biofilms are usually textured with fuzzy edges and nonhomogeneous gray level. Conventional image processing methods for analysis of shapes can not be applied in this case.

Unlike standard techniques, morphological techniques are based on concepts of geometry, algebra, topology and set theory, to characterize structural properties in images [9-12]. The central idea of these techniques is to examine the geometric structures in an image overlaying them with small patterns called structuring elements. Of all mathematical morphology analysis techniques, the most appropriate tool for segmented the bacteria in biofilms images.

This paper proposes a morphological segmentation in image of bacteria in biofilms in order to distinguish motile surface patterns per area. Disrupt these patterns of aggregation may help prevent development of biofilms. This method is rapid, reproducible, convenient, and can be used to control the pattern, spacing, and orientation between colonies of different bacteria in order to prevent biofilm development.

2. Materials and methods

2.1.1. Biospeckle patterns acquisition.

The proposed set-up for acquisition and storage of dynamic speckle patterns (biospeckle) is shown in fig. 1. An expanded HeNe laser (632.8nm and 30mW) illuminates the plate under study from the bottom through a ground glass diffuser. A CCD camera connected to a frame grabber registers a sequence of images (8 bits and 768 x 576 squared pixels) and stores it into the computer. The CCD height and objective were adjusted to focus the sample.

Using the experimental set up of Fig. 1, sequences images of speckle patterns were recorded. Time series corresponding to intensity level of each pixel were assembled (as many series as the image resolution = 768 x 576 pixels) to study the dynamics of the phenomenon.

To evaluate the dynamic within stationary periods, images sequences of 400 samples were registered using a 4 Hz sampling frequency, during 1min 40s. To distinguish motile surface patterns per area of colonization were applied techniques of Mathematical Morphology.

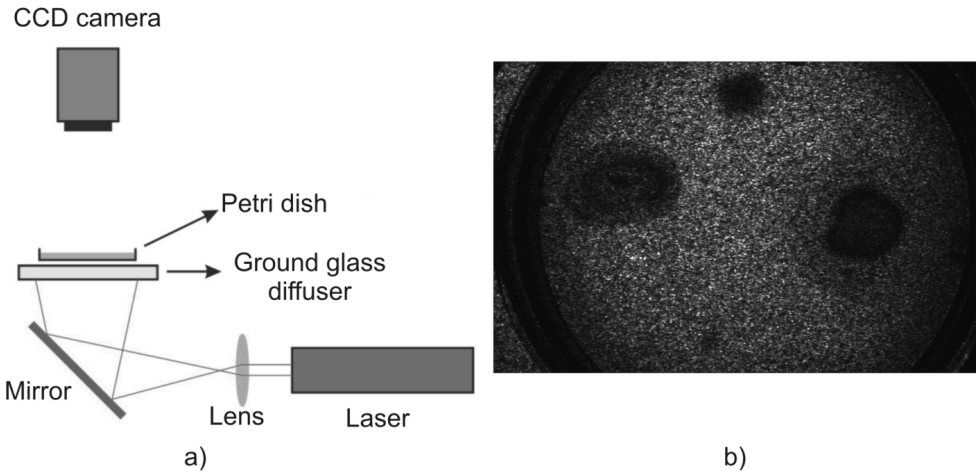


Figure 1. The dynamic laser speckle technique employed to easily discriminate filamentous fungi from motile bacteria in soft surface, such as agar plate a) Scheme of the setup for speckle imaging (the ground glass diffuser was removed and the mirror was replaced by a circular fluorescent tube over a dark surface for the white light imaging), b) example of speckle image.

2.1.2. Image processing

We used an RGB image obtained with the energy of filtered speckle images [5]. An RGB (red, green, blue) image is a three-dimensional byte array that explicitly stores a color value, between 0 (black) and 255 (white), for each pixel. A gray level image is obtained doing a weighted combination of each color channel in the RGB image ($I_{Gray} = 0.2989 \times R + 0.5870 \times G + 0.1140 \times B$).

Mathematical Morphology

The basic idea of Mathematical Morphology is comparing the objects contained in an image with a known object called structuring element [11,12].

The language of this important theory is set theory. Sets represent the shapes of the objects in an image. Every time the structuring element is superimposed on the image, a set operation is done for each pixel. The basic morphological operators of the MM are erosion and dilation [11]. A binary image can be modeled as a function X define on a subset of $G \subset \mathbb{Z}^2$ into the set $\{0,1\}$. For this work, X denotes a binary image.

Basic operators are defined, with respect to the structuring element B:

Erosion:

$$X \odot B = \{ y \in X \,/\, B_y \subseteq X \} \tag{1}$$

Dilation:

$$X \oplus B = \{ y \in f \,/\, B_y \cap X \neq \varnothing \} \tag{2}$$

where $B_y = \{ b + y \,/\, b \in B \}$ and $+$ represents the vector sum.

The remaining morphological operators are defined based on the combination of these two, such as opening and closing which in turn give rise to new filters [11,12]. Opening and closing are defined, respectively, as follows:
Morphological opening of the image X by the structuring element B is defined as:

$$\gamma_B(X) = (X \odot B) \oplus B \tag{3}$$

Morphological closing of the image X by the structuring element B is defined as:

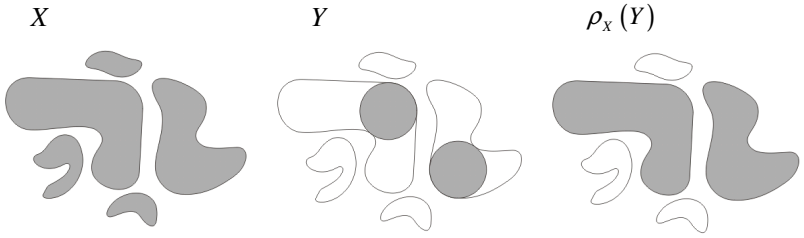
$$\Phi_B(X) = (X \oplus B) \odot B \tag{4}$$

The shape and size of the structuring element play a key role in the detection and extraction of features resulting from the image shape and size.
Conditional dilation of Y by a set X ($Y \subset X$), is defined as:

$$\delta_X(Y) = (Y \oplus B) \cap X \tag{5}$$

where B is a structuring element.

Reconstruction, $\rho_X(Y)$ where X is named mask, and Y is called marker $Y \subset X$, is obtained by iterating conditional dilation as: $\rho_X(Y) = \bigcup_{n \geq 1} \delta_X^n(Y)$, where n is the number of conditional dilations necessary to obtain the idempotency. Reconstruction is a very useful operator provided by Mathematical Morphology. The Reconstruction transformation is relatively well-known in the binary case, where it simply extracts the connected components of an image which are "marked" by another image [10]. Figure 2 shows an example of morphological reconstruction.



(a) (b) (c)
Figure.2: (a) Original image. (b) Marker image. (c) Reconstructed image.

The algorithm to distinguish motile surface patterns per area of colonization can be summarized as follows:

Step 1: Original RGB image is converted to gray level image (Fig. 3(b)). The weighted combination of each color channel (RGB) produce a gray - level image.

Step 2: Otsu binarization of the former resulting image. (Fig. 3(c))

Step 3: Area opening and area closing operators are applied to remove any pore (i.e., background connected component) with an area less than a threshold. Connectivity is given by the structuring element (square structuring element of size 3x3). (Fig. 3(d))

Step 4: Resulting image of the previous step is reconstructed from the border of the image. (Fig. 3(e))

Step 5: Image obtained in step four is subtracted from the one obtained is step three. (Fig. 3(f))

Step 6: Visualization. (Fig. 3(g))

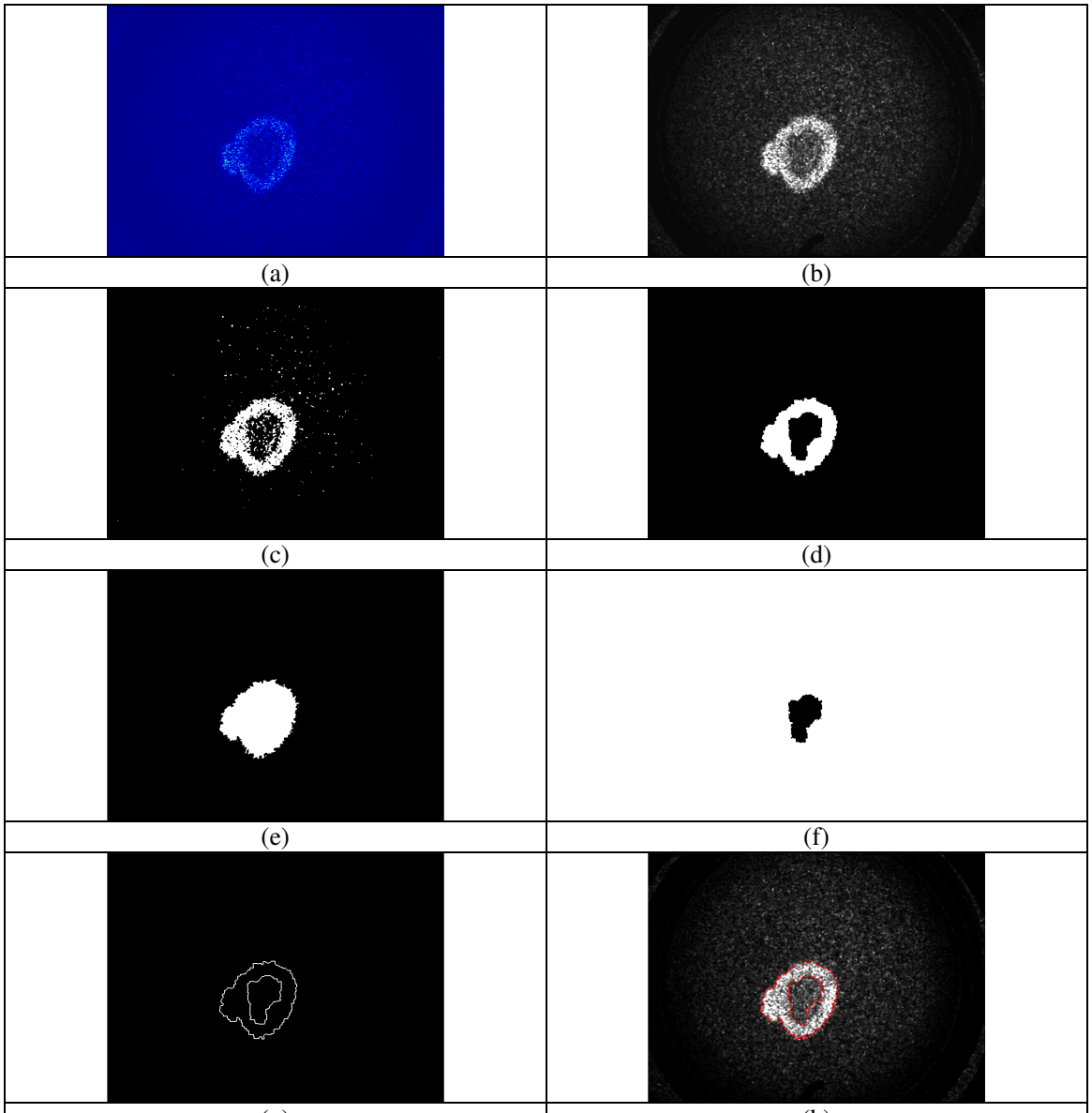


Figure. 3. a) Filtered RGB speckle images b) gray level images c) Binary image d) Filtered binary image e) reconstructed image f) g) h) visualization

3. Results and discussion

In figure 4 the first column are the resulting speckle filtered images [5] for chemotactic bacteria and for non-chemotactic and chemotactic bacteria in the same experiment. In the second column gray levels image are shown. These are obtained combining each color channel of the RGB image, and finally the last column shows in red the contours resulting after applying the calculation algorithm.
It can be observed that the algorithm locates the area of greatest bacterial activity. It is also shown that the geometry of growth does not affect the ability of the algorithm to discriminate the desired area.

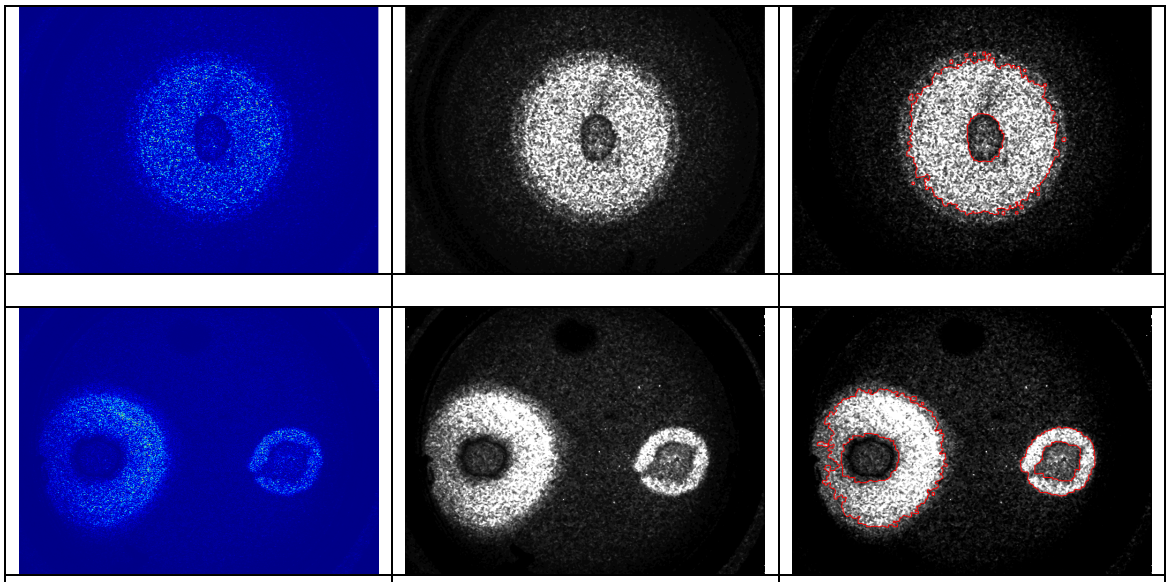


Fig. 4: a) Filtered RGB speckle images b) gray level images obtained combining each color channel of the RGB image c) visualization, contours obtained after applying the calculation algorithm.

4. Conclusions

The analysis of spatial patterns of bacteria during biofilm formation is essential for understanding the success of bacterial colonization. Therefore the quick identification of motile microorganisms attached per area is essential.
We distinguished motile surface patterns per area of colonization by applying image processing techniques in particular Mathematical Morphology. The methodology was effective for segmenting and detecting patterns of colonization known not to be completely spatially random. The presented method is fast, precise, robust, and can be used to control patterns, spacing, and orientation between colonies of different bacteria in order to prevent biofilm development.

5. References

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