Automated Biofilm Region Recognition And Morphology Quantification From Confocal Laser Scanning Microscopy Imaging

Jerzy S. Zielinski, Agnieszka K. Zielinska, Nidhal Bouaynaya, Justin G. Vaughan and Mark S. Smeltzer

***Abstract*—*Staphylococcus aureus* is an opportunistic human pathogen and a primary cause of nosocomial infections. Its biofilm forming capability is an adaptation strategy utilized by many species of bacteria to overcome stressful environmental conditions and provides both resistance to antimicrobial treatments and protection from the host immune system. This paper addresses a growing demand for an objective, fully automated method of biofilm structure description with standardized parameters that are independent of user input. In this study, we used watershed segmentation to analyze and compare confocal laser scanning microscopy (CLSM) images of two *S. aureus* strains with different biofilm-forming capabilities. Results are compared with manual calculations as well as the commonly used COMSTAT software.**

***Index Terms*—biofilm, *Staphylococcus aureus*, CLSM, mathematical morphology, watershed.**

I. Introduction

Staphylococcus aureus is an opportunistic human pathogen responsible for a wide range of diseases that vary in clinical presentation and severity. *S. aureus* can cause diseases ranging from minor skin infections to life-threatening conditions such as pneumonia, osteomyelitis and toxic shock syndrome. Recent significant changes in health care delivery and antimicrobial resistance patterns have caused a shift in the epidemiology of *S. aureus*. Recently, this has been evidenced by a dramatic increase in methicillin-resistant *S. aureus* (MRSA) infection rates which, at least in the United States, has led MRSA mortality rates to be higher than those of HIV. [1]

The public health concern caused by *S. aureus*-related infections has led to extensive efforts put into improving the efficacy of available therapies as well as introducing new pharmaceuticals. Both strategies are challenged by the fact that *S. aureus* infections are associated with formation of a biofilm, which limits the efficacy of therapy by creating a resistance to antimicrobials and by protecting the bacteria from the host immune system. In order to conduct studies on targeting biofilms therapeutically, it is necessary to be able to quantitatively measure biofilm morphological characteristics like area, biomass and thickness. In this paper, we consider a clinical isolate (UAMS-1), which forms robust, dense and uniformly distributed biofilm as well as its isogenic variant caring a mutation in the *sarA*- gene, limiting its ability to form a biofilm.

For the assessment of biofilm structure, CLSM has been described as an ideal technique [2]. By using several fluorescent stains or conjugated antibodies in combination with multichannel CLSM 3D, the location of different biofilm constituents can be recorded. Using these data sets, the three-dimensional architecture of the biofilms can be reconstructed and quantified with digital image analysis. There is a wide range of commercially available software capable of analyzing biofilm morphology, including COMSTAT, ImageJ, ISA3D and Volocity. However, they all rely on thresholding to segment the biofilm. Specifically, the automated segmentation procedure is implemented in two steps: (i) thresholding using user-dependent parameters [3] [4], followed by (ii) connecting volume filtration [5]. The purpose of this work is to create a fully automated method of biofilm segmentation and quantification that does not rely on user input or thresholding.

II. Quantifying biofilm structure

Quantitative parameters describing the biofilm physical structure have been extracted from three-dimensional confocal laser scanning microscopy images and used to compare different biofilm structures. Quantitative descriptive parameters of biofilm chosen for this study are: (i) area occupied by biomass in each cross section, (ii) biomass in biovolume, (iii) thickness distribution and (iv) average thickness.

III. Morphology quantification parameters

The following parameters are used to describe the biofilm structure:

i area occupied by biomass in each cross-section: measured as the total sum of all the unit areas (pixels) of each CLSM cross section categorized as occupied area.

$$S(z) = \oint C dx dy = \sum_i P_i(z) \qquad (1)$$

Jerzy S. Zielinski, is with Department of Applied Science, University of Arkansas at Little Rock, 2801 S. University Avenu, Little Rock, Arkansas 72204 (e-mail: jszielinski@ualr.edu).

Agnieszka K. Zielinska, is with Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, 4301 W. Markham St., Little Rock, AR 72205 (e-mail: akzielinska@uams.edu).

Nidhal Bouaynaya, is with Department of Systems Engineering, University of Arkansas at Little Rock, 2801 S. University Avenu, Little Rock, Arkansas 72204 (e-mail: nxbouaynaya@ualr.edu).

Justin G. Vaughan, is with College of Medicine, University of Arkansas for Medical Sciences, 4301 W. Markham St., Little Rock, AR 72205 (e-mail: jvaughan@uams.edu).

Mark S. Smeltzer, is with Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, 4301 W. Markham St., Little Rock, AR 72205 (e-mail: smeltzermarks@uams.edu).

Manuscript revised January 15, 2011.

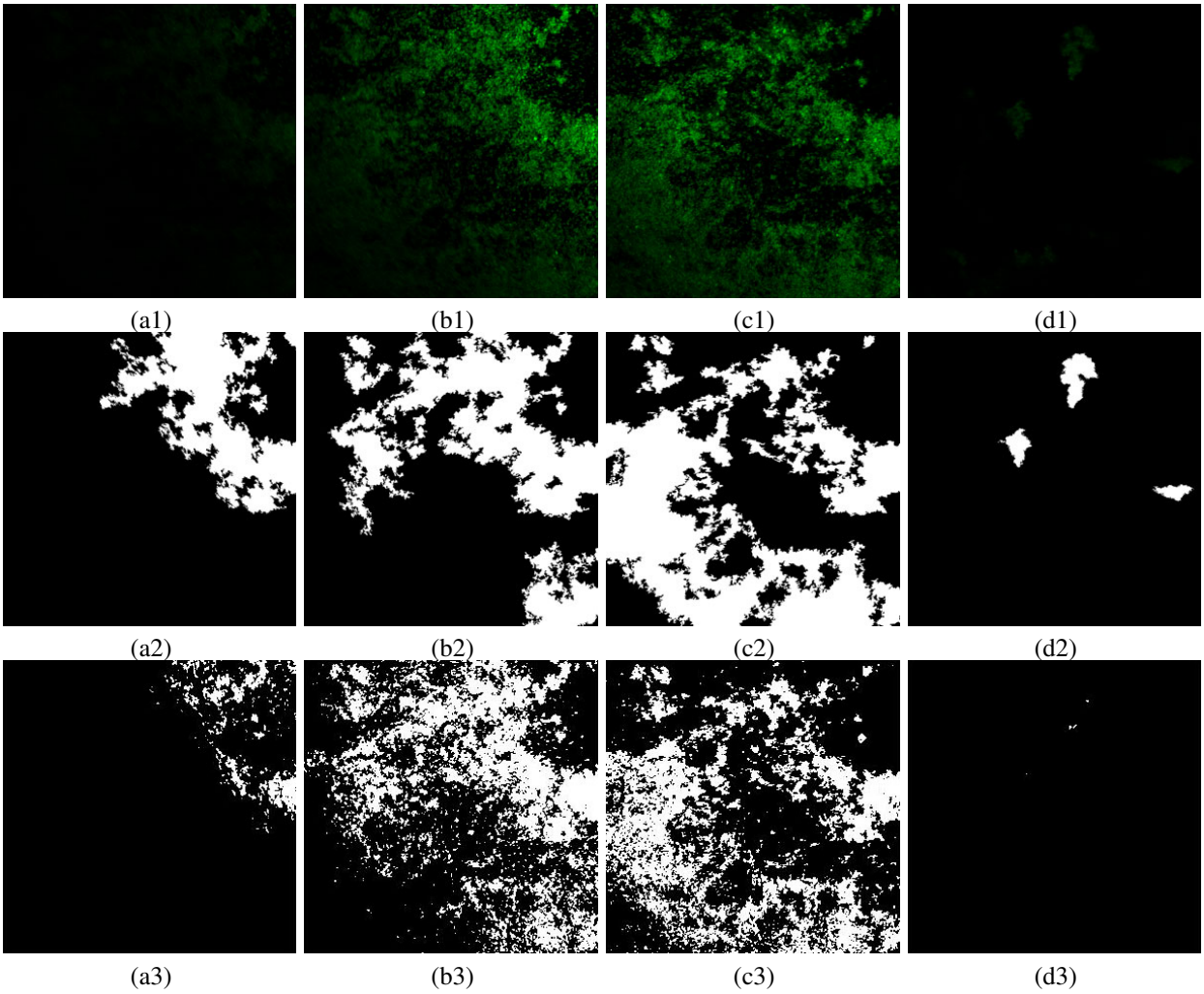

Fig. 1. Confocal images and their segmentations: A-D: images from the UAMS-1 *sarA*- mutant (section 1), their respective segmentations with watershed algorithm (section 2), and the widely used thresholding method (section 3)

where:

- $S(z)$ occupied area in cross section z,
- C closed contour of occupied area,
- $P_i(z)$ cell of a cross section recognized as occupied area

ii biomass in biovolume, V: measured from numeric integration of the area of microbial colonization profiles, following a method previously described in [6]

$$V = \int S(z)dz \approx [\frac{1}{2} \times S(z_1) + \sum_{m=2}^{m_e} S(z_m) + \frac{1}{2} \times S(z_{m_e})] \times \Delta z$$

where:

- m_e number of horizontal cross-sections,
- Δz z-step in the image stack.

iii thickness distribution: the number of occupied clusters in each cross-section over the total number of clusters in a cross-section of the CLSM 3D image.

iv average thickness: calculated as the average value of the height of all clusters of the biofilm rise from solid-substratum in the z direction between cross-sections.

Based on the four aforementioned "basic" parameters, other characteristics of the biofilm can be calculated. For example, after the biomass is segmented from the background, a number of features including roughness of the film, porosity, thickness, etc. can be obtained. Those parameters can be used together to uniquely describe the biofilm structure and to eventually differentiate between different biofilm strains.

IV. Image processing tool

The software suite of image processing operations was implemented under the Matlab programming environment (Matlab 2010a, The Mathworks, Inc). Matlab was chosen due to the convenience offered for matrix calculus. In order to

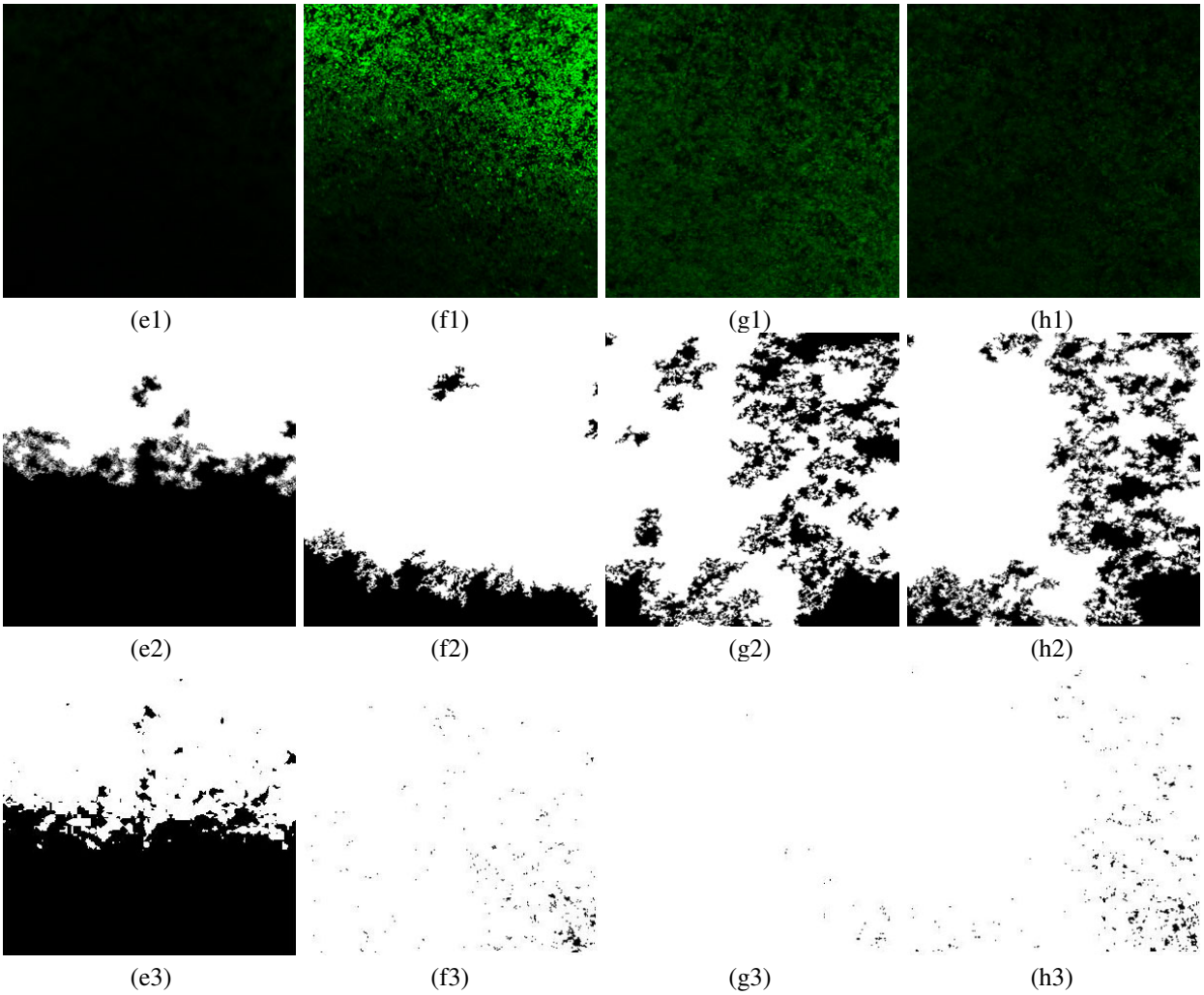

Fig. 2. Confocal images and their segmentations: E-H: images from the UAMS-1 (section 1), their respective segmentationswith watershed algorithm (section 2),and the widely used thresholding method (section 3).

evaluate our results, we used manually calculated data as a baseline and the widely used Matlab software COMSTAT for the comparison. In our approach, we use the watershed segmentation method based on Fernand Meyer's algorithm [7].

V. Preprocessing and used methodology

Segmentation is one of the most difficult image processing operations. The biofilm segmentation task is even harder because the biofilm is a disconnected structure. This difficulty may explain the use of simple thresholding in widely adopted biofilm analysis systems such as COMSTAT. Nonetheless, after trying several segmentation algorithms, it became apparent that the watershed transformation provides the most accurate segmentation of the biofilm structure. The watershed transformation finds "catchment basins" and "watershed ridge lines" in an image by treating it as a surface where light pixels are high (area of interest) and dark pixels are low (background). Segmentation using the watershed transformation works best if one can identify, or "mark," foreground objects and background locations. This marking process is done automatically with reference to the black background on the CLSM image. Marker-controlled watershed segmentation follows this basic procedure:

1) Compute a segmentation function. This is an image whose dark regions are the objects to be segmented.
2) Compute foreground markers. These are connected groups of pixels within each of the objects.
3) Compute background markers with a use of Gradient Magnitude as the Segmentation Function. These are pixels that are not part of any object.
4) Modify the segmentation function so that it only has minima at the foreground and background marker locations.
5) Compute the watershed transform of the modified segmentation function.

VI. Growth and CLSM of Static Biofilm [8]

Costar 3596 plates (Corning Life Sciences, Acton, MA) wells were coated overnight at 4oC with 20% human plasma (Sigma) in bicarbonate buffer. Overnight cultures of S. aureus grown in biofiolm media (TSB-NaCl/Glc) were diluted to an OD_{600} of 0.05 in fresh TSB-NaCl/Glc, and 200 μl of each

TABLE I
UAMS-1 *sarA*- RESULTS OF BIOMASS AND AVERAGE THICKNESS CALCULATIONS USING WATERSHED ALGORITHM AND COMSTAT SOFTWARE

	Manual calculations (baseline)	Watershed based algorithm	COMSTAT
Biomass ($\mu m^3/\mu m^2$)	5.0328	4.8320	3.48613
Average thickness (μm)	10.4750	8.4632	7.06501

TABLE II
UAMS-1 RESULTS OF BIOMASS AND AVERAGE THICKNESS CALCULATIONS USING WATERSHED ALGORITHM AND COMSTAT SOFTWARE

	Manual calculations (baseline)	Watershed based algorithm	COMSTAT
Biomass ($\mu m^3/\mu m^2$)	20.657	19.879	19.7659
Average thickness (μm)	24.5600	24.9567	23.6976

culture, after removing human plasma, was transferred to wells and incubated for 24 h at 37oC. The next day, the wells were gently washed three times with 0.85% (wt/vol) NaCl, followed by staining with mixture of SYTO-9 and propidium iodide diluted in 0.85% (wt/vol) NaCl (Cat# L7012; Invitrogen, Carlsbad, CA) for 18 min. After removing the stain, the wells were gently washed once with 0.85% (wt/vol) NaCl. Biofilm images were collected by CLSM using a LSM 510 META confocal scanning system (Zeiss, Thornwood, NY) and Axiovert 200 motorized inverted microscope (Zeiss). SYTO 9 (green, live cells) fluorescence was detected by excitation at 488 nm, and emission was collected with a 500- to 530-nm bandpass filter. Propidium Iodide (red; dead cells) fluorescence detected by excitation at 488 nm and emission collected with a 565-615 nm bandpass filter. All z-sections were collected at 1- μm intervals by using a C-Apochromat 40x/1.2W H2O objective lens. Image acquisition and processing was performed by using an LSM Image Browser (Zeiss).

VII. RESULTS

Images of the two different biofilms of strains UAMS-1 and its *sarA*- mutant and are displayed in Fig. 1 and 2 along with their respective watershed segmentations and threshold method results for comparison.

TABLE III
AVERAGE ERROR CALCULATED FROM MANUAL CALCULATIONS ACROSS ALL LAYERS IN CONFOCAL IMAGING WITH USE OF WATERSHED ALGORITHM AND COMSTAT

	sarA-Watershed	sarA COMSTAT	UAMS-1 Watershed	UAMS-1 COMSTAT
Area occupied by biomass	5%	7%	6%	3%
Thickness distribution	10%	15%	4%	3%

The biofilm parameters have been computed using the proposed watershed-based algorithm, and the widely used COMSTAT software. The ground truth values have been computed manually. The results appear in Tables I, II and III.

VIII. DISCUSSION AND CONCLUSION

The proposed algorithm efficiently segments and quantifies images not relying on a manual setup of a threshold. Average error of results obtained with watershed-based algorithm, calculated based on the manual analysis, was comparable to the one acquired with COMSTAT software. Watershed-based segmentation has proved to result in slightly more accurate quantification of the biofilm characteristics for biofilms with complex structures (i.e., not uniformly distributed), less dense and topographically developed biofilms, such as that seen in a *sarA*- strain. Dense biofilms, which are uniformly distributed like UAMS-1 were not as well recognized and will be the main target of further studies.

ACKNOWLEDGEMENT

This project is supported by the National Science Foundation EPSCoR RII Grant, Arkansas ASSET Initiative (# EPS-0701890), and the Arkansas Science & Technology Authority.

REFERENCES

[1] Nygaard TK; DeLeo FR; Voyich JM, "Community-associated methicillin-resistant staphylococcus aureus skin infections: advances toward identifying the key virulence factors.," *Curr Opin Infect Dis*, vol. 21, no. 2, pp. 147–152, 2008.

[2] Lawrence JR; Neu TR; Marshall KC, *Colonization, adhesion, agregation and biofilms*, American Society for Microbiology, 2002.

[3] J.B. Xavier; D.C. White ; J.S. Almeida, "Automated biofilm morphology quantification from confocal laser scanning microscopy imaging.," *Water Science and Technology*, vol. 47, no. 5, pp. 31–37, 2003.

[4] Staudt C; Horn H; Hempel DC; Neu TR., "Volumetric measurements of bacterial cells and extracellular polymeric substance glycoconjugates in biofilms.," *Biotechnol Bioeng.*, vol. 88, no. 5, pp. 585–592, 2004.

[5] Heydorn A; Nielsen AT; Hentzer M; Sternberg C; Givskov M; Ersbll BK; Molin S., "Quantification of biofilm structures by the novel computer program comstat.," *Microbiology*, vol. 146, pp. 2395–2407, 2000.

[6] Martin Kuehn; Martina Hausner; Hans-Joachim Bungartz;Michael Wagner; Peter A. Wilderer; and Stefan Wuertz, "Automated confocal laser scanning microscopy and semiautomated image processing for analysis of biofilms.," *Applied and Environmental Microbiology*, vol. 64, no. 11, pp. 4115–4127, 1998.

[7] Meyer; Fernand, "Topographic distance and watershed lines.," *Signal Processing*, vol. 38, pp. 113–125, 1994.

[8] Rice KC; Mann EE; Endres JL; Weiss EC; Cassat JE; Smeltzer MS; Bayles KW, "The cida murein hydrolase regulator contributes to dna release and biofilm development in staphylococcus aureus.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 19, pp. 8112–8118, 2007.