TECHNICAL NOTE

#40 Re-discovering Bacterial Biofilm Heterogeneity with MALDI Imaging Mass Spectrometry

Application

Biofilms are multicellular microbial communities where cells stick to each other and often adhere to a surface [1]. The study of biofilms is important given that most infections in the human body are caused by biofilms originated from one, or a combination of bacterial pathogens [1]. Biofilms exhibit tissue-like heterogeneity due to not only the different bacterial subpopulations, but also the spatially-defined differentiation of cells that supports the overall survival of the biofilm [2, 3]. During the process of infection in particular, concentration and distribution of nutrient metals fluctuates due to the efforts by the host to sequester essential minerals to limit pathogenicity (known as nutritional immunity [4]).

Identification of a particular bacterial subpopulation within a biofilm has been previously achieved by RNA FISH (fluorescence in situ hybridization) and reporter gene fusion techniques [5, 6]. The biggest limitation of these methods however, is that they require prior knowledge of the molecule of interest. Furthermore, the amount of targets that can be assessed at a single time is also restricted.

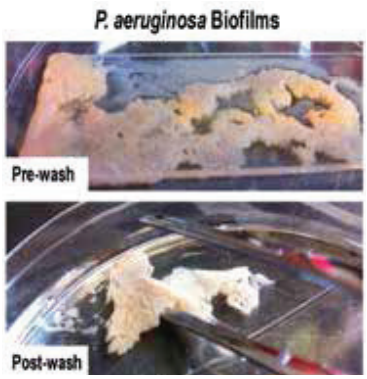
MALDI imaging mass spectrometry (MALDI IMS) has proven a powerful technology to detect analytes in tissue while preserving their spatial distribution, and thus comprises a unique approach to study bacterial biofilm heterogeneity.

Intended Use Of This Technical Note

This technical note describes the use of MALDI IMS to visualize protein distributions within *Pseudomonas aeruginosa* biofilms. HTX, the manufacturers, and the users that have accepted to share their data do not guarantee the performance of the work-flow, and each lab is responsible for applying the corresponding health and safety regulations. For research purpose only.

Background

P. aeruginosa is a biofilm-forming bacterium associated with pulmonary infections, particularly in patients with cystic fibrosis (CF) *P. aeruginosa* infects the respiratory tracts of 60-70% of CF patients by the age of 20 [7]. Here the user was interested in studying biofilm architecture (i.e. determination of subpopulations, as well as metal/nutrient/protein gradients) in P. aeruginosa by imaging protein expression in 1) biofilms grown in vitro, and 2) biofilms developed in CF human lung. In addition, the effect of calprotectin, a key player in nutritional immunity, on biologically-relevant protein profiles was examined.



Imaging Workflow

MALDI IMS of bacterial biofilms

Bacterial biofilms were grown over 5 – 6 days, in a Drip Flow Biofilm Reactor (DFR) (BioSurface Technologies) using glass microscope slides (VWR, USA; #48300-025) as growth surfaces. Below is a diagram of the experimental workflow, from biofilm culture, to IMS data acquisition (Fig. 1).

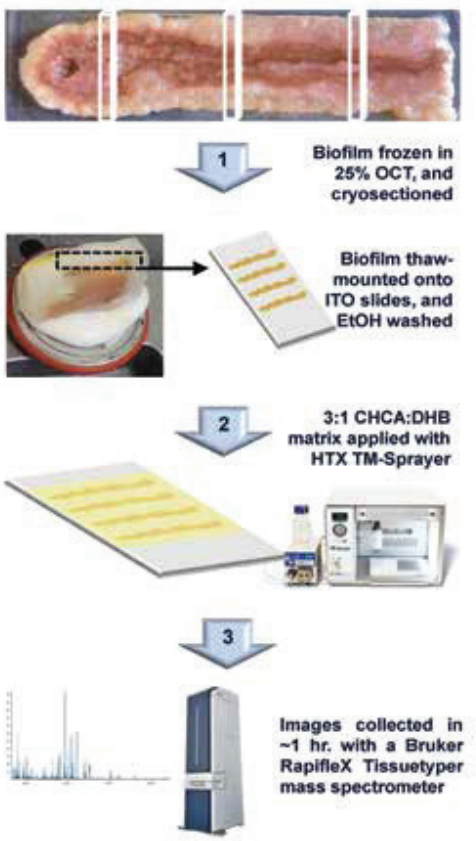


Figure 1. Summarized experimental work-flow for IMS of bacterial biofilms. See tables for further technical details and parameter optimization.

Biofilm sections were washed with 70% EtOH for 30 sec, 90% EtOH for 30 sec, 95% EtOH for 30 sec. Matrix was applied using an HTX TM-Sprayer as described below:

Flow Rate	0.2 mL/min (90% ACN)
Spray Nozzle Velocity	1,200 mm/min
Spray Nozzle Temperature	80°C
Track Spacing	2 mm
Number of Passes	8
Nitrogen Pressure	10 psi (0.7 bar)

Experimental Summary I.

Tissue type	Bacterial biofilm
Preservation	25% optimal-cutting temperature polymer (Tissue-Tek)
Tissue cut	12 µm thickness
Matrix deposition	DHB (15 mg/mL) and CHCA (5 mg/mL) in 9:1 ACN:H_2O with 0.2% trifluoroacetic acid
MALDI Laser	Gaussian beam profile Nd:YAG
Acquisition mode	Positive ion

Instrumentation and Supplies

Cryostat	Cryostar™ Thermo NX70
MALDI plate	ITO coated slides
Matrix	Sigma -Aldrich
Matrix Sprayer	HTX TM-Sprayer™
MALDI MS	BRUKER raplifleX MALDI Tissuetyper
Imaging software	BRUKER Daltonics flexImaging 4.1

IMS of biofilms were performed at 50 µm step size, in linear positive ion mode, with 50 x 50 µm pixels and laser on single beam mode. 50 laser shots were collected in random-walk mode at each pixel.

MALDI IMS of CF lungs

Biopsies from explanted CF lungs were mounted onto chilled ITO coated glass. Tissue was sequentially washed with 70% EtOH (30s), 100% EtOH (30s), 6:2:1 EtOH / chloroform / acetic acid (2min), 100% EtOH (30s), H2O (30s), and EtOH 100% (30s). Matrix was applied to washed sample sections using an HTX TM-Sprayer as described below:

Flow Rate	0.2 mL/min (90% ACN)
Spray Nozzle Velocity	1,200 mm/min
Spray Nozzle Temperature	80°C
Track Spacing	2 mm
Number of Passes	6
Nitrogen Pressure	10 psi (0.7 bar)

Experimental Summary II.

Tissue type	Human lung
Preservation	Cryogenic storage
Tissue cut	10 µm thickness
Matrix deposition	DHA (15 mg/mL) in 9:1 ACN:H_2O with 0.2% trifluoroacetic acid
MALDI Laser	Gaussian beam profile Nd:YAG
Acquisition mode	Linear positive ion

Instrumentation and Supplies

Cryostat	Leica CM 3050S Cryostat
MALDI plate	ITO coated slides
Matrix	Sigma -Aldrich
Matrix Sprayer	HTX TM-Sprayer™
MALDI MS	BRUKER raplifleX MALDI Tissuetyper
Imaging software	BRUKER Daltonics flexImaging 4.1

The MALDI MS was operated at 50 by 50mm pixels with the laser in single beam mode. 500 laser shots were collected per pixel, in 50 shot increments.

Results and MALDI MS Images

Fig. 2A shows an H&E stain of CF human lung tissue presenting significant inflammation. Gram-staining further revealed that bacteria with features consistent with P. aeruginosa and S. aureus co-colonized the infected airspace in the lung. A series of m/z protein ions were observed to localize in the inflamed airspace (Fig. 2B). Calprotectin was also found to co-localize in these areas [8].from a CF patient. Selected protein ion images collected with ultra-high speed MALDI-TOF IMS are shown if Fig. 1B-D.

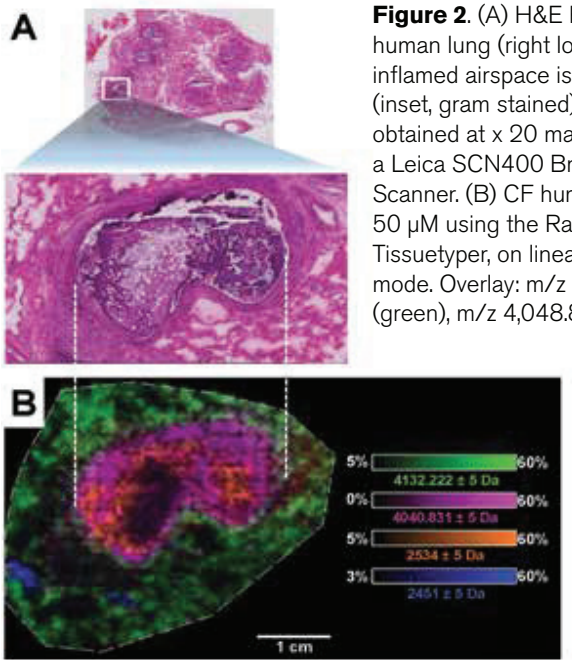


Figure 2. (A) H&E histology of CF human lung (right lower lobe). The inflamed airspace is shown enlarged (inset, gram stained). Optical images obtained at x 20 magnification using a Leica SCN400 Brightfield Slide Scanner. (B) CF human lung at 50 µM using the RapifleX MALDI Tissuetyper, on linear positive ion mode. Overlay: m/z 4,132.222 (green), m/z 4,048.831 (pink), m/z 2,534 (orange), and m/z 2,451 (blue). 100 shots/pixel in 20 µm steps, 4537 positions. Scale bar 1 cm.

Fig. 3A shows MALDI IMS of four different cross-sections of a control P. aeruginosa biofilm. MALDI images highlight the heterogeneity of m/z spatial distributions along, and within, the biofilm. Analysis of P. aeruginosa biofilms challenged with the host protein calprotectin (an antimicrobial protein that chelates essential nutrient metals) revealed metal-sensitive bacterial subpopulations within the biomass (Fig. 3B).

Bottom-up proteomics analysis of grossly-dissected biofilms allowed for the identification of hundreds of proteins related to metabolism, stress, protein synthesis, and other biological processes. MALDI IMS signals identified using bottom-up proteomics (Fig. 4) revealed distinct protein profiles in the center compared to the edge of the biofilm, as predicted by the presence of nutrient gradients (i.e. proximal vs. distal to nutrient influx).

These protein profiles were also found responsive to calprotectin exposure (Fig. 4A-C). The application

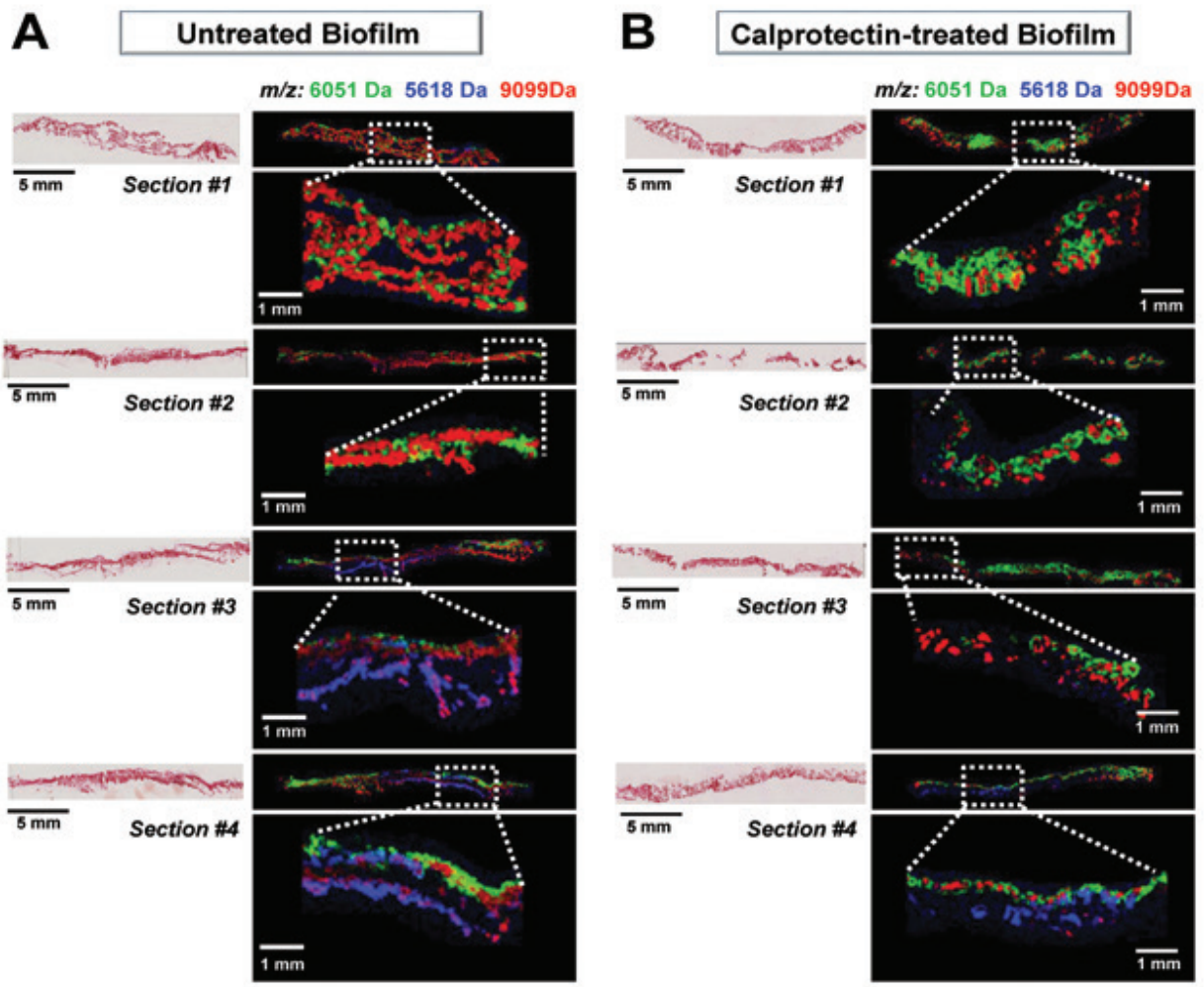


Figure 3. Four different cross-sections of Pseudomonas aeruginosa strain PA14 biofilms, untreated (A) or exposed to calprotectin in the media (B). Gram-Safranin stained images (post-analysis) are presented on the left, and MALDI IMS signals with differential biofilm localization are shown on the right. Overlay: m/z 6051 (green), m/z 5618 (blue), m/z 9099 (red). MALDI images collected at 50 µm, 66,571 pixels. Scale bar black, 5 mm. Scale bar white, 1 mm..

of MALDI IMS to the study of structural microbiology significantly furthers our understanding of how microbial communities respond to the host-imposed nutrient limitations during the process of infection.

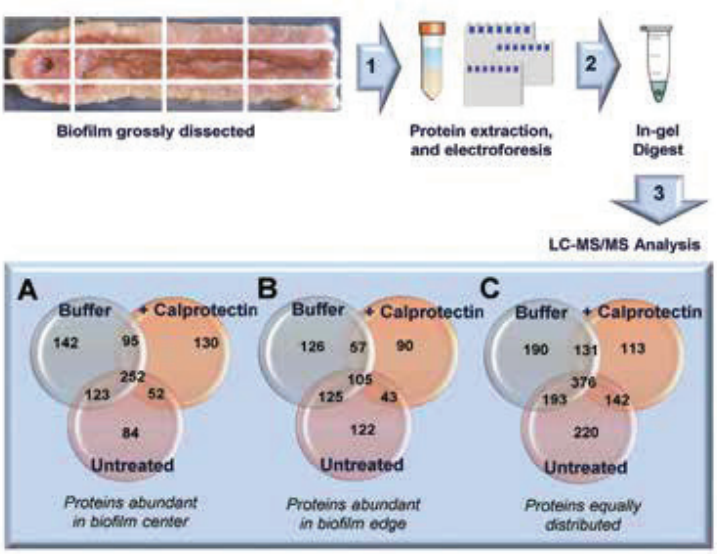


Figure 4. Summarized work-flow for proteomics analysis of PA14 biofilms. Central channel (A) or nutrient deplete (edge) regions (B) were untreated / treated with buffer / or exposed to calprotectin, dissected and lysed (80% ACN, 5% formic acid, 400 µL bacterial protein extraction reagent) for protein extraction. Protein samples were separated in a 10% Novex Bis-Tris gel at 200V, 5 min. Stained gel bands were excised and subjected to in-gel reduction, alkylation and tryptic digestion. Peptides were sequenced on a Thermo LTQ MS and the resulting spectra were searched against the Uniprot PA14 database using Sequest. Data were compiled using Scaffold version 4.4.3 [8]. (A) displays the number of proteins abundant in the biofilm center, (B) in biofilm edges, and (C) proteins that were equally distributed through the biofilm.

Conclusions

The combination of MALDI IMS, LA-ICP IMS and proteomics provides a powerful tool for the study of protein profiles (i.e. identification and spatial localization) and metal homeostasis within a bacterial biofilm, granting us the opportunity to re-discover biofilm architecture and heterogeneity, which seems tightly correlated with patterns of nutrient(s) availability.

The information and data collected by these technologies has high clinical implications given the frequent occurrence of bacterial co-infections in human disease.

References

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Acknowledgements

This Technical Note, including figures and illustrations, was produced by Maria J. Torres, East Carolina Diabetes and Obesity Institute, East Carolina University, based on a consultation with Jessica Moore, co-author of the study published in [7]. Tissue images and MS data were provided courtesy of Jessica Moore, and Jeff Spraggins, Mass Spectrometry Research Center, Department of Chemistry, Vanderbilt University School of Medicine, Nashville, TN, USA.