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Correlative Imaging of Structural and Elemental Composition of Bacterial Biofilms

Y Yang^{1,5}, R Heine¹, F Xu¹, H Suhonen², L Helfen^{1,2}, A Rosenhahn^{3,4}, T Gorniak^{3,4}, S Kirchen⁴, T Schwartz⁴, T Baumbach¹

¹Institute for Photon Science and Synchrotron Radiation /ANKA, Karlsruhe Institute of Technology (KIT), 76344 Eggenstein-Leopoldshafen, Germany

²European Synchrotron Radiation Facility (ESRF), 38043 Grenoble, France

³Analytical Chemistry-Biointerfaces, University of Bochum, 44801Bochum, Germany.

⁴Institute of Functional Interfaces (IFG), Karlsruhe Institute of Technology (KIT), 76344 Eggenstein-Leopoldshafen, Germany

⁵Institute of Geoscience, University of Freiburg, 79104 Freiburg, Germany.

thomas.schwartz@kit.edu

Abstract. Synchrotron-based phase contrast tomography (holotomography) and scanning hard X-ray fluorescence microscopy (SXF) are combined to characterize the three-dimensional (3D) structural and corresponding elemental distribution of bacterial biofilms of *Pseudomonas aeruginosa*. Samples were fixed without contrast agents or microtome sectioning. Within an intact microbial community single bacteria are clearly resolved, and their morphology can be directly visualized together with the elemental content. Such 3D set of complementary information at cellular level is essential for gaining a deeper understanding of biofilm evolution aiming to develop potential strategies on biofilm growth control and prevention.

1. Introduction

Bacterial biofilms are broadly defined as structured, multicellular communities of bacteria that are enclosed in an exopolymeric matrix and attached to a surface [1]. Many bacteria are well-known for their robustness against antimicrobial agents. This manifests itself particularly in health care where contamination of medical implants and wounds poses a severe threat to human health. But also industrial equipments like ventilation and oil wells suffer from biofilm formation [2]. Microbial organization has been investigated on the macro-scale and micro-scale [3-5] with special emphasis on their architecture and elemental composition. Biofilms show remarkable resistance to wetting [6], gas penetration [6], and uptake of heavy metals like copper, lead and zinc [7]. In this context it is important to understand which mechanisms allow this complex matrix to be robust to such an extent.

More insight into this matter is expected from the investigation of the structural organization of the bacteria in the biofilm and the measurement of the content of relevant trace elements and their distribution within the biofilm. Here we propose the application of a correlative imaging approach which enables both structural and elemental visualization of intact fixed biofilms. Due to the low



transmission of natural multilayer biofilms in the visible spectral range, conventional optical microscopy is hampered. Electron microscopy requires a large number of very thin sections due to their low penetration depth. For maintaining the integrity of the microbial communities, noninvasive multiple contrast hard X-ray imaging might be the method of choice.

Hard X-ray phase contrast tomography (holotomography) has outstanding performance in visualizing 3D cellular architectures of thick and non-transparent biological samples. The real part of the refractive index, i.e., the local variation in electron density, can be retrieved from the recorded Fresnel diffraction patterns based on a contrast transfer function approach [13]. For hard X-rays, the sensitivity of reconstructed phase maps can be two orders of magnitude better than conventional absorption contrast images [14]. Often, high resolution with good contrast is accessible without the use of sectioning or contrast agents, increasing the scientific relevance of the results.

For an elemental analysis of biological samples, synchrotron-based scanning hard X-ray fluorescence microscopy (SXFEM) is a suitable label-free probe. SXFEM with nano-scale resolution has emerged a few years ago [8] as a powerful chemical analysis tool in life sciences [9], material sciences [10] and environmental sciences [11]. By detecting characteristic fluorescence signals, this method is capable of mapping multiple elements simultaneously down to nano-scale resolution without labeling by dyes or heavy metals. Furthermore, low requirements for sample size and preparation, the high penetration power of hard X-rays and trace element sensitivity (sub-femtogram absolute detection limits) [12] renders this technique to be an excellent non-invasive way to investigate the elemental distribution within both sensitive and valuable small bulk samples.

Here we report about the results of a feasibility study aiming to image bacterial biofilms by a correlative approach [e16] combining 2D fluorescence mapping and 3D tomographic reconstruction of the electron density.

2. Materials and Methods

Gram-negative, aerobic rod bacteria *Pseudomonas aeruginosa* (environmental isolate) were selected as the representatives of the most-studied single species to form biofilms on substrates (Fig.1). A standard electron microscopy sample support, namely silicon nitride membranes (Si_3N_4) was chosen as substrate on which the bacteria were cultured in BM2 mineral medium (62 mM potassium phosphate buffer (pH 7), 7 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgSO_4 , 10 μM FeSO_4 , 0.4% (wt/vol) glucose) for 48 h. Planktonic, non adherent bacteria were removed by washing with phosphate buffered saline (pH 7.5) and subsequently the biofilms were fixed with 2.5 % glutaraldehyde solution for 1 h.



Figure 1. Optical micrograph (Nikon, Plan Fluor) of a fixed bacterial biofilm on a Si_3N_4 window. Magnification is $400\times$. Scale bar is $50\ \mu\text{m}$.

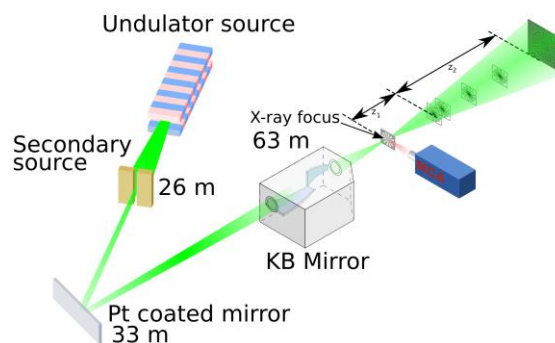


Figure 2. Sketch of the experimental setup at ID 22.

Both, the SXFEM and holotomography measurements have been performed at the nano-imaging end station of beamline ID22 at the European Synchrotron Radiation Facility (ESRF). The end station operates at energies between 17-29 keV and is dedicated to 2D and 3D nano-imaging with multiple

contrast mechanisms (see Fig. 2): propagation-based full-field phase contrast imaging (x-ray holography), scanning-probe fluorescence (covering atomic numbers $Z > 13$), and scanning-probe diffraction [17]. In our experiments, an undulator source produced X-rays of 17 keV incident energy. The rays were subsequently reflected from a Pt coated mirror to remove higher harmonics and, then focused by a pair of elliptically bent, graded multilayer mirrors (Kirkpatrick-Baez configuration). The measured focal spot size was 60×65 (vertical \times horizontal) nm^2 FWHM with a photon flux of 10^{12} photons/s at 17 keV. The high photon flux rendered a satisfying signal to noise ratio in a relatively short time.

For holotomography, 1999 phase contrast projection micrographs per tomographic rotation were recorded at four different propagation distances respectively. Each projection was acquired with 0.2 s exposure time. The pixel size of 25 nm in the sample is directly related to the physical detector pixel size of $0.756 \mu\text{m}$ and the geometrical magnification ($30.2 \times$) given by the sample position between the focal spot and the detector. Subsequently performing SXFM at the same sample position, the specimen was moved into the focus and raster scanned with a scanning pitch of 100 nm for 2D elemental mapping. Fluorescence spectra were collected with a dwell time of 0.1 s by a silicon drift detector (Vortex-EX, SII Nanotechnology). The spectra were normalized and fitted against the measured standards using PyMca for quantification of elemental contents [18].

3. Results and discussion

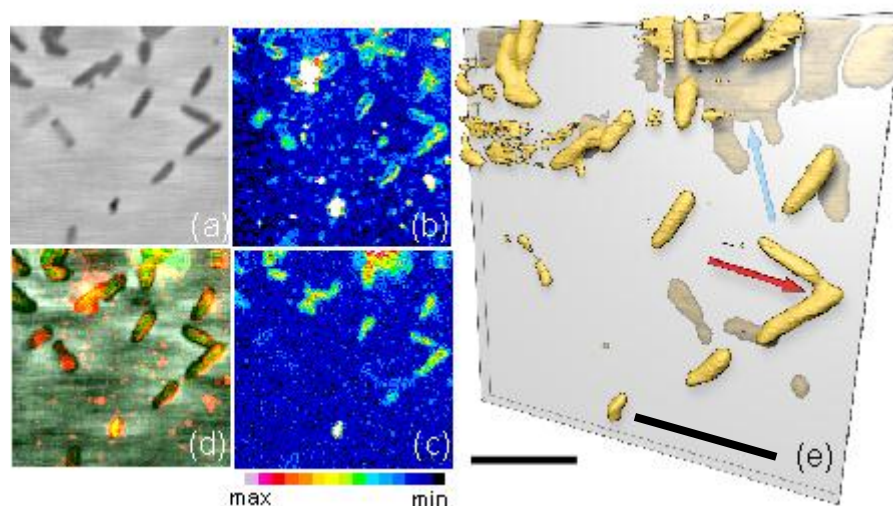


Figure 3. (a) Cross section of the reconstructed holotomogram showing single bacteria. (b)-(c) X-ray fluorescence images of the bacterial biofilm, with contrast adjusted independently (b: Fe, c: P). Scanned area $10 \times 10 \mu\text{m}^2$. (Scanning pitch: 100 nm/pixel, dwell time: 0.2 s/pixel, FWHM of beam: 100 nm). (d) Superposed image of 2D elemental compositions and the reconstructed cross section (red: Fe, green: P, gray: cross section (a)). (e) Corresponding volume rendering of the same area. Scale bar is $5 \mu\text{m}$

Fig. 3 shows exemplary results. The bacteria are well resolved in the fluorescence micrographs (see Figs. 3 (b) and (c) for the distribution of two selected elements) and their morphology is reproduced by the holotomogram (Figs. 3 (a) and (e)). In the volume rendering (Fig. 3 (e)) we observe two layers of bacteria that formed on both sides of the membrane, where different development states can be distinguished. On the foreside we find bacteria which are still in initial attachment (red arrow), on the back side of the membrane there are also bacteria in the state of irreversible attachment (blue arrow) with bacterial cells cemented to the substrate and forming nascent cell clusters [17]. Furthermore, when combined with the fluorescence images, the elemental composition of bacterial cells and the

clusters can be determined without ambiguity (compare Fig. 3 (d)). Phosphate is an indispensable component of many macromolecules, like proteins, nucleic acids, and lipids [2], so P appears spread over the whole cell as can be seen in Figs. 3 (c) and (d). The map of the Fe content (Figs. 3 (b) and (d)) shows higher concentrations along the contours of the cells indicating that Fe is richer along the cell membranes. One explanation for this distribution comes from the strong Fe binding agents called siderophores. They are suspected to be secreted by the bacteria to tightly bind Fe from the exterior of the cells and transport it into the cells as Fe is an essential trace nutrient for microbial life processes [18].

Conclusion

X-ray holotomography in combination with fluorescence microscopy provide a comprehensive protocol for biofilm research to simultaneously and correlatively investigate morphology and elemental composition. It is demonstrated as a versatile probe which benefits from both high resolution down to presently sub 100 nm pixel size and trace element sensitivity. Overall, this protocol enables visualization of spatial structures and elemental distribution of individual bacteria without inducing changes to the original microbial organization by slicing or chemical staining. This is especially useful for maintaining the natural state of a biofilm within its original habitat, opening the door for possible *in-situ* microbial research in the future.

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References

- [1] O'Toole G, Kaplan H B and Kolter R. 2000 *Annu. Rev. Microbiol.* **54** 49-79
- [2] Madigan M T, Martinko J M, Dunlap P V and Clark D P. 2009 *Pearson Press.* 677-679
- [3] Iltis G C, Armstrong R T, Jansik D P, Wood B D, and Wildenschild D. *Water Resour. Res.* **47** W02601
- [4] Lawrence J R, Swerhone G D W, Leppard G G, Araki T, Zhang X, West M M and Hitchcock A P. 2003 *Appl. Environ. Microbiol.* **69** 5543-5554
- [5] Neu T R, Manz B, Volke F, Dynes J J, Hitchcock A P, and Lawrence J R. 2010 *FEMS Microbiol Ecol.* **72** 1-21
- [6] Epsteina A K, Pokroya B, Seminarab A, and Aizenberga J. 2011 *PNAS.* **108** 995-1000
- [7] Teitzel G M and Parsek M R. 2003 *Appl. Environ. Microbiol.* **69** 2313-2320.
- [8] Vogt S, Maser J, Paunesku T, Stojićević N, Lai B and Woloschak G E. 2004 *Microsc Microanal.* **10** 1028-1029
- [9] Ortega R, Cloetens P, Devès G, Carmona A, Bohic S. 2007 *PLoS ONE.* **2** e925
- [10] Buonassisi T, Istratov A A, Heuer M, *et al.* 2005 *J. Appl. Phys.* **97** 074901
- [11] Endo S, Terada Y, Kato Y and Nakai I. 2008 *Environ Sci Technol.* **42** 7152-8
- [12] Bacquart T, Devès G, Carmona A, *et al.* 2007 *Anal Chem.* **79** 7353-9
- [13] Cloetens P, Ludwig W, Baruchel J, *et al.* 1999 *J. Phys. D.* **32** A145-A151
- [14] Cloetens P, Mache R, Schlenker M, and Lerbs-Mache S. 2006 *PNAS.* **103** 14626-14630
- [15] Bleuet P, Cloetens P, Gergaud P, *et al.* 2009 *Rev. Sci. Instrum.* **80** 056101
- [16] Xu F, Helfen L, Suhonen H, *et al.* 2012 *PLoS ONE.* **7(11)**:e50124
- [17] Martinez-Criado G, Tucoulou R, Cloetens P, *et al.* 2011 *J Synchrotron Radiat.* **19** 10-18
- [18] Solé V A, Papillon E, Cotte M, Walter Ph, Susini J. 2007 *Acta Part B* **62** 63-68
- [19] Sauer K, Camper A K, Ehrlich G D, *et al.* 2002 *J. Bacteriol.* **184**:1140-1154.
- [20] Cox, C D and Adams P. 1985 *Infect. Immun.* **48**:130-138.