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Confocal Raman microscopy and fluorescent *in situ* hybridization – A complementary approach for biofilm analysis

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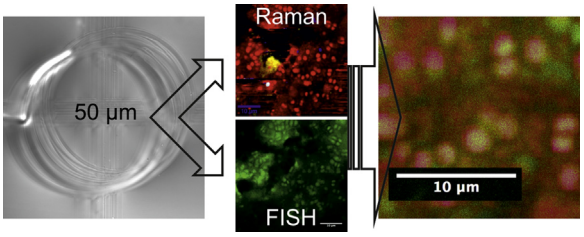
HIGHLIGHTS

- Raman microscopy provides *a priori* knowledge for fluorescent *in situ* hybridization.
- FISH identification and *in vivo* compatible Raman spectrum of each microbial cell.
- Mapping of microbes, chemical composition, biological and non-biological components.
- Cells appear larger in Raman than in FISH images due to respective signal origin.
- Technically easy, straight-forward mechanical approach based on micro-engravings.

GRAPHICAL ABSTRACT



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ABSTRACT

We combine confocal Raman microscopy (CRM) of wet samples with subsequent Fluorescent *in situ* hybridization (FISH) without significant limitations to either technique for analyzing the same sample of a microbial community on a cell-to-cell basis. This combination of techniques allows a much deeper, more complete understanding of complex environmental samples than provided by either technique alone. The minimalistic approach is based on laboratory glassware with micro-engravings for reproducible localization of the sample at cell scale combined with a fixation and de- and rehydration protocol for the respective techniques. As proof of concept, we analyzed a floc of nitrifying activated sludge, demonstrating that the sample can be tracked with cell-scale precision over different measurements and instruments. The collected information includes the microbial content, spatial shape, variant chemical compositions of the floc matrix and the mineral microparticles embedded within. In addition, the direct comparison of CRM and FISH revealed a difference in reported cell size due to the different cell components targeted by the respective technique. To the best of our knowledge, this is the first report of a direct cell-to-cell comparison of confocal Raman microscopy and Fluorescent *in situ* hybridization analysis performed on the same sample. An adaptation of the method to include native samples as a

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Activated sludge
Ammonium oxidizing bacteria

starting point is planned for the near future. The micro-engraving approach itself also opens up the possibility of combining other, functionally incompatible techniques as required for further in-depth investigations of low-volume samples.

1. Introduction

Fluorescent *in situ* hybridization (FISH) in combination with confocal laser scanning microscopy (CLSM) is a standard method for mapping microbial diversity (Moter and Göbel, 2000). rRNA-targeted oligonucleotide probes for FISH have been developed for the *in situ* detection of a multitude of microorganisms at different taxonomic levels and the method is widely used to describe microbial diversity in complex samples. For example, ammonia-oxidizing bacteria (AOB) are key players in the global nitrogen cycle, regulating the availability of ammonium by oxidizing it to nitrite. They are of economic importance worldwide especially in wastewater treatment and agricultural soil management. The FISH method has been used to describe the microbial diversity of AOB in samples ranging from environmental freshwater (French et al., 2012) and rice paddy soil (Briones et al., 2003) to man-made systems such as activated sludge tanks (Juretschko et al., 1998) and biofilm reactors (Nogueira et al., 2002). Presently, an extensive set of FISH probes targeting different taxonomic levels of AOB is available. The University of Vienna developed probeBase, an online data base containing all probes developed for FISH (Greuter et al., 2016; Loy et al., 2007).

However, despite its widespread success FISH is limited in several aspects. It is an invasive technique, allowing only end-point analyses since it invariably alters the sample due to the applied dyes, probes and fixations. The number of probes that can be used simultaneously on the same sample is limited by the color mixing of the attached fluorescent dyes. The fixation and pretreatment steps required for it are often strain dependent, resulting in a high risk of biased results in the case of samples containing many different or unknown species (de los Reyes et al., 1997; Macnaughton et al., 1994). By its very nature FISH cannot provide information about non-biological components often found embedded in extended samples of multilayered microbial communities such as biofilms (Kniggendorf and Meinhardt-Wollweber, 2011).

Confocal Raman microscopy (CRM) on the other hand may provide information on the two- and three-dimensional structure and the associated microorganisms in a microbial community along with information about possible non-biological components ranging from embedded mineral particles to variant chemical composition of the biofilm matrix itself (Sandt et al., 2008; Schwartz et al., 2009; Wagner et al., 2009). It can provide in-depth information about the protic content of a cell, providing insight into cell functions (Kniggendorf et al., 2014; Schultz and Levin, 2011) or cell identification (Gaus et al., 2006; Kniggendorf and Meinhardt-Wollweber, 2011; Maquelin et al., 2006) depending on the protein queried. Other than FISH, it is an *in vivo* method which does not require extensive preparation and *a priori* knowledge of the sample, and it is not limited in the number of species queried in a single study (Harz et al., 2009b; Kniggendorf and Meinhardt-Wollweber, 2011; Neu et al., 2010). However, analyzing Raman spectral images of complex microbial samples is a challenging task due to the large number of microbial species present (Stewart and Franklin, 2008), most of which have not been studied with Raman spectroscopy before. A positive identification of a microbial species or strain can be achieved by analyzing a resonantly enhanced Raman spectrum of its cytochrome-c. However, the specific cytochrome-c resonant Raman spectrum needs to be determined by measuring a known sample, thus the number of directly Raman-identifiable species is still relatively small (Kniggendorf and Meinhardt-Wollweber, 2011).

An analysis of the same microbial sample with both methods, Raman spectroscopy and FISH, targeted with cell-scale precision, would allow to obtain and assign specific Raman spectra for the vast pool of identified microbes known to FISH. On the other side, Raman spectroscopy can provide critical initial information about already Raman-identifiable microbes and non-biological constituents of the sample for a better targeted FISH analysis. Unfortunately, techniques based on fluorescent staining such as FISH are inherently incompatible with Raman spectroscopic techniques due to the quantum yield of the Raman effect being several orders of magnitude weaker than that of fluorescence. Alternative options for labeling the probes, such as quantum dots (Medintz et al., 2005) or even radionuclides (Pardue and Gall, 1969), do exist, but involve serious trade-offs regarding cost efficiency and experimental safety. Any combination of Raman spectroscopy and FISH with standard fluorescent staining resulted in very limited usability of Raman spectroscopy in the respective experimental context. For example, Huang et al. combined stable-isotope Raman spectroscopy and FISH for the cultivation-independent identification of microbial cells and structure-function analysis in complex microbial communities (Huang et al., 2007), (Huang et al., 2009). However, the advantage of Raman microscopy as a non-invasive method had to be traded and the resulting Raman spectra were not suitable for microbial identification.

In addition, many of the fixation protocols required for satisfactory FISH results are also detrimental to Raman microscopy. Of the three standard fixation procedures (Moter and Göbel, 2000), ethanol fixation, fixation with paraformaldehyde (PFA) solution, and heat treatment, only ethanol fixation does not affect the Raman spectrum immediately (Kniggendorf et al., 2011). However, it does cause a degradation of the spectrum over time, making ethanol fixed bacteria unsuitable for storage for subsequent Raman analysis. The heat treatment recommended for Gram positive bacteria causes an extreme background in the Raman spectrum, eliminating all but the strongest Raman lines (Kniggendorf et al., 2011). The treatment with paraformaldehyde solution recommended for Gram negative bacteria causes a significant loss in signal intensity over the whole Raman spectrum and an additional asymmetric loss at higher wavenumbers when applied on slides with a poly-L-lysine coating for better cell attachment. While different coating agents have yet to be tested with respect to the asymmetric loss in signal intensity, the overall loss in signal intensity has been observed also on uncoated glass slides, thus it may be considered intrinsic to PFA fixed samples (Kniggendorf et al., 2011).

In this paper, we combine confocal Raman microscopy (CRM) with subsequent Fluorescent *in situ* hybridization (FISH) without significant limitations to either technique. The mechanical approach is methodologically straight-forward and highly adaptable with respect to sample dimensions, cell sizes, and employed techniques. To the best of our knowledge, this is the first report of a