

Letter

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Nonlinear laser scanning microscopy of oral multispecies-biofilms: fixative induced fluorescence as a fast and economical in vitro screening method

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Abstract: In this letter we report a fast and easy method which could be used for initial screening of multispecies-biofilm development on putative new dental implant materials. Most staining methods require numerous washing steps that can result in detachment of loosely bound biofilms and therefore falsify the results. Thus, we used glutaraldehyde fixation, which induces autofluorescence through bacterial membrane protein cross-linking and concurrently stabilizes the biofilm structure. We analyzed the biofilms with nonlinear laser scanning microscopy and were able to (I) evaluate the multispecies-biofilm growth and (II) distinguish between bacterial species based on different two-photon autofluorescence intensities.

Keywords: autofluorescence; dental implant; glutaraldehyde; multiphoton microscopy; periimplantitis; Schiff base.

Multispecies-biofilms play an important role in the development and progression of periimplantitis, which is

considered as one of the major complications in dental implantology. If left untreated, the bacterial induced inflammation leads to bone loss and in the end to implant failure [1, 2]. To improve the clinical situation, current research efforts focus on the development of new implant materials with antimicrobial properties. However, these candidate materials have to be tested for their antimicrobial efficacy in sophisticated screening procedures that mostly include microscopic examination. For increased specificity and sensitivity, microorganisms are usually stained with fluorescent dyes or probes. However, these staining procedures often require numerous washing steps whereby loosely bound biofilms can be easily washed away and thus impeding data analysis and interpretation. Furthermore, DNA-intercalating dyes are often carcinogenic or at least toxic, making their handling and disposal more demanding. The aim of this study was therefore the establishment of a fast and easy method without complex staining procedures which can be used for initial screens. For this purpose we made use of a well-known property of aldehyde fixatives: the generation of fixative-induced fluorescence. This normally can be a problem if using specific probes or dyes because it complicates the distinction of real signals from the fixative-induced fluorescence in histological tissues [3]. During the fixation with glutaraldehyde, which was first described as a fixative for electron microscopy in 1963 [4], the aldehyde reacts with amino groups of proteins in the tissue via covalent cross-linking [5, 6]. Bacteria contain a large number of membrane proteins which can react with the aldehydes during the fixation process. Their product, a Schiff base, leads to a high autofluorescence of the cells while microscopic examination [7, 8]. The fixation procedure is easy to perform, cost efficient and increases the mechanical stability of biofilms [9–11]. We expected that a species-specific membrane protein composition would result in

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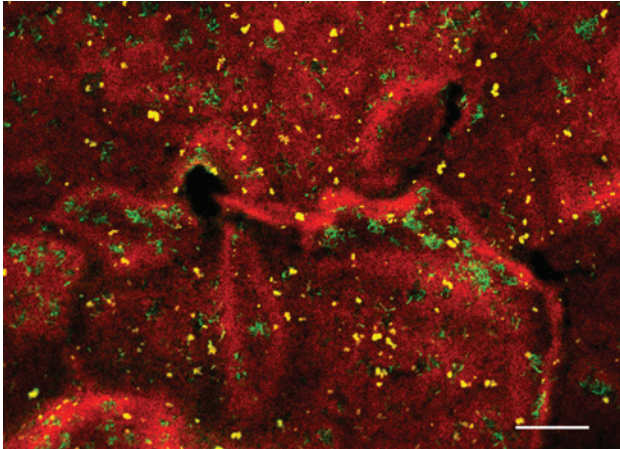


Figure 1: Exemplary CLSM image of a biofilm stained with urea-NaCl-FISH.

The biofilm contains the bacterial species *S. oralis* (Probe MIT-588 [13]; labeled with Alexa-405; red), *A. naeslundii* (Probe ANA-103 [13]; labeled with Alexa-488; green) and *V. dispar* (Probe VEI-217 [14]; labeled with Alexa-568; yellow). *S. oralis* dominates the biofilm, whereby *A. naeslundii* occurs as bigger aggregates and *V. dispar* as single cells or microcolonies. FISH was chosen in order to correlate the single species arrangement within the biofilm with the NLSM results of fixative-induced fluorescence. *S. oralis* ATCC 9811 was acquired from the American Type Culture Collection (ATCC); *A. naeslundii* DSM 43013 and *V. dispar* DSM 20735 from the German Collection of Microorganisms and Cell Cultures (DSMZ). The bacteria were precultured under anaerobic conditions (80% N₂, 10% H₂, 10% CO₂) in brain heart infusion medium (BHI; Oxoid, Wesel, Germany) supplemented with 10 µg/mL vitamin K at 37 °C. Subsequent to the biofilm experiments, each planktonic preculture was adjusted to an optical density at 600 nm (OD₆₀₀) of 0.1. To obtain a three-species biofilm, 300 µL of *S. oralis*, 300 µL of *A. naeslundii* and 300 µL of *V. dispar* were mixed with 2.1 mL BHI/vitamin K medium. From these mixtures, 150 µL were applied to individual wells of a 12-well µ-Chamber Slide (ibidi, Martinsried, Germany) and grown for 48 h under anaerobic conditions (80% N₂, 10% H₂, 10% CO₂) at 37 °C. Urea-NaCl-FISH was modified after [12]. Biofilm images were acquired with a confocal laser scanning microscope (CLSM) (C2si, NIKON, Tokio, Japan). The image was edited with ImageJ [15]. Scale bar: 30 µm.

different fluorescence intensities. Initially, we performed a multiprobe fluorescence in situ hybridization assay (urea-NaCl-FISH; modified after [12]). Due to probe labeling with different fluorescent dyes, this method allowed the simultaneous localization of three individual bacterial species within a biofilm. To analyze the biofilm with species-specific labels, confocal laser scanning microscopy (CLSM; C2si, NIKON, Tokio, Japan) was used. The urea-NaCl-FISH experiment (Figure 1) showed *Streptococcus oralis* as the most prevalent species (red). *Actinomyces naeslundii* occurred in bigger aggregates (green) whereas *Veillonella dispar* formed occasional spots or little microcolonies (yellow).

With this background knowledge, we started NSLM analysis of autofluorescence signals. Therefore, the

laser scanning microscope TriM Scope II (LaVision Biotec GmbH, Bielefeld, Germany) equipped with the titanium:sapphire femtosecond laser system Chameleon Ultra II (Coherent Inc., Santa Clara, CA, USA) was used. With such a microscope short laser pulses from the

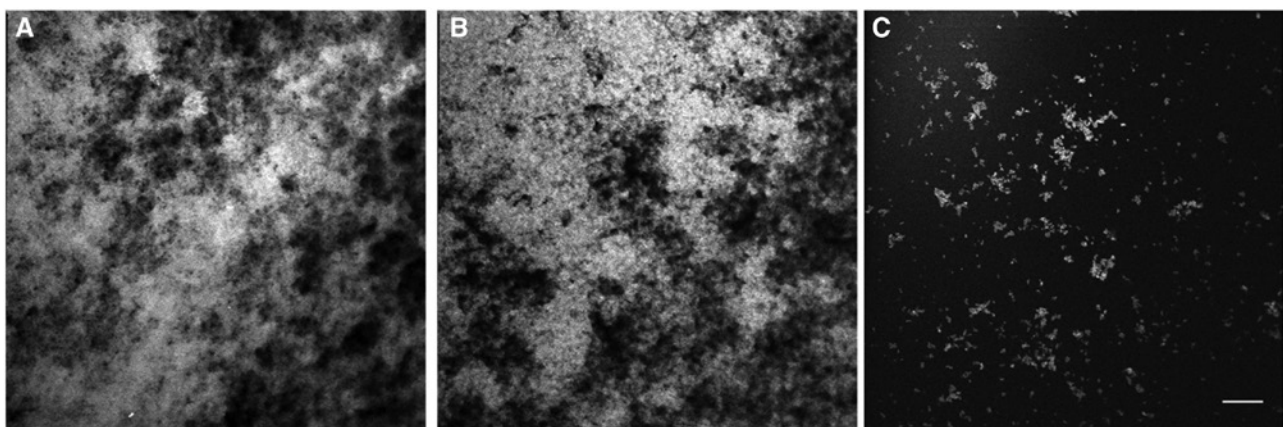


Figure 2: Exemplary NLSM images of 48 h old biofilms of (A) *S. oralis*, (B) *A. naeslundii*, and (C) *V. dispar*.

For the development of single-species biofilms, 300 µL of each species suspension were mixed with 2.7 mL fresh BHI/vitamin K medium to acquire a final OD₆₀₀ of 0.01. The subsequent cultivation was carried out as described before. After 48 h, the biofilms were fixed for 30 min using 2.5% glutaraldehyde (Roth, Karlsruhe, Germany) before the silicone cultivation chamber was removed. The glass slides were covered with Dulbecco's Phosphate Buffered Saline (PBS; Biochrom GmbH, Berlin, Germany) and a glass coverslip (ibidi, Martinsried, Germany) before microscopic examination. The experiments were prepared in triplicate. The images were edited with ImageJ [15]), Every image shows a representative imaging plane inside a biofilm. Scale bar: 30 µm.

near infrared are focused into the sample. Simultaneous absorption of two-photons within the focal volume excites fluorescent molecules inside the biofilm. After internal molecular conversion processes a transition from the excited state into the ground state finally leads to fluorescence. Since the excitation is restricted to the focal volume no pinhole (as in confocal microscopy) is necessary in the

detection beam paths. Thus, spatially localized excitation takes place and defined imaging planes can be scanned. In the experiments excitation of two-photon fluorescence was realized with a central laser pulse wavelength of 780 nm. In all cases, two-photon autofluorescence signals could be observed over a broad wavelength range (435–485 nm and 500–550 nm; detectable wavelength regimes

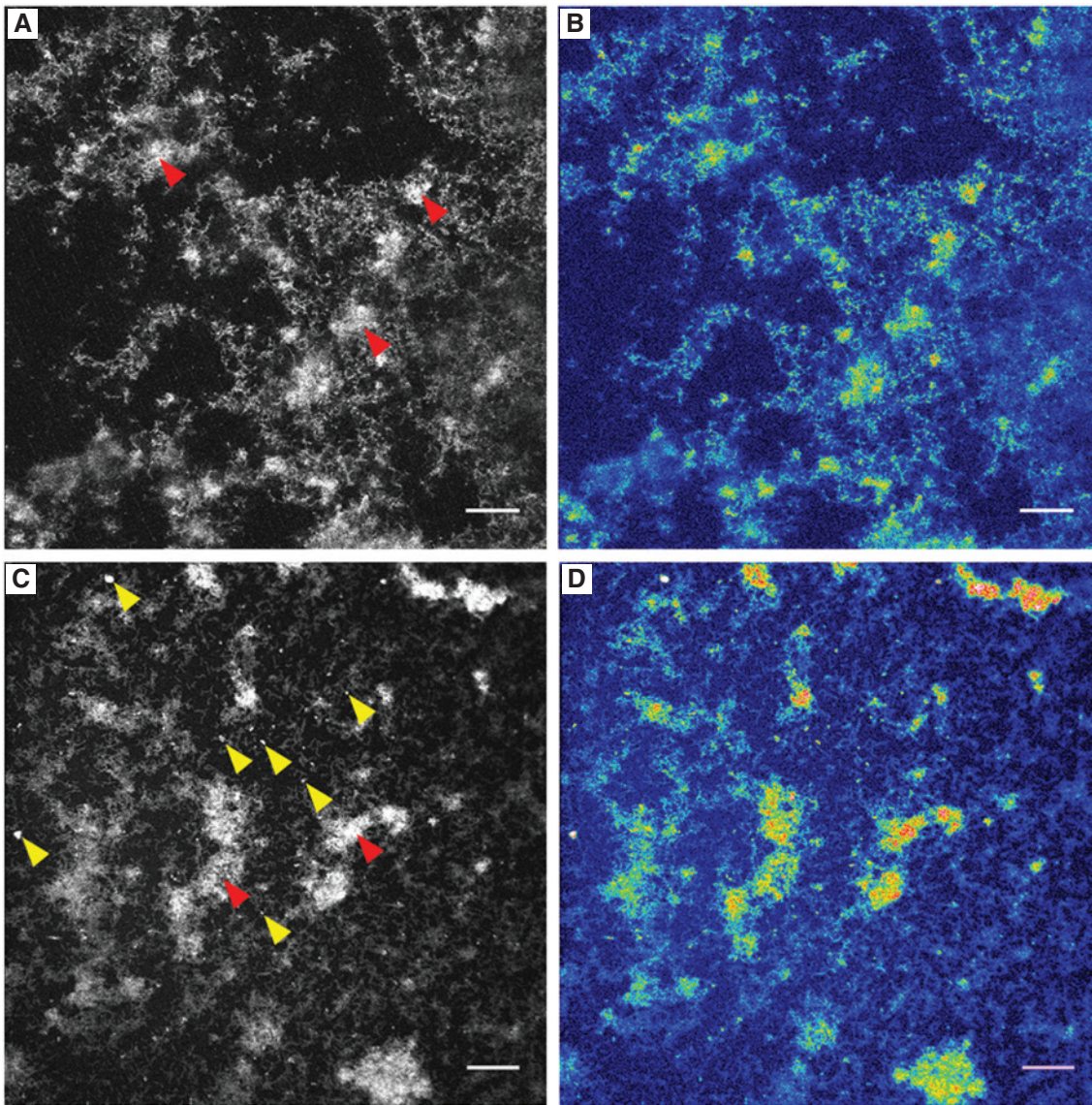


Figure 3: Exemplary NLSM images of 48 h old biofilms of (A) a two-species biofilm containing *S. oralis* and *A. naeslundii* and (B) a three-species biofilm containing *S. oralis*, *A. naeslundii* and *V. dispar*.

To obtain a two-species biofilm, 300 μL of *S. oralis* and 300 μL of *A. naeslundii* preculture ($\text{OD}_{600}=0.1$) were mixed together with 2.4 mL fresh BHI/vitamin K medium. To obtain a three-species biofilm, 300 μL of *S. oralis*, 300 μL of *A. naeslundii* and 300 μL of *V. dispar* were mixed with 2.1 mL BHI/vitamin K medium. Cultivation, fixation and microscopic examination were carried out as described before. Image pairs (A), (B) and (C), (D) show identical biofilm sections, both in grayscale (left) and pseudocolor (right; 16-color lookup table, ImageJ; [15]). Large aggregates with a brighter fluorescence signal can be seen in the two-species biofilm in (A), exemplarily marked with red arrows. These aggregates can also be seen in the three-species-biofilm in (C). In (B) and (D) these aggregates can be seen as green, yellow and red clusters. Additionally, within the three-species biofilm, brighter smaller spots or microcolonies can be seen in between, in (C) exemplarily marked with yellow arrows and in (D) as green, yellow and red spots. Scale bars: 30 μm .

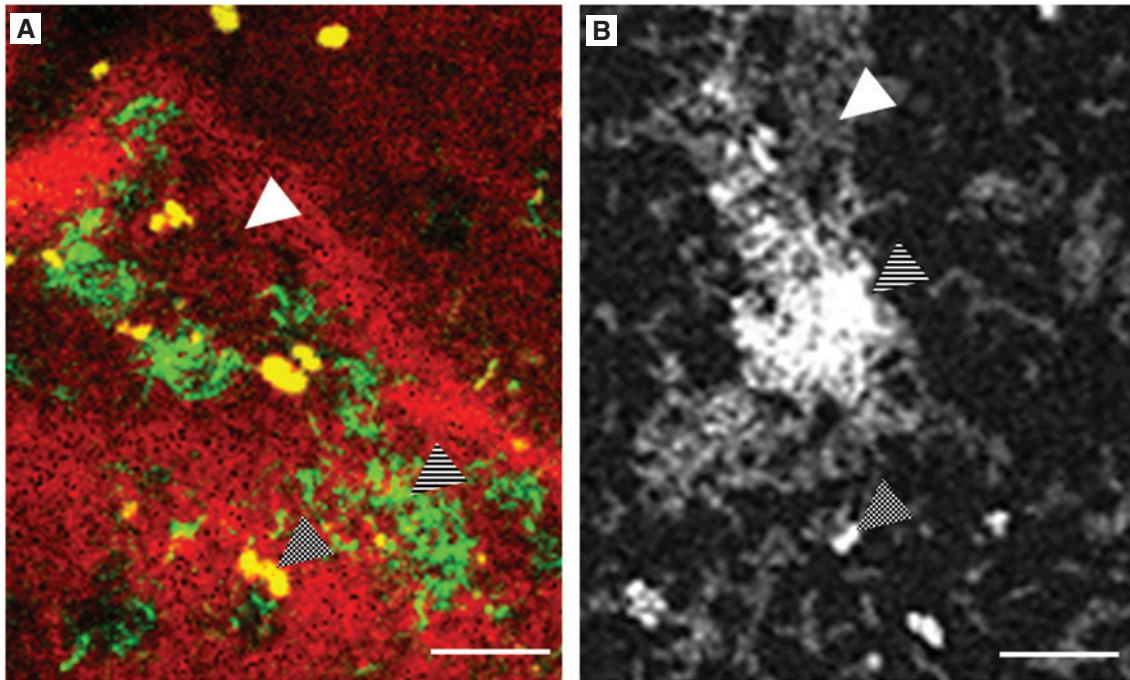


Figure 4: Comparison of urea-NaCl-FISH results (A) with autofluorescence image (B) of 48 h old three-species biofilm containing *S. oralis*, *A. naeslundii* and *V. dispar*.

The urea-NaCl-FISH experiment (A) demonstrated that the largest part of the biofilm shown in (B) belongs to *S. oralis* (white arrow). The brighter regions can be correlated to the aggregates of *A. naeslundii* (striped arrow) and the bright spots to *V. dispar* (pointed arrow). Scale bars: 10 μm .

defined by bandpass filters). The images from the two wavelength ranges were comparable and showed no difference in structural information. Therefore, only NLSM images on two-photon fluorescence signals in the range of 500–550 nm are displayed in this letter.

In a first step, single-species biofilms of *S. oralis*, *A. naeslundii* and *V. dispar* were investigated for their fixative-induced two-photon fluorescence patterns and are displayed in Figure 2. The 48 h old biofilms of *S. oralis* (Figure 2A) and *A. naeslundii* (Figure 2B) showed very dense and spongy structures compared to the thinner biofilm of *V. dispar* (Figure 2C). Inside the single species biofilms, no differences regarding the fluorescence intensities could be detected. In contrast, brighter fluorescing aggregations of bacteria could be detected within a dual-species biofilm in Figure 3A and in a three-species biofilm in Figure 3C (in both exemplarily marked with red arrows). The aggregates can also be seen in the images which were modified with a 16-color lookup table as green, yellow and red cluster (Figure 3B, D). In the three-species biofilm the third bacterial species occurs occasionally as brighter spots between the two others (Figure 3C; exemplarily marked with yellow arrows). We were able to correlate these two-photon autofluorescence images from NLSM with the results from urea-NaCl-FISH (Figure 1) and demonstrated

the similarities in Figure 4: *S. oralis* dominates the biofilm (exemplarily marked with a white arrow). The brighter fluorescing aggregates could be identified as *A. naeslundii* (exemplarily marked with a striped arrow) and the spots or microcolonies could be identified as *V. dispar* (exemplarily marked with a pointed arrow). We were able to distinguish between the three bacterial species based on different two-photon autofluorescence intensities.

In situ hybridization labeling is more laborious and costly than the glutaraldehyde fixation method. The latter is therefore more suited for standardized medium to high volume screening applications where cost-effectiveness and ease of use are given priority. A further advantage is the direct fixation of the biofilms without previous staining and washing procedures. We think that this method, once a biofilm-model is established, can be applied for the initial microscopic screening of new implant materials to get a first impression of their antimicrobial efficacy.

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