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Imaging of endodontic biofilms by combined microscopy (FISH/cLSM – SEM)

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Summary

Scanning electron microscopy is a useful imaging approach for the visualization of bacterial biofilms in their natural environments including their medical and dental habitats, because it allows for the exploration of large surfaces with excellent resolution of topographic features. Most biofilms in nature, however, are embedded in a thick layer of extracellular matrix that prevents a clear identification of individual bacteria by scanning electron microscopy. The use of confocal laser scanning microscopy on the other hand in combination with fluorescence *in situ* hybridization enables the visualization of matrix embedded bacteria in multi-layered biofilms. In our study, fluorescence *in situ* hybridization/ confocal laser scanning microscopy and scanning electron microscopy were applied to visualize bacterial biofilm in endodontic root canals. The resulting fluorescence *in situ* hybridization/confocal laser scanning microscopy and scanning electron microscopy and pictures were subsequently combined into one single image to provide high-resolution information on the location of hidden bacteria. The combined use of scanning electron microscopy and fluorescence *in situ* hybridization/confocal laser scanning microscopy has the potential to overcome the limits of each single technique.

Introduction

The scanning electron microscope (SEM) is an invaluable tool for describing biofilms because of its ability to provide an indiscriminate view on the surface topography at high resolution and magnification. However, even high-resolution SEM examinations of biofilms are often compromised by the fact that matrix embedded bacteria cannot be easily visualized (Marrie *et al.*, 1982). Furthermore it is known that biofilm bacteria often lose their characteristic shape and size making them difficult, if not impossible, to identify (Costerton, 1999; Webster *et al.*, 2004). These limitations pose a problem when indisputable proof of the existence of bacterial biofilms growing in natural environments is required. In general, the presence of bacteria in a matrix is a *sine qua non* for the presence of a biofilm. If bacteria cannot be demonstrated to be present, the proposed existence of a bacterial biofilm remains questionable. The use of fluorescence *in situ* hybridization (FISH) with confocal laser scanning microscopy (cLSM) allows bacteria to be specifically labelled in the extracellular matrix of biofilms using non-invasive imaging of fully hydrated biofilms (Böckelmann *et al.*, 2002; Lawrence *et al.*, 2003; Romero *et al.*, 2008). However, due to the limitations of light microscopy, non-labelled structures and topographical details cannot be adequately discerned (Pfeiffer *et al.*, 2003). The combined employment of FISH and cLSM with high-resolution SEM has been previously shown for a wide range of protists (Stoeck *et al.*, 2003). In this paper, we went one step further and overlaid the FISH/cLSM image on top of the corresponding SEM micrograph. This protocol enabled us to identify precisely the location of the matrix embedded biofilm in a root canal of an extracted human tooth and link the bacterial presence with high magnification details of the SEM imaging. We suggest that this approach will make it possible to detect and characterize bacterial communities, concealed in extracellular matrix, wherever they appear.

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Material and methods

Appropriate Institutional Review Board approval was obtained for this study (proposal #IR00000792). Informed consents were obtained from the patients. Five teeth were extracted due to failed endodontic treatment, fixed for 4 h in 4% paraformaldehyde, washed in Phosphate Buffered Saline (PBS) (pH 7.2) and stored in 50% ethanol at 4°C until use. The roots were split saggitally with a diamond disc (911H Hyperflex disc, Brasseler, Savannah, GA) under dripping sterile water to expose the root canals. For FISH, the hybridization protocol from Böckelmann was adapted (Böckelmann *et al.*, 2002). In short, for the identification of bacteria, the tooth fragments were hybridized for 90 min at 46°C in the dark with 50 μL eubacterial probe EUB338 (Cy3) (Integrated DNA Technologies, Coralville, IA) at a final concentration of 5 ng μL^{-1}, and finally washed at 46°C for 2×10 min with 2×500 μL washing buffer. For examination with the cLSM (LSM 5 PASCAL inverted, Carl Zeiss MicroImaging, Inc., Thornwood, NY, U.S.A.) using 10- and 20-fold objective lenses, the tooth fragments were positioned face down in sterile water in a slide chamber (Lab-Tek®, Electron Microscopy Sciences, Hatfield, PA, U.S.A.).

Following cLSM imaging, the tooth fragments were prepared for SEM. The specimens were dehydrated in a graded ethanol series, critical point dried, mounted on a stub with silver adhesive (Electron Microscopy Sciences, Hatfield, PA, U.S.A.), sputter coated with a 40-nm layer of platinum and examined with an SEM operating at 5 kV in the secondary electron mode (XL 30 S, FEG, FEI Company, Hillsboro, OR, U.S.A.). The magnifications of the resulting cLSM and SEM pictures were normalized and the images were overlaid using Adobe Photoshop (Adobe Systems, Inc. San Jose, CA, U.S.A.).

Results

When examined under SEM the different root canals were mostly covered with an amorphous matrix (e.g. Fig. 1A–C) and only few structures suggested the presence of embedded bacteria (Fig. 1B). Altogether, it was very uncertain if the amorphous matrix represented an extended biofilm with large amounts of bacteria or was a sterile product of underlying cells. To determine the presence of bacteria within the matrices, the specimens were labelled with the 16S rRNA directed EUB338 probe, which is employed to identify most, although not all prokaryotes (Daims *et al.*, 1999; Wagner *et al.*, 2003). The cLSM examination of the labelled root canals showed strong fluorescence signals in distinct regions (Fig. 1D). When corresponding FISH and SEM pictures of the same regions were overlaid, the signal of the EUB338 probe matched those areas where SEM micrographs indicated the presence of matrix (Fig. 1E). Optical sectioning of these regions with cLSM at higher magnifications revealed densely packed bacteria, often with short rod or coccus-like morphology (Fig. 1F).

Discussion

In this study, we present the combined use of FISH/cLSM and SEM on biofilms in infected root canals. The SEM images of the root canal revealed large regions of dense amorphous material without conclusive evidence that these structures were formed by a bacterial biofilm. The combined use of FISH/cLSM in conjunction with SEM examination and the subsequent overlay of the resulting images proved the existence of bacteria within the matrix and hence the presence of biofilm.

The technique of combined application of fluorescence microscopy and transmission electron microscopy (Biel *et al.*, 2003; Pfeiffer *et al.*, 2003; Robinson *et al.*, 2003) or fluorescence microscopy and SEM (Stoeck *et al.*, 2003; Schroeder-Reiter *et al.*, 2006; Micheva & Smith, 2007) to image the same structures has been used to provide unique information that is difficult or even impossible to obtain with a single imaging regimen (Robinson *et al.*, 2003). So far, the application of combined microscopy methods for the characterization of biofilms has been underexploited (Wrede *et al.*, 2008). Biofilms in general, and the highly hydrated extracellular polymeric substances (EPS) of biofilms in particular, suffer from artefact formation such as shrinking during the standard sample processing for electron microscopy (Webster *et al.*, 2004). Comparative visualization by light microscopy can help here to discriminate between artefacts and genuine structures (Costerton *et al.*, 1986; Lambe *et al.*, 1988). This is particularly useful where the large size or nature of the specimen does not lend itself to high pressure freezing or plunge freezing methods that minimize preparation artefacts.

The examination of specimens applying FISH/cLSM has the capacity to readily pinpoint the presence and relative abundance of bacteria whereas the SEM reveals the biofilm matrix and the exact topography. For example, the occurrence of biofilm in the lateral canal was brought to our attention only because the FISH/cLSM signal corresponded to a characteristic feature in the dentin on the SEM image, which was later identified as a lateral canal. The opacity of the tooth samples limited light transmissibility and made it impossible to identify the lateral canal using the cLSM's transmission channel. It was only when the tooth samples were examined by SEM that the lateral canal was identified. In their apical parts, root canals often branch into fine side canals (Chávez de Paz, 2007). The complete removal or killing of biofilms in these side canals during the endodontic treatment is one of the most demanding procedures in dentistry (Siqueira, 2001; Nair, 2004; Chávez de Paz, 2007) and has failure rates of between 5% and 15% (Peak *et al.*, 2001).

The EPS-embedded biofilms are not restricted to infected root canals. These structures are also present in other biofilm related diseases, such as infections of pacemakers (Marrie *et al.*, 1982), catheters, prosthetic heart valves, prostheses, cystic fibrosis or otitis media (Donlan & Costerton, 2002), which all represent topographically challenging environments.

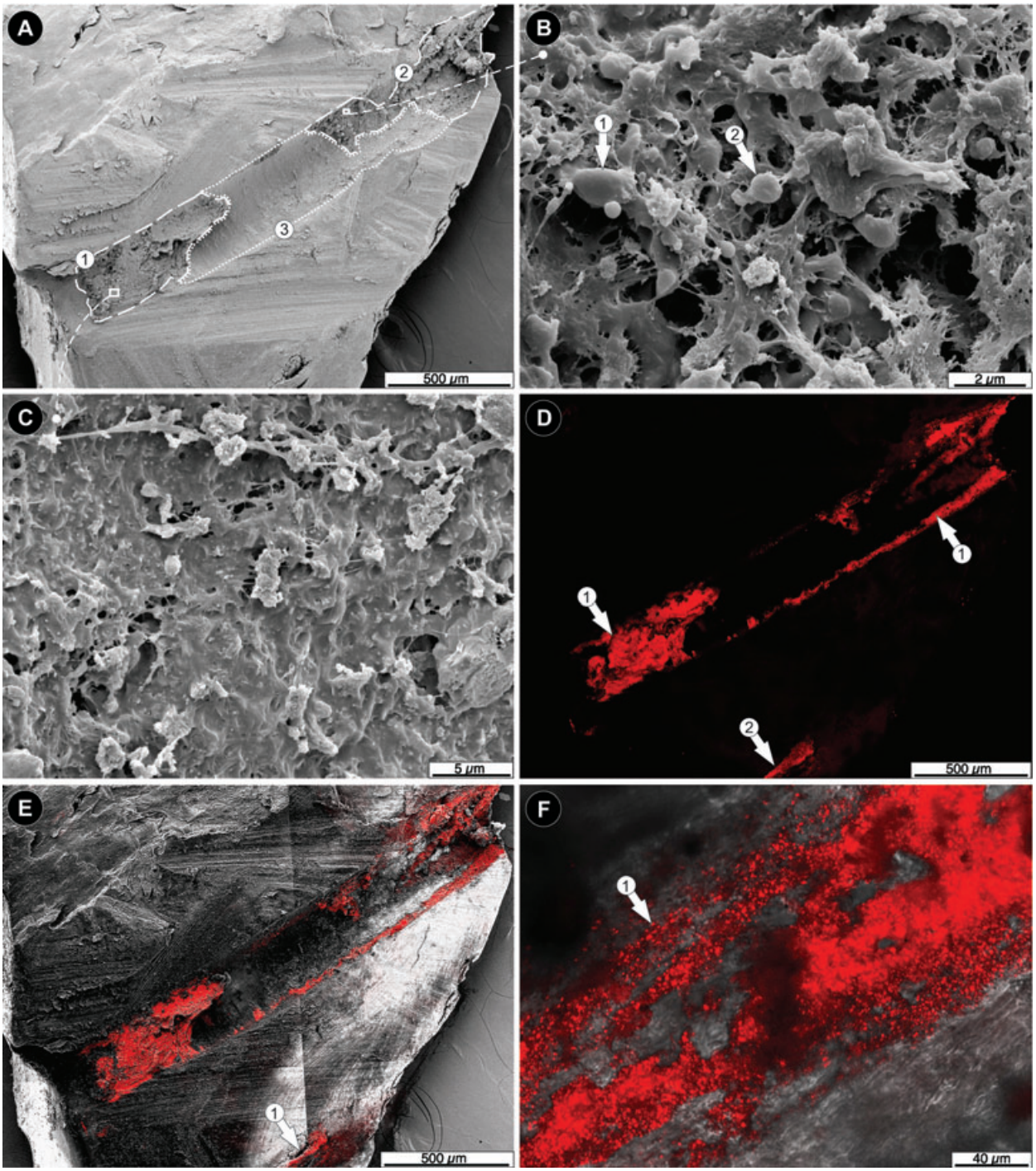

Fig. 1. (A) The micrograph provides an overview on one of the split root canals. Certain parts of the canal surface were covered with a thick matrix layer (encircled areas 1, 2) whereas other regions showed only sparse and comparatively thin islands of matrix (encircled area 3). (B) The size and shape of some structures of the matrix suggested the presence of bacteria (arrows 1, 2), but without conclusive evidence. (C) Other areas of the matrix consisted of so densely composed material that no traces of bacterial presence could be found. (D) After labelling the split tooth with the EUB338 (Cy3) probe, distinct parts of the canal showed a strong red fluorescence signal in the cLSM (arrows 1). The red signal was also clearly present at a lateral spot at some distance from the main root canal (arrow 2). (E) The overlay of corresponding SEM and FISH/cLSM (Fig. 1A and D) images of the same regions revealed matching areas of the FISH signal and the amorphous matrix, suggesting the presence of bacterial biofilm. The laterally located FISH signal was within a lateral canal in the root (arrow 1). (F) Higher magnifications of the FISH-labelled root canal surface with cLSM indicated a biofilm, composed of bacteria with short rod or coccus-like morphology (arrow 1).

In future studies, we aim to apply the method described above to identify the bacterial species in complex microbial communities present in pathogenic environments using specific rRNA FISH probes (Moter & Göbel, 2000). The method is also applicable for examining lectin binding of EPS (Böckelmann *et al.*, 2002; Lawrence *et al.*, 2003) using cLSM and combining the images with SEM and TEM data.

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