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Rapid Bacterial Detection during Endodontic Treatment

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Abstract
Bacteria present in the root canal (RC) space following an RC treatment (RCT) can lead to persistent infections, resulting in treatment failure and the need for reintervention or extraction. Currently, there are no standardized methods in use to clinically detect bacterial presence within RC spaces. The use of paper point sampling and fluorescence staining was shown to be a rapid method, able to detect residual bacteria following treatment. The study demonstrated that Calcein acetoxymethyl (AM) proved to be a suitable dye for detecting vital bacteria within mature endodontic biofilms, with an improved sensitivity over colony-forming unit counting in a stressed biofilm model. Furthermore, in a clinical trial with primary RCTs, 53 infected teeth were sampled in vivo, and increased detection of vital cells was found when compared with colony-forming unit counting, highlighting the sensitivity of the technique in detecting low cell numbers. By combining fluorescent staining and microspectroscopy with software-based spectral analysis, successful detection of vital cells from RCs was possible after 5 min of Calcein AM incubation. Application of this technology during RCT has the potential to reduce persistent infections through vital cell detection and additional treatment. Furthermore, this technique could be applied to antimicrobial research and disinfection control in clinical settings (ClinicalTrials.gov NCT03055975).

Keywords: biofilms, microscopy, diagnostic systems, endodontics, bacteria, fluorescence

Introduction
Root canal treatments (RCTs) are undertaken to eradicate bacterial infections from root canals (RCs) while retaining as much of the natural tooth as possible. One million RCTs are conducted under the British National Health Service each year, annually costing the General Dental Service £50.5 million (Lumley et al. 2008). Approximately 24% require secondary RCTs, due to persistence of microorganisms in the RC space (Siqueira 2001; Chávez de Paz 2007; Lumley et al. 2008; Anderson et al. 2012). During RCT, the debridement and removal of bacteria, as well as the shaping of the RC, take place (see Appendix for additional information). The main difficulties in RC preparations lie in the varying, unpredictable RC anatomy, which is often underestimated by conventional 2-dimensional radiographs (Peters 2004; Patel 2009). The resilient nature of bacterial biofilms, combined with often complex RC morphologies, makes disinfection challenging, leading to a considerable number of persistent infections (Lumley et al. 2008), which are one of the main causes of RCT failure (Chávez de Paz 2007).

Current clinical practices aimed at detecting bacteria in the RC space rely on subjective observations, such as clean, white dentine on the RC files. Reproducible detection and quantification of bacteria, with methods such as colony-forming unit (CFU) counting and polymerase chain reaction (Kato et al. 2007; Sathorn et al. 2007; Anderson et al. 2012), are time-consuming, making them unfeasible for routine intraoperative application. Another approach is to measure the autofluorescence from within the RC to differentiate infected from sound dentine. However, this relatively fast method relies on the fluorescence emission of biofilm by-products such as porphyrins and therefore lacks the ability to indicate the presence of vital cells and bacteria in particular. Furthermore, it remains unclear whether the sensitivity of such autofluorescence measurements would be sufficient to detect very low quantities of bacteria (Giana et al. 2003; Sainsbury et al. 2009; Ho et al. 2010). Sato et al. (2012) propose application of a system with live/dead staining and a membrane filter for bacterial counting. Unfortunately, the procedure, despite being comparatively rapid, takes 30 min and requires additional collection and preparation of dentine samples, making it unfeasible for routine use in a clinical setting. A recent approach by Tan et al. (2015) utilizes the detection of vital bacteria with adenosine triphosphate (ATP) activity. Although the ATP assay is rapid...
(-5 min) and sensitive, the technique described requires additional sampling and RC preparation before application of the ATP assay, increasing the overall time for treatment.

We have developed a new methodology based on the use of fluorescent dyes for the detection of vital bacteria, sampled with conventional endodontic paper points during RCTs. Fluorescence spectroscopy and microscopy are commonly used methods in biological and chemical sciences and are established tools in bacterial biofilm analysis and detection (Moter and Göbel 2000; Takenaka et al. 2008; Wakamatsu et al. 2014). In “live/dead staining,” the chosen dyes will contrast vital from dead cells via 2 fluorescent stains with different emission wavelengths. Ordinarily, they will exploit metabolic activity or compromised cell membranes and have a high affinity to cell structures such as nucleic acids (Tawakoli et al. 2013). With nonspecific vital cell staining, live cells in a range of bacteria involved in multispecies RCTs can be detected (Shen et al. 2010, 2011; Tawakoli et al. 2013; Wakamatsu et al. 2014).

In this study, we first used confocal microscopy to compare the suitability of a number of fluorescent dyes for rapid detection of vital cells in a model endodontic in vitro biofilm. As shown by Niazi et al. (Niazi et al. 2014; Niazi et al. 2015), this biofilm consists of strains of selected pathogens, confirmed with 16S RNA gene sequencing and quantitative viable counts. To demonstrate the possibility of bench-side detection, we coupled a spectrometer to a fluorescence microscope with a custom filter cube. Furthermore, with spectral unmixing, emissions from the fluorescent dyes were shown to be distinguishable from substrate autofluorescence, even at low fluorescence intensities. To demonstrate its clinical suitability, our methodology was applied to the detection of vital cells in a stressed in vitro grown biofilm as a controlled model system, as well as to ex vivo bacterial biofilms sampled with endodontic paper points during and post-RC patient treatments (Fig. 1). The use of established endodontic paper points minimizes additional clinical steps during RCTs and therefore does not affect the clinical treatment time.

The presented technique could be utilized to determine the end point of the endodontic chemomechanical debridement by assessing the removal of the endodontic pathogens from the main RC lumen in real time. Routine use could lead to a significant decrease in the need for retreatments, resulting in long-term monetary savings to the dental services and enhanced confidence in a positive outcome of the RCT.

Methods

Establishment of a Mature Endodontic In Vitro Biofilm

Replication of a multispecies biofilm consisting of typical RC pathogens required in vitro culturing of a model biofilm system, as described by Niazi et al. (Niazi et al. 2014; Niazi et al. 2015). The strains used for the formation of the biofilm were Propionibacterium acnes, Staphylococcus epidermidis, Actinomyces radicicoides, and Streptococcus mitis, which have been shown to be the predominant taxa from refractory endodontic lesions (Niazi et al. 2010). In addition, Enterococcus faecalis OMGS 3202, known to be involved in RCT failures, was added to the biofilm (Dahlen et al. 2000). Bacterial isolates were revived from storage at −80 °C and cultured at 37 °C in a MACS MG 1000 Anaerobic Workstation (80% N, 10% H, 10% CO₂; Don Whitley Scientific Limited) before being transferred to modified fluid universal medium for anaerobic growth on autoclaved hydroxyapatite (HA) discs (Clarkson Chromatography Products, Inc.). See Appendix and Appendix Table 1 for a detailed growth protocol and confirmation of present bacterial strains.

Characterization and Sensitivity of Fluorescent Stains to Vital Biofilms

The vast selection of fluorescent stains was narrowed to a number of stains with various binding and fluorescence characteristics: Syto 9 (L10316 FilmTracer Live/Dead; Life Technologies) with propidium iodide, FilmTracer Red-Orange (F10319; Life Technologies), and Calcein AM (CAM; sc-203865, Santa Cruz Biotechnology, Inc.). Details on the staining protocol and working solutions are given in the Appendix and Appendix Table 2. In addition to measuring the stained biofilms, measurements were taken of the background autofluorescence as well as of controls of stained HA discs, paper points (ProTaper Universal Paper Points; Dentsply), and nonvital biofilms. Images were analyzed by measuring the fluorescence intensity (Fig. 2) with Image J 1.47v (Schneider et al. 2012), as described in Appendix Figure 1.

Simultaneous Image Localization and Spectral Detection

Image localization and spectral analysis were carried out as described in the Appendix and Appendix Figure 2. In brief, a wide-field fluorescence microscope (Zeiss Axiovert 2000; Zeiss) was coupled to a fluorescence spectrometer (QE 65000; Ocean Optics), allowing for image acquisition and simultaneous spectral recording. Spectral unmixing software then enabled calculation of the proportion of the calcein signal (Pc) produced by vital cells. Spectral analysis was achieved with prerecorded base spectra, as shown in Figure 3.
Detection of Vital Cells in Stressed Biofilms In Vitro

To evaluate the detection sensitivity in comparison with gold standard CFU counting, biofilms grown on HA discs were exposed to 1% sodium hypochlorite (NaOCl) for 10 increasing durations (n = 5), before being thoroughly rinsed with phosphate-buffered saline. Following fluorescent staining, spectral analysis was carried out at 20 locations per disc. In addition, culturing and CFU counting was carried out for comparison with the fluorescence detection. A detailed protocol is outlined in the Appendix.

Detection of Ex Vivo Vital Cells from Patient RCs

Following ethical approval (05/Q0705/051), patients (n = 53) undergoing primary RC treatments were selected, and informed consent was taken. The teeth included in the study had been referred to a specialist center for the treatment of endodontic infection. They all responded negatively to vitality tests and presented preoperative apical radiolucencies. Endodontic paper points were used to sample the RC space at 3 time points during treatments: immediately after accessing the RC space, at the midpoint of the treatments (after instrumenting with a shaping file: S2, ProTaper Universal; Densply), and preobturation. Spectral unmixing software was used to analyze the fluorescently stained paper point samples (20 spectral readings at 500-µm intervals, starting from the tip). The proportional calcein signal (Pc) was calculated, and, based on controls, a threshold of 4.35% was chosen as being positive for bacterial detection (Fig. 4). Furthermore, CFU counts were then conducted on all the samples after 7 d and compared with the proportional calcein signal, as shown in Figure 5. Sampling and sample analysis details are outlined in the Appendix, and base spectra for spectral unmixing are shown in Appendix Figure 3.

Results

Characterization and Sensitivity of Fluorescent Stains to Vital Biofilms

Visualization of the vital biofilm was possible with all of the tested stains and stain concentrations. To compare the ability of each stain and concentration to specifically detect vital biofilms, as opposed to nonvital biofilms, we measured the ratio (Rv) between the fluorescence of vital and nonvital biofilms. Similarly, the ratio (Rf) between the fluorescence of vital biofilm and biofilm-free substrate was used to compare their ability to not stain the substrate.

Overall, CAM showed the highest ratios at all concentrations for all dyes (Fig. 2A). The maximum ratio Rv = 11.7 was observed at a concentration of 15 µg/mL (Fig. 2B). Above this concentration, the vital cell fluorescence intensity remained unchanged.

Only minimal changes were observed for Syto 9 and FilmTracer Red-Orange at any of the concentrations (Fig. 2A), where the maximum ratio Rf = 0.8 was observed at a concentration of 15 µg/mL (Fig. 2B). Above this concentration, the vital cell fluorescence intensity remained unchanged.

An analysis of variance Holm-Sidak statistics test showed that CAM staining produced a significantly higher (P < 0.001) vital:nonvital biofilm staining ratio when compared with both Syto 9 and FilmTracer Red-Orange (Fig. 2A, B). Staining vital
biofilms as compared with the paper point substrate was also shown to be significantly higher than Syto 9 and FilmTracer Red-Orange (P < 0.001; Fig. 2D). These experiments have established the most suitable stain and optimal concentrations for all future experiments, outlined in this paper as CAM at 15 µg/mL.

Detection of Vital Cells in Stressed Biofilms In Vitro

The optical biofilm sampling and detection method was compared with the gold standard of CFU counting through nutritionally stressed biofilms, which were exposed to NaOCl for increasing durations (Fig. 3). An immediate 3.3-fold drop in proportional calcein signal was observed after 2 s of NaOCl exposure (full scale in Appendix Fig. 7). As expected, increased exposure durations resulted in further decrease of the proportional calcein signal. However, detection of this signal remained possible for up to 40 s of exposure with NaOCl (Fig. 3C). After being exposed for 200 s, it dropped below to the nonvital positive control, indicating a complete loss in viability (Appendix Fig. 7).

In comparison with the calcein signal, the CFU counts showed a far stronger drop (861-fold) after 2 s of NaOCl exposure, and the stressed biofilms completely lost the ability to form colonies after 15 s of NaOCl exposure. These results indicate that the bacteria within the biofilm are losing the ability to form CFUs but remain in a detectable vital state for up to 40 s of exposure, as observed in other studies (Shen et al. 2010, 2011).

These measurements were repeated by sampling the same biofilms with endodontic paper points, and interestingly, detection of residual vital cells was improved. These results suggest that condensing the biomass into a small surface area maximizes the concentration of stained cells and increases the sensitivity of calcein detection. This advantage would be replicated during clinical sampling of the RC (Appendix Fig. 8).

Detection of Ex Vivo Vital Cells from Patient RCs

Samples were taken from 53 patients during RCTs with endodontic paper points and tested for vital bacteria with the described methodology. Sterile paper points identified a broad autofluorescence spectrum, which increased in intensity as the taper of the paper point increased. These measurements were repeated by sampling the same biofilms with endodontic paper points, and interestingly, detection of residual vital cells was improved. These results suggest that condensing the biomass into a small surface area maximizes the concentration of stained cells and increases the sensitivity of calcein detection. This advantage would be replicated during clinical sampling of the RC (Appendix Fig. 8).

Figure 3. Spectral analysis. (A) Normalized base spectra used for spectral unmixing: green = fluorescence in presence of vital cells (positive signal), red = substrate and nonvital biofilm. (B) Example of experimental spectra with fitted data. (C) Detection of vital bacteria based on the proportion of positive signal from spectral unmixing, CAM, Calcein AM; CFU, colony-forming unit; HA, hydroxyapatite.
Rapid Bacterial Detection

during the chemomechanical treatment, the majority of bacteria are removed. The proportional calcein signal distribution of the preobturation samples are shown in Appendix Table 3, indicating the majority of roots being below detection limits. Despite the lower numbers of RCs with detectable signal in the apex at the end of the treatment, clinically this results in 35.8% of teeth sampled showing a signal above detection threshold (Appendix Fig. 11). Furthermore, fluorescence detection proved to be more sensitive than CFUs in the detection of vital cells: a calcein signal was detected preobturation in 18.4% of roots, compared with 6.1% with culturing (Fig. 5).

Discussion

Persistent microbial infections and bacteria remaining in the RC space following treatment are one of the main causes of failure of RCTs (Siqueira 2001; Chávez de Paz 2007). This study aimed to develop an optical fluorescence-based chair-side method, which allows clinicians to objectively and rapidly detect residual bacterial contamination in the RC space. In vivo sampling with endodontic paper points combined with ex situ staining and analysis results in a rapid method that causes no disruption to the clinical workflow nor inconvenience or discomfort to the patient.

Unspecific vital cell stains enable the detection of a wide range of bacteria involved in multispecies infections of the RC space (Costerton et al. 1999; Davey and O’Toole, 2000; Hall-Stoodley et al. 2004; Shen et al. 2010). Characterization of various fluorescent dyes showed CAM to be the most suited in detecting vital bacteria in a mature, nutritionally stressed multispecies biofilm after just 5 min of incubation with minimal background staining. Low fluorescence ratios for $R_S$ and $R_V$ produced by the others stains can be attributed to staining of the absorbent paper point and to the 5-min incubation being an insufficient reaction time (Fig. 2). The biofilm used consisted of the predominant bacteria recovered from RCs of teeth affected by apical periodontitis (Niazi et al. 2010), including *E. faecalis* (Lima et al. 2001; Stuart et al. 2006).

We have developed a setup that combines fluorescence spectroscopy and microscopy for simultaneous localization and spectral analysis of vital bacteria with CAM. Applying spectral unmixing enables accurate quantification of the stained biofilm and the substrate (endodontic paper point or HA disc) autofluorescence. A proof-of-principle study subjecting biofilms to 1% NaOCl with increasing durations showed our method to be more sensitive to very low quantities of vital cells when compared with CFU counting. Bacterial biofilms have numerous mechanisms of reacting to environmental stress, including downregulating metabolism and entering a vital but nonculturable (VBNC) state (Mah and O’Toole 2001; Stewart and Costerton 2001; Shen et al. 2011), not detectable by CFU counting. However, as shown in Figure 3C, the bacterial metabolism is sufficient to hydrolyze CAM into the fluorescent calcein, enabling fluorescent detection even when the

**Figure 4.** Signal detection from a patient sample. The spectral unmixing is shown at 2 different points on the patient sample. The low signal from the sterile stained paper points and the determined detection threshold for sampling are indicated. PP, paper point.

**Figure 5.** Clinical trial consisting of 53 root canal treatments: percentage of roots in which a signal was detected at different time points throughout the treatment, as well as comparison with colony-forming unit (CFU) detection (n = 114).
cells have lost the ability to form CFUs. This is of great importance when aiming to detect bacteria deep within the nutrient-poor RC space, where surviving biofilms may enter a VBNC state while still causing inflammation (Shen et al. 2010).

Our clinical trial, consisting of 53 sampled RCTs, demonstrated the feasibility of this technique in a practical clinical setting. RCs were sampled and spectrally analyzed immediately after accessing the RC space, after shaping, and posttreatment. Spectral analysis of control stained sterile paper points led to a threshold of 4.35% (Fig. 4). A clear decrease in vital biofilm detection from the samples taken immediately after access was observed, as compared with samples taken during and posttreatment (Fig. 5). While the majority of preobturation samples did not exceed the detection threshold, remaining vital bacteria were detected at the apex of the RC space in 18.4% of roots. Furthermore, similar to the in vitro detection of bacteria in a VBNC state, the clinical trial showed that fluorescent analysis led to bacterial detection in more cases than CFU counting, which identified residual bacteria in only 13.2% of teeth. Interestingly, the detection of vital cells in 19 of 53 sampled teeth (35.8%) exceeds the percentage of RCT failures generally suggested by the literature, which are in the region of 20% to 25% when assessed through periapical radiographs (Ng et al. 2007; Lumley et al. 2008). However, there is substantial agreement between the percentage of positively sampled teeth in the present study and the rate of periapical pathosis observed in cone-beam computed tomography studies in primary RCTs, where detection at the 1 y recall was 37.5% (Patel et al. 2012). The correlation between fluorescence detection and treatment failure is currently being examined in a further clinical trial to demonstrate the relevance of residual bacteria. Sampled patients will be recalled 1 y postobturation for cone-beam computed tomography evaluation.

Since paper point sampling is restricted to the pathway created by the endodontic instruments, detection within inaccessible structures (e.g., lateral canals) may not be possible. However, endodontic paper points have the benefit of being highly absorbent and flexible, enhancing the detection of vital cells in the RC apex, as well as at the entrance to the lateral canals and walls, which remain untouched by mechanical instrumentation (Peters et al. 2003).

Our study demonstrates the potential advantages and clinical relevance of fluorescent staining in combination with spectral analysis. Compared with more conventional microbiology techniques, such as CFU counting and polymerase chain reaction (Kato et al. 2007; Sathorn et al. 2007; Anderson et al. 2012), the approach significantly reduces the processing time required, making it a viable technique for introduction into clinical trials. In addition, the technique successfully applied to detecting ex vivo vital bacteria in samples taken during RCTs. The potential to minimize persistent infections would reduce treatment costs and avoid an unnecessary secondary visit when no bacteria are detected. This technology also has the potential to be applied to other areas, clinically and in research. Research applications could include the evaluation of biofilm disinfection methods in areas of peri-implantitis and bone infection as well as general antibiotic and antimicrobial research. Clinically, the future development and application of more specific vital fluorescent stains could be applied to ex vivo detection from patients in a wide range of biological infections, ranging from wound to respiratory and implant-related infections and contaminations.

Our unbiased, rapid, and quantitative methodology was first proven on stressed in vitro biofilms. Furthermore, it was successfully applied to detecting ex vivo vital bacteria in samples captured during RCTs. The potential to minimize persistent infections would reduce treatment costs and avoid an unnecessary secondary visit when no bacteria are detected. This technology also has the potential to be applied to other areas, clinically and in research. Research applications could include the evaluation of biofilm disinfection methods in areas of peri-implantitis and bone infection as well as general antibiotic and antimicrobial research. Clinically, the future development and application of more specific vital fluorescent stains could be applied to ex vivo detection from patients in a wide range of biological infections, ranging from wound to respiratory and implant-related infections and contaminations.

**Author Contributions**

D.B. Herzog, contributed to conception, design, and data acquisition, drafted and critically revised the manuscript; N.A. Hosny, contributed to data analysis and interpretation, drafted and critically revised the manuscript; S.A. Niazi, contributed to data acquisition, critically revised the manuscript; G. Koller, contributed to conception, design, and data acquisition, critically revised the manuscript; R.J. Cook, F. Foschi, T.F. Watson, F. Mannocci, contributed to conception and design, critically revised the manuscript; F. Festy, contributed to conception, design, data acquisition, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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