REVIEW

Microscopic observation of bacteria: review highlighting the use of environmental SEM

L. Bergmans[1], P. Moisiadis[1], B. Van Meerbeek[1], M. Quirynen[2] & P. Lambrechts[1]

[1]Leuven BIOMAT Research Cluster and [2]Research Group for Microbial Adhesion, School of Dentistry, Oral Pathology and Maxillo-Facial Surgery, Catholic University of Leuven, Leuven, Belgium

Abstract

Bergmans L, Moisiadis P, Van Meerbeek B, Quirynen M, Lambrechts P. Microscopic observation of bacteria: review highlighting the use of environmental SEM. *International Endodontic Journal*, **38**, 775–788, 2005.

Throughout the years, various methods have been adopted to investigate bacteria involved in root canal infection and apical periodontitis. This paper reviews the most commonly used microscopic techniques and discusses their possibilities, limitations and sample preparation. In particular, a recently developed variant of scanning electron microscope (SEM), referred to as environmental SEM (ESEM), is highlighted due to its potential impact across the diverse field of biomaterials research. The performance of this ESEM technique for bacterial observation of endodontic pathogens was illustrated by a practical approach. The paper concludes with a discussion on the possible use of ESEM for testing endodontic treatment modalities under environmental conditions *in situ*.

Keywords: bacterial morphology, microscopic techniques, native state, root canal infection, sample preparation.

Received 18 January 2005; accepted 26 April 2005

Existing microscopic techniques for bacterial observation

Sampling and bacterial observation are often completed for research purposes. In general, these procedures are not part of the treatment strategy in daily endodontic practice. For bacterial observation related to research, different methods have been adopted throughout the years.

One of the first techniques to observe endodontic pathogens was compound-light microscopy in combination with histological staining and/or sectioning. Although not powerful enough to resolve many structures within the cell, this type of microscope can be used for first stage identification of bacteria by verifying cellular morphology (e.g. rod-, coccal- or spiral-shaped) and the reaction of an organism with the Gram stain (Brown/Brenn staining technique) (Ricucci & Bergenholtz 2003). In addition, markers such as antibodies (monoclonal or specific polyclonal) and nucleic acid probes have been developed for identification at the genus and species level of some bacteria associated with disease as an alternative to lengthy culturing techniques. Additional special equipment (i.e. dark-field illumination and phase-contrast microscopes) has been used to quantify the numbers of motile bacteria in the clinic directly after root canal sampling (Trope *et al.* 1992). Likewise, in periodontics, some laboratory protocols and a number of chair-side diagnostic tests are routinely used to evaluate large numbers of subgingival plaque samples for their content of a wide range of recognized pathogenic species. Such evaluations are critical in understanding treatment effects and are used by both researchers and clinicians (Greenstein & Polson 1985). The techniques often employed include culturing, dark-field and phase-contrast microscopy (Magnusson

Correspondence: Lars Bergmans, Leuven BIOMAT Research Cluster, Department of Conservative Dentistry, School of Dentistry, Oral Pathology and Maxillo-Facial Surgery, Catholic University of Leuven, Kapucijnenvoer 7, B-3000 Leuven, Belgium (Tel.: +32 (0)16 332747; fax: +32 (0)16 332752; e-mail: lars.bergmans@med.kuleuven.ac.be).

et al. 1985, Quirynen *et al.* 1995), or immunological assays and molecular methods (Socransky *et al.* 1998, Ximenez-Fyvie *et al.* 2000, Darby *et al.* 2001).

A second important approach to bacterial observation used the principle of electron microscopy (EM). The microscope involved constructs an image from a highly focused primary electron (PE) beam, which is scanned over the specimen in a square raster pattern. The PE have a much shorter wavelength than light, and therefore microscopes employing electron beams have 400 times the resolving power of an optical microscope thus revealing much more detail. Employing EM, it was demonstrated that even though bacterial species are diverse in form, their organization is fundamentally similar: small cells about 0.3–10 μm thick, enclosed within a membrane and encased within a rigid cell wall, with no distinct interior compartments. In fact, bacteria, which have a prokaryotic cell structure, do not have membrane-bound organelles within their cells and their DNA is mostly included in a single, closed, circular molecule.

The most common type of electron microscope is called the conventional scanning electron microscope (CSEM), and its associated technique has a long and distinguished record in the field of biomaterials. CSEM offers unique advantages such as high resolution and large depth of field, and related tools have evolved into complex integrated instruments that often incorporate several important accessories. Their principle improvement stems from the method of constructing an image by detecting electron signals generated by the incident beam and emitted from the specimen, whilst scanning across the surface. As a consequence, the whole microscope column, including the sample chamber, operates under high vacuum ($<10^{-5}$ torr) (1 torr = 133 Pa) to prevent gas scattering of either the incident beam or the produced electrons. The presence of a vacuum, however, implies that samples must not contain any volatile species; they must be solid and dry. Samples that are hydrated in their native state (e.g. biological tissues and cells) must be dried or frozen prior to observation. In addition, this category of samples exhibits low conductivity and has always been a challenge as surface charges generated by the incident electron beam must be drained away to prevent distortion of the image. Coating the specimen with a thin layer of an electrically conductive material will dissipate the electrons and prevent the build-up of charge. However, specimen preparation can introduce artefacts by altering specimen morphology whereas conductive coatings may obscure internal information by impeding the outgoing electron signals (Little *et al.* 1992). Finally, sample preparation implies that specimens do not preserve their native state. As a result, therapeutic endodontic strategies cannot be observed or tested *in situ*. The principle of CSEM and its technical issues will be discussed in a further section.

To overcome the limitations of CSEM, a second type of SEM called the environmental scanning electron microscope (ESEM) has been developed. The first commercial version of this product was made by the ElectroScan Corporation (Wilmington, MS, USA; later purchased by FEI/Philips Electron Optics) along with the work of G. D. Danilatos (Danilatos 1988, 1993a) more than a decade ago. In recent years, ESEM has begun to make impact across the diverse field of materials; an expansion which can be evidenced from the increased range of applications over a short span of time (Danilatos 1993b). The major advantage of ESEM is that hydrated and non-conducting samples, such as biological tissues and (bacterial) cells, can be imaged without prior dehydration or conductive coating. ESEM differs therefore from CSEM in two crucial aspects. First, instead of the sample being held under a high vacuum, a gaseous pressure is maintained in the specimen chamber whilst imaging is carried out, although the electron gun itself is kept at standard pressures of around 10^{-6}–10^{-7} torr. Around the sample, pressures of up to 10–20 torr can be tolerated and all operational parameters can be varied within a range, which is a function of pressure. In this way, if water vapour is the gas in the sample chamber, hydrated samples such as root canal bacteria can be imaged in their 'native' state. The second major difference between ESEM and CSEM is that insulators no longer need to be coated with a metallic layer before imaging. Because gas is present in the chamber, a mechanism exists to help dissipate the build-up of charge injected by the incident electron beam. Technically, ESEM is based on the integration of efficient differential pumping with a new design of electron-optics and detection systems. ESEM's physical principles and technical demands will be discussed comprehensively below.

A final type of EM, identified as the transmission electron microscope (TEM), offers unique properties such as high resolution. TEM involves the irradiation of whole specimens or ultra-thin sections (80–90 nm) which are thin enough to transmit at least 50% of the PE (Bancroft & Stevens 1996) using electron beam energies in the range of 60–350 keV. For amorphous materials, contrast is achieved by variations in electron scattering as the electrons traverse the chemical and

physical differences within the specimen. The emergent beam of transmitted electrons is focused by a system of lenses to form a magnified, two-dimensional image. The major advantage of TEM is its resolving power. The maximum obtainable resolution (1–2 nm for most biological material) is limited by the nature of the specimen and the techniques involved in specimen preparation. In prevention of artefacts, common preparation procedures of specimens for TEM involve relatively complex and long laboratory processing. Fixed and dehydrated specimens are generally embedded in an epoxy resin and stained with heavy metals (e.g. potassium permanganate or osmium tetroxide) to improve image contrast before ultra-thin sectioning using an ultramicrotome with glass or diamond knives (Nair *et al.* 2005). For endodontic microbial research, these sections have also been stained with tannic acid and ruthenium red staining prior to examination in the microscope (Haapasalo 1986, Sunde *et al.* 2002). Other preparation techniques that can be used are cryosectioning and freeze fracturing (followed by freeze etching and the production of a replica) (Haapasalo 1986, Matias *et al.* 2003). Eventually, the thickness of a section primarily determines the resolution obtainable in a TEM, and therefore the making of sections is very critical in preparing material for fine ultrastructural examination.

Bacterial observation using ESEM opposed to CSEM

CSEM technique and sample preparation

For SEM, basically two types of electron sources can be used to form the electron beam: (i) the thermionic emission (tungsten, Lanthanum Hexaboride or cerium filament) and (ii) the Field-emission (Fe). The latter requires ultra-high vacuum conditions and thus appropriate equipment. The electron beam, which typically has an energy ranging from a few kV to 50 kV, is focused by condenser lenses into a beam with a very fine spot size (~5 nm). The beam then passes through the objective lens where pairs of scanning coils deflect the beam over a rectangular area of the sample surface. As PE strike the surface they are inelastically scattered by atoms in the 'spot' and the beam energy is effectively spread over a certain distance into the sample. Interactions in this region lead to emission of electrons: (i) low-energy secondary electrons (SE or SE-I) (≤50 eV) produced by inelastic collisions with the orbital electrons and dislodged from the specimen itself and (ii) high-energy backscattered (or reflected) electrons (BSE) (>50 eV) that arise from elastic collisions between the PE and the atomic nuclei. These signals of electrons are collected and amplified by a positively biased grid or detector, and results of the analysis are displayed as a specific intensity on screen at a position that represents the position of the incident beam spot. Magnification results from the ratio of the area scanned on the specimen to the area of the screen. Increasing the magnification in a CSEM is therefore achieved quite simply by scanning the electron beam over a smaller area of the specimen. The most common image mode monitors the SE-signal. Because of their low energy these electrons must originate within a few nm (or less) from the specimen surface thus providing topographical information. The brightness of the resulting signal depends on the surface area that is exposed to the primary beam. This area is relatively small for a flat surface but increases for steep surfaces that tend to be brighter; so the final image is pseudo three-dimensional. The emitted SE-signal is detected by a scintillator-photomultiplier device, typically an Everhart–Thornley detector (E–T detector) (Everhart & Thornley 1960). In addition to SE, BSE-signals can also be detected. Because of their much higher energy these electrons may be scattered from fairly deep within the sample resulting in less topographical contrast than the case of SE. BSE have a definite direction. As such, they cannot be collected by a standard SE-detector unless the detector is directly in their path of travel.

Besides the emission and signalling effect, electrons can accumulate on the surface of non-conductive materials and charging will occur. At low beam energies, most SE are generated just below the surface of the specimen and most of them will backscatter into the vacuum. In contrast, high beam energies penetrate much deeper and most of the SE produced cannot escape, thus charging will more likely occur (Egerton *et al.* 2004). The negative field from the charging surface deflects the incident electron beam from its intended course and causes image drift. Elimination of specimen charging can be achieved by reducing the accelerating voltage below the charging point, or by conductive coating. When the microscope is used at low accelerating voltages, its resolution is greatly reduced. Therefore, the deposition of conductive films is generally preferred.

Apart from their non-conductive properties, biological specimens are, in their native state, hydrated at temperatures above 0 °C. Therefore, such specimens cannot be placed directly into a CSEM as water would

evaporate and interfere with the electron generation and detection system thus forming artefacts. In the case of delicate liquid-containing samples, which become hollow when dried, complete collapse often results. To avoid this problem, a complex and extensive series of processing steps is required for reliable observations of hydrated specimens under (ultra) high vacuum. Diverse laboratory protocols have been described in the literature and generally involve successive (pre)fixation, dehydration/drying or freezing, coating with an electron-conductive material and viewing (Watson *et al.* 1980, Bancroft & Stevens 1996, Van Meerbeek *et al.* 2000). The main aim of fixation is to preserve the structure of the tissue in an as near life-like condition as possible. Most popular fixative solutions used today are aldehyde fixatives (glutaraldehyde or formaldehyde) made up in phosphate or cacodylate buffers. Dehydration refers to the removal of water from the fixed tissue mass by an organic solvent and is generally carried out in an ascending series of low concentration aqueous ethanol or acetone solutions to an absolute dehydration agent. Once dehydration is complete, the specimen must be dried in a way that causes minimal distortion and disruption of the tissue architecture. Simple air-drying (including evaporation by heat or under vacuum) should be avoided. The most widely used method to prepare biological tissue is known as 'critical-point drying' (CPD). This process involves replacement of the absolute ethanol in the tissues with a transitional fluid, most commonly carbon dioxide (CO_2). The liquid CO_2 is then removed by conversion to its gaseous form by raising the temperature and pressure to the critical point. CPD avoids artefact formation by never allowing a liquid/gas interface to develop; in this way the tissue is not exposed to surface tension forces. An alternative technique called freeze-drying (FD) removes water as vapour directly from ice (sublimation) without passing through the liquid state. FD is so effective in processing chemical components that small living organisms can be preserved and kept alive during and after the process: they pass into a dormant state from which they can be revived by the addition of water (e.g. nutritional supplements industry). In addition to CPD and FD, chemical drying methods, such as HMDS-drying using hexamethyldisilazane have been advocated for biological specimen preparation (Perdigão *et al.* 1995). Besides specimen dehydration/drying, one can also freeze the specimen (Cryo-SEM) in liquid nitrogen or liquid nitrogen cooled Freon. Freezing prevents the vapour from interfering with the electron generation and detector systems, provided sufficiently low temperatures are reached and maintained during examination. This technique enables a large range of specimens to be successfully examined without dehydration, although it has some limitations including the possibility of ice crystal damage due to a failure to freeze the tissue rapidly enough.

Once a specimen is mounted on a stub with a conductive carbon or silver-based adhesive, it should be coated as soon as possible. The typical method for deposition is sputter coating. The specimen is inserted into a vacuum chamber and a thin film (15–20 nm) of conductive material (most commonly gold, gold/palladium, or platinum) is deposited. Throughout the entire procedure, proper methods of manipulating microorganisms should be adopted. The requirements depend on whether bacteria are processed in suspension, in tissues, in tissue culture, or on agar (*for tutorial see* Watson *et al.* 1980).

ESEM technique (no sample preparation required)

The ESEM, owing to technical modifications, permits variation of the specimen chamber environment through a range of pressure-, temperature- and gas-related compositions. Therefore, hydrated (free water or damp) and insulating samples may be examined without preparation (Donald 2003). The ESEM technique associates two technical modifications compared with CSEM: (i) separation of the high vacuum column from the low vacuum specimen chamber using pressure limiting apertures (PLAs) and (ii) a new type of detector: the 'gaseous detection device' (GDD) (Danilatos 1990). Basically, a special system containing pumps and valves creates a series of differentially pumped vacuum/pressure zones separated by PLAs. These apertures are sufficiently large to allow the electron beam to pass through, but still small enough and specially orientated to severely limit gas flow from one compartment to the next. In the sample chamber, common pressures are as high as 10–20 torr.

As indicated above, a high vacuum is usually required to stop the electrons from being scattered by gas molecules leading to degradation of image quality. ESEM, however, is different because the total distance with a significant presence of gas, through which the electrons travel, is kept as short as possible. Thus, only some of the incident electrons will undergo large-angled collisions with the gas molecules. As a result a very small fraction of PE is removed from the beam originating in the column, and a sharp central probe is

maintained and superimposed on quite a broad 'skirt' (Danilatos 1988). The gas is, however, not a passive participant in the imaging process; it plays a key role in signal detection. As electrons (SE or BSE) are emitted, they also have to travel through the gas. SE in particular, because of their low energies, have a high collision cross-section with the gas molecules and ionization of the molecules has a significant probability of occurring. Each ionizing collision gives rise to a daughter electron (or environmental-SE) which, like the original SE, is drawn towards the positively charged GDD and further collisions can occur. This phenomenon is known as the proportional cascade multiplication of the imaging current. As well as environmental-SE produced in the ionizing collisions, positive ions are produced simultaneously. These positively charged particles are attracted to the sample surface and suppress the effects of negative charges as seen with insulating samples. This mechanism of charge suppression allows the imaging of microbial cells in their native, uncoated state, with a relatively large choice in accelerating voltages and without causing charging-related artefacts.

As stated, ESEM permits a small amount of gas (up to 10–20 torr) around the sample. In principle this means that hydrated samples can be kept fully hydrated, but in practice achieving this takes some thought especially when using biological materials with high inherent water content. During the whole imaging process, it is important to be aware of (i) the thermodynamic and kinetic stability of the sample and (ii) the chamber pressure and temperature. The phase diagram for water shown in Fig. 1 indicates the partial pressure of water vapour necessary to stabilize liquid water at a given temperature. If one tries to work at room temperature, it can be seen that the water vapour in the chamber is impossibly high, and imaging under these conditions is currently not practical. To achieve saturation, it is desirable to drop the sample temperature to just above freezing, and then only a moderate gas pressure in needed to stabilize water (Tai & Tang 2001). Usually this is done by the use of a Peltier cooling stage.

ESEM for the observation of endodontic pathogens: a practical approach

The rationale of this practical approach was to illustrate the performance of ESEM when observing three common endodontic pathogens in their native state *in situ*. Examination under environmental conditions (ESEM) was therefore compared with inspection under conventional high vacuum (CSEM).

Specimen selection and inoculating procedure

Six carious-free, extracted human teeth were selected and stored in a 0.5% solution of chloramine in water at 4 °C until use. Crowns of the teeth were isolated and prepared in such a way that mounting them in the specimen stub was possible with a flat surface positioned horizontally. This surface was cut parallel to the coronal plane as near as possible to the pulp extensions using a rotating diamond saw (Isomet Saw; Buehler Ltd, Evanston, IL, USA) under water-cooling. To ensure complete removal of the smear layer, specimens were immersed in an ultrasonic bath with 2.5% NaOCl for 4 min followed by 17% EDTA for 4 min. Next, residual crystals from NaOCl or EDTA were removed in three washes with physiologic saline for a period of 2 min each. Samples were kept in 0.9% refreshed physiological saline and refrigerated at 4 °C until further use.

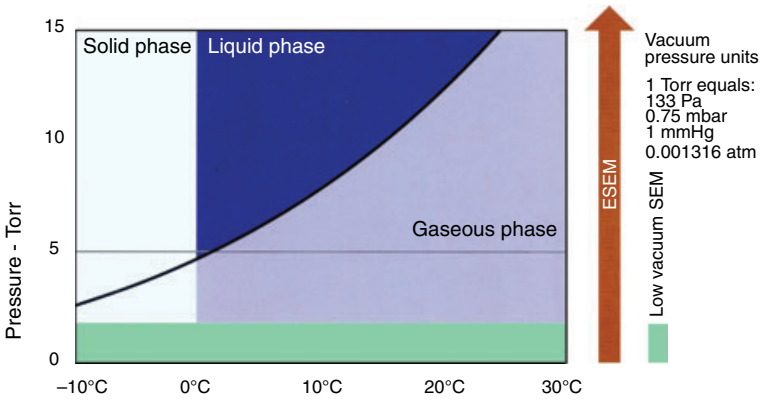

Figure 1 Liquid water can be brought into thermodynamic equilibrium with the vapour phase. The phase diagram for water indicates the partial pressure of water vapour necessary to stabilize liquid water at a given temperature. Thus, typical working conditions in the ESEM might be something like 2 °C and 5.3 torr of water vapour (Source: FEI Instruction brochure for Philips XL30 ESEM-FeG).

Prior to inoculation, dentine samples were autoclaved at 134 °C for 15 min and placed in sterile bijou bottles with the flat surface up. Next, bijou bottles were filled with brain–heart infusion (BHI) broth (37 g L^{-1}; Oxoid Ltd, Basingstoke, UK) inoculated with strains of *Streptococcus anginosus* (LMG 14502) (2/6 samples), *Enterococcus faecalis* (LMG 7937) (2/4 remaining samples) or *Fusobacterium nucleatum* (LMG 13131) (two remaining samples) from the Belgian Coordinated Collections of Microorganisms (BCCM™). The initial concentration of the suspension was standardized at a cell density of 4×10^8 cells mL^{-1}. A 20 μL test of this solution was spread on blood agar (BA) plates (Blood Agar Base II®; Oxoid Ltd), supplemented with haemin (5 mg mL^{-1}), menadione (1 mg mL^{-1}), 5% sterile horse blood, and 0.8% (w/v) Bacto Agar (Difco Laboratories, Detroid, MI, USA), and incubated for 24 h to confirm vitality of the microorganisms at the point of inoculation. Filled bijou bottles were incubated under anaerobic conditions (Concept 300 Anaerobic Workstation; Ruskin Technology, Bradford, UK) for 2 days prior to observation with ESEM or CSEM (one of each strain per method).

Observation with ESEM

Three samples selected for ESEM (one of each strain) were taken and 20 μL tests of the respective inoculation solutions were spread on BA plates and incubated for 7 days (under anaerobic conditions: 80% N_2, 10% CO_2 and 10% H_2) to confirm vitality of the microorganisms in solution at the end of the experiment. ESEM samples were directly viewed in environmental 'Wet' mode with a Philips XL30 ESEM-FeG (FEI/Philips Electron Optics, Eindhoven, the Netherlands) equipped with a Schottky Field-emission electron gun. Two PLAs separated the microscope chamber from the FeG column, thereby creating three regions that were separately pumped. The actual chamber pressure and the temperature of the specimen were controlled from a MS Windows graphical user interface and regulated to bring liquid water into thermodynamic equilibrium with the vapour phase. When working at a temperature of 4 °C, a gas pressure of 3–5 torr, and the inclusion of a few drops of water before the pump-down procedure, optimal conditions to maintain a relative humidity of 80–85% were expected. A Peltier-cooled specimen stage allowed regulating specimen temperature before and during the observation. A gaseous secondary electron detector (GSED) was mounted below the final lens assembly to permit secondary electron imaging in a gaseous environment. Observations were done at 5–10 kV.

Throughout the experiment it became however clear that a pure culture of *F. nucleatum* could not be imaged (reason still unknown). The procedure was therefore successfully repeated using a mixed culture of *F. nucleatum* and *E. faecalis*. Resultant ESEM micrographs of all strains were presented in Figs 2–4.

Parallel observation with CSEM

Three samples selected for CSEM (one of each strain) were taken and 20 μL tests of the respective inoculation solutions were spread on BA plates and incubated for 7 days (under anaerobic conditions: 80% N_2, 10%

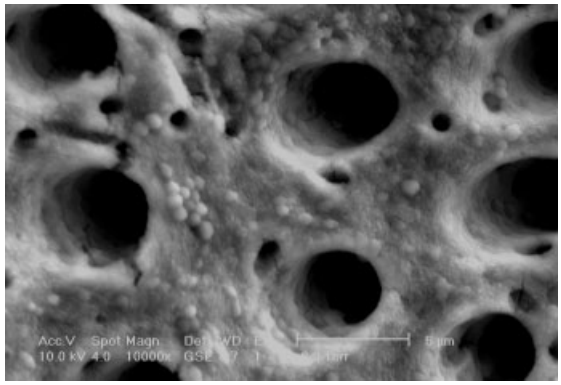

Figure 2 Pioneer specimens of *S. anginosus* adhered to the surface and could multiply to form micro-colonies (co-aggregation) thus resulting in confluent growth (10 000×, Fe-ESEM).

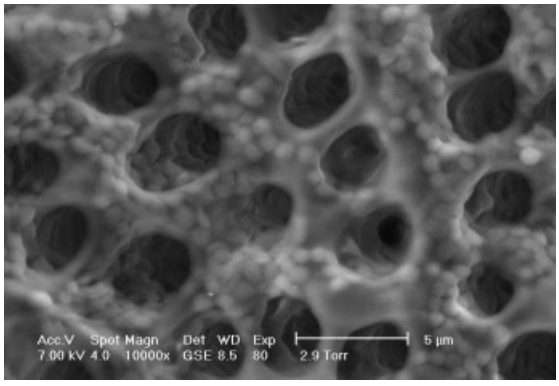

Figure 3 Confluent growth of *E. faecalis* has resulted in the formation of a biofilm. This organized three-dimensional structure is enclosed in a matrix of EPS (10 000×, Fe-ESEM).

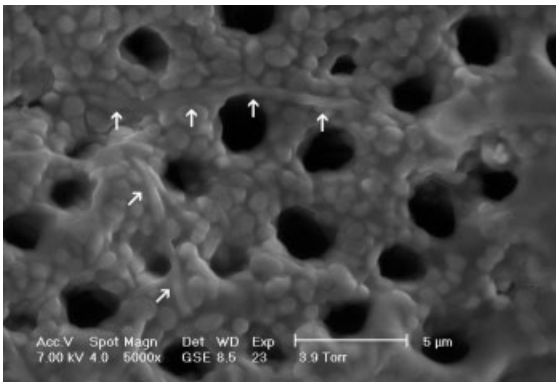

Figure 4 *Fusobacterium nucleatum* species (arrows) were visible using ESEM only in mixed culture with *E. faecalis*. Even then their cells were not as clear distinguished as the cocci ones (5000×, Fe-ESEM).

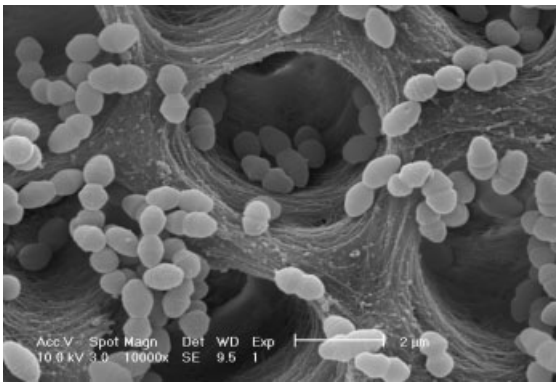

Figure 6 Some specimens of *E. faecalis* have adhered to the dentine surface whilst others invaded the dentinal tubules. (10 000×, Fe-CSEM).

CO_2 and 10% H_2) to confirm vitality of the microorganisms in solution at the end of the experiment. CSEM samples were fixed for 12 h at 4 °C in 2.5% glutaraldehyde in 0.1 mol L^{-1} sodium cacodylate buffer at pH 7.4, rinsed in 0.2 mol L^{-1} sodium cacodylate buffer at pH 7.4 for 1 h with three changes, rinsed with distilled water for 1 min, and then dehydrated in a series of ascending ethanol baths. Dehydrated samples were air-dried following a bath of hexamethyldisilazan, mounted on aluminium stubs with carbon adhesive tabs or with silver paint, sputter-coated with gold (Sputtering device 07 120; Balzers Union, Liechtenstein), and observed with a Philips XL20 Fe-SEM (Philips Co., Eindhoven, the Netherlands) used in conventional high vacuum mode with a conventional SE-detector. Observations were done at 10 kV. As for ESEM, the procedure was repeated using a mixed culture of *F. nucleatum* and *E. faecalis*. Resultant CSEM micrographs of all strains were presented in Figs 5–7.

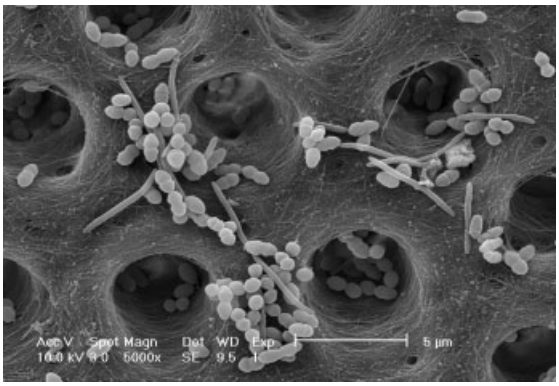

Figure 7 This CSEM micrograph shows colonies of *F. nucleatum* and *E. faecalis*. Both species could clearly be distinguished (5000×, Fe-CSEM).

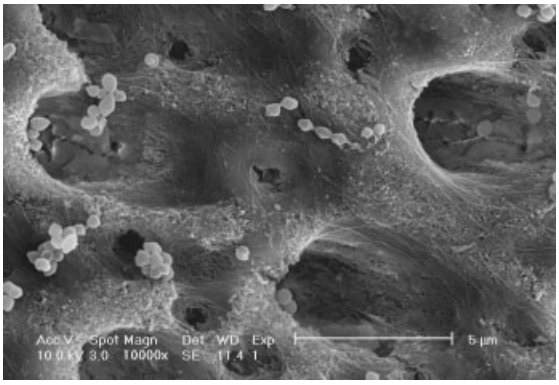

Figure 5 Cells of *S. anginosus* have colonized the dentine. The CSEM image is of higher resolution and less noisier than the analogous ESEM image (Fig. 2). Details of the dentine structure such as collagen fibres could be observed (10 000×, Fe-CSEM).

Validation of the ESEM technique for bacterial observation

Image quality of the ESEM micrograph

Good image quality is essential for correct interpretation of obtained information. ESEM micrographs are composed of grey values, and both contrast and resolution are important. According to the ESEM principle, an image is built by detection of electron

signals emitted from a specimen during surface scan. Therefore, when examining image quality, many factors have to be considered. The most important are: the electron optics, the specimen and the detector device.

Regarding formation of the beam, the Fe type of electron source is generally preferred above thermionic ones. Fe-sources are much brighter (allowing working at low kV) and produce a very narrow electron beam, thus increasing image contrast and resolution. Nevertheless, Fe-sources require an ultra-high vacuum (10^{-10} torr) in the emission chamber and should be integrated in proper systems. Two of such systems (Philips XL30 ESEM-FeG and Philips XL20 Fe-SEM) were used in our practical approach.

As previously mentioned, ESEM instruments incorporate a special design of electron-optics column that allows a number of pressure stages to be maintained along the path of the generated beam. As a result, the electron beam originating at the (ultra-) high vacuum source will experience an increasing gas pressure on its travel towards the specimen surface. This condition is fundamentally connected to the principle of ESEM and has been thoroughly investigated. A conclusion appeared in 1988 when Danilatos (1988, 1993a) demonstrated that, due to scattering and distribution in a gas, a fraction of the electrons is removed from the original (in high vacuum) beam and redistributed in a very broad 'skirt' surrounding the remaining intact fraction (unaffected Gaussian distribution) at the centre. This finding is important because it means that the resolving power of EM can be maintained in the low presence of a gas. According to some manufacturers' publicity, today's ESEM offer the ability to image specimens at very high spatial resolutions; as high as 2–5 nm in some cases. As indicated below, achieving this optimal resolution for moist biological samples in practice is often far from easy. Nevertheless, for many classes of samples the inconvenience of losing resolution is more than outweighed by the ability to carry out completely new experiments.

Signalling electrons are formed on collisions between PE from the incident beam and atoms from the specimen under investigation. The first type of emitted electrons, called secondary electrons, arises in the near-surface region of the sample thus providing topographical information. As for the CSEM, topographical detail largely depends on the diameter of the beam 'spot'. The magnitude of the resultant signal depends on the energy and current of the incident beam: the emission of SE increases as the energy of the PE increases until a certain limit is reached. Beyond this limit the beam is activating electrons deep below the surface; these electrons usually recombine before reaching the surface for emission.

Aside from SE, the incident beam results in the emission of backscattered electrons (BSE), which may be scattered from fairly deep within the sample thus resulting in less topographical detail than for the case of SE. Interestingly, the magnitude of the resultant signal is a weak function of the atomic number (Z) of compositional elements. In this way BSE-imaging is practical in distinguishing structural elements within the sample thus providing compositional (internal) information. At this deeper level, the size of the spot has been broadened by multiple scattering effects and only a small number of generated BSE will be detected by the SE-detector due to their definite direction. As a result, the BSE-signal is typically of lower resolution and noisier than the SE-signal. To partly solve this problem, an additionally integrated BSE-detector can be used. Accordingly, BSE-signals from cell structures can be enhanced by the introduction of heavy metal stains (e.g. potassium permanganate or osmium tetroxide).

Despite the fact that generation of electron signals seems rather simple, obtaining good results during ESEM observation of biological tissues in their native state is not that easy (Gilbert & Doherty 1993, Gwinnett 1994, Thiberge *et al.* 2004). The first topic of discussion is the fact that the presence of a watery film on the sample surface seems to limit topographical observation. Indeed, it has been reported that at high magnification surface details cannot be imaged; at low magnification contrast of the ESEM image is at least reduced (Tai & Tang 2001, Habold *et al.* 2003). In this way the inter-cellular EPS-matrix, which forms a hydrated and negatively charged layer as present in biofilms, could negatively affect image formation. Likewise, it has been reported that BSE-signals originating within cells are impeded by cytoplasmic water when hydrated biological specimens are directly examined in an atmosphere of water vapour at reduced temperatures. Critical-point drying and freeze-drying could increase the BSE-signal in this type of situation (Collins *et al.* 1993). Another point for discussion is the chemical composition of the sample. Biological tissues and cells are composed of low atomic number (Z) elements such as carbon in cells ($Z = 6$) and oxygen in water ($Z = 8$). As we are imaging differences in scattering between elements with kindred atomic numbers, the resulting contrast will often be low and may depend strongly on the local concentration of

heavier ions. As indicated above, deposition of metals with high atomic number on to tissue structures enhances the electron density of the specimen. When applied on to specific sites within cells, this type of staining can enhance image contrast and reveal much more information. As a final point, some investigators have suggested the use of computer software employing an image-processing algorithm to filter out contrast variations. This procedure can be used in order to optimize image contrast and thus to extract image formation that would otherwise not be perceptible (Habold *et al.* 2003). Even so, the most reliable approach to obtain good image quality is by adopting the finest available hardware whilst using optimal conditions for scanning.

Assuming that the formation of an image is not that easy, image interpretation may not be simple either. Indeed, many users have declared that understanding the contrast observed in an ESEM image is a far from trivial matter. Various studies have indicated that there are sources of contrast in ESEM not generally seen in the CSEM, at least in part because the presence of the standard metallic coating obscures the true emission signal from the sample (Peters 1982, Collins *et al.* 1993, Schnarr & Futing 1997, Griffin 2000, Toth & Phillips 2000, Thiberge *et al.* 2004). Obviously, the 'additional' contrast arises from variations in the yield of generated electrons by local electronic structure differences and by the recombination of some emitted electrons with ions from the gas (Stokes *et al.* 1998, Toth *et al.* 2002). As a result, one knows that contrast is related to both charge movements within the sample and instrument operating parameters. A unifying interpretation of contrast is so far not complete.

Without doubt, an important and final factor controlling image quality is the detector device. Fundamentals of ESEM created the challenge to detect an electric flow in a gas, which has led to the invention of a new type of detector: the GDD (Danilatos 1990). To stress the possibility of SE-detection in a gaseous environment, ElectroScan introduced the acronym ESD (environmental secondary detector), which is currently known as GSED. The primary function of the GSED is to discriminate (*most*) noise forming electrons by using a suppressor electrode and a detector ring. Throughout the years, optimum designs and geometrical positioning of the detectors have been determined. In particular, proper detection of BSE has been rather difficult due to scattering through angles approaching 180°. By making the BSE-detector surrounding the SE-detector directly above the specimen, an improved detector system was obtained (Collins *et al.* 1993).

To this point, no attention has been paid to other signals that can be generated when the incident beam scans across the specimen surface in both CSEM and ESEM. In fact, beside the emission of SE and BSE, the absorption of PE-energy may give rise to both X-rays and photons of visible light (an effect known as cathodoluminescence or CL). The description of image formation in EM is equally applicable to all emitted signals except for the detection system which is different each time. Collection and detection of these signals can be used to produce further information on the specimen that is investigated (Danilatos 1988, 1993a, Sigee 1998, Griffin & Browne 2000). Energy-dispersive X-ray spectroscopy (EDX or EDAX), for example, including computer analysis of the wavelength and energy spectra, can be used to measure accurately the nature and quantity of different elements is the material. This technique, however, is of little use for the microbiologist because light elements such as carbon produce too weak X-ray signals. SE-imaging stays by far the most common because it can be used with almost any specimen.

Nowadays, various 'environmental systems' are commercially available. The original ESEM (which is a trademark of FEI/Philips Electron Optics) detects charge flow in a gas, but as other manufacturers enter the market (selling so-called 'variable pressure instruments' or VP-SEM, which typically are not yet able to operate at such high pressures for technical reasons), a variety of signals are being used for detection (Danilatos 1993a, Donald 2003). For all systems, one limitation seems to lie in the purity of the gaseous detector. Concentrating on the 'true' ESEM, which detects the electron signal, none of the existing SE-detectors is really capable of detecting a pure SE-signal, and BSE-detectors are even worse. For other kinds of ESEM, information about detectors and their 'dominant' signal detection is even more limited. In most systems, a subsequent electronics circuitry is used to improve the final image by further amplifying, integrating and averaging the incoming signal.

Observation of bacteria in their 'native' state

The clear advantage of ESEM for (bacterial) cell observation is the ability to view biological specimens directly without dehydrating or coating, i.e. in their native state (Collins *et al.* 1993). More than reducing preparation time, direct application of ESEM enables

delicate specimen to be viewed with minimal mechanical disruption (McKinlay *et al.* 2004) and without obscuring internal contrast. However, when validating the ESEM technique for bacterial observation one has to remember that the cells of interest are living microorganisms. To decide on the real nondestructive character of ESEM, the viability of bacteria during the entire scanning procedure has to be investigated. This section will therefore focus on two viability-related aspects: beam-radiation damage and the environmental conditions in the specimen chamber.

As yet it is not known whether living (bacterial) cells can be viewed but not killed by the electron beam (National Research Council 1990, Donald 2003). In fact, this question is rather complex and a lot of issues are involved. It all starts with the delivery of high-energy PE to the specimen surface by the incident beam. Upon arrival, a variety of interactions between these electrons and atoms of the sample occur, leading to the emission of imaging signals along with the incident of radiation damage. The intensity and relation of the different interaction types depend on the electron beam, the environment, and the specimen itself. In general, radiation damage is more likely in TEM than SEM (Egerton *et al.* 2004), and ESEM is more predisposed than CSEM due to the typical nature of the samples and the presence of liquid water (Royall *et al.* 2001). Regarding this paper, the potential damage of ESEM on biological cells (e.g. bacterial cells) will be further explored.

The electron beam used in ESEM can cause temporary or permanent changes in the surface or bulk structure of a biological specimen. Important are the inelastic collisions (Coulomb interactions) of PE with the atomic electrons, which can result in specimen heating and radiolysis, including structural damage and mass loss (Egerton *et al.* 2004). Heating is not expected to be a major problem in bulk specimens because the heat flow is radial in three dimensions, leading to a relatively small temperature rise. On the contrary, the amount of generated heat will be much higher in very small or thin objects. Radiolysis implies the appearance of ionization and bond scissions. Chemical bonds are broken and the molecules change in shape and shift in position, causing structural changes and perhaps the removal of light atoms (particularly hydrogen, nitrogen and oxygen). Organic specimen containing relatively weak bonds are typically highly beam-sensitive and very prone to radiolysis. In addition, liquid water within the specimen acts as a source of small, highly mobile free radicals (hydroxyl radicals), which accelerate specimen degradation (Royall *et al.* 2001). With a bulk specimen, damage is produced close to the surface, within the electron range. For microscopic specimens, the damaged part will be more important considering the smaller total volume. Although lowering the temperature of a specimen does not change the inelastic cross-section, it does reduce the sensitivity of an organic specimen to structural damage and mass loss (Egerton *et al.* 2004). Of course, cooling the specimen will also help to prevent heating. Each of the above-mentioned processes is dose-dependent. Note that in electron microscope literature, 'dose' usually means electron exposure: the product of incident current density (i.e. energy disposition per unit volume) and exposure time. Lowering the irradiation dose can be achieved by lowering the acceleration voltage and/or current intensity of the beam, or by changing the scanning mode (e.g. lowering the level of magnification) (Donald 2003). The tolerable level of structural and compositional damage is given by the objectives of the experimental set-up, and will limit the amount of information that can be extracted by ESEM.

Besides beam radiation damage, other factors may affect, perhaps to a lower degree, the viability conditions of bacteria throughout the ESEM-scanning procedure. Amongst them are factors affecting the metabolism and growth of microorganisms in their natural habitat (i.e. temperature, redox potential, pH and nutrients). Thus, to examine the true *in situ* environmental conditions of the ESEM-specimen chamber, one should consider these factors as well. According to the specifications on the strains, the optimal temperature for the bacterial cultures used in our practical approach was 37 °C. The temperature of the specimen during ESEM was about 4 °C, therefore much lower. Besides temperature, the redox potential or anaerobic condition of the environment can be considered. Although anaerobiosis is frequently described in rigid terms, sharp distinctions cannot be made and a wide spectrum of oxygen tolerances occurs. *Enterococcus faecalis* and *S. anginosus* are facultative anaerobes tolerating the presence of low oxygen concentrations with an optimum of 5%. *Fusobacterium nucleatum* belongs to the group of obligate anaerobes (i.e. they require reduced conditions for their normal metabolism) and oxygen concentration is considered the main factor limiting their growth. During our practical approach, the gaseous environment in the ESEM-specimen chamber contained a mixture of water vapour and ambient air at reduced pressures. The true

oxygen concentration, however, was not determined and neither was its implication on the viability of the cells. To better guarantee a low redox potential during ESEM, it might be helpful to surround bacteria with transport fluids that contain reducing agents. A third important factor for bacterial wellness is the surrounding pH. Many microorganisms require a pH around neutrality for growth and are sensitive to extremes of acid or alkali. The medium used in our practical approach was a BHI broth with a pH of 7.4 (±0.2 at 25 °C) which was required by our strains for growth. The extent to which this pH was influenced by the low temperature conditions or the radiation of the beam was not determined. At least specimens were not rinsed before ESEM scanning, so part of the medium remained on the substrate and could promote a good environment full of nutrients.

Other non-destructive techniques: the competitors of ESEM

In addition to ESEM, other microscopic techniques have been explored and used in study designs for (bacterial) cell observation in a non-destructive way, i.e. at a state most closely approximating the native state. This section reviews these approaches by discussing their principles and applications.

A first technique, which can be seen as a modified technique for SEM of biological cells and tissues, has been proposed very recently by the name of 'Wet SEM' (Thiberge *et al.* 2004). Wet SEM relies on a membranous partition (i.e. a polyimide membrane of 145 nm in thickness) that protects the sample from the vacuum whilst being transparent to the electron beam. In this way, hydrated samples can be maintained in fully physiological conditions and imaged with little loss of resolution compared with CSEM. This technique can now be used to probe inside whole cells, giving information on organelles and internal structure. Also tissue sections can be imaged, giving structural information on the connectivity and organization of cells and extracellular structures *in situ*.

A second non-invasive technique called laser scanning confocal microscopy (LSCM) is now being used to determine the true architecture of plaque and the location of selected bacteria within the biofilm (Marsh 2004). A conventional confocal microscope generates thin (>0.35 μm) optical slices up to 200 μm below the surface of semi-transparent materials such as cells without dehydration damage due to vacuum. Stacks of sections taken at successive focal planes can be reconstructed to produce a three-dimensional view. Following the confocal principle, resolution of optical microscopy is significantly improved, lying somewhere between that of conventional light microscopy and TEM/SEM (Watson 1991). The simplest technique for using confocal microscopy is to label the chemical structure within biological tissues with fluorescent dyes (confocal fluorescence microscopy). In addition, new imaging techniques such as multi-photon pulsed-laser excitation of dyes give the potential of greater depth penetration and improved resolution (LSCM). In general, the major advantage of this technique is that it does not require special specimen processing, as observations can be carried out under near environmental conditions. In this way, LSCM has shown that biofilms may have a more open structure than previously thought from studies involving CSEM (Marsh 2004).

Another technique for bacterial observation that does not require special specimen processing is atomic force microscopy (AFM). This contact type of scanning-probe microscopy has been used to obtain micrographs of dried bacteria in ambient air, and living ones in their culture medium at resolutions similar to SEM (Robichon *et al.* 1999). Using AFM, a specimen placed on an XYZ piezoelectric translator is scanned below a stylus mounted on a cantilever spring, and surface features cause the stylus to deflect the cantilever during scanning. This bending movement is measured by observing the angular deflection of a laser beam reflected at the back-end of the cantilever. By feedback, the Z motion of the XYZ translator is controlled, and the contact force between the tip and the specimen surface is kept almost constant. In the end, a true 3D image of the sample surface is reconstructed from collected data. AFM can be used in contact mode or in tapping mode. The latter technique consists of lightly tapping the tip on the surface during scanning, which dramatically reduces imaging forces and increases resolution. Especially soft and fragile specimens are better scanned in tapping mode without risk of sample damage, whilst maintaining the highest resolution. Tapping mode imaging can be conducted in a fluid environment whilst tip and object are submerged, and consequently it is possible to obtain micrographs of living bacteria in culture broth (Robichon *et al.* 1999). However, interpretation of the obtained results has been difficult because the nature of the interaction forces that come into play between the tip and the sample are not fully understood, as is the extent of deformation of the 'soft' cell membrane structure by the hard AFM tip.

Future directions and applications

Most advances in science are dependent upon the development of appropriate techniques to demonstrate them. As ESEM provides a way to image root canal bacteria directly in their native state, effects of therapeutic endodontic strategies on the appearance and distribution of pathogens could be monitored *in situ* (e.g. bacterial cells growing as biofilms) in real time. To improve the possibility for such experiments, ESEM ancillary equipment (e.g. a micro-injector to supply a flow of liquid) can be fully integrated. Major techniques such as laser sources may be interfaced as well. *In situ* experiments can be carried out and recorded under ambient levels of light. This is because, unlike the E–T detector of CSEM, the GSED is not light or heat sensitive.

With the given information on microscopic techniques in mind, some guidelines for testing endodontic treatment strategies using ESEM can be formulated. First of all, an intact appearance of bacterial morphology does not mean that those bacteria are (still) alive. Both radiation damage and environmental conditions can cause cell death, even without signs of deterioration such as shrinkage detectable by ESEM. To determine the bactericidal effect of therapeutic actions, other techniques (e.g. bacterial culturing and fluorescent microscopy) should be used in conjunction with ESEM. Further, bacteria under stressful conditions (e.g. starvation or low temperatures), instead of going into a lytic state, may lower their metabolic activity and go into a dormant state from which they may recover afterwards. This way of protection may influence the susceptibility of pathogens for various irritants. A recurrent question that arises during discussion of the applicability of ESEM information to real world processes is the effect of reduced pressure in the ESEM chamber in comparison with actual processes that occur at ambient (760 torr) or elevated pressure (Prack 1993). This is a real concern when you consider that both the state and mutual interaction (e.g. wetting properties) of substances can be different. To undermine this problem, the actual treatment steps can be performed both inside and outside the specimen chamber. Software for precise location fixing will allow relocating the same area on the specimen surface before and after treatment. Finally, when scanning specimens in their culture medium, some elements of this medium can result in the image background being fouled (Robichon *et al.* 1999). However, these contaminating elements may be distinguished from bacteria by their shape, height and size.

To conclude, the ESEM technique, young in comparison with the conventional methods and therefore still with its theoretical challenges, looks set to allow the observation of root canal bacteria in their native state without prior preparation or conductive coating. More than reducing preparation time, its direct application provides a way to explore the effect on bacterial appearance of endodontic treatment modalities using the *in situ* testing environment of ESEM. Moreover, with modern instruments being capable of rapidly switching between high vacuum and low vacuum mode, the conventional and the environmental SEM approach can be used in parallel.

Acknowledgements

This investigation was part of the project OT/2002/49 (Research Fund K.U.Leuven), and further supported by the Fund for Scientific Research Flanders (FWO) and the National Lottery (Environmental SEM, F6/12-AV.F461, G.0335.01, Ludo Froyen), and by a fund of the Toshio Nakao Chair for Adhesive Dentistry inaugurated at the Catholic University of Leuven with Bart Van Meerbeek and Paul Lambrechts awarded as chairholders. The authors are indebted to Rudy De Vos for skilful assistance with conventional and environmental SEM-FeG.

References

Bancroft JD, Stevens A (1996) *Theory and Practice of Histological Techniques*. New York: Churchill Livingstone.

Collins SP, Pope RK, Scheetz RW, Ray RI, Wagner PA, Little BJ (1993) Advantages of environmental scanning electron microscopy in studies of microorganisms. *Microscopy Research and Technique* **25**, 398–405.

Danilatos GD (1988) Foundations of environmental scanning electron microscopy. In: Hawkes PW, ed. *Advances in Electronics and Electron Physics*. Boston: Academic Press, **71**, 109–250.

Danilatos GD (1990) Theory of the Gaseous Detector Device in the ESEM. In: Hawkes PW, ed. *Advances in Electronics and Electron Physics*, Vol. 78. Boston: Academic Press, pp. 1–102.

Danilatos GD (1993a) Introduction to the ESEM instrument. *Microscopy Research and Technique* **25**, 354–61.

Danilatos GD (1993b) Bibliography of environmental scanning electron microscopy. *Microscopy Research and Technique* **25**, 529–34.

Darby I, Mooney J, Kinane D (2001) Changes in subgingival microflora and humoral immune response following perio-

dontal therapy. *Journal of Clinical Periodontology* **28**, 796–805.

Donald AM (2003) The use of environmental scanning electron microscopy for imaging wet and insulating materials. *Nature Materials* **2**, 511–6.

Egerton RF, Li P, Malac M (2004) Radiation damage in the TEM and SEM. *Micron* **35**, 399–409.

Everhart T, Thornley RFM (1960) Wide-band detector for micro-microampere low-energy electron currents. *Journal of Scientific and Industrial Research* **37**, 246–8.

Gilbert LC, Doherty RE (1993) Using ESEM and SEM to compare the performance of dentin conditioners. *Microscopy Research and Technique* **25**, 419–23.

Greenstein G, Polson A (1985) Microscopic monitoring of pathogens associated with periodontal diseases. A review. *Journal of Periodontology* **56**, 740–7.

Griffin BJ (2000) Charge contrast imaging of material growth and defects in environmental scanning electron microscopy – linking electron emission and cathodoluminescence. *Scanning* **22**, 234–42.

Griffin BJ, Browne JR (2000) Fiber-optic based spectral cathodoluminescence: simple and economic option for use in conventional and environmental SEM. *Microscopic Microanalysis* **6**, 42–8.

Gwinnett AJ (1994) Chemically conditioned dentin: a comparison of conventional and environmental scanning electron microscopy findings. *Dental Materials* **10**, 150–5.

Haapasalo M (1986) Bacteroides buccae and related taxa in necrotic root canal infections. *Journal of Clinical Microbiology* **24**, 940–4.

Habold C, Dunel-Erb S, Chevalier C, Laurent P, Le Maho Y, Lignot JH (2003) Observations of the intestinal mucosa using environmental Scanning electron microscopy (ESEM); comparison with conventional scanning electron microscopy (CSEM). *Micron* **34**, 373–9.

Little BJ, Wagner PA, Ray RI, Pope R, Scheetz R (1992) Biofilms: An ESEM evaluation of artifacts introduced during SEM preparation. *Journal of Industrial Microbiology* **8**, 213–22.

Magnusson I, Liljenberg B, Yoneyama T, Blomqvist N (1985) Sampling of subgingival microbiota for dark-field microscopy. *Journal of Clinical Periodontology* **12**, 209–15.

Marsh PD (2004) Dental plaque as a microbial biofilm. *Caries Research* **38**, 204–11.

Matias V, Al-Amoudi A, Dubochet J, Beveridge J (2003) Cryotransmission electron microscopy of frozen-hydrated sections of *Escherichia coli* and *Pseudomonas aeruginosa*. *Journal of Bacteriology* **185**, 6112–8.

McKinlay KJ, Allison FJ, Scotchford CA, Grant DM, Oliver JM, King JR et al. (2004) Comparison of environmental scanning electron microscopy with high vacuum scanning electron microscopy as applied to the assessment of cell morphology. *Journal of Biomaterials and Materials Research* **69**, 359–66.

Nair P, Henry S, Cano V, Vera J (2005) Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after 'one-visit' endodontic treatment. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics* **99**, 231–52.

National Research Council (1990) *Health Effects of Exposure to Low Levels of Ionizing Radiation, Biological Effects of Ionizing Radiation Report V*. Washington, DC: National Academic Press.

Perdigão J, Lambrechts P, Van Meerbeek B, Vanherle G, Lopes AL (1995) Field emission SEM comparison of four postfixation drying techniques for human dentin. *Journal Biomedical Materials Research* **29**, 1111–20.

Peters KR (1982) Conditions required for high quality high magnification images in secondary electron-I scanning electron microscopy. *Scanning Electron Microscopy* **4**, 1359–72.

Prack ER (1993) An introduction to process visualization capabilities and considerations in the environmental canning electron microscope (ESEM). *Microscopy Research and Technique* **25**, 487–92.

Quirynen M, Bollen C, Vandekerckhove B, Dekeyser C, Papaioannou W, Eyssen H (1995) Full- vs. partial-mouth disinfection in the treatment of periodontal infections: short-term clinical and microbiological observations. *Journal of Dental Research* **74**, 1459–67.

Ricucci D, Bergenholtz G (2003) Bacterial status in root-filled teeth exposed to the oral environment by loss of restoration and fracture or caries – a histobacteriological study of treated cases. *International Endodontic Journal* **36**, 787–802.

Robichon D, Girard JC, Cenatiempo Y, Cavellier JF (1999) Atomic force microscopy imaging of dried or living bacteria. *C.R. Académie Sciences Paris* **322**, 687–93.

Royall CP, Thiel BL, Donald AM (2001) Radiation damage of water in environmental scanning electron microscopy. *Journal of Microscopy* **204**, 185–95.

Schnarr H, Futing W (1997) Some aspects of optimizing contrast for the investigation of joint materials in the ESEM. *Scanning* **19**, 143–57.

Sigee DC (1998) Environmental SEM and X-ray microanalysis of biological materials. *Mikrochimica Acta* **15**, 283–93.

Socransky S, Haffajee A, Cugini M, Smith C, Kent R Jr (1998) Microbial complexes in subgingival plaque. *Journal of Clinical Periodontology* **25**, 134–44.

Stokes DJ, Thiel BL, Donald AM (1998) Direct observation of water-oil emulsion systems in the liquid stated by environmental SEM. *Langmuir* **14**, 4402–8.

Sunde P, Olsen I, Debelian G, Tronstad L (2002) Microbiota of periapical lesions refractory to endodontic therapy. *Journal of Endodontics* **28**, 304–10.

Tai SS, Tang XM (2001) Manipulating biological samples for environmental scanning electron microscopy observation. *Scanning* **23**, 267–72.

Thiberge S, Nechushtan A, Sprinzak D, Gileadi O, Behar V, Zik O et al. (2004) Scanning electron microscopy of cells and tissues under fully hydrated conditions. *Proceedings of the National Academy of Sciences USA* **10**, 3346–51.

Toth M, Phillips MR (2000) The role of induced contrast in images obtained using the ESEM. *Scanning* **22**, 370–9.

Toth M, Daniels DR, Thiel BL, Donald AM (2002) Quantification of electron-ion recombination in an electron-beam irradiated gas capacitor. *Journal of Physic Developments* **35**, 1796–804.

Trope M, Tronstad L, Rosenberg ES (1992) Dark-field microscopic spirochete count in the differentiation of endodontic and periodontal abscesses. *Journal of Endodontics* **18**, 82–6.

Van Meerbeek B, Vargas M, Inoue S et al. (2000) Microscopy investigations. Techniques, results, limitations. *American Journal of Dentistry* **13**, 3–18.

Watson TF (1991) Applications of confocal scanning optical microscopy to dentistry. *British Dental Journal* **171**, 287–91.

Watson LP, McKee AE, Merrell BR (1980) Preparation of microbiological specimens for scanning electron microscopy. *Scanning Electron Microscopy* **2**, 45–56.

Ximenez-Fyvie L, Haffajee A, Socransky S (2000) Microbial composition of supra- and subgingival plaque in subjects with adult periodontitis. *Journal of Clinical Periodontology* **27**, 722–32.