Serial sectioning of teeth and microscopy in cariology research

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In-vitro studies commonly establish the validity of a detection system by using a ‘‘gold standard’’ against which the diagnostic method can be evaluated. The most common gold standard used for caries lesions is the histological evaluation of hard tissue sections. In order to evaluate serial sections histologically, elaborate equipment is required. Undecalcified teeth with enamel and dentin cannot be sectioned with conventional histological procedures due to the risk of section damage. There are a number of shortcomings in histological methods, e.g. section damage during preparation of samples or the section might not relate to the actual investigation site in question. This publication presents in detail the tools, auxiliary forms and geometric procedures to embed and serially cut teeth in a three dimensional orientation, which allows accurate location of each section from within the tooth. A macro photographic reproduction technique of histological slides is also presented. This method has been applied successfully in dental education and for cariology studies.

Keywords: Dentistry; cariology; tooth hard section; histology

1. Introduction

In dentistry, in-vitro studies commonly establish the validity of a detection system by using a ‘‘gold standard’’ against which the sensitivity and specificity of the diagnostic method can be calculated. The most common gold standard used for caries lesions is the histological evaluation of hard tissue sections. There are large variations on this theme and at its simplest the teeth are hemisected at the place to be examined. When hard tissue sections are prepared their thickness can vary considerably (250–1000 mm). When examining histological sections of teeth a high resolution of fine structures is required and either decalcified and appropriately stained sections or undecalcified ground sections are used as a gold standard to meet the aims and objectives of many studies. Research into the hard tissues of enamel and dentine requires a histological technique which preserves this mineralized tissue and takes into account the hardness and brittleness of the tissues [1, 2]. To address this teeth need to be embedded in a resin material prior to serial sectioning and a technique is required which allows orientation of the sections into a three dimensional arrangement. To achieve this, problems associated with moisture content of a natural tooth have to be overcome, together with a three dimensional grid or measuring system, so as to locate accurately each section from within the tooth.

To view undecalcified sections under a microscope two lighting techniques can be used, namely with oblique incident light or with transmitted light. Incident light enables the specimens to be more than 100 µm in thickness but transmitted light requires specimens to be thinner in the order of 100 µm or less. The evaluation of specimens in oblique incident daylight allows color temperature to show tooth substances with their natural interplay of colors and optical peculiarities largely intact. Observation in transmitted light provides a visual impression that is quite different from this. There are a number of shortcomings in histological methods, many of which are operator errors. For instance there may be section damage during preparation of samples and the section might not relate to the actual investigation site in question. In addition, assessment of lesion depth from a section is also difficult as it is often unclear where caries affected tissues end and pulp–dentine complex reactions begin. It is therefore important to have a meticulous histological methodology and more than one validator which pose a problem in large multi-national studies.

As with visual examination, digital reproduction can be carried out using the microscope with oblique incident light or with transmitted light. Depending on the desired detail resolution, the lens' enlargement must be combined with an adequate pixel resolution of the camera's chip [3].

The publication aims to present in detail the tools, auxiliary forms and geometric procedures to embed and serially cut teeth in a three dimensional orientation, which allows accurate location of each section from within the tooth. A macro photographic reproduction technique of histological slides is also presented. This method has been applied in cariology studies [4-7] and for teaching purposes in our dental school.
2. Material and methods

2.1. Storage of Extracted Teeth

A series of extracted human premolars and molars was used. After extraction, the teeth were stored in a saturated thymol solution (1 g of thymol crystals added to 1000 ml of water) at room temperature until they were further processed. To prepare the tooth for dehydration, its root was resected approximately 1.5 mm below the cementum-enamel junction, thus opening the pulp chamber and making the dentine near the pulp more accessible to dehydration and infiltration.

2.2 Digital Macro Photographs of the Occlusal Surface

Macrophotography was used to document the initial clinical features of the occlusal aspect of each tooth such as its macromorphology and the optical properties of the tooth surface. In this study glass scales (Leitz Comp., Wetzlar, Germany) were reproduced alongside images of the tooth to act as co-ordinates for precise location of investigation sites on the occlusal surface. This enables structures to be localized in the image according to their position and extension (Fig. 1). Further, this makes it possible to assign results to a third plane, for example, as depth profiles from corresponding histological sections.

2.3. Dehydration, Resin infiltration and Mounting

The teeth were dehydrated in increasing concentrations of alcohol from 40 % to 60 %, 80 %, 100 % and once again 100 % for 12 hours in each concentration using agitation to remove the water. The teeth were then embedded using a two stage infiltration under normal conditions, firstly by immersing the teeth in a mixture of ethanol and methacrylate monomer in proportions of 1:1 for 24 hours, followed by a pure monomer, Technovit® 7200 VLC (Heraeus Kulzer, Germany), for 36 hours. The resin infiltrated tooth was then polymerized using visible light from fluorescent tubes that replicate daylight (Histolux, Kulzer Exakt, Germany) for 10 hours. Acrylic cylinders were prepared with the following dimensions: height H= ~25mm, external diameter D= ~16mm, internal diameter d= ~13mm, and a borehole depth/height h= ~15mm (Fig. 1). The crowns were glued to the base of the borehole by their mesial approximal surface. Thus the longitudinal fissure of the tooth crown ran along the long axis of the cylinder. A depth marker in the form of a right-angled triangle made from coloured foil (~ 100 µm thick) was aligned parallel with the cut root face and occlusal surface of the tooth. The mesial approximal surface of the tooth was also glued to this mounting surface. Thus each section would contain a part of the triangle in the form of a fine coloured line. The length ML of this line in millimeters, which was directly measurable in the specimen, yields an indirect measure of the height of the section above the base of the triangle and the mounting surface of the tooth (Fig. 1). The dimensions of the triangle were: base length b=9mm, height a=18mm, and a gradient angle of ß=63.5°. The geometric relationship (H= 2*(9–ML)) was used to calculate the height H of each section above the mounting surface. The acrylic glass cylinder with the tooth crown and depth marker was then filled with Technovit® Monomer and polymerized (Fig. 1, upper right).

2.4. Serial Sectioning

After polymerization the sample was screwed onto an acrylic plate (100x50x55mm) which adheres to the vacuum suction plate of the diamond band saw (Exakt, Germany), stabilizing it against any stress. For each tooth the distal approximal surface of the tooth crown was exposed at points by the first cut, and then polished. This also starts to cut into the triangle of the depth marker. A microscope slide was then glued onto the cut and polished section face and then the first section of the specimen was cut with the prescribed thickness. This process was repeated for all sections until the mesial approximal surface and the base of the depth marker were reached. A cutting band, 300 µm thick with grade D64 diamonds was used (Exakt, Germany). The most up-to-date cutting technology, contact point cutting, was used in this study. In this technique the sample executes a reciprocal rotary motion around an adjustable angle in front of the diamond band. This reduces the contact of the sample to the cutting band nearly to a point and not a line, as with conventional technologies. The necessary feed from one section to the next amounts to approximately 650 µm. The feed of the specimen is set by way of a digital measuring sensor with a resolution of ±1 µm.

When all sections of the specimen had been cut, the grooves and artifacts left by the diamond band were removed by grinding and the specimens were reduced to coplanar surfaces of the desired thickness. First they were ground with a fine-grained diamond grinding wheel and then precision ground using paper coated with 2500 and 4000 grade Al2O3. The side bearing the specimen was fastened to a vacuum slide holder and a sliding carriage kept it coplanar while moving it across the grinding wheel. An adjustable micro-probe ended the grinding process when the desired thickness had been reached. To protect the polished surfaces from soiling and damage, they were coated with a rapid light-curing, viscous adhesive (Technovit® 7230, Heraeus-Kulzer, Germany) and covered with thin mineral glasses (~140 µm thick).
2.5 Visual Examination of Slides under a Stereo Microscope

The sections were unstained, undecalcified, more than 100 µm thick and were viewed under a stereo microscope using incident light. The source of light reproduced that of daylight (5600° K) and did not heat the section. The substrate beneath the specimen did not have any structure and was of a light-absorbent, deep black color.

2.6 Macro-Photographic Digital Reproduction

Photographic images of the sections were taken using a digital camera with a macro-lens (EOS 30D, MP5; f=65mm, Canon Comp., Krefeld, Germany), manual focus and a nominal image scale of M=1:1 to M=5:1. For imaging, the sections were illuminated using an oblique incident light from a ring-shaped fluorescent tube which reproduced daylight in front of a deep black background, or transmitted light. The images were post-processed in order to isolate the object from its background, finely adjust the tonal values and enhance the edge definition.

3. Results

To illustrate the potential of this sectioning technique, ten sections from a single molar tooth with fissure caries are illustrated (Fig. 2). Beneath each section the length of foil ML can be seen, from which the distance of each section to the mesial approximal surface is calculated. The enamel and the dentine, as well as the caries lesions can clearly be distinguished.
3.1. Application to scientific studies

The above described method was used to validate the International Caries Detection and Assessment System (ICDAS-II) for detection of occlusal caries [4]. Carious lesions may occur at different sites on the occlusal surfaces of teeth, hence the use of the coordinate system ensured accurate location of each section and thus that the lesion on the section originated from the investigation site in question [6].

Dentists have a number of methods at their disposal for the clinical detection of dental caries on occlusal surfaces. Apart from purely visual and visual-tactile caries diagnosis, these include amongst others laser or light fluorescence-based methods. Our accurate histological technique was therefore used to determine the diagnostic accuracy and reproducibility of a commercially available laser fluorescence device (DIAGNOdent, KaVo, Germany) [7]. Additionally the serial sections of the investigated teeth were measured by means of the laser fluorescence device in order to determine whether there was any relationship between the fluorescence readings taken at the surface of the tooth and those taken from a histological section within the “body” of the lesion, and hence reflecting the bacterial load. It could be shown that there is a moderate relationship of laser fluorescence readings taken on the surface of a tooth to that within the lesion determined on the section, as the fluorescence measurement is inextricably linked to bacterial products, although the relationship was found to be moderate [7].

As it was stated before, histology is frequently used as a gold standard to validate caries detection methods. But poor assessment consistency could lead to apparent changes in diagnostic accuracy. In multi-center, multi-examiner studies electronic transfer of information would be convenient, provided there is no deterioration in quality. In a study using serial teeth sections it could be shown that viewing digital images of tooth sections for the assessment of caries lesion depth produces intra- and inter-examiner reproducibility values which are comparable to viewing the sections directly under a microscope. This has potential benefits for multi-centre studies since viewing digital images of sections on a computer monitor would be more convenient if sections are to be viewed by a number of people in different locations.

Fig. 2 Series of sections of a molar with fissure caries. Thickness of the specimens: ~ 200 μm.

The distance of each section to the mesial-approximal surface is coded indirectly from the length of the marker ML and H can thus be calculated.

The sections are ordered from the mesial surface (1) to the distal surface of the tooth (10).
Serial histological sections may also be used as a gold standard to obtain the diagnostic accuracy of detection methods for approximal caries diagnosis such as radiography. For this purpose the teeth would be sectioned in the mesio-distal direction (Fig. 3). This technique was used for a study which evaluated the agreement of approximal caries diagnosis obtained with different types of digital radiography systems when the radiography images were imported into a reference system [8].

3.2. Application to dental education

One important task which can be answered by means of accurate histology is the evaluation of failures in endodontic treatments. This is mainly of interest when the clinical signs (e.g. radiography) would not provide the dentist with proper information about why an endodontic treatment was not successful and thus the tooth had to be extracted. For this purpose the tooth is usually cut from the crown to the root in horizontal order. By producing serial sections dentinal cracks and even longitudinal fractures can be seen microscopically [9]. This is of interest especially in the dental education of postgraduates when consideration is given to possible causes for failure in endodontic treatments.

Dental students come into direct contact with oral hard and soft tissues in the clinical study phase. The differentiated knowledge of the normal histology of oral tissues is the basis for being able to understand and critically classify the procedures of examination, diagnosis and the following therapies to be learnt. With the procedures described dental students have the possibility in our clinic to get the pathology-histological image files of teeth and of special dental preparations in a digital form in pre-clinical microscope courses and in clinical courses (CD-ROM resp. online access for students of Philipps-University Marburg, Germany). Through the digitalization of hard tissue sections virtual microscopy can be offered on a wider scale.

A long term aim is to construct a database which is freely available to students. This has been piloted recently and examples are available on: http://zahnhistologie.de/vm/pulpabzess/ and http://www.uni-marburg.de/fb20/zahnerhaltk/lehre/Download/downloadhistopraeparate.

Besides this, students in our dental school have the possibility to produce serial sections of teeth by themselves in specially organized workshops. From the didactical point of view, this is a quite important step in improving the understanding of the students regarding to cariology and histology.

4. Discussion

Preparing histological specimens of undecalcified hard tooth substance cannot be accomplished using conventional histological cutting techniques because the dentine is too hard and the enamel so brittle. However, decalcification of specimens leads to entire loss of the enamel and with it interesting structures. Therefore in studies that investigate caries diagnosis, methods are required which enable specimens of a useful thickness and resolution to be prepared while leaving the enamel and dentine in their natural state. To reduce the risk of section damage, cutting and precision grinding of the tooth specimens needs to be done with the teeth embedded in artificial resin. These hydrophobic materials cannot adhere to the tooth nor infiltrate it in its hydrated state. In increasing concentrations of alcohol, water is replaced by alcohol in stages. Resecting the tooth’s root reduces the diffusion paths and exposure times [10]. The alcohol which had permeated the dehydrated dental hard tissue is then replaced by the monomer of the artificial resin during infiltration. Complete infiltration is an essential condition for being able to fix the samples to slides and process them for cutting after polymerization, so that they will be stabilised against stress and free of artifacts [11]. The twostage soft polymerization for ten hours in visible light stabilises the infiltrated tooth for further processing.

Including a geometrically defined depth marker made of a coloured foil next to the tooth inside the body of the specimen served as a gauge on each slide in each specimen. The length measured in the specimen was an indirect measure for calculating the height of the section above the reference plane. Thus the question of whether a specimen was accurately located within a series of sections of a block of samples never arises.

The sawing technique enabled sections with a primary thickness of approximately 100 to 200 µm to be made in series. The microscopic cracks in the enamel could be adequately minimized through precision grinding. Moving the slide with the sample in a coplanar manner above the grinding wheel with an adjustable limit switch reliably limited the
final thickness. The quality of the sample's surface could be varied by selecting various grades of abrasive paper [12-14].

The procedure involves a high experimental effort and needs many years of laboratory routine. In perspective newer or alternative procedures that do not work destructively are the objective. Computed tomography (CT), invented in 1973 [15], is a well-known medical technique for the examination of internal structures nondestructively. X-ray microtomography (XMT), developed originally in 1982 [16], is a miniaturized version of CT that has a resolution of microns as opposed to millimeters. Since XMT produces a full 3D X-ray attenuation map of the scanned object, there is no compression of 3D information into two dimensions, i.e. the resultant image is a true representation with no superimposition, as in intraoral radiographs, for example [17]. In addition, there is no physical sectioning using XMT, and thus, there is no loss of information between sections, as in the case of conventional microscopic techniques, such as optical microscopy and contact microradiography. Hence, by taking XMT slices of the whole object and stacking the slices, an accurate 3D map of the object can be produced. This method has been used to generate 3-dimensional volume data sets of the X-ray absorption of human teeth in vitro in order to facilitate the understanding of the morphology and progression of dental caries as well as the morphology of the carious tooth pulp [17, 18]. This learning method is considered to be useful for the education and training of dental students [18].

Different other methodologies have also been indicated to assess the depth of carious lesions, such as microrafocal radiography [19] and confocal laser scanning microscopy (CLSM) [20]. As for the histological examinations sectioning of teeth are required for these methods as well.

For purposes of documentation, and in the event that examiners in different places are involved in assessment, digital reproduction of histological sections is the best choice for information transfer. In recently conducted studies we used the Leica Zoomsystem Z6 APO M 420 (with QWin Standard V 3.4.0 software) for digital photography of the teeth and sections [21].

5. Conclusion

The procedures described in this report provide a step by step account of the embedding, sawing and grinding technique for undecalcified teeth. This produces slides demonstrating the histological aspects of enamel and dentine, and caries in relation to these tissues, which can serve as gold standard to assess specificity and sensitivity of different methods for caries diagnosis, as well as for aspects for quality assurance of therapeutic interventions like restorations and post extraction diagnoses. The histological technique allows accurate allocation of section to individual investigation sites on the occlusal surface. The macro photographic digital reproduction of slides allows transfer of images to facilitate cooperation of working groups as well as for teaching purposes.

References


