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Microtomographic Examination of Teeth After Application of Selected Contrasting Agents

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Abstract

The present research was aimed at imaging predentine, structure of the walls of the dentinal tubules, and distribution of collagen fibres on which the dentinal tubules are built, using microtomography.

Methodology: Teeth were first demineralised and subsequently contrasted with uranyl acetate and osmium tetroxide. In the next stage, these contrasted teeth were analysed by X-rays with the use of Nanotom S. The Fiji Is Just ImageJ and VG Studio Max programs were used to conduct numerical analysis of the data. Then the 3D model was made.

Results: The teeth serving as reference material were not subjected to contrasting agents. The images obtained via microtomography were poorly differentiated. Teeth contrasted with uranyl acetate: the spatial image of the entire tooth became very clearly visible. Teeth contrasted with osmium: the preparations differ in terms of contrast. This preparation enables the differentiation of sharper details throughout the tooth model.

Conclusions: It was possible to show vessels and odontoblast spikes in the pulp chamber. It was also possible to follow the course of the dentinal tubules and to link the structures of the walls of the tubules with collagen fibres in the 3D image, with using Nanotom S microtomograph.

Keywords: collagen, dentine, microtomography, osmium, uranyl acetate, bioprinting

Citation
Przemysław Kustra (2023), Microtomographic Examination of Teeth After Application of Selected Contrasting Agents. *Digital Medicine and Healthcare Technology* 2023(2), 1–15.

DOI
https://doi.org/10.5772/dmht.16

Published
5 June 2023

1. Introduction

Modern technologies comprise research tools which enable the enhancement of knowledge in all areas of medicine, including histology. The tissues of the tooth, including dentine, are the subject of investigation. The analyses concern, inter alia: the path of collagen fibres, the distribution of hydroxyapatite crystals with respect to these fibres, which is undoubtedly related to the mechanics of the tooth and its resistance to directional forces. The dependence of mechanical parameters on

histological structure has already been the subject of previous studies; nevertheless, the problem is relatively poorly understood. Therefore, the authors have undertaken the problem anew, while extending the methodology of research significantly [1].

In available analyses of hard mineralised dental tissues, detailed morphological structure is described on the basis of radiological examinations or examinations performed using various types of microscopes, e.g. scanning microscopes, or other similar methods based on the evaluation of surfaces in a two-dimensional image, e.g. using an atomic force microscope, electron microscope (transmission and scanning), spectroscope (using near-infrared light), secondary ion mass spectroscope, confocal microscope, metallographic microscope, or optical microscope [2–9]. On the basis of the these examinations, it is possible to assess only the surface which emerges after cutting through the sample; this means that this type of analysis does not permit a detailed reconstruction of the region being studied. In a case where an analysis of several successive layers is taken into account, the probable spatial arrangement of structures is assessed. It follows that all models of teeth are based on probability analysis. In addition, in the course of preparing models for microscopic examination, some destruction of the sample is unavoidable, which consequently may result in errors during analysis. Therefore, it is appropriate to use modern imaging devices, which ensure multidimensional continuity of measurement and additionally depict the spatial distribution of the organic structure [7, 10–15].

An exploration of the issue of decomposition of organic substances in mineralised tissues can contribute to clarifying the distribution of forces during the process of loading of structures, as well as the area of mineralisation processes [1].

2. Materials and methods

The development of a method of comparison of selected techniques for the imaging of tooth structure using a microtomograph.

The material for the study comprised healthy teeth (without e.g. pathological carious lesions on the surface of the enamel or cementum), removed for orthodontic, periodontic, prosthetic, or endodontic (excluding root canal treatment) reasons.

Following removal, the teeth were fixed in 3% glutaraldehyde buffered to pH 7.4, using a cacodylate buffer, for a period of 12 h. The fixing process was carried out at a temperature of 4 °C. The teeth fixed in this manner were then rinsed in distilled water for approximately 5 h. Subsequently the material was subjected to demineralisation in 5% nitric acid (HNO_3) for a period of 24 h. After this time, the teeth were again rinsed in distilled water until a neutral pH was obtained for the rinsing liquid.

At this stage, ten teeth were designated for use as reference material. The remaining teeth were divided into two groups. One group (10 teeth) was contrasted

in a supersaturated aqueous solution of uranyl acetate for 24 h, then rinsed in distilled water for 24 h. The other group (10 teeth) was contrasted with an aqueous solution of 1% osmium tetroxide, also for 24 h, and rinsed in distilled water for 24 h. A third group comprised teeth without contrast (10 teeth). These teeth remained in distilled water and were used in the next stage for microtomographic examinations. In the group of 10 teeth, there were incisors, premolars, molars. The number of ten was dictated by the financial scope and the test on the repeatability of the study.

A Nanotom S microtomograph (General Electric, USA) was used for the study. This device is capable of scanning samples whose dimensions do not exceed 10 × 10 × 15 cm, and which weigh less than 2 kg. The Nanotom S microtomograph functions with a maximum resolution of 0.5 μm (detail detectability to 100 nm). A high-power lamp (57 W) and a large range of available voltages (from 5 to 180 kV) enable measurement of a wide range of materials, including biological tissues (3).

The paper presents photos from 3D reconstruction, with a resolution of 6 μm. In the course of the research described in this paper, radiographic images with resolutions of approximately 2–5 μm (as well as 0.5 μm, in the case of the investigation of (microsection) were also obtained). The examination of each tooth took about 8 h. During rotation, a series of 2,400 projections was made, on the basis of which it was possible to create a computer reconstruction of the object. It should be noted that geometric measurement results in a more accurate representation of the internal structure [16].

Longer measurement time contributes to a better signal-to-noise ratio in the image, as well as higher resolution. This kind of measurement can take as long as several hours (depending on the type of microtomograph). There are also restrictions related to the size of the sample; the maximum allowable diameter is approximately 3–10 cm. For this reason, the scope of research capacity is limited to in vitro studies.

To calibrate the apparatus, paper strips were used along with the contrast agent. It was shown that the higher the power of the device, the higher the resolution of the resulting images (Figure 1a).

With the help of RVG radiovisiography (Kodak 1200, Paris, France, 2014), complete images and cross sections were made of each tooth, both before demineralisation and contrasting of teeth and after these processes, with the aim of using these images with the contrast agent in a classic radiological examination (Figure 1b).

It took more than two years to calibrate the method. This included, for example, cuts where all structures were known. This helped define digital points and create digital images with the ability to equate to cuts.

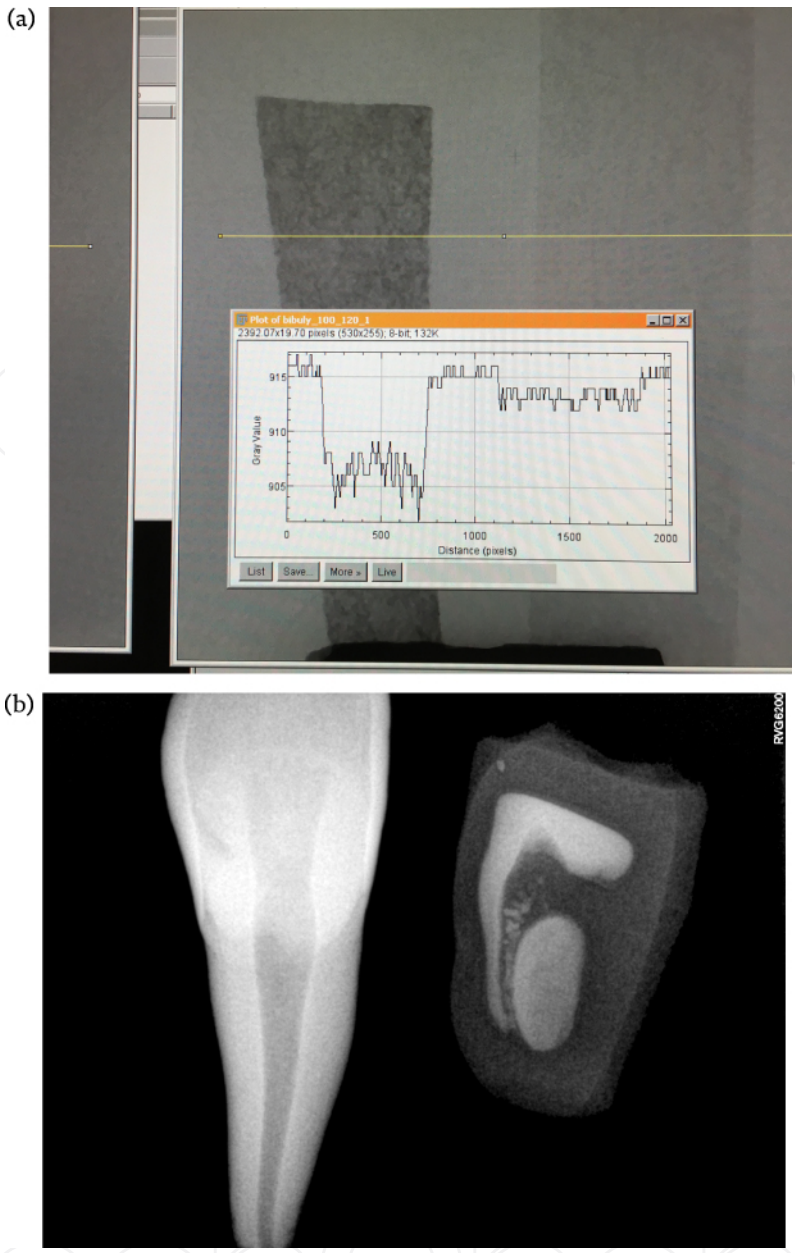

Figure 1. (a) Test of the effect of contrasting. Tissue paper with the contrasting agent e.g. uranyl acetate; image on a Kodak 2200 X-ray apparatus. (b) Radiological image created using radiovisiography. The tooth on the left is uncontrasted and mineralised; the tooth on the right is contrasted and non-mineralised.

3. Results

The teeth serving as reference material were not subjected to contrasting agents. The images obtained via microtomography were poorly differentiated, difficult to interpret, and blurred, with a greyish background and with granules poorly defined in both dentine and cement areas. Identically poor definition of granules, with

blurring of details, was evident in the walls of the pulp chamber. This problem equally concerns dentine, cement, and the contents of the pulp chamber and root canal (Figure 2). Similar difficult-to-read images can be found in other publications [11].

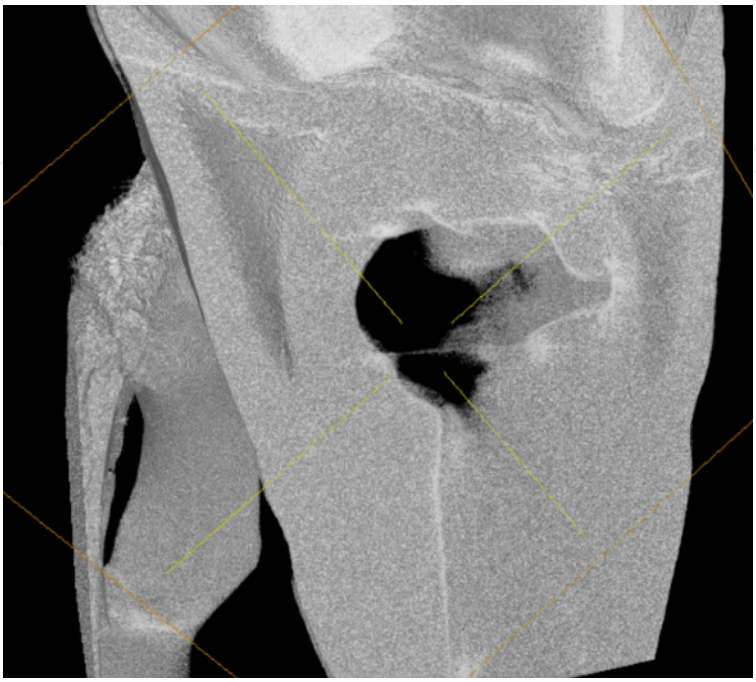

Figure 2. Tomographic image of the sample without contrasting of the tooth. Resolution 6 μm, enlargement 8.33.

3.1. Teeth contrasted with uranyl acetate

The spatial image of the entire tooth became very clearly visible (Figure 3). It is possible to discern the clearly defined borderline between the crown and the cervical margin of the tooth. The surface of the crown from the dentine side (enamel was removed in the demineralisation process) is visible, with typical depressions where bundles of enamel prisms are located.

The tomographically obtained cross sections of teeth are much clearer compared to images without this contrast agent. The wall of the lower part of the pulp chamber and its tapering lumen are very clearly visible. Dentine with numerous sharply marked granules covers the exposed surface. Round granules, similar in terms of diameter, are very numerous, and appear lighter on a darker background (Figure 4). Numerous depressions corresponding to the mouths of the dentinal tubules are visible in the wall of the pulp chamber (Figure 5). The mouths of tubules can be observed at other locations within the pulp chamber; inside them, content can be detected, which may correspond to dentinal tubules with appropriate spatial orientation (Figure 6).

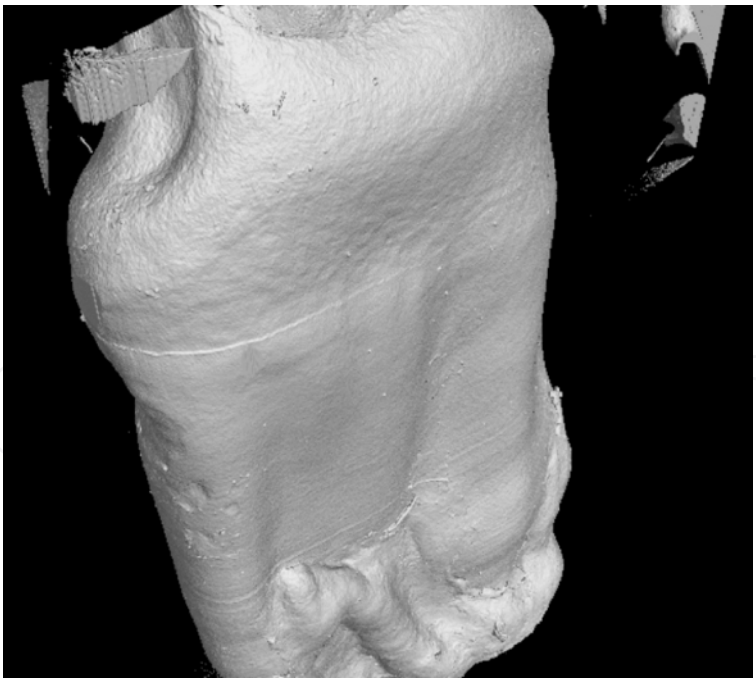

Figure 3. Spatial image of a tooth after contrasting with uranyl acetate, following earlier mineralisation. Resolution 6 μm, enlargement 8.33.

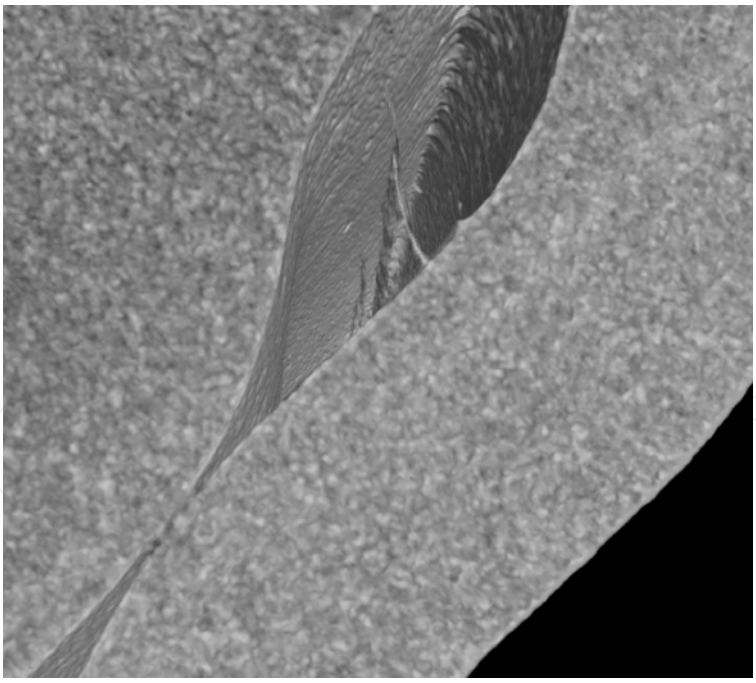

Figure 4. Image of a tooth in oblique microtomographic cross section after contrasting with uranyl acetate, with the pulp chamber clearly marked. Resolution 6 μm, enlargement 8.33.

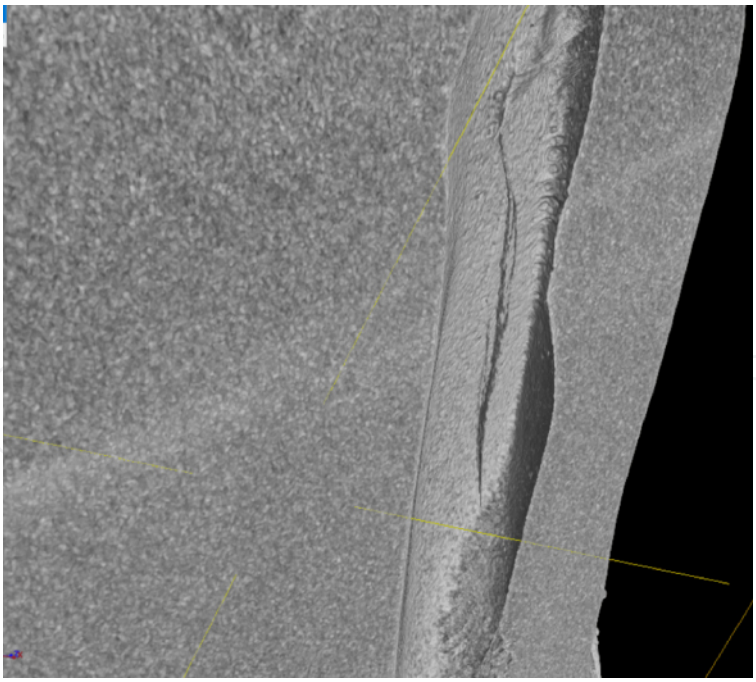

Figure 5. Image of the wall of the pulp chamber in a microtomographic cross section after contrasting with uranyl acetate. The mouths of the dentinal tubules are visible. Resolution 6 μm, enlargement 8.33.

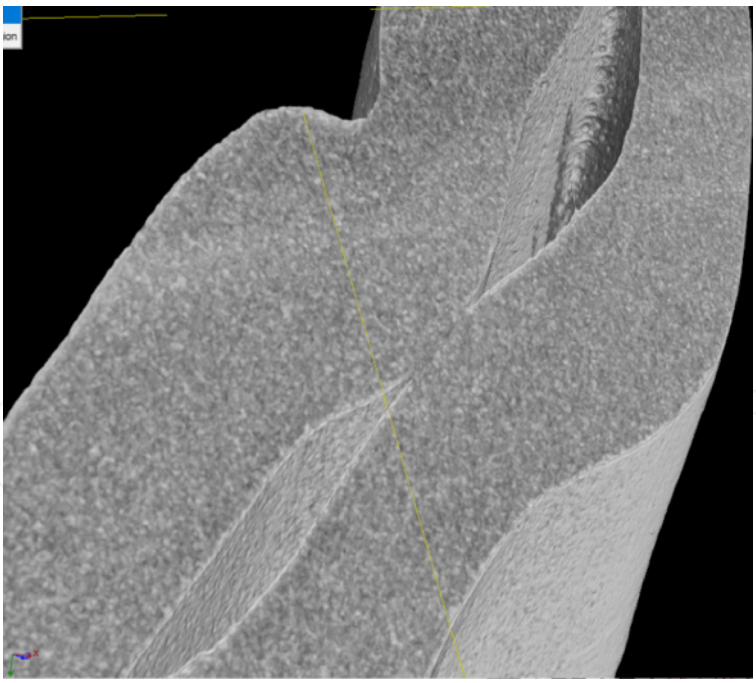

Figure 6. Image of the wall of the pulp chamber in a microtomographic cross section after contrasting with uranyl acetate. The mouths of the dentinal tubules, in which odontoblast processes are closed, are visible. Resolution 6 μm, enlargement 8.33.

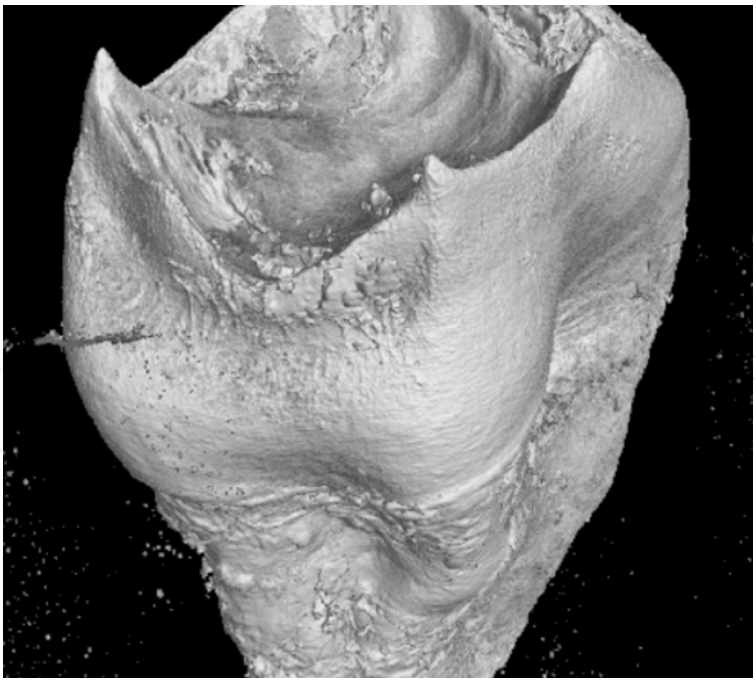

Figure 7. Spatial image of a tooth after demineralisation and contrasting with osmium tetroxide. Resolution 6 μm, enlargement 8.33.

3.2. Teeth contrasted with osmium

The preparations differ in terms of contrast. This preparation enables the differentiation of sharper details throughout the tooth model (Figure 7).

The tooth is very clearly outlined, with a recessed enamel seat and a line delineating the enamel-cement border. The surface of the cement is also clearly visible from the periodontal side.

More details are supplied by images obtained from tomographic longitudinal and transverse sections of teeth. In the longitudinal cross section of the tooth, the chamber is visible in the middle part, whereas in the root part, two tubules can be seen. Visible in the pulp chamber of the tooth are blood vessels which branch depending on their path in relation to the tooth's cross-sectional plane; cross sections of the vessel can be observed along with its inner contents.

Around the area of the pulp chamber is a more strongly contrasted zone corresponding to predentine, from which parallel dentinal tubules emerge (Figure 8).

Figure 9 presents an oblique cross section of the lower part of the pulp chamber of the examined tooth. Vascular sections, running longitudinally, are visible in the location marked with arrows, with the interior wall exposed. The wall of the chamber from its interior is markedly plicated, with small folds which serve as the exit sites of tubules in the depth of the dentine. In the interior of the root canal are

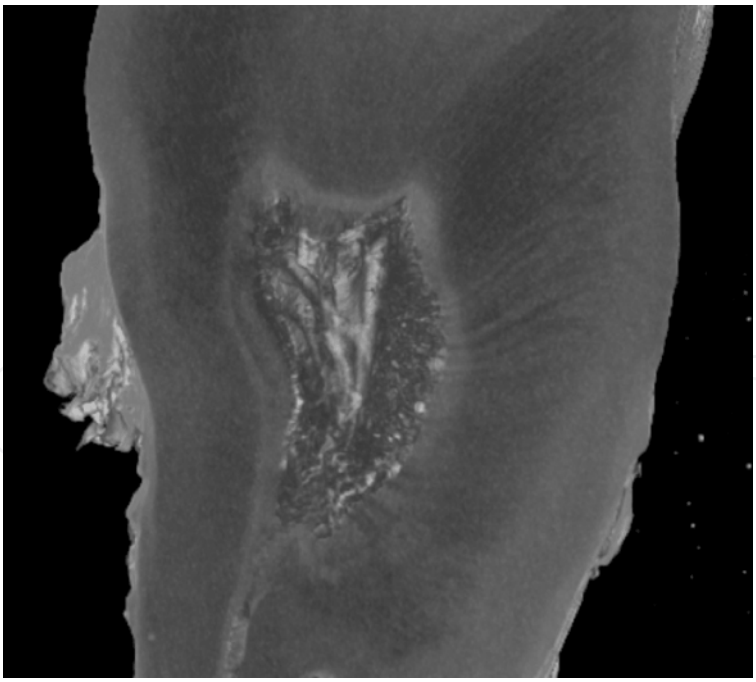

Figure 8. Image of a tooth in longitudinal microtomographic cross section with marked contrast after contrasting with osmium tetroxide. Branching blood vessels are visible in the interior of the middle part of the pulp chamber. Resolution 6 μm, enlargement 8.33.

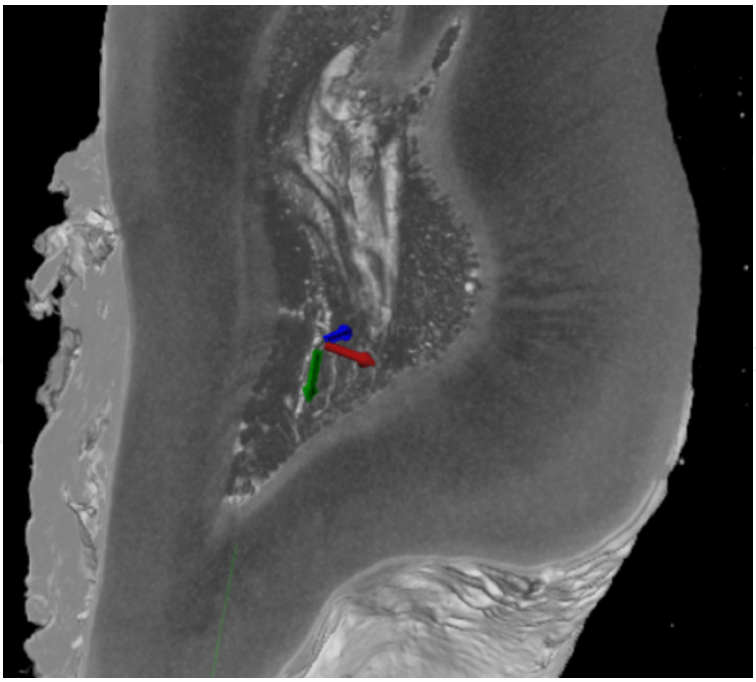

Figure 9. Image of a tooth in oblique microtomographic cross section after contrasting with osmium tetraoxide. Blood vessels are visible in the pulp chamber. Arrows mark the clearly visible lumen of blood vessels. Resolution 6 μm, enlargement 8.33.

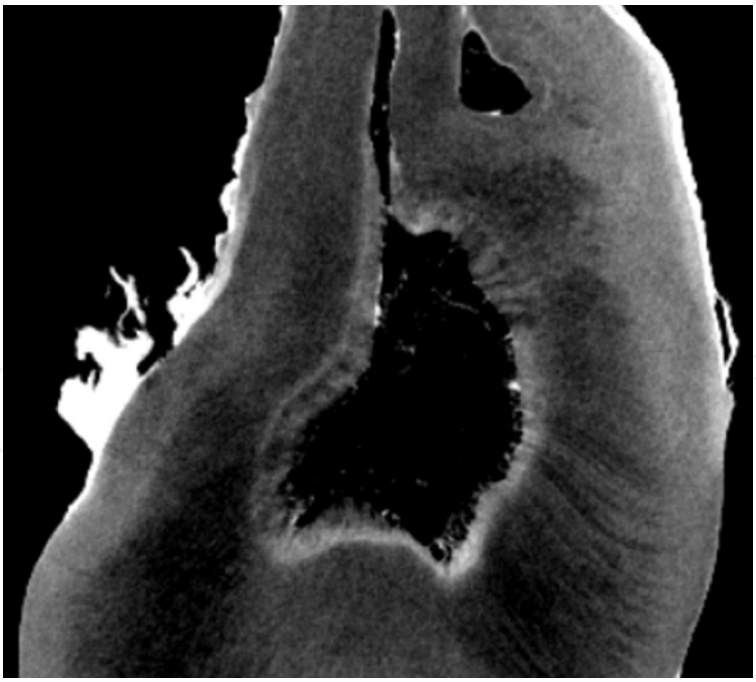

Figure 10. Image of a tooth in a sagittal microtomographic cross section. Good image contrast following contrasting with osmium tetroxide. Clearly visible pulp chamber and tubules in the root section. Dentinal tubules are visible running from the pulp chamber to the periphery. Resolution 6 μm, enlargement 8.33.

numerous small plications in various cross sections in relation to the plane of the tomographic section of the tooth. Also visible in this cross section is the border between dentine and cement, which is characterised by a more external location.

The contrasted mouths of the dentinal tubules are clearly visible in the dentine in the area around the pulp chamber. Also, in a more distal section towards the periphery of the tooth, the paths of the dentinal tubules, running parallel in relation to each other, are visible. In this image, the tubules are directed towards the perimeter from the crown to the enamel-dentine line. Along this longitudinal path, the lumen can be seen in the tubules. Clearly contrasted granules can be observed in the walls of the tubules in accordance with the direction of their path; these granules are arranged in orderly fashion, perpendicular to the long axis of the dentinal tubule. Taking into account the affinity of osmium to collagen fibres, it can be assumed that fibre bundles surround the lumen of the dentinal tubule.

On the right-hand side, in the crown, tubules are visible, running in a similar manner as well as in a differently-oriented path in regular and oblique cross sections (Figure 10). Another cross section (Figure 11), made at higher magnification in the pulp chamber, shows numerous thick odontoblasts located opposite the mouths of the dentinal tubules.

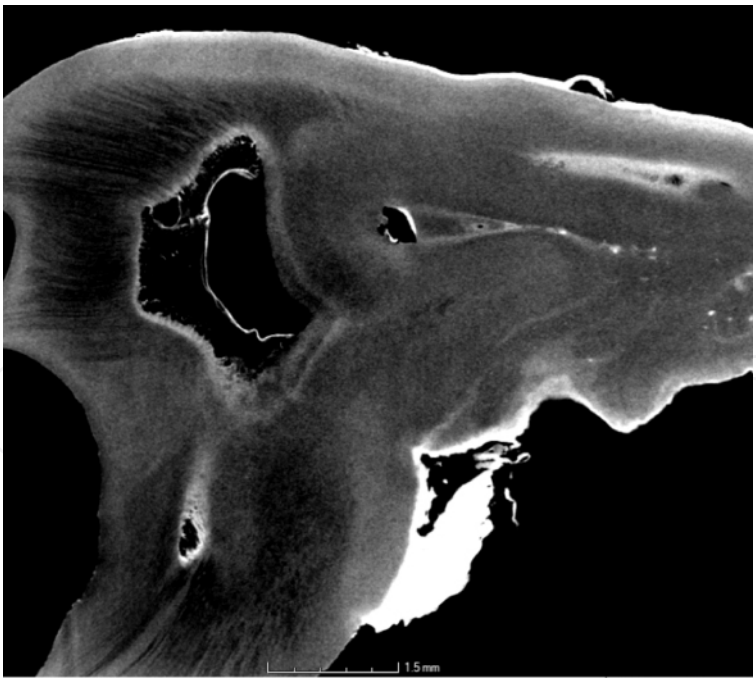

Figure 11. Oblique microtomographic cross section of a tooth. High-contrast image following contrasting with osmium tetraoxide. Visible dentinal tubules in the longitudinal section with granular wall structure and odontoblast processes visible in the pulp chamber opposite the mouths of the dentinal tubules. Resolution 6 μm, enlargement 8.33.

In the dentine itself, it is possible to observe the paths of the tubules running parallel to one another in the direction of the enamel-dentine junction. In another location, on the left side of the pulp chamber, tubules are visible in regular and oblique (sagittal) cross sections.

Three dimensional model (shape) of organic structure of the tooth was reproduced based on multiple registered projections using reconstruction algorithms. The spatial models of the part of organic dentine has probably shown the process of different activity of the fibres of the odontoblasts in the production of collagen fibres.

4. Discussion

Microtomographic examinations are used to analyse the structure and properties of materials such as bones and tissue scaffolds, as well as in many fields unrelated to medicine [3, 17]. The results presented in this article are the first to be obtained by means of a microtomographic examination of teeth using the technique of demineralisation and contrasting of the sample. In the course of examining each sample, as many as 2,400 radiological images were created. It is worth noting that heretofore it was possible to analyse the structure of a tooth only by means of

microstudies carried out with the use of X-rays. These studies focused exclusively on mineralised tissues [7, 16, 18, 19]. However, currently special contrasting agents can be used and samples can be appropriately prepared for the imaging of organic elements [8, 13]. The reagents used in the transmission electron microscopy technique are uranyl acetate and osmium tetraoxide [20].

Special contrasting enabled the study of structures (the maximum capacity of the device is 100 nm) without (and this is particularly important) compromising the continuity of the sample. At present, a spatial model of the structure of a fish bone [1] has been created using this technique. In the case of the investigation of dentine, the use of a microtomograph may be useful for evaluating organic structures. This method enables a more profound analysis of histological structure [8, 21].

When studying teeth, there can be no loss of water from the samples, as this leads to distortion of the resulting images. Such samples were discarded from the study. Correct images obtained during the study were changed to zero-one models (01), thanks to which the structures subjected to analysis were assessed not only by means of the visible image, but also with the help of the software program. The Fiji Is Just ImageJ program (Wayne Rasband of the National Institutes of Health, USA) and VG Studio Max (Volume Graphics, Heidelberg, Germany) were used to view the acquired images and to conduct numerical analysis of the data (that is, each point of the radiological image of the tooth was, according to the obtained resolution, converted to a digital point, with all data, e.g. density; this gave a lot of data due to the large number of points). In this respect, the finite element method is helpful [9, 13]. Although it has been used for many years, new applications in research methodology are still being found for this method, because it can be used in combination with data from more and more modern imaging devices, such as microtomographs [22]. It is worth noting that, along with the improvement of research devices, the results of calculations are becoming more accurate. The use of the methods and devices described above in dentistry may contribute to an improvement in the quality of treatment for patients, as they enable a better understanding of tooth mechanics, potentially resulting in the modification and refinement of materials to fill tissue defects [7, 8, 19, 22, 23].

Part of the dentine adjacent to enamel, approximately 20 μm wide, is built of mineralised collagen fibres, oriented nearly perpendicular to the dentine-enamel junction [21]. The presence of a soft dentine zone validates the research with scanning electron microscope in which a hypothesis concerning the structural adaptation of dentine to the transfer and minimisation of mechanical and thermal stress was put forward [3, 17]. This area is darker and less contrasted compared to the inner part of the dentine. This zone was visible in all ten teeth examined using microtomograph [4–6, 16].

According to previous studies, the number of dentinal tubules increases significantly in the soft zone of the dentine, while the volume of inter-tubular dentine decreases. Based on the obtained images, it can be concluded that this is not a uniform process. Thus it is worth considering the potential mechanical functions of this relatively soft dentine zone in conjunction with the enamel-dentine junction and the presence of alternating zones with variable levels of mineralisation [3–6, 16, 17].

A comparison of the images obtained from non-contrasted teeth and those contrasted with uranyl acetate and with osmium tetraoxide shows that contrasting using the latter agent enhances the quality of images, enabling the acquisition of a much greater number of details of tooth structure and making it possible to study the arrangement of the vessels in their multi-dimensional paths within the space of the pulp chamber [24].

In relevant cross sections, particularly those contrasted with osmium, which best enables the acquisition of good, readable images, it is possible to examine the inside wall of the pulp chamber, showing the pre-dentine zone, and to study the distribution and analyse the longitudinal, oblique, and transverse paths of the tubules. In addition, the study will help to determine the future directions of bioprint technology: bioprint techniques and the quality of biomaterials [24].

Finally, it should be stated that the contrasting method is recommended in the tomographic examination of teeth.

5. *Conclusions*

On the basis of the research, the following conclusions can be drawn:

- The new model of the Nanotom S microtomograph, with a resolution below 1 μm, enables significant improvement in the quality of the spatial model.
- Thanks to the applied method of contrasting the organic component of the tooth with osmium tetraoxide and uranyl acetate, it was possible to show vessels and odontoblast spikes in the pulp chamber. It was also possible to follow the course of the dentinal tubules and to link the structures of the walls of the tubules with collagen fibres in the 3D image.
- Visible morphological structures can be imaged and presented spatially, not just in two dimensions.
- Images obtained in the study may facilitate the interpretation of the results of other studies, such as tests of strength, stiffness (gives the opportunity of understanding, e.g. stiffness), and elasticity of hard tooth tissues, which can contribute positively to a new perspective when planning clinical trials, in vitro.

- The results of the obtained research may significantly contribute to the development of bioprint technology, as they show new detailed data on the structure of soft tissues in the pulp chamber of the tooth.

Conflict of interest

The author declares no conflict of interest.

Acknowledgments

We would like to thank all of the engineers servicing "Nanotom", who participated. I also wish to thank Prof. Dr. Hab. med. Roman Pawlicki.

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