

Computerized Reconstruction of Pulpal Blood Vessels Examined under Confocal Microscope

SUMMARY

The purpose of this study was the evaluation of 3 different histological methods for studying pulpal blood vessels in combination with 2 types of confocal microscope and computer assisted 3-dimensional reconstruction. 10 human, healthy, free of restorations or caries teeth that were extracted for orthodontic reasons were used. From these teeth, the pulp tissues of 5 were removed, fixed in formalin solution, dehydrated and embedded in paraffin. Serial cross sections 5µm thick were taken from 3 of the above mentioned pulpal tissues and stained with CD34 according to the immunohistochemical ABC technique, while the rest 2 were stained with CD34 and Cy5 by means of immunofluorescence after serial cross sectioning of 10µm. 5 of the 10 teeth were fixed, decalcified, serial cross sectioned (30µm thickness) and stained with eosin. The physical sections were examined under 2 types of confocal laser microscope. Serial images were taken for each section, alignment of the images was followed and finally 3-dimensional reconstructions of the pulpal vessels were achieved.

The combined use of immunofluorescence, confocal microscope and automatic segmentation proved to be a useful method for the detailed study of pulpal vasculature. The above method provides deep knowledge of the form and spatial relationship even of the smallest pulpal blood vessels with neighbouring structures like odontoblasts, which are essential for the fully understanding of their role and function within the dental pulp.

Key words: CD34; Cy5; Immunofluorescence; 3D reconstruction

**Anna Digka¹, Kleoniki Lyrroudia²,
Lucie Kubinova³, Georgia Karayannopoulou⁴,
Ioannis Marras⁵, Ioannis Pitas⁵**

¹Private Dentist, Thessaloniki, Greece

²Aristotle University of Thessaloniki
Department of Endodontology, Dental School
Thessaloniki, Greece

³Academy of Sciences of the Czech Republic
Institute of Physiology

Department of Biomathematics
Prague, Czech Republic

⁴Aristotle University of Thessaloniki
Medical School, Thessaloniki, Greece

⁵Aristotle University of Thessaloniki
Department of Informatics, Thessaloniki, Greece

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Introduction

Dental pulp is the only soft dental tissue, surrounded by dentin, and it consists of odontoblasts, fibroblasts, antigen-presenting cells, stem cells, connective tissue fibres, and a broad vascular and nervous plexus^{1,2}. The inner layer of the entire vascular system consists of endothelial cells (ECs). CD34 is a cell surface protein that is expressed by hematopoietic cells (HCs) and ECs. Antibodies against CD34 are extensively used as a pan-endothelial marker in immunohistochemistry^{3,4}. Cy5-conjugated anti-CD34 has been used in immunofluorescent confocal microscopy⁵. Eosin, on the other hand, is a fluorescent red dye resulting from the

action of bromine on fluorescein. It can be used to stain cytoplasm, collagen and muscle fibres for examination under microscope.

A variety of methods have been used until today in order to study the structure of the vascular plexus of the dental pulp. One of the above mentioned methods was that of resin casts in order to follow the dental pulp vessels' distribution⁶, while later on, 3-dimensional reconstruction by using serial cross sectioning and light or transmission electron microscopy were used^{7,8}.

The purpose of this study was the evaluation of 3 different histological methods for studying pulpal blood vessels in combination with 2 types of confocal microscope and computer assisted 3-dimensional reconstruction.

Material and Methods

Tissue Specimens

The research was approved by the Ethical Committee of the Aristotle University of Thessaloniki, Greece. 10 upper premolars from young patients (range 16-18 years), extracted for orthodontic reasons, were used. The teeth were free of caries and without restorations.

5 teeth were fixed immediately after extraction in a 10% formalin solution buffered at a pH of 7.2 for 48 hours. A longitudinal groove was prepared along the external surface of each tooth thereafter and a mechanical fracture was achieved in order to have an exposure of the dental pulp tissue. The exposed pulp tissue was gently removed and was put once again into a fresh formalin solution of the same concentration for 48h. The specimens were finally embedded in paraffin blocks and serial cross sections 5µm thick were taken from each specimen that was used for immunohistochemistry, while 10µm thick serial cross sections were taken for the immunofluorescence procedure. Immunohistochemistry for CD-34 was applied on the sections of 3 specimens. Immunofluorescence for CD-34 and Cy-5 was applied on 2 remaining specimens.

5 teeth were decalcified after the fixation period. Serial cross sections 30µm thick were taken from the above specimens and eosin stained.

All serial cross sections were examined under confocal microscope.

Antibodies-Immunohistochemistry

The avidin:biotinylated enzyme complex (ABC) technique was used in the cases selected to be treated following the immunohistochemical method. The sections were deparaffinised, endogenous peroxidase was inhibited by using 1.6% hydrogen peroxide in methanol, washing with distilled water was done and heating in a microwave oven followed in a citrate buffer 10mM pH 6 for 21min.

Rinsing with Tris-buffered saline (TBS) (DAKO) was done thereafter and incubation with rabbit serum (DAKO) in TBS 1/5 for 20min was performed. After that, an incubation with mouse monoclonal primary antibody CD34 (Novocastra) in 1/20 dilution was performed overnight. The sections were rinsed with phosphate-buffered saline and incubation was followed with Biotinylated Rabbit Anti-Mouse immunoglobulins (DAKO) in 1/200 dilution for 30min. Washing with TBS and incubation with StrepABC complex HRP (DAKO) at 1/100 dilution for 30min followed. Colour was developed with chromogen 3, 3-diaminobenzidine (DAKO) for 5 min, while washing with TBS and nuclear counterstaining with Harris haematoxylin for 45 sec was achieved. Normal mouse serum replaced the primary antibody in negative control sections.

Antibodies-Immunofluorescence

The specimens selected for immunofluorescence, were serial cross sectioned (10µm thick). The sections were deparaffinised and treated with 2.73% hydrogen peroxide and 0.1% sodium azide diluted with distilled water. After trypsin digestion the sections were first incubated with CD34 monoclonal antibody diluted at 1/20 with TBS containing 55% foetal calf serums for 12h at 4°C and then with Cy TM5-conjugated secondary antibody diluted at 1/100 with phosphate-buffered saline for 12h at 4°C. The slides were mounted in Mowiol.

Decalcification and Eosin Staining

After fixation with a 10% formalin solution, the teeth were decalcified by using a 5% solution of trichloroacetic acid. Decalcification was controlled roentgenographically. After decalcification teeth were transferred into 10% formalin with 0.5% of eosin for 3 days, dehydrated and embedded in glycol-methacrylate. Thereafter, 30µ thick serial sections were taken.

Laser Scanning Confocal Microscopy

- Observation of the sections 5µm thick stained with CD34 was done by using Confocal Laser Scanning Microscope (CLSM) Bio-Rad MRC 600 (using excitation wavelength of 488nm), equipped with an oil immersion Nikon CFN Plan Fluor objective (40x, N. A. = 1.3). From each physical section, 11-14 images were taken at various overlapping locations of each optical section. 6 serial optical sections, 1µm apart, were taken from each paraffin section.
- Observation of the sections 30µm thick, stained with eosin, was also done by using CLSM Bio-Rad MRC 600 equipped with an oil immersion Nikon CFN Plan Fluor objective (40x, N. A. = 1.3). From each physical section, 15-30 images were taken at various overlapping locations of each optical section. 6 serial optical sections, 5µm apart, were taken from each glycol-methacrylate section.
- The sections for immunofluorescence were observed in a Leica TCS SP2 AOBS CLSM using 20x oil immersion objective (HC PL APO CS, NA=0.70). For Cy-5 detection, excitation with helium-neon laser at a wavelength of 633 nm and emission at 649-732 nm was applied in one channel (red), while in the second channel (green), the autofluorescence images were acquired at the excitation wavelength of 514 nm and detection range 572-709 nm.

3-Dimensional Reconstruction and Visualization

2 CLSMs were used, namely Bio-Rad MRC 600 and Leica TCS SP2 AOBS. CD34 and eosin staining was used for imaging the cross-sections. As no single microscopy image could cover the entire section, a number of overlapping images were taken from each serial optical section. Consequently, digital image stitching was

performed using the GlueMRC software to obtain one image per physical section⁹. 3-dimensional reconstruction and visualization of the vascular plexus were achieved using either surface or volume rendering.

- a. For surface rendering, vessel segmentation was achieved automatically using colour and texture information^{10,11}, or using active contour approaches¹². Semi-automatic segmentation has also been used in case that better segmentation accuracy was needed. The alignment of serial sections was performed automatically through image registration¹³ with manual fine tuning. Then the segmented serial sections were interpolated¹⁴ and the vessel surfaces were triangulated. Finally, 3D visualization of the obtained vessel surfaces was achieved by using surface rendering¹⁵.
- b. For surface rendering fully automatic image registration^{16,17} and alignment algorithms were used to register the serially acquired optical slices with respect to a reference slice^{18,13}. Such algorithms rely on a global energy function¹⁹ with variables the rigid transformation parameters (2D translation and rotation) of a physical slice with regard to its neighbourhood. A volumetric rendering algorithm, using opacity, shading, depth and light effects, was then applied²⁰ for better visualization and morphological analysis of the 3D reconstructed data. Image warping techniques can be used to cover eventual spurious holes in the tissue²¹.

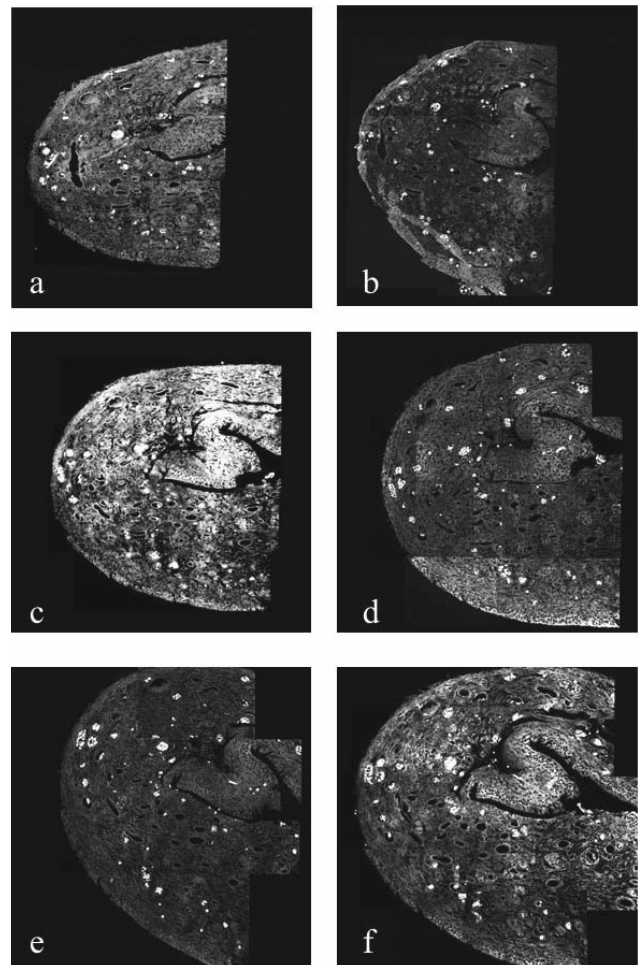


Figure 1. Serial paraffin cross sections (a-f), 5µm thick, stained for CD34 with immunohistochemical ABC technique and studied by confocal scanning laser microscope BioRad MRC 600

Results

- a. By using immunohistochemistry (CD34) and confocal microscope BioRad MRC 600, the structure of the dental pulp and its pulpal vessels was shown (Fig.1). 3-dimensional reconstruction of the above case is observed in figure 2. The course and spreading of the pulpal vessels, as well as the presence of vessels of different sizes are shown in the above 3-dimensional reconstruction.
- b. By using eosin staining of decalcified teeth and BioRad MRC 600, the appearance of the pulpal vessels' plexus is clearly seen (Fig. 3). 3-dimensional reconstruction of the pulpal vessels' plexus is shown in figure 4. The distributions of the vessels within pulpal tissue as well as the presence of larger and smaller vessels and their spatial relationship inside dental pulp are shown in figure 4. Spreading of vessels' plexus covers the entire pulpal space, while in the wider root canal the vascular plexus appears to be denser and the presence of larger vessels is more intense than in the narrower root canal.

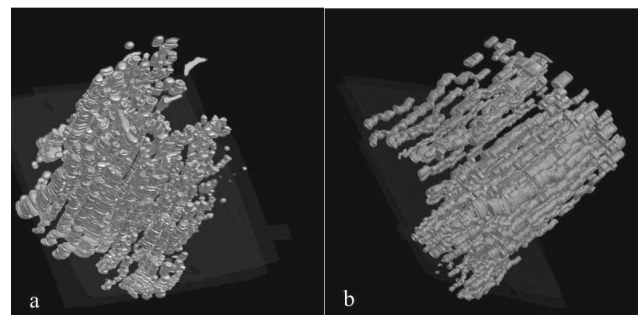


Figure 2. 3-dimensional volume reconstructions of 6 serial paraffin cross sections, 5µm thick, stained for CD34 with immunohistochemical ABC technique and imaged using the confocal scanning laser microscope BioRad MRC 600. The course and spatial arrangement of the dental pulp vessels are shown

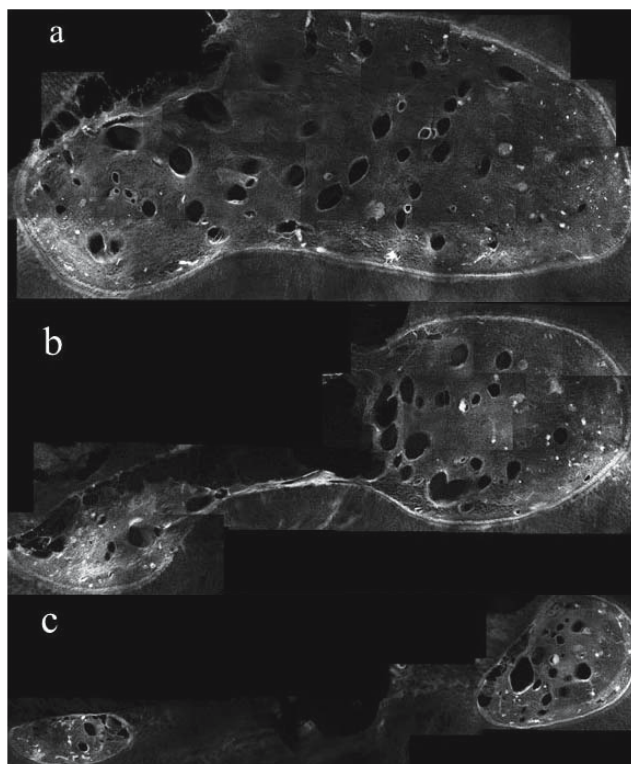


Figure 3. 3 histological sections of decalcified and glycol-methacrylate embedded specimen, 30µm thick, stained with eosin and studied by confocal scanning laser microscope BioRad MRC 600, from the coronal (a), middle (b) and apical (c) part of the pulp

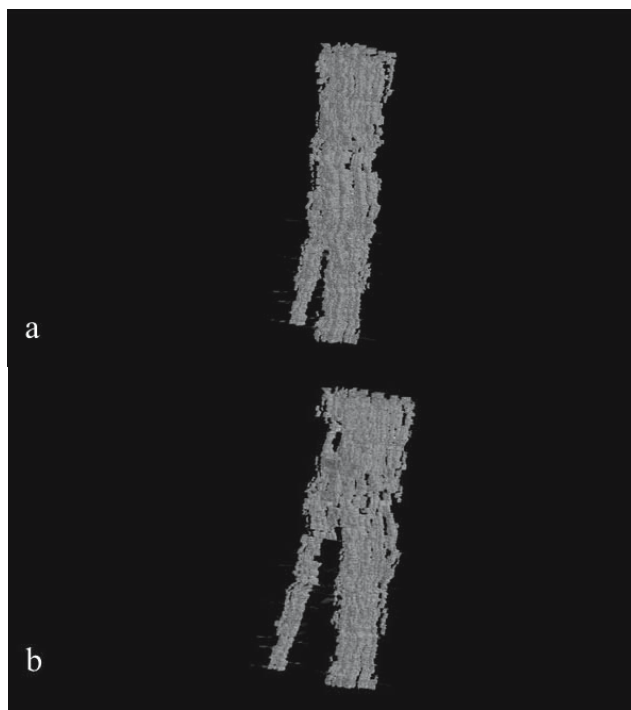


Figure 4. 3-dimensional volume reconstructions of serial cross sections of a decalcified and glycol-methacrylate embedded specimen, 30µm thick, stained with eosin and imaged using the confocal scanning laser microscope BioRad MRC 600. The course and spatial arrangement of the dental pulp vessels in both root canals are shown

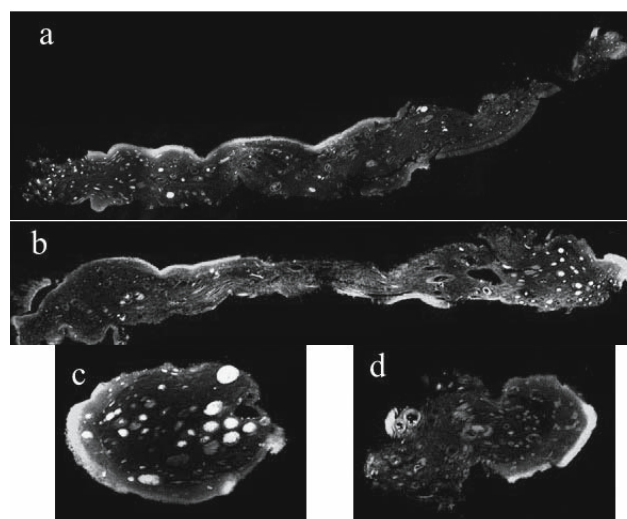


Figure 5. 3 histological paraffin sections, 10µm thick, stained for CD34-Cy5 by immunofluorescence and studied by Leica TCS SP2 AOBS confocal laser scanning microscope, from the coronal (a), middle (b) and apical (c and d) part of the pulp, x40. Dental pulp vessels' wall is shown intense red, the odontoblasts green and the erythrocytes intense yellow

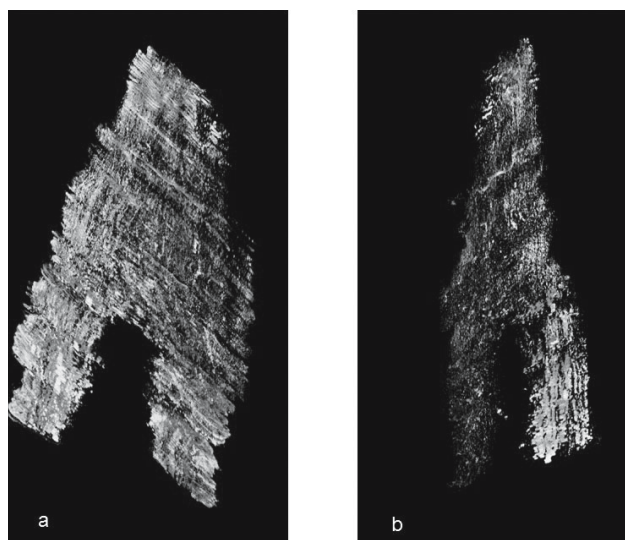


Figure 6. 3-dimensional volume reconstructions of 300 serial cross sections stained for CD34-Cy5 by immunofluorescence and imaged using the Leica TCS SP2 AOBS confocal laser scanning microscope. a: In this three-dimensional volume reconstruction, the course of the vessels in the dental pulp is shown (orange-red color) and their spatial arrangement with the odontoblasts (green colour); b: 3-dimensional volume reconstruction of the same specimen after the appliance of filters software for opacity. In this reconstruction only the course of the dental pulp vessels is shown

- c. The most impressive and detailed image regarding the pulpal vessels' plexus is seen by using the immunofluorescence (CD34-Cy5) method (Fig. 5). A 3-dimensional reconstruction of the whole pulpal vessels' plexus is shown on figure 6. A number of 300 physical serial cross sections were taken from the above pulp tissue that was examined under Leica

TCS SP2 AOBS CLSM. In the above 3-dimensional reconstruction, the presence of the vessels appears extremely intense. Inside the root canals, the presence and apical-coronal course of larger vessels is obvious, while in the coronal part of the pulp the huge number of smaller vessels is rather impressive (Fig. 6b). Furthermore, not only the course, distribution of pulpal blood vessels and their spatial relationship inside the pulp are visualized (Fig. 6a), but their relationship with the other pulpal cells, like odontoblasts, are shown as well (vessels are shown red, the odontoblasts green and the erythrocytes inside the vessels yellow).

Discussion

In our previous study⁵, combining immunohistochemical and immunofluorescence application for the detection of dental pulp vessels, immunofluorescence (CD34-Cy5) proved to be superior to immunohistochemical ABC technique. Recently a combination of immunohistochemistry for both pulpal blood vessels and nerves has been described (CD34 and S100) applied in ground tooth sections²².

The corrosion cast method, used in the past⁶, enables observation of several characteristic types of vasculature. However, it concomitantly has limitations in the study of biological functions, as no vascular cells' wall remains on the resin casts. The great advantage of our method in comparison with that of resin casts is that the dental pulp tissue retains its integrity and the connective tissue surrounding the dental pulp vessels remains intact. Furthermore, it is of great value that serial cross sections can be stored and examined under both conventional light and confocal microscope and the digital images can also be stored and re-examined in the near future. The reconstructions of vessels are based only on image processing of the pulpal sections and not on dissolving the surrounding connective tissue.

The method of serial cross sectioning and computerized 3D reconstruction has been used by other research groups²³⁻²⁹ for examination of different tissues. The above method has been employed as well, in order to study the distribution of pulpal blood vessels and nerves by using a limited number of serial dental pulp paraffin sections^{7,8}.

The quality and success of 3D reconstruction depends greatly on the number and thickness of the serial sections that are used, the histological method that is followed and the method of the reconstruction that is applied. Thus, in this study confocal microscope in combination with eosin staining, immunohistochemical application of CD34 and immunofluorescence CD34-Cy5 was used.

Confocal microscope is a powerful tool for visualizing fluorescent specimens. The principal advantage of confocal microscopy over conventional wide-field microscopy is that it reveals in more detail the 3-dimensional structure of the specimen under examination. Fluorescent specimens viewed with a conventional wide-field fluorescent microscope appear blurry and lack contrast because fluorophores throughout the entire depth of the specimen are illuminated, and fluorescence signals are collected not only from the plane of focus but also from the areas above and below. A confocal microscope selectively collects light from a thin ($<1\mu\text{m}$) optical section at the plane of focus in the specimen. Structures within the focal plane appear more sharply defined than with a conventional microscope because there is essentially no flare of light from out-of-focus areas. A 3-dimensional view of the specimen can be reconstructed from a series of optical sections at different depths. The capability for optical sectioning makes confocal microscopy well suited for studying the structure and function of cells using immunofluorescence reagents, organic dyes, fluorescent-fusion proteins and fluorescence in situ hybridization³⁰.

The use of confocal microscope proved to be more useful than the light microscope immunofluorescence because all the details of the pulpal vascular plexus in the space can be observed. Confocal microscope provides the capability to use thicker serial sections (in the cases of eosin staining $30\mu\text{m}$), a fact that results to a less number of serial sections for examination and also enables us to examine a larger amount of histological sections and longer parts of dental pulp in less time.

In this study, due to confocal microscope, the decalcified and eosin stained specimens gave us the ability to use thicker ($30\mu\text{m}$) sections. This procedure provides better visualization of the pulpal vessels' course and distribution in the space although it appears the great disadvantage of the lack of specificity concerning vessels' detection, since eosin isn't an endothelial marker.

Immunohistochemistry by using CD34 on paraffin sections that were much thinner ($5\mu\text{m}$) provide the specific vessels' detection, since CD34 is considered to be a pan- endothelial marker. 3D reconstructions from the above sections also reveal the vessels' course and distribution within the entire volume of pulp tissue.

Both the above methods have the disadvantage that are very time-consuming, since in every section the contours of each vessel should be manually delineated for segmentation, procedure that restricts the number of dental pulp tissue specimens that can be examined and reconstructed.

Immunofluorescence for CD34-Cy5 specimens in combination with confocal microscopy and volumetric rendering provided the best 3D reconstruction result in our work. This method combines tissue specificity and rather thick paraffin sections ($10\mu\text{m}$) and requires no manual

segmentation of the vessels. Therefore, it is time efficient and also retains in the 3D reconstructions the histological structure of the surrounding tissues. Thus, in the above 3D reconstructions the vessels' spatial arrangement with the neighbouring structures can be shown.

Single sections cannot give information concerning the spatial arrangement of a structure or an arrangement of several structures and their mutual relationships. Knowledge of the form of an organ and the perception of its spatial arrangement with the neighbouring structures is essential for the fully understanding of its form and function. The volume reconstructions that we have achieved can be visualized in 3 dimensions, rotated in a proper perspective angle for a better convenience to obtain an accurate and fully perceptive 3D representation of the volume of interest.

The above possibility gives our method great value since it allows a fully comprehension of the course and spreading of detailed pulpal blood vessels' plexus in the space and at the same time enables us to study histological sections from which the volumes have been created.

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Correspondence and request for offprints to:

Anna Digka
36 Ermou Str.
54623, Thessaloniki
Greece
E-mail: annadiga@hotmail.com