Online Supplementary Material

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Immunostaining of Pulpal Nerve Fibre Bundle/Arteriole Associations in Ground Serial Sections of Whole Human Teeth Embedded in Technovit®9100

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Materials and Methods

Specimens and fixation

Four healthy retained third molars from three male individuals (Cross-sections: *specimen 1*: 14 years, *specimen 2*: 16 years, *specimen 3*: 15 years, longitudinal sections: *specimen 4*: same individual as specimen 2) were removed by osteotomy and immediately cooled down to 4° C. The patient´s consent was obtained. Two to three hours after removal the teeth were sliced into horizontal sections of about 2.7 mm thickness with a D64-coated diamond band saw (Exakt, Hamburg, Germany) at lowest speed while rinsed with physiological saline. The sections were fixed in 3.7% formol/tap water for 1 to 3 days, flushed with tap water overnight and then dehydrated in increasing concentrations of isopropanol finally reaching a 70% solution, which was used for storage.

Specimen preinfiltration and infiltration

Dehydration was completed by taking the specimens for 1h through 80% isopropanol, then through two changes of 90% for 1h and through three changes of 100% isopropanol for 1h each, followed by application of xylene first for 1h and then overnight.

For embedding, the Technovit® 9100 kit provided by Heraeus Kulzer, Wehrheim, Germany, was used. It was, however, impossible to obtain artefact-free embedding results for rat spleen and liver or human tooth specimens without two decisive modifications of the working procedure described by the manufacturer. These were vacuum infiltration (or facilitating infiltration by evaporation of acetone) and polymerisation in a newly designed special pressurization device (Fig. 1A,B).

A certain portion of the base solution provided with the Technovit® 9100 kit was destabilized by applying a maximum of 3 to 4 l of the solution to a chromatography column filled with 50g basic Al_2O_3 (Roth, Karlsruhe, Germany, No. X908.1). The effluent was then stored in brown glass bottles at -20°C.

The preinfiltration steps were performed according to the manufacturer's instructions applying first a 1:2 mixture of xylene and stabilized Technovit base solution, then stabilized base solution containing dibenzoylperoxide (1g per 200ml solution) and finally destabilized base solution with dibenzoylperoxide. All preinfiltration steps were performed for 1h; the first two steps were carried out at room temperature (RT) and the third step at 4°C. These steps could be omitted if acetone evaporation (see below) was used to facilitate infiltration.

The most important procedure of Technovit® 9100 embedding is the infiltration step, which is not adequately described in the manual supplied with the kit. The infiltration solution consists of base solution, dibenzoylperoxide and polymethylmethacrylate (PMMA) powder (for example: 250 ml base solution, 1g dibenzoylperoxide, 20g

PMMA powder). The PMMA powder is added to the base solution, but does not easily dissolve and thus the solution needs to be stirred for several (4-6) hours. For practical purposes 150 ml of destabilized base solution were mixed with 20g of PMMA powder and stirred at RT until a perfectly clear solution was obtained. This solution was stored at 4°C overnight. Benzoylperoxide was added the next day followed by additional destabilized base solution yielding a total of 250 ml. The infiltration solution was then aliquoted and stored at -20°C. As the solution is of high viscosity, enhancing infiltration is absolutely mandatory for good embedding results. Thus, the samples were placed in partially open glass containers in a desiccator and kept under a vacuum of 400 mbar ($4 \cdot 10^4$ Pa) for 6 days at 4°C using a pressure controlled vacuum pump. Prolonged infiltration is decisive, because previous experiments had shown that specimens of about 2-3 mm thickness needed to be infiltrated for more than 4 days. Insufficient infiltration is indicated by white-appearing air-filled spaces in the interior of the sample after polymerisation.

Subsequent experiments, which were performed after completion of the study described in this paper, showed that the infiltration step could even be shortened and facilitated. Vacuum infiltration was replaced by transferring the specimens from the last xylene step to acetone for 6 h with one change of acetone and then further to acetone:solution A (see below) 1:3 in a 180 ml screw-top jar without lid on a balance placed in a totally opened fume hood for about 15 h. Both liquids were carefully weighed before mixing. The screw-top jar was left open until one third of the fluid weight was lost. The specimens were then placed in pure solution A for 24 h in the tightly screwed jar and finally transferred into the pressurized containers for polymerization. All infiltration steps were carried out at room temperature.

Polymerization

For final embedding of the specimens two stock solutions were prepared according to the manufacturer's instructions. Stock solution A consisted of 500 ml destabilized base solution, 3g benzoylperoxide and 80 g PMMA powder prepared in two steps as described for the infiltration solution and stored at -20°C. Stock solution B was composed of 4 ml of the solution of N,N,3,5 tetramethylaniline (corresponding to 4.3 g) provided with the kit, 2 ml of 1-decanthiol solution (corresponding to 1,7 g) and 44 ml of destabilized base solution. 30 ml of base solution were mixed with 4 ml tetramethylaniline solution, then 2 ml decanthiol solution were added and mixed. Finally, the container was filled with base solution up to 50 ml. Solution B was also aliquoted and stored at -20°C.

Shortly before starting the embedding procedure, the stock solutions were removed from the refrigerator. One part of stock solution B was added to nine parts of stock solution A and mixed with a glass rod for exactly 1 min. Care was taken to quickly proceed with the following embedding procedure.

The embedding moulds provided by Heraeus Kulzer are totally insufficient for polymerization of Technovit® 9100. Thus, a special device (Fig. 1A,B) was constructed, which permits pressurization during embedding of the specimens in metal-sleeved Teflon tubes with motile bottom and top stoppers. The individual infiltrated tooth slice was placed in one of the six tubes of the pre-cooled device, which contained a pre-polymerized mill-cut mould of Technovit® 9100 to secure the specimen for horizontal embedding. For upright embedding the specimen was glued to a cylindrical piece of pre-polymerized Technovit® 9100 with Loctite® 4305 isocyanate glue which was cured for 20 sec with a blue light lamp. The polymerisation solution was then poured into the tube up to a mark and the top stopper was inserted. The stopper contains a screw-hole for the escape of air and for observing whether it touches the surface of the solution during lowering. If this position was reached, a screw was inserted into the hole and tightened. After filling and closing all six tubes in this way, a pressure

plate with an affixed spring for each tube was screwed in and tightened until a pressure of about 6 bar ($6 \cdot 10^5$ Pa) had built up. The pressure exerted by the springs and the motile stoppers guaranteed that shrinking of Technovit® 9100 during polymerization did no longer produce bubbles outside the specimen or a total stop of polymerization due to intruding oxygen. Using the Heraeus Kulzer embedding moulds inevitably leads to such problems.

A large number of polymerization experiments at different temperatures had revealed that antigens detectable in fixed specimens were well preserved after polymerization at temperatures between 4°C and -18°C. Although polymerization at 4°C was well suited for soft tissues such as isolated pulp or gingival specimens, it did, however, lead to bubble formation in the vicinity of hard substances. This is most likely due to polymerization shrinkage in the interior of the specimen while the Technovit at the surface of the embedding tube has already hardened. Thus, prolonged polymerisation at -18°C for 4 days was preferred for tooth slices. After this period the device was warmed to 4°C and then to room temperature for 1h each, followed by 24h incubation at 37°C to promote evaporation of Technovit monomer droplets from the specimen surface. The metal sleeves were removed from the teflon tubes at 4°C and the specimens were pushed out of the tubes with a special lever device.

Slides, gluing, sawing and grinding

Selection of slides: Mineral glass slides of 2 mm thickness with ground edges (Gerhard Menzel GmbH, Thermo Fisher Scientific, Braunschweig, Germany) were used. This type of slides provided enough mechanical stability for the sawing and grinding procedures, thermostability for antigen retrieval prior to immunostaining and chemical stability for deplastination. Ground edges were essential to avoid injuries during these procedures. The thickness of the slides was individually measured and the slides were then sorted into groups of similar thickness to alleviate further handling.

Encoding of slides: For permanent documentation of the procedures and parameters text was engraved onto the underside of the slides at both sides of the specimen with a water-cooled diamond ball bit (Komet, Lemgo, Germany, Type 001.214.006) operated by a CNC (computerized numerical control) machine and free software.

Slide pretrement: The surface was cleaned by washing the slides in inox metal carriers in a conventional dishwasher for 2h using normal dish tabs at 65°C. After drying these carriers were wrapped and welded in plastic foil.

Preparation of a Technovit® 9100 cylinder with embedded sample: After polymerization the surface of the sample was exposed with a first cut using a diamond band saw (Exakt, Hamburg, Germany). Then the total sample cylinder length was reduced to about 18 mm with a second cut. The two section planes needed to be highly parallel to fit the specimen holder of the Leica® 1600 sawing microtome. To achieve this, the sample surface was finally ground coplanar in a grinding machine (Exakt, Hamburg, Gemany) with grid 3000 abrasive paper.

Gluing: Immunostaining leads to extreme mechanical, chemical and optical demands with respect to the glue used for attaching the sections to the glass slide surface. A wide range of glues was tested and only a high temperature silicone glue came up to the expectations, namely Novasil® SP 6364 (Hermann Otto GmbH, Fridolfing, Germany). An aliquot of Novasil was first centrifuged for 3 min at 3000 rpm before application to remove air bubbles. Then one droplet of silicone was brought onto the sample surface using a 1.5 mm dental ball carrier. Finally, the glass slide was slowly placed on the sample surface taking care to avoid inclusion of new air bubbles between glass and sample. Slide and sample were compressed for 12h in an adhesion pressure device (Exakt, Hamburg, Germany) until Novasil had hardened. After this procedure the silicone layer between sample and slide was on average 10 ± 4 µm thick. The processing time of Novasil is about 6 min until a superficial film occurs.

Sawing: A saw microtome (type 1600, Leica, Bensheim, Germany) with a 300 µm thick cutting blade coated with fine grid diamonds was used to separate the slide holding a section of about 100 µm primary thickness from the remaining sample cylinder. The microtome was operated at about 600 rpm and rinsed with tap water. The cutting speed was set to about 2.7 mm/min. A vacuum holder was used to fix the 2 mm thick glass slide at the end of the cutting process and to prevent it from being catapulted out of the machine.
The thickness of the resulting sections was defined and variations minimized by the following procedure: Two strips of adhesive tape of about 80 µm thickness were glued onto both ends of the glass slide beside the Technovit® 9100 cylinder containing the sample. Then several lines were drawn on the tape surfaces with a permanent marker. Thus, the first contact between the tape strips and the rotating cutting edge was easily recognized, when the lines disappeared. Simultaneous abrasion of the lines indicated coplanar slicing conditions.
The process of gluing and sawing was repeated until the entire sample was processed. A sample of 2 mm thickness usually yielded 5 cross-sections.

Grinding: The initial sample thickness was reduced from about 100 µm to about 23 µm (excluding the silicone film) by manual grinding. Wetted abrasive paper discs of decreasing particle size were used starting with grid 1200 (No. 41840, Exakt, Hamburg, Germany), followed by grid 2500 (No. 41810, Exakt, Hamburg, Germany), and finally by grid 3000 (No. 404175, Patho-Service, Exakt, Hamburg, Germany). The progress of surface smoothening was controlled microscopically. Final polishing was executed with paper of grid 4000 (No. 41610, Exakt, Hamburg, Germany). The actual sample thickness was measured in short intervals with a digital micrometer gauge of ±1 µm resolving power. To avoid wedge-shaped removal of sample material the adhesive tape procedure described above was repeated after each grinding step, successively using tapes of 60 µm, 50 µm and finally 40 µm thickness (No. 57315, 3M, Neuss, Germany). The initially clear and transparent tape strips lost transparency when grinding contact occurred. This technique securely prevented sample loss by too extensive grinding.

Antibodies and immunohistological methods

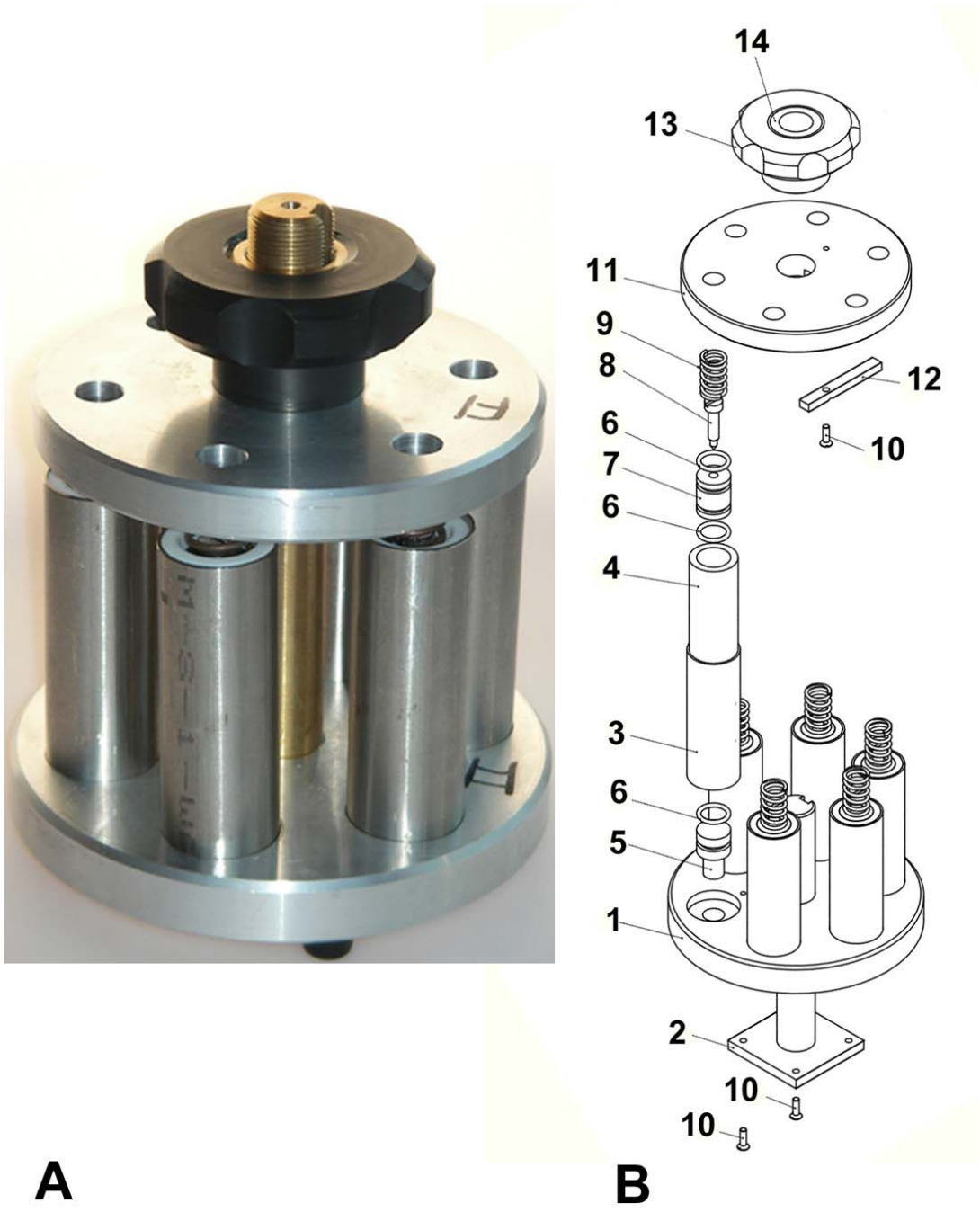
Both topics are described in the main text.

Supplementary online overlay video

An overlay video sequence of a typical nerve fibre bundle/arteriole combination was generated from a series of 13 ground and immunostained sections from specimen 2. To achieve this, the combination was relocated at the individual levels by digitally superpositioning the sections to obtain optimally fitting surface contours of the tooth.

Counting of myelinated nerve bundles and their association with arterioles

Two of the authors (B.S., V.S.) counted S-100-positive nerve bundles with and without arterioles by consensus evaluation of digital images at 10 - 15 MPixel resolution showing tooth pulp cross-sections in a 24" display. The diameter of the structures was assessed by moving a transparent ring-shaped digital measuring device with subsequent digital documentation within the image.



Online Suppl. Fig. 1. External aspect **(A)** and technical drawing **(B)** of the newly designed device for polymerizing Technovit® 9100. Components in **(B)**: **1** - bottom pressure plate, **2** - drawbar, **3** - steel jacket, **4** - pressure cylinder, **5** - piston, **6** - O ring, ISO 3601, 12mm x 2mm, **7** - vented piston, **8** - venting screw, **9** - compression spring, ISO 10243, 1.6mm x 12.5mm x 21.5mm, **10** - M3 countersunk slot head screw, ISO 2009, 10mm, **11** - upper pressure plate, **12** - pilot pin, **13** - screw for upper pressure plate, **14** - thread insert
Items **4**, **5**, and **7** consist of teflon.