TURUN YLIOPISTO

Manual for hard tissue research laboratory

Hard tissue sectioning

Syventävien opintojen kirjallinen työ

Hammaslääketieteen laitos, Suupatologian ja suuradiologian oppiaine

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Abstract:
Histology is a branch of science that studies different kinds of tissues. In the human body there are four main types of tissues and one of them is called connective tissue. This tissue type includes all the hard tissues of the human body, like bones and teeth. In dentistry hard tissue studies are necessary when examining bones and teeth or for example implant materials. More widely, hard tissue studies have a great meaning in musculoskeletal and material research. Hard tissues consist of minerals from which calcium phosphates are the most important. Calcified tissues are among the most difficult for histological research due to their hardness and they present some special challenges when preparing tissue sections.
The preparation of high-quality hard tissue sections is an extended process which demands some specialized expertise in histological laboratory techniques. Routinely, hard tissue samples must be decalcified to be able to prepare tissue sections. In this process calcium salts are eliminated from the sample by using decalcifying acids like formic acid, or chelating agents, like ethylenediamine tetra-acetic acid (EDTA). However, by using this method the tissue structures may be damaged. When preparing hard tissue sections without decalcifying the tissue in advance, sufficiently low temperatures must be used so that enzyme activity and tissue antigenicity remain immunohistochemically expressible.
In this manual, the most promising way of performing histological hard tissue studies is shown by using Technovit 9100 New embedding method (Heraeus Kulzer GmbH). This method is especially suitable for the studies of bone and teeth. The actual cutting phase is done by using a hard tissue microtome. Sections made by using this method are suitable for different kinds of histological stainings, enzyme histochemistry and immunohistological studies like in situ hybridisation.

Avainsanat: hard tissue, histology

Tiivistelmä:

Histologia on tieteen haara, jossa tutkitaan erilaisia kudoksia. Ihmiskehossa esiintyvät kudokset jaetaan neljään kudostyyppiin, joista yksi ryhmä on side- ja tukikudokset. Tämä kudosryhmä pitää sisällään kaikki kovakudokset, kuten luut ja hampaat. Hammaslääketieteessä kovakudosten histologian tutkiminen on tärkeää, kun halutaan tutkia hampaita, luita tai implanttimateriaaleja. Kovakudostutkimusta hyödynnetään esimerkiksi diagnostiikassa ja materiaalitutkimuksissa. Kovakudokset koostuvat mineraaleista, joista kalsiumfosfaatit ovat tärkeimpiä. Kalsiumfosfaatteja sisältävät kudokset ovat kovuutensa vuoksi haastavia histologisen tutkimuksen kohteita.
Onnistuneen kovakudosleikkeen valmistusprosessi on pitkä. Lisäksi se vaatii tekijältään erityisosaamista ja asettaa tiettyjä vaatimuksia laboratoriolle sekä sen varustukselle. Rutiinisti histologisia kovakudosleikkeitä valmistetaan dekalsifikaatioksi kutsutun työvaiheen avulla, jossa kalsiumin suolat poistetaan kudoksesta dekalsifioivilla hapoilla, kuten muurahaishapolla, tai kelatoivilla yhdisteillä kuten etyleenidiamiinitetraetikkahapolla (EDTA). Menetelmä voi kuitenkin vaurioittaa kudosrakenteita. Valmistettaessa kovakudosleikkeitä ei-kalsifioiduista kovakudoksista on huolehdittava riittävän alhaisesta lämpötilasta, jotta kudoksen entsyymiaktiivisuutta ja antigeenisyyttä voidaan jatkossa tutkia immunohistologisin tutkimuksin.
Tässä kovakudoslaboratorioon tarkoitetussa oppaassa esitellään hyväksi osoittautunut kovakudoshistologian menetelmä, jossa hyödynnetään Technovit 9100 New - resiinivalua. Menetelmä sopii erityisen hyvin hammas- ja luututkimuksiin. Leikkeen sahaukseen käytetään timanttivannesahaa. Valmiita leikkeitä voidaan tutkia perinteisin värjäysmenetelmin sekä entsyymihistokemiallisin ja immunohistologisin tutkimuksin.

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1 Introduction

Performing hard tissue histology is challenging in many ways. Different tissue sample structures and sizes require applying different technical variations. Due to density and lower permeability of hard tissue, longer tissue processing times are also required, often taking weeks. In dentistry, a variety of sample structures from soft tissue to hard mineralized enamel or implant materials present some special challenges.

The classic method for hard tissue studies is based on softening the tissue by decalcification followed by embedding in paraffin wax. By decalcification calcium salts are eliminated from the sample by using decalcifying acids like formic acid, or chelating agents, like ethylenediamine tetra-acetic acid (EDTA). However, within this technique the enamel of the tooth, mostly composed of minerals, is lost (Williamson, 2015). Decalcification is also time consuming and it may affect on immunohistological antigenicity and nucleic acid recovery (Freemont, et al., 2016).

Generally, paraffin wax has been the most practical embedding media for majority of tissues. However, for hard tissue studies and some diagnostic applications resin embedding is more suitable as paraffin wax can’t provide enough support for the tissue resulting fragmentation during sectioning. Especially with nondecalcified teeth, cortical bone and tissue implants sectioning can be difficult and more supportive resin embedding is crucial. (Suvarna, et al., 2019).

The hard tissue sectioning technique presented in this manual is the cutting-grinding technique. This technique can be used for nondecalcified hard tissue samples and it’s also suitable for samples including implants made of different metals. First the tissue sample is embedded in plastic and hard tissue plastic blocks are created through polymerization. The aim of using plastic is to support the tissue from the inside and the outside which enables future microtomy. Next, a plexiglass and a microscope slide are glued onto the block. After that the resulting “block sandwich” is clamped into the cutting machine and a 100-200 µm thick section is cut with a high precision diamond saw. The final thickness of 20-100 µm is achieved by grinding and polishing.

In preparing hard tissue sections there are a few main steps to follow after extraction of the sample: fixation, dehydration, preinfiltration and infiltration, embedding, trimming, attachment, sectioning, grinding and polishing. Steps from tissue fixation to embedding are together called the embedding procedure. The rest of the steps are for the preparation of tissue sections.

2 Resin embedding in hard tissue studies

2.1 The use of resins

Resins provide remarkably strong support in tissue preparation for sectioning (Suvarna, et al., 2019). The resin embedding media that are used in histology are grouped in acrylic, epoxy or polyester resins. They consist of at least one resin monomer plus additional components such as hardener, flexibilizer, plasticizer, accelerator or filler/regulator.

Depending on the resin used, there are many desirable features in resin properties, especially from the hard tissue study point of view. While being hard enough for tissue support resin also has good plasticity, flexibility and specimen adhesion, making sectioning easier. There are no internal structures and they are transparent to light and electrons. This allows high-quality sections for light microscopy and even for electron microscopy as resin can also maintain the three dimentional structure of the cells under electron bombardment. By using resin embedding method one could also save important labile substances like enzymes, that normally might get lost by standard tissue processing and paraffin wax embedding. Even immunohistological studies, including in situ hybridizations, are possible by choosing a resin that can be removed from the section so that the cellular targets for labelling are exposed. (Suvarna, et al., 2019).

2.2 Technovit 9100 New

Technovit 9100 New (Heraeus Kulzer GmbH) is a commersial resin embedding system based on acrylic resin, methyl methacrylate (MMA). Technovit 9100 New is ideal for undecalcified hard tissues and implant materials. It polymerizes in the absence of oxygen and at low temperatures (- 2 to -20°C). (Willbold & Witte, 2010). In addition, it is soluble so it can easily be removed from the specimen prior to further histological and immunohistological studies (Suvarna, et al., 2019).

The Technovit 9100 New embedding kit consists of several components: (i) a stabilized basic solution, (ii) PMMA-powder, (iii) hardener 1, (iv) hardener 2 and (v) regulator. The basic solution is composed of monomers and it can be used in stabilised or destabilised form. The chemical polymerisation takes place in the absence of oxygen and with the aid of a catalyst including benzoyl peroxide (Hardener 1) and N, N-dimethylaniline (Hardener 2). (Anon., 2001). In general, benzoyl peroxide aided polymerization reaction starts at 50-60°C but with the amine component it can start at low temperatures (Suvarna, et al., 2019). The other components are PMMA-powder, which is a filler made of PMMA-micropellets, and a regulator. They allow a controlled polymerisation without large

increases in the temperature during polymerisation. The PMMA-powder also has a role in reducing shrinkage during polymerisation and in improving polymerisation quality. (Anon., 2001).

3 Principles of hard tissue sectioning and staining using Technovit 9100 New embedding method

3.1 Tissue specimen handling

After collection of study samples, all the unnecessary tissue is being extracted from the sample. In some cases, samples are dissected into smaller pieces so that the future tissue processing including dehydration and infiltration becomes more efficient. All the samples received in a laboratory must be identified adequately to ensure accurate specimen tracking.

3.2 Fixation

Tissue samples must be placed in fixative, most commonly formalin, as soon as possible after harvesting. Fixation is the most important step of the sample preparation process. In fixation, all the metabolic processes are stopped by using suitable reagents, so the tissue stays in its actual condition. It also prevents tissue autolysis so that the optimal preservation of morphological details is achieved. (Suvarna, et al., 2019).

Fixation can be done with several different reagents that can be divided into two categories: cross-linking and denaturing fixatives. In this manual formaldehyde (formalin) is used which is a cross-linking, most commonly used fixative for histological examination. The optimal time for fixation depends on temperature, the fixative and the type and size of the sample. Tissues like bones and teeth usually need more time to be fully fixed than softer tissues. Generally, a small hard tissue sample is completely fixed after 24 hours. When applying longer fixation times, the fixative should be changed two or three times. (Willbold & Witte, 2010). After fixation the fixative will be washed out.

3.3 Dehydration

Before embedding the specimen in plastic, it needs to be dehydrated for removing the residual fixative and free water. Dehydration occurs at room temperature in an increasing organic solvent series, in this case made of alcohol. Incomplete dehydration impairs the tissue penetration of further reagents, leaving the specimen soft and non-receptive to resin infiltration. When the dehydration is complete, alcohol will be removed from the specimen with xylene.

3.4 Preinfiltration, infiltration and embedding

After the specimen has been cleared from all the alcohol with xylene, it needs to be strictly preinfiltrated (phases 1-3) and then infiltrated with suitable plastic solutions. The aim of using plastic is to gain sufficient internal and external support for the tissue. Generally, the density and the hardness of the supporting embedding media and the specimen should be as similar as possible. Infiltration must be sufficient to displace the xylene, otherwise the polymerization may not be uniform and the tissue will be soft and crumbly making microtomy difficult. (Suvarna, et al., 2019). The time needed depends on the size and consistency of the specimen.

After the infiltration phase, the specimen will be set to a plastic embedding mould which will be filled with the final polymerization solution. The mould is placed into a vacuum desiccator to remove gas bubbles. It is important to get all the air removed, otherwise the polymerization or sectioning may fail. The polymerization process takes place in a freezer in the absence of oxygen and at low temperatures (-20°C). The time for polymerization depends on the specimen size and consistency and the temperature. The bigger the polymerization volume, the lower the temperature must be. In addition, the polymerization is more complete and the polymerization shrinkage is minor the lower the temperature is. When the polymerization is complete the hardened block can be pressed out of the mould. The block is then kept under a laboratory fume hood for final evaporation.

3.5 Trimming, attachment and sectioning

The resulting specimen block is roughly trimmed with a high precision diamond band saw into a shape more suitable for attaching it to a plexiglass and a microscope slide. When there is a plexiglass and a microscope slide glued on the opposing sides of the block, the whole block complex is then clamped in a cutting machine and cut in half by using a high precision diamond saw with a result of a 100-200µm thick specimen section on the microscope slide. This section is then partially ground and polished to a thickness of 20-100µm with a high precision grinding machine.

3.6 Deacrylation and staining

When the sectioning process is completed, the resin embedding media is usually removed from the specimen. After that the specimen is ready for further studies. Examples of hard tissue stainings are presented in Figures 1-4 below.

Figures 1-4. Hematoxylin-eosin staining of hard tissue samples made with Technovit 9100 New embedding -method. Figure 1 shows a non-decalcified tooth sample where the enamel layer is preserved. Figure 2 shows porcine gingival tissue and bone block-implant culture model for the study

of tissue attachment to the implant surface. Figures 3 and 4 show biopsies from rat jaws with experimental implants. The histological sections with two different kind of hard tissues and soft tissue in same samples are high-quality.

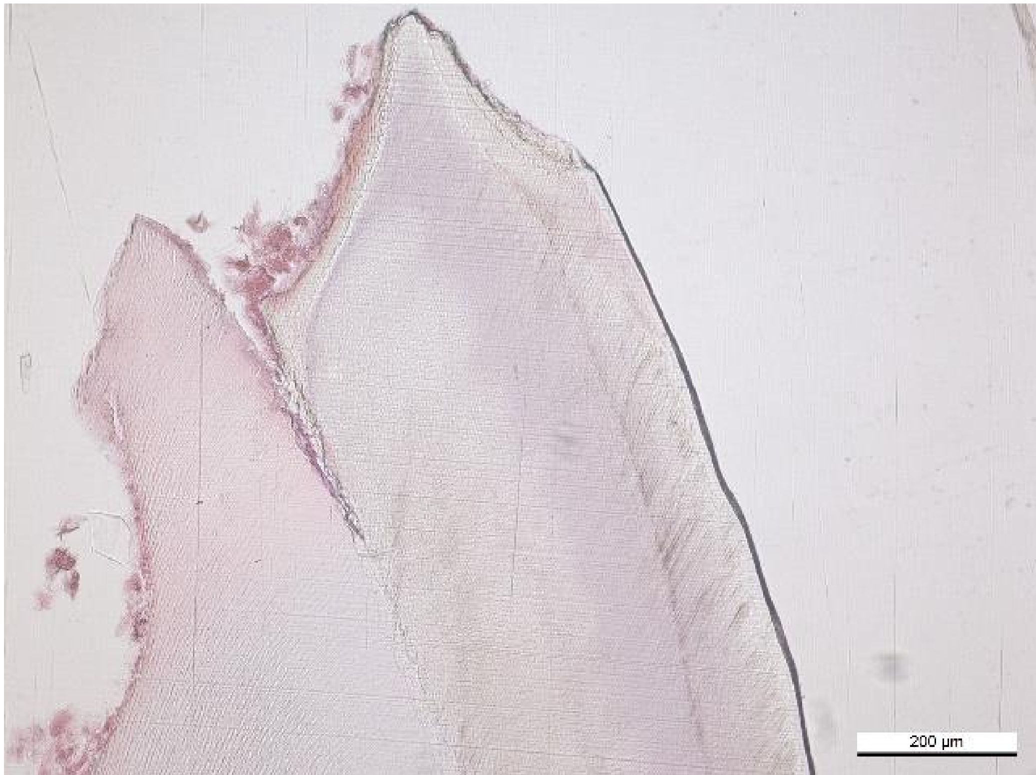


Figure 1.

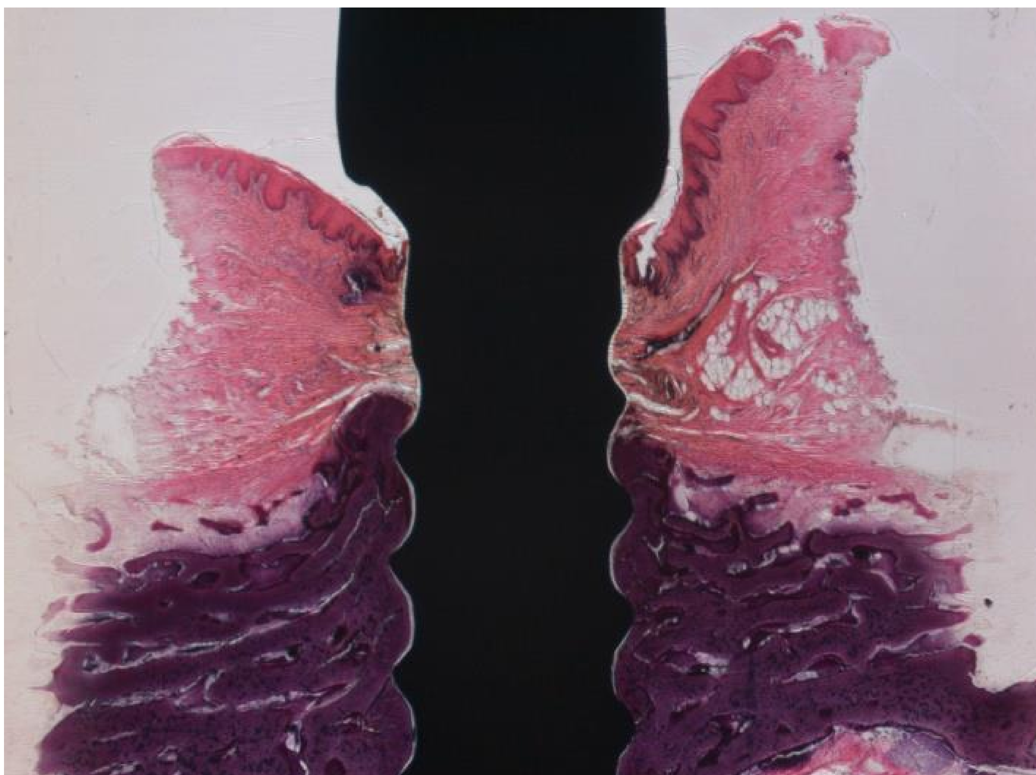


Figure 2.

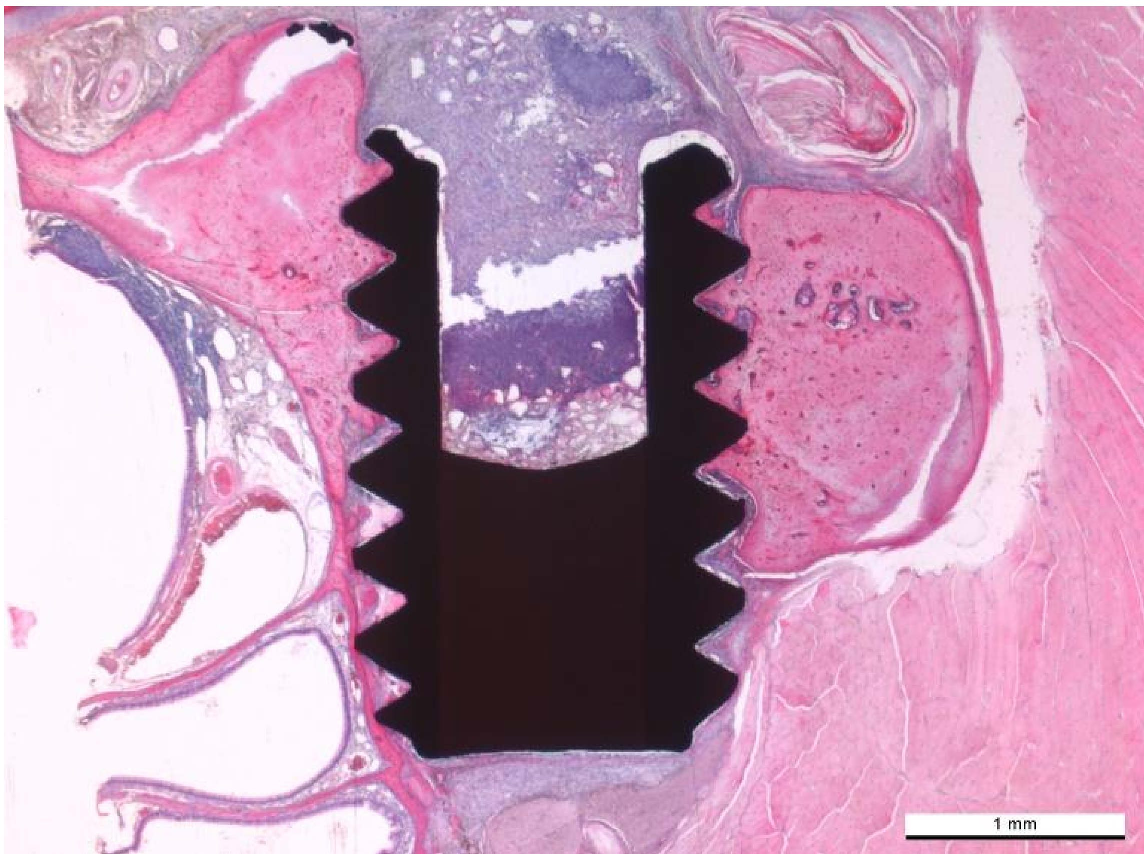


Figure 3.

Figure 4.

4 Laboratory instructions

4.1 Preparation of Technovit 9100 New solutions

Note that many resin components are toxic and there are possible health and safety risks, especially contact dermatitis. It is important that all the solutions used are handled properly according to safety regulations (Suvarna, et al., 2019).

Start the process by destabilizing the basic solution under a laboratory fume hood. The basic solution is destabilised by using chromatography column with aluminium oxide powder. Fill the chromatography column with 50g aluminium oxide, pour in the stabilized Technovit basic solution and let it slowly flow through it. One column prepared as above is sufficient to destabilise 4 litres of basic solution. Store the final destabilised basic solution immediately 100ml at the time in glass containers and cool right away. It can be stored light protected at +4°C for up to 4 weeks or at -20°C for half a year.

Next, prepare the pre-infiltration, infiltration and stock solutions A and B under a laboratory fume hood according to following Technovit 9100 instructions.

	Basic solution	**PMMA powder**	**Hardener 1**	**Hardener 2**	**Regulator**	**Xylene**
Pre-infiltration 1	200ml (Stabilized)					200ml
Pre-infiltration 2	200ml (Stabilized)		1g			
Pre-infiltration 3	200ml (Destabilized)		1g			
Infiltration	ad* 250ml (Destabilized)	20g	1g			
Stock solution A	ad* 500ml (Destabilized)	80g	3g			
Stock solution B	ad* 50ml (Destabilized)			4ml	2ml	

* Start with dissolving all the solid substances into only a part of the basic solution. Once all the substances have dissolved, the final volume is obtained by adding basic solution until the wanted volume is reached. Use volumetric glass flasks and glass stirrers.

Pre-infiltration solution 1 is made by mixing together 200ml of stabilized basic solution and 200ml of xylene. For pre-infiltration solution 2, mix 1g of hardener 1 together with 200ml of stabilized basic solution and for pre-infiltration solution 3, 1g of hardener 1 with 200ml of destabilized basic solution. Next, start preparing the infiltration solution by first adding 1g of hardener 1 to approximately 200ml of destabilized basic solution. Then, add in 20g of PMMA powder and mix until the mixture is entirely homogeneous. For achieving the final solution volume of 250ml, ad in destabilized basic solution as much as needed. For the stock solution A, mix 80g of PMMA powder to approximately 400ml of destabilized basic solution and while mixing thoroughly, ad in 3g of hardener 1 and mix until a homogeneous mixture is achieved. For achieving the final solution volume of 500ml, ad in destabilized basic solution as much as needed. Lastly, prepare the stock solution B by mixing 4ml of hardener 2 with approximately 30ml of destabilized basic solution and then, while thoroughly mixing, ad in 2ml of regulator. Add in more destabilized basic solution until the desired final solution volume of 50ml is reached.
Store the pre-infiltration solutions 1 and 2 light protected in a fridge (+4°C) and the other solutions light protected in a freezer (-20°C) for up to half a year or use immediately for your project.

4.2 Dehydration

Studied specimens arrive the laboratory in specific sample cassettes that are typically placed in 10% buffered formalin for fixation. Usually, the time needed for sufficient fixation is about 24 hours at room temperature.

Start working under a laboratory fume hood. Move the sample cassettes out from the formalin and place them to an empty container. Collect the formalin waste in their own waste container. Next, wash the specimens by placing the cassette container into a sink under running tap water. The time needed for washing depends on specimen size – the bigger the specimen the more time is needed. For example, an hour is enough for small specimens like teeth.

After washing, it is time for dehydration by placing them overnight in an increasing series of alcohol and finally xylene. Start with placing the cassettes in 70% alcohol and store overnight at room temperature. Next, repeat with 96% alcohol, twice with 100% alcohol and twice with xylene.

4.3 Pre-infiltration and infiltration

Following dehydration, place the specimen cassettes in the pre-infiltration solution 1 and store overnight at room temperature. Next, repeat respectively with the pre-infiltration solution 2. The final pre-infiltration step is done by placing the cassettes in the pre-infiltration solution 3 and storing them overnight in a fridge (at +4°C). For infiltration, place the cassettes in the infiltration solution and store 2-4 weeks in a freezer (at -20°C). For teeth and specimens including implants, it is recommended to extend the infiltration time to 4 weeks. For specimens including only soft tissues 48 hours is enough.

4.4 Polymerization

Right before embedding, prepare 50ml of polymerization mixture by mixing together cooled stock solutions A and B in a beaker by adding 5ml of stock solution B to approximaly 40ml of stock solution A. Ad more stock solution A until 50ml is reached. Mix carefully using a glass stirrer.

Make suitable sticker markers for specimen identification. Pour the polymerization mixture into the pre-cooled embedding moulds. One mould is big enough for 15ml of mixture. Place the infiltrated specimens positioned the sectioning surface down carefully into the centers of the embedding moulds with plastic forceps. Do not use any metal instruments. Place the moulds into a pre-cooled vacuum desiccator that is located under a laboratory fume hood. Pay attention to the specimen orientation so that their identity won't get mingled. Evacuate the specimens at -4°C and in 200-400mbar for approximately 30min or at least as long as there are no more air bubbles forming. Finally, let the pressure out from the vacuum desiccator and put the lids on to the moulds. Place the sticker markers on to the lids for specimen tracking. For polymerization, place the moulds carefully on a tray and place it in a freezer (-20 ± 0,5°C). The time needed for polymerization depends on the specimen size and consistency. Typically, 2 weeks is enough. During that time, do not open or touch the moulds. When the polymerization is complete, press the hardened blocks out of the moulds, place the sticker markers on to the blocks and keep them under a laboratory fume hood for about 48 hours for final evaporation.

4.5 Trimming and attachment

Next, prepare a band saw machine for trimming: set a trimming support table, 0,2mm thick diamond band saw and a weight of 25-50g in place. Turn water cooling on. The sectioning surface facing down, trim all the excess material off from the blocks so that the trimming surfaces become as parallel as possible with the specimen surfaces. By planning this step carefully, the future gluing and sectioning becomes easier. Pay special attention to surfaces where a plexiglass and a microscope slide are going

to be glued on as those two surfaces need to be parallel with the wanted sectioning plane and each other. While trimming, let the weight do its work and don't force the saw to move quicker across the block by pushing, because that could misdirect the saw. When the trimming is done, make sure the glue surfaces are parallel by measuring the thickness of the block from different measuring points. Correct by grinding if needed.

Next, a so-called block plexiglass in made. For attaching the specimen block on to a plexiglass following gear is needed: a plexiglass, a pasteur pipette, glue (Technovit 7210 VLC), a timer and an UV light utilizing gluing machine. First, write the specimen information on the upper edge of the plexiglass. Attach the plexiglass upside down up to the gluing machine with a vacuum. Then, with a pasteur pipette spread some glue on to the other block surface where the specimen is. Bring the glue side of the block against the plexiglass and UV light cure for 15min.

When the block plexiglass is ready, the part of the specimen that is going to be on the final microscope slide is going to be exposed by grinding. For grinding, a grinding machine and discs of varied degrees of coarseness are needed. Start with a grinding disc that is coarse, for example P800 from Exakt Technologies. Wet the discs under warm tap water before use. Attach the block plexiglass on to the grinding machine and set a 100 g weight for a start. After a moment, more weight can be added. Check the results at regular intervals, which is easily done by examining the surface against light from different angles. When the wanted sectioning surface is almost reached change the grinding disc to a more fine one, for example K1200 from Exakt Technologies. After having exposed the wanted surface, let the specimen dry at room temperature for 24h. During that time, prepare the microscope slide as follows: take a new microscope slide and with a small piece of grinding paper, for example a silicon carbide P500 paper from Exakt Technologies, roughen it a little from the middle of it for better attachment. After that there should be slight grinding marks showing. Wash the slide under water with soap, rinse with 100% alcohol and drain until it is dry.

4.6 Making a block sandwich

Before gluing the microscope slide on to the specimen block, measure the thicknesses of the slide and the block plexiglass. Take the measurements approximately from the middle of the slide and the block. Write them down on a specimen form as later showed on "Sectioning". Next, clean the surfaces that are going to be glued together with 100% alcohol. Put one drop of RC Primer A (Heraeus Kulzer GmbH) in the middle of the microscope slide and let it dry for 1 minute. Attach the slide upside down on to a UV light utilising gluing machine. Spread Technovit 7210 VLC glue on top of the tissue block and bring it against the microscope slide. Make sure there are no air bubbles in the glue and UV light

cure for 15 minutes. Hence, the specimen block is glued between a plexiglass and a microscope slide and a block sandwich is made. Finally, from the middle of the specimen block, measure the thickness of the block sandwich and write it down on a specimen form.

4.7 Sectioning

The goal is to have a specimen section thickness of 20-100μm. To achieve this, a little mathematics is needed. Based on the thicknesses of the microscope slide, the block plexiglass and the whole block sandwich, calculate the thicknesses of the glue, the microscope slide plus glue and eventually the final thickness of the microscopy slide with the specimen on it.

SPECIMEN FORM			
Researchers:			
Samples:			
Block name / number			
Specimen info			
Date			
Specimen name / number			
Aim for final thickness of the specimen (μm)			
Preliminary info			
Microscope slide (μm)			
Block sandwich (μm)			
Block plexiglass (μm)			
Glue (μm)			
Microscope slide + glue (μm)			
Aim for final thickness of the microscopy slide			
Thickness right after sectioning (μm), average of 3			
After grinding and polishing (μm)			
800			
1200			
2500			
4000			
Final thickness of the specimen (μm)			

Figure 5. Specimen form

When the final thickness of the microscopy slide is calculated, it is separated from the block by sawing. Clamp the block plexiglass side of the block sandwich onto a diamond band saw machine. Place the microscope slide near to the saw and set a small padding under it. Turn the machine and water cooling on and adjust the saw placement right next to the microscope slide so that a slight even voice of saw hitting the slide can be heard. When the right saw placement is found, set a weight of 25-50 g to the saw and let the saw go through the specimen block on its own. Don't push the machine to move quicker. Let the microscope slide drop on the padding when it is ready and turn the machine off. After sectioning, measure the thickness of the resulting microscope slide from 3 different measuring points. Calculate their average value and write it down on the specimen form.

4.8 Grinding and polishing

The final thickness of the microscope slide is achieved by grinding and polishing by using a grinding machine and grinding discs of varied degrees of coarseness. Depending on how much there is to grind to achieve the final thickness, choose a suitable grinding disc for a start. If there is a lot to grind, choose a more coarse disc. Wet the disc under warm tap water and set it in place. Before attaching the microscope slide on, turn the grinding machine on to ensure it has wetted properly. Turn the machine off and set a weight of 100-200 g depending on how much there is to grind. The bigger the weight the faster the machine grinds.

Attach the microscope slide upside down to the grinding machine by vacuum and start grinding. Check the results at regular intervals by measuring the thickness. Initially the measurement can be taken from the middle of the specimen, but when the final thickness is approaching the measurement should be taken from the edge of the specimen. Respectively, the closer the aimed thickness gets the more fine the grinding disc should be. For example, P500 and P800 silicone carbide discs are suitable for grinding at the beginning, K1200 is valid for a phase somewhere between and P2500 and K4000 are for finishing and polishing. When the wanted thickness is reached and there are no scratches in the specimen, the microscopy slide is ready for further studies.

4.9 Deacrylation

Before further studies, the specimen has to be deacrylated. Working under a laboratory fume hood, place the microscopy slide twice in xylene for 10 minutes, twice in methoxy methyl acetate for 1 hour, twice in acetone for 2 minutes and finally twice in distilled water.

5 Working process – tooth sectioning

Teeth were extracted, fixed in 10% buffered formalin for 24 hours and received in the laboratory in sample cassettes. Specimens were washed under running tap water for an hour (figure 6) and then placed overnight in increasing series of alcohol (figure 7) and finally xylene at room temperature for dehydration as earlier explained.

Figure 6. Washing the specimens

Figure 7. The specimens placed in alcohol

Following dehydration, specimens were pre-infiltrated and infiltrated as earlier explained. Sticker markers were made for specimen tracking. For embedding, the polymerisation mixture was made after which 15ml of it was poured into each pre-cooled embedding mould. With plastic forceps, the infiltrated specimens were taken out from their cassettes and then placed into the centers of the embedding moulds facing the sectioning surfaces down (figure 8). The moulds were then placed into a pre-cooled vacuum dessicator for air bubble removing. The specimens were evacuated in -4°C in 200-400mbar for 30min after which it was confirmed that there were no more air bubbles forming. The pressure was let out of the vacuum dessicator and the moulds were closed with their lids marked with the sticker markers (figure 9). The moulds were then stored in a freezer (-20°C) for polymerization for 2 weeks.

Figure 8. Infiltrated teeth and embedding moulds filled with the polymerisation solution

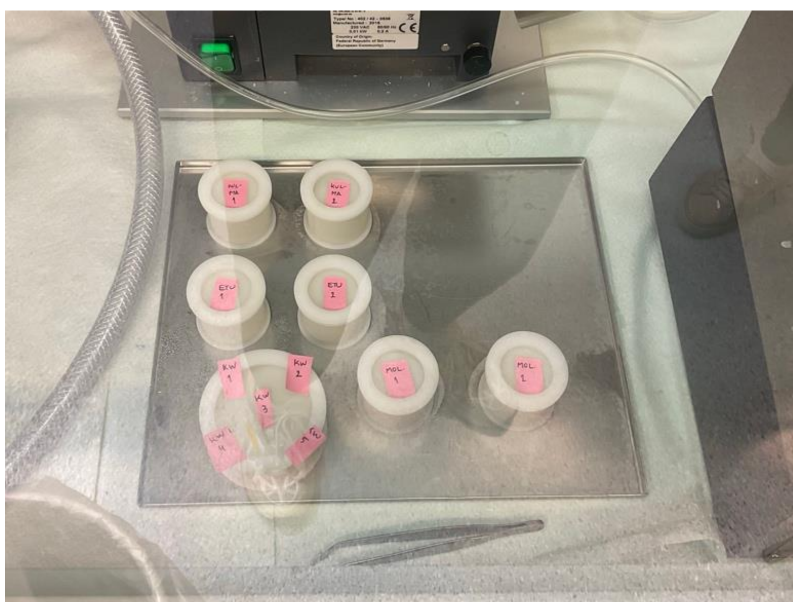


Figure 9. The embedding moulds placed on a tray and ready for polymerization

After 2 weeks, the hardened blocks were pressed out of their moulds under a laboratory fume hood and the sticker markers were placed on top of them (figure 10). The blocks were then kept under the laboratory fume hood for 48 hours for final evaporation.

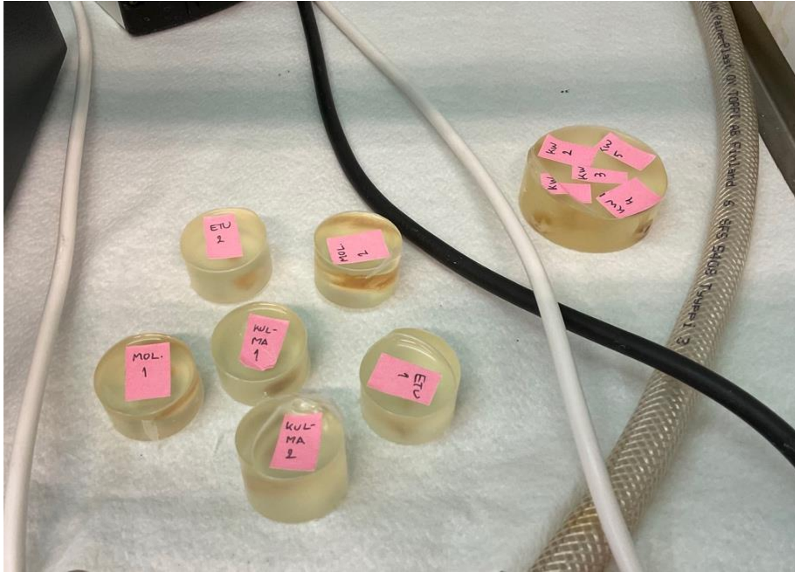
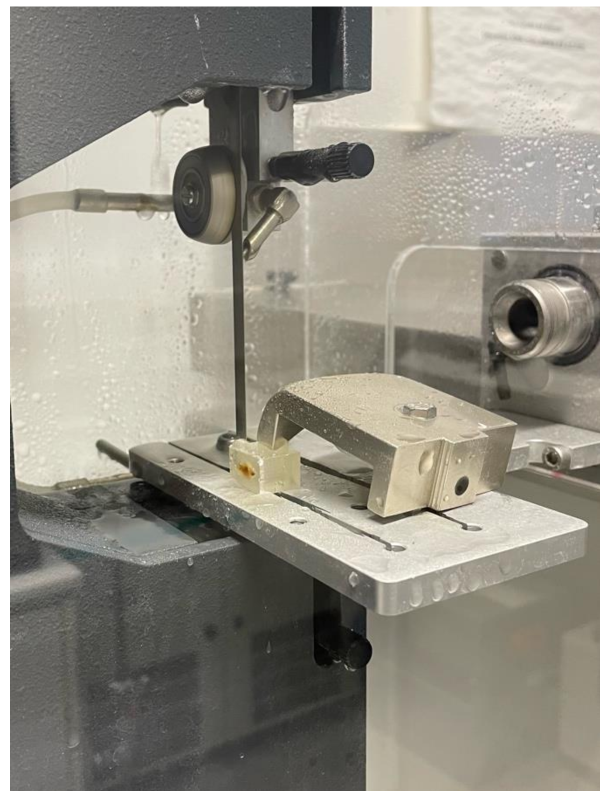


Figure 10. The polymerized and marked blocks pressed out of their moulds

For trimming, a block trimming mount, a weight (25 g) and a 0,2mm thick diamond band saw were set in place to a diamond band saw machine (Exakt Technologies). Then, the blocks were trimmed to shapes and sizes more suitable for gluing plexiglasses and microscope slides on (Figures 11 and 12). To make sure that the gluing surfaces were parallel enough the block thicknesses were measured from different measuring points.



Figures 11 and 12. Trimming

Next, the block plexiglasses were made one by one by gluing the block on a plexiglass using Technovit 7210 VLC glue (Heraeus Gulzer GmgH) and an UV light utilizing gluing machine (Exakt Technologies) (figure 13 and 14). The wanted specimen surfaces were then exposed by grinding the blocks using Exakt 400 micro grinding system (Exakt Technologies) with a 100 g weight in it (figure 15). The grinding was started by using a P800 silicone carbide grinding paper (Exakt Technologies) which was changed to a K1200 silicone carbide grinding paper (Exakt Technologies) when the specimen surfaces ware almost entirely visible. When the tissue surfaces were completely detectable (figure 16), the specimen were let to dry for 24 hours. During that time new microscope slides were roughened from the middle using a small piece of P500 silicone carbide grinding paper (Exakt Technologies), washed under running tap water with soap and rinsed with 100% alcohol after which they were left to drain.

Figure 13. The gluing set-up for attaching the specimen block onto the plexiglass

Figure 14. A completed block plexiglass

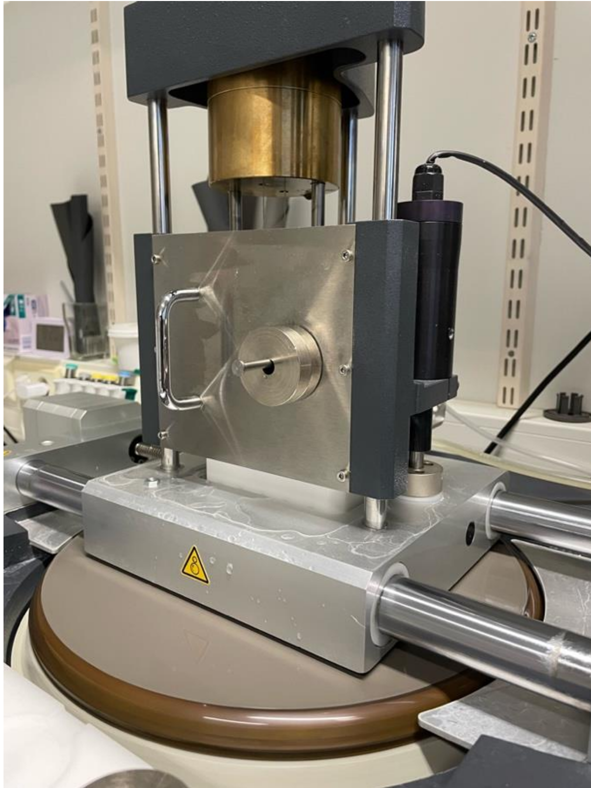


Figure 15. The grinding set-up

Figure 16. Confirming that the wanted specimen surface is exposed by examining it against light

After all the block plexiglasses were ready, block sandwiches were made one at the time. Starting with the first one, the thicknesses of the microscope slide and the block plexiglass were measured and written down on the specimen form (figure 20). The surfaces of the microscopy slide and the block were cleaned with 100% alcohol. One drop of RC Primer A (Heraeus Kulzer GmbH) was put in the middle of the microscope slide and it was let to dry for 1 minute. Technovit 7210 VLC glue was spread on top of the tissue block and the microscope slide was glued onto the block plexiglass using a UV light utilising gluing machine (Exakt Technologies) for 15 min (figure 17). After that, the thickness of the resulting block sandwich was measured and written down on a specimen form (figure 18). Then, the rest of the block sandwiches were made.

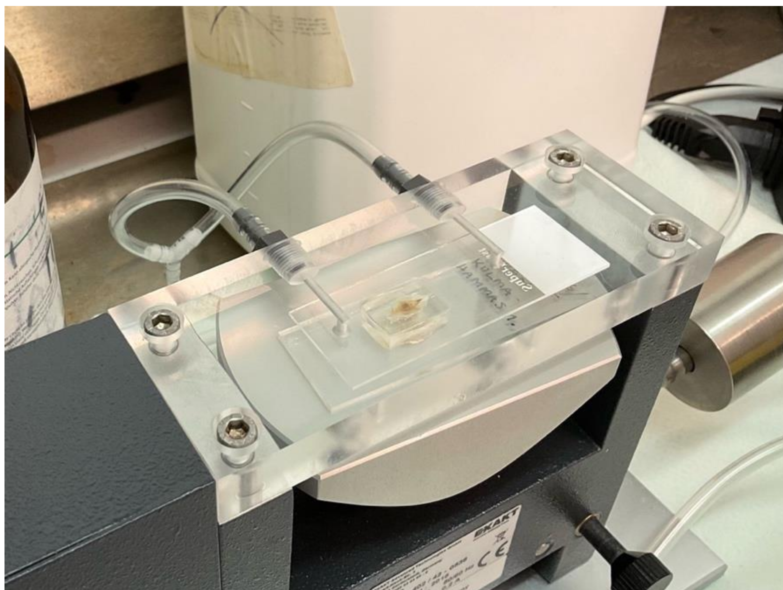


Figure 17. The gluing set-up for the first block sandwich

Figure 18. The thickness of the first block sandwich was measured

When all the block sandwiches were ready, the whole process was completed with one block sandwich at the time. Starting with the first one, the final thickness of the microscopy slide was calculated on the specimen form as the aim for section thickness was 20µm (figure 20). Then, the block sandwich was clamped onto a diamond band saw (Exakt Technologies) by vacuum and the microscope slide was separated from the block by sawing (figure 19).

Figure 19. The specimen plexiglass was separated from the block by sawing

After sectioning, the thickness of the resulting microscope slide was measured from 3 different measuring points and their average value was written down on the specimen form. According to specimen form calculations, there were 183µm to grind down for reaching a final specimen thickness of 20µm as the thickness of the specimen plexiglass was 1210µm (Figure 20).

SPECIMEN FORM			
Researchers:			
Samples:			
Block name / number	Kulmahammas 1		
Specimen info	1		
Date			
Specimen name / number			
Aim for final thickness of the specimen (µm)	20		
Preliminary info			
Microscope slide (µm)	999		
Block sandwich (µm)	7320		
Block plexiglass (µm)	6313		
Glue (µm)	8		
Microscope slide + glue (µm)	1007		
Aim for final thickness of the microscopy slide	1027		
Thickness right after sectioning (µm), average of 3	1210		
After grinding and polishing (µm)			
800	1072		
1200	1040		
2500	1030		
4000	1028		
Final thickness of the specimen (µm)	21		

Figure 20. The specimen form calculations

Using the Exakt 400 micro grinding system (Exakt Technologies) with x 100 g weight in it and a P800 silicone carbide paper (Exakt Technologies) the specimen was ground down to 1072µm after which a K1200 silicone carbide grinding paper (Exakt Technologies) was changed. From that moment, the measurements were taken from the edge of the specimen. When the measurement of 1040µm was reached, a P2500 silicone carbide polishing paper (Exakt Technologies) was changed and the specimen was polished until the measurement was 1030µm. Lastly, the final polishing was done by using a K4000 silicone carbide polishing paper (Exakt Technologies).

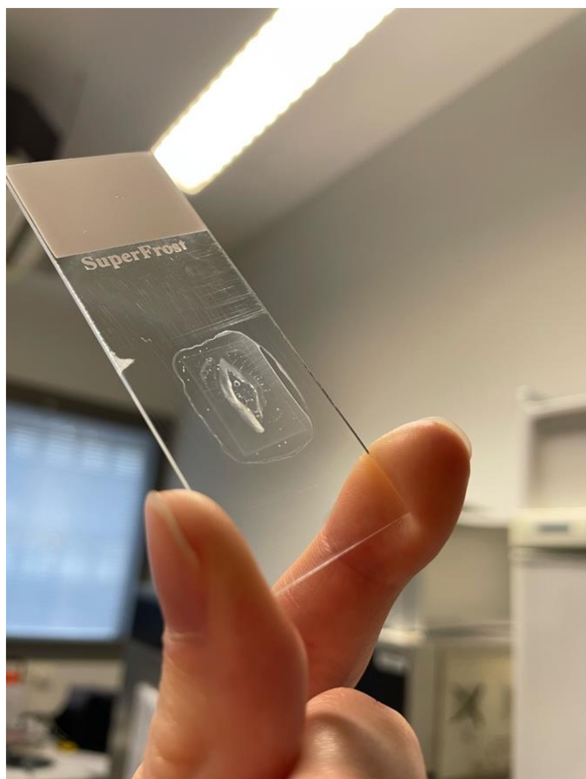


Figure 21. The ground and polished specimen.

Figure 22. H&E stained specimen and the original block plexiglass

Correspondingly, the rest of the block sandwiches were sectioned, ground and polished. Finally, the resulting microscopy slides were deacrylated and H&E stained for further studies. The remaining block plexiglasses were stored for future sectionings.

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