NSH

Hard Tissue Troubleshooting Guide on Tissue Processing Schedules and Embedding Methods for Hard Tissue

Developed by the NSH Hard Tissue Committee- November, 2017 – Sarah Mack, Chair

Hard Tissue Committee
11-30-2017

INTRODUCTION

This guide was requested by histotechnicians having difficulty sectioning bone and soft tissue. This guide provides information on processing schedules as well as embedding methods for tissue embedded in plastics i.e. methyl methacrylate (PMMA/MMA), transmission electron microscopy (TEM) resins (Epon, Spurrs), paraffin for decalcified bone and OCT™ embedding for either calcified or undecalcified bone frozen sections. Processing and embedding samples are key steps in producing quality sections. The guide will discuss in detail what Hard Tissue Committee (HTC) members have experienced with plastics, decalcified bone for paraffin microtomy, and either decalcified or calcified bone for cryomicrotomy. HTC members have graciously provided these troubleshooting hints. HTC wishes to thank Carol Bain, Gayle Callis, Scott Hooten, Sarah Mack, Philip Seifert and Carol Whitinger for their contributions.

Sarah Mack, Hard Tissue Committee Chair

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Submissions are based on the author's experience with hard tissue. References when used are provided within each submission.

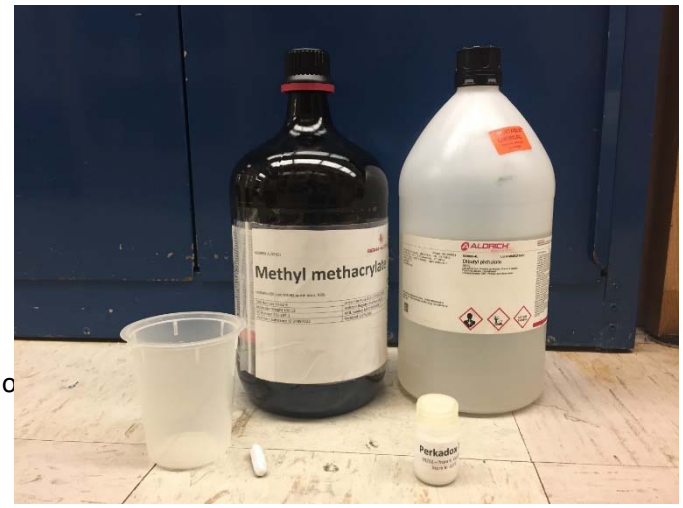
PMMA Embedding

Submitted by Carol Bain, HTL(ASCP)

These are the steps after the samples have been already processed: dehydrated and infiltrated with monomer.

You will need:

- Monomer – methyl methacrylate (MMA)
  - CAS # 80-62-6 – Sigma-Aldrich - product # M55909
- Plasticizer - Dibutyl phthalate (DBP)
  - CAS # 84-74-2 – Sigma-Aldrich - product # 524980
- Initiator – Perkadox16
  - Akzo Nobel - https://www.akzonobel.com/our_key_markets/product-finder/product_finder_detail.aspx?id=12349
- A plastic (polypropylene) graduated cylinder
- A plastic (polypropylene) beaker
- A magnetic stirrer
- A magnetic stir bar



Embedding Solution

95 ml ..... MMA
5 ml ..... DBP
0.25 g..... Perkadox 16
UNDER THE EXHAUST HOOD
Add MMA and DBP in plastic beaker with magnetic stir bar o
Start mixing
Add slowly 0.25g of perkadox 16
Don't forget to label beaker
Cover with foil
Mix overnight at room temperature
The solution will change color from clear to a light yellow/amber.

Preparing pre-polymerized base-molds:

- You will need
- Embedding solution
- Polypropylene containers with screw top
    - Example: C576 - SecurTainer™ II #4, product # C576-20MA

- A pipette
- Water bath at 40°C

Fill containers with about 5 ml of embedding solution (around 0.75cm deep)
Cap them TIGHTLY
Place them in the water bath overnight. The water level should be no higher than the embedding solution level. The containers should not float.

Embedding

- Fill prepolymerized base mold container with around 15-20 ml of embedding solution
- Place processed sample in container, position it according to your ROI.
- Cap container tightly. Identify sample properly. Sharpie ink will wash off. Use paper labels written with pencil.
- Let it sit at room temperature for 1 to 2 days. If you have a vacuum oven, or a vacuum chamber, place it under vacuum overnight, at room temperature.
- DO NOT INTRODUCE HEAT. This might trigger a very fast polymerizing reaction which will overheat (polymerization is exotermic) and boil.
- Check if MMA polymerized by inverting the container (still capped).

Rat Knees

Submitted by Scott Hooten, HT(ASCP)CM

Following is the procedure is for rat knees. The decal procedure I use for other types bone, depending on the bone type, might take a little longer.

I use a Gryphon Diamond Blade Band saw, it is a wet saw for cutting flooring tile, to trim the calcified bone down to the size I want it for decalcification. We do this to minimize the amount of decal solution we have to use, and it helps get better fixation of the bone marrow. The band saw is a nice small size so it fits in the fume hood when I need it and when I don't I keep it tucked away under a bench top. For decal I use Cal-Ex II, it is formic acid and formaldehyde, so you get some fixation while decalcifying. For the rat knees it usually takes about a week to two weeks. I change the decal solution every other day, and I am lucky and have a radiograph machine right next to my fume hood so I take radiographs every couple days to check and see if the decal is complete.

I am usually working with whole knees, so once decal is done I trim them and place the pieces into cassettes. I used to do our normal soft tissue cycle on the processor, but it always gave me issues with cutting, so I recently extended the processing cycle and that seems to have fixed my problems.

Here is my program from my tissue processor. Every step is 1 hour and 30 minutes long.

- 70% Alcohol vacuum no
- 95% Alcohol vacuum no
- 95% Alcohol vacuum yes
- 100% Alcohol vacuum no
- 100% Alcohol vacuum yes
- 100% Alcohol vacuum yes
- Xylene vacuum no
- Xylene vacuum yes
- Paraffin vacuum no
- Paraffin vacuum yes
- Paraffin vacuum yes
- Paraffin vacuum yes

When sectioning I do everything pretty much the same as soft tissue. Cut my ribbon and float it on about water bath heated to around 46 degrees. If I am having issues with sections staying on my slides during the stain run, I add a little Chrome Alum-gelatin adhesive, Bond-IT from Mercedes Medical, to my water bath.

Research Bone Automated Tissue Processing Schedule

Submitted by Sarah Mack

			Mouse Hindlimb		Mouse Spine		Rabbit Femur Condyle		Rat Shoulder	
Station	Reagent	Temp C	P/V	Infiltration	P/V	Infitration	P/V	Infiltration	P/V	Infiltration
1	70% Alcohol	RT	Yes	60 min	Yes	60 min	Yes	60 min	Yes	40 min
2	80% Alcohol	RT	Yes	60 min	Yes	60 min	Yes	60 min	Yes	60 min
3	95% Alcohol	RT	Yes	60 min	Yes	60 min	Yes	45 min	Yes	60 min
4	95% Alcohol	RT	Yes	60 min	Yes	75 min	Yes	60 min	Yes	75 min
5	100% Alcohol	RT	Yes	60 min	Yes	60 min	Yes	60 min	Yes	90 min
6	100% Alcohol	RT	Yes	60 min	Yes	60 min	Yes	60 min	Yes	90 min
7	100% Alcohol	RT	Yes	60 min	Yes	95 min	Yes	60 min	Yes	120 min
8	Xylene	RT	Yes	60 min	Yes	60 min	Yes	90 min	Yes	60 min
9	Xylene	RT	Yes	60 min	Yes	60 min	Yes	135 min	Yes	60 min
10	Xylene	RT	Yes	60 min	Yes	95 min	Yes	60 min	Yes	60 min
11	Paraffin	58*	Yes	60 min	Yes	60 min	Yes	75 min	Yes	90 min
12	Paraffin	58*	Yes	60 min	Yes	60 min	Yes	75 min	Yes	90 min
13	Paraffin	58*	Yes	60 min	Yes	60 min	Yes	75 min	Yes	90 min
14	Paraffin	58*	Yes	60 min	Yes	95 min	Yes	120 min	Yes	90 min
15	Wax Drain	60*								
16	Cleaning Xylene									
17	Cleaning EtoH									
Run Time				14 hrs		15hr 45 min		23hr 15 min		17hr 55 min

P/V: Pressure and Vacuum, used in all processing programs.

Research Murine Bone Embedding Methods

Submitted by Sarah Mack

Murine Ankle Embedding

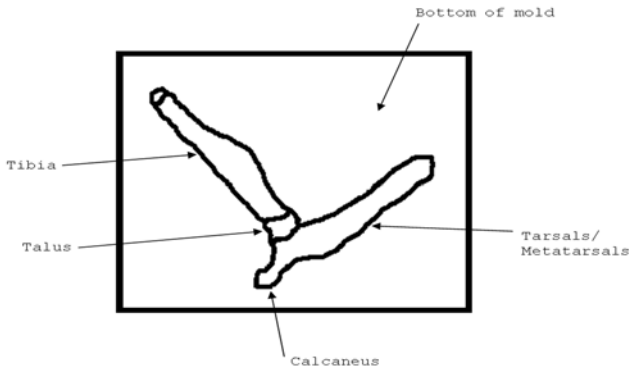
Technique: To section paraffin-embedded adult mouse ankle joints. Specific emphasis is placed on the tibia-talus joint, Flexor Digitorum Longus (FDL) tendon, and Flexor Hallucis Longus (FHL) tendon.

Procedures:

Embedding

- Ensure ankles have been skinned and trimmed close to the bones. The toenails may be removed easily with a scalpel while taking care only cut into the phalanges and not into the metatarsals (the FDL and FHL both insert into the distal metatarsal regions).
- Specimens must be allowed 20 minutes to completely melt in the paraffin reservoir before attempting embedding (if not embedded immediately following processing).
- Identify the medial and lateral sides of the ankle, tibia, calcaneus, and tarsals/metatarsals (digits). The prominent tendon connecting the gastrocnemius and soleus to the calcaneus is the tendocalcaneus (Achilles' Tendon). This runs close to parallel with the tibia and helps locate the FDL and FHL. Examining the plantar surface of the foot, 3 white tendons are easily visible. The FDL is most medial following close to the FHL. Both wrap underneath the calcaneus from the distal tibia.
- Handle specimens by lightly gripping the tarsal region or proximal tibia only.
- Embed specimen with medial side facing the bottom of the mold in a 'V' shape. (Fig. 1)
    - Tibia-talus – Align tibia parallel with bottom of mold. Primary focus should be on the tibia, but tendocalcaneus is another good aiding landmark.
    - FDL/FHL – Align tendons parallel with bottom of the mold. A straight edge or #11 scalpel can be used to trim away soft tissues and digits that make flat embedding difficult. Cut close and parallel with the tendon and embed on the cut edge.

Figure 1. Orientation of Ankle for Embedding



Hind Limb – Embedding

Technique: To section paraffin-embedded adult mouse hind limb. Specific emphasis is placed on the articulating region in the medial compartment of the knee, growth plate, cortical bone, and trabecular bone.

Procedures:

Embedding

- Ensure hind limbs have been skinned and trimmed close to the bones. Handle with care so as to avoid dislocation of the knee when removing muscle. A sharp scalpel and patience will help remove muscle without any damage to the joint, cortical bone, or periosteum.
- Specimens must be allowed 20 minutes to completely melt in the paraffin reservoir before attempting embedding (if not embedded immediately following processing).
- Locate femur and tibia and identify the medial and lateral sides as well as anterior and posterior of the limb. Fibula is located on lateral side and slightly posterior. Patella and patellar tendon are located directly anterior of the knee and are easily identified by their "pearl white" color. Locating these will help with orientation.
- Lay limb on its medial side. The limb should naturally lay slightly posterior. You can visualize this by examining the patellar tendon's position (i.e. tendon facing up = laying posterior; tendon facing down = laying anterior; tendon parallel with bottom of mold = laying directly medial.
- For articular cartilage:
    - Begin with holding the knee perfectly medial and angle joint ~20^{o} toward posterior. The tibia and femur will have a slight downward slope, but usually will not be resting on the bottom of the mold.
    - Holding this position, carefully move mold to cooling plate. Do not let go with forceps until a thin layer of wax has solidified on the bottom of the mold to hold the specimen's position.
- For growth plate, cortical bone, and/or trabecular bone:
    - Hold knee in exactly medial position. The patellar tendon should parallel with the bottom of the mold. Check alignment of the tibia and femur and try to visualize a straight line through both bones. Make this line parallel to the bottom of the mold.
    - Holding this position, carefully move mold to cooling plate. Do not let go with forceps until a thin layer of wax has solidified on the bottom of the mold to hold the specimen's position.
- All hind limbs should be oriented in a 'V' shape inside mold. (Fig. 1)

Vertebra – Embedding

Technique: To section paraffin embedded adult mouse spine.

Procedures:

Embedding

- Specimens must be allowed 20 minutes to completely melt in the paraffin reservoir before attempting embedding (if not embedded immediately following processing).
- Due to the curvature of the spine, no more than groupings of 2-3 vertebrae should be attempted per block. Separate the regions of the spine for optimal orientation.
- Remove excess muscle from specimens to identify ventral and dorsal structures.
- Orient and embed specimens with ventral side "up" (vertebral bodies and disks should be facing opposite the bottom of the mold). The spinous processes should be exactly perpendicular to the bottom of the mold and touching the metal.
- Do not apply pressure to the vertebrae during embedding as this causes distortions visible in the intervertebral discs.

Kowamoto Tape Transfer Protocol for Frozen Calcified Tissue Sections

Submitted by Carol Whitinger, BS, HT (ASCP)

PPE – Caution! Hexane and dry ice can cause immediate freezer burn. Use lab coat, safety glasses or goggles, and gloves.

Prep – Place the knife holder for tungsten carbide blades (Leica #14041933993) in the cryostat to chill overnight. For the best sections, use the customized Kawamoto knife holder that fits into the knife holder for a standard (non-disposable) blade.

Embedding

Supplies

- Cryosection preparation kit (Section-Lab Co. Ltd., Japan) containing embedding container with handle, tissue basket, and hammer & base.
- SCEM or SCEM L-1 – Super Cryo Embedding Media
- Hexane (otherwise known as Isopentane or N-methyl butane)
- Dry ice
- Appropriate containers for both the hexane and the dry ice

Protocol

- Pour hexane over a small container of dry ice until there is enough cold hexane to freeze a block (see Freezing Tissue and Blocks for Cryosectioning).
- Fill an appropriately sized embedding container with an embedding medium.
- Slightly freeze the embedding medium until the bottom and sides start to turn white.
- Snap freeze the tissue using the wire basket from the kit – First place the basket in the hexane, then drop the tissue into the basket. Do not submerge the forceps or tool that was holding the tissue or the tissue will freeze to it.
- Orient the frozen tissue in the slightly frozen embedding medium and freeze the entire block, but do not let hexane overflow onto the top of the mold. Freeze the mold completely.
- Remove the block from the mold with the hammer and base provided with the kit.
- Wrap block in foil pre-labeled with ID number (use alcohol-resistant marker on lab tape) and store at minus 80°C.

Sectioning

Supplies

- Cryofilm transfer tape of choice - adhesive tape sheets, cutting mat, and rolling cutter (or razor blade) from the kit
- Double-sided tape (preferably only 5 mm wide, Scotch brand)
- Slides, non-charged
- Cryofilm fitting tool (rubbing stick from kit)
- Blades, Tungsten carbide tipped (Leica TC-65 disposable blades or similar)

- Cryostat and tools for sectioning
- OCT – Optimal Cutting Temperature medium or similar
- 1X phosphate buffered saline (PBS), if coverslipping
- Aqueous mounting media (ProLong®, Life Technologies is suggested)

Protocol

- Set cryostat to -25°C. Place the following supplies into the cryostat to chill:
    - Forceps, brush, and any other instruments generally used when cryo sectioning
    - Slides with double-sided tape applied at the correct width
    - Blade(s) - place in holder with writing side facing you. Tighten well, but do not overtighten, the two screws. Alternatively, using the Kawamoto knife holder, tighten the screws with the Allen wrench.
    - Cryofilm tape windows
    - Blocks to section equilibrated to the temperature of the cryostat

- Set knife angle to 12-15°. Typical section thickness is 8-10 µm, but 5 µm can be achieved.
- Freeze a block to a metal chuck using OCT. Orient the block on the microtome so that the 'good' part will be at the top of the block (which will be the end of the sectioning stroke).
- Face the block until you're ready to take a section. Brush any debris off of the block face.
- Remove backing from a chilled cryofilm tape window.
- Lay the tape window smoothly on the block face with the sticky side (usually the gold side) toward the block.
- Rub the tape window firmly in an upwards motion (away from the knife!) with the rubbing stick to fully adhere the block face.
- Slightly loosen the tape at the top of the block so it isn't cut by the knife.
- Cut the section being sure to keep the beginning of the tape window in FRONT of the knife, slightly pulled up from the block.
- Lay the section sticky side up onto a slide with the double-sided tape. The section should fit perfectly between the strips of tape. Press one gold end onto the tape, gently pull the section flat, and press the other gold end onto the tape to completely secure the section.
- Store slide at -80°C or remove the slide from the cryostat and dry for approximately 30 minutes prior to immediate use.
- Fix the tissue with a few drops of 4% paraformaldehyde before further processing, which can be done right on the tape. Stain with (Mayer's) Hematoxylin and Eosin or other stains as needed.
- In order to coverslip the section, cut it away from the taped ends with the rolling cutter or fresh razor blade. Lay the section on a fresh slide with a little xylene or PBS depending on the mounting medium used. Coverslip using aqueous or resinous mounting media.

References:

1. Noriaki Ono, University of Michigan Dental School; noriono@umich.edu
2. User instructions from the Multi-Purpose Cryosection Preparation Kit
3. *Use of a new adhesive film for the preparation of multi-purpose fresh-frozen sections from hard tissues, whole animals, insects and plants*, Arch Histol Cytol, 66 (2): 123-143 (2003); Tadafumi Kawamoto, Radioisotope Research Institute, Tsurumi University, School of Dental Medicine, 2-1-3 Tsurumi, Tsurumi-Ku, Yokohama, Japan 230-8501; kawamoto-t@tsurumi-u.ac.jp
4. Maekawa and Kawamoto, Breeding Science 52: 57-60 (2002)
5. Finetec website

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Processing Bone for Parrafin and PMMA Embedding (Research)

Submitted by Gayle M. Callis HTL, HT, MT(ASCP)

AUTOMATED PARAFFIN PROCESSING OF DECALCIFIED BONE:

Bone must be totally fixed in Neutral Buffered Formalin before decalcification. Decalcification endpoint test is done on all bones for both acid or EDTA decalcification. For information on fixation and decalcification, consult the NSH Hard Tissue Troubleshooting Guide on Grossing, Decalcification and Fixation**.**

Hints:

Running tap water rinse to remove decalcifying agents from bone before processing, 1h to 4 h.

Store rinsed bones in 70% alcohol or return to NBF until processing. Keep storage in 70% alcohol minimal to prevent dry, hard bone at microtomy.

If decalcified bone has been stored in 70% ethanol, start processing in 70% ethanol. You can shorten time in 70% alcohol station since storage in this alcohol has started dehydration.

Overstuffing cassettes with any bone causes poor exchange of processing solvents, clearant and paraffin infiltration. This results in mushy bones at microtomy. Avoid 'waffle weave' pattern from cassette lid on top of bone sample which is seen at embedding. No more than 3 mm to 4 mm thick bone in a regular tissue cassette.

NOTE: When trimming a block, if bone looks opaque, is mushy, overly dry, or excessively hard, you may have any one of the following 1) inadequate dehydration, 2) inadequate clearing or 3) inadequate paraffin infiltration ***or a combination of all three steps.*** Revaluate each processing step to make correction(s).
Do not add temperature to solvent stations on processer. This causes dry, hard bone at microtomy.
Xylene hardens bone but is less sensitive to water. For less hard bone at microtomy, use a single alphatic hydrocarbon xylene substitute for clearing during processing. Put xylene in first clearing station, and Clearite 3 Richard Allan/Thermo Scientific, Kalamazoo MI USA in last two clearing stations***.*** ***Optional is three Clearite 3 stations with time added i.e., 15 to 30 min per station to compensate for lack of water sensitivity as compared to xylene.*** Recommendation: always increase time in xylene substitutes more sensitive to water carryover.

Do not use recycled xylene in clearing stations.

Fresh, recently changed solvents always improve processing. Maintain proper solvent levels in all processor containers.

Newer tissue processors are very efficient resulting in over-processing i.e. over-dehydration, over-clearing. We shortened bone processing schedule from older VIP 1000 (K series) schedule for the new VIP6 model. ***Do pilot study processing runs to determine optimal processing time/schedule for murine and rat bones.***

SAMPLE: MURINE BONE PROCESSING USING A VIP6 AUTOMATED TISSUE PROCESSOR Sakura Finetek Usa, Carpentiniera Ca, Usa.

Small bones need a shorter processing schedule than large animal bones. Exception: since murine paws have many densely packed bones, we used a longer processing schedule for adequate dehydration, clearing and paraffin infiltration. Whole mouse skull with brain needs longer processing schedule than murine femur, tibia, bisected skull w/brain i.e., 2 h per station.

MURINE WHOLE FEMUR, KNEE, TIBIA

VIP6 Automated Tissue Processor. Sakura Finetek USA Carpentieria

Station	Reagent	Time	Temp	P/V
1	70% ethanol	1 h	Ambient	On
2	80% ethanol	1 h	Ambient	On
3	95% ethanol	1 h	Ambient	On
4	95% ethanol	1 h	Ambient	On
5	95% ethanol	1 h	Ambient	On
6	100% ethanol	1 h	Ambient	On
7	100% ethanol	1 h	Ambient	On
8	100% ethanol	1 h	Ambient	On
9	Xylene or *(Clearite 3)*	1 h *(1 ½ h)*	Ambient	On
10	Xylene Substitute**	1 ½ h	Ambient	On
11	Xylene Substitute**	1 ½ h	Ambient	On
12	Paraffin*	1 h	60ºC	On
13	Paraffin	1 h	60ºC	On
14	Paraffin	1 h	60ºC	On

Bone NBF fixed, decalcified in 10% to 15% formic acid, tap water rinsed 1 h to 4 h before processing starting in 70% ethanol. Decalcified bones can be stored in 70% ethanol until processing.
P/V: Pressure/Vacuum ON
*Paraffin: Preference Tissue Prep 2 Fisher Scientific/Thermo Scientific Kalamazoo MI, a harder paraffin for bone
Xylene Substitute: Clearite 3 Richard Allan/Thermo Scientific Kalamazoo MI, ***OR* single aliphatic hydrocarbon of your choice.
If Xylene is used instead of a single aliphatic hydrocarbon xylene substitute, use a) two 1 h changes OR b) three changes ***(30 min, 30 min, 1 h)*** **for 2 h total time in xylene**).
Minimum is 3 paraffin changes

WHOLE RAT KNEES: Increase time by 5 min to 30 min in each station.

WHOLE ADULT MOUSE SKULLS WITH BRAIN: 1 ½ h to 2 h in all stations. Remove skin and ears without damaging nasal openings. Lower jaw can be disarticulated and removed if desired. NBF fixation a week minimum, 10% to 15% formic acid decalcification using decalcification endpoint testing to ensure complete calcium removal. Process in mega cassettes but embed with regular cassettes. *Mega-cassettes do not fit snugly in microtome block holder.*
Bones larger than murine i.e. 5 mm to 1 cm thick slabs from sheep, bovine, canine, human femoral head can require up to 4 h per station.

Figure 1: Special Embedding Method. Large, 5 mm thick bones at thickest part of sample, whole mouse skull, rat knee. Use 24 x 40 Peel a Way molds. Keep molds and cassettes warm in embedding center. Molds need to be pliable, and cassettes will not have paraffin hardening too fast. Fill mold with paraffin, embed thicker bone, add a labelled cassette, carefully push cassette down to bone. Maintain cassette at an even level using forceps. The cassette should almost touch the bone. Move mold to cold plate on embedding center. If block is a bit uneven, adjust angles, etc. on microtome for sectioning. It takes some practice!

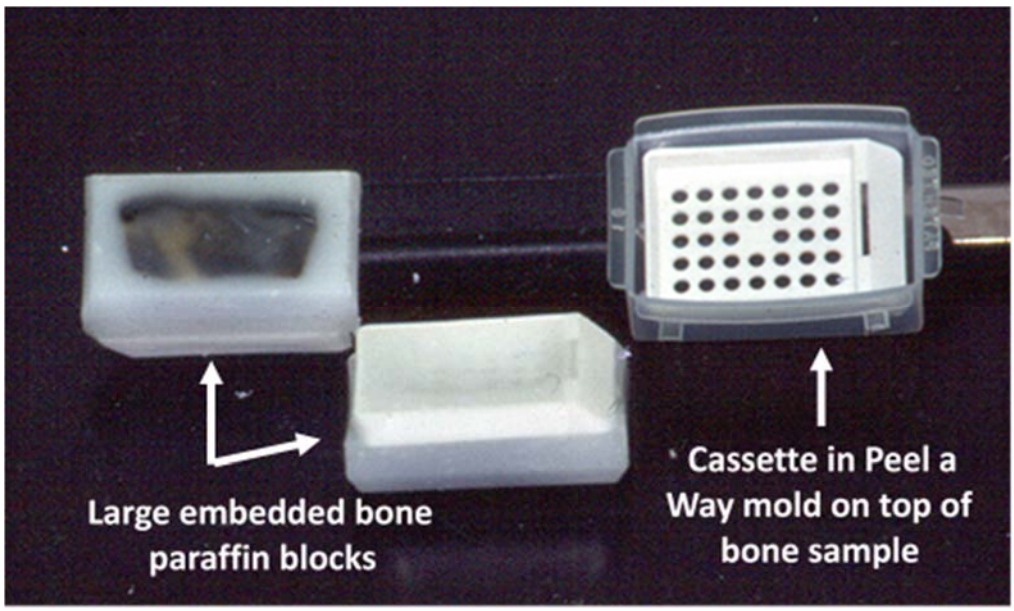


Figure 2: In general, embedding bone at a slight angle allows blade/knives to have less resistance during sectioning. Cutting across a longer bone edge causes blades to jump and chatter.

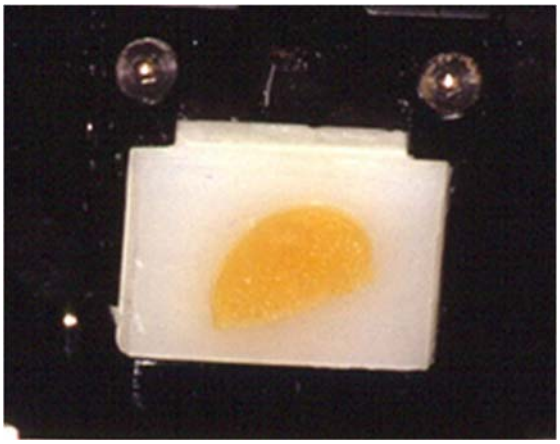


Figure 3: Whole knees are embedded in "half- moon" orientation so blade passes through curve of knee first. Cartilage is softer than bone. If this knee orientation was reversed, cartilage can separate from bone surface. Note tissue left to protect knee joint and articular cartilage surfaces.

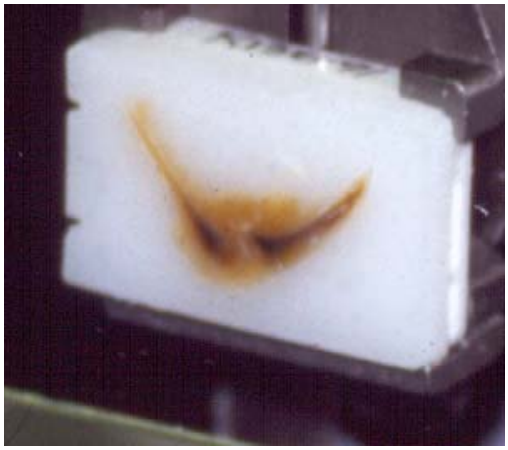
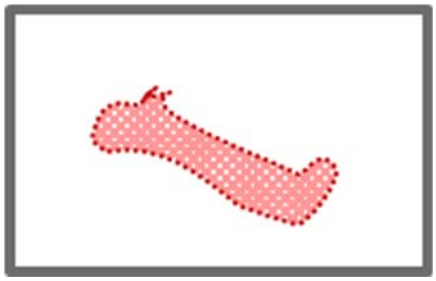
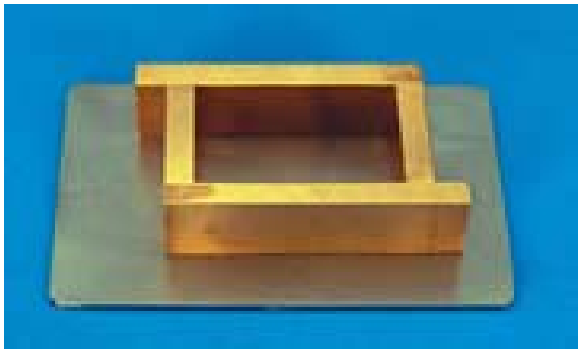


Figure 4: Femur (either end) at an angle for same reason stated as Figure 2.

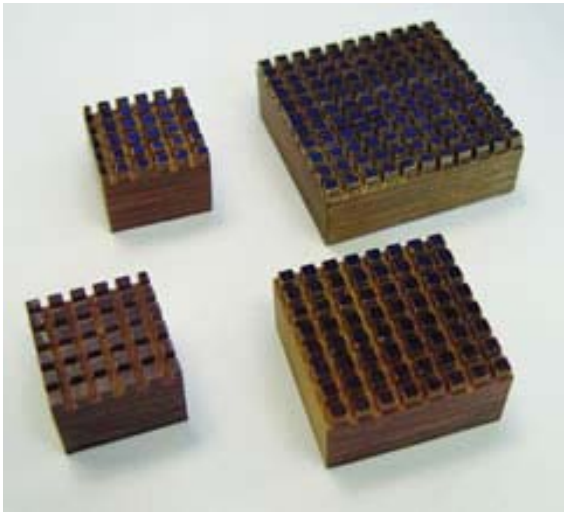


EMBEDDING LARGE BONE SLABS: Use metal laboratory pans, baking pans or adjustable L molds. EMS at www.emsdiasum.com



Put pan a large 60 °C oven. Fill pan with melted paraffin, embed bone in pan. L mold can be filled in heated cassette storage area, even at RT, the moved to embedding center cold platform to harden paraffin.

Place an oven heated 60 °C block holder (hard resin) on top of bone. EMSwww.emsdiasum.com. Add more paraffin. Special large microtome clamps are needed to hold these blocks.



Remove pan from oven and place either on a solid ice block, a preference, or embedding center cold plate.

POLYMETHYL METHACRYLATE (PMMA/MMA) PROCESSING AND EMBEDDING

PMMA mixtures and some hints are compliments of Diane Sterchi.

Sterchi DL. An evaluation of methyl methacrylate mixtures for hard tissue processing. 1995 J Histotechnol 18:45-49.

Sterchi DL, Eurell JC. New method for preparation of undecalcified bone sections. 1989 Stain Tech 64(4)

Gamble M., Bancroft J. Theory and Practice of Histological Techniques. 5th, 6th, and 7th Ed. Bone Chapters

TRIMMING BONE SAMPLE:

Expose as much of the bone and/or bone marrow as possible. Reduce size of bone, bisect, cut slabs or open both ends of whole bone specimen to allow good dehydration, clearing and infiltration with unpolymerized PMMA mixtures.

FIXATION: 10% neutral buffered formalin, change daily or every other day. Complete fixation 5 days to two weeks. Dependent on size and number of bones in container. Fixative is changed frequently.

CAVEAT: Some implant materials are soluble in processing solvents and monomer. Do solubility tests before proceeding to the next steps.

DEHYDRATION: Rinse with tap water then store in 70% ethanol, or process immediately. Dehydration and clearing was done using a Wheaton O Ring vacuum desiccator and vacuum pump. We had a powerful inhouse water vacuum system which eliminated any monomer fumes during processing. Buy extra O rings which expand in solvent fumes i.e. xylene, methacrylate monomer. Reusable O rings return to normal shape by letting them sit inside a hood. ***A tissue processor can be used for dehydration and clearing, but do NOT go into paraffin. A FUME HOOD IS REQUIRED FOR ALL STEPS FOR WHEN USING A VACUUM PUMP XYLENE CLEARING, MONOMER INFILTRATION, EMBEDDING AND POLYMERIZATION @ RT.***

DEHYDRATION PROTOCOL @ RT: 2 h to 4 h per change depends on the size of the bone. Larger bones may take up to a week in each change for complete dehydration. Extra changes are always allowed especially with large whole bones.

70% ethanol x 2 changes
80% ethanol x 2 changes
95% ethanol x 2 changes
100% ethanol x 2 to 3 changes

CLEARING @ RT: Xylene 2 h to 4 h (Alternative: 50% Absolute ethanol/50% methyl methacrylate monomer.

PMMA INFILTRATION: Recipes for mixtures are found in referenced publications (Sterchi published methods.) Note: Benzoyl peroxide is 99% water free (Sigma-Aldrich), store at 4 °C. Warm to RT before opening jar.

CAVEAT: Avoid water contamination. Any chemical or mixture stored in refrigerator must warm to RT before *opening containers to avoid water condensation.*

Use two PMMA mixtures for infiltration then embed in PMMA embedding mixture. **First** PMMA (monomer) change does not contain plasticizer dibutyl phthalate. **Second** PMMA (monomer) change contains a polymethylmethacrylate powder MW 996,000 Sigma-Aldrich and dibutyl phthalate. When infiltrating with any mixture, use vacuum up to 6 h, cap and store at 4 °C. You will see bubbles coming off the bone during vacuuming. Release and draw vacuum several times during a day mimics automated vacuum/pressure processing.

When making PMMA mixtures, mix @RT until mixture is clin disposable beaker, no more than 500 ml at a time. Change infiltration mixtures after a week.

Infiltration mixtures can suddenly polymerize so after vacuum during a day, move infiltration to 4 °C after vacuuming overnight or longer. Always warm samples to RT before removing lid for an infiltration change.

Refrigerators should be explosion proof for safety.

HINT: For extra hard PMMA, eliminate dibutyl phthalate. This is for some metal implants to be sawn, ground and polished for thick sections.

EMBEDDING: See Sterchi publications for embedding mixture recipe.

Embedding mixture contains methyl methacrylate monomer, benzoyl peroxide, poly methyl methacrylate powder and dibutyl phthalate. Stir mixture overnight, embed RT samples in glass or plastic vials, large plastic Rubbermaid style containers. Specimen bottles used for formalin fixation with perfectly flat bottoms are excellent, come in 40 ml, 60 ml, 90 ml sized with O-ring style lids.

A pre-polymerized PMMA layer in embedding mold can be made to hold the bone off bottom of mold/container. Embedding mixture interfaces with the layer during polymerization. Use the second infiltration mixture to make layers, and stack containers in a hood while layers polymerize – a good way to utilize waste 2nd infiltration mixture.

Open embedding molds allow evaporation of monomer. This changes the ratio of embedding mixture components, and results is terrible plastic blocks. Cover open molds tightly, and place in a sealed container for polymerization. Sealed containers make the best embedding molds.

POLYMERIZATION:

Use containers with lids, plastic preferred. Embedded bones are put under vacuum for 8 hours or until no air bubbles are seen. Cap container, and allow PMMA to polymerize. This can be done at RT, or if in small vials, in a 38 °C water bath. Gradually increase temperature to 45 °C. over several days. Some smaller blocks will polymerize is a day, even overnight. Preference: Specimens in large 250 ml Nalgene jars with lids (outside threads), place in hood @ RT. It can take a week or longer to polymerize. We avoided any extra heat or rapid polymerization with bubbles in and around bone will happen. ***NEVER USE A HEATED OVEN OR INCUBATOR FOR POLYMERIZATION,*** as these have uneven hot air currents.

FINALIZE POLYMERIZATION (CURING): Top of block will be sticky. Harden block by placing container with block in a 37 °C oven until block is hard. Go to a 60 °C oven for 4 h to 6 h. After curing, go to a freezer for 2 h, then remove block. Glass vials should be wrapped before breaking and remove any glass shards.

REMEMBER: PMMA is not a STAT protocol. Patience and attention to details is required. Be very careful handling and disposing of methyl methacrylate. It is a central nervous system toxin. Avoid fumes and contact with skin and wear double to triple nitrile gloves. A fume hood is required.

Troubleshooting guide on tissue processing and embedding of bone and teeth
For Transmission Electron Microscopy (TEM)
Submitted by Philip Seifert

I. Tips And Troubleshooting For Hard Tissue Samples

Tissue processing and embedding for conventional transmission electron microscopy (TEM) incorporates methods for soft tissues with additional modifications and considerations. Mineralized tissue requires proper fixation, dissection and trimming for optimal epoxy resin infiltration and embedment required for ultramicrotomy. The steps discussed here will start from post-fixation with osmium tetroxide and if required, after demineralization. Gentle agitation of tissue samples in Steps A-E may be used on either rotation or shaker equipment and an EM processor under chemical fume hood ventilation.

A. Post osmium tetroxide rinse

1. 0.1M sodium cacodylate buffers or deionized water are routinely used.
2. Time: Use at least two rinses for 2 minutes each, minimum.
    a. Storage in buffer under refrigeration for days may be held until subsequent processing steps.

B. *En bloc* stain (optional)

1. After uranyl acetate solution (0.5% to 2%) in distilled water or sodium maleate buffer (pH 6.0) is generally used at room temperature for times of 30 minutes to 2 hours.
2. Due to the low radioactivity and high toxic hazards of uranyl acetate, appropriate PPE and radioactivity monitoring is required or should be considered throughout the storage, handling, use, and disposal.
3. Storage of uranyl acetate should be 4 °C, shielded from light and locked in a box within the refrigerator for restricted access.
4. Nonradioactive uranyl acetate substitutes for *en bloc* staining should be evaluated and tested prior to use. [Reference 3]

C. Graded alcohol dehydration

1. The ethyl alcohol gradient starts at 25% ethyl alcohol then proceeds into 50%, 75%, 90%, 95%, and 3x 100% for times of 5 to 15 minutes at room temperature.
2. Extended alcohol dehydration times and changes may be necessary according to the size and type of mineralized sample type.

D. Transitional solvent

Acetone and propylene oxide are routinely used as transition solvents from ethyl alcohol into infiltration epoxy resin.

1. Temperature: room temperature
2. Time: several changes with total times of 15 minutes to 2 hours
3. Note: Propylene oxide is a highly flammable solvent with significant health hazards.
    a. Use appropriate PPE, prepare and use propylene oxide solutions under fume hood ventilation and follow established hazardous waste handling and disposal methods.

E. Epoxy embedding media

Epoxy Epon 812 replacement (comprised of Epon-812 substitute, DDSA, NMA and DMP-30) and Spurr's (comprised of NSA, ERL 4221, DER 736 and DMAE) are routinely used as infiltration and embedding resins for conventional TEM.

1. Proper solution preparation and storage; Prepare epoxy resin prior for use, the 'pot life' is generally estimated as around 24 hours. There are different epoxy resin mixture formulations ranging from soft, medium and hard based on the composition of epoxy monomers, plasticizers and chemical accelerators. The medium hardness formulation is generally acceptable for semi-thin and thin sections of most tissues during ultramicrotomy. Use of the hard formulation may result in additional support of the hard mineralized material during ultramicrotomy of undemineralized tissue yet may increase chatter sectioning artifact.
2. Use full mixture with accelerator for all infiltration and final polymerization steps.
3. Use disposable plasticware, syringes for volume measurements, and magnetic stirrer for mixture preparation.
4. Storage of epoxy resin in sealed syringes and frozen for use as initial resin infiltration may be used within a few weeks. Always thaw for several hours prior to use. Frozen epoxy resin mixtures are not recommended for final resin infiltration and polymerization steps.
5. Temperature: Room temperature to 30 ^{o}C under fume hood ventilation.
6. Time: several changes with total times of 12 hours to 2 days depending upon size, and bone sample type.
7. Vacuum may be applied during infiltration steps to aid in epoxy Epon resin infiltration. Vacuum infiltration is not recommended for Spurr's resin due to the volatile nature of the Spurr's resin monomers, which can alter the formulation and resin characteristics.
8. Epoxy resins health hazards and handling;
    a. Use appropriate PPE, prepare and use under a fume hood. Follow established hazardous waste handling and disposal methods for all used

solutions and mixtures. Epoxy resin components are suspect carcinogens.

F. Sample Embedment and Oven Curing

1. Using a dissecting stereo microscope, gently transfer each sample onto absorbent tissue into either a mold or capsule and orient to plane of cut.
    a. Epon epoxy resins can be fully cured uncovered in molds or capsules
    b. Spurr's resin should always be covered or capsules capped to fully cure and avoid a sticky, soft top interface.
2. A convection oven without fan ventilation should be used for curing epoxy resins;
    a. Temperature: 60 °C to 70 °C
    b. Time: 24-48 hours.

G. Troubleshooting

1. Soft or brittle polymerized resin blocks
    a. Review resin mixture preparation method, formulations, laboratory humidity and oven temperature.
    b. Spurr's resin should cure fully covered or capped.
        i. Use either the heated oven or laboratory microwave for curing < 1 minute.
2. Air dry artifacts during processing
    a. Sample exposed to air during solution and mixture transfer during manual processing.
        i. Keep solution or mixture covering over the sample in vial prior to transfer or delivery of new solution during processing steps.
    b. EM processor setup malfunction due to solution levels
        i. Check solution and mixture levels in containers of EM processor.
3. Incomplete infiltration
    a. Interior core of tissue sample has the risk of poor infiltration for TEM compared to periphery. May potentially be rectified through;
        i. Review of tissue collection, fixative solution preparation, perfusion versus immersion fixation, and post-fixation trimming procedure in detail.
        ii. Use freshly prepared dehydration i.e., gradient, alcohols, and resin solutions.
        iii. Propylene oxide and acetone transition solvents should be fresh and stored to avoid moisture from air. Tissue should always remain immersed in transition solvent and quickly transferred into resin infiltration solutions.
        iv. Precisely trim tissue smaller in dimensions to ~1 mm to 3 mm.

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