MethodsX 13 (2024) 103046

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/methodsx

Optimized Methyl methacrylate embedding of small and large undecalcified bones. ☆,☆☆

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ARTICLE INFO

Method name:
Methyl methacrylate embedding of small and large bones

Keywords:
MMA
Plastic
Bone
Histology

ABSTRACT

Methyl methacrylate (MMA) plastic embedding has been long established as a technique for the processing and histological assessment of bones. It provides the added benefit over paraffin in that it does not require decalcification of the tissue in order visualize the cellular detail, thus preserving vital information about the amount of unmineralized osteoid present in addition to the degree of mineralization in the bone. It also allows for the incorporation of dynamic histomorphometric analysis through the retention of fluorescent labels incorporated into the bone. Efficient infiltration of hard tissue is essential to the processing of bones and producing quality slides suitable for achieving usable quantifiable histology out the other end. This technique:

- Updates previously published MMA embedding protocols to reflect utilization of stabilized acrylamides (over the unstabilized reagents of the past)
- Outlines the techniques that are important for embedding both small (*mus*), medium (*rattus*), and large (porcine, lagomorph, human) histological samples.
- Updates the clearing and infiltration processes utilized and validates quality of the sample preparation though histological staining to confirm preservation of cellular detail, mineralization information, and enzymatic activity

Specifications table

Subject area:	Biochemistry, Genetics and Molecular Biology
More specific subject area:	*Bone histology*
Name of your method:	*Methyl methacrylate embedding of small and large bones*
Name and reference of original method:	Kacena MA, Troiano NW, Wilson KM, Coady CE, Horowitz MC. Evaluation of two different methyl methacrylate processing, infiltration, and embedding techniques on the histological, histochemical, and immunohistochemical analysis of murine bone specimens. *J Histotech*, 27:119–130, 2004. https://doi.org/10.1179/his.2004.27.2.119
Resource availability:	*Jung gigatome:* https://www.gigatome.de/04_products.html *Tungsten carbide blades:* https://dornandhart.com/product/tungsten-carbide-d-profile-knife-for-rotary/

☆ **Related research article:** Bone Journal reference: BON 117198 Article reference: BON_BONE-D-24-00173 (Accepted for publication on July 7, 2024).

☆☆ **For a published article:** T. A. Nelson , S. Tommasini , J. A. Fretz, Deletion of the transcription factor EBF1 in perivascular stroma disrupts skeletal homeostasis and precipitates premature aging of the marrow microenvironment, Bone 187 (2024) 117198. https://doi.org/10.1016/j.bone.2024.117198

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https://doi.org/10.1016/j.mex.2024.103046
Received 4 September 2024; Accepted 11 November 2024
Available online 13 November 2024

Background

Bone pathology and biology often can only be completely understood through an analysis of non-decalcified tissues. Obtaining high quality bone sections from these mineral-laden matrices, however, is not easily accomplished, and requires specific conditions to obtain proper fixation, efficient dehydration, optimal infiltration, and calibrated polymerization. As a result, embedding mineralized tissues for histological assessment is a slow and nuanced process. We report here an update of the processing protocols we utilize to obtain efficient and reproducible sections from small (mouse), medium (rat), and large (rabbit) non-decalcified bone specimens in methyl methacrylate (MMA). The protocols are separated for processing based on the size of the samples. Longer infiltration and hardening times are required for larger/thicker/more highly mineralized bones. Resultingly, small samples can be processed more rapidly than larger samples. Importantly, these protocols provide updates to the reagents to consider the safety of the researchers and include a transition from destabilized to stabilized MMA. Monomer preparation for destabilized reagents involves a complex process involving hydroxyquinone, sodium hydroxide, phase separations, and clearance though CaCl [2]. Destabilized plastics also have the potential to exothermically polymerize with explosive consequences within closed containers. Inclusion of commercially available stabilized MMA is not only safer, but also easier to handle methodologically.

Method details

A note: It is not specifically indicated at each step, but proper ventilation should be employed when utilizing the chemicals in this protocol. Many are hazardous, extremely volatile, and should be mixed and handled with proper PPE within a fume hood.

Tissue preparation

1. Femora were dissected free of adjacent soft tissue.
2. Bones were placed directly into an excess of 70% ethanol (5 ml for a mouse femur; 15 ml for a rat femur; 50 ml for a rabbit sample) and kept at 4°C for at least 24 h (mouse), 48 h (rat), or 48–96 h (rabbit). If the bones are not processed immediately, they can be stored in this solution at 4°C for a month (unpublished observation and Frost [3]).
3. Bones were transferred to a tissue processor for dehydration and clearing using the following program:

Solution	Duration	Temp	Vacuum
70% EtOH	1.5 h	Ambient	P/V*
95% EtOH	1.5 h	Ambient	P/V
95% EtOH	2.0 h	Ambient	P/V
100% EtOH I	3.0 h	Ambient	P/V
100% EtOH II	4.0 h	Ambient	P/V
100% EtOH III	4.0 h	Ambient	P/V
50/50 100% EtOH/Toluene	3.5 h	Ambient	P/V
Toluene I	2.0 h	Ambient	P/V
Toluene II	2.0 h	Ambient	P/V
Toluene III	2.0 h	Ambient	P/V

Basic MMA solution

All plastics used for embedding utilize a common basic MMA consisting of 85% (by volume) methyl methacrylate monomer (MilliporeSigma # M55909), and 15% plasticizer dibutylphthalate (MilliporeSigma # 524980).

The hardness of the MMA matrix is not adjusted. We have found that the use of 85%/15% MMA/DP is optimal for most situations and matches the density of bone from laboratory and experimental farm animals quite well.

Preparation of pre-polymerized bases

Bases are made by combining 2.5 g of 70% active (water weight) Benzoyl Peroxide (PolySciences # 21446) in 100 mL of basic MMA solution. A 1–2 cm thick base is poured into the container of an appropriate size. Small containers need a 1 cm thick base (4–5 mL in a 20 mL vial). Larger samples need a thicker base (2 cm). Bases are left to sit on the benchtop for 24 h and then transferred to a radiant heat oven to polymerize at 37°C for 5 days. The bases function to elevate the samples within their containers, and also assist in nucleating polymerization. Examples of bases and how they elevate the sample are demonstrated by Fig. 1A,B).

Infiltration and polymerization

Small (mouse) Bones

1. Infiltration begins with basic MMA solution. Bones are placed into glass vials or plastic (Polycarbonate) jars large enough to accommodate the bones (6 ml of solution in a 20 ml vial). Sample containers are loosely capped and stored under vacuum in a glass desiccator at 4°C for 3 days.

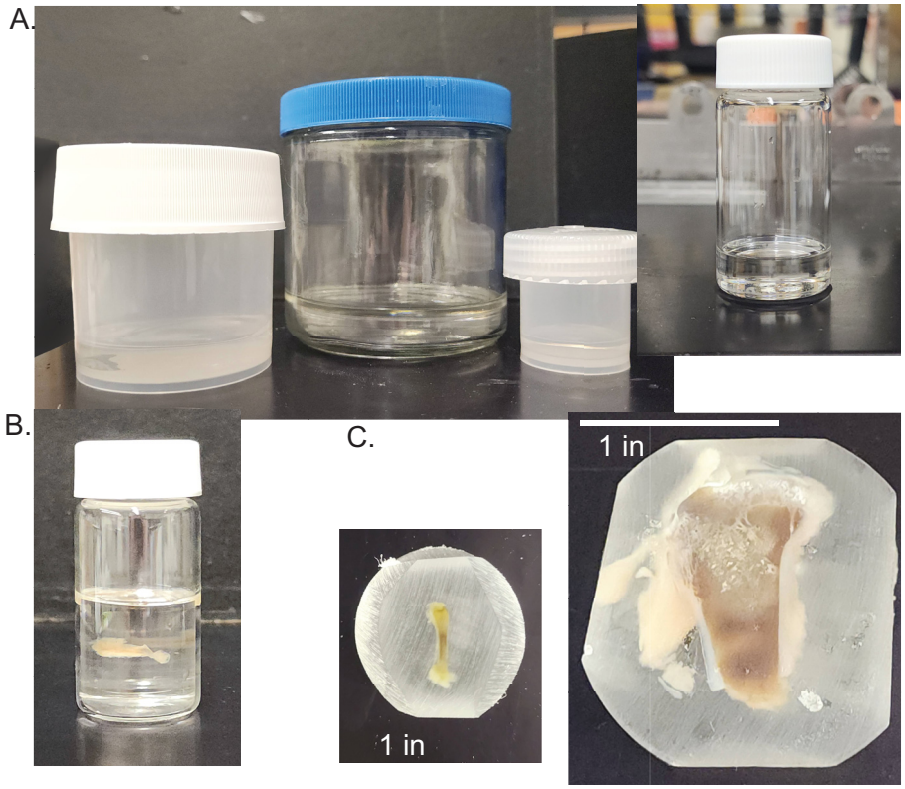

Fig. 1. (A) MMA bases in containers of various sizes to accommodate the proportions of different bones. (B) Mouse tibia elevated off the bottom of the vial in a polymerized MMA block. (C) MMA blocks after grinding and polishing.

2. After infiltration samples are transferred to vials containing pre-polymerized bases (see above) and topped with basic MMA mixed with 2.5% benzoyl peroxide. Containers are filled with enough solution to allow for at least 1.5 cm of liquid above the top of the bone (9 ml of solution in a 20 ml vial).
3. Tightly capped samples are left at room temperature for 48 h.
4. Then, the vials are transferred to an open shallow container to cover the lower 1/3 of the container with ddH2O. This water bath helps dissipate heat generated during polymerization. Vials are placed in a 37°C radiant heat oven for 4 days to complete polymerization.
5. Samples are freed from containers by wrapping them in cloth for safety purposes, and then breaking the glass.

Medium (rat) and Large (rabbit/human) Bones

1. Infiltration begins with basic MMA solution. Bones are placed into glass vials or plastic (Polycarbonate) jars large enough to accommodate the bones (See Fig. 1). Sample containers are loosely capped and stored under vacuum in a glass desiccator at 4°C for 3 days.
2. Bones are transferred to vials containing basic MMA mixed with 1% benzoyl peroxide. The samples are loosely capped and stored for an additional 3 days within the vacuum desiccator at 4°C.
3. Bones are transferred to vials containing basic MMA mixed with 2.5% benzoyl peroxide. The samples are loosely capped and stored for an additional 3 days within the vacuum desiccator at 4°C.
4. After infiltration is complete, samples are transferred to vials containing pre-polymerized bases (see above) and topped with basic MMA mixed with 2.5% benzoyl peroxide . Containers are filled with enough solution to allow for at least 1.5 cm of liquid above the top of the bone.
5. Tightly capped samples are left at room temperature for 4–10 days until the majority of the MMA solution is soft-set and no longer liquid.
6. The vials are then transferred to a 37°C oven for 4 days to complete the polymerization and hardening process.
7. Samples are freed by cutting the plastic container away.

Sectioning

1. Blocks were cut to size with a hand saw and sanded on a Buehler Metaserve™ Grinder-Polisher with 80 (Buehler #36-08-0080) or 240 (Buehler #36-08-0240) grit grinding paper for coarse grinding and polishing, respectively. Blocks are polished to bring the desired plane for sectioning close to the surface of the plastic.

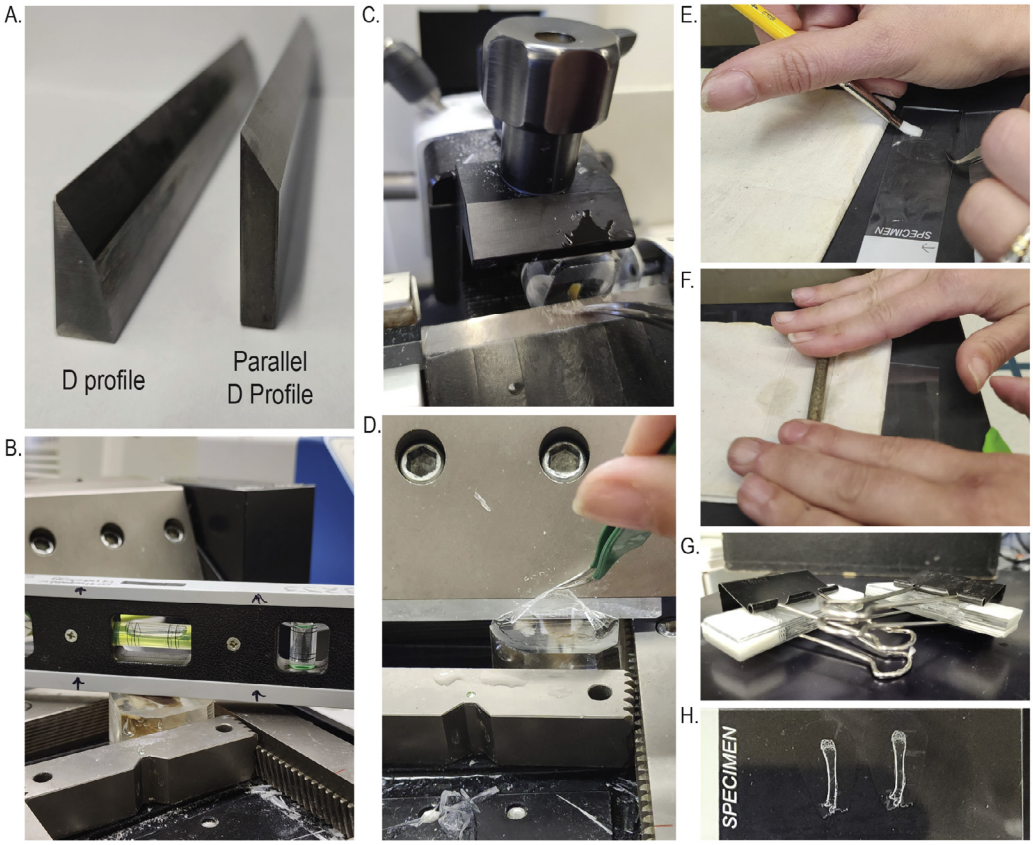

Fig. 2. (A) Differences in the shapes of tungsten-carbide blade profiles. (B) Leveling MMA blocks in the gigatome. (C) Collecting sections off the face of the microtome. (D) Collecting sections from the knife of the gigatome. (E) Flattening sections on the surface of the slides. (F) Rolling sections flat. (G) Stacked slides in clamps to adhere sections. (H) Adhered sections of mouse femur.

2. **Small:** Prepared specimen blocks were clamped directly into a Leica 2165 Microtome (Leica, Heidelberg, Germany) block holder and trimmed using a tungsten-carbide knife, D-Profile (Dorn and Hart Microedge, Loxley, AL). (The different knife shapes are illustrated in Fig. 2A.)
   Medium/Large: Prepared specimen blocks are clamped into a Jung Polycut E or Gigatome (Roggenburg, Germany) mounted with a Parallel D-Profile tungsten-carbide blade (Dorn and Hart Microedge, Loxley, AL), and the block face was leveled (Fig. 2B).
3. The block face and knife were moistened with 40% EtOH to facilitate sectioning.
4. Once the desired depth is reached, 5 μm sections were removed from the knife blade with fine forceps (Fig. 2C&D). And placed on a chrome-alum-coated microscope slide in a puddle of 70% EtOH. (Thicker 8μm sections were cut to visualize mineralization labels like calcein and alizarin red). The protocol for gel-coating slides is detailed below in the following section.
5. The sections were then teased and flattened with a fine paintbrush (Fig. 2E).
6. Sections were then covered with a strip of clear plastic (cut from the packaging of Fisherbrand glass pipettes), and rolled smooth to remove the remaining wrinkles and excess EtOH (Fig. 2F).
7. Slides were stacked and pressure was applied using large binder clips (Fig. 2G).
8. Sections were incubated overnight at 37˚C to adhere sections to the slides.
9. After adherence the plastic cover is gently peeled away from the dried sections. The slides are now ready to be used (Fig. 2H).

Chrome-alum gel slide preparation

Solution A
9.0 g of gelatin, type A, 275 Bloom (Fisher, G8)
1000 mL dH_2O
Dissolve the gelatin by warming the water to 60˚C and mixing with a magnetic stirrer.
Solution B
4g of chromium potassium sulfate dodecahydrate (Fisher #C337)
100 mL dH_2O

This solution can be stored at 4°C for several months.

1. Combine 1000 mL of solution A with 38.5 mL of solution B
2. Warm solution A+B to 75°C in a stainless steel staining dish.
3. Clean slides are dipped for 2 min.
4. Slides are stood on edge to drain.
5. Slides are transferred to 60°C to dry overnight.

Deplasticizing and Rehydrating Sections

Removing the MMA through out the sections is the essential first step to performing any subsequent stains on the samples. It is achieved as follows:

1. Slides are submerged in three changes of Cellosolve™ (2-ethoxyethyl acetate, MilliporeSigma #109967) for 30 min each.
2. Slides are transferred to 70% EtOH for 5 min.
3. Slides are transferred to 40% EtOH for 5 min.
4. Sections are transferred to dH_2O for 5 min.

Staining protocols

Hematoxylin and Eosin (H&E) staining

Our protocol is adapted from the protocol of Sheehan and Hrapchak [4].

1. Deplasticized slides are stained for 10 min in filtered Harris formula hematoxylin (Fisher #245-651).
2. Slides are rinsed with running tap water until it runs clear (5 min).
3. Sections are differentiated in acidic alcohol (70% EtOH with 1% acetic acid) for 5 s.
4. Slides are rinsed with running tap water for an additional 5 min.
5. Slides are blued in weak ammonia solution (10 mL ammonium hydroxide in 1000 mL of dH_2O) for 30 s.
6. Slides are washed for an additional 5 min in running tap water.
7. Slides are transferred to 80% EtOH and partially dehydrated for 1 min.
8. They are stained with alcoholic eosin Y (Epredia #71211) for 2 min.
9. Excess eosin is washed away with sequential washes in 100% EtOH. The first is for 1 min, the second for 3 min and the third for 5 min.
10. Sections are then fully dehydrated with three exchanges of xylene (Fisher #016371).
11. Sections are mounted with Epredia media #4112.

Toluidine blue

This method is adapted from Baron et al. [5] (page 22).

1. Citric acid buffer was prepared by combining 1.58g citric acid (Fisher #A104), 0.75g of disodium phosphate (JT Baker #3828-01), 1000 mL dH_2O, and adjusting the pH to 3.7.
2. 2 g of Toluidine blue O (Fisher #T161) were added to 100 mL of buffer, mixed thoroughly, and then filtered. The pH was checked to be sure it remained at pH 3.7.
3. Deplasticized and rehydrated slides were immersed in stain for 15–20 min at room temperature.
4. The slides were rinsed in three changes of citrate buffer and blotted dry.
5. Slides were allowed to fully dry on the benchtop for at least 20 min.
6. Sections were dehydrated rapidly through two changes of tert-butanol (Fisher #A401), one change of 50%/50% tert-butanol/toluene, and two final changes of toluene (Fisher #T324).
7. Slides were mounted with Epredia media #4112.

(Mineralized tissue is medium blue/purple, unmineralized osteoid is pale blue, and cartilagestains dark blue/purple.)

Alkaline phosphatase (ALP) staining

Our protocol is adapted from that of Baron et al. [5] (pages 32–33).

1. Stock Tris buffer was prepared as follows: 12.1 g Trizma base (American Bioanalytical #AB02000) was dissolved in 500 mL of dH_2O.
2. “Working Tris buffer” is made by combining 25 mL of stock Tris buffer, 1 mL of 0.2 M hydrochloric acid, and 74 mL of distilled water. The pH was adjusted to 9.4 with sodium hydroxide or hydrochloric acid.
3. Slides were incubated at room temperature in working Tris buffer for 1 h
4. The staining solution was prepared fresh as follows:
    a. Mix A: 40 mg of Naphthol ASBI phosphate (MilliporeSigma #N2250) was dissolved in 2 mL of N,N-dimethylformamide (MilliporeSigma #319937)
    b. Mix B: 40 mg of Fast Blue RR salt (MilliporeSigma #F0500) was dissolved in a second 2 mL of N,N-dimethylformamide

c. Combine A and B and then add 0.195 g $MgCl_2$. This admixture is then added to 35 mL of working Tris buffer, mixed well, and filtered.

5. The slides are removed from the buffer, blotted, and the stain is dropped directly onto slides in a humidification tray. The sections are incubated for 1 hour at 37°C.
6. After color development, the slides are rinsed with water, mounted with aqueous CrystalMount™ (Accurate Chemical & Scientific Corp #BMDM02), and hard set by utilizing a slide warmer set to 60°C. Slides are then cover slipped using Epredia #4112.

(ALP-positive cells will appear purple. The surrounding tissue will have a pale brown color.)

Tartrate-resistant acid phosphatase (TRAP) staining

Our method is an adaptation of the Cole and Walters protocol [6] with a Naphthol reagent substitution.

1. TRAP incubation buffer was made by combining 9.2 g anhydrous sodium acetate (MilliporeSigma S-2889), 11.4 g tartaric acid (MilliporeSigma, T-6521), 2.8 mL glacial acetic acid, dH_2O to 1000 mL final volume. The pH is adjusted to 4.7–5.0 with sodium hydroxide and acetic acid.
2. Working TRAP buffer was created by combining 120 mg of Fast Red Violet LB (MilliporeSigma #F3381) and 200 mL of "incubation buffer" from step 1. This is stirred over low heat (warm to 37°C). Then, 20 mg of Naphthol AS-MX phosphate (#N4875) is dissolved in 1 ml of N,N-dimethylformamide (MilliporeSigma #319937), and poured into the warm working buffer.
3. Deplasticized sections are incubated in working TRAP solution for 1 hour at 37°C for enzymatic color development.
4. After incubation, slides are washed 3 times with dH_2O (5 min each).
5. Slides are counterstained with Fast Green FCF (Fisher #BP123)(0.02% solution in dH_2O) for 3 min.
6. The sections were then quickly rinsed in dH_2O.
7. The slides were dehydrated by sequential passage through two incubations each of 95% EtOH (2 min each), 100% EtOH (2 min each), and 3 incubations for 2 min each of Xylene.
8. Slides are cover slipped with Epredia media #4112.

(TRAP-positive cells are reddish pink. The surrounding tissue stains green.)

Alizarin red staining

Our protocol is adapted from the Armed Forces Institute of Pathology [7] (page 198).

1. Deplasticized slides are first counterstained with 0.2% Light Green (Electron Microscopy Sciences #26386-07) for 1 min.
2. The slides are then quickly rinsed by dipping in dH_2O.
3. They are next immediately incubated in Alizarin red staining solution (2 g Alizarin red S (MilliporeSigma #A5533) in 100 mL dH_2O and the pH is adjusted to 4.1–4.3 with ammonium hydroxide) for 5 min.
4. After incubation, the slides are rinsed by quickly dipping them sequentially in dH_2O, acetone, 50%/50% acetone/xylene, 100% xylene (3xs)
5. Slides are mounted with Epredia media #4112.

(Mineralized tissue appears red and the surrounding tissue appears green.)

Von Kossa staining

This protocol is performed as previously reported by Baron et al. [5] (page 23).

1. A 5% solution of silver nitrate (Fisher #S486) is made and filtered.
2. Deplasticized slides are incubated in the silver nitrate solution at room temperature for 30 min in the dark.
3. The slides are then washed with three changes of dH_2O for 5 min each.
4. 5% sodium carbonate- formaldehyde solution is prepared as follows: 75 mL of dH2O and 25 mL 37% formaldehyde (Fisher #33314) are combined, then 5 g of sodium carbonate (Sigma #S-7795) was dissolved in the mixture.
5. Slides are differentiated in the sodium carbonate-formaldehyde solution for 2 min.
6. The slides are then washed for 10 min in running tap water.
7. Counterstaining is achieved with methyl green pyronin (Sigma #HT70116) for 20 min.
8. The slides are washed in two 1 min changes of dH_2O.
9. The samples are then dehydrated in on change of 95% EtOH for 1 min, and two changes of 100% EtOH for 1 min each, then cleared through two changes of xylene, (2 min each).
10. Coverslips are applied using Epredia media #4112.

(Mineralized tissue appears black and osteoid appears red to light pink.)

Safranin O staining

This protocol is adapted from the Armed Forces Institute of Pathology [7] (page 197).

1. Deplasticized slides are placed in Weigert's solution for 7 min to stain nuclei. Weigert's is made by mixing equal parts Weigert Solution A (Electron Microscopy Sciences #26044-06) and B (Electron Microscopy Sciences #26044-16).
2. Slides are washed for 10 min with running tap water to remove excess stain.
3. The sections are then incubated in 0.01% Fast Green solution (0.1 g Fast Green, FCF (Fisher #BP123 in 1000 mL dH_2O) for 5 min.

4. Samples are quickly dipped in 1% acetic acid (10 mL glacial acetic acid in 1000 mL dH_2O).
5. The slides are then stained with 0.1% Safranin O for 5 min (0.1 g Safranin O, MilliporeSigma #S-8884 in 1000 mL dH_2O).
6. The slides are rinsed quickly in dH_2O.
7. They are then incubated in 1% acetic acid for 5 min.
8. The sections are next sequentially passed through sequential washes (2 min each) of 95% ethanol (twice), 100% EtOH (twice), xylene (3 times).
9. Mounting was accomplished with Epredia media #4112.

(Cartilage/proteoglycans stain red while the surrounding tissue stains pale green.)

Goldner's trichrome staining

This protocol is adapted from the work of Luna [8] (pages 151–152).

1. Deplasticized sections were first treated with Bouin's solution (MilliporeSigma #HT10132) overnight at ambient temperature.
2. Slides were then washed with running tap water for 10 min until excess color was washed away.
3. The sections were then incubated with Weigert's hematoxylin (mixed the same as above in Safranin O) for 10 min.
4. The samples were again rinsed with running tap water for 10 min.
5. Slides were next stained with Ponceau Acid Fuchsin (Electron Microscopy Sciences #26386-04) for 5 min.
6. They were then quickly rinsed in 1% acetic acid solution (see Safranin O above).
7. Then, they were transferred to Phosphomolybdic Acid-Orange G (Electron Microscopy Sciences #26386-06) solution for 10 min to decolorize the collagen.
8. The slides were quickly rinsed again in 1% acetic acid.
9. They were then stained with Light Green (Electron Microscopy Sciences #26386-07) for 5 min.
10. A final incubation in 1% acetic acid for five min happens next.
11. The sections are then blotted (but not dried) to remove excess liquid.
12. Dehydration is completed through 5 min incubations in 95% EtOH (three times), 100% EtOH (three times), and xylene (three times).
13. The sections are mounted with Epredia media #4112.

(Mineralized matrix stains green, cartilage stains pink, and osteoid stains red.)

Method validation

To validate this method sections of MMA-embedded bones from mouse, rat, and rabbit were processed, sectioned, and stained as described above. The images in Fig. 3 illustrate how the various matrix and cell identities stain with each protocol. They also demonstrate the quality of cellular detail that can be obtained using these protocols.

For Toluidine blue staining, mouse femora exhibit few microfractures through the entirety of the mineralized tissue, and exceptional cellular detail can be seen at higher magnification (Fig. 3A). The stain clearly delineates unmineralized osteoid from the adjacent mineralized scaffold. The clearly identifiable cuboid osteoblasts and amorphous multi-nucleated osteoclasts are also easily discernible and highlighted. For these reasons, Toluidine blue is preferred by our laboratory for determining the basic cellular detail from MMA sections.

Information on mineralization can also be achieved by the application of Von Kossa and Alizarin Red stains (Fig. 3B & J, respectively). These, however, only interact with the mineralized portion of the matrix and do not provide as clear cellular detail for osteoblasts and osteoclasts. The Green counterstain of Alizarin Red does not make any cell types obviously identifiable. Von Kossa, by contrast, employs a counterstain that can differentiate osteoid, and osteoblasts, but smaller osteoclasts can be difficult to identify at times.

H&E by contrast (Fig. 3E, F), still contains excellent cellular detail, but does not always delineate a difference between the mineralized matrix and the osteoid. While thick osteoid fronts are identifiable with H&E, thinner layers are not easily distinguished from the adjacent cells or mineralized matrix. While most mineralized bone stains uniformly pink with eosin (Fig. 3E, rat femur), there is also a propensity for transitional bone near the growth plate to have light staining by eosin (Fig. 3F, mouse femur). Conversely Hematoxylin sometimes reacts with the mineralized regions (visible under the edge of the marked osteoid in Fig. 3f') creating a purple haze within the mineralized bones.

There are specific enzymatic activities of the osteoblast (ALP, Fig. 3D) and the osteoclast (TRAP, Fig. 3C), and these can be visualized by stains implementing colorimetric changes precipitated by these respective phosphatases. This protocol for MMA embedding is gentle enough to preserve both enzymes in a conformation and condition that allows for the continued catalysis of the respective reactions for each. Neither stain allows for the discrimination of mineralized tissue from osteoid, nor for the identification of other cell types, but they can provide verification of the presence of osteoblasts and osteoclasts on the bone surfaces.

Safranin O, alternatively, provides information about the quality of the cartilage present (Fig. 3G-I). This is a standard stain employed by paraffin-embedded decalcified sections. Here too, similar information can be obtained regarding the proteoglycan content of the cartilage with MMA as is obtained from paraffin-embedded decalcified bones employing this stain. It does not provide information on the osteoid content, nor does it make osteocytes easy to visualize (Fig. 3H).

Goldner's Trichrome stain is helpful for discriminating cartilage from mineralized bone and is visualized in Fig. 3K. Like Eosin, the Light Green stain within mineralized matrix can have low penetrance within the transitional bone adjacent to the growth plate

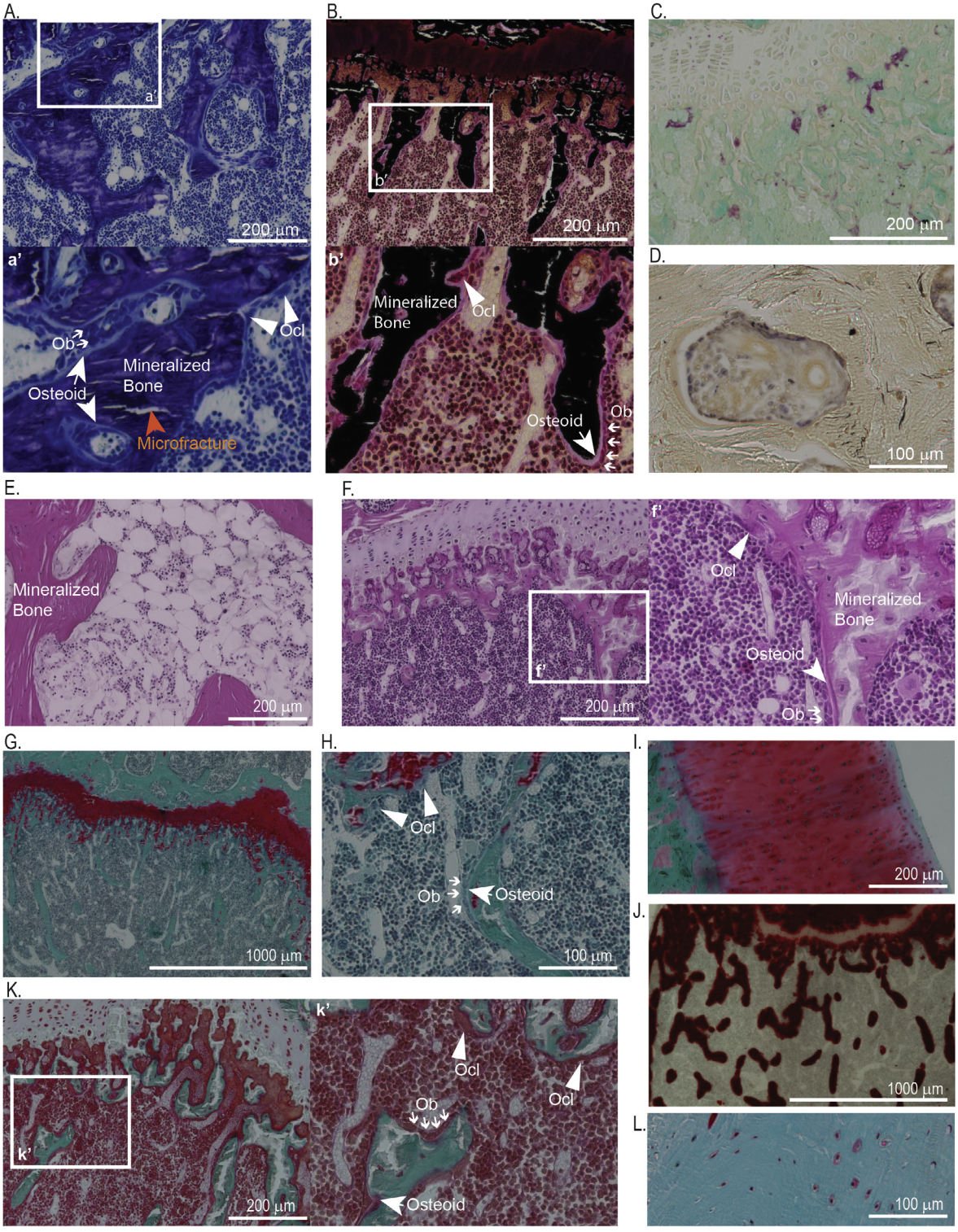

Fig. 3. (A) Tol blue staining of mouse femur. Ob (osteoblast), Ocl (osteoclast). (B) Von Kossa Staining of mouse femur. (C) TRAP staining of osteoclasts in a mouse femur. (D) ALP staining of rat femur. (E) H&E staining of rat femur. (F) H&E Staining of mouse femur. (G & H) Safranin O staining of mouse femur. (I) Safranin O staining of Rabbit articular cartilage. (J) Alizarin Red staining of mouse femur. (K) Goldner's Trichrome stain of mouse femur. (L) Goldner's Stain in cortical mouse bone displaying osteocytic osteolysis in a mouse model of chronic kidney disease [1].

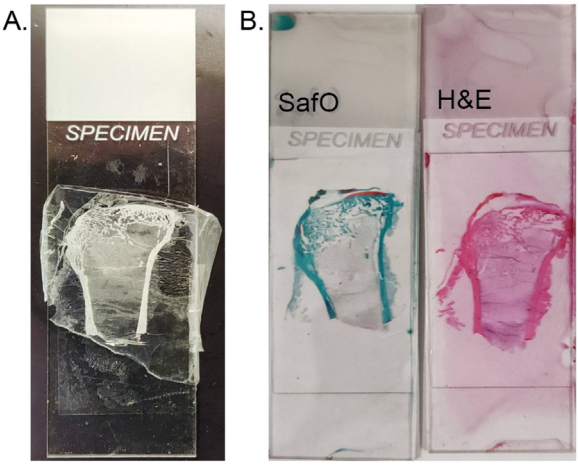

Fig. 4. (A) MMA sections of rabbit bone overhanging the edges of a standard 1 × 3 inch laboratory slide. (B) Section quality after Safranin O and H&E staining of these overhanging sections.

(Fig. 3K). Osteoid will stain a deep red, while cartilage is pink and mineralized bone is green. Goldner's is especially helpful in visualizing severely osteopenic bones, and those bones exhibiting osteocytic osteolysis as the lacunae will be surrounded by a pink halo (Fig. 3L). It is harder to discriminate osteoid in Goldner's from the adjacent osteoblasts than in Toluidine Blue stain.

Limitations

Please note that the fixation utilized by this protocol only involves 70% EtOH. There is no formalin/formaldehyde reagent-mediated step. Cellular detail is preserved better without the addition of a crosslinking fixation [9]. This is an important distinction from standard paraffin embedding that can be easily overlooked and will negatively affect the results. Should researchers prefer to prepare their bones with 10% neutral buffered formalin the following modification can be applied at the time of dissection. The bones should be stripped free of the surrounding soft tissue, then fixed for 16–24 h (mouse), 48 h (rat), 48–96 h (rabbit). The samples should then be rinsed thoroughly under cool running tap water (60 min), transferred to 70% ethanol, and stored at 4°C for a further 24 h (mouse), 48 h (rat) or 48–72 h (rabbit) before proceeding to dehydration and clearing. It is important to assure that a buffered solution is utilized for these steps to neutralize any formic acid that may accumulate during the incubation and adversely affect the mineralization or proteoglycan quality, or result in the deposition of formaldehyde hematin upon reactions with hemoglobin [10]. Improvements in the quality of VK and Tol Blue staining as well as osteoclast cellular detail, over that obtained with buffered formalin, can be obtained by inclusion of the non-crosslinking formaldehyde alternative Histochoice® (Sigma #H2779) [11]. This benefit of HIstochoice is offset by a decrease in the TRAP activity [11]. Should Histochoice® be used as a fixative, the same times that are outlined here for neutral buffered formalin should be used.

The protocol outlined in this paper also calls for the use of toluene inside of a tissue processor. Some laboratories may not want to use this in their machines as xylene is more traditionally implemented. We have found, however, that toluene provides superior preservation of the cellular detail compared to xylene for bones.

It is imperative that the rate of polymerization be controlled when implementing work with MMA. Use of a radiant heat oven is important to provide even heat to all surfaces of the samples while they are being cured. Hardening is also an exothermic reaction. If a large volume of MMA is transferred to the radiant heat oven before it has been partially or fully solidified the quality of the sample will be affected. In the most mild of situations there may be a shifting of the position of the bone within the vial. In more extreme situations bubbles can from within and surrounding the sample ruining the tissue or the quality of the block. In the most excessive situations rapid polymerization can generate excessive pressure causing the vial to break or even explode.

Additional limitations of this procedure are the size of the bones that can be processed, and the need for specific instruments to handle larger specimens. Mouse bones can generally be accommodated on a benchtop microtome equipped with the appropriate blades and sample clamps. While acquiring these mounts and blades requires a moderate capital investment, processing larger bones mandates the use of a gigatome and a significant purchasing cost. The gigatome can accommodate a block up to 80 × 100 mm. Allowing for the necessary collar of MMA around all sides of the section, a maximum size of the tissue would be around 60 × 80 mm. Placing these large sections will also require the use of oversized slides (4 × 3 inch slides will allow for this, and are available). Additionally, the larger the blocks turn out to be the more difficult it becomes to manipulate and effectively smooth the sections onto slides without introducing wrinkles or tears into the sections.

In order to image and visualize the sections the laboratory will also have to be equipped with microscopes with oversized stages to hold these larger slides, or slide imaging systems with holders that can accommodate the larger slides. Due to the dimensions of bones, it is possible that larger bones can sometimes be placed on a slide that is smaller than the block. An example of this is in

Fig. 4 with a rabbit humerus placed on a standard 1 × 3 inch microscope slide. The extraneous MMA overhanging the edge of the slide will be dissolved during the deplasticization process without impacting the quality of the resulting sections.

Third, there are some staining limitations to the use of this protocol. The use of gelatin-coated slides does create some background haze for some stains. This is demonstrated in Fig. 4B by the Eosin haze present with the H&E stain. This is not a large issue at high magnification, but occasionally there will be a visible line of background stain that can traverse the tissue section.

Not all stains interact with the gelatin. Only select stains like Eosin and Fast Green will generate nonspecific haze. Other protocols, like all the reagents used in the Toluidine Blue or the Von Kossa staining protocols, have no reactions with the gelatin. This protocol outlined here has optimized the gelatin hardness and concentration to minimize off-target staining, yet still maintain adherence by the MMA sections. For smaller samples the nonspecific staining can be avoided by substituting gelatin-coated slides with silane-coated slides (many brands are commercially available). Adherence to silane slides can be enhanced by baking the pressed sections overnight at 60°C. (This will decrease the enzymatic activity of the sections.) Extreme care needs to be used with these slides when washing the tissue. Additionally, the larger the tissue is, the more prone the sample will be to partial or complete lifting from silane-coated slides.

The last set of limitations for this protocol involves the end goal of the research being performed. While the embedding process is gentle, as demonstrated by the preserved enzymatic activity of both TRAP and ALP utilized for the stains described above, the chemicals that are used for handing of plastics affects the ability of these sections to be used for immuno-histochemistry. Antibody epitope sensitivity will vary on a case-by-case basis, but antibodies that work in paraffin-embedded sections do not always guarantee epitope recognition will be retained in MMA sections. We have not optimized our protocol for the implementation of IHC, and instead suggest the use of paraffin embedding for this. Inclusion of a formalin or Histochoice® fixation step can be beneficial for improving preservation of antigenicity in MMA [11], but the applicability of an antibody to MMA sections is not listed on descriptions from providers, and the optimal fixation and processing needs to be determined and modified on an individual antigen basis. Furthermore, as platforms and specific chemistries continue to be developed for spatial genomic and proteomic analysis of tissue sections, MMA-embedded sections are generally unable to be utilized for these purposes with the protocols that exist at this time.

Ethics statements

All animals used complied with the ARRIVE guidelines and were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

CRediT author statement

Jackie A. Fretz: Conceptualization, methodology, writing- original draft preparation, visualization.
Nancy W. Troiano: Methodology, validity tests, writing- reviewing and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

We thank the Yale Department of Orthopaedics for continuing to maintain and operate the Orthopaedics Research Histology and Histomorphometry Laboratory for the past 40+ years. Mouse bones visualized here were group housed in pathogen-free conditions within the Yale facilities on corncob bedding with 12 hour light/dark cycles. Yale Medical School IACUC approved the purchase and use of all animals (protocol #10543). Sprague-Dawley bones imaged in this paper were supplied by Dr. Robert Canales at the University of Florida and used here with his permission [12]. Rabbit bones imaged in this report were collected following terminal surgeries performed by the Yale YARC Veterinary Staff.

This research did not receive any specific grants from funding agencies in the public, commercial, or not-for-profit sectors.

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