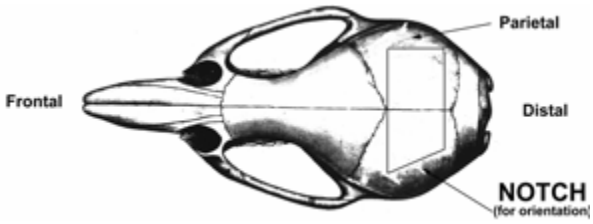
MCI Core Procedures for Bone Preparation

1. Bone Dissections

Femur is preferred for µCT and histology. You can use the tibia for RNA isolation (See separate instructions.).

Calvaria. Remove the skin. Cut off the entire top of the skull and remove the brain. Carefully cut out the parietal bone (use very sharp scissors to avoid shattering the bone). The skull should not be cut down the center suture (leave intact). Make a small diagonal cut on one distal side of the bone to denote the back as is illustrated in the diagram below:



Add the bone to 4% Paraformaldehyde/1X PBS, pH7.4, 4oC, in a plastic tube. Fill the tube to the top with the fixative; label each sample both on the inside foil and the top of the cap; tighten and parafilm the cap. In the event there is breakage, this will prevent the sample number from being effaced by the alcohol. Refrigerate until ready to mail. The bones should be mailed the same day or the next day, on ice.

Femur. Dissection should involve removing the entire limb. Once the limb has been removed from the pelvis, the remaining skin should be peeled off the limb and the foot removed. Carefully separate the femur from the tibia. Use gentle dissection to remove most of the connective tissue overlaying the bone. Do not scrape down to the bare bone. Remove majority of muscle but leave some on to keep the periosteum intact.

Since the analysis is done on the distal portion, extra care must be used to preserve damaging the condyles. Try to keep intact with the epipyhsis (cartilage cap). Excise femur at the joint in the upper extremity of the hip socket and the tibia at the ankle. If the mouse is large, separate the tibia and femur at the knee joint with a scalpel. Dissect quickly so that the limb can be placed into fixative as soon as possible.

Note: Other long bones prepare as for femurs. For µCT analysis of vertebrae, dissect individual vertebra to bone. Identify and maintain vertebral level (e.g. L3). For histomorphometry only, several vertebral units may be processed together.

Place in fixative immediately upon dissection. Label each sample on the outside of the tube and include a pencil written label on the inside as well. Secure the lid and then cover with parafilm to prevent ethanol leakage.

Include an inventory of all samples in the shipment.

2. Fixation solutions

Recommended for preserving bone enzyme alkaline phosphatase (bone formation) and acid phosphatase (bone resorption):

2A - Standard 4% PARAFORMALDEHYDE (PFA)

UNDER HOOD

100g PFA
2250ml ddH_2O
250ml (10x) PBS

Heat H_2O to 75°C
Add PFA (with constant stirring) to hot water in fume hood
Stir until solution becomes clear
Add 10x PBS
Cool solution slowly and pH to 7.4
Store at 4°C
Fix bones in 4% PFA at 4°C for 2-3 days before proceeding.

NOTE: Large specimens (≥6months) should be fixed 3-4 days (no longer than 5 days). It is best to put sample in vials (loosely capped in desiccators in the cold room (no desiccant) hooked to house vacuum for better penetration.

2B - PLP Fixative (For Alkaline Phosphatase Enzyme Preservation)

Stock Solutions:

SS1 8% Paraformaldehyde (8% PFA):
Dissolve 8gm of paraformaldehyde in 100mL double-distilled water (ddH_2O). Heat the mixture to 60-65 °C. Add 0.270gm glucose. Adjust the pH 7.4 with NaOH.

SS2 0.2*M* Lysine –HCL:
Dissolve 36.5 grams L-lysine (Sigma Cat# L5626) in one liter ddH_2O.

SS3 0.1*M* Dibasic Sodium phosphate:
Dissolve 7.1gram dibasic sodium phosphate (Sigma Cat# S0876) in 500 ml ddH_2O.

SS4 0.1*M* Sodium phosphate:
Dissolve 6.9 gram sodium phosphate (monobasic not anhydrous, Sigma Cat# S9638) in 500 ml ddH_2O.

SS5 FINAL Sodium phosphate buffer: Take 125.0 ml of 0.1*M* Dibasic Sodium phosphate add 0.1*M* Sodium phosphate dropwise to bring the pH 7.4.

Preparation of PLP Fixative at the time of use

1. **Take 125 ml of 0.2*M* L-Lysine-HCL (SS2) and add 0.1*M* Dibasic sodium phosphate (SS3) dropwise to bring the pH 7.4.**

2. **Add FINAL sodium phosphate buffer to above to bring the final concentration of L-Lysine 0.1M pH 7.4 for a total volume of 250mL. This is Lysine Phosphate buffer (LPB) which is good for three weeks.**

3. **To Lysine Phosphate buffer add 8% Paraformaldehyde Solution (SS1) (3:1 LPB: SS1). This LPB +PF and must be fresh. The final concentration of L-Lysine is 0.075M and PFA 2%.**

4. **To 100 ml of LPB +PFA add 0.124 grams of Sodium Meta periodate (Sigma Cat#S1878). This is FINAL PLP fixative and must be used within 24 hrs.**

References:

Dengshun Miao and Andrew Scutt
Histochemical Localization of Alkaline Phosphatase Activity in Decalcified Bone and Cartilage
J. Histochem. Cytochem. 2002 50: 333-340. [Abstract] [Full Text]

IAN W. MCLEAN and PAUL K. NAKANE
Periodate-Lysine-Paraformaldehyde Fixative A New Fixative For Immunoelectron Microscopy
J. Histochem. Cytochem. 1974 22: 1077-1083. [Abstract] [PDF]

3. Dehydration for Histomorphometry and µCT

After fixing bones in 10% Formalin (clinical specimens) or 4% Paraformaldehyde in PBS for 3-5 days 4°C, bones may be gradually dehydrated to 70% Ethanol for histomorphometry and MicroCT

1X PBS	**1 Hour**	**4°C**
1X PBS	"	"
1X PBS	"	"
0.9 % NSS	**15 minutes**	"
0.9 % NSS/50% ETOH	"	"
50% ETOH	**quick**	**RT**
50% ETOH	**20 minutes**	"
50% ETOH	"	"
50% ETOH	"	"
70% ETOH	"	

Store – ship to us in 70% Ethanol. If you are carrying by hand on a plane – drain off most of the alcohol and leave a moist film - tighten cap and seal with parafilm.

DYNAMIC HISTOMORPHOMETRY involves sub-peritoneal injections of fluorochromes on days 7 and 2 prior to sacrificing the animals. We recommend 2 calcein injections, 10 mg/kg body weight of the animal or one calcein and one and xylenol orange, 90 mg/kg body weight. Bones should be fixed in 70% ethanol. These bones will be embedded in methyl methacrylate.
Rationale: if you want to see the fluorochromes, bones cannot be decalcified. Therefore, an embedding medium with the same density as bone was developed, methyl methacrylate (water insoluble) or glycol methacrylate (water soluble). Immunohistochemistry cannot be performed on these samples.

If you want both dynamic and static histomorphometry, then bones from one side of the animal can be prepared for paraffin embedding and the other side for methylmethacrylate. Remember to use the two different fixatives and clearly label the samples.

4. Histology

After µCT imaging – bones are rehydrated for decalcification by us in neutral EDTA for paraffin sectioning. This will allow you to do IHC or in situ hybridixation .

However if your mice have a fluorescence tag in cells or dynamic bone labeling (multiple calcein injections), you must let us know as these bones have to be cut in plastic without demineralization see above for different fixation – directly into 70% ethanol.