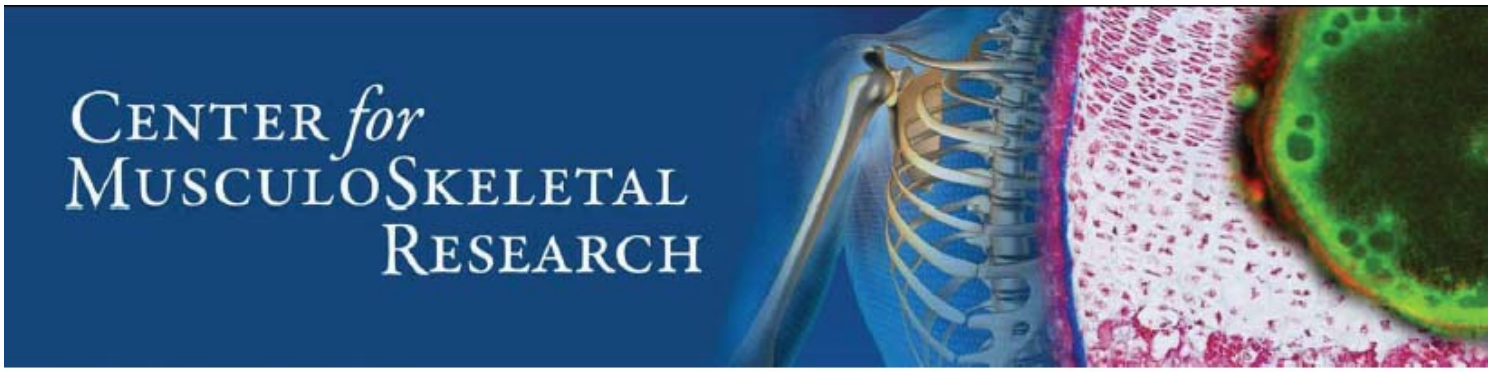


Histology, Biochemistry, and Molecular Imaging Core (HBMI)

Directors:	Brendan F. Boyce, M.D.
	Jennifer H. Jonason, Ph.D.
Staff:	Sarah Mack (Manager)
	Kathy Maltby
	Martin Chang

Histology, Biochemistry, and Molecular Imaging Core (HBMI)

Mission Statement

The primary mission of the HBMI Core is to provide efficient and high quality histological, biochemical, cellular, and molecular services to investigators throughout the Center for Musculoskeletal Research using both tissue and cellular models.

Research and Service Programs:

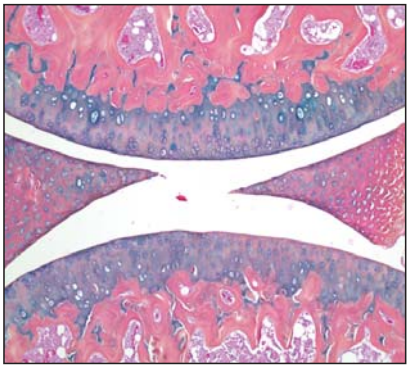
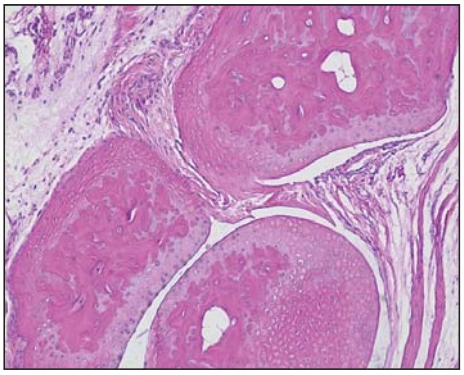
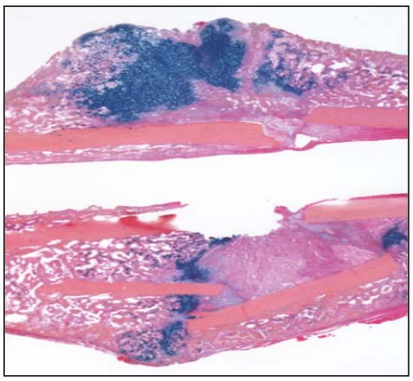
1) Histology, Immunohistochemistry (IHC), and *In Situ* Hybridization (ISH)

2) Microscopy, Histomorphometry, and Imaging

3) Biochemistry, Cellular, and Molecular Biology

Histology, IHC, and ISH Program

- Service *and* Training Program
  - Service Program
    - Fill out appropriate paperwork and submit to Core for tissue decalcification, processing, embedding, sectioning, and staining.
    - Turnaround time is currently 2-3 months!
  - Training Program
    - Submit samples to Core for decalcification, processing, and embedding. Learn to section and stain on your own.
    - Training sessions held on Wednesdays following Center Meeting.
      - *Plan on entire day!*
    - Keep in mind learning the mechanics of the microtome and the landmarks of your tissue will take time!
      - *Plan to practice!*
    - Use of microtome will depend upon approval by both PI and Sarah

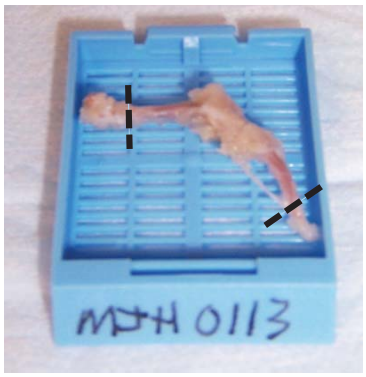
Histology 101

- **Paraffin and frozen tissue grossing and processing.**
- **Paraffin and frozen embedding and sectioning.**
- **Histological staining both routine and special stains.**

Grossing of Skeletal Tissue
Prior to Fixation

- **Hindlimbs: Remove all skin, fur, and muscle tissue, except for tissue immediately surrounding the knee joint.**

- **Cut open proximal femur and distal tibia prior to fixation, for three days.**



Grossing of Skeletal Tissue
Prior to Fixation

- **Femur Fracture: Remove as much muscle tissue as possible without disturbing the fracture callus. Three day fixation, removal of pins, followed by overnight fixation.**

Grossing of Skeletal Tissue
Prior to Fixation

- **Vertebrae: Remove as much skin and muscle tissue as possible. Fix tissue for 4-5 days, or alternatively perfusion fixation followed by 2 day standard fixation.**

- **If you are not sure about grossing, please ask for help.**

Details of Tissue Fixation for Paraffin Processing

- **Dissect skeletal tissue and remove the skin and muscle.**
- **If tissue is not grossed thoroughly, fix will not penetrate effectively.**
- **Fix in 10% Neutral Buffered Formalin (NBF) according to the timetable listed.**

Tissue Fixation for Paraffin Processing

- **Postnatal/Adult Mouse Limbs 3 days in 10% NBF or 4% PFA**
- **Postnatal/Adult Mouse Spines 4 to 5 days in 10% NBF or 4% PFA**
- **Postnatal Calvaria 2 days in 10% NBF or 4% PFA**
- **Fixation time varies according to tissue type and size**

Thoroughly Wash Tissue

- **Wash three times in 1X PBS for 5-10 minutes each, wash three times in distilled water for 5 minutes each.**

- **Store in 70% Ethanol , for minimal amount of time, or NBF both at 4 degrees, until tissue can be decalcified.**

Decalcification of Samples

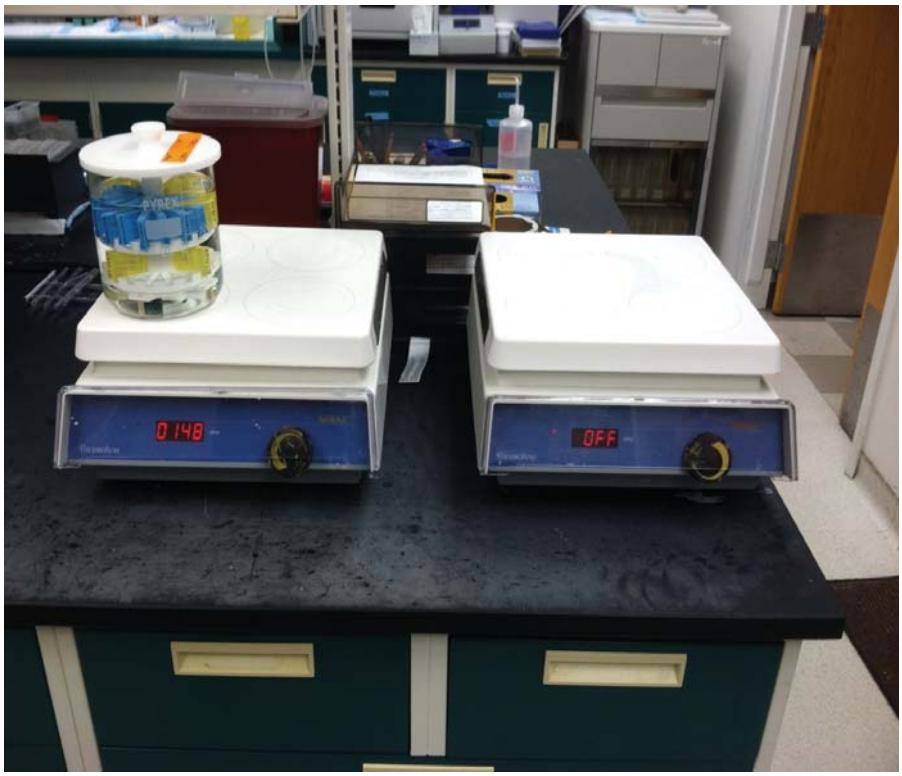
- **Formic Acid can be made and used at concentrations of 5% to 20%. It is also available commercially as Immunocal *TM* (Decal Chemical Corporation) at a 5% concentration, which we use.**

- **Our core uses EDTA at a 14% concentration, with a pH of 7.3 and the solution is changed daily.**

- **All samples are placed on a stir plate with a stir bar, stirring lightly or on a rocker, rocking gently.**

- **Decalcifying will not be adequate if samples are not grossed and fixed well.**

Using Stir Plate For Decalcifying



Types of Decalcifying Agents.

- **Formic acid allows for good cellular detail and works well with the common skeletal stains we use such as, Alcian Blue/Hematoxylin/Orange G, Safaranin O/Fast Green.**

- **When performing enzymatic stains such as Tartrate Resistant Acid Phosphatase (TRAP) stain or Beta-galactosidase staining use EDTA.**

- **EDTA is a chelating agent that aids in the removal of calcium and mineral from cartilage or bone. However if it is used for extended amounts of time it can have adverse affects on proteoglycans found in the extracellular matrix of cartilage and bone.**

Post Fixation

- **Some samples may be too large for proper fixation as the focus is on the joints, muscle and connective tissue.**
- **Too much soft tissue and muscle does not allow for adequate fixation.**
- **Large tissue needs to be post-fixed following decalcification to allow proper dehydrating and infiltration during processing.**
- **Post fix is determined by Core.**

Tissue Rinsing after Decalcification

- **Due to the amount of salt containing solutions that the bone is subjected to it must be rinsed thoroughly before processing.**

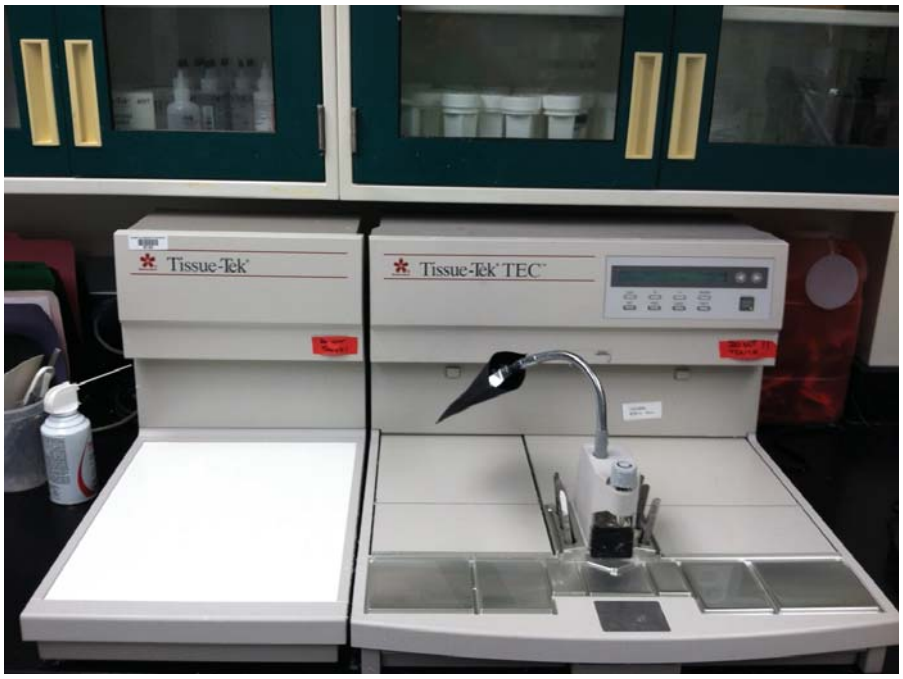
- **We rinse tissue in 1 X PBS three times for 5 minutes each, distilled water three times for five minutes each, 50% ethanol one time for 5 minutes, 70% ethanol for 5 minutes and store in 70% in 4 degrees, until processing at the end of the day. If tissue can not be processed immediately, store it in 10% NBF at 4 degrees.**

Paraffin Processing of Bone

Paraffin Processing of Bone

- **Bone processing programs require longer processing cycles , minimally 1 hour per station. Larger samples such as rat, rabbit and dog need even longer.**
- **A series of alcohols dehydrate tissue. We use xylene as a clearant agent.**
- **We use a paraffin with a plastic polymer, Paraplast Plus, as it infiltrates bone tissue better.**
- **A desired sample size is 3 to 4 mm thick and the size of a penny. No "stuffing" of cassettes allowed.** ☹

Bone Embedding



Paraffin Embedding

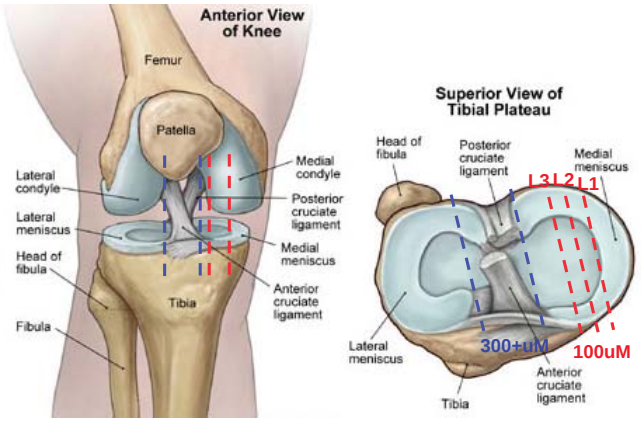
- **Embedding is a critical step in bone histology, as it is with any sample.**
- **If bone is embedded at the wrong angle or is not flat, it may cause the bone to chunk out during sectioning.**
- **Embed all samples in the same orientation, one specimen per block.**
- **Embed your tissue as flat as possible. If there is a cut side, place cut side down in mold.**

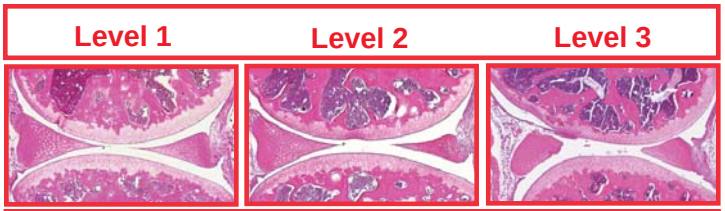
Paraffin Embedding Continued

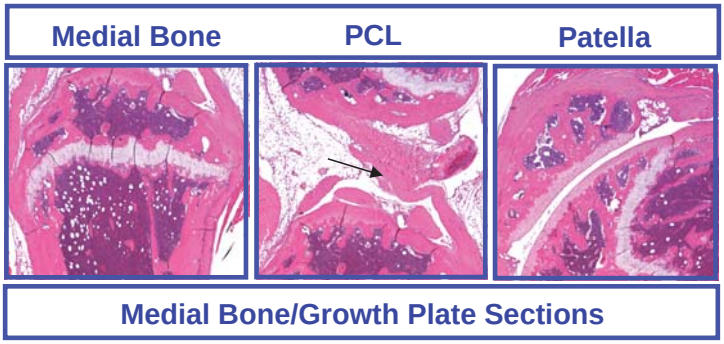
- **We embed mouse legs, femur and tibia in a smile orientation ☺, medial side down, with tibia/fibula connection up as we are focusing on the skeletal muscle.**
- **Mouse spines are embedded at a slight angle.**
- **Mouse ankles are embedded distal tibia, after being trimmed, allowing it to be embedded flatter.**

Bone Embedding

Anatomy and Microtomy of the Hindlimb: Articular Cartilage vs. Medial Bone/Growth Plate







Mouse knee joint regions utilized for "articular cartilage" sections.

Mouse hindlimb regions utilized for "medial bone/growth plate" sections.

Paraffin Sectioning

Paraffin Sectioning

- **When trimming into or facing blocks, be very careful! Do not trim in at more than 10 µm and change your blade when necessary.**
- **Trimming in too fast will cause bone to chunk out and may cause joint damage.**
- **We cut between 3 and 5 µm and generally cut 3 levels per block.**

Paraffin Sectioning Continued

- **After you have faced in to the area of interest, place your blocks on an ice try with a thin layer of water. The colder the block, the easier the bone is to section.**

- **Use slides that are charged/plus slides to help bone adhere to the slide.**
- **Because bone can be tricky to embed, you must be willing to angle your chuck/block holder allowing you to get to the proper level or plane.**

Paraffin Sectioning Continued

- **Know what the end result is desired to be before you start!**
- **You can't always go back and recut!**
- **Watch for chatter and knife lines, cutting bone requires changing blades frequently.**
- **Cut onto warm/hot water bath 44º to 48º .**
- **Bake sections at 56º to 60º for 1 hour to overnight before staining.**

Frozen Tissue Fixation and Decalcification

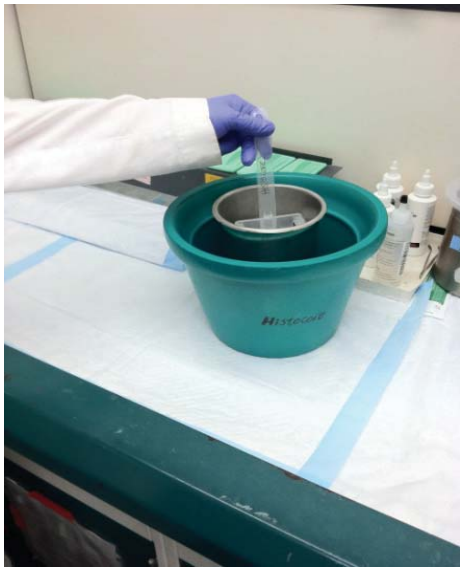
- **Dissect skeletal tissue and remove the skin and muscle.**
- **Fix tissue in 4% Paraformaldehyde (PFA), or 10% NBF, dependant on end result.**
- **Decalcify tissue according to end point usage.**

Embedding Frozen Samples

- **Run tissue through 10%, 20% and 30% sucrose made in PBS, gradient, overnight in each solution.**

- **Infiltrate in OCT for 30 minutes at room temperature, change to fresh OCT and freeze according to lab protocol.**

Processing Frozen Samples



Snap Freezing

- **Snap freezing refers to the ultra-low temperature freezing method used to prepare high-quality cryosections.**
- **Ice-crystals that form during a slow freezing process cause distortion in tissue morphology and can lead to more difficult sectioning.**
- **Dry ice (-80^{0}C) can cool a standard sized specimen submersed in O.C.T. within 3 minutes typically, but is still not cold enough to eliminate crystal formation.**

Bone Cryosectioning and CryoJaneTM

Frozen Sectioning

- **Minimizing fixation means better antigenicity.**
- **A down fall to frozen sectioning can be the decreased quality of bone and cartilage sections.**
- **We currently use the Leica CM 1850 cryostat with the Leica CryoJane tape transfer system. This method has greatly improved the morphology of our adult frozen sections.**

Why Frozens?

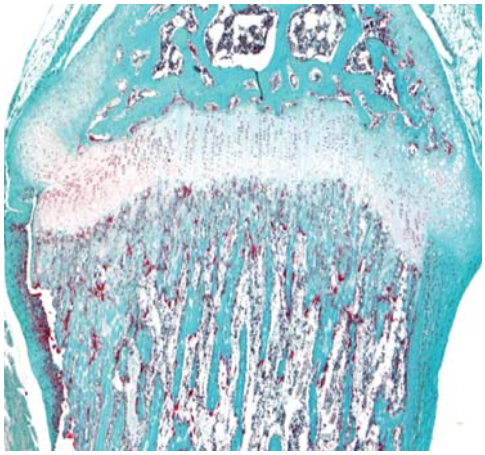
- **Preserves enzymes, proteins and lipids.**

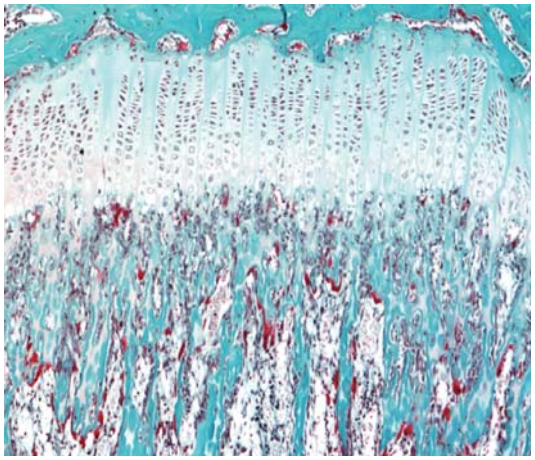
1. **Oil Red O stain identifies simple lipids that can be used only on frozen sections.**

2. **Proteins tagged with fluorescent markers can be visualized with frozen sections: GFP**

Routine Staining of Paraffin Sections

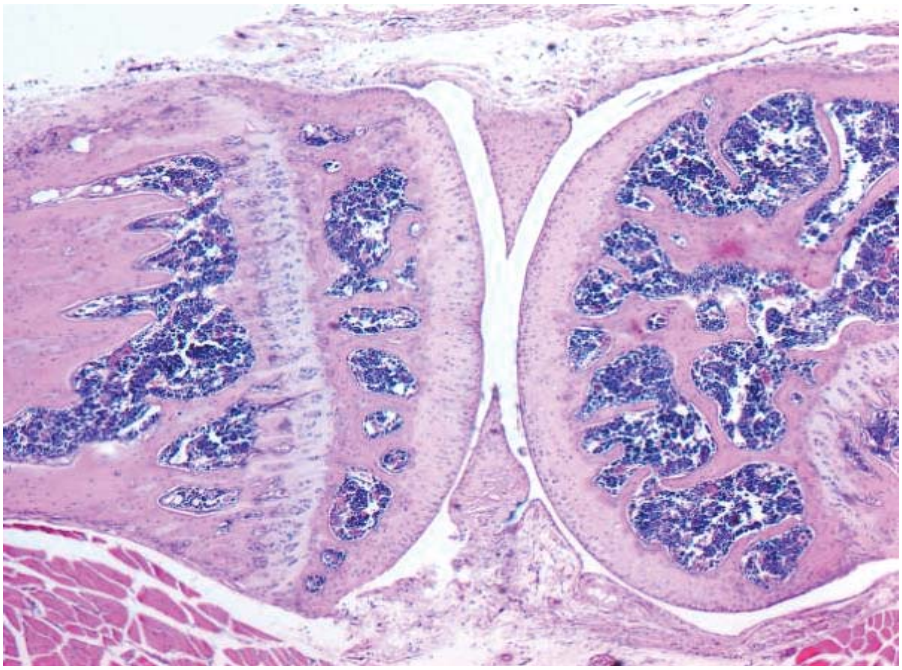
- **Tartrate-resistant acid phosphotase (TRAP): used to identify osteoclasts. Colors are more intense with EDTA decal.**





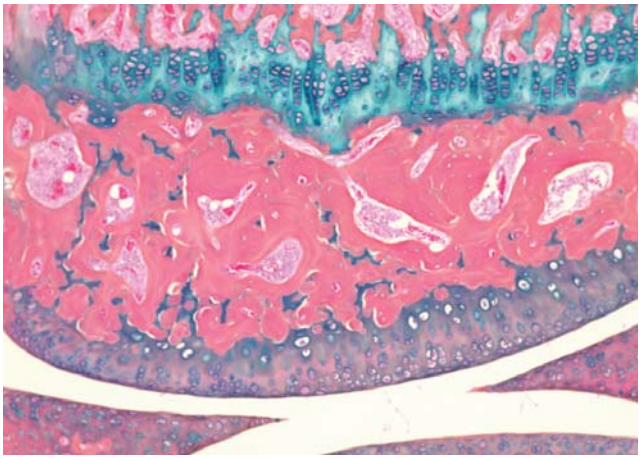
Hematoxylin and Eosin (H&E):

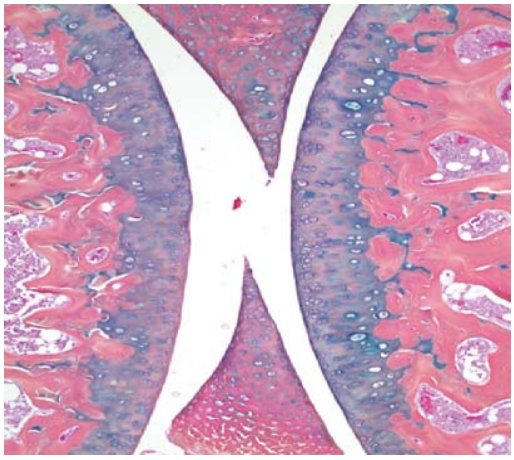
- **Basic nuclear and cytoplasmic stain**



Alcian Blue/Hematoxylin/Orange G (ABH):

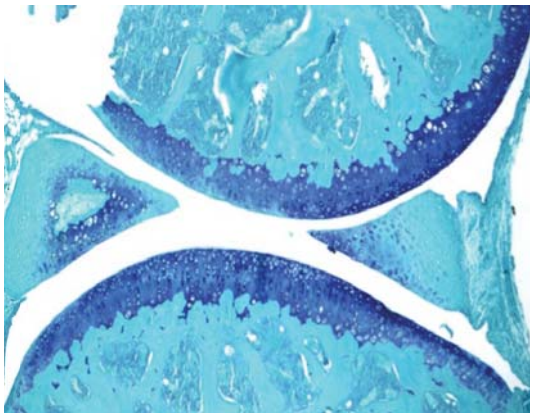
- **Used for growth plate and articular cartilage staining. Colors are more intense when samples are decalcified with formic acid.**

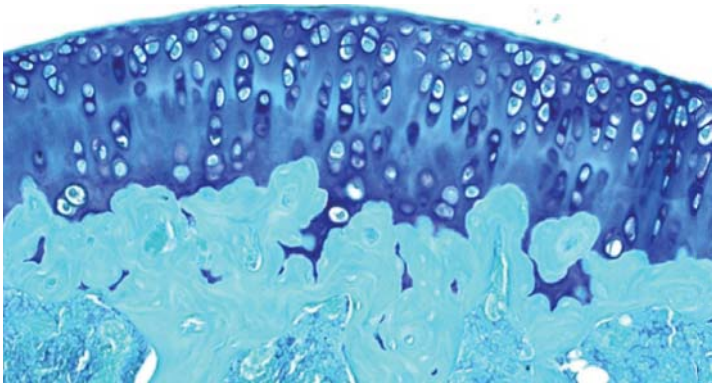




Toluidine Blue (TolB)

- **Used for staining cartilage. It is sensitive to pH, temperature and light.**





The Take Home Message

- **If tissue is not grossed thoroughly, fix will not penetrate effectively.**
- **Decalcifying will not be adequate if samples were not grossed and fixed well.**
- **Large tissue needs to be post-fixed to allow proper dehydrating and infiltration during processing.**
- **If one of these steps is not done right, there will be a domino affect.**

Submitting Your Samples to the Core

- **Fill out a work order form, have your PI sign the justification form.**
- **Never leave your samples with out speaking with someone in the Core.**

Help Us Help You

- **Let the Core know of any new projects, so everyone can be prepared.**
- **Follow protocols, which are available from the Core and are located on the Core website, to eliminate variables.**
- **When in doubt, ask for help.**

Contact Information:

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- **Kathy Maltby: kathleen_maltby@urmc.rochester.edu**
- **Martin Chang: martin_chang@urmc.rochester.edu**

- **Center for Musculoskeletal Research Core Website:**

http://www.urmc.rochester.edu/musculoskeletal-research/core-services/histology/