WS#92 Practical Approaches to Processing Bone: A Clinical / Research Comparative Overview

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Handout
WORKSHOP 92

PRACTICAL APPROACHES TO PROCESSING BONE: A CLINICAL / RESEARCH COMPARATIVE OVERVIEW

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IMAGING MODALITIES – PURPOSES AND FUNCTIONS

Radiography (X-Rays) – Generated electrons are absorbed by tissue to create images which can be on film or a digital representation of a Two-Dimensional image. Relatively Low Resolution images of differing contrast. Best for hard / dense materials. (See P.15)

DEXA – Dual Energy X-ray Absorptiometry. Two-Dimensional x-ray based technology that affords quantitative estimates of mineral content and areal bone density. Primarily used to determine bone density or fat mass in vivo.

pQCT – Peripheral Quantitative Computerized Tomography. Three-Dimensional serial x-ray images are produced from multiple two-dimensional images assembled by computer algorithms (software). 3-D images as thin as 0.11 mm. Provides differential volume of cortical vs trabecular bone either in vivo or ex vivo, a plus for evaluation of drug therapy. Also provides a true volumetric bone density, in vivo. If sample is ex vivo the specimen may be submitted in any fixative suitable for downstream analysis.

Micro CT – Microscopic Computerized Tomography. Three-Dimensional volumetric, serial x-ray images as thin as 10 microns can provide quantitative static histomorphometric indices of bone architecture, geometry and strength. Provides static histomorphometric parameters of bone structure. Submit specimen in any fixative suitable for intended study.

MRI – Magnetic Resonance Imaging. Magnetic fields altered by radio waves specific for hydrogen atoms can be focused on sequential 0.5 mm cubes of tissue to create multiple series of 2D or 3D computer software assembled images of all types of tissue. Added value for soft tissue, bone and tumor related studies in vivo.

PET – Positron Emission Tomography. A high resolution analytical imaging scintillation camera that responds to compounds labeled with positron emitting radioisotopes (such as FDG) as molecular probes to image and measure biochemical processes in vivo. These probes are usually gamma emitting analogs of glucose and are specific for distinct tissues.

IVIS – In Vivo Imaging System. Relies on implanted, engineered, bioluminescent expressing cells to capture images in real time. Cells / target structures may be surface or up to 1 cm deep. Tomographic reconstruction can be represented in 3D. Valuable for tumor and infection studies optimally in live animals. Can be used ex vivo.

BIOMECHANICAL TESTING – PURPOSES AND FUNCTIONS

Instron and Material Testing Systems (MTS) units are most prominent. These machines exert and read specific forces via load cells and transducers providing data pertaining to a variety of orthopaedic applications on tissue as well as hardware. Testing modalities in either venue include pull-out strength, three and four point bending, torsion and load-to-failure (crush). Incremental degradation as well as failure points can be calculated.
HISTOMORPHOMETRY – DEFINITION, HISTORY AND PURPOSE

Histomorphometry is defined as the quantitative measurement and characterization of microscopic images using a computer, manual or automated digital image analysis system. Typically, the primary measurements made are: area, length (or perimeter), distance between points or lines and number. These referents, such as tissue volume, bone volume, bone surface, and osteoid surface are used to derive other functional indices such as trabecular thickness, trabecular number and trabecular ratio. In addition, the number of particular cells (osteoblasts; osteoclasts) per millimeter of bone surface can be determined. Bone histomorphometry is the only procedure which allows for the accurate assessment of the cellular components and the accuracy of the skeleton.

Histomorphometry provides accurate measures of:
Bone turnover
Static and Dynamic tissue and cell activities of modeling an remodeling
Bone quality (microarchitecture, texture of bone matrix – lamellar or woven bone
Micro damage and the presence or absence of mineralization defects
Mechanisms underlying bone mass changes at the tissue and cellular levels

Dynamic measurements determine rates of cellular activity. Static measurements provide an assessment of the architecture of the bone and a baseline determination of cell number and location. Applications of histomorphometry include the evaluation of the effects of treatments and also as a research tool for the elucidation of the cellular basis for a particular skeletal phenotype.

SPECIMEN RECEIPT / MANAGEMENT

As with most clinical diagnostic applications, specimen imaging and some ex-vivo testing is the first performed function. These functions can be performed on fresh, fixed or fresh frozen material depending on the projected “end game” analysis required.

SPECIMENS FOR BIOMECHANICAL TESTING – As per the myriad of bone studies we have performed, there appears to be no substantive difference in data generated between fresh samples and frozen samples which were thawed (and kept moist) prior to and during testing. Formalin fixed or 70% ethanol fixed bone samples can be successfully tested generating valuable data. However there must be consistency from sample to sample in regard to how the sample was treated prior to testing. Data from identical test groups may not be deemed significant if one was tested after being thawed and one was tested after having been formalin fixed. We have obtained excellent histologic preps in both plastic and decalcified paraffin from material which was frozen, then thawed prior to fixation in 10% NBF.

SPECIMENS DIRECT TO HISTOLOGY – We prefer samples submitted directly for histology (decalcified paraffin or non-decalcified plastic) to be fixed ASAP in 10% NBF. If samples are fluorescent labeled in vivo for dynamic Histomorphometry we prefer these samples to be submitted in ethanol. (This does NOT apply to Bone Marrow Biopsies)
GROSSING TECHNIQUES

Femoral Head / Knee resection  Saw / Blade
Tumors / Tumor Composite  Saw / Blade
Composite Soft Tissue / Bone  Blade - Post Decal “Downsizing”

Safe use of saws and blades in the grossing process.
Always wear Goggles, Mask, Gloves
Use wood blocks to hold and advance the specimen to saw blade
Clean standard saw blade with wood and 70% ethanol after each specimen
Clean diamond saw blade with glass followed by 70% ethanol

Round white cassette = 3.3 cm ID x 0.6 cm  Standard cassette = 3.0 x 2.5 x 0.4 cm
Super cassette = 6.3 x 4.9 x 1.3 cm  Mega cassette = 3.0 x 2.5 x 1.0 cm
Bag = 7.5 x 9.5 cm  caution - staples usually rust

Mega and Super Cassettes available from: Sakura Finetek, Torrence CA
Nylon Bags available from: Thermo Fisher Scientific, Pittsburgh PA

FIXATION

PARAFFIN AND NON-LABELED MMA MINERALIZED STUDIES - Our preferred fixative for decalcified paraffin specimens, as well as MMA intended samples which have NOT been labeled in vivo for dynamic histomorphometry is 10% neutral buffered Formalin.

At room temperature and ambient pressure, Formalin penetrates approximately 1 mm per 24 hours (Lillie).
Raising the temperature to 37°C can double this rate.
Vacuum can increase the room temperature rate by approximately 2.5 X.
10X tissue volume is the recommended volume of NBF

Consider:  Standard cassettes = 4 mm deep  "Mega" cassettes = 10 mm deep.

Typical 10% NBF fixation times in our facility:
5mm slab sections of tumors, Femoral heads, Knees = 3-5 days.
Rat Tibia osteogenic zones = 2-3 days  Mouse Tibia, Femur, Spine = 1-2 Days
Rabbit osteomyelitic composites = 5-7 days depending on soft tissue content.

DYNAMIC HISTOMORPHOMETRY – Because in-vivo labels (tetracycline; alizarin red; calcein) for dynamic histomorphometry are soluble in water, a low water content or non-aqueous fixative is necessary to preserve the labeled fronts. We prefer these samples be submitted in 95% ethanol. We have found that up to 48 hours in 95% ethanol followed by absolute ethanol provides clear labeled fronts and quality cellular morphology. The absolute ethanol will preserve the label for up to one year if the material is stored in the dark. Formalin mordanting of slides may be required for some immuno procedures.
DECALCIFICATION
Crystals of Inorganic Salts (Calcium) + Organic Matrix = BONE

Decalcification = The removal of the inorganic components from the hard tissue, i.e., the dissolution of the hydroxyapatite complex: $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$.

\[
\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + 8\text{H}^+ \rightleftharpoons 10\text{Ca}^{++} + 6\text{HPO}_4^{--} + 2\text{H}_2\text{O}
\]

The usual mechanism for this process is immersion in acid or an acid-chelator complex.

The "End Point" of the decalcification process is reached when all inorganic material is removed from the tissue. Additional exposure can extract organic components from the tissue, especially from cells. Stronger solutions will increase this risk. This is the primary cause of poor staining associated with decalcified tissue.

End Point determination methods:
1. Radiography - most accurate (Carson), but time consuming and costly (see P.15).
2. Probing & Bending – convenient - may not determine optimal end point
3. Chemical testing - very accurate - "False Negatives" may occur – limited decalcifants
4. Timed Immersion – Serviceably accurate once familiar with decalcifier and tissue type

Stronger acids or stronger concentrations of a given acid type will promote faster decalcification. Doubling the acid concentration will not automatically reduce the decalcification time by half. Usual time reduction averages 20 – 25%. Doubling the change rate of a given concentration can reduce the decalcification time by up to 33%. Combining the two adjustments can achieve up to 40% reduction in decalcification time.

Commercial Acid Decalcifants:
Most formulas in commercially available decalcifiers involve either a 1-1.5N Hydrochloric Acid / Chelator complex or Formic acid from 5-15% with or without a fixative.

5% Formic Acid Decalcification:
Slower than most commercial decals - Faster than EDTA
Accurate End Point determination via Ammonium oxalate test (*see below)
Works well for all stated applications - Enhances Immunohistochemistry??

*Ammonium Oxalate End Point Test:
Add 1 part 5% Ammonium Oxalate to 5 parts “used” Formic Acid in a test tube. If calcium is still present in the fluid, it will form a Calcium Oxalate precipitate within 15 minutes. If not, the specimen is considered to be free of calcium.
EDTA (Ethylenediaminetetraacetic Acid)

EDTA decalcification protocols are quite varied depending on the strength and buffering of the composition. It requires significantly longer time than other common methods. End Point Determination is often more complicated as well as less accurate. Here is a sampling of known EDTA protocols we have been successfully associated with:

Example 1:  This is the directive for one of the “group proprietary” 10 % EDTA compositions: “The specimens are best decalcified in their submitted containers but they can be placed in plastic cassettes for bulk decalcification if suspended. The EDTA solution is stirred continuously and changed every 3-5 days. The end point of the decalcification is best determined by x-ray. The guidelines below have been determined by experience for some of the specimens we receive."

- Mouse femur and tibia  4-6 weeks.
- Rat Tibia              6-8 weeks.
- Larger Specimens      8+ weeks.

Example 2:
Decalcification with EDTA:
1. Samples were fixed with 10% formalin for 3 days.
2. Gently wash tissue with running water and ddH2O.
3. Suspend tissue in 10% EDTA-disodium (pH 7.4) or 14% EDTA-tetrasodium, pH 7.4(Sigma) at room temp. With gentle shaking. Change EDTA-solution every 4 days.
4. Check end-point with x-ray. It usually takes 2 weeks for rat “DO” tibia and 4-6 weeks for the whole rat tibia/femur.

* The volume of decalcification solution used should be 20 times (I prefer 50 times) greater than that of the specimen.

* EDTA tetrasodium salt, 14%: dissolve in 800 mls water and 10M NaOH, adjust the pH with glacial acetic acid, not HCl!

Example 3:
Some of our groups submit EDTA decalcified material on a “don’t ask; don’t tell” basis.

Example 4:
When we are compelled to decalcify with EDTA we use a commercially prepared EDTA / Sucrose decalcifier from Newcomer Supply which we immerse in 20X specimen volume with daily changes. The “typical” mouse tibia will decalcify in approximately one week. End Point is confirmed by radiography.

TRAP and certain enzyme histochemistry procedures benefit from EDTA decalcification. Good results via TRAP have been achieved on Formic Acid decalcified material, but the crispness is not as good as samples decalcified in EDTA.

Sources of Pre-Packaged EDTA decalcifiers include:
- Newcomer Supply, Middleton WI
- Polyscientific R&D, Bay Shore NY
### PROCESSING - PARAFFIN

Upon end point of decalcification:

<table>
<thead>
<tr>
<th>Step</th>
<th>Solution</th>
<th>Whole Mouse Knees</th>
<th>Joints, Femoral Heads, Large Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Wash in tap H₂O</td>
<td>2 hours minimum</td>
<td>3 hours</td>
</tr>
<tr>
<td>2.</td>
<td>70% ETOH</td>
<td>1-2 hrs - Overnight (park)</td>
<td>6 hours with agitation</td>
</tr>
<tr>
<td>3.</td>
<td>95% ETOH</td>
<td>1-2 hours with agitation</td>
<td>Overnight</td>
</tr>
<tr>
<td>4.</td>
<td>100% ETOH I</td>
<td>1-2 hours with agitation</td>
<td>3-6 hours with agitation</td>
</tr>
<tr>
<td>5.</td>
<td>100% ETOH II</td>
<td>1-2 hours with agitation</td>
<td>3-6 hours with agitation</td>
</tr>
<tr>
<td>6.</td>
<td>100% ETOH III</td>
<td>2-4 hours with agitation</td>
<td>Overnight</td>
</tr>
<tr>
<td>7.</td>
<td>Methyl Salicylate I</td>
<td>1-3 hrs – Overnight (park)</td>
<td>3-6 hours with agitation</td>
</tr>
<tr>
<td>8.</td>
<td>Methyl Salicylate II</td>
<td>1-3 hrs with agitation</td>
<td>3-6 hours with agitation</td>
</tr>
<tr>
<td>9.</td>
<td>Methyl Salicylate III</td>
<td>3 hrs – Overnight (park)</td>
<td>3-6 hours with agitation</td>
</tr>
<tr>
<td>10.</td>
<td>Methyl Salicylate IV</td>
<td>N/A</td>
<td>Overnight</td>
</tr>
<tr>
<td>11.</td>
<td>Paraffin I*</td>
<td>1-2 hrs</td>
<td>3-6 hrs</td>
</tr>
<tr>
<td>12.</td>
<td>Paraffin II*</td>
<td>1-2 hrs</td>
<td>3-6 hrs</td>
</tr>
<tr>
<td>13.</td>
<td>Paraffin III*</td>
<td>1-3 hrs</td>
<td>3 hrs - Overnight</td>
</tr>
<tr>
<td>14.</td>
<td>Paraffin IV* (Option)</td>
<td>1-3 hrs</td>
<td>3 hrs - Overnight</td>
</tr>
<tr>
<td>15.</td>
<td>Embed in fresh EM400. After skin forms, cure in refrigerator at 5°C.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Times for steps within same solution type can be interchanged.

Specimens must be visually clear prior to paraffin infiltration.

*Our infiltration paraffin is 50/50 of "Surgipath EM400 Embedding Compound" and "Fisher TissuePrep" paraffin @ 60-67°C with 15 – 18 lb vacuum

Baseline suggested times are extrapolated from experience with listed tissue types. Extra large cortical bone/tumor composites and exceptionally thick samples may require additional time or additional steps. (park) steps are suggested steps for manual process overnight holding. (unless you wanna work all night)
### PROCESSING – MMA - FIXATIVE**10% NBF

<table>
<thead>
<tr>
<th>SOLUTION</th>
<th>DURATION</th>
<th>DAY</th>
<th>DATE</th>
<th>TIME IN</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% EtOH</td>
<td>8-24 HRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80% EtOH</td>
<td>8-24 HRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>95% EtOH</td>
<td>8-24 HRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100% EtOH #1</td>
<td>8-24 HRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100% EtOH #2</td>
<td>24 HRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100% EtOH #3</td>
<td>24 HRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACETONE</td>
<td>6-8 HRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XYLENE</td>
<td>24 HRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50:50 XYLENE/MMA I</td>
<td>24 HRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMA I refrigerator w/vacuum^</td>
<td>24 HRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMA II refrigerator w/vacuum^</td>
<td>24 HRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMA III refrigerator w/vacuum^</td>
<td>24 HRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMA III refrigerator /vacuum ^fresh</td>
<td>24 HRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMA III refrigerator /vacuum ^fresh</td>
<td>24 HRS</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Embed in fresh MMA III sealed container in 37°C waterbath.

### PROCESSING – MMA - FIXATIVE**95% EtOH

<table>
<thead>
<tr>
<th>SOLUTION</th>
<th>DURATION</th>
<th>DAY</th>
<th>DATE</th>
<th>TIME IN</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% EtOH #1</td>
<td>12 hrs or o.n.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100% EtOH #2</td>
<td>12 hrs or o.n.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100% EtOH #3</td>
<td>12 hrs or o.n.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACETONE</td>
<td>8 hours</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XYLENE</td>
<td>Overnight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMA I vacuum room temp</td>
<td>Overnight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMA II w/vacuum. Place in frig O.N.^^</td>
<td>8 hours</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMA III refrigerator w/vacuum, Place in refrigerator O.N., ^^</td>
<td>Overnight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMA III refrigerator Fresh w/vacuum</td>
<td>8 hours</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMA III refrigerator Fresh w/vacuum Place in refrigerator O.N.^^</td>
<td>Overnight</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Next Day Embed in fresh MMA III in waterbath @ 37°C until polymerized

<table>
<thead>
<tr>
<th>SOLUTION NAME</th>
<th>COMPONENT 1 Methyl Methacrylate</th>
<th>COMPONENT 2 N-Dibutyl phthalate</th>
<th>COMPONENT 3 Benzoyl peroxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMA I*</td>
<td>80.0 ml</td>
<td>20.0 ml</td>
<td>none</td>
</tr>
<tr>
<td>MMA II*</td>
<td>80.0 ml</td>
<td>20.0 ml</td>
<td>2.0 gm</td>
</tr>
<tr>
<td>MMA III*</td>
<td>80.0 ml</td>
<td>20.0 ml</td>
<td>3.0 gm</td>
</tr>
</tbody>
</table>

*Mix all day. Store in flammable refrigerator. Bring to room temperature before use. See Page 11 for further details. All Components from Sigma-Aldrich.

DEHYDRATION – PARAFFIN & MMA
Density and quality of a bone specimen is as important as size and thickness when considering extended time in ETOH. Smaller pieces of cortical bone may take longer to dehydrate than larger pieces of cancellous bone or bone containing large quantities of fatty marrow. The generic concept is that alcohols are upgraded to insure that there is no significant water accumulation during use. When dehydrating large cancellous or marrow containing bone samples, the concept of fat accumulation is equally as critical. Fat is soluble in alcohol and fat laden alcohol does not dehydrate as efficiently contributing to poor clearing and infiltration. Relative fat content of ETOH can be ascertained by pouring the used ETOH into distilled water as demonstrated. If defatting in ETOH during MMA processing is inadequate immersion in Acetone may be required (see protocol P8). We have established that prolonged paraffin processing dehydration or long term storage of MMA intended material in ETOH does not adversely affect the “final product”.

**CLEARING – PARAFFIN & MMA**

Xylene clearing is adequate for routine size and density decalcified bone specimens. It is convenient because of its compatibility with automated processors. Xylene generally tends to make tissue brittle and this effect is especially true of decalcified bone. The extended clearing times required for large samples further compounds the effect. Methyl Salicylate does not contribute to making tissue brittle even after extended times (e.g.; one week). Methyl Salicylate is more water tolerant than Xylene, which can increase the margin of error for incomplete dehydration. Complete clearing of large specimens is best established by visual confirmation. Contact the manufacturer of automated processors to see if Methyl Salicylate is compatible with component parts before using. Pathologists and researchers have commented that cells look “crisper” and immunos appear “clearer” in sections processed through Methyl Salicylate. While an alcohol to Methyl Salicylate carryover is not critical, Methyl Salicylate to paraffin carryover can be detrimental to block quality and sectioning ease. Care should be taken to totally drain the specimen of excess Methyl Salicylate before transferring to paraffin.

The xylene and / or acetone immersion during the MMA protocol acts both as defatting and clearing steps. Xylene making the material “more brittle” is not significant in MMA processing.

**Sources for Methyl Salicylate include:**

- **Aldrich Chemicals, Milwaukee, WI**  
  Cat# M8 050
- **Fisher Scientific, Fairlawn, NJ**  
  Cat# 03695
- **Mallinckrodt, Paris, KY**  
  Cat# 2064
- **Polysciences, Warrington, PA**  
  Cat# 05943

**INFILTRATION - PARAFFIN**

We have found that superior paraffin infiltration into bone is often facilitated by higher temperatures. We generally infiltrate large samples at up to 65-67 degrees with vacuum at 15-18 lbs. The use of blended or polymer augmented paraffins can reduce the risk of paraffin degradation due to high temperatures. We prefer to store our paraffin blend at room temperature and melt it prior to use. Decalcified bone samples infiltrated “over the weekend” have not exhibited any reportedly undesirable results. Composite bone / soft tissue samples where the primary target area of study is the bone can exhibit compromised soft tissue after extended infiltration times under these conditions. Methyl Salicylate content in the final infiltration paraffin will adversely affect the polymerization of the tissue especially with large marrow fields and you will smell the Methyl Salicylate in the tissue during sectioning. We feel that the Methyl Salicylate fumes from contaminated “Paraffin I” in the vacuum oven can be picked up by the “clean” paraffin if stored together in the heated oven for long periods.

**INFILTRATION – MMA**
Our MMA protocol (page 8) is the current hybridization of several past procedures. We adhere closely to the stirring and temperature advisories especially when combining component parts for the infiltration and polymerization fluids. If the material to be polymerized is exceptionally dense or hard we will adjust the MMA monomer : N-Dp ratio as high as 95:5. Our standard manufacture volume is 300 ml regardless of the solution (I, II or III). Samples can be stored in MMA III in the refrigerator for up to 5 days during infiltration. It is most critical to bring the container to room temperature before opening to keep the introduction of moisture to the fluids via condensation to a minimum.

EMBEDDING - PARAFFIN

We embed in Surgipath EM 400, stored at room temperature and melted to 63 - 67 degrees prior to use. Non-standardized specimens require non-standardized molds. Decalcified bone specimen orientation is not exactly “by the book”. It is generally better to place the longest aspect of the specimen at an angle to the blade (even perpendicular to the blade) rather than parallel to it. It is generally better to place multiple same sized specimens on an angle in the block rather than in a straight line parallel to the blade. It is generally better to orient a wedge shaped specimen so as to cut the narrowest portion first. This is critical to generating quality, chatter-free sections for large or very dense samples.

We prefer the “old fashioned” embedding rings to cassette bottoms for embedding even standard sized (less than 24 x 36 mm) specimens. We feel that the combination of the extra paraffin combined with the universal clamp provides more stability than the cassette / cassette holder combination. For larger blocks we melt the back of the block to one of the metal custom block holders we have. Do not store paraffin block / metal holder composite in the refrigerator. They will separate on forceful contact with the blade.

POLYMERIZATION – MMA

We have abandoned the use of glass containers in favor of polypropylene containers as molds. We have found that the use of pre-polymerized “blanks” (molds which have a 6-10 mm layer of previously polymerized MMA) afford a more consistent platform for sample orientation. The pre-polymerized layer can be “softened” at the time of specimen placement prior to addition of the actual polymerization fluid. The sealed polymerization container is placed in a 37 degree C water bath which is filled with Armor Beads (MidSci.com) to ensure even temperature control. Smaller containers (20 ml) will generally polymerize overnight. Larger containers (50 ml) depending on the volume of fluid, may take up to three days. If the block is well polymerized but the “meniscal surface” is sticky, the block can be “finished” by opening the container and placing it in a 37 degree C oven.

SECTIONING - PARAFFIN

For horizontal sledge microtomes, usually required for blocks in excess of 50 x 60 mm, our experience indicates little difference in the quality of slides produced by motorized vs manual units. The most important contributors to quality sections include accurate decalcification, thorough processing, stability of components (block/block holder, knife/knife holder), sharpness of knife, angle of specimen orientation.

In our experience, disposable blades are sufficient for light to medium duty specimens (bone marrow cores, debridements, some osteonecrosis cores, etc.). We prefer re-sharpenable steel blades as most of our specimens are much larger than routine. We sharpen our blades on a Hacker H/I 76 at a grind angle of 38 degrees with a strop angle of approximately 40 degrees decreasing the speed of the machine to one revolution per pass during the strop step.

Not all angle markings are consistent for each microtome manufacturer. We prefer 6.2 – 6.5 on our Leitz 1512 and 15 on our Lipshaw 80A sledge when using a steel blade. When using a disposable blade we use a less acute angle of 5.5 on the Leitz scale.
Icing is critical for quality bone sectioning. The temperature of the ice makes the paraffin harder and the water shed by the melting ice tends to soften the tissue face, making for a more homogeneous composite. Icing reduces chatter especially in large blocks. As a “last resort before re-infiltration” to eliminate chatter: ice and trim the block in a less than optimal approach. Be sure that the optimal approach is within 30 degrees of your trim approach. When ready to section, rotate the knife (or block depending on your microtome) to that optimal approach, re-ice and cut. This will change the cadence of the chatter lines and usually vastly improve your section.

Section thickness when pertaining to decalcified bone can be a wide ranging topic. Bone marrows are preferred to be 2 - 4 microns; bone tumors: 3-6 microns depending on the disease process; general debridements and joints: 4 - 6 microns; orthopaedic specimens requiring specific architectural detail: 4 - 7 microns. Composite rodent knee whole mounts will exhibit less cartilage wrinkling at 4 microns.

We stock slides up to 100 x 80 mm with requisite cover glasses. We also stock 5” x 7” plate glass for extreme samples.

We float our sections out on 41 - 48 degree distilled water. Thinner and composite sections = lower temperature; thicker sections or those containing more cortical bone = higher. We have a two step drying process for sections for sections 5 microns or thicker: Once retrieved off the water using a 62 degree C warmer for approximately 10 minutes or until the section is deemed to be “flat enough” then transferring the slide to a second warmer which is approximately 72 degrees C until the section is “completely flat”.

Sources for Large Slides and Coverglass include:
Arthur Thomas, www.thomassci.com
Brain Research, Boston, MA
Erie Scientific, Portsmouth, NH
Richard-Allan, Kalamazoo, MI

SECTIONING - MMA

We begin by cutting the container away from the polymerized block and sawing the block to the requisite size on a tabletop, water cooled band saw. The “back” of the block is sanded on a tabletop grinder, thoroughly dried and affixed to a pre made methacrylate holder which goes into the microtome chuck (J. Histotech 31:4 pp 179 – 182. 2008). We section the blocks on a Leica RM2255 Microtome fitted with a D-profile tungsten knife. Once the tissue is exposed we generally trim no more than 10 microns thick. Sections are taken at four to five microns at slow speed with the aid of painting the block face with 30% ethanol. A drop of Haupt’s Gelatin Solution (1 gm Gelatin; 2 gm Phenol; 15 ml Glycerine in 100 ml 30 degree C distilled water) is placed on the slide. Immediately, the section is placed atop the drop of Haupt’s and two drops of 50% ethanol are placed atop the section for up to 5 minutes. During this time, any teasing or stretching of the section is done. When deemed wrinkle free, a pre cut piece of Kisol Folie (Delaware Diamond Knives, Wilmington DE) is placed over the section and depressed to extrude any bubbles. Section-to-slide adherence is facilitated by heat so the slide is placed on a 40 degree C slide warmer. After a minimum of two minutes on the slide warmer the face of the slide is covered with bibulous paper and stacked into a slide press (Energy Beam Sciences, East Granby, CT) and placed overnight in a 37 degree C drying oven.

COMMON SECTIONING PROBLEMS

In our collective experience, most problems blamed on insufficient decalcification were ultimately traced to some other portion of the process. Generic problems are usually
attributed to dull knives, loose knives or blocks or a lack of adequate icing. Sometimes a seemingly perfect sectioning process will yield less than desirable results.

Most “exploding marrow fields” are usually not the result of insufficient fixation or decalcification, but poor infiltration caused by the failure of dehydration to get all the fat out. So even though the problem is infiltration, the cause of the problem is with the dehydration. Sometimes extended clearing before re-infiltration may help. Simply re-infiltrating may not have a significantly positive effect.

Try this systematic approach to eliminate chatter: ice and trim the block in a less than optimal approach. Be sure that the optimal approach is within 30 degrees of your trim approach. When ready to section, rotate the knife (or block depending on your microtome, but knife is preferable) to that optimal approach, re-ice and cut. This will change the cadence of the chatter lines and usually vastly improve your slide.

**BONE MARROWS**

All the above information pertaining to the production of the optimal Orthopaedic slide is applicable to the preparation of the optimal bone marrow slide as well. What seems to be most responsible for most of the unhappiness with the quality of bone marrow preparations is most often derived from time constraints. Quality as a primary goal is being superseded by turnaround times and resource restrictions which are now “industry standard”. The techniques are there; IF we are given the time to utilize them.

**SAMPLE PROTOCOLS**

**Example Protocol X** (pertinent highlights)

1. Bone Marrow core to be fixed in B-Plus Fixative (Zinc Formalin) minimum of two hours, maximum of 8 hours.
2. Pathology Resident will decalcify core in Cal Ex (1.35N HCl / EDTA solution) Time to be judged by size and volume (*collectors said to use varying gauge needles*)
3. Decalcified core will be placed immediately on Biopsy processor (6 hr cycle)

**Example Protocol Y** (pertinent highlights)

1. Fix core (12 ga, 2.3 mm) minimum 2 hrs in Acetic Zinc Formalin then cassette
2. Rinse 15 minutes in running DI water (“running DI water”?)
3. Decalcify in Surgipath II Decal (HCl / EDTA) for 30 minutes (EDTA<5%)
4. Rinse 15 min in running DI water
5. Hold in 10% NBF on processor for processing (3.5 – 5 hrs)
6. Process on 4 hr cycle

**Example Protocol Z** (pertinent highlights)

1. Fix for one hour in B-Plus; Transfer to lab in 10% NBF
2. Three Changes of tap water
3. Decalcify in 10% Formic Acid for 5 to 6 hours
4. Wash 25 minutes in tap water
5. Process on 14 station, 6 hr cycle

Considerations:
Optimal results suggest thorough fixation be done BEFORE decalcification

B5 was generally accepted as the best bone marrow fixative because it was faster than 10% NBF (2 hrs) but eventually abandoned due to Mercury content. Zinc now replaces Mercury in such fixative formulas.

B-Plus fixative recommends “at least 2 hrs” for bone marrows (ref protocol Z)

Applied Culling formalin penetration study (0.8 mm / hr) was done for soft tissue

Clinic Residents may transport specimens to lab PRIOR TO immersion in fixative

Dr. Manfred Dietel’s 2009 tumor / tumor bank tissue fixative recommendations

Surgipath II Decalcifier suggests 1 hr immersion (ref protocol Y)

Quality EDTA decalcification usually is done at 10 – 14% concentration

Protocol Z 6 hr process includes 3 xylene and 4 paraffin stations (Ideal)

It is argued that surgeons and clinicians have superseded Pathologists as the driving force behind turnaround times and acceptable quality in Histopathology

Histologist involvement in grossing puts a vested interest in the production of the block

Pathology is trending from confirmational & diagnostic to “prognostic”

Manfred Dietel NSH 2009: Standardized procedures to guarantee reliable results to the benefit of patients are imperative for the importance of histological diagnoses AND predictive accuracy.

A role for Flow Cytometry?

Procedural standardization to ensure uniform production can only be achieved through mutual cooperation amongst Clinicians, Histotechs and Pathologists (listed alphabetically)

**STAINING**

When it comes to staining decalcified paraffin embedded bone Bancroft states: "Properly decalcified tissue needs no staining modifications". However, the manipulative
modifications when staining slides as large as 5” X 7” can be extensive. Coverslipping can be an adventure on these larger slides. With decalcified paraffin preps the term “deparaffinize and hydrate to distilled water” is pretty much understood.

With MMA preps the process is more involved. Slides are removed from the press and stripped of the bibulous paper and Kisol Folie. “Deplasticization” takes place in: four five minute changes of 2-Methoxyethyl acetate (Sigma Aldrich, ST Louis, MO); followed by three changes of Absolute Ethanol; Two changes of 95% ethanol; 70% ethanol then water all changes are three minutes each. Many stains developed for paraffin applications can be used on MMA preps with simple modifications to times and reagent strength and attention paid to variable expressions of mineralized vs. non-mineralized components.

HEMATOXYLIN-EOSIN (Paraffin)
Gill's II Hematoxylin (Polyscientific R&D, Cat. # S-210) 5 minutes
Working Eosin Phloxine Stain (Polyscientific R&D, Cat. # S-176) 1 minute

SAFRANIN-O / FAST GREEN (Paraffin)


MASSON TRICHRONE (Paraffin & MMA)

VON KOSSA (GMA & MMA)

MODIFIED GRAM STAIN FOR BACTERIA IN BONE (Paraffin, GMA & MMA)

IMMUNOHISTOCHEMISTRY (DECALCIFIED BONE)
In our facility, we perform all processing and sectioning and immunohistochemistry as required by the PI. In addition, we can (and do) perform specific IHC, EHC, ISH procedures. Numerous investigators utilize our protocols in their lab or have the staining performed in our facility. We have a published and long history of immunostaining using a variety of reagents including (but not limited to) BMPs, Apoptosis, TUNEL, VEGs, PCNA, OPN, TNFs, Twist, OCT, TGF, Collagens, Smad, Nanog, Perisotin, Pecams, Integrin, CD-34, 31.

IMMUNOHISTOCHEMISTRY (UNDECALCIFIED MMA EMBEDDED BONE)
In our hands, most immuno procedures which can be successfully performed on decalcified paraffin embedded bone will yield satisfactory results on MMA embedded samples pending selected protocol modifications, most notable being overnight incubation at 4 degrees C.

SPECIMEN RADIOGRAPHY (X-Ray) PRINCIPLES
Radiographs in histology laboratories are used as documentation and diagnostic tools.
An X-ray picture results from moving electrons colliding with matter. These electrons may bounce off, be absorbed by, or pass through the target matter. Bounced electrons may scatter and be absorbed by surrounding matter. Only electrons that reach the film will produce the shadow effect seen on the X-ray film. The X-Ray energy is regulated both by the number of electrons generated as well as the speed these electrons are traveling.

**TERMINOLOGY**

*Milliamperage (mA) = number of electrons, brightness, low voltage.*
*Kilovoltage (kV) = wavelength, speed of electrons, contrast, high voltage.*

**VARIABLE kV**

Low kV long wavelength, low penetrating power, easily absorbed.
High kV short wavelength, more energy, more penetrating power, less absorption.

**X-RAY GENERATING MACHINES IN THE LABORATORY INCLUDE:**

1. Faxitron – *Faxitron X-Ray Corp. Wheeling, IL*
   Varied mA; Max. 110 kV; Timer options; Beam Sensor.

2. AXR 110 Minishot System - *Associated X-Ray Corp., East Haven, CT*
   Fixed 3 mA; Max. 110 kV; Digital timer; No beam sensor.

**FILM**

*Kodak PPL Oncology Portal Pack / Automated Process*

**PROCEDURES:**

A generic approach to specimen radiography settings is: Energy / Unit of time
Energy = Number of Electrons (mA) at a particular speed (kV)

With a fixed mA machine this settings concept is simplified to kV / Sec.

Orthopaedic Pathology specimens - resected joints, bone tumors
Slab Thickness: 5 mm = Ideal
Exposure settings with fixed 3mA machine: avg. 28kV (soft) to 35kV (hard) for 20 Sec. ^^

Rat Femur / Tibia
Exposure settings: 30 kV; 20 sec ^^

Rabbit osteomyelitic bone
Exposure settings: 33 kV; 20 sec ^^

Mouse Tibia, Femur, Spine
Exposure settings: 27 kV; 20 sec ^^

^^ Note: Times are expressed for X-Ray units with no beam sensors. Beam sensor units will terminate the exposure when the requisite number of electrons has been detected. Most dense material must be placed over beam sensor.

**ADDITIONAL REFERENCES**


27. Frances L. Swain, Center for Orthopaedic Research, University of Arkansas for Medical Sciences. E-mail: swainfrancesl@uams.edu

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