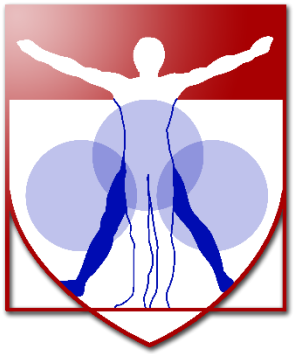
Histology Core Learning Lunch: Tips and Tricks for Top Quality Musculoskeletal Histology

October 28th, 2021



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CENTER for
MUSCULOSKELETAL
DISORDERS

Overall Aims

- The mission of the Penn Center for Musculoskeletal Disorders Histology Core is to provide comprehensive, high quality histology services to musculoskeletal researchers at the University of Pennsylvania and the broader research community.

- The Specific Aims of the core are:
  - To provide guidance and training on the capabilities, advantages, and disadvantages of the various methodologies to assess musculoskeletal tissue structure and composition through formal educational enrichment programs and one-on-one interactions.
  - To provide expertise and service for histological and histomorphometric assays of musculoskeletal tissues.
  - To develop new histologically-based techniques that will be applicable to musculoskeletal research.
  - To provide funding for development of new projects and collaborations and to develop preliminary and/or feasibility data for investigators.

Personnel

Ling Qin, Ph.D.
Co-Director
Associate Professor of Orthopaedic Surgery
Calcified and Frozen Histology
311A Stemmler Hall

Lachlan Smith, Ph.D.
Co-Director
Associate Professor of Orthopaedic Surgery
Paraffin Histology
378A Stemmler Hall

Waixing Tang, Ph.D.
Technical Director
344 Stemmler Hall

Facilities and Equipment

Paraffin

- 2 x tissue processors
- 4 x paraffin microtomes
- Embedding station

Frozen

- 3 x cryostat microtomes
- Cryojane/cryofilm technique for undecalcified sectioning

Plastics

- Processing and embedding
- Polycut microtome

Facilities and Equipment

Imaging and Analysis

- Double headed teaching microscope
- Brightfield and fluorescence microscopes with high resolution cameras for image capture
- Histoquantitation workstation (Bioquant)
- Slide scanner (Axio Scan.Z1, full service only)

Services Offered

Self Service

- Virtual training is required for using core equipment (email Dr. Tang for access to training videos).
- Users can perform:
  - Paraffin embedding
  - Paraffin sectioning
  - Plastic sectioning
  - Frozen sectioning (additional training for cryofilm)
  - Histoquantitation (Bioquant)
  - Microscopy and imaging (bright field and fluorescence)
- Dr Tang is available to supervise and assist.

Services Offered

Consumables included in self-service:

- Paraffin wax
- OCT embedding medium
- Tools (forceps, bushes, pens etc)

For an additional fee:

- Cryofilm
- Slide boxes

Users Supply:

- Blades
- Slides

Services Offered

Full Service – Complete Project Delivery

- Free consultation and protocol development
- Project completed by core technician
  - Paraffin processing and embedding (1 week)
  - Paraffin sectioning (2 weeks)
  - Plastic sectioning (4 weeks)
  - Plastic sample processing and embedding (4 weeks)
  - Frozen sectioning (cryojane and cryofilm) (2 weeks)
  - Routine histochemical staining (2 weeks)
  - Slide scanning (1 week)
  - Immunohistochemistry (4 weeks)
- Accelerated service for urgent projects
- Budget preparation, requires PI sign-off

Services Offered

Sample Submission

Histology Core Service Request Form

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Please Fill Out Applicable Info
Email to Dr Waixing Tang and arrange sample drop off:
waixing@pennmedicine.upenn.edu

Name: _____ *Email:* _____

Project Description (1-2 sentences describing project goals - required unless paraffin processing only):

_____

_____

Species: _____ *Tissue:* _____ *P.I.:* _____

P.I. Email: _____ *Drop-Off Date:* _____ *Date Needed:* _____

**Samples for Paraffin Processing must be fixed e.g. in formalin, decalcified (if desired), rinsed, put in 70%EtOH prior to drop off*

Paraffin

Processing (Tissue Processor)

☐ Yes # of samples: _____

Processor Cycle (if known) : _____

If unknown consult the Core first

Embedding: Sectioning:

☐ Yes ☐ Yes [Complete Adjacent Table]

Staining: ☐ Yes [Complete Adjacent Table]

Frozen

Embedding: Sectioning:

☒ Yes ☐ Yes [Complete Adjacent Table]

Staining: ☐ Yes [Complete Adjacent Table]

Sectioning & Staining

Sample No.	Section Thickness (um)	# of sections per slide	# of Slides	Stain Type [if desired]

Services Offered

Usage Charges

- Free consultation and protocol development
- Competitive charges for full and self service histology

  www.med.upenn.edu/pcmd/histologymain.html
- Generous subsidies offered for Penn users

Feedback

- Online surveys
- Ideas for new services

www.med.upenn.edu/pcmd/histologymain.html

Essential Steps for High Quality MSK Histology

Steps

1. Choose paraffin, frozen or plastic
2. Fixation
3. Decalcification (if necessary)
4. Processing
5. Sectioning
6. Staining
7. Imaging

- Histology is as much an art as a science, and is highly application-specific
- Always start with a technical consultation and practice samples

Paraffin Histology

Advantages

- High quality sections
- Blocks can be stored indefinitely at room temperature
- Great tissue/cell morphology

Disadvantages

- Always requires decalcification for mineralized tissues
- May not be suitable for immunofluorescent staining as bone has higher auto-fluorescence.
- IHC usually detects one protein
- Thin sections only
- Antigen retrieval is always required for immunohistochemistry
- Alters physical properties of the tissue (i.e. not suitable for AFM)

Frozen Histology

Advantages

- Fast (no paraffin processing, dewaxing needed)
- Better preservation of antigenicity for IHC
- Preserves endogenous fluorescence
- Decalcification is not required (cryofilm)
- Simultaneously detect multiple proteins in immunofluorescent staining
- Allows thick sections
- Preserves physical properties of the tissue (e.g. for AFM)

Disadvantages

- Poorer section quality
- Long term storage problematic (Store in -80C for only 1 year)

Plastic Histology

Advantages

- Superior morphology
- Standard procedure for bone static and dynamic histomorphometry
- No decalcification

Disadvantages

- Time consuming
- Require a high level of skills
- No IHC can be performed

Fixation

Purpose

- Preservation of tissue architecture
  - Inactivates proteolytic enzymes
  - Prevents bacterial decomposition
  - Stabilizes and strengthens tissue

Choice of Fixative

Q: 10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA)?

- ***10% NBF***
  - Convenience: Can be stored at working concentrations
  - Rapid fixation/better for larger samples: Contains methanol, which penetrates tissue more rapidly to initiate fixation
  - Downside: Degrades over time with formic acid by product
- ***4% PFA***
  - Less convenient: Typically, must be prepared fresh. PFA is toxic.
  - Can be better for immunohistochemistry: absence of additives such as methanol can improve antigen retrieval through better control of crosslinking

Fixation

Common Questions

Q: How much fixative volume do I need?

- Submerge in fixative volume **at least** 10-20 times of sample volume

Q: How long do I need to fix my sample for?

- Fixation time should be at least 1 hour per 1 mm of tissue thickness
- Best if sample thickness does not exceed 5mm

Q: Can I fix my samples for too long?

- Yes! Prolonged fixation may make immunohistochemistry/antigen retrieval more difficult due to excessive crosslinking. Can also make samples brittle. Formic acid byproduct may degrade tissue

Q: Should I fix my samples under agitation?

- Gentle agitation can help replenish fixative around the tissue and accelerate fixation

Fixation

Common Questions

Q: Does temperature matter?

- Yes! Higher temperatures may speed up fixation but also accelerate degradation of unfixed regions. Fix at room temperature or 4°C.

Q: Do I have to fix my samples fresh, or can they be frozen and fixed later?

- Fixation of fresh samples is normally preferable
- If unavoidable, flash freeze to prevent ice crystal formation and store at -80 °C.
- For practical reasons, samples sometimes have to be frozen and stored at -20°C prior to fixation. Minimize freeze thaw cycles.

Q: Do I need to fix my samples when I am cryo-sectioning?

- Yes: Fixation will maintain morphology, prevent decomposition, and lead to better results. Sections are most commonly fixed on slides after sectioning.

Fixation

Common Questions

Q: Should I refresh the fixative solution?

- Not critical, but refreshing the fixative solution after the first hour can accelerate fixation and improve results

Q: What about non aldehyde-based fixatives, such as organic solvents?

- OK for some very specific applications (such as plastic embedding)
- Rapid tissue penetration
- Tissue shrinkage, damage cell membranes and organelles

- When fixation is complete, wash off excess fixative in water or PBS and store in 70% ethanol for a few days until processing

Decalcification

- Extend fixation times for bone specimens before commencing decalcification.
- Three types of decalcifying agents:

  strong acid; weak acid; and chelating agents.
- Neutral EDTA (14%) is the best for research applications where very high-quality morphology is required.
- Factors affecting the rate of decalcification:

  a) Solution volume (> 20-fold of tissue volume)

  b) Solution change frequency

  c) pH (high pH accelerate decal but might damage tissue)

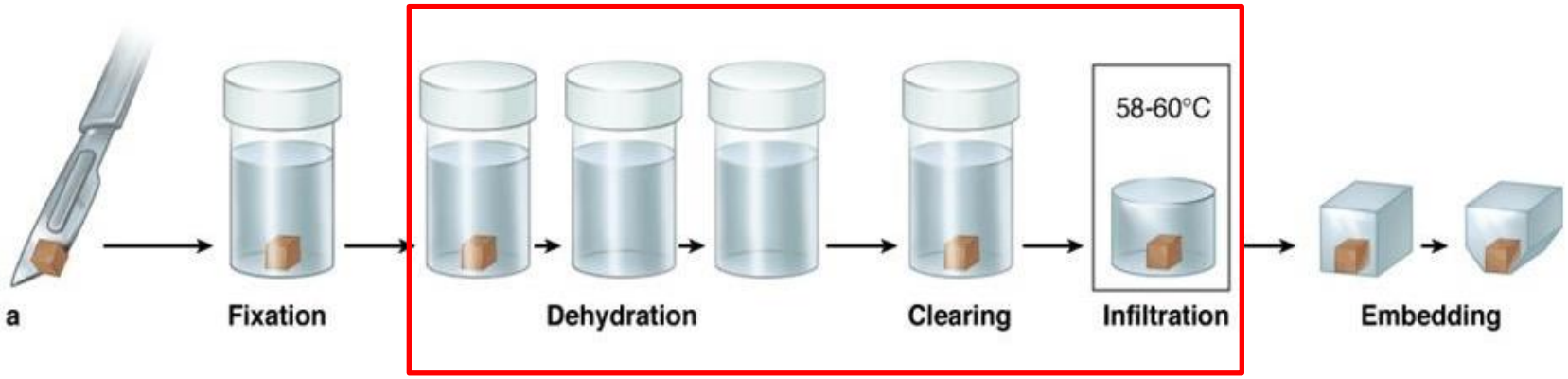
  d) Temperature

  e) Gentle agitation
- End point determination: X-ray examination; needle punching; chemical

Paraffin Processing

Steps:

- Dehydration through graded ethanols
- Cleared in xylene
- Infiltrated with paraffin



https://paraffinwaxco.com/

Purpose:

- Stabilizes the tissue to enable sectioning

Paraffin Processing

Preparation and Processing Time:

- Automated tissue processor: uses vacuum to improve infiltration for faster processing
- Fixed and decalcified tissues should be placed in cassettes and presented to the core in 70% ethanol
- Select the processing cycle in consultation with core technical director
- Processing times are based on sample type and thickness
  - E.g. 3mm, 5mm, 10mm, custom
  - Thicker samples take longer to process
- For a new sample type, start with a practice

Paraffin Processing

Problem:

- Tissue feels soft or mushy during embedding
- Tissue bounces out of paraffin block during sectioning
- Tissue does not adhere to slide or falls off easily
- Tissue sections disintegrate when placed in the flotation bath

Cause: Under processing, i.e. inadequate dehydration and paraffin infiltration

Solutions:

- Process using a longer cycle to improve dehydration and paraffin infiltration. As a rough guide, processing times should be selected based on tissue thickness
- Work with core Technical Director to select the optimal processing cycle. Core processing cycles have been optimized for tissue size and type
- For new sample types, start with a practice sample
- If samples are still under processed reagents may need to be changed

Paraffin Processing

What about Hand Processing?

- Time consuming, inferior infiltration
- May be preferred for very small or delicate samples
- May be preferred for temperature sensitive samples, such as biomaterials with low melt points (<60°C)
- Recommend Citrisolve as clearing agent (less toxic than xylene)
- Protocols available

Notice New Problems with Routine Samples?

- If you suddenly notice problems with samples you process routinely, please immediately notify the core. This may indicate a problem with the machine.

Paraffin Sectioning

<u>Problem:</u> Samples difficult to section

- Musculoskeletal tissues can be difficult to section due to the presence of multiple tissues with different structures and densities. For example, samples that contain a mixture of cartilage, fibrocartilage and bone such as joints and intervertebral discs

<u>Solutions:</u>

- Chill blocks in an ice bath. This will make the densities of the different tissues more homogeneous
- Moisten cutting face with a delicate paintbrush between sections. This will make tough tissue such as fibrocartilage easier to cut.
- Increase sectioning thickness to up to 10 microns

Paraffin Sectioning

<u>Problem:</u> Samples difficult to section

- Musculoskeletal tissues can be difficult to section due to the presence of multiple tissues with different structures and densities. For example, samples that contain a mixture of cartilage, fibrocartilage and bone such as joints and intervertebral discs

<u>Solutions:</u>

- If samples “shred” when sectioning, they may be inadequately fixed or under-processed
- If samples are “brittle” when sectioning, they may be over-fixed or over-processed
- If samples are “crunchy” when sectioning, they may not be completely decalcified

Frozen Sectioning

Problem: Tissue folding or curling when sectioning

Solutions:

- Use a fine tissue paintbrush to gently flatten the curled tissue
- Make sure that your cutting blade is sharp, use a new blade
- Use an anti-roll plate, which is a glass slide that fits right over the cutting stage, and allows the cut tissue to stay flattened as it is cut

Problem: Tissue cracking during sectioning

Solution:

- Check your cryostat temperature and make sure it's not too cold. Adjust the temperature by increasing it 5-degree until you no longer get tissue cracks in your sections.

Frozen Sectioning

Problem: **Tissue smudges or streaks when sectioning**

Solution:

- Check your cutting blade carefully. Sometimes frozen debris or OCT is stuck on the blade and can cause streaks. Clean, reposition or change the blade.

Problem: **Tissue is not sticking to the glass slide**

Solutions:

- The temperature difference is what allows the tissue to stick to the slide when you try to mount it on glass. If you are not getting this result, it could be that the temperature of the glass slide is too low, or the glass slides have been kept inside the freeze chamber.

Plastic Sectioning

Problem: Sections have too many wrinkles

Solutions:

- Cut thinner sections.
- Leave the sections in 70% ethanol for 7 to 10 minutes compared to the usual 3 minutes.
- Press the section using your finger under the plastic film evenly and for several seconds.
- Roll sections lightly and carefully 3 to 5 times on the slide.

Problem: Sections fall off slides

Solutions:

- Use a higher concentration of gelatin (3-4%) to coat your slides.
- Reducing the washing time, especially for the alcohol solutions.
- Don't start staining immediately following removal from baking in the 24°C oven. Leave them at room temperature for a few days.
- Be sure to transfer the slides very gently between staining steps.

Antigen Retrieval

- Methylene bridges formed during fixation cross-link proteins and mask antigenic sites. Antigen retrieval methods break these methylene bridges and expose antigenic sites, allowing antibodies to bind

Heat induced epitope retrieval

- Heat-induced epitope retrieval is most often performed using a pressure cooker, a microwave, or a vegetable steamer. Some labs use a water bath set to 60°C and incubate the slides in retrieval solution overnight. This is useful when working with tissue sections that fall off the slide when heated at higher temperatures; in particular bone, cartilage, and skin

Enzymatic retrieval

- The enzyme to use will be indicated on the antibody datasheet. If not, trypsin is useful for a wide range of antigens that require retrieval post-formalin/PFA fixation

Experimentally identify the best antigen retrieval method for each antibody.

More information:

- See learning prior lunch slides on IHC for musculoskeletal tissues

Online Resources

https://www.med.upenn.edu/pcmd/histologycore-resources.html

Prior Learning Lunch Slides:

- Cryofilm in Orthopaedic Research
- Optimizing Immunohistochemistry for Challenging Tissues
- Introduction to Whole Mount Imaging
- Introduction to Histomorphometry
- RNA Scope for Mineralized Tissues

Protocols:

- Fixation, decalcification and processing for paraffin histology
- Cryofilm technique for sectioning calcified tissue
- Chitosan adhesive film solution preparation (for cryofilm)
- Alcian blue and picrosirius red staining
- Safranin O and fast green staining

Tips and Tricks:

https://www.med.upenn.edu/pcmd/histologycore-histologytips.html

Final Thoughts

- For new tissue types and applications, schedule a consultation and start with a practice sample
- Protocol development is free!!