UNIT FOR LABORATORY ANIMAL MEDICINE
PATHOLOGY CORE
UNIVERSITY OF MICHIGAN

Necropsy/Tissue Collection and Tissue Fixation/Trimming Sample Guidelines

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I need necropsy and/or histology, pathology services in support of my research project - how do I request these?

For a new project, please first submit a Pathology Consultation request through MiCORES, describing your project and your needs. This will give us an understanding of your project and end goals and help us suggest a planfor tissue collection and/or analysis. Support is available for necropsy, bloodwork, histology, digital slide generation, pathology interpretation or any combination of the above. Histology or bloodwork results can be supplied directly to you for interpretation, or our pathologists can interpret results and generate a report tailored to the research question. Raw data and slides will be provided as well as the report.

Back to Top

How do I reserve space for large or small animal tissue necropsy/ tissue collection only?

Our necropsy rooms* at NCRC (small or large animal) or ARF (small animal) are available by reservation to perform necropsy and tissue collection yourself. You must receive an orientation in order to be granted approval ofspace use; please e-mail ULAM-PathologyCore@umich.edu for more detail. There is no fee to use the room unless our technician is needed for assistance. There are 3 available concurrent slots for both the small animal and large animal rooms at NCRC, 1 available slot for ARF. You may reserve time in 1-hour increments. You may schedule as early as 30 days, and up to 24 hours, prior to your chosen day(s); likewise, you may cancel your reservation up to 1 hour prior. Please follow clean-up guidelines as in orientation- you will be charged technician time if the room is not left in suitable condition for the next user.

*Our necropsy rooms are not approved spaces for survival surgery - please contact ULAM ASOR for survival surgeries. Terminal procedures may be performed in the room with approval and addition to your IACUC protocol.Rooms must also be added to your IACUC protocol if you are performing euthanasia in the space. For necropsy or tissue collection only (i.e., after euthanasia elsewhere) addition of the rooms to an IACUC protocol is not required.

Back to Top

UNIT FOR LABORATORY ANIMAL MEDICINE
PATHOLOGY CORE
UNIVERSITY OF MICHIGAN

My lab animal(s) died or became ill unexpectedly and I am concerned about a spontaneous disease condition unrelated to the experiment. How do I obtain a diagnostic necropsy?

Diagnostic necropsy to identify spontaneous disease is available to labs through our veterinary residents withouta recharge. Contact the veterinary resident assigned to your building via e-mail at ULAM-vetres@umich.edu or by phone at 6-1696. She/he will perform the necropsy and with the help of the Core's pathologists, will interpret the results in a diagnostic report. For research necropsies related to experimental questions or to assist in troubleshooting research-related complications, please see questions below regarding our research necropsy service.

NOTE: the diagnostic necropsy service is part of the ULAM veterinary care program and the purpose is to identify spontaneous disease of concern to colony health. This service cannot be used to investigate research questions or to repeatedly necropsy animals in cases of attrition due to ordinary causes (e.g., old age, background losses within expected levels). Slides/blocks for diagnostic cases are retained by the diagnostic veterinary service and are not returned. If you would like slides/blocks returned, please request a research necropsy (recharge applies). Research necropsies are submitted directly to the Core using MiCores, and slides/blocks are returned to the investigator. Questions about the applicability of a diagnostic vs research necropsy should be directed first to the veterinary resident orclinician for your area.

Back to Top

My lab animal(s) died or became ill unexpectedly and I suspect a research complication. How do I request help determining the cause of death/illness?

Research necropsies are available on a recharge basis to investigate research complications or to addressplanned research questions.

DO NOT FREEZE carcasses- this will cause tissue freeze artifacts and preclude histology! Either necropsy immediately, place in 4°C (refrigerate) for necropsy within 24 hrs. For mice or rats that are found dead or euthanized after hours, **at minimum** you should open the thorax, abdomen, and skull (to allow fixative penetration) and place in a jar large enough to accommodate 20:1 volume ratio of fixative: tissue. Please note thatthe interval between death and fixation will affect the severity of tissue autolysis and may impact findings.

Be sure to include any relevant history upon submission as to the experimental background, strain, or relevantclinical signs information. This will help us interpret results most accurately.

Follow instructions for submission below.

Back to Top

UNIT FOR LABORATORY ANIMAL MEDICINE
PATHOLOGY CORE
UNIVERSITY OF MICHIGAN

How do I submit a necropsy request in MiCORES?

Samples for research (recharge) necropsy requests may be submitted through MiCORES: https://umich.corefacilities.org Necropsy, histology, digital slide, and/or pathology interpretation requests are under Anatomic Pathology services. Make sure to click "Add Selected Services" located beneath each group of services, and thoroughly review the charges and paymentsection (including short code) before you submit your request. Indicate your preferred sample drop-off location and time at the bottom of your MiCORES request. Wait until you receive confirmation of your drop-off time, then drop off your samples labeled with your MiCORES Service ID at your requested location. Please be punctual.

More specific guidance on submission is available in our separate MiCORES guidance document.

Back to Top

How quickly do I need to fix tissues after collection?

Tissues should be placed in fixative immediately upon collection. Tissues most sensitive to autolysis (e.g., GI tract) should be collected first- changes of autolysis (degradation) begin to occur within 10-15 minutes of euthanasia for the GI. If you are collecting from large groups of animals, then you should euthanize them one at a time for tissue collection; do not euthanize the whole group first and then collect! In general, we estimate that complete necropsy with collection of all major tissue types for a single mouse by a single, trained collector takes approximately 20-30 minutes per animal. Training and/or fee-for-service necropsy tissue collection by our technicians are available upon request.

Bone samples must be fixed (24-72 hours, depending on the species) before undergoing decalcification.

Back to Top

What is decalcification?

For paraffin sectioning, bone samples must be decalcified (i.e, softened). Acids and chelating agents (e.g., EDTA) are two common types of decalcifying agents. The core uses weak organic acids (Immunocal™ or other formic acid-based solution), which allows for quick decalcification while preserving epitopes. Samples should be well rinsed (~10 minutes) after the decal process is complete. Samples requiring decalcification may be submitted in fixative for us to decalcify or you may decalcify these yourself. Please inquire if you are decalcifying tissues yourself. Certain types of decalcifying solutions are not compatible with certain downstream histology processes (particularly immunohistochemistry) and time necessary for decalcification varies with tissue type. The core may return or further decalcify incompletely decalcified samples.

Sectioning of non-decalcified bone samples requires plastic embedding, not paraffin. We do not offer plastic sectioning.

Back to Top

UNIT FOR LABORATORY ANIMAL MEDICINE
PATHOLOGY CORE
UNIVERSITY OF MICHIGAN

What is the proper tissue size for fixation? How large can my tissues be?

For cassetted tissues the thickness should be **3-5 mm in width** (~ thickness of a nickel) and **no larger than 2 cm square** (postage stamp sized)

- Formalin only penetrates ~0.5 mm/hr. Large tissues will degrade or fix unevenly and microscopy or IHC may not be possible
- Tissues trimmed thicker than the height of the cassette will create artifact and cassette indentations
- Do not use sponges unless you need to keep the tissue flat (GI, skin) or if it is at risk of falling through the cassette (adrenal gland)

For larger tissues that are not cassetted at necropsy, the tissue should be trimmed to 0.5-1.0 cm thickness, then immersion fixed, followed by trimming for cassettes after fixation.

Poor technique at tissue trimming can markedly affect your slide quality, sometimes in an irreversible way. Our trained technicians are available to trim organs for you or to provide you training or advice.

*If our core is trimming tissues for you, please describe the target structure(s) for your tissues at submission or set up a time to be present during trimming.

Back to Top

How should I fix and trim specific organs?

Standardized trimming guidelines for many organs exist. A good resource for mouse/rat tissue are the global standardized tissue trimming guidelines for toxicologic pathology, available through goRENI. Some models have standardized guidelines for tissue trimming in the primary literature. We are able to provide customized tissue trimming recommendations for many applications. See below for some commonly submitted tissue.

- GI-tract: Remove the tissue first (rapidly degrades) and either infuse with formalin using a needle OR open the intestinal tract to allow formalin to penetrate.
    - Cross-sections or short longitudinal sections (~1-2 cm) can be used. For longitudinal sections limit to 4 per cassette, for cross-sections limit to 6 per cassette.
    - "Swiss rolls" of the small intestine or colon (in mice) **must be** performed at the time of tissue harvest and cassetted prior to fixation to ensure proper orientation. The small intestine and colon must be in **separate** cassettes, although we can put one section of each on the same slide - **specify** this in your request. For adult mice, the small intestine may not be able to fit as a Swiss roll in its entirety in one cassette. Remember that the tissue should not be touching the edges of the cassette (no larger than 2 cm^2).
    - For large animals do not submit the entire GI tract or large sections of it whole - you must open and assess the tract at necropsy and fix only smaller samples of relevant areas

- Mouse lung: Insufflate with formalin using a needle within the trachea (not the same needle used to insufflate the GI!). Fill to approximately the volume of the chest cavity (do not overfill).
- The lung may be infused with fixative, then immersion fixed and cassetted whole with the ventral side down)
- Mouse liver: do not fix the mouse liver as a whole organ! Cut it open or (minimally) separate the lobes
- Mouse brain: do not trim before fixation. Either fix first by immersion in adequate volume or perfusion fix. Trim only after fixation - guidelines for trimming landmarks are available by request.
- Mouse kidney: can fix whole and trim after fixation. For larger species kidneys should be trimmed at necropsy prior to fixation.
- Mouse or rat eyes: cassette whole (do not trim). Other species- please inquire.
- Skin: Skin should be fixed flat and embedded on edge after processing. Strips of skin no wider than 2 cm should be collected in the direction of hair growth (for proper orientation of follicles) and fixed flat. This is most easily achieved by placing the underside (subcutis) down on a piece of cardboard or a tissue sponge. Sponges can be cassetted and cardboard can be cassetted or immersed in formalin. Don't use corrugated cardboard, waxed cardboard, or tissue paper. Skin can be trimmed into smaller strips after fixation (see section on sponges below).
- Large animal organs should be sampled and sectioned into smaller pieces - for certain tissues (lung, heart, brain) where the architecture must be preserved during fixation, special procedures apply- please inquire.

Sponges should be used only if specimens are exceedingly small (at risk of slipping through cassette holes) or for tissues that need to be kept flat or in a certain orientation during processing. Do not use sponges in other cases - if used with larger tissues this will cause artifacts. Some common uses for sponges are below:

- Skin: Skin must be fixed flat (see section above). After fixation, the skin can be cut into thin strips and cassetted between two sponges to keep them flat during processing. Our technicians will embed these strips on edge at embedding.
- Small tissues like adrenal glands and arteries should be placed between sponges to prevent loss of samples.

Tissue dye: avoid the use of tissue dye if possible as it may contaminate other tissues in the jar. If used, it needs to be used very sparingly and allowed to dry before immersing the tissue. To indicate orientation, it is often sufficient to supply a diagram or specify the target of interest and tissue dye is unnecessary - please inquire.

Poor technique at tissue trimming can markedly affect your slide quality, sometimes in an irreversible way. Our trained technicians are available to trim organs for you or to provide you training or advice. If our core is trimming tissues for you, please describe the target structure(s) for your tissues at submission or set up a time to be present during trimming.

Back to Top

Which organs can I cassette together?

Only tissues of similar density should be cassetted together. Cassetting hard and soft tissues together will result in artifacts during sectioning. In general, try to limit to 4 tissues per cassette for optimal results. Try to avoid cassetting tissues of vastly different sizes or thicknesses together since one tissue may "cut through" before reaching the target area of the second tissue. A suggested cassetting scheme for complete mouse necropsy is below. We can make recommendations for other project-specific cassetting schemes, please inquire.

Suggested cassetting scheme for complete mouse necropsy, all major organs:

1. Brain (2-3 coronal sections)
2. Whole lung (insufflated, ventral side down)
3. Liver (1-2 sections), kidneys (long or transverse cross-sections)
4. Heart, skeletal muscle
5. Spleen, thymus, lymph nodes (usually mesenteric)
6. Stomach, urinary bladder
7. Small intestine, colon
    a. no more than 4 pieces if longitudinal, 6 if cross-section
    b. remove fecal pellets from colon before fixation
8. Cecum (must remove contents before fixation)
9. Testes/epididymis or ovaries/uterus/cervix/vagina
10. Bone marrow (sternum, with ribs cut off): decalcify AFTER fixation
11. +/-Skin (strips cut in direction of hair growth and fixed flat)
12. +/- salivary glands/cervical lymph nodes, adrenal glands
13. +/- tumor or other tissue of interest

Back to Top

How much fixative do I need to use?

For immersion fixation, aim for a fixative: tissue volume ratio of 20: 1 (minimum 10:1). If too little formalin is used, then tissues will degrade and microscopic analysis will not be possible. Remember that formalin only penetrates ~0.5 mm/hr so if your tissues are too thick, they will degrade centrally before fixation regardless of the volume of formalin present. Fix in WIDE-MOUTHED, FLAT-BOTTOMED jars: narrow neck vials make it hard to remove tissue, cone-bottomed tubes deform the tissue to the shape of tube. Pre-filled 10% neutral buffered formalin biopsy jars are available (ex. Fisher Scientific).

Lower amounts of formalin may be adequate if the tissue has been first perfused. For some tissues (large animal brain) special techniques apply - formalin penetrates this tissue quicker and so it can be fixed whole but in a large volume of formalin and with several formalin changes. Alternatively, perfusion can be used.

Back to Top

What container should I use for fixation and submission?

Please fix your specimen in WIDE-MOUTHED, FLAT-BOTTOMED, plastic jars. Narrow neck vials make it hard to remove tissues or cassettes and cone-bottomed tubes deform the tissue to the shape of tube. Glass jars risk breakage. Avoid submitting tissues in microcentrifuge tubes (some exceptions include: mouse arteries, spleen, and aortic root), and remember to label containers with the type of fixative (e.g., 70% ethanol or 10% NBF) and the date/time of the start of fixation. PBS is not recommended for long-term tissue storage—please submit samples in formalin or transfer your samples to 70% ethanol prior to submission. Make sure the tissue samples are fully submerged in the fixative.

Label each primary container with a unique sample ID and label your entire submission with your service ID from MiCORES. We will label your cassettes, blocks, and slides with your sample ID - to avoid handwriting issues, please submit a sample list in the fillable grid or an uploaded spreadsheet at the time of MiCORES entry. We will cross-check against your samples.

Place the primary container(s) in a secondary container (ex. sealable plastic bag). You will need tertiary containment (ex. Styrofoam cooler) will be needed for transport but the samples can be left at our lab in secondary containment.

Back to Top

UNIT FOR LABORATORY ANIMAL MEDICINE
PATHOLOGY CORE
UNIVERSITY OF MICHIGAN

Which Fixative Should I Use?

For most routine histology we recommend fixation in 10% neutral buffered formalin for a minimum of 24 hours of fixation at 20:1 volume: tissue ratio. 10% NBF is the most commonly used cross-linking fixative and provides excellent tissue morphology. For histology, the tissue can remain in fixative almost indefinitely before processing. For immunohistochemistry, transfer to 70% ethanol is recommended after 24-48 hours. PBS is not recommended for long-term tissue storage. Please submit your samples in formalin or transfer to 70% ethanol prior to submission. Certain immunohistochemistry reactions are sensitive to fixation and must use alternative fixatives or fresh frozen tissues – this depends on the antibody. Please contact us with your specific antibody target if you have questions. Please refer to the fixative chart below for more information.

Commonly used fixatives for routine histology

10% Neutral Buffered Formalin (NBF)*	4% Paraformaldehyde**	Others:
COMMON USES: • Most common general use fixative for paraffin embedded tissues • Suitable for most routine immunohistochemistry with appropriate retrieval PROS: • Can be ordered as working solution and stored at RT CONS: • Degrades with age and produces formic acid	COMMON USES: • Short fixation or cryo-embedded tissues, in-situ hybridization • Certain neuroscience and immunohistochemical applications PROS: • Lacks methanol for methanol-sensitive procedures CONS: • Has to be freshly prepared • More $$ than 10% NBF	Glutaraldehyde: used for electron microscopy; NOT suitable for routine light microscopy (makes tissues dry and brittle) Davidson's fixative: suitable for eye fixation (special procedures apply) 70% ethanol: A precipitating (not cross-linking) fixative; gives inferior morphology for tissue evaluation; for immunohistochemistry tissues are often transferred to 70% ethanol after 24-48 hrs to avoid over-fixation

*Formalin is an aqueous solution of formaldehyde (which is a gas at room temperature). The traditional original stock solution of formalin was made at 37.5% concentration.

"10% neutral buffered formalin" refers to a 1:10 dilution of this stock solution, so 10% NBF really equals 3.75% formalin! Nowadays 10% NBF is sold pre-made at the appropriate working concentration (3.75% formalin) so a) you don't need to dilute it and b) it is essentially the SAME concentration of formaldehyde as 4% paraformaldehyde!

**4% paraformaldehyde is made from polymerized (solid) formaldehyde that is dissolved with heat into aqueous solution. While it has approximately the SAME concentration of formaldehyde as 10% NBF (see above) it does not contain methanol. Methanol is typically added to 10% NBF as a stabilizing agent – formaldehyde breaks down over time to methanol and adding methanol at the beginning slows down this process. The lack of methanol in 4% PF makes it more suitable for certain applications but decreases its stability.

How long should the tissues stay in fixative (minimum/maximum)?

For immersion fixation in formalin, fix tissues for at least 24 hours. Tissues can be left in formalin indefinitely for standard histology (H&E, special stains). For immunohistochemistry, tissues should be switched to 70% EtOH after 48 hours. Ideally, tissues should be processed to paraffin blocks soon after fixation- paraffin is the most stable format for long-term tissue and antigen preservation. Long-term storage in ethanol can be detrimental as well, as this tends to dry out tissues and make them brittle. Please plan ahead (avoid submission on a Friday or before a long holiday) to allow us time to process your submission to paraffin as soon as possible.

*For immunohistochemistry remember that UNDERFIXATION is as problematic as over-fixation. Under-fixation will result in degradation of protein epitopes and IHC will not work. We recommend that you stick with 24-48 hrs. of fixation, followed by paraffin embedding. Certain epitopes are sensitive to fixation and may require fresh frozen sections or will only work with certain fixatives. Decalcification also affects IHC and protocols optimized for soft tissues may not work well for decalcified bone. Please see our IHC FAQs or inquire for further guidance.

Back to Top

What if I need more guidance?

Our core may be able to offer one-on-one training depending on the request and staff availability. Alternatively, you may contact the ULAM Training Core for technique training. For assistance with euthanasia and sample collection, please contact ULAM Technical Services. A good reference for organ-specific trimming are the standardized tissue trimming guidelines issued by the Registry of Industrial Toxicologic Pathology (RITA) https://reni.item.fraunhofer.de/reni/trimming/index.php?lan=en. We also have organ-specific guidelines for particular projects within our core – please inquire for organ or project-specific tissue trimming guidance.

Please plan ahead and inquire before submission if you have questions. Mistakes made at necropsy and tissue trimming will affect the quality of your results and may be irreversible. The core reserves the right to return or re-trim (with charge) improperly trimmed tissues. Improper trimming will delay your project and/or generate unsatisfactory results.

Back to Top