PRACTICAL HISTOPATHOLOGY IN MOUSE MODELS OF HUMAN DISEASE:
GUIDES TO PHENOTYPING THE GENETICALLY ALTERED MOUSE

http://mousepheno.ucsd.edu/

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3693904/
1. Approval to conduct experiments on animals, following ethical guidelines
2. Use of necropsy facilities in designated scientific buildings
3. Transport of cages appropriately, using covered boxes
4. Transport of and disposal of carcasses, using approved methods
5. Consultation with veterinary personnel for non-routine procedures
6. Consultation with personnel in ACP’s BSB laboratory for evaluation of blood and chemistry parameters
7. Consultation with Histology core personnel prior to mouse necropsy
Finish serum chemistry analyses before proceeding to histology

<table>
<thead>
<tr>
<th>Test</th>
<th>Mean (mg/dL)</th>
<th>Std Dev (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>196.7</td>
<td>91.2</td>
</tr>
<tr>
<td>Urea nitrogen</td>
<td>21.4</td>
<td>4.4</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.2</td>
<td>0.2</td>
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<tr>
<td>Bicarbonate</td>
<td>15.9</td>
<td>3.2</td>
</tr>
<tr>
<td>Chloride</td>
<td>107.5</td>
<td>4.2</td>
</tr>
<tr>
<td>Sodium</td>
<td>150.5</td>
<td>3.9</td>
</tr>
<tr>
<td>Potassium</td>
<td>5.0</td>
<td>1.1</td>
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<tr>
<td>Calcium</td>
<td>9.0</td>
<td>0.4</td>
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<tr>
<td>Direct bilirubin</td>
<td>0.1</td>
<td>0.1</td>
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<tr>
<td>Total bilirubin</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Albumin</td>
<td>1.4</td>
<td>0.2</td>
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<tr>
<td>Total protein</td>
<td>4.2</td>
<td>0.3</td>
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<tr>
<td>Phosphorus</td>
<td>8.0</td>
<td>1.6</td>
</tr>
<tr>
<td>AST (SGOT)</td>
<td>72.7</td>
<td>36.3</td>
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<td>ALT (SGPT)</td>
<td>38.7</td>
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<tr>
<td>Alkaline phos, total</td>
<td>101.5</td>
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<tr>
<td>Lipase</td>
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<tr>
<td>Cholesterol</td>
<td>98.6</td>
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<tr>
<td>HDL-chol.</td>
<td>78.6</td>
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<tr>
<td>LDL-chol.</td>
<td>12.2</td>
<td>8.6</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>67.8</td>
<td>26.1</td>
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Tests for Kidney function

Tests for Liver function
Finish hematology analyses before proceeding to histology

<table>
<thead>
<tr>
<th>Hematology</th>
<th>n = 7</th>
<th></th>
<th>n = 4</th>
<th></th>
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<tbody>
<tr>
<td>WBC (K/μL)</td>
<td>6.37</td>
<td>3.03</td>
<td>3.82</td>
<td>2.06</td>
<td></td>
<td></td>
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<tr>
<td>Neutrophils (%)</td>
<td>23.90</td>
<td>10.72</td>
<td>41.34</td>
<td>27.53</td>
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<tr>
<td>Neutrophils (K/μL)</td>
<td>1.35</td>
<td>0.52</td>
<td>1.84</td>
<td>1.83</td>
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<tr>
<td>Lymphocytes (%)</td>
<td>71.04</td>
<td>12.19</td>
<td>54.64</td>
<td>28.29</td>
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<tr>
<td>Lymphocytes (K/μL)</td>
<td>4.74</td>
<td>2.79</td>
<td>1.82</td>
<td>1.13</td>
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<tr>
<td>Monocytes (%)</td>
<td>4.47</td>
<td>1.29</td>
<td>3.51</td>
<td>0.59</td>
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<tr>
<td>Monocytes (K/μL)</td>
<td>0.26</td>
<td>0.07</td>
<td>0.14</td>
<td>0.10</td>
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<tr>
<td>Eosinophils (%)</td>
<td>0.47</td>
<td>0.59</td>
<td>0.39</td>
<td>0.27</td>
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<tr>
<td>Eosinophils (K/μL)</td>
<td>0.02</td>
<td>0.03</td>
<td>0.02</td>
<td>0.02</td>
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<tr>
<td>Basophils (%)</td>
<td>0.12</td>
<td>0.22</td>
<td>0.12</td>
<td>0.08</td>
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<tr>
<td>Basophils (K/μL)</td>
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<td>0.01</td>
<td>0.00</td>
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<td>RBC (M/μL)</td>
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<td>0.50</td>
<td>8.13</td>
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<tr>
<td>HGB (g/dL)</td>
<td>12.0</td>
<td>0.8</td>
<td>10.9</td>
<td>0.9</td>
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<tr>
<td>HCT (%)</td>
<td>40.0</td>
<td>2.1</td>
<td>34.8</td>
<td>1.2</td>
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<tr>
<td>MCV (fL)</td>
<td>44.5</td>
<td>1.8</td>
<td>42.8</td>
<td>1.1</td>
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<td>MCH (pg)</td>
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<td>MCHC (g/dL)</td>
<td>30.1</td>
<td>1.7</td>
<td>31.3</td>
<td>2.4</td>
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<tr>
<td>RDW (%)</td>
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<td>1.3</td>
<td>21.2</td>
<td>3.8</td>
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<td></td>
<td></td>
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<tr>
<td>PLT (K/μL)</td>
<td>765</td>
<td>142</td>
<td>942</td>
<td>279</td>
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<tr>
<td>MPV (fL)</td>
<td>5.16</td>
<td>0.27</td>
<td>5.41</td>
<td>0.47</td>
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</table>
When tissues are removed from the body, different preservation methods will help ensure optimal evaluation in order to determine the significance of the pathologic changes induced by disease.
An example of different ways to process tissues

- **Skin**
  - Culture medium (alpha MEM + antibiotics)
  - For primary fibroblast culture
  - Storage: 10 ml screw top tubes
  - Shipping temperature: RT

- **RNA later**
  - For RNA gene expression
  - Storage: Tight screw top plastic container, -20/-80
  - Shipping temperature: On dry ice

- **Flash Freeze**
  - In OCT compound plastic mold
  - For Frozen Histology
  - Frozen sample in mold into labeled plastic bag, -20/-80
  - Shipping temperature: On dry ice

- **Flash Freeze**
  - Cryotube
  - For DNA Biochemistry
  - Frozen sample in cryotube into labeled plastic bag, -20/-80
  - Shipping temperature: On dry ice

- **Buffered Formalin**
  - For Paraffin Histology
  - Tight screw top labeled plastic container
  - Room Temp
  - Shipping temperature: RT
The various tissues and organs that are examined using microscopy:

1. NEURAL
2. HEART / Blood vessels
3. LUNGS
4. LIVER
5. PANCREAS
6. SALIVARY GLAND
7. STOMACH
8. SMALL INTESTINE
9. COLON
10. SPLEEN
11. TONSIL
12. THYMUS
13. LYMPH NODES
14. BONE MARROW
15. KIDNEY
16. BLADDER
17. TESTIS
18. PROSTATE
19. UTERUS
20. OVARY
21. BREAST
22. PLACENTA
23. SKIN
24. SKELETAL MUSCLE
25. SMOOTH MUSCLE, ADIPOSE
26. CARTILAGE
27. BONE
28. THYROID/ Parathyroid
29. ADRENAL
30. PITUITARY
31. ---Eyes
32. ---Sinuses

Assess for metastasis.
Examples of Human Mouse Differences: in blood counts

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cell life span</td>
<td>120 days</td>
<td>43 days</td>
</tr>
<tr>
<td>White blood cells</td>
<td>Mostly neutrophils</td>
<td>Mostly lymphocytes</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td>Abundant megakaryocytes</td>
</tr>
<tr>
<td>Markers</td>
<td>CD markers</td>
<td>Different names</td>
</tr>
<tr>
<td>A few of many differences</td>
<td><strong>Human</strong></td>
<td><strong>Mouse</strong></td>
</tr>
<tr>
<td>----------------------------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>Brain</td>
<td>Gyri/sulci</td>
<td>Lissencephalic brain</td>
</tr>
<tr>
<td>Tonsil</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Lungs</td>
<td>3 right lobes 2 left lobes</td>
<td>Many right lobes 1 left lobe</td>
</tr>
<tr>
<td>Stomach</td>
<td>Glandular</td>
<td>Squamous + glandular</td>
</tr>
<tr>
<td>Colon</td>
<td>Proximal/distal difference</td>
<td></td>
</tr>
<tr>
<td>Cecum</td>
<td>Merges</td>
<td>large</td>
</tr>
<tr>
<td>Appendix</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>A few of many differences</td>
<td>Human</td>
<td>Mouse</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td>Many lobes</td>
</tr>
<tr>
<td>Kidney glomeruli</td>
<td>Gender difference</td>
<td></td>
</tr>
<tr>
<td>Seminal vesicles</td>
<td>Prominent</td>
<td></td>
</tr>
<tr>
<td>Uterus</td>
<td>Bi-cornuate</td>
<td></td>
</tr>
<tr>
<td>Ovary</td>
<td>Several follicles develop</td>
<td></td>
</tr>
<tr>
<td>Placenta</td>
<td>Distinct</td>
<td>Different</td>
</tr>
<tr>
<td>Brown fat</td>
<td>Not prominent</td>
<td>Prominent</td>
</tr>
<tr>
<td>Adrenals</td>
<td></td>
<td>Gender difference</td>
</tr>
<tr>
<td>Salivary glands</td>
<td>3 separate sites</td>
<td>3 grouped together, with gender difference</td>
</tr>
</tbody>
</table>
Mouse Lungs collapse on opening the thorax—Un-inflated lungs cannot be examined accurately by microscopy.

Identify the trachea (shiny cartilagenous rings) and insert a blunt needle. And INFLATE the lungs with OCT:PBS 1:1 to FREEZE for use as frozen sections in immunohistochemistry. Or Inflate with fixative and transfer to 70% alcohol for processing, embedding and paraffin sectioning.
Separate out each of the mouse lung lobes and embed flat in order to identify abnormalities.
Examples of mouse lung sections

Well inflated

not inflated

OCT infiltrated lung prior to freezing
Frozen section
Good morphology

non-OCT infiltrated lung,
Frozen section,
poor morphology
It is important to determine whether the spleen undergoes Fixation (flat between sponges)

Or

Whether it is cryo-protected for correct freezing for immunohistochemistry

Or

just placed in the freezer for extracts
All organs need cryoprotection before freezing, for microscopic examination by frozen sections, to prevent freeze artefact.

Frozen sections of mouse spleen with H&E to review morphology:

OK to use

freeze artefact, do not use
Tissues that are removed from the body have to be processed correctly for histology

Frozen tissue: Using specific freezing protocols
Snap-freeze tissue which is then stored at minus 80
Use the cryo microtome or cryostat
To do frozen sections

Fixed Tissue: in 10 volumes of fixative for 24 hours and then transfer to 70% alcohol
For processing and embedding into paraffin wax for storage at room temperature
To cut paraffin sections
Freeze for protein, lipid, sugar, DNA/RNA etc. extracts

Isolate cells for culture

Immerse this sections in appropriate fixative to Process into paraffin blocks

Freeze for use in immunohistochemistry

Process for EM

Dry ice in 2-methyl butane

- Fix thin slices in correct fixative
- Dehydrate in graded alcohols
- Infiltrate with xylene
- Infiltrate with hot paraffin wax
- Make blocks for sections
- Store at room temperature
- Deparaaffinize sections by
  - removing wax in xylene,
  - rehydrate in decreasing concentrations of alcohol to water

OCT surrounds fresh tissue in plastic mold

Frozen for histology

Frozen tissue or Fixed Paraffin-embedded tissue can then be sectioned for histology into 3--30 micron sections
Materials needed for flash freezing tissue for histology
Video of freezing technique

www.mousephenom.ucsd.edu

http://mousephenom.ucsd.edu/movies/freezing.MOV
IMMUNOHISTOCHEMISTRY ASSAYS best on frozen sections but paraffin sections may also be used
Caveat: Effect of different fixatives on preserving epitopes in frozen sections

If the tissue is paraffin embedded, some mouse monoclonals do not recognize the epitope, in spite of using retrieval techniques.
FIXATIVES

• Fix Thin slices of tissue, or inflated lungs, or tissue in sponges
• use 4% freshly made paraformaldehyde for 24 hours before immersion in 70% alcohol to submit to histotech
• Or 10% buffered formalin for 24 hours before immersion in 70% alcohol to submit to histotech
• Or Bouin’s solution--has picric acid (yellow), acetic acid and formalin--fixes fast, makes tissues hard if left in it for more than 6 hours, many antibodies do not detect epitopes after Bouin’s fixation
• Or Zinc containing fixatives, preserve epitopes for immunostaining
Four commonly used Fixatives for tissue processing in histology

10% Neutral buffered formalin

4% paraformaldehyde is made fresh in the fume hood before use

Zinc formalin fixation requires special processing

Bouin’s fixative is quick but it hardens tissues, if fixed for too long, so move specimens to 70% alcohol in 6 hours.

Use 10 volumes of fixative for each samples, overnight in labeled cassettes, before transfer to 70% alcohol for processing embedding and sectioning, staining for microscopic analysis
Make 4% paraformaldehyde in the chemical hood with heating and with NaOH and PBS, cool and freeze in aliquots

Add 4 g paraformaldehyde

Wear a mask and be careful while weighing it out, it disperses easily

To 50 ml of water

Heat to 65 degrees

Add 6 ul of 10M NaOH

Solution will clear

Filter via Whatman paper

Add 10 ml of 10X PBS

Make up volume to 100 ml

Store at 4 degrees for upto 24 hours

Or freeze in aliquots
Use simply labeled cassettes, using indelible pencil, to fix thin slices of organs or rolls of intestine, in 10 volumes of fixative, for less than 24 hours, before transferring to 70% alcohol, for processing into paraffin blocks.

Do not use a “Sharpie “ to label cassettes.

Use Sponges in cassettes for to flatten certain organs such as: Spleen, Thymus, Pancreas, adipose tissue, skin, small organs such as adrenals, ovaries, lymph nodes to orient them FLAT for good sections.
If you need to **FREEZE** FIXED tissue for histology:

If the animal has been **perfusion fixed** -- the organs have to SINK (Descend to bottom of tube) in 30% sucrose/PBS

Before blotting well to remove extra sucrose, to **freeze in OCT** for histology examination

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Materials that are needed to use to freeze tissue for histology
Mouse Small Intestine
Well-fixed, processed, sectioned and stained

Effects of poor fixation
Bones have to be de-calcified after fixation

Decalcification solutions:

HCl; Formalin + HCl; EDTA only - for slow decalcification for IHC
Importance of Orientation of tissues:

- Coronal sections
- Sagittal sections
- Transverse sections
Correct orientation to gain the most information during histopathologic examination, an example of a section of mouse embryo
HEMATOXYLIN AND EOSIN STAINS

**H&E** = hematoxylin and eosin.

**Hematoxylin** colors nuclei blue

**Eosin** colors the cytoplasm pink

**HISTO**: HISTOLOGY SECTIONS FOR VIEWING UNDER THE MICROSCOPE, using BRIGHTFIELD illumination

Always review sections using the basic **hematoxylin and eosin** (H&E) stain before proceeding to perform an immunohistochemical assay in order to check out the morphology of the tissue and to determine that what you are looking for is present in the section to be immunostained and that the section has no other abnormalities.
This is a photo of an unstained section on a slide, which needs histochemical stains to help with identification of the tissue.
An example of a section of Mouse lung frozen section stained only with hematoxylin
An example of a section of Mouse lung stained with **Hematoxylin** and **Eosin** to demonstrate morphology.
Examples of Different Histochemical stains to demonstrate different components in a section of Human Colon

H&E is standard to assess morphology

A Trichrome stain to demonstrate collagen (blue)

PAS (periodic Acid Schiff) for carbohydrates—here demonstrated fuschia colored mucin in goblet cells of colon epithelium
Reticulin stain to highlight supporting support

Normal mouse liver

Liver with invading cancer
Oil Red O stain of FROZEN section of mouse liver showing moderate amounts and large amounts of fatty accumulation in hepatocytes

Control: adipose tissue
Commonly used “Blue” stains in histochemistry:

--hematoxylin: for nuclei

--Trichrome: for collagen and for scarring/fibrosis

--Alcian Blue: for mucin and for cartilage

--Nissl: for nuclei in neurons

--Luxol Fast Blue (LFB): for myelin
Commonly used “Red” stains in histochemistry:

--Eosin: stains cytoplasm and depending in the tissue type, can vary in intensity

--PAS: Periodic Acid Schiff—for carbohydrate containing material, in mucin and basement membranes

--Alizarin Red: stains bone

--Safranin O: stains cartilage

--Oil Red O—to identify lipid containing cells, has to be done on frozen sections
White Matter paraffin sections of Mouse Brain: Myelin stain

Luxol Fast Blue (LFB) for myelin
Commonly used “Black“ stains in histochemistry:

--PTAH: Phosphotungstic Acid Hematoxylin, to identify striations in skeletal muscle and also for collections of abnormal fibrin in clotting disorders

Elastic: to identify elastic fibers

Reticulin: to identify reticulin supporting fibers
Human Skeletal muscle with Phosphotungstic Acid Hematoxylin PTAH stain to demonstrate striations
Human aorta: H&E and Elastic stain
This is a large vessel with abundant elastic fibers to contribute strength
Silver stain to demonstrate reticulin supporting tissue
EPITHELIUM is the term given to the cells that
- cover the exterior surface of the body,
- lines both the internal closed cavities of the body,
- lines body tubes that communicate with the exterior (alimentary, respiratory, genitourinary)
- comprise the various organs (liver)

Epithelium can be
- impervious (epidermis or bladder),
- secretory (stomach),
- absorptive (intestines),
- be a transport system (trachea),
- receive sensory stimuli (taste buds of the tongue)
Epithelium can be impervious (epidermis or bladder)
Stratified Squamous epithelium—stacked up like plates

Squamous epithelium function helps with shear forces that are encountered as in
- Skin, with anuclear keratin layer
- Esophagus (No keratin layer)
- Cervix (no keratin layer)
- External ear canal
- Anus
Epithelium can be secretory (stomach), absorptive (intestines),

Columnar epithelium—is so termed because the cells are arranged like columns.

The height of the cell is greater than the width.

Epithelial lining of intestine
Ovary with developing follicles

Primordial follicles lined by flat squamous epithelium

Primary follicles lined by cuboidal epithelium

Cuboidal epithelium

Stromal fibroblasts
Pseudo-stratified Columnar epithelium

Human trachea

Mouse trachea
Transitional epithelium of bladder (mouse)
Junctional zone epithelium: Where epithelium of one kind changes naturally to another

Mouse Stomach: half is squamous epithelium

Mouse cervix: external is squamous epithelium
Junctional zone epithelium: Where epithelium of one kind changes naturally to another

HUMAN STOMACH

Mouse Stomach: half is squamous epithelium
Normal breast ducts and alveoli have an Inner layer of cuboidal epithelial cells (keratin+) and an Outer layer of myoepithelial cells (smooth muscle actin).

Keratin is a marker for most epithelial cells.

Frozen sections of Human Tonsil
H&E for morphology
IgG negative control

squamous epithelium
anti-Keratin (epithelial)
lymphoid cells demonstrate
anti-CD45 binding

scale bar = 500 microns
Vimentin is a marker for stromal fibroblasts and blood vessels.
1. Frozen sections are useful for Immunohistochemistry.  T/F
2. Frozen sections are useful for Morphologic examination.  T/F
3. Paraffin sections are made after fixation.  T/F
4. Paraffin sections can be used for immunohistochemistry  T/F
5. Length of fixation affect the ability to detect antigens in paraffin sections.  T/F
6. Bone has to be fixed and decalcified for two-three days before processing into paraffin blocks, for sectioning and staining.  T/F
1. Your genetically altered mouse died last night. You want to know the cause of death. Should you fix and look paraffin sections or plan to sacrifice littermate controls and gene altered animals at specified time points, harvest organs, fix and examine paraffin sections?

2. The animal has been perfused with PBS and then with fixative. Can the tissue from various organs be now frozen and sectioned for immunohistochemistry?