Chapter 3

Necropsy and Sampling Procedures in Rodents

Laurence Fiette and Mohamed Slaoui

Abstract

Necropsy is a major step of most studies using laboratory animals. During necropsy, tissue and organ changes noticeable grossly can be recorded, and important tissue samples can be stored for subsequent evaluation. It is therefore important that the personnel in charge of this key experimentation step be adequately trained and aware of the study endpoints.

Key words: Necropsy, Histology, Macroscopy, Sampling, Rodents, Mouse, Rat

1. Introduction

Necropsy is a postmortem procedure that consists of observation of macroscopic changes of tissue and organ *in situ* with naked eyes and of collection of key organs and tissues samples for further analyses.

Necropsy techniques have been developed to diagnose diseases in animals and are classically used in health status monitoring of domestic and laboratory animals. These postmortem procedures are also considered as an important step in biomedical research using laboratory animals and in particular in toxicity studies where histopathology is a major endpoint. In all cases, necropsy is the ultimate examination of the animal body. It allows detecting, describing, and reporting of any gross finding that could be key to understand changes noted during the *in vivo* part of the experiment. It is therefore important that the personnel in charge of the postmortem procedure have access to the animal history including clinical examination and behavioral changes that preceded necropsy as well as to the results of any imaging or laboratory investigations. On the other hand, the necropsy personnel should have access to the experiment study plan to harvest tissue important samples to meet the objectives of the study.

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Since necropsy can be performed only once, it is crucial to follow a precise procedure that allows identification of gross abnormalities and adequate sampling of organs.

The necropsy procedure consists of a series of systematic operations that allow examining all body organs and cavities without altering the characteristics of any tissue or organ of the animal. The collection of samples for histology or other complementary analyses also follow precise rules.

Histopathology is a major endpoint of many experiments. It is therefore crucial that tissues be sampled and preserved in a standardized manner especially when the microscopic findings need to be compared group wise. The amount of tissue that needs to be sampled should be representative of the whole organ since the probability of detecting lesions is primarily dependent on the amount of the tissue examined. For large organs of the body, such as the lungs or the liver, it is therefore necessary to define the number of sections and the specific lobe/area to be sampled. The anatomic characteristics of each organ should be considered. For example, the kidney is composed of a cortex, a medulla, and a pelvis. Each of these anatomical regions has distinct histological characteristics that need to be evaluated, as microscopic changes may reside only in one or two of these structures. For organs comprising a lumen or a cavity (such as intestines, urinary bladder, uterus, or heart), the amount and type of structures present on a transversal section may considerably vary and may subsequently compromise and sometimes irreversibly hamper adequate microscopic evaluation. Therefore, the plane of section should be considered and carefully standardized during sampling. For tissues with multiple or complex anatomical structures (such as the brain, nasal cavity, or intestines), it is advised to collect multiple sections to examine all structures of interest.

The probability of detecting lesions in the histological slide is also influenced by the technical procedures such as method of preservation and preparation. It is essential that the postmortem degradation process (otherwise termed as autolysis) be controlled. This is achieved by thorough immersion of tissue samples in a fixative like formalin. Other fixation techniques are available and should be always considered depending on each experiment-specific endpoints.

The purpose of this chapter is to describe the different steps of the necropsy, and to give general guidelines for sampling procedures in Rodents although these can be easily adapted to other laboratory animal species. As the knowledge of anatomy basics and species differences is required to perform the necropsy, a basic anatomical description will also be given. It is extremely useful to be aware of the subsequent histological processing of organs/tissues for histology to understand the rules of sampling and trimming. Hence, this chapter is intimately linked to the next

one dedicated to Histopathology procedures: from sampling of tissues to histopathological evaluation.

Well-illustrated descriptions of necropsy protocols are available (1–5), some of them on the Web (6, 7) that would complement this chapter. Handbooks on the anatomy of Rodents are also useful (8, 9). The reader is also strongly encouraged to refer to the three excellent publications from the RITA/NACAD group about organ sampling and trimming in rats and mice that give guidelines in a very attractive manner, with excellent full-color macrophotographs and microphotographs from the corresponding Hematoxylin and Eosin (H&E)-stained slides (10–12).

2. Materials

1. Fume hood.
2. Rubber gloves (vinyl gloves in case of allergy to latex), protective clothing, eyeglasses, and mask.
3. Dissecting board preferentially in plastic, which could be easily cleaned and autoclaved.
4. Blunt ended forceps. Serrated forceps should be avoided as they may damage small animal tissues.
5. Small dissecting scissors, surgical scissors, and microsurgical (ophthalmologic type) scissors (These are very useful especially during extraction of the central nervous system).
6. Syringes (1 mL, 5 mL, 10 mL) and needles (a 21-gauge needle is suitable for infusion of the lung with fixative).
7. Scalpels (new blades and handle).
8. Plastic bags and paper towels.
9. Containers for histological specimens, cassettes, and labels. All containers should be adequately identified before start of necropsy.
10. Specific containers for other specimens (bacteriology, virology, mycology, parasitology, chemistry) should be available.
11. Tubes for liquid samples.
12. Euthanasia solution or suitable source of CO_2 and container.
13. Fixative (usually 10% neutral buffered formalin). Unless otherwise specified, the fixative mentioned in the text will be 10% neutral buffered formalin. Ready-to-use 10% neutral buffered formalin is commercially available from major suppliers. However, this fixative can be easily prepared. A detailed procedure is described in Table 1. Formalin is now included

in the list of human carcinogens and will be abandoned in the near future. Different commercial alternatives are proposed and are currently under testing in many laboratories.

14. Decalcifying solution (in case bones should be examined). There are several decalcifying solutions. A 26% formic acid solution (TBD2® Shandon Lipshaw) is routinely used in histopathology laboratories.
15. Ethanol 95% and 70%.

Table 1
Protocol for preparation of formalin and modified Davidson's fixatives

Protocol for preparation of formalin 10%, buffered	
Formaldehyde (37–40%)	100 mL
Distillated water	900 mL
Monosodium phosphate anhydrous	4 g
Disodium phosphate anhydrous	6.5 g
Final solution 3.7–4% formaldehyde	
To be used preferentially within 6 months after preparation. Keep at room temperature	
Protocol for preparation of paraformaldehyde 4% (PFA 4%)	
Paraformaldehyde (powder)	4 g
Distillated water	80 mL
Put 6 µL of sodium bicarbonate	
Put 10 mL of PBS ×10	
Filtrate on a paper	
Add PBS to obtain 100 mL	
Adjust the pH to 7.2–7.4	
The solution should be heated to facilitate the dissolution of the PFA, but below 65°C and in a fume hood	
Store at 4°C up to 24 h	
Can be also frozen in aliquots	
Protocol for preparation of modified Davidson's fixative	
30% of a 37–40% solution of formaldehyde	
15% Ethanol	
5% Glacial acetic acid	
50% Distilled H_2O	

16. Saline solution or Phosphate Buffer Solution (PBS).
17. Weigh scale.
18. Material to take photographs.
19. Metric scale.
20. Recording necropsy cards.

During necropsy, it is recommended to place the instruments in a stainless steel instrument holder with 70% ethanol. Used needles and scalpel should be placed in a special container for harmful material.

There are several hazards related to handling and dissection of laboratory animals during necropsy that should be considered. The chemical risk is one of them. For example, formalin causes eye, skin, nose, and respiratory tract irritation; it is also classified as a strong skin sensitizer and carcinogen in humans. Therefore, formalin should not be handled without gloves or outside a fume hood (13, 14).

Laboratory Rodents can spontaneously carry and transmit several diseases to man (also called zoonoses) (15). Although laboratory Rodents are usually tested for these agents, there is always a risk to contract one of these diseases. In addition, the allergic risk remains important especially for people with known animal allergies. Working with laboratory animals can lead to exposure to allergens via inhalation, direct skin and eye contact with animal dander, hair, urine, serum, or saliva. It is therefore essential that all necropsy personnel uses protective equipments (16, 17) and that cadavers and all waste be eliminated appropriately.

3. Methods

3.1. General Recommendations

3.1.1. Necropsy Protocol and Specimen Collection

It is strongly recommended to carefully read the experimental study plan or protocol. The list of organs that should be examined and sampled differs from one study to another depending on the aim and duration of the study. The following procedure allows examining and collecting most tissues and organs in Rodents with the main purpose of histopathology evaluation of tissue samples. It is very much inspired from necropsy performed in toxicity studies. The major steps are as follows:

- Examination of the live animal
- Euthanasia
- Exsanguination
- Opening of the abdominal cavity
- Opening of the thoracic cavity

- Opening of the skull
- Examination of muscles and skeleton.

This protocol should be followed step by step. It is the more convenient way to remove, examine, and sample each organ or tissue. Alternative methods are available; they will be mentioned in the notes.

Organs should always be immersed in the fixative immediately after their removal, macroscopic examination, or weighing. It is sometimes required to perform necropsy on animals found dead. Variable degrees of autolysis will inevitably take place, but it is still useful to perform the autopsy. Carcasses of found dead animals can be placed in a refrigerator (but NOT frozen). Necropsies should be performed as soon as practicable (if possible within the same day).

3.1.2. Weighing of Organs

One important part of necropsy is to describe the size of organs. This requires that the dissector has in reference the normal size of the organ. Small variation in size can be challenging even for experienced pathologist especially when organ sizes need to be compared among several individuals and groups. To accurately compare organ sizes, it is most helpful to record individual organ weights. These can be compared either as absolute organ weights or as ratios of organ weight to total body weight or to brain weight. Following the recommendations from the Society of Toxicologic Pathology (STP) (18) liver, kidneys, heart, brain, adrenal glands, testes, spleen, and thymus should be weighed routinely in all general toxicology studies with multi-dose administration in Rodents (Table 2). Thyroid and pituitary gland should be weighed in rats but not in mice, as handling of these minute tissues may induce artifacts that can complicate microscopic assessment. Epididymides and prostate weighing is recommended in rat studies but only on a case-by-case basis in mouse as well as other organs including female reproductive organs in Rodents. Organs weights are not recommended in carcinogenicity studies.

In all cases, organs should be weighed free of surrounding fat and connective tissues. It is important to remove these tissues in a standardized manner and without inducing any damage or artifact to the tissue.

3.1.3. Organ Sampling for Histology

Most of the tissue samples will be immersed in the fixative. Small tissues can be kept in histology cassettes at the time of dissection. The same cassette, adequately identified, can be used for the paraffin embedding and paraffin block preparation. The number of cassettes and hence paraffin blocks can be considerably reduced by combining a few tissues and organs in one cassette. For example, several small and large intestines can be grouped (19, 20) (Table 3).

Table 2
List of organs for weighing

Rat	Mice
Liver	Liver
Kidney	Kidney
Heart	Heart
Adrenal glands	Adrenal glands
Brain	Brain
Testes	Testes
Prostate	Spleen
Epididymes	Thymus
Spleen	Thyroid/parathyroid
Thymus	Pituitary gland
Thyroid/parathyroid	
Pituitary gland	

Sellers, R., et al., in *Toxicologic Pathology* **35**, 751–755 (2007)

3.1.4. Examination and Recording of Macroscopic Observations

The description of macroscopic findings should be sufficiently detailed to give a mental representation of gross changes. Therefore, all criteria should be used to describe the gross changes of an organ: location, appearance (color, shape, consistency), demarcation from surrounding tissues, number, distribution and severity, size, which should always be measured in two or three dimensions (cm or mm), or in volume (mL).

3.1.5. Orientation

It is critical for the necropsy personnel to correctly use the terms that define the orientation of an organ or a section (Fig. 1):

1. Dorsal refers to the back of the animal whereas ventral refers to the abdomen.
2. Cranial refers to the skull, whereas caudal refers to the tail.
3. Rostral refers to the organs or structures situated toward the front of the head.
4. Lateral pertains to a side, medial is related to, situated in, or extending toward the middle, closer to the body's midline.
5. Proximal is located nearer to a point of reference; distal is located far from a point of reference (origin, point of attachment, or midline of the body).

Table 3
Example of blocking scheme

Cassette	Tissues
1	Heart Aorta Vena cava
2	Muscle skeletal (diaphragm, tongue and soleus)
3	Lung: entire (all lobes)
4	Thyroid with trachea (specimen immediately caudal to larynx, cross section)
5	Kidneys (cross-sections of left and right kidneys) Urinary bladder
6	Ureters
7	Adrenal glands
8	Stomach: glandular and nonglandular portions Duodenum Jejunum
9	Ileum (with Peyer's patch) Cecum Colon Rectum
10	Salivary glands (Mandibular, sublingual and parotid) Cervical lymph nodes
11	Pancreas Mesenteric lymph node Thymus Spleen
12	Liver: left lateral (largest) lobe (one section cut from hilus to periphery), median lobe (one section to include gall bladder)
13	Ovaries Oviducts Uterus (two cross sections through horns and one longitudinal section through body and cervix) Vagina (cross section)
14	Testes (cross sections, left and right) Epididymides Seminal vesicles with coagulating glands (cross sections or longitudinal sections depending on size) Prostate (cross section)
15	Skin ventral inguinal specimen (one section parallel to hair growth, to include mammary glands)
16	Clitoral or preputial glands
17	Femur with knee and tibia with bone marrow
18	Sternum with bone marrow
19	Brain (2 transversal sections through cerebrum including olfactory lobes and hippocampus, and one through cerebellum and pons)
20	Spinal cord
21	Pituitary
22	Eyes with optic nerves and lacrimal glands

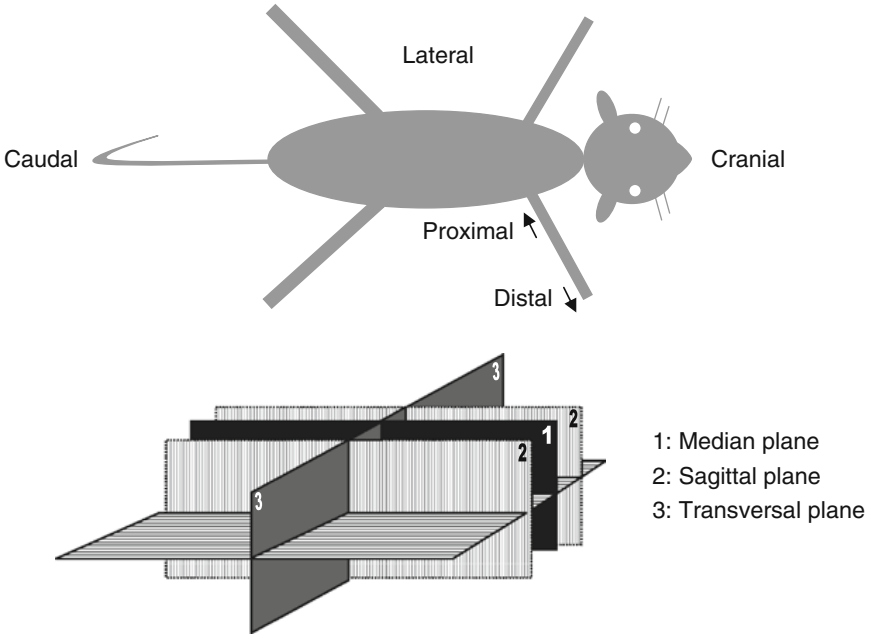

Fig. 1. Anatomical orientation descriptors usually used at necropsy.

The major planes of sections are defined as follows:

1. Median plane, passing longitudinally through the middle of the body from front to back, dividing it into left and halves right.
2. Frontal (coronal) planes, passing transversally through the body from side to side, perpendicular to the median plane, dividing the body into front and back parts.
3. Sagittal planes are vertical planes passing through the body parallel to the median plane dividing the body into left and right portions.
4. Transverse plane (dorsoventral), passing vertically through the body, perpendicular to the sagittal planes, and dividing the body into front and back portions.
5. Horizontal plane, passing through the body, perpendicular to both the frontal and median planes, dividing the body into upper and lower parts.
6. Vertical plane, perpendicular to a horizontal plane, dividing the body into left and right or front and back portions.

3.2. Examination of the Live Animal

1. Observe the behavior of the animal and check its response to external stimuli.
2. Observe the fur and the skin to detect any changes.

3.3. External Examination

1. Weigh the animal. As indicated in Subheading 3.1.2, this will be useful to calculate organ weight to body weight ratios.
2. Examine the animal before proceeding to the necropsy.

3. Assess the general condition of the animal (emaciated, thin, adequate/good condition, obese).
4. Check and record any external lesions (skin lesions, fur loss or discoloration, and subcutaneous masses). Record sizes and surfaces whenever possible and record their gross appearance and precise location.
5. Check and record the color changes of the skin and mucosae (gingival, genital mucosae, conjunctiva).
6. Examine the eyes, mouth, teeth, and nasal openings and record any abnormality.
7. Examine the ano-genital region to look for signs of diarrhea, rectal, or vaginal prolapse and record any abnormality.
8. Gently palpate the abdomen to reveal abdominal masses or presence of fluid.
9. If the abdomen is distended by fluid, take a sample with a sterile needle and syringe for further evaluation.
10. Palpate any mass and record its consistency (soft, fluctuant, firm, or hard).

3.4. Euthanasia

Various methods of euthanasia are available for Rodents. The selected method should induce death quickly with minimal animal pain or distress and should not interfere with the gross observation and microscopic evaluation of the tissues. Euthanasia protocols should be approved by the local ethical committee.

We describe in this chapter the euthanasia method which is commonly used in toxicological studies: exposure to carbon dioxide (CO_2). This method requires only a source of CO_2 and a polycarbonate box of a size that is well-matched with the size of rodents. The euthanasia procedure is as follows:

1. Place a wet sponge in one of the corners of the box.
2. Place the lid with the CO_2 tube attachment on the box.
3. Charge the chamber with CO_2 gas for 1–2 min.
4. Place animals in the box.
5. Turn gas on low so as not to frighten the animals.
6. Administer CO_2 until deep sedation is observed.
7. Death is induced by maximal exsanguination from the abdominal aorta (this can be the occasion to collect blood for hematology, coagulation, and clinical chemistry parameters).
8. Check heart beat and respiration to verify death.

3.5. Incision of the Skin and Examination of the Subcutaneous Tissues/Organs

Skin, subcutaneous tissue, mammary glands, salivary glands, superficial cervical lymph nodes, extraorbital lacrimal gland, clitoral glands, preputial glands, penis, and prepuce.

3.5.1. Incision of the Skin and Examination of the Subcutaneous Tissue

1. Pin the animal on the dissection board, ventral side up and head in front of you. Remember that from this point, the left side of the animal is on your right side and vice versa.
2. Moisten the skin and hairs with 70% alcohol.
3. Incise the skin with a scalpel on the midline, from the mandibles to the pubis. In males, incision should end on both sides of the penis.
4. Reflect the skin on both sides of the incision.
5. Examine the skin and the subcutaneous tissue. Record any lesion; confirm skin changes observed in the previous step.

3.5.2. Examination and Sampling of the Mammary Glands

1. Examine the mammary glands: the mouse has five pairs of mammary glands (three thoracic and two abdominal), while the rats have six pairs (three thoracic and three abdominal). There are six or seven pairs in hamsters (21, 22). In females, the mammary tissue extends from the salivary gland region to the base of the tail. When lactating, the mammary gland occupies almost all the abdominal and thoracic ventral subcutaneous area in the mouse and in the rat.
2. Harvest the mammary gland from the inguinal region where the mammary tissue is abundant in both rats and mice. Take a transverse section (1 cm × 3 cm) including the associated nipple and skin (see Note 1). This applies to males as well as to females.

3.5.3. Examination and Sampling of the Superficial Lymph Nodes

Examine the superficial lymph nodes. Under normal conditions, lymph nodes are grayish organs, bilateral, and have the size and shape of a small pea. Major superficial lymph nodes, located in the subcutaneous area are the cervical superficial, *axillary* in the axillary fossa, *brachial*, *inguinal*, and *popliteal*. The peripheral lymph nodes that are most often examined are the *mandibular, axillary,* and/or *popliteal lymph nodes* (see Note 2). The location of these lymph nodes is detailed in Table 4 (23).

3.5.4. Examination, Removal, and Sampling of Salivary Glands, lacrimal glands, clitoral or preputial glands

1. Examine the three pairs of salivary glands located on both sides in the cranioventral region of the neck: *mandibulary glands*, *sublingual glands*, and *parotid glands* (24, 25). The mandibulary gland is the largest one, located in the ventral region of the neck. Sublingual glands are situated on the top of mandibulary glands. Parotid glands are the most lateral ones; they extend to the base of the ear.
2. Collect salivary glands by gently grasping the tip of the closest salivary gland to the thorax with forceps. Then slowly pull toward cutting the surrounding tissues with scissors and immerse in fixative.

Table 4
Nomenclature and location of major lymph nodes

	Name	Location
Superficial	Mandibular	Rostromedial to the sublingual and mandibular salivary glands
	Axillary	Axillary fossa
	Brachial	Proximity to the angle of the capsula, upon the biceps, underneath the pectorals
	Inguinal	Adherent to the skin of the groin
	Popliteal	Between the adductor muscle and the semimembranous muscle behind the knee
Deep	Deep cervical	Behind the salivary glands hidden in the connective tissue encircling the trachea
	Lumbar, caudal	Anterior to the bifurcation of the abdominal aorta
	Mediastinal	Posterior face of the thymus
	Mesenteric	Lengthened shape, with the mesentery, close to the ascending portion of the colon
	Pyloric (pancreatic)	Anterior end of the pancreas, near the pylorus
	Renal	Between the aorta and hilum of the kidneys
	Sacral	Posterior to the bifurcation of the abdominal aorta
	Sciatic	Below the sciatic nerve on the back

3. Examine extraorbital lacrimal glands. These glands are located on the ventro-lateral aspect of the head. They appear as flat, brown-gray glands next to the parotids.
4. Remove extraorbital glands on both sides by gently grasping the tip of the gland with forceps, isolate the gland from its attachment in the eye socket using scissors, and immerse the two glands in fixative (see Note 3).
5. Examine clitoral glands in females or preputial glands in males. Clitoral/preputial glands are modified sebaceous glands that are included in the subcutaneous adipose tissue. They can be found cranial to the vulva in females and lateral to the penis in males. Preputial glands are leaf-shaped and dark-gray color with a soft consistency.
6. Remove whole glands on both sides by gently grasping the tip with forceps to isolate them from the surrounding tissue. Immerse the clitoral/preputial glands in fixative.

3.5.5. Examination of the Penis

Examine the penis and the prepuce in males. These organs will be harvested with the remaining genital organs.

3.6. Opening of the Abdomen and Examination of Abdominal Organs

Abdomen is opened and abdominal organs such as peritoneum, spleen, mesenteric lymph nodes, pancreas, digestive tract, liver, adrenal glands, kidneys, and genital organs are examined.

3.6.1. Opening of the Abdominal Cavity

1. Grasp the abdominal wall with forceps near the sternal xyphoid appendix and lift firmly.
2. Make a small incision to let air into the abdomen. This will allow abdominal viscera to be separated from the abdominal wall.
3. Cut the abdominal wall on the midline with scissors from the pelvis to the xyphoid appendix. Make sure not to cut any of the abdominal organs that lie underneath.
4. Reflect the abdominal wall on the sides.
5. Examine the abdominal serous membrane (*peritoneum*) and look for the presence of abnormal contents such as serous fluid, blood, or fibrin as well as to any adhesion between abdominal wall and abdominal organs.
6. Check the position of the different organs *in situ*.
7. Check fat deposits and score the nutritional conditions (1. Obese, 2. Good nutritional condition, 3. Poor nutritional condition, 4. Bad nutritional condition, 5. Emaciation, absence of fat in the body deposits). The amount of fat is dependent on the age and strain of the animal: adults and aged animals tend to have more fat deposits in the abdominal cavity.

3.6.2. Removal, Examination, and Sampling of the Spleen

1. The spleen is situated on the left superior abdominal quadrant. To remove it, grasp the connective tissues and fat surrounding the spleen with the forceps. Cut them along the hilus of the spleen and cut the gastrosplenic ligament as well.
2. Examine the spleen. It is a lengthened, oval, slightly curved shape organ, has a dark-red color, and is soft in consistency with a thin transparent capsule (26). It is attached to the stomach by the gastro-splenic ligament.
3. Should spleen be weighted, it should be carefully freed from all remnants of connective and adipose tissues, in particular along the hilus.
4. The entire spleen can be sampled and immersed in fixative although a 2-mm thick transverse mid-section can be sufficient.

3.6.3. Removal of the Digestive Tract and Mesenteric Lymph Nodes

Abdominal part of the esophagus, stomach, small intestine (duodenum, jejunum, ileum), large intestine (cecum, colon, rectum), and mesenteric lymph nodes.

1. Dissect the anus free from the surrounding skin.

2. Insert the tip of heavy duty scissors between the colon and the pelvis to cut the pelvic arch on both sides. Take off the resulting bone chip to facilitate the subsequent removal of the genital tract and rectum.
3. Hold the rectum with forceps and lift it upwards.
4. Isolate the rectum from the vagina in females.
5. Gradually extract the intestines in a caudal-cranial direction, while cutting the insertion of the mesentery as close to the intestines as possible.
6. Cut the esophagus just below the diaphragm in the abdomen leaving a small portion of esophagus connected to the stomach.
7. Isolate the whole digestive tract together with the pancreas.
8. Examine the deep abdominal lymph nodes (23, 27) for nomenclature and location before unzipping the intestines. The mesenteric lymph nodes are usually the largest lymph nodes of the abdominal cavity. They drain the duodenum, ileum, cecum, and colon.
9. Starting at the rectum, progressively unzip the coiled intestines by pulling gently with scissors.
10. Samples of intestine segments as well as mesenteric lymph nodes should be immersed in the fixative as soon as possible.

3.6.4. Examination and Sampling of the Pancreas

1. In Rodents, the pancreas is very diffuse and completely enclosed in the mesentery. It is tan in color and therefore easily identifiable from the surrounding fat. It has one *left lobe* located close to the spleen in the great omentum, and one *right lobe* adjacent to the duodenum.
2. Lift up the pancreas attached to the spleen and separate them from the intestines by cutting its insertion with scissors.
3. Isolate the spleen from the pancreas with the scissors.
4. Take a sample from the *left pancreatic lobe* to have the largest surface. The *right pancreatic lobe* will be sampled with the duodenum.

3.6.5. Examination and Sampling of the Stomach

5. In small Rodents (mice, rats, hamsters, and gerbils), the stomach has two distinct regions: the proventricular or anterior region that has a white appearance, and the glandular region which has a reddened and thicker wall (28, 29). Both are clearly demarcated by a limiting ridge in these species but not in the guinea pigs.
6. Isolate the stomach from the esophagus and the intestines.
7. In mice, open the stomach along the greater curvature, mount on a plastic card, fix it with pins, and immerse it in the fixative. In rats, open the stomach of the rat along or para-median

to the greater curvature and placed on a corkboard. Remove the gastric content and, if necessary, clean carefully the mucosa with saline solution or fixative. Spread out the stomach and pin it on the cardboard. This procedure avoids folds in the mucosa and therefore is essential for the macroscopic orientation and allows reproducible microscopic evaluation of the thickness of gastric mucosa.

3.6.6. Examination and Sampling of the Intestines

1. The small intestine has three different regions: *duodenum*, which is very short (1 cm) in Rodents, *jejunum* and *ileum* that are not recognizable grossly. The large intestine is composed of the *cecum*, *colon,* and *rectum* (short segment in Rodents, included in the pelvis) (30). Lymphoid tissue associated with the small intestine (jejunum and ileum) is also called gut-associated lymphoid tissue (GALT) or Peyer's patches. These appear as slightly elevated lighter fields in the intestine's wall and can be discernible as prominent areas when activated.
2. Separate carefully the intestines from the mesentery during necropsy (or after fixation).
3. Examination of Intestines should comprise at least the content, wall thickness, color, aspect of mucosa, or any noticeable lesion in each intestinal segment.
4. If the microscopic evaluation of the whole intestine and GALT on a single section is preferred, the so-called "Swiss roll method" can be performed (31, 32) (see Note 4).
5. Routine intestine samples for histopathology evaluation usually consists of one transverse section (2–3-mm thick) from each part of the bowel without opening it: duodenum (1 cm distal to the pyloric sphincter), jejunum (central section), ileum (1 cm proximal to cecum), cecum, colon (central section), rectum (2 cm proximal to the anus). For small intestines, it is also essential to sample the GALT. Standardized sampling procedure of the bowel segments is necessary to guarantee examination of each required segment.
6. After sample collection for histopathology, the remaining intestine should be opened longitudinally and examined for abnormalities. To better examine the mucosa, a gentle rinse of the ingesta with saline solution may be necessary. This latter procedure is time consuming and should be considered on a case-by-case basis only.

3.6.7. Removal, Examination, and Sampling of the Liver

1. The liver has four lobes: *medial lobe*, *right medial lobe*, *right lateral lobe*, *caudate lobe*, plus a *papillary process* (see Note 5). There is no gallbladder in the rat. The liver is normally dark-reddish and has a hard, but friable consistency. A thin transparent capsule covers the liver.

2. The liver is a fragile organ that should be handled with caution.
3. Remove the liver gently out the way with the forceps.
4. Grasp the xyphoid process firmly with the forceps.
5. Puncture the diaphragm with scissors and trim it completely away from the ribs.
6. Pull upward to create a negative pressure in the thorax.
7. Using the diaphragm as a handle, pull the liver out of the abdominal cavity. Separate the liver from the diaphragm by cutting the falciform and coronary ligaments that attach the liver to the diaphragm.
8. Before weighing the liver, remove all remnants of the diaphragm and ensure that the small lobes are present to weigh the entire organ.
9. Sampling of the liver (see Note 6). In the rat, take a piece from the left lateral lobe (transverse) and the right medial lobe (transverse); in the mouse from the left lateral lobe (transverse), from the left and right medial lobe including gall bladder (longitudinal-vertical preferentially to keep the gallbladder with the liver). In both species, a transverse section from the caudate lobe is optional. Size of samples should be as large as possible, but all pieces should fit into one cassette.
10. Immerse the two or three liver samples in the fixative. It is advised to keep the remaining liver tissue in fixative.

3.6.8. Removal, Examination, and Sampling of the Kidneys and Adrenals Glands

1. Remove the kidney and adrenals, grasp the caudal part of the ureter with forceps near its opening, and keep the adrenals attached to the kidneys. Adrenal glands and kidneys are located deep in the retroperitoneal space; they should be recognized as early as possible during the process.
2. Separate the adrenals from kidneys.
3. Examine each adrenal gland. These glands are small white structures within the perirenal fat. In male, adrenal glands tend to be large, often rose-colored and translucent while in females, they are smaller and, due to high lipid content, have an opaque pale color (24, 33, 34). Adrenals have an external *cortex* and central *medulla*.
4. Check their shape, volume, as well as the presence of nodular formations.
5. Before weighing adrenal glands, remove carefully all remnants of fat and connective tissues. As for all paired organs, unless otherwise specified in the study plan, the weight of the pair is recorded.

6. Immerse the adrenal glands in the fixative. Due to their relative small size in Rodents, they will be embedded *in toto*.
7. Examine the kidneys. Kidneys are located on the dorsal wall of the abdominal cavity. They are bean-shaped pair organs, with a hilus in the concave margin from which main vessels, nerves, and ureters exit. Their color is brownish red and their consistency is firm. The right kidney is the more cranially located, usually larger and heavier than the left one (35, 36). The kidneys are surrounded by a capsule and have three different regions: *cortex*, *medulla*, and *papilla*.
8. Before weighing, kidneys must be freed of all remnants of connective and adipose tissues.
9. Kidneys can be immersion fixed *in toto* or after trimming (e.g., take a longitudinal section from the left kidney and a transverse section from the right kidney).

3.6.9. Removal, Examination, and Sampling of the Urinary Bladder

1. Examine the urinary bladder (12, 37).
2. The urinary bladder can be freed of urine after incision of the wall and then sampled *in toto*. Alternatively, the bladder can be sampled after instillation of the fixative. In the mouse, instill the fixative (0.05 mL) through the bladder wall after ligation of the urethra with a ventral knot. In the rat, it is more convenient to instill the fixative (0.2 mL) with a needle inserted via the urethra. In both rats and mice, fixative instillation should not be performed when the bladder is distended with urine.
3. Then continue the fixation by immersion in a container of fixative.
4. It may be helpful also to mark the ventral side of the bladder with a stick of silver nitrate.

3.6.10. Removal, Examination, and Sampling of the Male Genital Organs

1. Remove the testes. They are oval-shaped paired organs, a few millimeters in diameter that lay inside the scrotum. They are covered by a smooth and transparent membrane *(tunica albuginea)*. They are grayish-white with a soft elastic consistency. When the abdominal cavity is opened, testes are often found outside the scrotum, in an intra-abdominal position. Grasp them delicately by the inguinal fat pad and cut them away from the viscera. If the testes are still in the scrotum, open the scrotum and extract the testes with the epididymides by cutting the fibrous ligaments anchoring the tail of the epididymis to the scrotum. Cut the vas deferens.
2. Examine the testes (38–41). Check the shape, volume, weight, consistency, and presence of masses.
3. Weigh the testes individually or as a pair. Weigh the epididymis (in rats only) separately if needed. Before weighing the testes

and epididymes, all remnants of connective and adipose tissues should be removed.

4. Place the testes with the epididymis on a piece of cardstock. Orient testis and epididymis in the same plane so that they can be trimmed simultaneously later (see Note 7). Fix the testes and epididymis as a whole, without cutting the testes before fixation to prevent them from rupturing (see Note 8).
5. It is suggested to fix testes and epididymis in Davidson solution instead of formalin (Table 1).
6. Remove the male accessory glands: *seminal vesicles*, *coagulating glands* (dorsocranial lobe of the prostate), *prostate* (two ventral lobes and two dorsolateral lobes). The dorsolateral and ventral lobes lie in a vertical axis above each other with urinary bladder and seminal vesicles in between.
7. Remove the group of adjacent organs consisting of prostate, urinary bladder, seminal vesicles, and coagulating glands.
8. Examine the male accessory glands (38, 39, 42, 43). Check any size or color changes or presence of nodular masses.
9. Before weighing the prostate (rats only), all remnants of connective and adipose tissues should be carefully removed.
10. Immerse these organs into the fixative *in toto* if weights are not required to prevent leakage of the glandular secretions.
11. Remove the penis, prepuce, and urethra.

3.6.11. Removal, Examination, and Sampling of the Genital Organs in Female

1. Dissect the vulva and vagina free from the skin and cut the supporting ligaments of the vagina, uterus, and oviducts.
2. Cut the ligaments and isolate the ovaries with the oviduct and the whole genital tract.
3. Examine the ovaries and oviducts. Ovaries are small oval reddish organs found within the fat tissue caudally to the kidneys and attached to the inferior poles of the kidneys, and to the posterior wall of the abdomen by ligaments. Check the shape, volume, consistency and presence of any gross lesions (24, 38, 44, 45).
4. If ovaries are to be weighed, isolate them from the oviducts before weighing.
5. Examine the uterus and the vagina. Record any enlargement, fluid, or mass (24, 38, 44, 45).
6. If uterus has to be weighed, uterine horns and the cervix should be weighed together but separated from the vagina.
7. The *uterine body* (fused part of the uterus) and the vagina should be placed with their dorsal aspect on cardboard before fixation.

3.7. Opening of the Thorax and Examination of Thoracic Organs

The thorax is opened and the thoracic organs such as tongue, larynx, trachea, thymus, mediastinal lymph nodes, lungs, heart, and thyroid gland (with parathyroids) are examined.

3.7.1. Opening of the Thorax

1. Open the thorax by first lifting the sternal xyphoid process with forceps. Then cut the ribs starting from the xiphoid process and up to the first rib to remove the sternum and rib cage to reveal the thoracic organs.
2. The sternum is a convenient organ for bone marrow examination after decalcification. Take a piece containing two to three sternebrae and immerse this sample into fixative.
3. Examine the thoracic serous membrane (*pleura*) and presence of abnormal contents such as serous fluid, blood, fibrin, or adhesions between organs.
4. Check the position of the different organs *in situ*.

3.7.2. Removal of the Tongue, Trachea, Esophagus, and Thoracic Organs

1. Cut the muscles of the lower jaw with a scalpel.
2. Cut the soft palate and pharynx.
3. Grasp the tip of the tongue with forceps and retract gently to remove the tongue, larynx, trachea, and esophagus from the head and neck.
4. Continue retracting to remove the heart and the lungs from the thorax. Use scissors to perform a blunt dissection to free these tissues.
5. Cut the thoracic aorta and posterior vena cava at the level of the diaphragm

3.7.3. Isolation, Examination, and Sampling of the Heart and Aorta

1. Isolate the heart from the lungs by delicately cutting the main vessels with scissors.
2. Examine the heart without opening the inner cavities (*atria* and *ventricles*) (24, 46, 47). Before weighing the heart, all blood should be removed. In Rodents, this can be easily achieved by placing the base of the heart over a piece of cleaning paper.
3. Immerse the heart *in toto* in the fixative.
4. Take a section from the *thoracic aorta* (in the middle of the last 1 cm caudal segment). This region is closely attached to dorsal vertebrae and can easily be removed.

3.7.4. Isolation, Examination, and Sampling of the Thymus

1. Isolate the thymus by gently grasping one of the two lobes in its inferior part and cut the ligament connecting it to the pericardium.

2. Examine the thymus (48). In rodents, the thymus is an oval-shaped lobulated organ with a whitish-translucent color. With age, the thymus shrinks but remains grossly visible.
3. Before weighing the thymus, all remnants of connective and adipose tissues should be carefully removed.

3.7.5. Examination and Sampling of the Tongue

1. Examine the tongue (28).
2. Make a transversal incision to sample half the tongue and immerse it in the fixative (see Note 9).

3.7.6. Examination and Sampling of the Thyroids (Parathyroids), Trachea, and Esophagus

1. Examine the thyroid gland. Thyroid gland has two symmetric oval lobes, adherent to the lateral and dorsal surfaces of the trachea and has a translucent, tan yellowish color. The parathyroids are located around or within the thyroids but *are not visible grossly* (49, 50), (see Note 10).
2. Take the *larynx* including *epiglottis*, *ventral pouch*, and *cricoid cartilage* (rats only) and immerse it in the fixative.
3. If the thyroid is weighed (rat only), carefully remove all remnants of connective and adipose tissues. Immerse the thyroids in the fixative. It is advised to weigh the thyroid gland after 1 or 2 min of fixation.
4. Then take a transverse sample section of trachea and esophagus. If the thyroids are not weighed, leave them on the trachea. Take a transverse section of the esophagus, trachea, including the thyroids (and parathyroids) (oral studies). Tracheal content should be noted and reported.

3.7.7. Examination and Sampling of the Lungs

1. Examine the lungs (51, 52). In Rodents, lungs have three right lobes (*right cranial lobe*, *right middle lobe, right caudal lobe*), an *accessory lobe,* and one *left lobe* (see Note 11).
2. The lung parenchyma has normally a smooth surface, a nice light pink color (but that depends on air and blood present if not inflated), and a spongy consistency. Examine all lung lobes and note any changes.
3. For optimal microscopic evaluation, instillation of the lung by the fixative is strongly recommended. This can be performed easily through the trachea. A 21-gauge needle is placed on a 3-mL syringe filled with the fixative. The needle is introduced in the trachea at its open end. Clamp gently around the needle with forceps and inflate the lung by depressing the plunger of the syringe very slowly until excess fixative refluxes up the trachea. The lung with the trachea should be then immersed in the fixative. If the procedure is correctly executed, there is no need to place a ligature on the trachea (see Note 12).

3.8. Head and Central Nervous System

Brain, cerebellum, spinal cord, pituitary gland, eyes, Harderian glands, nasal cavities, and Zymbal's gland.

The most optimal way the fix to central nervous system is intracardiac perfusion of fixative (see Note 13). However, fixation of the nervous system by immersion allows acceptable preservation of tissues and is routinely used in toxicity studies.

3.8.1. Removal of the Brain and Spinal Cord

1. Cut the skin over the head with a median-longitudinal incision from the nape to the snout.
2. Reflect the two edges of the skin and pull them to better observe the entire skull.
3. Remove any excess tissue or muscle from the cranium and neck.
4. Keeping the head firmly with large forceps, insert the tip of heavy duty scissors in the left eye socket (avoid any damage to the eyeball), and cut the nasal bone transversely at the level of the nasal septum between the two orbital cavities. The used scissors for this operation should be exclusively dedicated to this step.
5. Then, with the ophthalmologic scissors, cut progressively the parietal, interparietal, and occipital bones in a craniocaudal direction on both sides to isolate a bone cap and reveal the brain. Be very careful to avoid damaging the brain beneath the skull bones during this operation.
6. Pull the skullcap caudally with forceps and take it away.
7. Use the small ophthalmologic scissors to cut the vertebrae. Start from the occipital bone level and then, with the tip of the scissors, alternate right and left side cuts of the vertebral bodies to progressively remove the vertebral arches. In this way, the spinal cord will be uncovered in the vertebral canal.
8. Raise the brain by gently introducing forceps under the frontal lobe of the encephalon, then cut intracranial vessels and nerves at the brain base.
9. Gently handle the brain between the thumb and forefinger and cut successively the spinal nerves coming from the ventral aspect of the spinal cord on each side of the brain, and hold the detached segment of the spinal cord. In the area of the cauda equina (where nerves are numerous), cut the spinal cord transversely (see Note 14).
10. Isolate the brain and the spinal cord by a scalpel frank transversal section at the junction between the medulla oblongata and the brain.

3.8.2. Examination of the Brain and Spinal Cord

1. Examine the brain (cerebrum and cerebellum) (53, 54). The brain is covered by the meninges: the *dura mater* is fibrous and in direct contact with the skull, the *pia mater* is highly

vascularized and adheres intimately to the surface of the brain, the *arachnoid* is a transparent membrane located between the *dura* mater and the *pia* mater, but too thin to be visualized grossly.

2. The cerebrum has two *cerebral hemispheres*, separated by a longitudinal fissure. There are no cerebral circumvolutions in Rodents that have a lissencephalic brain. Rodent brains have two large *olfactory bulbs* located in the front. The cerebellum shows thin convolutions and lays in the caudal part of the brain.
3. On the ventral part of the brain, the *optic chiasma*, the *median eminence* (representing a part of the hypothalamus), the *pons*, and the *medulla oblongata* can be observed.
4. The brain is a very fragile tissue, especially if not fixed. It is therefore important not to handle this organ with the forceps but rather lift it carefully using the scalpel blade.
5. Before weighing the brain, carefully remove all remnants of connective tissue (see Note 15). To achieve accurate brain weights, the spinal cord should be cut off at a consistent level.
6. It is preferable that the brain (undetached from the cerebellum) be immersion fixed *in toto*. The brain is an anatomically complex tissue. Therefore, microscopic examination of the brain should be performed at standardized section levels: transverse section of the cerebrum at the optic chiasma, cerebrum at the base of the posterior hypothalamus, midcerebellum, and medulla oblongata (12). Therefore, the brain slices are routinely prepared after fixation.
7. Examine the *vertebral canal* on the cadaver.
8. Examine the spinal cord.
9. Take three transverse sections of the spinal cord for microscopic examination at the upper cervical, mid-thoracic, and lumbar levels. Put all three segments in a cassette and immerse in the fixative (see Note 16). As for the brain, spinal cord is a very fragile tissue and should not be handled with the forceps.

3.8.3. Examination, Removal, and Sampling of the Pituitary Gland

1. Examine the pituitary gland (24, 55). The pituitary gland can be easily seen on the ventral aspect of the cranial cavity after removal of the brain. It appears as a small spherical gland, covered by a thin layer of *dura matter* and located behind the optic chiasma in the *sella tursica*, a small depression of the sphenoid bone.
2. The fixation *in situ* of the gland is recommended before removal or weighing. Weighing after fixation provides accurate weight measurements and improves morphology. Fixation can be performed by a few drops of fixative on the ventral aspect of

the cranial cavity or by immersion of the remaining skull in the fixative; the gland can be removed later (11) (see Note 17).

3. Weighing of the pituitary gland is not required in mice and usually performed after fixation.

3.8.4. Removal, Examination, and Sampling of the Eyes, Optic Nerve, Harderian Gland, and Lacrymal Glands (Internal/External)

1. Remove the eyes by sinking a pair of curved forceps behind the orbit. Gently grasp the optic nerve, isolate the eye by pulling it outward and cutting its attachments to the socket (see Note 18).
2. Examine the eyes (56).
3. Immerse the eyes with the optic nerve in the fixative (see Note 19).
4. Examine the Harderian glands (or retroorbital gland). The Harderian gland lies intraorbitally behind the eyes and embraces the back of the ocular globe. It is a gray-colored gland under normal conditions, cone-shaped in the rat, and horseshoe-shaped in the mouse.
5. Remove the Harderian glands and immerse them in the fixative.

3.8.5. Sampling of the Nasal Cavity and Zymbal's Glands

1. Remove the skin and muscles and immerse the head in the fixative if further histological examination of the nasal cavity and paranasal sinuses, nasal cavity, nasopharynx, and paranasal sinuses is needed (57, 58).
2. Zymbal's glands are modified sebaceous glands located at the base of the external ear in anterio-ventral position (59). A section through the base of the skull after gentle decalcification will allow histological examination of these glands.

3.9. Muscles and Skeleton

Bone (femur, sternum), skeletal muscle (biceps femoris), peripheral nerve (sciatic nerve), bone marrow (femur, sternum).

1. Remove the skin from the hind leg.
2. Transversally cut the biceps femoris with a scalpel (12, 27, 60). Gently grasp one edge with the forceps and immerse it into the fixative.
3. Take a sample of the sciatic nerve (1 cm long) (12) (see Note 20). Gently grasp one edge with forceps and fix it on a card board.
4. Remove the distal portion of one femur, the knee joint with the proximal portion of the adjacent tibia. This will allow microscopic examination of the bone, joint tissues, and bone marrow (12, 61, 62). For routine bone and bone marrow microscopic examination, a sternum section should be sampled (see Note 21).
5. Immerse the samples in the fixative.

4. Notes

1. A longitudinal section, vertical to the direction of the hair flow can be taken to examine the skin and mammary glands; in this case, the nipple is not included if the lymph node is enclosed.
2. In case of parenteral application, one lymph node draining the application site and another distant one could be collected.
3. The extraorbital glands can be removed and embedded together with the salivary glands.
4. "Swiss roll" technique: this technique allows examining the whole intestine and the GALT on a single section. However, these transverse sections when they are made properly will often provide a better morphology. Strip the intestines off the mesentery, open it with a pair of scissors, and gently rinse the intestine to remove the content. Then recoil the intestine except cecum on cotton swabs and put in the fixative. After fixation, detach the spooled intestine and proceed to embedding. This procedure is not required in routine microscopic examination of the intestines. Bear in mind that with this technique, the intestinal mucosa and the lymph follicles will often be cut tangentially.
5. Nomenclature of hepatic lobes can differ. We have elected to use the anatomical terms we recommend: medial lobe, right medial lobe, right lateral lobe, caudate lobe, and papillary process.
6. If major bile duct is required, take a section through the left lateral lobe of the liver.
7. Optionally epididymides can be isolated from the testes, fixed, and trimmed separately
8. In short-term studies, fixation of the testes with modified Davidson's or Bouin's solutions is highly recommended to detect less extensive early and subtle changes (63).
9. The longitudinal vertical section of the tongue covers a large part of the dorsum including the dorsal prominence. The section also includes the lingual lesser salivary glands and should be slightly lateral to the median sulcus. A transverse section of the tongue is recommended if blood sampling from the tongue is performed.
10. There are variations in position and number of parathyroids in Rodents. In the rat, there is one pair of parathyroids, with a variable position but usually on the anterior lateral aspect of the thyroid lobes. In the mouse, there are usually two parathyroids (sometimes more than two), situated bilaterally just under the capsule near the dorsolateral border of each thyroid

lobe. They are rarely at the same level and may be deeply embedded in the thyroid tissue.

11. Nomenclature of lung lobes can differ. We have elected to use the anatomical terms that we recommend: right cranial, right middle, right caudal lobe, accessory lobe, and left lobe.
12. Alternative procedure for the lung in oral studies in the rat: right lobes embedded, ventral surface down and in the mouse: whole lung embedded, ventral surface down.
13. The optimal way to fix the central nervous system is to perfuse it *in situ* while the animal is deeply anesthetized. The procedure is as follows: anesthetize the animal (for example) with intraperitoneal injection of Nembutal working solution (1.6 mL stock solution with 8.4 mL PBS), 0.1 mL/10 g body weight. Then, pin the mouse on a dissection board ventral side up. Trim back the skin from the thorax to the mandible. Open the thorax following the necropsy protocol (herein described). If blood is needed, collect it by cardiac puncture at this time. With small scissors, cut the right auricle (64). Blood will start to flow from the heart. Insert a 23-gauge needle within the left ventricle, with the syringe containing PBS. Perfuse the PBS with steady pressure, strong enough to wash the blood out from the body, but not so much that it causes damages. The liver (and other organs) would normally go pale very quickly. The lungs should not bulge; if they do, your syringe has perfored the interventricular septum. Slowly remove the needle and repeat the perfusion. After injecting 15 mL of PBS, change the syringe and perfuse 4% paraformaldehyde in the same way. If fixation is successful, the body will stiffen from the tip of the tail to the tip of the nose. Proceed as usual for tissue collection.
14. Alternatively remove the vertebral bodies with the spinal cord.
15. Changes in brain weights are rarely associated with neurotoxicity. The utility of brain weight rests in the ability to calculate organ to brain weight ratios which is helpful when terminal body weights are affected or to normalize organ weight data.
16. To avoid artifactual vacuolation in the white matter, do not store specimens from nervous tissue in alcohol.
17. The pituitary gland can be trimmed *in situ* as transverse section of skull. This procedure is not recommended.
18. Each eye can be removed from the socket together with the optic nerve and the Harderian gland after fixation of the head.
19. For long-term studies, formalin fixation of the eyes is generally sufficient. For other study types, fixation in Davidson's fixative is recommended to avoid detachment of the retina.
20. Alternatively, the skeletal muscle and sciatic nerve can be sampled together. In this case, the gracilis, adductor, semimembranous,

and semitendinous muscles are removed from the medial aspect of the thigh to get access to the sciatic nerve running along the medial surface of the biceps femoris muscle. After fixation, transverse and longitudinal sections are prepared.

21. The bone marrow is generally examined concurrently with the bone tissue after decalcification. On this specimen, it is possible to evaluate the cellularity, the number of megakaryocytes, and the stromal compartment. If evaluation of the iron content and more precise cytology are needed, examination of a bone marrow smear of a core sample from the femur may be useful. This method requires training to obtain satisfactory results. Prepare bone marrow smears as fresh as possible to avoid blood clotting. Cut off the proximal and distal epiphyses with scissors. Then blew air from one end into the marrow cavity and collect the marrow cast onto a glass slide. The smear is prepared conventionally with a cover glass. A smear of adequate quality contains grossly visible particles. Alternatively, aspiration with a pipette containing anticoagulated serum or a small paint brush or a cotton bud from the longitudinally opened femur can also be performed (this procedure implies that the contro-lateral femur, knee joint, and proximal tibia should be sampled).

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