

Thomas J. Steinbach  
Daniel J. Patrick  
Mary Ellen Cosenza *Editors*

# Toxicologic Pathology for Non- Pathologists

 Humana Press

**TOXICOLOGIC PATHOLOGY  
FOR NON-PATHOLOGISTS**

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Edited by

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## Preface

This book is based on the successful American College of Toxicology (ACT) and Society of Toxicologic Pathology (STP) “Pathology for Non-Pathologists” short course that is held every other year in the United States. This course is primarily geared toward toxicologists who want to expand their understanding of toxicologic pathology in order to be better study directors; however, it has also proven to be of great interest to other drug development scientists and regulatory reviewers.

In 2003, a small group of ACT members felt that a practical pathology course for non-pathologists/toxicologists would be useful to aid experienced toxicologists and regulatory officials. Because of the breadth of topics to present, multiple days would be needed to properly cover the topics of interest. A decision was made to hold the first course separate from the ACT annual meeting. During the first year, these members selected appropriate topics, recruited knowledgeable instructors, and identified companies that could provide financial support and meeting space. The inaugural 2004 course committee included one of the editors of this book, Mary Ellen Cosenza, as well as Leigh Ann Burns-Naas, Debbie Hoivik, Laura Dill Morton, Jerry Hardisty, Winston Evering, Isaac Hayward, Paul Howroyd, Stuart Levin, Douglas Wolf, and Farrel Fort. The Society of Toxicologic Pathology agreed to formally partner with the ACT on the initial and subsequent short course efforts, and this partnership has steadily strengthened the collaboration between these two organizations.

The first committee members felt there was a need to start the short course with an overview of general pathology concepts that included fundamental vocabulary and the basics of pathophysiological processes (e.g., degenerative, regenerative, hyperplasia, hypertrophy, neoplasia, etc.; see this book’s appendix on SEND terminology and definitions). These concepts cover findings typically seen in toxicology studies. The course would then cover organ system pathology. Some of the other important topics included addressing biomarkers, correlation of clinical pathology endpoints (chemistry and hematology) with microscopic changes, and well-known pathology findings for classes of toxic substances. The first course was held at Baxter Healthcare Corporation in Deerfield, Illinois. Other sponsors that year included GlaxoSmithKline, National Institute of Environmental Health Sciences, and Pfizer. Due to growing attendance over the years, the course moved to larger locations and is now held at a hotel conference center.

The course has benefitted from outstanding course speakers and dedicated course organizers who are often members of both the ACT and STP. Dan Patrick has helped organize the course and secure presenters since 2010 and met Tom Steinbach when he agreed at the last minute to be a substitute presenter when one of the scheduled speakers couldn’t attend the course in 2012; their camaraderie began at that point and continues. Tom has been a course organizer since 2014. Repeatedly, Tom and Dan have been asked about the possibility of sharing previous course notes, course slides, and recommendations on textbooks from individuals who couldn’t attend the course. These frequent inquiries made it clear that there was a need to reproduce some of the important education from the course in an easy-to-understand reference book. About 2 years ago, Tom and Dan set out to develop such a

book. The overall goal would be to help non-pathologists understand, contextualize, and communicate the pathology data and interpretations from the study pathologist in a practical and usable format. They also wanted to include a highly respected non-pathologist to help ensure that the product would fulfill these goals, and they were fortunate that their first choice, Mary Ellen Cosenza, accepted. The editors reached out to some of the highly regarded speakers from past courses as well as respected and well-known colleagues with expertise in specific organ systems or other specific aspects of toxicologic pathology. They were extremely happy in the outstanding group of pathologists who agreed to take this project on and volunteer many hours of their busy lives to write these chapters.

So, that is a brief summary on how this book before you came to be. We are incredibly indebted and grateful to the many authors who contributed their time and expertise in this final product that we are very proud of. David Sabio of EPL Inc. is also commended for producing many of the high quality medical illustrations and images. We sincerely hope that the original intent of helping non-pathologists understand, converse in, and apply a basic understanding of pathology in their day-to-day careers is fulfilled.

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# Chapter 1

## Introduction to Toxicologic Pathology

George A. Parker

### Abstract

Toxicologic pathology involves microscopic examination of organ and tissue specimens from laboratory animals that have been exposed to candidate drugs, devices, or various chemical or biological agents. The goals are to identify organ system toxicity, dose levels that produce toxicity, and biomarkers of toxicity. A variety of investigative techniques are employed in the detection of histomorphologic alterations, most commonly light microscopic examination of histologic tissue sections and preparation of reports containing subjectively based diagnostic terms and interpretations that convey the identity and anticipated significance of observations. Contemporary clinical pathology evaluations are performed to help identify changes in bodily fluids which may precede and/or accompany histological alterations and further characterize these changes and their adversity. Clinical pathology evaluations also help identify potential clinical biomarkers of xenobiotic-associated tissue damage.

**Key words** Pathology, Histopathology, Toxicologic pathology, Toxicology, Safety assessment

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### 1 Overview of Pathology and Pathologists

The pathology evaluation of toxicology studies is a common source of toxicity data that can substantially alter the development or marketing of drugs and chemicals, to the degree that a decision for continued development may be based largely on the pathology data. The histopathology data result from subjective analysis of histological sections performed by pathologists who may be in a separate division, company, or geographic location from the personnel who conduct the in-life study. Study directors and other responsible individuals may come from a background that included little or no exposure to pathology. The combination of (a) the subjective nature of the analysis, (b) lack of familiarity with pathology, and (c) the potentially significant impact of the pathology analysis often results in concerns among those who are responsible for the overall study conduct or product development. Toxicology stakeholders may benefit from an understanding of the challenges within the science and practice of pathology. A major goal of this book is to provide insight into the basic nature of pathology and

the pathology analysis that is performed in the conduct of non-clinical toxicology studies for non-pathologists.

Histopathology consists of a form of subjective image analysis that is performed on thin sections of tissue that are stained by various dyes to allow visualization of the tissue components. Without these stains, the tissue sections have few interpretable microscopic features. When viewing tissue sections by light microscopy, it is important to remember that the histologic presentation is not precisely the same as the *in vivo* tissue structure and that all differences in color as seen microscopically are merely artifacts introduced by the histotechnology procedures. The tissue specimen, as seen microscopically, typically originated from a laboratory animal that was exsanguinated at the time of necropsy; thus it lacks the blood perfusion that characterizes the living tissue. The specimen was preserved by immersion in a fixative, commonly neutral buffered formalin, dehydrated through graded alcohol solutions, cleared with xylene, and infiltrated with and embedded in paraffin. Thin paraffin sections collected from the paraffin block were deparaffinized by immersion in xylene or a xylene substitute, rehydrated through graded alcohols up to water, stained to allow visualization of the tissue, and protected by application of a thin glass coverslip using a mountant. These technical processes may introduce tissue artifacts that must be distinguished from histopathological changes. There is some truth to the smug assertion that histopathology is the study of tissue artifacts.

The key to success in this endeavor is the training and experience in the observer, *i.e.*, the pathologist (Seaton 2014). Untrained microscopists, regardless of their level of effort, enthusiasm, and general scientific ability, are rarely able to function above a rudimentary level in histopathological evaluation. Anyone with normal visual acuity is able to see structures in histological sections, but interpreting the significance and meaning of those microscopic observations relative to toxicity and overall effect on the health status of the animal requires a considerable amount of background training and experience. The importance of this perspective in the interpretation of microscopic observations has been emphasized on numerous occasions, *e.g.*, a highly publicized situation in which multiple long-term safety assessment studies in rats were initially thought to result in test substance-related lymphoma involving the lungs. It was eventually determined that the proliferative lymphoid tissue in the lungs of affected rats represented a known component of the response to *Mycoplasma pulmonis* infection (Schoeb and McConnell 2011a, b; Schoeb et al. 2009). Though infectious and parasitic diseases are not as common in modern laboratory animal colonies as in previous times, the possible involvement of these diseases in toxicology studies must be considered. Exacerbation of cryptic or indolent infectious diseases is particularly likely in studies of test materials that are intentionally or incidentally immunomodulatory. These disease interferences are a real-life problem, not

merely an academic exercise (Hutto 2010). In some instances the laboratory animals used in toxicology studies may have a substantial prevalence of potentially significant infectious diseases. Polyomavirus infection was reported in 12 of 57 immunosuppressed cynomolgus monkeys (van Gorder et al. 1999), and 95% of rhesus macaques in one colony were seropositive for rhesus cytomegalovirus (Andrade et al. 2003). The cited rhesus macaque colony had been closed to incoming animals for more than 70 years, indicating the latent rhesus cytomegalovirus infection was endemic. An exhaustive presentation of possible infectious or parasitic disease interferences in all of the common laboratory animal species is beyond the scope of this chapter. Table 1 presents a list of possible disease interferences in nonhuman primates that may illustrate the potential magnitude of this issue (Haley 2012; Parker and Snyder 2017).

Toxicologic pathologists are most commonly graduates of veterinary medical colleges who undertake additional training in pathology. The veterinary pathology training programs encompass all aspects of pathology in domestic, laboratory, and zoo/wildlife animals. The training programs typically prepare candidates to complete board certification examinations as administered by the American College of Veterinary Pathologists, the European College of Veterinary Pathology, the Japanese College of Veterinary Pathologists, and the Royal College of Pathologists in the UK. Residency programs in veterinary pathology typically involve three to four years of full-time training following completion of veterinary college. Individuals who successfully complete the requirements to become board-certified in veterinary pathology indicate that status by abbreviations such as DACVP, DECVP, DJCVP, or FRC-Path. In addition to board certification in pathology, many pathology trainees also complete a master's or Ph.D. degree in pathology or a related biomedical area. Many pathology training programs consist of combination residency and graduate programs that result in Ph.D. or master's degrees in addition to preparation for board certification examinations. Non-veterinarian toxicologic pathologists are uncommon in North America, but are encountered with greater frequency in Europe/UK and Japan. In the formative days of toxicologic pathology, a number of physicians with an interest in comparative medicine became involved in toxicologic pathology and made remarkable contributions to the field. Many physician researchers remain highly involved in the field of toxicologic pathology, but modern organizations related to toxicologic pathology are largely populated by veterinarians with specialty training in pathology. It should be noted that Japan also has a board certification procedure, administered by the Japanese Society of Toxicologic Pathology (JSTP) that is open to individuals who are not veterinarians. Some of the certifying organizations, e.g., ACVP, have separate board certification procedures for anatomic and clinical pathology.

**Table 1**  
**Selected pathogens of nonhuman primates used in toxicology studies**

<b>Viral agents</b>	<b>Bacterial agents</b>	<b>Fungal agents</b>	<b>Protozoan and metazoan parasites</b>
Adenovirus	<i>Campylobacter</i>	<i>Aspergillus</i> spp.	<i>Acanthamoeba</i> spp.
Cercopithecine herpesvirus I (B virus)	<i>E. coli</i> , enteropathogenic	<i>Candida albicans</i>	<i>Balantidium coli</i>
Cynomolgus polyomavirus	<i>Helicobacter pylori</i>	<i>Dermatophyte</i> spp.	<i>Blastocystis</i> spp.
Cytomegalovirus	<i>Helicobacter heilmannii</i> -type		<i>Cryptosporidium</i> spp.
Hepatitis A virus	<i>Lawsonia</i>		<i>Cyclospora</i> spp.
Lymphocryptovirus	<i>Moraxella catarrhalis</i>		<i>Demodex</i> spp.
Measles virus	<i>Mycobacterium tuberculosis</i>		<i>Endolimax nana</i>
Polyomavirus	<i>Rhodococcus equi</i>		<i>Entamoeba coli</i>
Rhesus rhabdovirus	<i>Salmonella</i> spp.		<i>Enterocytozoon bieneusi</i>
Simian immunodeficiency virus	<i>Shigella</i> spp.		<i>Giardia duodenalis</i>
Simian parvovirus	<i>Yersinia</i> spp.		<i>Oesophagostomum</i> spp.
Simian type D retrovirus			<i>Plasmodium</i> spp.
Simian varicella virus			<i>Pneumonyssus semicola</i>
Simian virus 40			<i>Sarcocystis</i> spp.
			<i>Schistosoma</i> spp.
			<i>Strongyloides fuelleborni</i>
			<i>Toxoplasma gondii</i>
			<i>Trichomonas</i> spp.
			Trichuristrichiura

Modified from Haley (2012) and Parker (2016)

Following completion of basic training in veterinary pathology, achievement of board certification status, and possibly completion of an additional academic degree, novice pathologists enter employment in private industry, academia, or government service. Those entering private industry may be employed by pharmaceutical firms, contract research organizations (CROs), or various facets of the chemical industry, including agricultural chemical firms. The early career pathologist, though fully trained and accredited, typically requires a period of training and close supervision while attaining additional familiarity in toxicologic pathology. The early career

pathologist must learn there are additional expectations involved other than simply observing and interpreting pathological changes. In most industrial situations the toxicologic pathologist is expected to function as part of a team, which may be different from previous experiences in academia or traditional diagnostic veterinary pathology. Integration into the investigative team requires the pathologist to become familiar with regulatory requirements, procedures, and policies related to the in-life phase of toxicology studies. Learning to provide these complex services in compliance with good laboratory practice (GLP) regulatory requirements is a major step in the development of the early career pathologist. In addition, most corporations have an internal culture that must be navigated to achieve the goal of providing accurate, timely, and cost-effective pathology services in support of the firm's mission.

Pathology as it relates to nonclinical safety assessment toxicology is broadly divided into anatomic and clinical pathology. The anatomic pathology area typically includes necropsy, histotechnology, and histopathology and often includes organ weight analysis, electron microscopy, and other special morphology-based analyses. Clinical pathology includes the traditional clinical pathology analyses, a multitude of special assays, and various biomarker assays. Close coordination between clinical pathology and anatomic pathology reporting is required in order to avoid conflicts between these two major forms of pathology interpretation. Ideally, the pathology reporting includes correlation between clinical observations, necropsy observations, clinical pathology data, organ weights, and histopathological findings.

---

## 2 Necropsy

The necropsy, or postmortem examination, of animals from toxicology studies may be performed by pathologists, but more commonly is performed by non-pathologist personnel who are specifically trained in these procedures. Pathologists may or may not be in attendance for the necropsy examinations, but pathologists are typically available if questions or issues arise. The necropsy examinations are performed in accordance with study protocols as well as guidelines delineated in the facility's standard operating procedures (SOPs). Facility SOPs typically provide very specific guidelines regarding specimen collection and fixation as well as the terminology used in recording necropsy observations. Standardization of necropsy terminology is necessary for tabulating necropsy observations relative to experimental treatments.

Phosphate-buffered formalin is commonly used as a fixative, which prevents postmortem degeneration (autolysis) of tissue specimens. Non-buffered formalin should be avoided, as the slightly acidic non-buffered solution enhances the formation of

acid hematin from hemoglobin contained in erythrocytes. Presence of brown acid hematin in histological sections is considered a technical defect though, in reality, its presence rarely precludes the possibility of competent histopathological interpretations.

All involved in necropsy planning or conduct should be familiar with the technical details of formalin fixation, as failure to understand the terminology can result in serious failures in tissue preservation. Pure formaldehyde is a gas, which is dissolved in water to form formaldehyde solution. This formaldehyde solution is sometimes referenced as “pure formaldehyde,” though it consists of only 37–39% formaldehyde. Formaldehyde solution is diluted with water to form a 10% solution known as formalin. Various buffers are added to yield solutions such as neutral buffered 10% formalin, but it must be recognized that this final use solution is typically only 3.5–3.7% formaldehyde. Formaldehyde solution may contain up to 15% methanol as a stabilizing agent, which may interfere with ultrastructural studies (Bolon et al. 2013). As a result of these variables, it is difficult to know the exact formaldehyde content of the initial formaldehyde solution or the resultant neutral buffered formalin. In situations where a more exact concentration of formaldehyde is needed, or exclusion of methanol is necessary, it is customary to prepare fresh solutions of paraformaldehyde (polyoxymethylene) for use in tissue fixation.

Formaldehyde solution and formalin are clear solutions that cannot be visually differentiated from water, saline, or other clear solutions. In bygone days it was customary to sniff the formalin container as it was opened to verify the presence of pungent formaldehyde. This practice must be discouraged, due to the potential health effects of formaldehyde exposure. A reliable, centrally controlled procedure (e.g., addition of a small amount of eosin) for identification of formaldehyde-containing fixatives must be instituted in order to ensure that (a) fixative solutions actually contain formaldehyde and (2) fixatives are not used for some other purpose where the formaldehyde content may have detrimental effects.

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### 3 Histotechnology Procedures

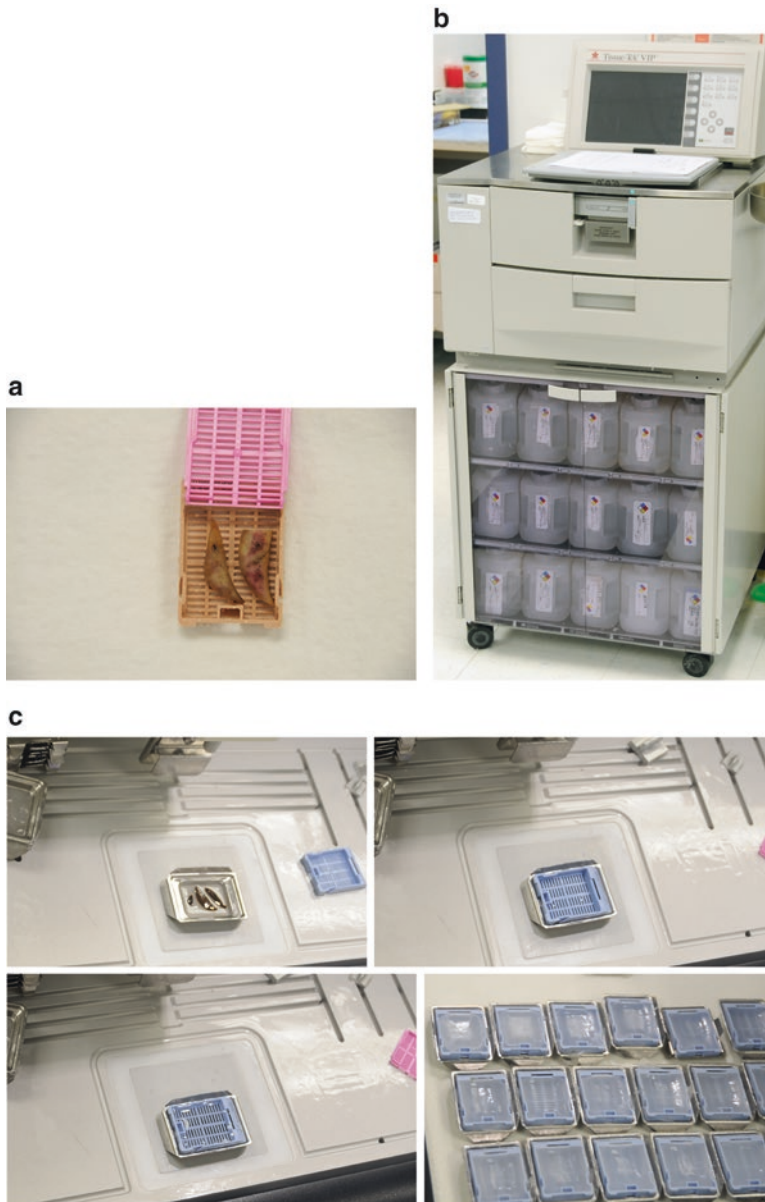
These procedures for preparation of histological sections are part of the field of histotechnology, though in our common workday terminology they are often referenced as “histology procedures.” Knowledgeable scientists in toxicology and pathology know that histology is actually defined as the biomedical specialty that deals with the study of tissue structure, as exemplified in multiple textbooks (Bloom et al. 1994; Young et al. 2006), rather than the technical procedures that result in histological sections.

Conversion of fixed tissue specimens into histological sections suitable for light microscopic examination involves sequen-

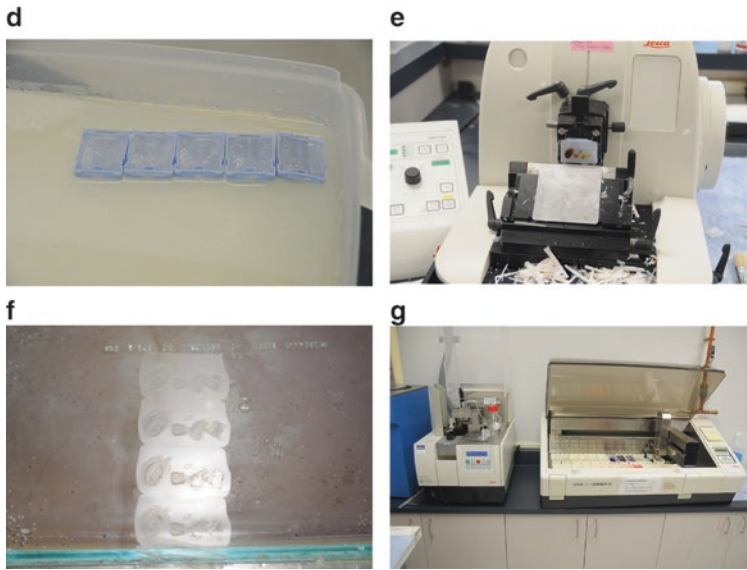


tial steps known as gross trimming, processing, embedding in a matrix such as paraffin wax, microtomy, staining, and coverslipping. While these activities are the province of the histotechnology staff, it is useful for toxicologists and pathologists to have a general knowledge of the processes involved in preparing histological sections (Fig. 1a–g).

1. In the gross trimming step, the fixed tissue specimens from necropsy are trimmed to a dimension that will fit into process/embed cassettes (see additional steps below) and are sufficiently thin to allow ready penetration of fluids. It is common practice to trim tissue specimens to approximately 3 mm thickness. The surface of the tissue specimen typically occupies no more than 50% of the surface area of the cassette in order that sufficient embedding medium remains to securely hold the tissue specimens for microtomy.
2. The term “processing,” which has special meaning in histotechnology, starts with passage of trimmed tissue specimens through sequential alcohol solutions of increasing concentration, with the goal of removing water from the tissue. Following dehydration, the tissue specimens are immersed in a clearing agent such as xylene or xylene substitutes to remove fat from the specimen, and the specimen is finally infiltrated with paraffin or other molten embedding medium. Tissue processing can be accomplished by manually moving tissues through glass jars containing the appropriate fluids, but in modern histotechnology laboratories, these procedures are performed by robotic tissue processors that typically have a single reaction chamber containing tissue specimens. The various processing fluids are sequentially introduced into the sealed reaction chamber, which is subjected to cycles of vacuum and pressure to aid penetration of fluids into the tissue specimens and heated to maintain the molten status of the final embedding media. All these activities are controlled by computer programs that can be varied to optimize the histological processing of various types of specimens.
3. Embedding of tissue specimens into paraffin blocks is necessary to hold the tissue specimens in a solid matrix that will allow preparation of the thin slices (“histological sections”) that are typically 3–6  $\mu\text{m}$  in thickness. This is accomplished with the aid of an embedding center, which maintains a vat of molten paraffin that is dispensed into a cassette mold held on a small hot plate to maintain the paraffin in molten status while the tissue specimen is properly aligned in the future block. Once the specimen is properly aligned, the cassette mold is moved to a chilled plate that quickly cools the molten paraffin to a solid state.



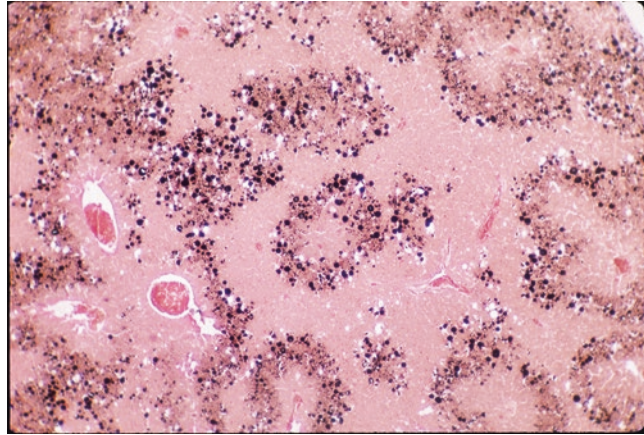
**Fig. 1** (a) Formalin-fixed tissue specimens are gross trimmed to a size that will allow the specimens to fit into a process-embed cassette. Tissue specimens are approximately 3 mm in thickness, which would theoretically allow preparation of 600 serial sections of 5  $\mu\text{M}$  thickness. In reality, technical issues make it impossible to collect and preserve each individual section. A major loss of tissue occurs as the paraffin block is faced to allow preparation of full-face sections. Paraffin blocks should be faced as few times as possible to avoid depletion of the tissue specimens. If special stains are anticipated, the full listing of stains should be determined in advance and the paraffin blocks should be faced only once, ideally at the same time the initial sections for routine staining are prepared. (b) Trimmed tissue specimens are subjected to histological processing in an automated tissue processor. “Processing” has specific meaning in a histology laboratory, where it refers to sequential passage of tissue specimens through graded alcohols to remove water, a clearing agent such as xylene to remove lipids, and infiltration with a molten embedding medium such as paraffin. Modern tissue



**Fig. 1** (continued) processors have a processing chamber that holds the specimens in process-embed cassettes. Reagents are pumped from the various reservoirs, here shown at the bottom of the instrument. The processing chamber is sealed and typically has alternating cycles of pressure and vacuum to aid in replacement of air with the processing fluids. The processing chamber must also be heated to maintain embedding media in a molten state. The tissue processor instrument has computerized control that allows the use of multiple processing cycles for different types or sizes of tissue specimens. (c) Following processing the paraffin-infiltrated tissue specimens are embedded in a solid block of paraffin, using the labeled process-embed cassette as a mold that determines the final size of the paraffin block. The embedding center has a heated plate that maintains the paraffin in a molten state, which allows manipulation of tissue specimens to proper alignment in the final block (upper left image). Once proper alignment of the specimens is achieved, the process-embed cassette is placed on the embedding mold (upper right image), and the embedding mold is filled with liquid paraffin (lower left image) and finally moved to a refrigerated plate on the embedding center (lower right image). This results in rapid cooling of the block containing the tissue specimens. (d) Microtomy is aided by cooling the paraffin blocks on wet ice. (e) The paraffin block containing tissue specimens is clamped in a microtome, which moves the block up and down across the face of a knife blade. The microtome causes the block to advance at specified intervals, resulting in thin ribbons of paraffin containing tissue sections. (f) The paraffin ribbon containing tissue sections is floated on a warm water bath to allow dissipation of any wrinkles that may have been introduced during microtomy. One or more sections are picked up onto glass slides for staining or other uses. If multiple ribbons are placed on the water bath simultaneously, great care must be taken in maintaining the identity of the individual ribbons. (g) The autostainer on the right side of the image consists of multiple reservoirs filled with reagents used for staining tissue sections. The instrument is computer-controlled; thus it can move racks of slides through any designated series of fluid reservoirs, as opposed to older instruments that were limited to a linear pathway of progress. The initial steps in the staining process are essentially a reversal of the tissue processing steps, with passage through xylene or xylene substitutes to remove paraffin and graded alcohols to return the tissue specimens to an aqueous state. Autostainers result in uniform staining of tissue sections only if proper attention is given to rotation and replenishment of the various fluids used in the instrument. The coverslipping robot on the left side of the image dispenses mountant medium and places a very thin glass coverslip on the surface of the slide. Once the mountant has dried, the stained tissue section is protected and ready for microscopic examination. More modern versions of autostainers and coverslipping robots are connected, with the autostainers mechanically passing racks of stained slide through to the coverslipping robot

4. Microtomy, often considered the centerpiece activity of histotechnology, is accomplished by clamping the solid paraffin block containing embedded tissue specimens into a microtome. The microtome moves the paraffin block up and down across the face of an extremely sharp knife edge and advances the block toward the knife by whatever incremental distance represents the desired thickness of the final histological section. Sequential sections from the block adhere side-to-side, forming a “ribbon” that is floated on a warm water bath that allows the ribbon to flatten, thus removing folds and wrinkles from the histological section. Labeled glass microslides are inserted into the water beneath the floating ribbon, and one or more sections are lifted onto the microslide for subsequent staining.
5. The staining process requires removal of the paraffin by immersion of the microslides in xylene or xylene substitute and rehydration of the tissue specimens by sequential immersion in decreasing concentrations of alcohol, ending with water, i.e., a reversal of the steps employed in tissue processing. Once in an aqueous phase, the tissue specimens are subjected to various staining procedures that allow visualization of tissue components. Staining with hematoxylin and eosin (H&E) is most commonly used in nonclinical toxicology studies, though numerous histochemical and immunohistochemical stains are available for specific purposes. Routine staining in modern facilities typically is accomplished by computer-driven autostainers that can be programmed to perform a variety of staining tasks.
6. The stained tissue section lying on a glass microslide is very fragile and must be protected by applying a thin glass coverslip that essentially encases the tissue specimen in a small glass box. The coverslip is adhered to the microslide by use of various mounting media that are fluid when held in a container, but quickly solidify when placed in a thin film between the coverslip and the underlying microslide. All these materials are engineered to allow the greatest possible light transmission and the least possible refractive index, resulting in a stained tissue specimen that visually appears to be floating in air. Coverslip application in modern laboratories is accomplished by coverslipping robots, some of which are linked to the antecedent autostaining robot.

The great majority of the histological sections used in toxicology studies are prepared from paraffin-embedded specimens, but there are occasions where other embedding materials are required. For example, the use of xylene or xylene substitutes in histological processing removes neutral lipids from the specimens; therefore, frozen sections have traditionally been used for demonstration of neutral lipid accumulations. The requirement for frozen sections for lipid staining can be circumvented by use of osmium post-



**Fig. 2** This H&E-stained histological section was prepared from a specimen of rat liver that was treated with osmium tetroxide following fixation, prior to histological processing. Osmium complexes with lipids in the tissue, rendering the lipids insoluble in the solvents used in routine histological processing. This eliminates the need for frozen sections for demonstration of fatty change in tissues. The dense black staining indicates osmium-complexed lipids in the tissue. Note the distinct pattern of distribution relative to the lobular microarchitecture of the liver. Osmium staining for neutral lipids, 2× objective magnification

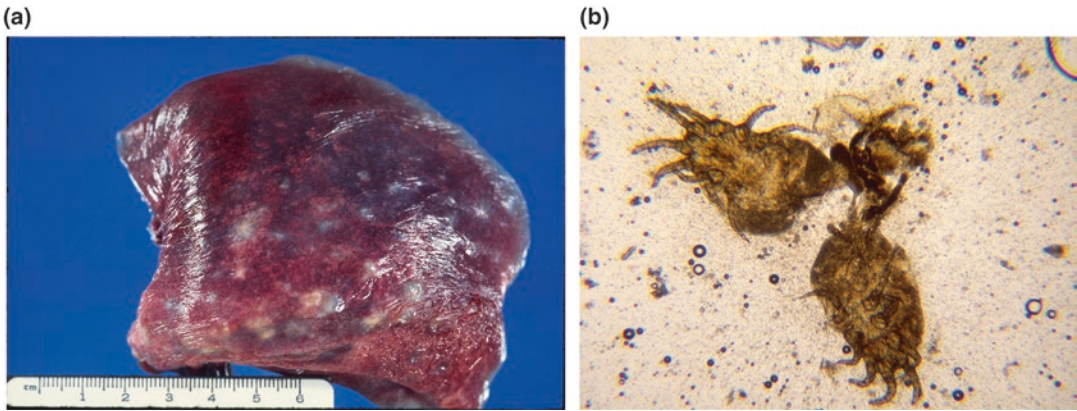
fixation of formalin-fixed specimens, followed by routine hematoxylin and eosin staining. Osmium complexes with neutral lipids, rendering them insoluble in standard histologic solvents, resulting in dense black deposits of osmicated lipids in the H&E-stained sections (Fig. 2).

Modern histotechnology procedures most commonly are centered on the use of process/embed cassettes, which are perforated plastic containers that hold tissues as they are subjected to the various steps of histological processing and eventually serve as molds and labels for paraffin blocks. In modern laboratories the cassettes are commonly labeled by computer-driven robots. When circumstances dictate, the plastic process/embed cassettes may be labeled with pencils, *but not the commonly used felt-tip pens that are in abundant supply in most laboratories*. The ink used in many felt-tip pens is soluble in one or more of the solvents used in histotechnology. Even the pens that are purchased specifically for use in histotechnology should be viewed with suspicion until the ink is tested and found to be insoluble in the laboratory's current histology solvents.

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## 4 Routine Microscopy

Standard toxicology study protocols typically refer to “routine light microscopy” as the process used to perform the histopathological evaluation. A more precise term for this form of microscopy



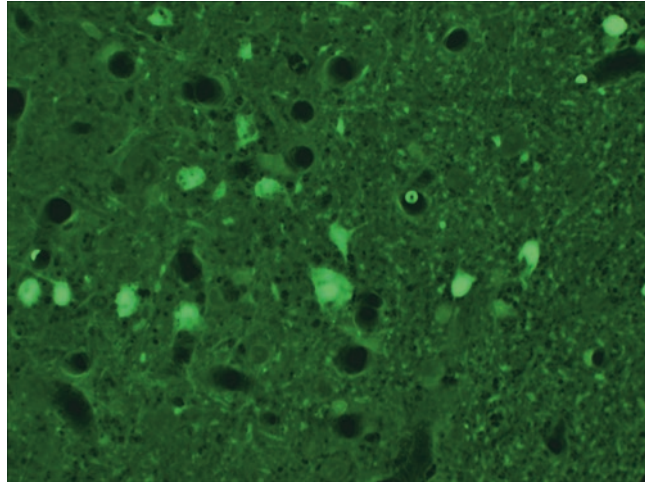
**Fig. 3** (a) This lung specimen from a rhesus macaque has multiple white to gray discolored areas that represent inflammation associated with the presence of lung mites (*Pneumonyssus semicola*) (Andrade et al. 2003; Leonovich 2010). (b) Incident light microscopy of material expressed from the lung lesions shown in Fig. 8a revealed the causative lung mites

is “transmitted visible light microscopy,” which indicates a light source in the visible spectrum is placed beneath the specimen and the light beam passes through the specimen to the eyes of the observer. In “reflected visible light microscopy,” the light beam is incident upon the surface of the (typically opaque) specimen and then reflected back to the eyes of the observer. This latter form of visible light microscopy is commonly applied in metallurgical studies and the dissecting microscopes that may be used for subgross examination of specimens from toxicology studies (Fig. 3a, b).

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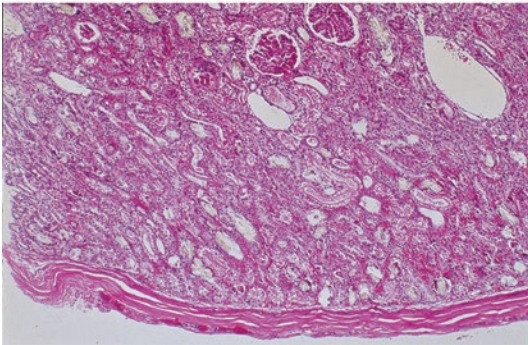
## 5 Specialized Microscopy

Other alterations in the light path or characteristics of the light beam result in different forms of microscopy that are variably applicable to toxicology studies. Substitution of the visible light beam by ultraviolet (UV) light, coupled with application of fluorochromes to the specimen, results in fluorescence microscopy, which is widely used in localization of specific molecules to cells or regions of histological specimens. Fluorescence microscopy is also utilized in Fluoro-Jade staining (Schmued and Hopkins 2000), which accentuates the autofluorescence seen in degenerating neurons (Fig. 4). In polarization microscopy, polarized lenses are placed in the incident light beam above and below the specimen. Rotation of the lenses to a point where they are at  $90^\circ$  to each other effectively blocks the light path to the observer. If the specimen contains crystalline material, it may rotate the light beam to a plane where it passes the upper polarized lens, thus allowing light to reach the eyes of the observer and revealing the presence of crystals in the tissue specimen (Fig. 5a, b). Some types of crystalline mate-

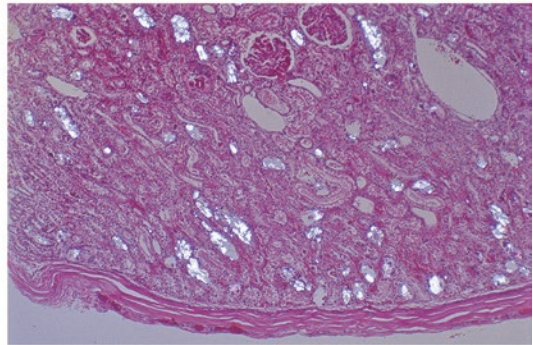


**Fig. 4** Fluorescence microscopy performed on a brain sections stained with Fluoro-Jade B reveals intense fluorescence in degenerating neurons in a model of experimentally induced neurotoxicity. Fluoro-Jade B stain, UV fluorescence microscopy, 40× objective magnification

(a)

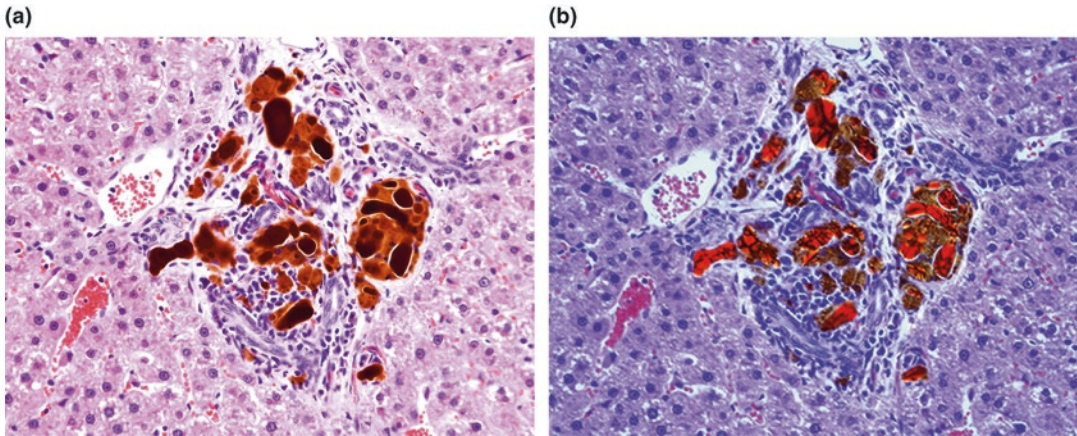


(b)



**Fig. 5 (a)** The H&E-stained section of the kidney from a dog has faintly discernible light brown material within cortical tubules. The dog was submitted to necropsy following consumption of automobile antifreeze. H&E stain, 10× objective magnification. **(b)** Examination via partially polarized light reveals bright, multicolored birefringent material within cortical tubules. Partially polarized light was used to permit visualization of the background renal structure. With fully polarized microscopy, the crystalline material would appear in a black background. The intratubular material is typical of the oxalate crystal accumulation seen in renal tubules following ingestion of ethylene glycol, which is used in automobile antifreeze. H&E stain with polarization microscopy, 10× objective magnification

rial have specific polarization characteristics, such as the red color and “Maltese cross” configurations seen with porphyrin crystals in tissues (Greijsdanus-van der Putten et al. 2005) (Fig. 6a, b). In phase-contrast microscopy, the microscope converts phase shifts, which are invisible to the human eye, into variations in brightness that are visible. This allows visualization of biological structures



**Fig. 6 (a)** This H&E-stained histological section of the liver is from a dog treated with a drug candidate. The biliary tract has mixed inflammatory cell infiltration and a prominent accumulation of unidentified amorphous brown material. H&E stain, 40× objective magnification. **(b)** Examination of the same microscopic field via partially polarized light reveals the brown material to have a distinct red color. Some of the pigment accumulations have intersecting dark bands that are generally consistent with “Maltese crosses.” The image was captured using partially polarized light in order that the background hepatic architecture would be visible. With completely polarized light, the red color was more intense and the “Maltese cross” configurations were more distinctive. The overall histologic presentation is consistent with hepatic accumulation of porphyrin pigment. H&E stain with polarization microscopy, 40× objective magnification

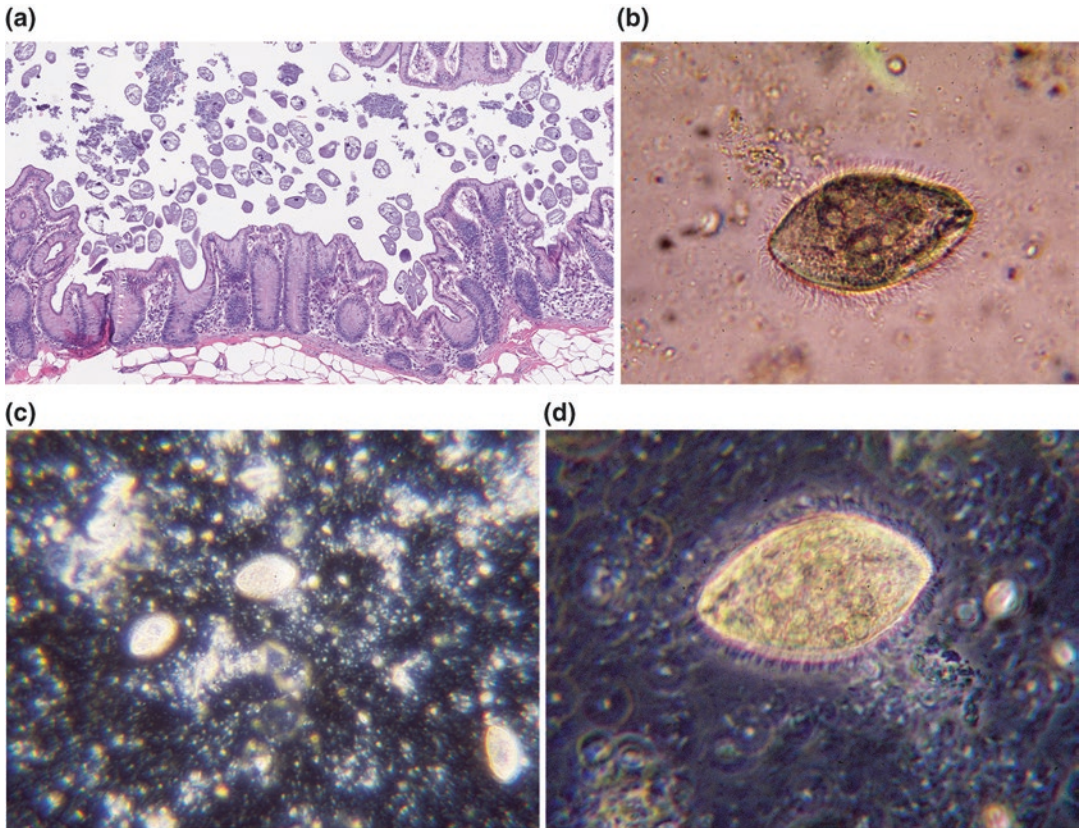
that are not visible by other means and may be performed on living organisms contained with liquid media (Fig. 7a, b). In dark-field microscopy, the direct path of the visible incident light is blocked from reaching the observer’s eye; thus only light that is scattered by the specimen reaches the eye of the observer. The result appearance is brightly illuminated specimens “floating” in a dark background (Fig. 7c). A combination of phase-contrast and dark-field microscopy may be employed (Fig. 7d).

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## 6 Histochemistry

Histochemical stains other than routine hematoxylin and eosin, commonly known as “special stains,” involve the use of various chemical reactions to demonstrate tissue components, microbes, etc., in histological sections. Typical histochemical stains involve a series of chemical treatments that may range from very simple to highly complex and often involve subjective evaluations of staining progress by histologic technicians/technologists. Due to this subjective input, performance of histochemical stains involves a mixture of art and science. As such, this work is typically assigned to the more experienced histology lab personnel. Some examples of special stains include PAS (periodic acid-Schiff) for polysaccha-

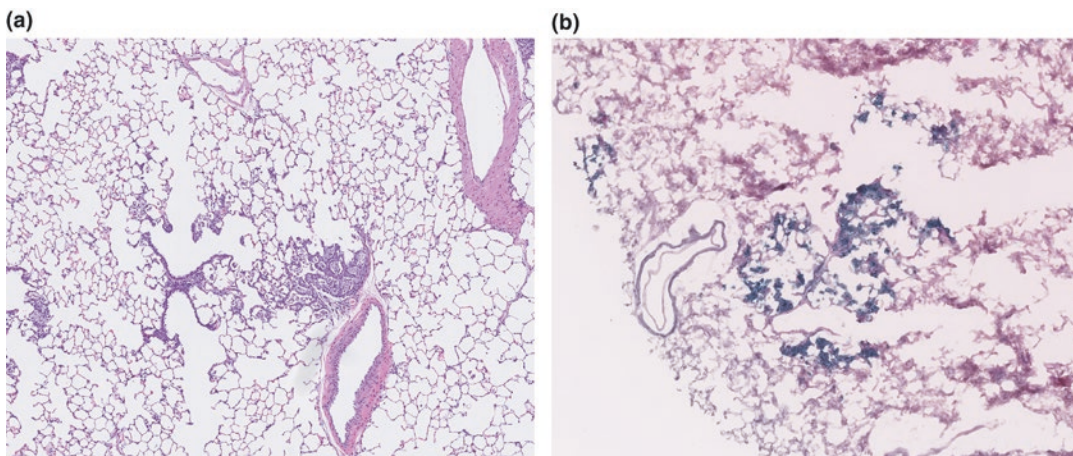




**Fig. 7** (a) The H&E-stained section of the colon from a rhesus macaque has numerous intraluminal organisms that are consistent with *Balantidium coli*. Very little of the internal structure of the organisms is visible in histological sections, which are essentially two-dimensional. H&E stain, 20× objective magnification. (b) Phase-contrast microscopy performed on a wet mount of colon contents from a rhesus macaque reveals surface cilia and internal structures in a *Balantidium coli* organism. Wet mount, phase-contrast microscopy, 100× objective magnification. (c) Dark-field microscopy performed on a wet mount of colon contents from a rhesus macaque reveals a number of ovoid organisms that are consistent with *Balantidium coli*. Wet mount, dark-field microscopy, 40× objective magnification. (d) Combined phase-contrast and dark-field microscopy on a wet mount of colon contents from a rhesus macaque reveals additional structural details of the *Balantidium coli* organisms. Phase-contrast and dark-field microscopy, 100× objective magnification

rides, von Kossa for calcium, Perls' Prussian blue for iron, and Sudan black B for lipids (Fig. 8a, b).

Specific requirements of the histopathology evaluation may necessitate the use of special tissue fixatives. For example, it is common practice to use Davidson's or modified Davidson's fixatives for preservation of eye and testis specimens (Latendresse et al. 2002). It is critically important that those involved in the design of toxicology studies should determine any specific tissue collection or preservation requirements well in advance of the planned necropsy, as procurement of the necessary reagents may involve delays.



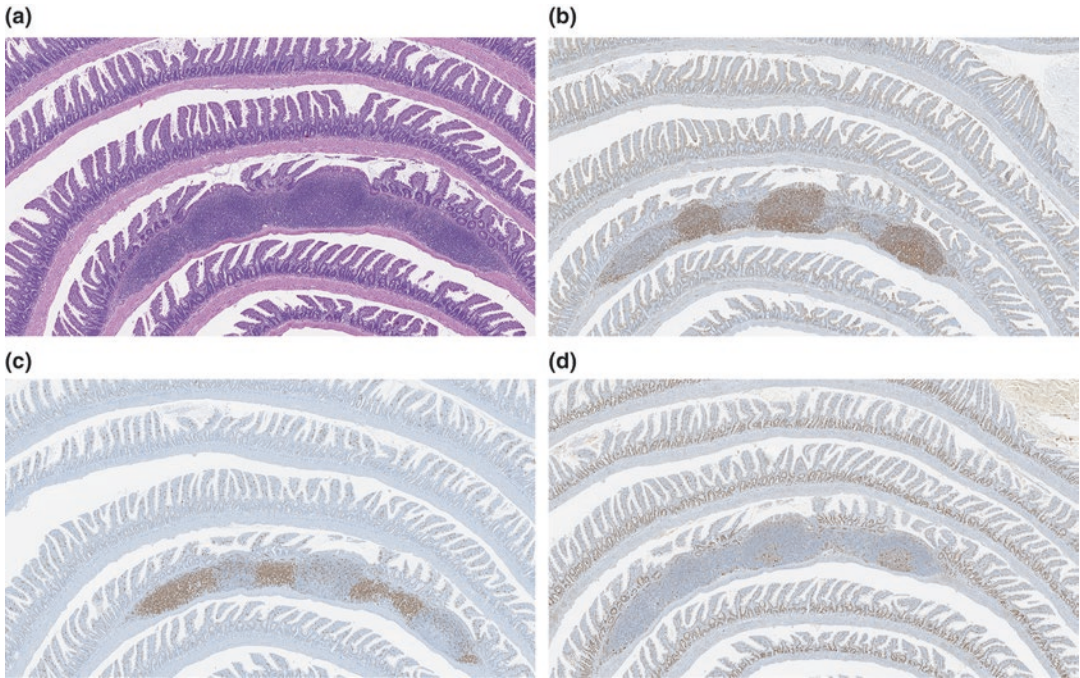
**Fig. 8** (a) The histological section of the lung is from a rat that received a test article in corn oil vehicle via oral gavage. Note the focally extensive infiltration of inflammatory cells near distal airways. H&E stain, 5× objective magnification. (b) A frozen section of the lung, from the same animal shown in Fig. 1a, stained with Sudan black B reveals the presence of corn oil in association with the inflammatory cell infiltrates. Reflux and aspiration of minor quantities of test article/vehicle is seen with some frequency in gavage studies and should be considered a possible basis for unexplained pulmonary findings in gavage studies (Crabbs et al. 2013)

Failure to meet these fixation requirements may have a disastrous effect on meeting the specific goals of the study. For further information on this complex topic, readers are referred to histotechnology texts (Carson 1997; Sheehan and Hrapchak 1980) or reference publications on histochemistry (Thompson 1966).

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## 7 Immunohistochemistry

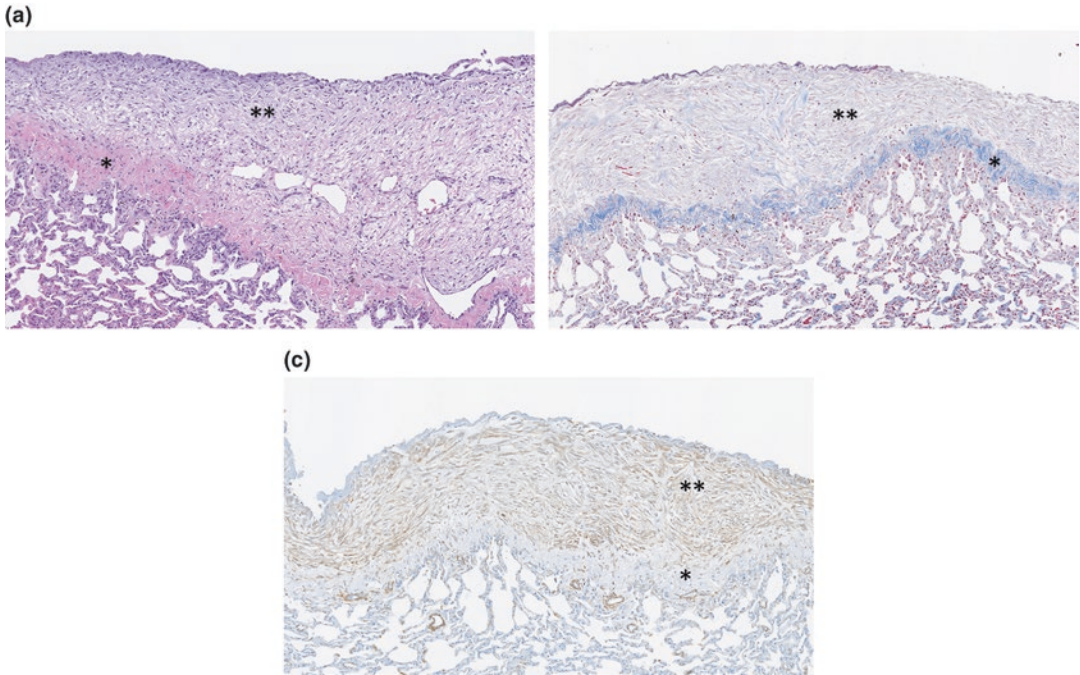
Traditional histopathology involves microscopic evaluation of the histomorphology of tissues, which largely ignores the major contribution of physiological and biochemical abnormalities in many pathological processes. The development of immunohistochemistry (IHC) was a major advance in the field of histopathology, as it allowed detection of many of the molecules that define cell types and cellular structures as well as signaling and effector molecules that contribute to many pathological processes (Figs. 9a–d and 10a–c). Immunohistochemistry depends on the non-covalent bonding of primary antibodies that recognize the three-dimensional presentation of antigenic epitopes. The primary antibodies may be directly labeled with a fluorochrome that is visualized via fluorescence microscopy or an enzyme such as horseradish peroxidase that converts a chromogen to a product that is visible by routine visible light microscopy. More commonly, a “sandwich technique” is employed whereby the primary antibody is recognized by a fluorochrome- or enzyme-labeled secondary antibody, followed by



**Fig. 9** (a) The image is from a Swiss roll preparation of the small intestine from a rat collected at postnatal day 28. Note the plaque-like array of deeply stained lymphoid follicles that constitute a mucosal lymphoid aggregate (“Peyer’s patch”). Examination of the routinely stained histological section suggests intact mucosal immune system structures, but provides little information regarding the functional subcategories of immune cell populations. H&E stain, 5× objective magnification. (b) An immunohistochemical stain directed at CD45RA reveals a dense population of brown-stained CD45RA+ B cells in lymphoid follicles of the Peyer’s patch. CD45RA IHC stain with 3, 3'-diaminobenzidine chromogen and hematoxylin counterstain, 5× objective magnification. (c) An immunohistochemical stain directed at CD3 reveals a dense population of brown-stained CD3+ lymphocytes in the spaces between lymphoid follicles in the Peyer’s patch. These aggregates of CD3+ T cells are equivalent to the paracortex zone of lymph nodes. Note the regularly spaced population of surveillance T cells in the superficial intestinal mucosa, as well as the substantial population of T cells scattered throughout the follicles. This intrafollicular T-cell population is critically important in development of B-cell-mediated immune responses. CD3 IHC stain with 3, 3'-diaminobenzidine chromogen and hematoxylin counterstain, 5× objective magnification. (d) An immunohistochemical stain directed at Ki67 proliferation marker reveals marked proliferative activity in the intestinal crypts and within germinal centers at the base of lymphoid follicles within the Peyer’s patch. Presence of active germinal centers indicates intact responsiveness to antigenic stimulation and initiation of a humoral immune response. Ki67 IHC stain with 3, 3'-diaminobenzidine chromogen and hematoxylin counterstain, 5× objective magnification

fluorescence microscopy or enzyme-chromogen interaction in chromogenic IHC (Fig. 11).

The common use of IHC staining as a second-tier investigative technique in pathology evaluation warrants specific consideration of tissue preservation requirements for IHC. Aldehyde-mediated cross-linking of amino acids starts to occur soon after formalin immersion, and with prolonged formalin immersion, the protein cross-linking may block access of the antibodies used in IHC

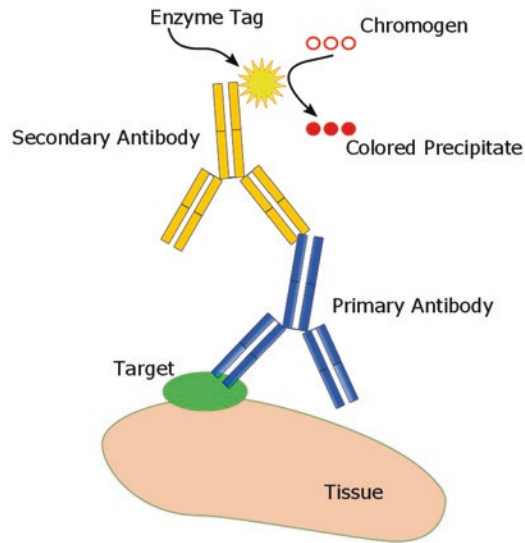


**Fig. 10** (a) The histological section is from a rhesus macaque that was subjected to 11 Gy ionizing radiation 53 days prior to necropsy. The original pleural layer (\*) consists of a dense layer of pink-stained fibrous connective tissue, while the superficial surface has a thick layer (\*\*\*) of immature fibrous connective tissue. H&E stain, 10× objective magnification. (b) A Masson's trichrome stain performed on the same tissue specimen shown in Fig. 3a shows a thin layer of mature collagenous tissue (\*) in the original pleural surface, with a smaller amount of collagenous tissue in the immature superficial layer (\*\*). Masson's trichrome stain, 10× objective magnification. (c) An immunohistochemical stain directed at alpha-smooth muscle actin ( $\alpha$ SMA) shows little  $\alpha$ SMA staining in the original mature pleural layer (\*), but there is abundant  $\alpha$ SMA staining in the superficial layer of immature fibrous connective tissue (\*\*). Presence of abundant  $\alpha$ SMA immunoreactivity suggests the involvement of myofibroblasts in the pathogenesis of the radiation-induced pleural fibrosis. Alpha-smooth muscle actin IHC staining, 10× objective magnification

staining. This technical defect may be overcome by judicious use of antigen retrieval processes, but those processes are imperfect and introduce an additional variable into the experiment. If there is any expectation that IHC staining will be required during the pathology evaluation, immersion of tissue specimens in aldehyde-based fixatives should be limited to approximately 48 hours, after which the tissue specimens should be transferred to 70% ethanol.

## 8 In Situ Hybridization

In situ hybridization (ISH) is somewhat similar to IHC, but base pairing between nucleic acids replaces antigen-antibody binding in the initial recognition step. An additional strength of ISH over IHC



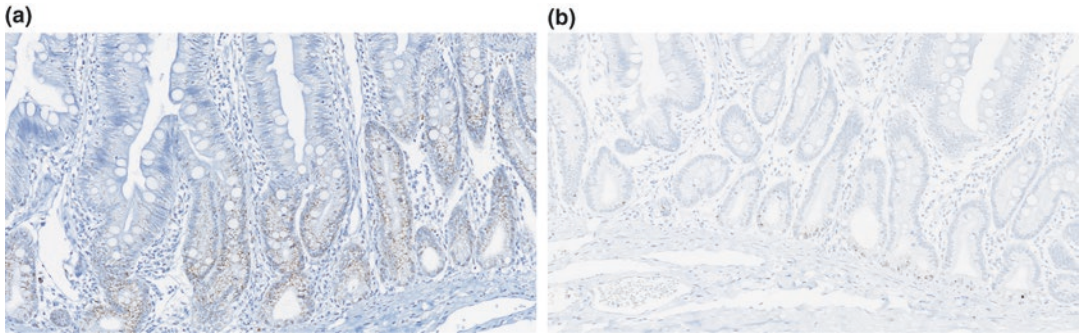
**Fig. 11** In the “sandwich technique” of chromogenic immunohistochemical staining, the target molecule on the tissue is detected by a primary antibody to that molecule. A secondary antibody directed at the primary antibody is tagged with an enzyme such as horseradish peroxidase. After sequential steps that allow binding of the primary and tagged secondary antibodies, the section is flooded with a chromogen such as 3, 3'-diaminobenzidine (DAB) that reacts with the enzyme to form a colored precipitate. The end result is a colored deposit at the site of primary antibody binding. In fluorescent immunohistochemical staining, either the primary or secondary antibody is tagged with a fluorochrome which is directly visualized by fluorescence microscopy. Different fluorochromes emit different wavelengths upon UV stimulation; thus it is possible to demonstrate multiple molecular targets simultaneously. (Artwork compliments of Cynthia L. Swanson, M.S.)

is the possibility of repeated temperature-based nucleic acid dissociation-association cycles, which results in amplification of faint signals. In situ hybridization may be used to visualize production of messenger RNA; thus it is particularly valuable in detection of early cellular responses or responses where production of signaling or effector molecules is very low or the existence of those molecules is highly transient due to the action of reversal or control pathways (Fig. 12a, b).

## 9 Ancillary Morphological Assay Procedures

### 9.1 Tissue Microarray

Tissue microarray (TMA) is a histological technique whereby small cores of tissues are oriented in an array in a single paraffin block, thus allowing multiple tissues to be efficiently analyzed by various

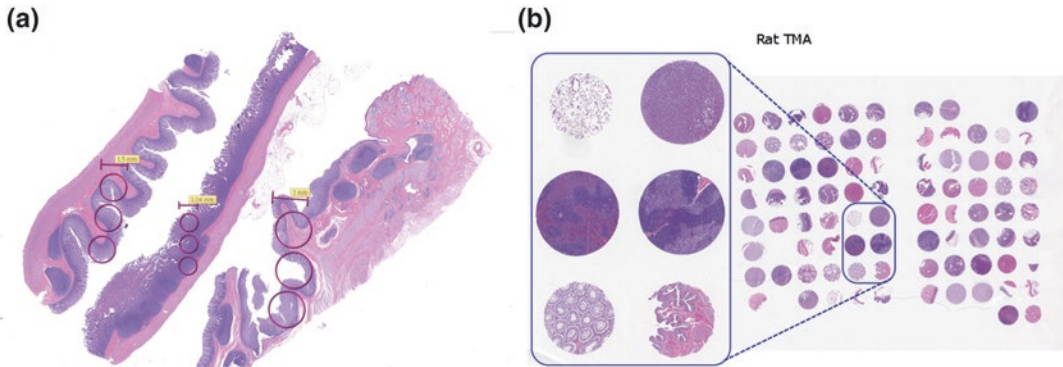


**Fig. 12** (a) Chromogenic in situ hybridization (ISH) allows visualization of nucleic acid target sequences in tissues, as opposed to the peptide targets that are typically revealed by immunohistochemistry. Messenger RNA (mRNA), a common target for ISH, is very labile in fixed tissue specimens; therefore, it is desirable to demonstrate the presence of the mRNA product of an invariably present “housekeeping” gene in order to confirm that mRNA species were adequately preserved in the specimen. This ISH-stained section of the small intestine from a rhesus macaque reveals an abundance of the PPIB mRNA target. PPIB in situ hybridization, 20× objective magnification. (b) Chromogenic in situ hybridization (ISH) staining directed at Lgr5 mRNA expression reveals a population of positively stained cells in the deep aspect of small intestinal crypts of a rhesus macaque, thus confirming the presence of an Lgr5-positive population of intestinal stem cells. Lgr5 in situ hybridization staining, 20× objective magnification

histochemical stains, immunohistochemistry, or in situ hybridization. Tissue microarrays may represent a broad selection of tissues, or selected tissues that represent one or more organ systems. It is common practice to prepare TMAs based on multiple neoplasms, e.g., multiple lung carcinoma specimens from individual subjects. Most often TMAs contain duplicate or triplicate samples from individual tissue specimens, thus allowing confirmation of any observations in individual specimens. The number of tissue cores included in each TMA is determined by the bore size of the punch used to create the tissue cylinders. Standard TMA blocks commonly have 30, 60, or 90 individual tissue cylinder cross-sections (Fig. 13a, b). Tissue microarrays are routinely employed in basic biomedical research and are applied with some frequency in toxicology investigations (Morgan et al. 2002; Irwin et al. 2004; Luebke et al. 2006). MALDI analysis (see below) is also possible on TMA sections (Mascini et al. 2015; Powers et al. 2014).

## 9.2 Tissue Cross-Reactivity

Tissue cross-reactivity (TCR) studies are based on immunohistochemistry staining of multiple tissues, typically using a primary antibody that is prepared to indicate the presence of a bound test material such as a biopharmaceutical molecule. The underlying principle is the expectation that binding of a test material to an unexpected tissue site may be a harbinger of an untoward reaction in a tissue other than the pharmaceutically relevant tissues. The initial procedure for TCR studies was outlined in the US FDA publication entitled “Points to Consider in the Manufacture and



**Fig. 13** (a) Histological sections of the small and large intestine of a cynomolgus macaque demonstrate the relative size of the tissue cores that result from use of 1.04-, 1.5-, and 2-mm microarray tissue punches. Though smaller cores allow a greater number of tissue samples on a microslide, the small cores may not be completely representative of the entire tissue. Selection of core diameter requires judgments related to tissue structure and the overall goals of the analytical procedure. (b) This H&E-stained tissue microarray contains numerous tissue specimens; thus it is a very efficient tool for use in many tissue-based analytical procedures. It is standard practice to include multiple sections of a tissue microarray block on a single slide, thus allowing duplication of analytical procedures with a minimum of reagent and labor investment

Testing of Monoclonal Antibody Products for Human Use,” first published in 1983 and revised in 1987, 1994, and 1997. Earlier versions of TCR tests included tissues from humans, rats, and non-human primates, but more recent versions are focused largely on human tissues. More details on the TCR assay are available in multiple publications (Bussiere et al. 2011; Hall et al. 2008; Leach et al. 2010).

### 9.3 Laser-Capture Microdissection

Laser-capture microdissection (LCM) is a general term for a number of histological techniques that allow selective capture of individual cells or tissues from within a broader expanse of the histological section. These procedures involve the use of very fine (e.g., 1  $\mu\text{m}$  diameter) laser beams to cut histological tissue sections as they are viewed via light microscopy. The areas of interest may be translocated to a capture container, or may be retained on the slide as extraneous areas are deleted. One procedure involves application of a thin film of a plastic-type material to the surface of a histological section, followed by directing a very fine laser beam to the area of film that overlies the cell or tissue of interest. Interaction of the laser beam with the film and underlying tissue causes the cell or tissue to become adhered to the film. The selected subpopulation of cells or tissues is subsequently detached from the film into a separate container. After collection, the cells or tissues of interest are used for various investigational purposes, often involving molecular biology assays. The following references present the salient features of LCM (Emmert-Buck et al. 1996; Espina et al.

2006) as well as examples of its application in toxicology (Cullen et al. 2010; Dunnick et al. 2016).

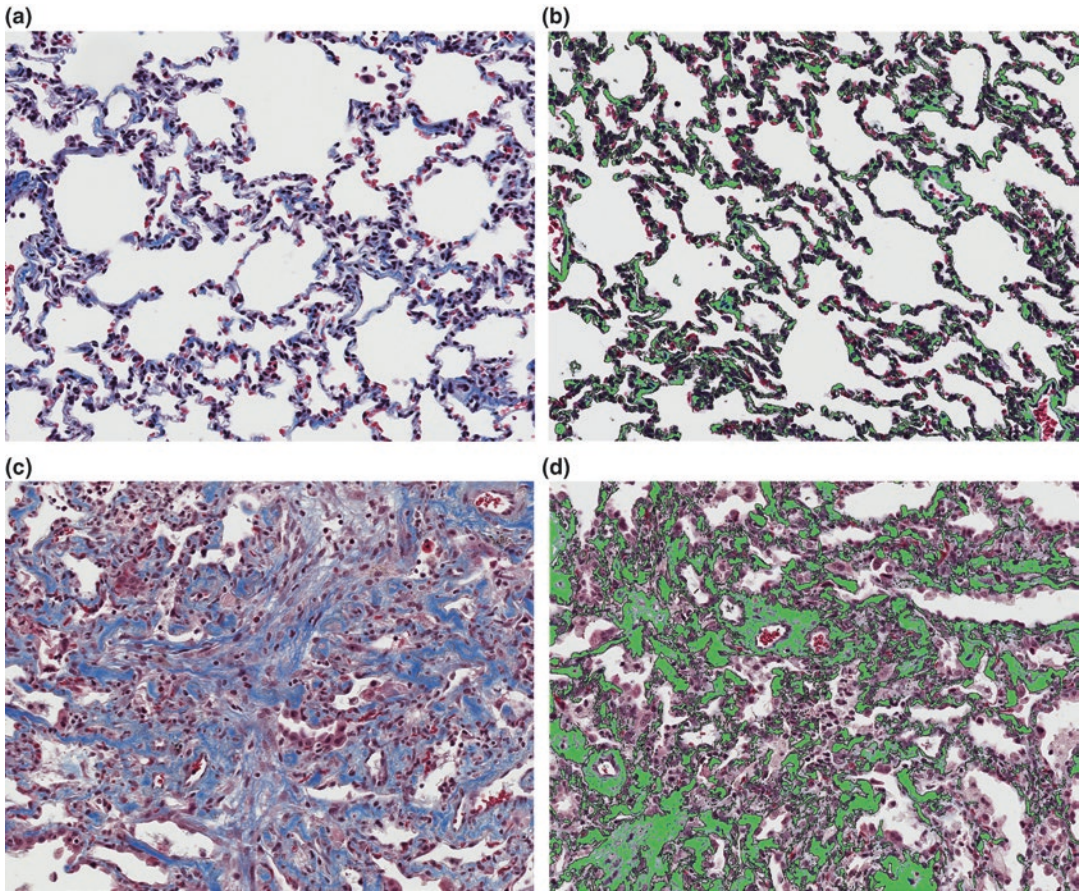
#### **9.4 Morphometric Analysis**

In some situations the standard subjective analysis of histological sections is not sufficiently precise to accomplish established goals. A greater level of precision may be possible with carefully selected counts or measurements, though these procedures tend to be labor-intensive and costly (Fig. 14a–d). Historically, morphometric analysis involved preparation of photomicrographic prints with superimposed measurement lines or grids to introduce the quantitative element to the analysis. More recently, morphometric analysis has been performed on digital images or scanned (“virtual”) slides, using software that greatly facilitates the capture of morphometric data. In performing morphometric analysis, the analyst must remain cognizant of the underlying requirement for technically adequate histological preparations that are representative of the pathologic entity of interest. No degree of analytical precision will yield meaningful data if the substrate specimens fail to meet these basic requirements. Those proposing to undertake the additional expense of morphometric analysis must also decide whether the additional level of precision would contribute significantly to the overall decision-making process. As a commonly encountered example, in routine circumstances, it would make little sense to employ histological morphometric analysis to precisely characterize the degree of xenobiotic-associated hepatocellular hypertrophy when the degree of that liver alteration is revealed with adequate precision by the organ weights collected at necropsy.

A specific form of morphometric analysis consisting of linear measurements of various brain structures is incorporated into developmental neurotoxicity study guidelines (US Environmental Protection Agency OPPTS 870.6300) and the neurotoxicity arm of extended one-generation developmental toxicity studies (EOGRTS) (Organisation of Economic Cooperation and Development Test Guideline 443). These linear measurements are a response to the recognition that chemically mediated alteration in brain size may occur in the absence of detectable histological alterations. See Chapter, “[Pathology of Juvenile Animals](#),” for a presentation of linear measurements used in the analysis of developmental neurotoxicity studies.

Linear measurements of the height of thyroid follicular epithelial cells, and areal measurements of the colloid content of thyroid follicles, may provide a more quantitative indication of the effects of thyrotoxicants (Fig. 15a, b). Semi-automated counting of ovarian follicles, particularly when stained by selective immunohistochemical staining procedures, may offer advantages over manual counting when definitive estimates of ovarian follicle populations are needed (Picut et al. 2008) (Fig. 16a, b).

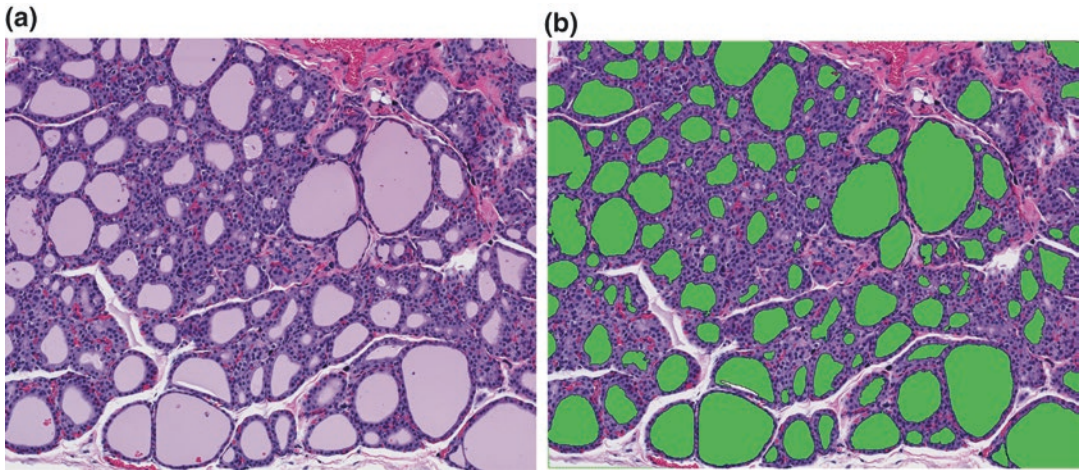




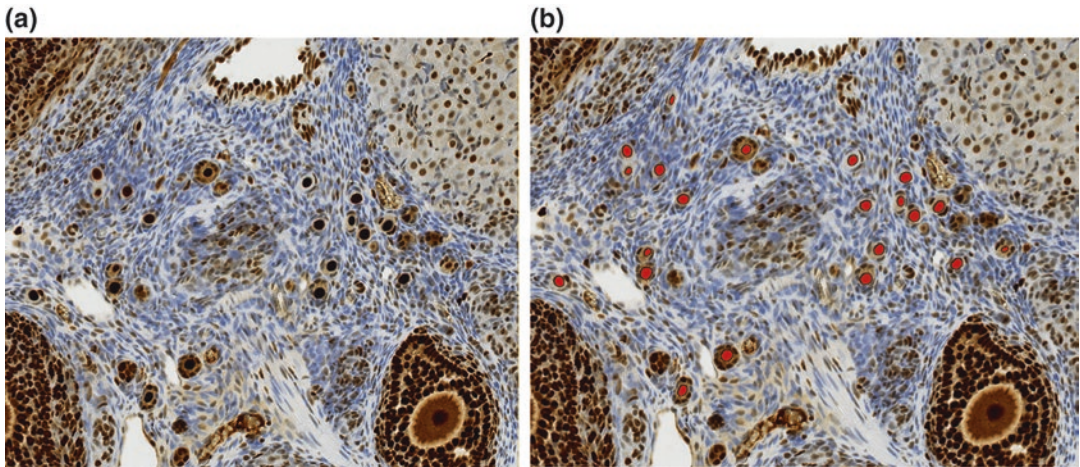
**Fig. 14** (a) A section of normal lung tissue from a rhesus macaque has a small amount of blue-stained collagenous tissue within alveolar walls. Masson's trichrome stain, 20× objective magnification. (b) In this type of morphometric analysis, the blue color of the Masson's trichrome-stained collagenous tissue in the lung is selected by the analytical program and "painted" a primary color for subsequent analysis. In this rudimentary example, the number of green-colored pixels would be expressed per the total area of the lung image. In a real-life example, it would be necessary to compensate for the amount of lung tissue versus the air-filled spaces to arrive at a meaningful estimate of the amount of collagenous tissue in the lung. (c) This section of lung tissue is from a rhesus macaque collected 119 days after receiving 10 Gy of whole-body ionizing radiation with 5% bone marrow shielding. Note the larger amount of blue-stained collagenous tissue within alveolar walls, as compared to the naïve control animal in the previous images. Masson's trichrome stain, 20× objective magnification. (d) The blue-stained collagenous tissue is "painted" green by the analytical program, and the amount of collagen in the lung is estimated by counting the green pixels, which equates to relative area of collagen deposition

### 9.5 Stereological Analysis

Stereological analysis in a broad sense consists of techniques for statistically based analysis of three-dimensional objects or spaces. As applied to toxicologic pathology, stereological analysis most commonly applies to counting or measuring objects or structures in entire organs. Rather than relying on exhaustive step-sectioning of the entire organ, histological sections collected at intervals are



**Fig. 15** (a) This H&E-stained section of the thyroid gland was collected from a rat at postnatal day (PND) 61. Note the variation in size of the pink-stained colloid accumulations within follicles. H&E stain, 10× objective magnification. (b) In a basic color segmentation-type analysis, the light pink follicular colloid is “painted” a distinctive color such as green, which can then be subjected to various forms of morphometric analysis. Common forms of analysis would include overall colloid content relative to the total tissue or mean size of colloid accumulations. H&E stain with color segmentation morphometric analysis, 10× objective magnification



**Fig. 16** (a) This section of rat ovary was subjected to immunohistochemical staining for proliferating cell nuclear antigen (PCNA), which selectively stains the small round primordial and primary follicles as well as several other structures in the section (Picut et al. 2008). (b) The morphometric analytical program detected the population of primordial and primary follicles via a combination of color segmentation, size of the structures, and the “roundness” of the structures. A variety of structures were selectively stained by the PCNA IHC staining, but the morphometric analytical process displayed excellent fidelity in selecting only primordial and primary follicles

used to estimate the population of the entire organ, as based on the Cavalieri principle (Gundersen and Jensen 1987). In its simplest form, the Cavalieri principle states that the three-dimensional volume of a structure can be estimated by preparing cross-sections of the structure, performing multiple two-dimensional area measurements of those cross-sections, and multiplying by the linear interval between the two-dimensional sections. Preparation of histological sections for stereological analysis is based on a process known as systematic uniform random sampling (SURS) (Gundersen and Jensen 1987). Various probes are then used to interrogate the specimens in order to arrive at statistically relevant counts or measurements (Boyce et al. 2010a, b). Many journals in the neuroscience area require unbiased stereological analysis if structure-based counts or measurements are end-points. Similar requirements are proposed for studies of respiratory tissues (Knudsen and Ochs 2011). Though not typically mandated by regulatory requirements, stereological analysis should be considered in situations where definitive counts or measurements are required. For example, studies involving potential therapies for Parkinson's disease commonly require definitive enumeration of tyrosine hydroxylase-positive "dopaminergic" neurons in the substantia nigra region of the brain (Fig. 17). Stereological analysis is required in situations involving neuronal population counts, as studies have shown that simple visual examination of histological sections may be inadequate for detection of toxicologically relevant alterations in neuronal numbers (de Groot et al. 2005). *It is critical for those involved in study design to understand that stereological analysis must be planned in advance of specimen collection and should not be attempted retrospectively after routine studies have been completed.*

While the immediate application of stereological analysis in toxicologic pathology is based on analysis of histological sections, it should be noted that other forms of planar, two-dimensional tissue presentations (e.g., computerized tomography) could also be subjected to stereological analysis techniques.

### **9.6 Matrix-Assisted Laser Desorption Ionization (MALDI)-Mass Spectrometry Imaging (MSI)**

MALDI-MSI represents an additional morphology-based investigational technique in the progression from routine light microscopy, special histochemical stains, immunohistochemistry (IHC), and in situ hybridization (ISH). MALDI coupled with mass spectrometry imaging (MALDI-MSI) utilizes frozen or formalin-fixed, paraffin-embedded (FFPE) specimens coated with a matrix, which is then probed with a two-dimensional laser to collect a mass spectrum of the area of interest. The associated computer program assembles the spectral data upon an optical image, thus allowing determinations of the molecular content of the areas of interest (Maronpot et al. 2017). MALDI-MSI coupled with gene expression analysis has the potential to render exquisite insight into the basic nature of pathological processes (Brown et al. 2016). MALDI