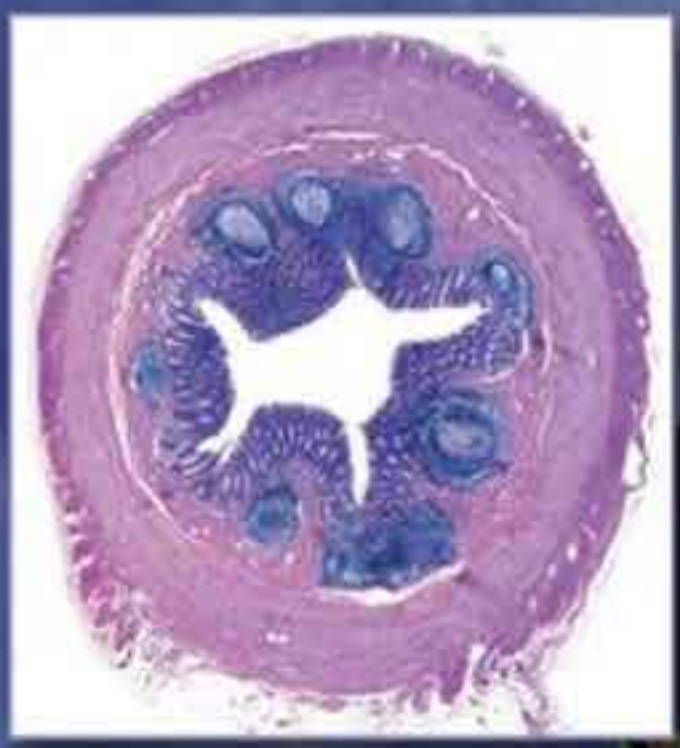
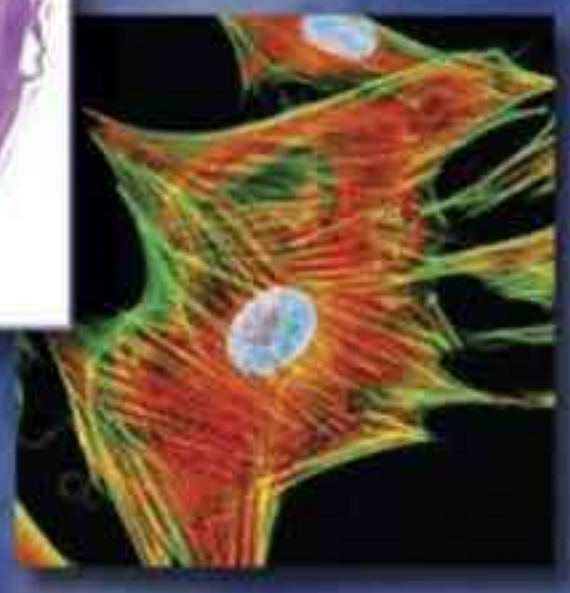
Seventh Edition

HISTOLOGY

A TEXT AND ATLAS

with Correlated Cell and Molecular Biology





Wojciech Pawlina

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Histology

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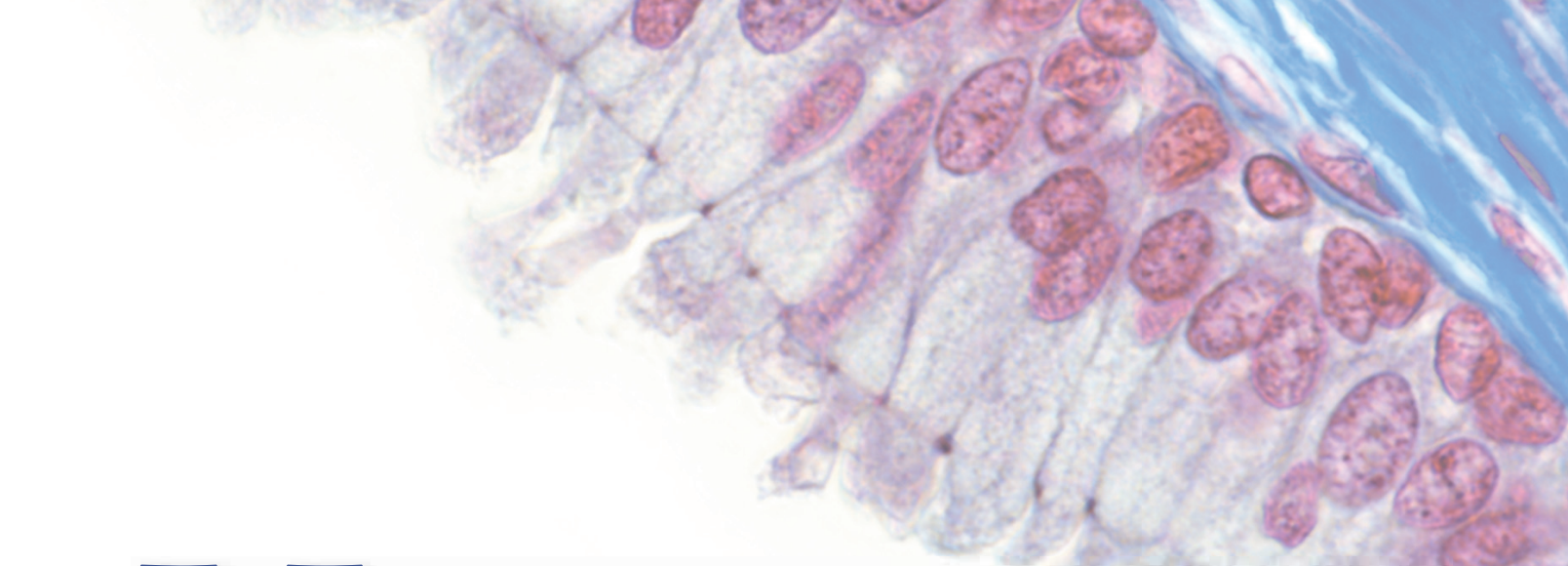
Wojciech Pawlina
Discussing histology education in his eosin-colored tie



Histology

A Text and Atlas

With Correlated Cell and Molecular Biology

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This edition is dedicated to **Teresa Pawlina**, *my wife, colleague, and best friend whose love, patience, and endurance created a safe haven for working on this textbook*

and

to my children **Conrad Pawlina** *and* **Stephanie Pawlina Fixell** *and her husband* **Ryan Fixell** *whose stimulation and excitement are always contagious.*

Preface

This seventh edition of *Histology: A Text and Atlas with Correlated Cell and Molecular Biology* continues its tradition of introducing health science students to histology correlated with cell and molecular biology. As in previous editions, this book is a combination "text-atlas" in that the standard textbook descriptions of histologic principles are supplemented by an array of schematics, tissue and cell images, and clinical photographs. In addition, the separate atlas sections now conclude each chapter to provide large-format, labeled atlas plates accompanied by legends that highlight and summarize the elements of microscopic anatomy. *Histology: A Text and Atlas* is, therefore, "two books in one."

The following significant modifications have been made to this edition:

"Histology 101" sections have been added at the end of each chapter. These new sections contain essential information for a quick review of the material listed in a bullet-point format and are perfect for students who find themselves on the eve of quizzes or examinations. These reader-friendly sections are designed for fast information retrieval with concepts and facts listed in separate boxes.

All figures in this book have been carefully revised and updated. Many schematics and flowcharts have additionally been redrawn. More than one-third of all figures have been replaced by new drawings designed to show the latest interpretation of molecular, cellular, and tissue concepts based on recent discoveries in molecular research. All drawings maintain a uniform style throughout the chapters with a palette of eye-pleasing colors. Several conceptual drawings have been aligned side by side with photomicrographs, a feature carried over from the sixth edition that was widely agreeable to reviewers, students, and faculty members.

Cellular and molecular biology content has been updated. Text material introduced in the sixth edition has been updated to include the latest advancements in cellular and molecular biology, stem cell biology, cellular markers, and cell signaling. The seventh edition focuses on target concepts to help students with overall comprehension of the subject matter. To accommodate reviewers' suggestions, the seventh edition integrates new information in cell biology with clinical correlates, which readers will see as new clinical information items in blue text and clinical folders. For example, within the adipose tissue discussion, the reader might also discover a cell biology topic regarding white-to-brown fat transdifferentiation. Also added is a basic discussion on virtual microscopy, a new approach used in the majority of U.S. histology courses.

Reader-friendly innovations have been implemented. Similar to the previous edition of this book, the aim is to provide more ready access to important concepts and essential information. Changes introduced in the sixth edition, such as bolded key terms, clinical information in blue text, and a fresh design for clinical correlation folders, were all enthusiastically approved by the new generation of textbook users and have been maintained in this edition. Important concepts have been revised and are listed as sentence headings. Dominant features of cells, tissues, and organs have been summarized into short phrases and formatted into bulleted lists clearly identifiable in the body of the text by oversized, colored bullets. Essential terms within each specific section are introduced within the text in eye-catching, oversized, bold, red font. Text containing clinical information and the latest research findings is presented in blue, with terminology pertaining to diseases, conditions, symptoms, or causative mechanisms in oversized bolded blue. Each clinical folder contains updated clinical text with more illustrations and drawings easily found within each chapter and visually appealing to keep readers turning page after page.

More features have been added. In understanding that students are pressed for time and require stimulation when reading several hundred pages of text, we continue to enhance this textbook with pedagogic features, including:

- "Histology 101" sections at the end of each chapter
- Summary tables including a review table on the characteristics of lymphatic organs
- More Clinical Correlation and Functional Considerations Folders, which contain clinical information related to the symptoms, photomicrographs of diseased tissues or organs, short histopathologic descriptions, and treatment of specific diseases
- Updated and relabeled atlas plates
- New figures, illustrations, and high-resolution digital photomicrographs, more than one-third of which have been redrawn for greater clarity and conceptual focus
- A bright, energetic new text design that sets off the new illustrations and photos and makes navigation of the text even easier than before

As in the last six editions, all changes have been made with students in mind. We strive for clarity and concision to aid student comprehension of the subject matter, familiarity with the latest information, and application of newfound knowledge.

Wojciech Pawlina

Acknowledgments

First and foremost, I wish to thank the creator of this book, Dr. **Michael H. Ross**, my mentor, colleague, and dear friend for his confidence in my ability to carry on with this project, so the future generations of students studying histology would benefit from his visionary idea of integrating text and atlas into a single book. While preparing this seventh edition, I have very much missed him, frequently recalling our meetings and discussions. He will forever be present in my heart and thoughts.

Changes to the seventh edition arise largely from comments and suggestions by students who have taken the time and effort to send me e-mails of what they like about the book and, more importantly, how the book might be improved to help them better learn histology. I have also received thoughtful comments from my first-year histology students who always have an eye for improvement. I am grateful to them for the keen sense by which they sharpen this work.

Many of my colleagues who teach histology and cell biology courses all over the world have, likewise, been helpful in creating this new edition. Many have suggested a stronger emphasis on clinical relevance, which I strive to continually engage as new research makes itself known. Others have provided new photomicrographs, access to their virtual slide collections or new tables, or have pointed out where existing diagrams and figures need to be redrawn.

Specifically, I owe my thanks to the following reviewers, who have spent time to provide me with constructive feedback in planning this seventh edition.

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A few colleagues have made especially notable contributions to this textbook. I am extremely grateful to Drs. **Joaquin Garcia** and **Joseph Grande** from Mayo Clinic College of Medicine for providing original histologic images of the highest quality of several clinical specimens; to Dr. **Arthur Hand** from the University of Connecticut School of Dental Medicine for providing exceptional images of dental tissues; to Dr. **Michael Hortsch** from the University of Michigan Medical School for providing guidance in obtaining permission to use their outstanding virtual microscopy slide collection; to Dr. **Kenneth Lerea** from New York Medical College for providing text on cell signaling mechanisms; to Dr. **Nirusha Lachman** from Mayo Clinic College of Medicine who provided me with ideas for improvements; and to the many other clinicians and researchers who gave me permission to use their original unique photographs, electron micrographs, and photomicrographs in this edition. They are all acknowledged in the appropriate figure legends.

I was fortunate that one of the most talented medical illustrators, **Rob Duckwall** from the Dragonfly Media Group (Baltimore, Maryland), continued to work on this edition to complete our three-edition long marathon project of replacing all the illustrations in this book. His dedication, effort, and achievement, in my humble opinion, are comparable to those made on behalf of the Sistine Chapel. Duckwall is the Michelangelo of this Histology Sistine Chapel. His commitment and willingness to work on our artist–author team provided an unprecedented creative dynamic that has made all the difference. I fondly recall the time when we discussed the physics of endolymph flow in the internal ear early (*really* early—1:00 AM) on a Saturday morning and the midnight chats on how to elevate zipper lines between two dome-shaped cells in the bladder. Thank you, Rob, for your professionalism, quality of work, and attention to detail. You have made each and every drawing an unparalleled work of art.

I also wish to extend my special thanks to **Jennifer Clements**, the Art Director, for providing me with the support for relabeling and replacing images in the text and atlas sections of this book. Her bright and outgoing nature was a welcome addition to our weekly progress conference calls. My appreciation also goes to **Greg Nicholl**, Product Development Editor, who had the most challenging work: putting all the leads together to create a tangible product. Greg has provided the needed expertise during the development process. While he was immersed in all the rules, regulations, page counts, details with page design, and deadlines, I reminded him on several occasions that in biological sciences 2 + 2 does not always = 4. My thanks and appreciation goes out to **Sara Cleary** for providing expertise with copy-editing. A special thanks goes to **Crystal Taylor**, Senior Acquisition Editor, for her support throughout the development of this book. Her vigilance and thorough attention to detail is much appreciated. Finally, my sincere appreciation goes to **Harold Medina**, the Project Manager of Absolute Service, Inc., and his team of talented compositors lead by **Syrah Romagosa** for an excellent and creative job in bringing this challenging publication to fruition.

Contents

1

Methods

OVERVIEW OF METHODS USED IN HISTOLOGY

The objective of a histology course is to lead the student to understand the microanatomy of cells, tissues, and organs and to correlate structure with function.

Histology *[Gr., ιστός, histos = tissue; λογία, logia = science]*, also called **microscopic anatomy**, is the scientific study of microscopic structures of tissues and organs of the body. Modern histology is not only a descriptive science but also includes many aspects of molecular and cell biology, which help describe cell organization and function. The methods used by histologists are extremely diverse. Much of the histology course content can be framed in terms of light microscopy. Today, students in histology laboratories use either **light microscopes** or, with increasing frequency, **virtual microscopy**, which represents a method of viewing a digitized microscopic specimen on a computer screen or mobile device. In the past, more detailed interpretation of microanatomy was done with the **electron microscope (EM)**—both the **transmission electron microscope (TEM)** and the **scanning electron microscope (SEM)**. Now, the **atomic force microscope (AFM)** can also provide images, which are comparable or higher in resolution to those obtained from TEM. Both EM and AFM, because of their greater resolution and useful magnification, are often the last step in data acquisition from many auxiliary techniques of cell and molecular biology. These auxiliary techniques include:

- histochemistry and cytochemistry,
- immunocytochemistry and hybridization techniques,
- autoradiography,
- organ and tissue culture,
- cell and organelle separation by differential centrifugation, and
- specialized microscopic techniques and microscopes.

The student may feel removed from such techniques and experimental procedures because direct experience with them is usually not available in current curricula. Nevertheless, it is important to know something about specialized procedures and the data they yield. *This chapter provides a survey of methods and offers an explanation of how the data provided by these methods can help the student acquire a better understanding of cells, tissues, and organ function.*

One problem that students of histology face is understanding the nature of the two-dimensional image of a histologic

slide or an electron micrograph and how the image relates to the three-dimensional structure from which it came. To bridge this conceptual gap, we must first present a brief description of the methods by which slides and electron microscopic specimens are produced.

TISSUE PREPARATION

Hematoxylin and Eosin Staining with Formalin Fixation

The routinely prepared hematoxylin and eosin–stained section is the specimen most commonly studied.

The slide set given each student to study with the light microscope consists mostly of formalin-fixed, paraffin-embedded, hematoxylin and eosin (H&E)–stained specimens. Nearly all of the light micrographs in the Atlas section of this book are of slides from actual student sets. Also, most photomicrographs used to illustrate tissues and organs in histology lectures and conferences are taken from such slides. Other staining techniques are sometimes used to demonstrate specific cell and tissue components; several of these methods are discussed below.

The first step in preparation of a tissue or organ sample is fixation to preserve structure.

Fixation, usually by a chemical or mixture of chemicals, permanently preserves the tissue structure for subsequent treatments. Specimens should be immersed in fixative immediately after they are removed from the body. Fixation is used to:

- terminate cell metabolism,
- prevent enzymatic degradation of cells and tissues by autolysis (self-digestion),
- kill pathogenic microorganisms such as bacteria, fungi, and viruses, and
- harden the tissue as a result of either cross-linking or denaturing protein molecules.

Formalin, a 37% aqueous solution of formaldehyde, at various dilutions and in combination with other chemicals and buffers, is the most commonly used fixative. Formaldehyde preserves the general structure of the cell and extracellular components by reacting with the amino groups of proteins (most often cross-linked lysine residues). Because formaldehyde does not significantly alter their three-dimensional structure, proteins maintain their ability to react with specific antibodies. This property is important in immunocytochemical staining methods (see page 7). The standard commercial solution of formaldehyde buffered with phosphates (pH 7) acts relatively slowly but penetrates the tissue well. However, because it does not react with lipids, it is a poor fixative of cell membranes.

In the second step, the specimen is prepared for embedding in paraffin to permit sectioning.

Preparing a specimen for examination requires its infiltration with an **embedding medium** that allows it to be thinly sliced, typically in the range of 5 to 15 μm (1 micrometer [μm] equals 1/1,000 of a millimeter [mm]; see Table 1.1). The specimen is **washed** after fixation and **dehydrated** in a series of alcohol solutions of ascending concentration as high as 100% alcohol to remove water. In the next step, **clearing**, organic solvents such as xylol or toluol, which are miscible in both alcohol and **paraffin**, are used to remove the alcohol before infiltration of the specimen with melted paraffin.

When the melted paraffin is cool and hardened, it is trimmed into an appropriately sized block. The block is then mounted in a specially designed slicing machine—a **microtome**—and cut with a steel knife. The resulting sections are then mounted on glass slides using **mounting medium** (pinene or acrylic resins) as an adhesive.

TABLE 1.1 Commonly Used Linear Equivalents

1 picometer	=	0.01 angstrom (Å)
1 angstrom	=	0.1 nanometer (nm)
10 angstroms	=	1.0 nanometer
1 nanometer	=	1,000 picometers (pm)
1,000 nanometers	=	1.0 micrometer (μm)
1,000 micrometers	=	1.0 millimeter (mm)

In the third step, the specimen is stained to permit examination.

Because paraffin sections are colorless, the specimen is not yet suitable for light microscopic examination. To color or stain the tissue sections, the paraffin must be dissolved out, again with xylol or toluol, and the slide must then be rehydrated through a series of solutions of descending alcohol concentration. The tissue on the slides is then stained with **hematoxylin** in water. Because the counterstain, **eosin**, is more soluble in alcohol than in water, the specimen is again dehydrated through a series of alcohol solutions of ascending concentration and stained with eosin in alcohol. Figure 1.1 shows the results of staining with hematoxylin alone, eosin alone, and hematoxylin with counterstain eosin. After staining, the specimen is then passed through xylol or toluol to a nonaqueous mounting medium and covered with a coverslip to obtain a permanent preparation.

Other Fixatives

Formalin does not preserve all cell and tissue components.

Although H&E–stained sections of formalin-fixed specimens are convenient to use because they adequately display general structural features, they cannot elucidate the specific chemical composition of cell components. Also, many components are lost in the preparation of the specimen. To retain these components and structures, other fixation methods must be used. These methods are generally based on a clear understanding of the chemistry involved. For instance, the use of alcohols and organic solvents in routine preparations removes neutral lipids.

To retain neutral lipids, such as those in adipose cells, frozen sections of formalin-fixed tissue and dyes that dissolve in fats must be used; to retain membrane structures,

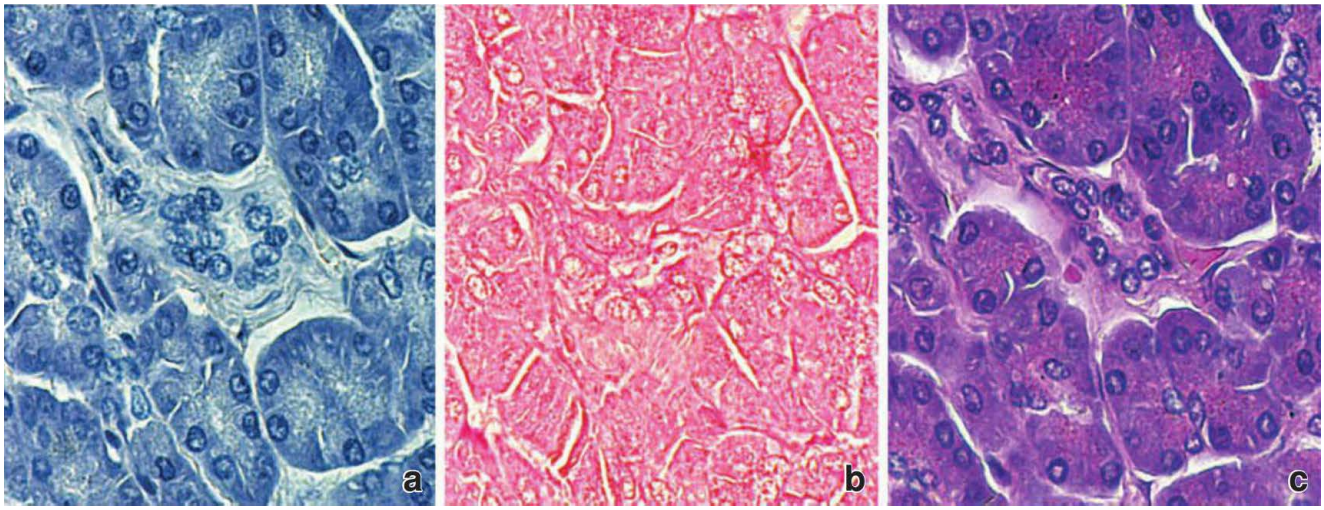

FIGURE 1.1 ▲ Hematoxylin and eosin (H&E) staining. This series of specimens from the pancreas are serial (adjacent) sections that demonstrate the effect of hematoxylin and eosin used alone and hematoxylin and eosin used in combination. **a.** This photomicrograph reveals the staining with hematoxylin only. Although there is a general overall staining of the specimen, those components and structures that have a high affinity for the dye are most heavily stained—for example, the nuclear DNA and areas of the cell containing cytoplasmic RNA. **b.** In this photomicrograph, eosin, the counterstain, likewise has an overall staining effect when used alone. Note, however, that the nuclei are less conspicuous than in the specimen stained with hematoxylin alone. After the specimen is stained with hematoxylin and then prepared for staining with eosin in alcohol solution, the hematoxylin that is not tightly bound is lost, and the eosin then stains those components to which it has a high affinity. **c.** This photomicrograph reveals the combined staining effect of H&E. ×480.

special fixatives containing heavy metals that bind to the phospholipids, such as permanganate and osmium, are used (Folder 1.1). The routine use of **osmium tetroxide** as a fixative for electron microscopy is the primary reason for the excellent preservation of membranes in electron micrographs.

Other Staining Procedures

Hematoxylin and eosin are used in histology primarily to display structural features.

Despite the merits of H&E staining, the procedure does not adequately reveal certain structural components of histologic sections such as elastic material, reticular fibers, basement membranes, and lipids. When it is desirable to display these components, other staining procedures, most of them selective, can be used. These procedures include the use of orcein and resorcin-fuchsin for elastic material and silver impregnation for reticular fibers and basement membrane material. Although the chemical bases of many staining methods are not always understood, they work. Knowing the components that a procedure reveals is more important than knowing precisely how the procedure works.

HISTOCHEMISTRY AND CYTOCHEMISTRY

Specific chemical procedures can provide information about the function of cells and the extracellular components of tissues.

Histochemical and cytochemical procedures may be based on **specific binding** of a dye, use of a **fluorescent dye–labeled antibody** with a particular cell component, or the **inherent enzymatic activity** of a cell component. In addition, many large molecules found in cells can be localized by the process of **autoradiography**, in which radioactively tagged precursors of the molecule are incorporated by cells and tissues before fixation. Many of these procedures can be used with both light microscopic and electron microscopic preparations.

Before discussing the chemistry of routine staining and histochemical and cytochemical methods, it is useful to examine briefly the nature of a routinely fixed and embedded section of a specimen.

Chemical Composition of Histologic Samples

The chemical composition of a tissue ready for routine staining differs from living tissue.

The components that remain after fixation consist mostly of large molecules that do not readily dissolve, especially after treatment with the fixative. These large molecules, particularly those that react with other large molecules to form macromolecular complexes, are usually preserved in a tissue section. Examples of such large macromolecular complexes include:

- **nucleoproteins** formed from nucleic acids bound to protein,
- **intracellular cytoskeletal proteins** complexed with associated proteins,
- **extracellular proteins** in large insoluble aggregates, bound to similar molecules by cross-linking of neighboring molecules, as in collagen fiber formation, and
- **membrane phospholipid–protein (or carbohydrate) complexes**.

These molecules constitute the structure of cells and tissues—that is, they make up the formed elements of the tissue. They are the basis for the organization that is seen in tissue with the microscope.

FOLDER 1.1 Clinical Correlation: Frozen Sections

Sometimes, the pathologist may be asked to immediately evaluate tissue obtained during surgery, especially when instant pathologic diagnosis may determine how the surgery will proceed. There are several indications to perform such an evaluation, routinely known as a ***frozen section***. Most commonly, a surgeon in the operating room requests a frozen section when no preoperative diagnosis was available or when unexpected intraoperative findings must be identified. In addition, the surgeon may want to know whether all of a pathologic mass within the healthy tissue limit has been removed and whether the margin of the surgical resection is free of diseased tissue. Frozen sections are also done in combination with other procedures such as endoscopy or thin-needle biopsy to confirm whether the obtained biopsy material will be usable in further pathologic examinations.

Three main steps are involved in frozen section preparation:

- **Freezing the tissue sample**. Small tissue samples are frozen either by using compressed carbon dioxide or by immersion in a cold fluid (isopentane) at a temperature of −50°C. Freezing can be achieved in a special high-efficiency refrigerator. Freezing makes the tissue solid and allows sectioning with a microtome.
- **Sectioning the frozen tissue**. Sectioning is usually performed inside a cryostat, a refrigerated compartment containing a microtome. Because the tissue is frozen solid, it can be cut into extremely thin (5 to 10 μm) sections. The sections are then mounted on glass slides.
- **Staining the cut sections**. Staining is done to differentiate cell nuclei from the rest of the tissue. The most common stains used for frozen sections are H&E, methylene blue (Fig. F1.1.1), and PAS stains.

The entire process of preparation and evaluation of frozen sections may take as little as 10 minutes to complete. The total time to obtain results largely depends on the transport time of the tissue from the operating room to the pathology laboratory, on the pathologic technique used, and the experience of the pathologist. The findings are then directly communicated to the surgeon waiting in the operating room.

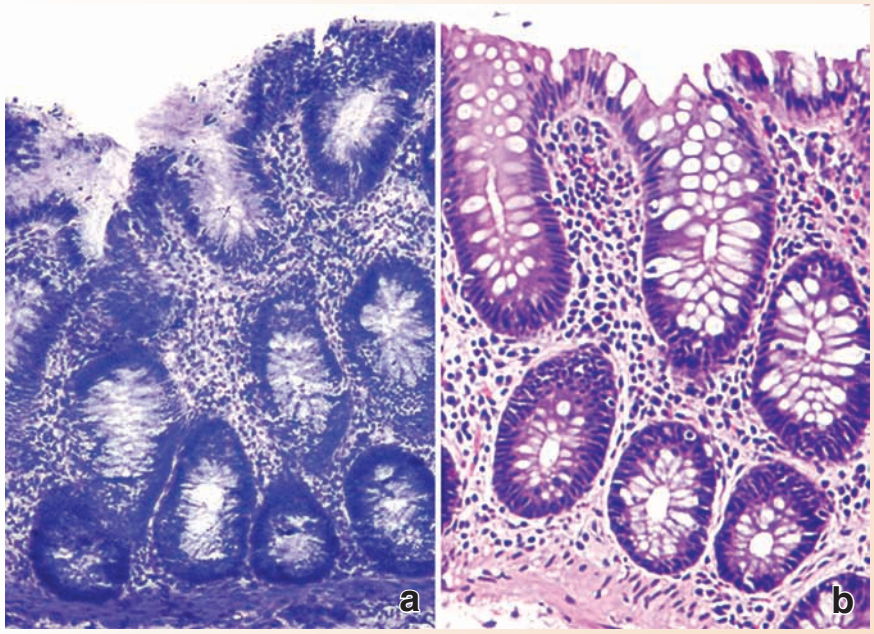

FIGURE F1.1.1 ▲ Evaluation of a specimen obtained during surgery by frozen-section technique. a. This photomicrograph shows a specimen obtained from the large intestine that was prepared by frozen-section technique and stained with methylene blue. ×160. **b.** Part of the specimen was fixed in formalin and processed as a routine H&E preparation. Examination of the frozen section revealed it to be normal. This diagnosis was later confirmed by examining the routinely prepared H&E specimen. ×180. (Courtesy of Dr. Daniel W. Visscher.)

In many cases, a structural element is also a functional unit. For example, in the case of proteins that make up the contractile filaments of muscle cells, the filaments are the visible structural components and the actual participants in the contractile process. The RNA of the cytoplasm is visualized as part of a structural component (e.g., ergastoplasm of secretory cells, Nissl bodies of nerve cells) and is also the actual participant in the synthesis of protein.

Many tissue components are lost during the routine preparation of H&E–stained sections.

Despite the fact that nucleic acids, proteins, and phospholipids are mostly retained in tissue sections, many are also lost. Small proteins and small nucleic acids, such as transfer RNA, are generally lost during the preparation of the tissue. As previously described, neutral lipids are usually dissolved by the organic solvents used in tissue preparation. Other large molecules also may be lost, for example, by being hydrolyzed because of the unfavorable pH of the fixative solutions. Examples of large molecules lost during routine fixation in aqueous fixatives are:

- **glycogen** (an intracellular storage carbohydrate common in liver and muscle cells), and
- **proteoglycans** and **glycosaminoglycans** (extracellular complex carbohydrates found in connective tissue).

These molecules can be preserved, however, by using a nonaqueous fixative for glycogen or by adding specific binding agents to the fixative solution that preserve extracellular carbohydrate-containing molecules.

Soluble components, ions, and small molecules are also lost during the preparation of paraffin sections.

Intermediary metabolites, glucose, sodium, chloride, and similar substances are lost during preparation of routine H&E paraffin sections. Many of these substances can be studied in special preparations, sometimes with considerable loss of structural integrity. These small soluble ions and molecules do not make up the formed elements of a tissue; they participate in synthetic processes or cellular reactions. When they can be preserved and demonstrated by specific methods, they provide invaluable information about cell metabolism, active transport, and other vital cellular processes. Water, a highly versatile molecule, participates in these reactions and processes and contributes to the stabilization of macromolecular structure through hydrogen bonding.

Chemical Basis of Staining

Acidic and Basic Dyes

Hematoxylin and eosin (H&E) are the most commonly used dyes in histology.

An **acidic dye**, such as **eosin**, carries a *net negative charge* on its colored portion and is described by the general formula [Na^+dye$^-$].

A **basic dye** carries a *net positive charge* on its colored portion and is described by the general formula [dye$^+Cl^-$].

Hematoxylin does not meet the definition of a strict basic dye but has properties that closely resemble those of a basic dye. The color of a dye is not related to whether it is basic or acidic, as can be noted by the examples of basic and acidic dyes listed in Table 1.2.

Basic dyes react with anionic components of cells and tissue (components that carry a net negative charge).

Anionic components include the phosphate groups of nucleic acids, the sulfate groups of glycosaminoglycans, and the carboxyl groups of proteins. The ability of such anionic groups to react with a basic dye is called **basophilia** *[Gr., base-loving]*. Tissue components that stain with hematoxylin also exhibit basophilia.

The reaction of the anionic groups varies with pH. Thus:

- At a *high pH* (about 10), all three groups are ionized and available for reaction by electrostatic linkages with the basic dye.
- At a *slightly acidic to neutral pH* (5 to 7), sulfate and phosphate groups are ionized and available for reaction with the basic dye by electrostatic linkages.
- At a *low pH* (below 4), only sulfate groups remain ionized and react with basic dyes.

Therefore, staining with basic dyes at a specific pH can be used to focus on specific anionic groups; because the specific anionic groups are found predominantly on certain macromolecules, the staining serves as an indicator of these macromolecules.

As mentioned, **hematoxylin** is not, strictly speaking, a basic dye. It is used with a **mordant** (i.e., an intermediate link between the tissue component and the dye). The mordant causes the stain to resemble a basic dye. The linkage in the **tissue–mordant–hematoxylin complex** is not a simple electrostatic linkage; when sections are placed in water, hematoxylin does not dissociate from the tissue. Hematoxylin lends itself to those staining sequences in which it is followed by aqueous solutions of acidic dyes. True basic dyes, as distinguished from hematoxylin, are not generally used in sequences in which the basic dye is followed by an acidic dye. The basic dye then tends to dissociate from the tissue during the aqueous solution washes between the two dye solutions.

Acidic dyes react with cationic groups in cells and tissues, particularly with the ionized amino groups of proteins.

The reaction of **cationic groups** with an acidic dye is called **acidophilia** *[Gr., acid-loving]*. Reactions of cell and tissue components with acidic dyes are neither as specific nor as precise as reactions with basic dyes.

Although electrostatic linkage is the major factor in the primary binding of an acidic dye to the tissue, it is not the only one; because of this, acidic dyes are sometimes used in combinations to color different tissue constituents selectively. For example, three acidic dyes are used in the **Mallory staining technique**: aniline blue, acid fuchsin, and orange G. These dyes selectively stain collagen, ordinary cytoplasm, and red blood cells, respectively. Acid fuchsin also stains nuclei.

In other multiple acidic dye techniques, hematoxylin is used to stain nuclei first, and then acidic dyes are used to stain cytoplasm and extracellular fibers selectively. The selective staining of tissue components by acidic dyes is attributable to relative factors such as the size and degree of aggregation of the dye molecules and the permeability and "compactness" of the tissue.

Basic dyes can also be used in combination or sequentially (e.g., methyl green and pyronin to study protein synthesis and secretion), but these combinations are not as widely used as acidic dye combinations.

TABLE 1.2 Some Basic and Acidic Dyes

Dye	Color
Basic Dyes	
Methyl green	Green
Methylene blue	Blue
Pyronin G	Red
Toluidine blue	Blue
Acidic Dyes	
Acid fuchsin	Red
Aniline blue	Blue
Eosin	Red
Orange G	Orange

A limited number of substances within cells and the extracellular matrix display basophilia.

These substances include:

- **heterochromatin** and **nucleoli** of the nucleus (chiefly because of ionized phosphate groups in nucleic acids of both),
- **cytoplasmic components** such as the ergastoplasm (also because of ionized phosphate groups in ribosomal RNA), and
- **extracellular materials** such as the complex carbohydrates of the matrix of cartilage (because of ionized sulfate groups).

Staining with acidic dyes is less specific, but more substances within cells and the extracellular matrix exhibit acidophilia.

These substances include:

- most **cytoplasmic filaments**, especially those of muscle cells,
- most **intracellular membranous components** and much of the otherwise unspecialized cytoplasm, and
- most **extracellular fibers** (primarily because of ionized amino groups).

Metachromasia

Certain basic dyes react with tissue components that shift their normal color from blue to red or purple; this absorbance change is called *metachromasia*.

The underlying mechanism for **metachromasia** is the presence of **polyanions** within the tissue. When these tissues are stained with a concentrated basic dye solution, such as **toluidine blue**, the dye molecules are close enough to form dimeric and polymeric aggregates. The absorption properties of these aggregations differ from those of the individual nonaggregated dye molecules.

Cell and tissue structures that have high concentrations of ionized sulfate and phosphate groups—such as the ground substance of cartilage, heparin-containing granules of mast cells, and rough endoplasmic reticulum of plasma cells—exhibit metachromasia. Therefore, toluidine blue will appear purple to red when it stains these components.

Aldehyde Groups and the Schiff Reagent

The ability of bleached basic fuchsin (Schiff reagent) to react with aldehyde groups results in a distinctive red color and is the basis of the periodic acid–Schiff and Feulgen reactions.

The **periodic acid–Schiff (PAS) reaction** stains carbohydrates and carbohydrate-rich macromolecules. It is used to demonstrate glycogen in cells, mucus in various cells and tissues, the basement membrane that underlies epithelia, and reticular fibers in connective tissue. The Schiff reagent is also used in **Feulgen stain**, which relies on a mild hydrochloric acid hydrolysis to stain DNA.

The PAS reaction is based on the following facts:

- Hexose rings of carbohydrates contain adjacent carbons, each of which bears a hydroxyl (−OH) group.

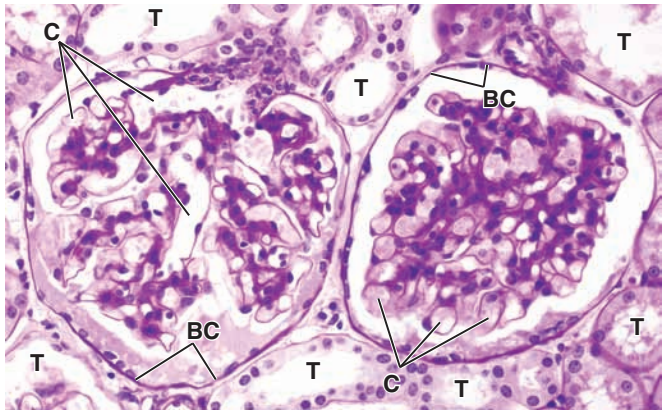

FIGURE 1.2 ▲ Photomicrograph of kidney tissue stained by the PAS method. This histochemical method demonstrates and localizes carbohydrates and carbohydrate-rich macromolecules. The basement membranes are PAS-positive as evidenced by the magenta staining of these sites. The kidney tubules (*T*) are sharply delineated by the stained basement membrane surrounding the tubules. The glomerular capillaries (*C*) and the epithelium of Bowman's capsule (*BC*) also show PAS-positive basement membranes. The specimen was counterstained with hematoxylin to visualize cell nuclei. ×320.

- Hexosamines of glycosaminoglycans contain adjacent carbons, one of which bears an −OH group, whereas the other bears an amino ($-NH_2$) group.
- Periodic acid cleaves the bond between these adjacent carbon atoms and forms aldehyde groups.
- These aldehyde groups react with the Schiff reagent to give a distinctive magenta color.

The PAS staining of basement membrane (Fig. 1.2) and reticular fibers is based on the content or association of proteoglycans (complex carbohydrates associated with a protein core). PAS staining is an alternative to silver-impregnation methods, which are also based on reaction with the sugar molecules in the proteoglycans.

The Feulgen reaction is based on the cleavage of purines from the deoxyribose of DNA by mild acid hydrolysis; the sugar ring then opens with the formation of aldehyde groups. Again, the newly formed aldehyde groups react with the Schiff reagent to give the distinctive magenta color. The reaction of the Schiff reagent with DNA is **stoichiometric**, meaning that the product of this reaction is measurable and proportional to the amount of DNA. It can be used, therefore, in spectrophotometric methods to quantify the amount of DNA in the nucleus of a cell. RNA does not stain with the Schiff reagent because it lacks deoxyribose.

Enzyme Digestion

Enzyme digestion of a section adjacent to one stained for a specific component—such as glycogen, DNA, or RNA—can be used to confirm the identity of the stained material.

Intracellular material that stains with the PAS reaction may be identified as glycogen by pretreatment of sections with diastase or amylase. Abolition of the staining after these treatments positively identifies the stained material as glycogen.

FOLDER 1.2 Functional Considerations: Feulgen Microspectrophotometry

Feulgen microspectrophotometry is a technique developed to study DNA increases in developing cells and to analyze *ploidy*—that is, the number of times the normal DNA content of a cell is multiplied (a normal, nondividing cell is said to be *diploid*; a sperm or egg cell is *haploid*). Two techniques, **static cytometry** for tissue sections and **flow cytometry** for isolated cells, are used to quantify the amount of nuclear DNA. The technique of static cytometry of Feulgen-stained sections of tumors uses microspectrophotometry coupled with a digitizing imaging system to measure the absorption of light emitted by cells and cell clusters at 560-nm wavelength. In contrast, the flow cytometry technique uses instrumentation able to scan only single cells flowing past a sensor in a liquid medium. This technique provides rapid, quantitative analysis of a single cell based on the measurement of fluorescent light emission. Currently, Feulgen microspectrophotometry is used to study changes in the DNA content in dividing cells undergoing differentiation. It is also used clinically to analyze abnormal chromosomal number (i.e., ploidy patterns) in malignant cells. Some malignant cells that have a largely diploid pattern are said to be well differentiated; tumors with these types of cells have a better prognosis than tumors with *aneuploid* (nonintegral multiples of the haploid amount of DNA) and tetraploid cells. Feulgen microspectrophotometry has been particularly useful in studies of specific adenocarcinomas (epithelial cancers), breast cancer, kidney cancer, colon and other gastrointestinal cancers, endometrial (uterine epithelium) cancer, and ovarian cancer. It is one of the most valuable tools for pathologists in evaluating the metastatic potential of these tumors and in making prognostic and treatment decisions.

Similarly, pretreatment of tissue sections with deoxyribonuclease (DNAse) will abolish the Feulgen staining in those sections, and treatment of sections of protein secretory epithelia with ribonuclease (RNAse) will abolish the staining of the ergastoplasm with basic dyes.

Enzyme Histochemistry

Histochemical methods are also used to identify and localize enzymes in cells and tissues.

To localize enzymes in tissue sections, special care must be taken in fixation to preserve the enzyme activity. Usually, mild aldehyde fixation is the preferred method. In these procedures, the reaction product of the enzyme activity, rather than the enzyme itself, is visualized. In general, a **capture reagent**, either a dye or a heavy metal, is used to trap or bind the reaction product of the enzyme by precipitation at the site of reaction. In a typical reaction to display a hydrolytic enzyme, the tissue section is placed in a solution containing a substrate (AB) and a trapping agent (T) that precipitates one of the products as follows:

$$AB + T \xrightarrow{\text{enzyme}} AT + B$$

where AT is the trapped end product and B is the hydrolyzed substrate.

By using such methods, the lysosome, first identified in differential centrifugation studies of cells, was equated with a vacuolar component seen in electron micrographs. In lightly fixed tissues, the acid hydrolases and esterases contained in lysosomes react with an appropriate substrate. The reaction mixture also contains lead ions to precipitate (e.g., lead phosphate derived from the action of acid phosphatase). The precipitated reaction product can then be observed with both light and electron microscopy. Similar histochemical procedures have been developed to demonstrate alkaline phosphatase, adenosine triphosphatases (ATPases) of many varieties (including the Na^+/K^+ ATPase that is the enzymatic basis of the sodium pump in cells and tissues), various esterases, and many respiratory enzymes (Fig. 1.3a).

One of the most common histochemical methods (often used in conjunction with immunocytochemistry) employs horseradish peroxidase for enzyme-mediated antigen detection. A widely used substrate for horseradish peroxidase is the 3,3′-diaminobenzidine (DAB), a colorless organic compound that produces a brown insoluble product at the site of enzymatic reaction (Fig. 1.3b). The product of this enzymatic reaction can be easily localized in cells, yielding high-resolution images in both light and electron microscopy.

Immunocytochemistry

The specificity of a reaction between an antigen and an antibody is the underlying basis of immunocytochemistry.

Antibodies, also known as **immunoglobulins**, are glycoproteins that are produced by specific cells of the immune system in response to a foreign protein, or **antigen**. In the laboratory, antibodies can be purified from the blood and conjugated (attached) to a fluorescent dye. In general, **fluorescent dyes (fluorochromes)** are chemicals that absorb light of different wavelengths (e.g., ultraviolet light) and then emit visible light of a specific wavelength (e.g., green, yellow, red). **Fluorescein**, the most commonly used dye, absorbs ultraviolet light and emits green light. Antibodies conjugated with fluorescein can be applied to sections of lightly fixed or frozen tissues on glass slides to localize an antigen in cells and tissues. The reaction of antibody with antigen can then be examined and photographed with a fluorescence microscope or confocal microscope that produces a three-dimensional reconstruction of the examined tissue (Fig. 1.4).

Two types of antibodies are used in immunocytochemistry: polyclonal antibodies that are produced by immunized animals and monoclonal antibodies that are produced by immortalized (continuously replicating) antibody-producing cell lines.

In a typical procedure, a specific protein, such as actin, is isolated from a muscle cell of one species, such as a rat,

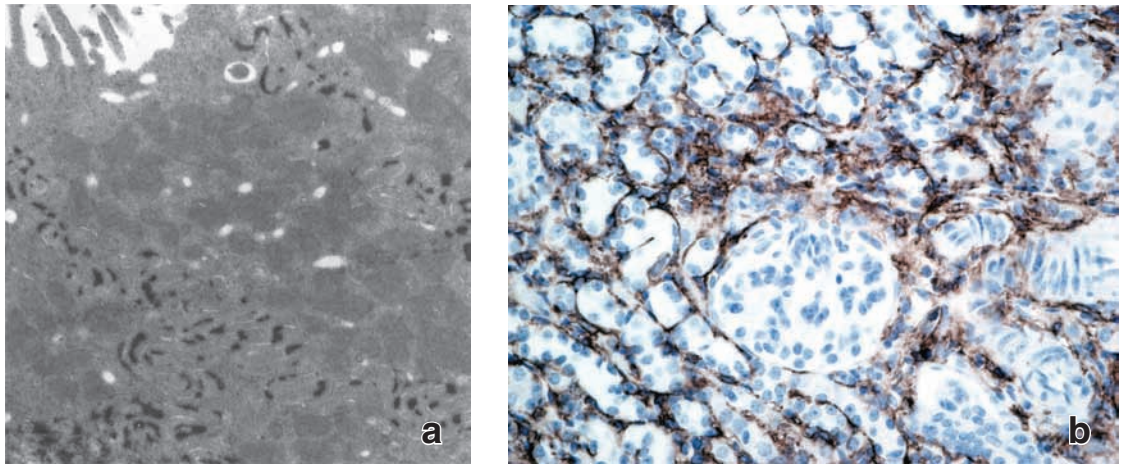

FIGURE 1.3 ▲ Electron and light microscopic histochemical procedures. a. This electron micrograph shows localization of membrane ATPase in epithelial cells of rabbit gallbladder. *Dark areas* visible on the electron micrograph show the location of the enzyme ATPase. This enzyme is detected in the plasma membrane at the lateral domains of epithelial cells, which correspond to the location of sodium pumps. These epithelial cells are involved in active transport of molecules across the plasma membrane. ×26,000. **b.** This photomicrograph shows macrophages stained with a histochemical method using peroxidase-labeled antibodies and DAB reagent. A paraffin-embedded section of mouse kidney with renal vascular hypertension disease was stained for presence of F4/80+ specific marker protein expressed only on the surface of macrophages. Initially, sections were exposed to primary rat anti-mouse F4/80+ antibodies followed by incubation with secondary goat anti-rat IgG antibodies labeled with horseradish peroxidase. The specimen was washed and treated with a buffer containing DAB. A brown precipitate (product of DAB oxidation by horseradish peroxidase) is localized in the areas where macrophages are present. The specimen was counterstained with hematoxylin to visualize cell nuclei. ×400. (Courtesy of Dr. Joseph P. Grande.)

and injected into the circulation of another species, such as a rabbit. In the immunized rabbit, the rat's actin molecules are recognized by the rabbit immune system as a foreign antigen. This recognition triggers a cascade of immunologic reactions involving multiple groups (clones) of immune cells called **B lymphocytes**. The cloning of B lymphocytes eventually leads to the production of anti-actin antibodies. Collectively, these **polyclonal antibodies** represent mixtures of different antibodies produced by many clones of B lymphocytes that each recognize different regions of the actin molecule. The antibodies are then removed from the blood, purified, and conjugated with a fluorescent dye. They can now be used to locate actin molecules in rat tissues or cells. If actin is present in a cell or tissue, such as a fibroblast in connective tissue, then the fluorescein-labeled antibody binds to it and the reaction is visualized by fluorescence microscopy.

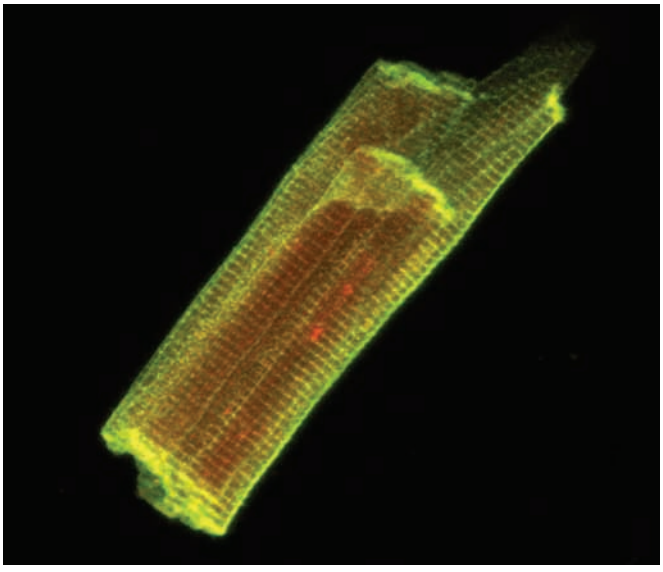

FIGURE 1.4 ▲ Confocal microscopy image of a rat cardiac muscle cell. This image was obtained from the confocal microscope using the indirect immunofluorescence method. Two primary antibodies were used. The first primary antibody recognizes a specific lactate transporter (MCT1) and is detected with a secondary antibody conjugated with rhodamine (*red*). The second primary antibody is directed against the transmembrane protein CD147, which is tightly associated with MCT1. This antibody was detected by a secondary antibody labeled with fluorescein (*green*). The *yellow* color is visible at the point at which the two labeled secondary antibodies exactly co-localize within the cardiac muscle cell. This three-dimensional image shows that both proteins are distributed on the surface of the muscle cell, whereas the lactate transporter alone is visible deep to the plasma membrane. (Courtesy of Drs. Andrew P. Halestrap and Catherine Heddle.)

Monoclonal antibodies (Folder 1.3) are those produced by an **antibody-producing cell line** consisting of a single group (clone) of identical B lymphocytes. The single clone that becomes a cell line is obtained from an individual with **multiple myeloma**, a tumor derived from a single antibody-producing plasma cell. Individuals with multiple myelomas produce a large population of identical, homogeneous antibodies with an identical specificity against an antigen. To produce monoclonal antibodies against a specific antigen, a mouse or rat is immunized with that antigen. The activated B lymphocytes are then isolated from the lymphatic tissue (spleen or lymph nodes) of the animal and fused with the myeloma cell line. This fusion produces a **hybridoma**, an immortalized individual antibody-secreting cell line. To obtain monoclonal antibodies against rat actin molecules, for example, the B lymphocytes from the lymphatic organs of immunized rabbits must be fused with myeloma cells.

Both direct and indirect immunocytochemical methods are used to locate a target antigen in cells and tissues.

The oldest immunocytochemistry technique used for identifying the distribution of an antigen within cells and tissues is known as **direct immunofluorescence**. This technique uses a fluorochrome-labeled **primary antibody**

FOLDER 1.3 Clinical Correlation: Monoclonal Antibodies in Medicine

Monoclonal antibodies are now widely used in immunocytochemical techniques and also have many clinical applications. Monoclonal antibodies conjugated with radioactive compounds are used to detect and diagnose tumor metastasis in pathology, differentiate subtypes of tumors and stages of their differentiation, and in infectious disease diagnosis to identify microorganisms in blood and tissue fluids. In recent clinical studies, monoclonal antibodies conjugated with immunotoxins, chemotherapy agents, or radioisotopes have been used to deliver therapeutic agents to specific tumor cells in the body.

(either polyclonal or monoclonal) that reacts with the antigen within the sample (Fig. 1.5a). As a one-step procedure, this method involves only a single labeled antibody. Visualization of structures is not ideal because of the low intensity of the signal emission. Direct immunofluorescence methods are now being replaced by the indirect method because of suboptimal sensitivity.

Indirect immunofluorescence provides much greater sensitivity than direct methods and is often referred to as the "sandwich" or "double-layer technique." Instead of conjugating a fluorochrome with a specific (primary) antibody directed against the antigen of interest (e.g., a rat actin molecule), the fluorochrome is conjugated with a **secondary antibody** directed against rat primary antibody (i.e., goat anti-rat antibody; Fig. 1.5b). Therefore, when the fluorescein is conjugated directly with the specific primary antibody, the method is direct; when fluorescein is conjugated with a secondary antibody, the method is indirect. The indirect method considerably enhances the fluorescence signal emission from the tissue. An additional advantage of the indirect labeling method is that a single secondary antibody can be used to localize the tissue-specific binding of several different primary antibodies (Fig. 1.6). For microscopic studies, the secondary antibody can be conjugated with different fluorescent dyes so that multiple labels can be shown in the same tissue section (see Fig. 1.4). Drawbacks of indirect immunofluorescence are that it is expensive, labor intensive, and not easily adapted to automated procedures.

It is also possible to conjugate polyclonal or monoclonal antibodies with other substances, such as enzymes (e.g., horseradish peroxidase), that convert colorless substrates (e.g., DAB) into an insoluble product of a specific color that precipitates at the site of the enzymatic reaction. The staining that results from this **immunoperoxidase method** can be observed in the light microscope (see Fig. 1.3b) with either direct or indirect immunocytochemical methods. In another variation, colloidal gold or ferritin (an iron-containing molecule) can be attached to the antibody molecule. These electron-dense markers can be visualized directly with the electron microscope.

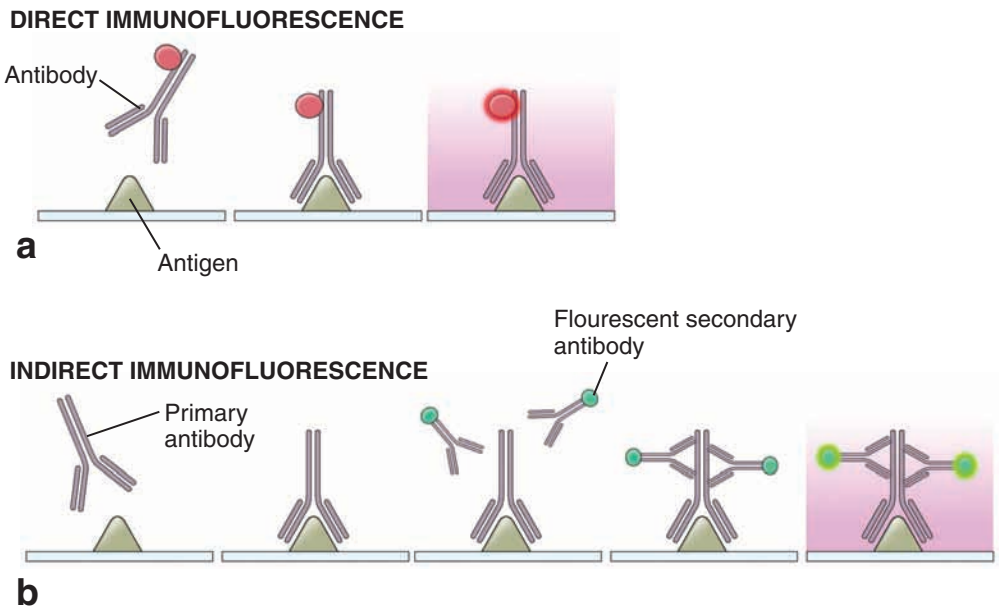

FIGURE 1.5 ▲ Direct and indirect immunofluorescence. a. In direct immunofluorescence, a fluorochrome-labeled primary antibody reacts with a specific antigen within the tissue sample. Labeled structures are then observed in the fluorescence microscope in which an excitation wavelength (usually ultraviolet light) triggers the emission of another wavelength. The length of this wavelength depends on the nature of the fluorochrome used for antibody labeling. **b.** The indirect method involves two processes. First, the specific primary antibodies react with the antigen of interest. Second, the secondary antibodies, which are fluorochrome labeled, react with the primary antibodies. The visualization of labeled structures within the tissue is the same in both methods and requires the fluorescence microscope.