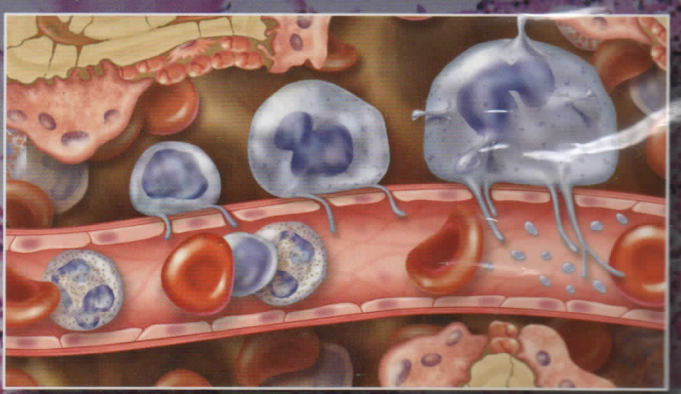
JUNQUEIRA'S Basic Histology TEXT & ATLAS

INTERNATIONAL EDITION

ANTHONY L. MESCHER



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Junqueira's Basic Histology

TEXT AND ATLAS

Anthony L. Mescher, PhD

Emeritus Professor of Anatomy and Cell Biology
Indiana University School of Medicine
Bloomington, Indiana

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CHAPTER 1

Histology & Its Methods of Study

Histology explores the body's tissues and how these are arranged to produce functional organs. The subject involves all aspects of tissue biology, from cellular biology to microscopic anatomy, focusing on how cell activities, shapes, and groupings are optimized for specific organ functions.

Tissues have two interacting components: cells and extracellular matrix (ECM). The ECM involves many kinds of macromolecules, most of which form complex structures, such as collagen fibrils. The ECM supports the cells and contains the fluid transporting nutrients to the cells and carrying away their wastes and secretory products. Cells produce the ECM locally and are in turn strongly influenced by matrix molecules. Many matrix components bind to specific cell surface receptors that span the cell membranes and connect to structural components inside the cells, forming a continuum in which cells and the ECM function together in a well-coordinated manner.

During embryonic development, cells and their associated matrix become functionally specialized and give rise to fundamental types of tissues with characteristic structural features. Organs form by an orderly combination of these tissues, and their precise arrangement allows the functioning of each organ and of the organism.

The small size of cells and matrix components makes histology dependent on the use of microscopes and molecular methods of study. Advances in biochemistry, molecular biology, physiology, immunology, and pathology are essential for a better knowledge of tissue biology. Familiarity with the tools and methods of any branch of science is essential for a proper understanding of the subject. This chapter reviews common methods used to study cells and tissues, focusing on microscopic approaches.

PREPARATION OF TISSUES FOR STUDY

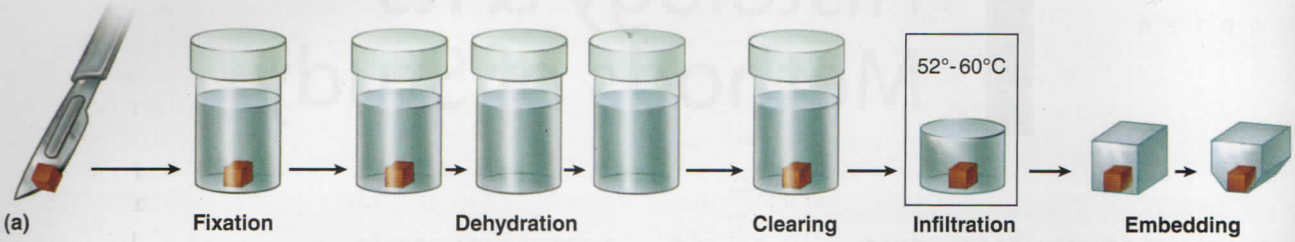
The most common procedure used in histologic research is the preparation of tissue slices or "sections" that can be examined visually with transmitted light. Because most tissues and organs are too thick for light to pass through, thin translucent sections are cut from them and placed on glass slides for microscopic examination of their internal structures.

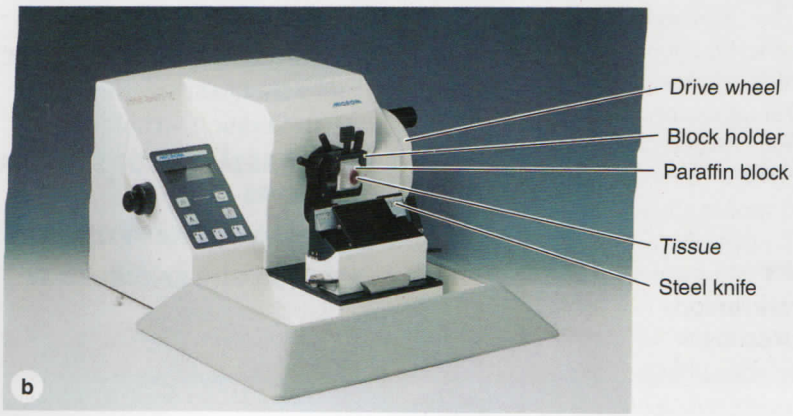
The ideal microscopic preparation is preserved so that the tissue on the slide has the same structural features it had in the body. However, this is often not feasible because the preparation process can remove cellular lipid, with slight distortions of cell structure. Figure 1–1 summarizes the initial steps used in tissue preparation for light microscopy.

Fixation

To preserve tissue structure and prevent degradation by enzymes released from the cells or microorganisms, pieces of

FIGURE 1–1 Sectioning fixed and embedded tissue.





Most tissues studied histologically are prepared as shown, with this sequence of steps **(a)**:

- **Fixation:** Small pieces of tissue are placed in chemical solutions that preserve cell and tissue structure by cross-linking proteins and inactivating degradative enzymes.
- **Dehydration:** The tissue is transferred through a series of increasingly concentrated alcohol solutions, ending in 100%, which removes all water.
- **Clearing:** Alcohol is removed in organic solvents in which both alcohol and paraffin are miscible.
- **Infiltration:** The tissue is then placed in melted paraffin until it becomes completely infiltrated with this substance.
- **Embedding:** The paraffin-infiltrated tissue is placed in a small mold with melted paraffin and allowed to harden.
- **Trimming:** The resulting paraffin block is trimmed to expose the tissue for sectioning (slicing) on a microtome.

Similar steps are used in preparing tissue for transmission electron microscopy (TEM), except special fixatives and dehydrating solutions are used with smaller tissue samples and embedding involves epoxy resins which become harder than paraffin to allow very thin sectioning.

(b) A **microtome** is used for sectioning paraffin-embedded tissues for light microscopy. The trimmed tissue specimen is mounted in the paraffin block holder, and each turn of the drive wheel by the histologist advances the holder a controlled distance, generally from 1 to 10 µm. After each forward move, the tissue block passes over the steel knife edge and a section is cut at a thickness equal to the distance the block advanced. The paraffin sections are placed on glass slides and allowed to adhere, deparaffinized, and stained for light microscope study. For TEM, sections less than 1 µm thick are prepared from resin-embedded cells using an ultramicrotome with a glass or diamond knife.

organs are placed as soon as possible after removal from the body in solutions of stabilizing or cross-linking compounds called **fixatives**. Because a fixative must fully diffuse through the tissues to preserve all cells, organs are usually cut into small fragments before fixation to facilitate penetration. To improve cell preservation in large organs, fixatives are often introduced via blood vessels, with vascular perfusion allowing fixation rapidly throughout the tissues.

One widely used fixative for light microscopy is formalin, a buffered isotonic solution of 37% formaldehyde. Both this compound and glutaraldehyde, a fixative used for electron microscopy, react with the amine groups (NH_2) of proteins, preventing their degradation by common proteases. Glutaraldehyde also cross-links adjacent proteins, reinforcing cell and ECM structures.

Electron microscopy provides much greater magnification and resolution of very small cellular structures, and fixation must be done very carefully to preserve additional "ultrastructural" detail. In these studies glutaraldehyde-treated tissue is then immersed further in buffered osmium tetroxide, which preserves (and stains) cellular lipids as well as proteins.

Embedding & Sectioning

To permit thin sectioning, fixed tissues are infiltrated and embedded in a material that imparts a firm consistency. Embedding materials include paraffin, used routinely for light microscopy, and plastic resins, which are adapted for both light and electron microscopy.

Before infiltration with such media, the fixed tissue must undergo **dehydration** by having its water extracted gradually by transfers through a series of increasing ethanol solutions, ending in 100% ethanol. The ethanol is then replaced by an organic solvent miscible with both alcohol and the embedding medium, a step referred to as **clearing** because infiltration with the reagents used here gives the tissue a translucent appearance.

The fully cleared tissue is then placed in melted paraffin in an oven at 52–60°C, which evaporates the clearing solvent and promotes **infiltration** of the tissue with paraffin, and then **embedded** by allowing it to harden in a small container of paraffin at room temperature. Tissues to be embedded with plastic resin are also dehydrated in ethanol and then infiltrated with plastic solvents that harden when cross-linking polymerizers are added. Plastic embedding avoids the higher temperatures needed with paraffin, helping to avoid tissue distortion.

The hardened block with tissue and surrounding embedding medium is trimmed and placed for sectioning in an instrument called a **microtome** (Figure 1–1). Paraffin sections are typically cut at 3–10 μm thickness for light microscopy, but electron microscopy requires sections less than 1 μm thick. One micrometer (1 μm) equals 1/1000 of a millimeter (mm). Other spatial units commonly used in microscopy are the nanometer (1 nm = 0.001 μm = 10^{-6} mm) and the angstrom (1 Å = 0.1 nm or 10^{-4} μm). The sections are placed on glass slides and stained for light microscopy or on metal grids for electron-microscopic staining and examination.

›› MEDICAL APPLICATION

Biopsies are tissue samples removed during surgery or routine medical procedures. In the operating room, biopsies are fixed in vials of formalin for processing and microscopic analysis in a pathology laboratory. If results of such analyses are required before the medical procedure is completed, for example, to know whether a growth is malignant before the patient is closed, a much more rapid processing method is used. The biopsy is rapidly frozen in liquid nitrogen, preserving cell structures and at the same time making the tissue hard and ready for sectioning. A microtome called a **cryostat** in a cabinet at subfreezing temperature is used to section the block with tissue, and the frozen sections are placed on slides for rapid staining and microscopic examination by a pathologist.

Freezing of tissues is also effective in histochemical studies of very sensitive enzymes or small molecules because freezing, unlike fixation, does not inactivate most enzymes. Finally, because clearing solvents often dissolve cell lipids in fixed tissues, frozen sections are also useful when structures containing lipids are to be studied histologically.

Staining

Most cells and extracellular material are completely colorless, and to be studied microscopically, tissue sections must be stained (dyed). Methods of staining have been devised that make various tissue components not only conspicuous but also distinguishable from one another. Dyes stain material rather selectively, often behaving like acidic or basic compounds and forming electrostatic (salt) linkages with ionizable radicals of macromolecules in tissues. Cell components, such as nucleic acids with a net negative charge (anionic), have an affinity for basic dyes and are thus termed **basophilic**; cationic components, such as proteins with many ionized amino groups, stain more readily with acidic dyes and are termed **acidophilic**.

Examples of basic dyes include toluidine blue, alcian blue, and methylene blue. Hematoxylin also behaves like a basic dye, staining basophilic tissue components. The main tissue components that ionize and react with basic dyes do so because of acids in their composition (eg, DNA, RNA, and glycosaminoglycans). Acidic dyes like eosin, orange G, and acid fuchsin stain acidophilic components of tissues, such as mitochondria, secretory granules, and collagen.

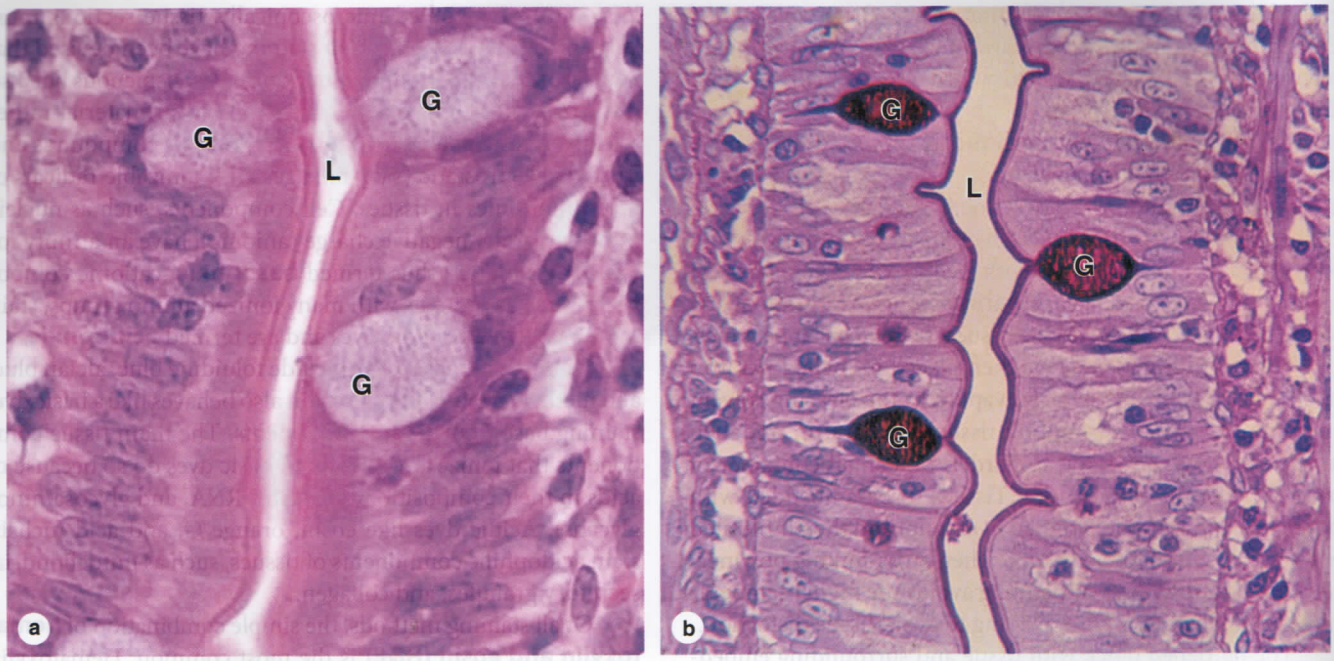
Of all staining methods, the simple combination of **hematoxylin and eosin (H&E)** is the most common. Hematoxylin stains DNA in the cell nucleus, RNA-rich portions of the cytoplasm, and the matrix of cartilage, producing a dark blue or purplish color. In contrast, eosin stains other cytoplasmic structures and collagen pink (Figure 1–2a). In H&E, eosin acts as a **counterstain**, usually a single dye applied separately to distinguish additional features of a tissue. More complex procedures, such as trichrome stains (eg, Masson trichrome), allow greater distinctions among various extracellular tissue components.

The **periodic acid–Schiff (PAS) reaction** utilizes the hexose rings of polysaccharides and other carbohydrate-rich tissue structures and stains such macromolecules distinctly purple or magenta. Figure 1–2b shows an example of cells with carbohydrate-rich areas well-stained by the PAS reaction. The DNA of cell nuclei can be specifically stained using a modification of the PAS procedure called the Feulgen reaction.

Basophilic or PAS-positive material can be further identified by enzyme digestion: pretreatment of a tissue section with an enzyme that specifically digests one substrate. For example, pretreatment with ribonuclease will greatly reduce cytoplasmic basophilia with little overall effect on the nucleus, indicating the importance of RNA for this cytoplasmic staining.

Lipid-rich structures of cells are revealed by avoiding the processing steps that remove lipids, such as treatment with heat and organic solvents, and staining with **lipid-soluble dyes** such as **Sudan black**, which can be useful in diagnosis of metabolic diseases that involve intracellular accumulations of cholesterol, phospholipids, or glycolipids. Less-common methods of staining can employ **metal impregnation** techniques, typically using solutions of silver salts to visualize certain ECM fibers and specific cellular elements in nervous tissue. The Appendix lists important staining procedures used for most of the light micrographs in this book.

FIGURE 1–2 Hematoxylin and eosin (H&E) and periodic acid–Schiff (PAS) staining.



Micrographs of epithelium lining the small intestine, **(a)** stained with H&E, and **(b)** stained with the PAS reaction for glycoproteins. With H&E, basophilic cell nuclei are stained purple, while cytoplasm stains pink. Cell regions with abundant oligosaccharides on glycoproteins, such as the ends of the cells at the lumen (**L**) or the scattered mucus-secreting goblet cells (**G**), are poorly stained by H&E. With PAS, however, cell staining is most intense at the lumen, where projecting microvilli have a prominent layer of glycoproteins at the lumen's lining (**L**), and in the mucin-rich granules of goblet cells. Cell surface glycoproteins and mucin are PAS-positive due to their high content of oligosaccharides and polysaccharides, respectively. The PAS-stained tissue was counterstained with hematoxylin to show the cell nuclei. (a. ×400; b. ×300)

Slide preparation, from tissue fixation to observation with a light microscope, may take from 12 hours to 2½ days, depending on the size of the tissue, the embedding medium, and the method of staining. The final step before microscopic observation is mounting a protective glass coverslip on the slide with clear adhesive.

LIGHT MICROSCOPY

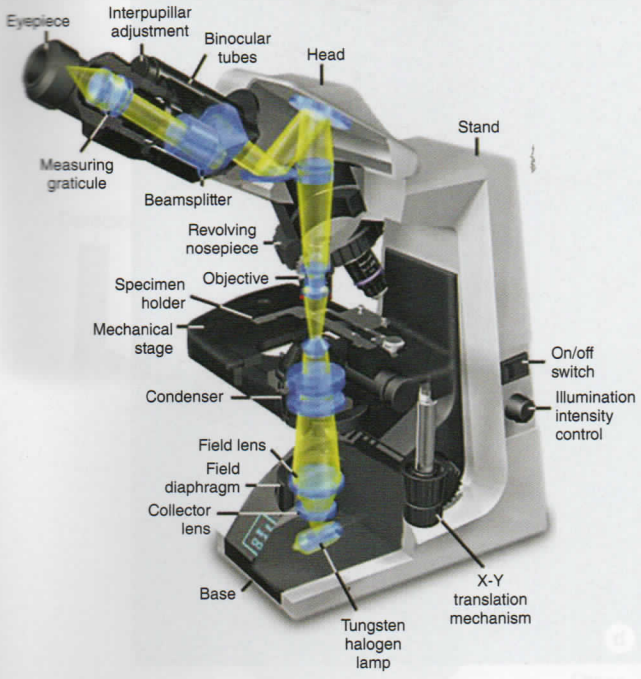
Conventional bright-field microscopy and more specialized applications like fluorescence, phase-contrast, confocal, and polarizing microscopy are all based on the interaction of light with tissue components and are used to reveal tissue features.

Bright-Field Microscopy

With the **bright-field microscope**, stained tissue is examined with ordinary light passing through the preparation. As shown in Figure 1–3, the microscope includes an optical system and mechanisms to move and focus the specimen. The optical components are the **condenser** focusing light on the object to be studied; the **objective** lens enlarging and projecting the image of the object toward the observer; and the **eyepiece** (or **ocular** lens) further magnifying this image and projecting it onto the viewer's retina or a charge-coupled device (CCD) highly sensitive to low light levels with a camera and a monitor. The total magnification is obtained by multiplying the magnifying power of the objective and ocular lenses.

The critical factor in obtaining a crisp, detailed image with a light microscope is its **resolving power**, defined as the smallest distance between two structures at which they can be seen as separate objects. The maximal resolving power of the light microscope is approximately 0.2 μm, which can permit clear images magnified 1000–1500 times. Objects smaller or thinner than 0.2 μm (such as a single ribosome or cytoplasmic microfilament) cannot be distinguished with this instrument. Likewise, two structures such as mitochondria will be seen as only one object if they are separated by less than 0.2 μm. The microscope's resolving power determines the quality of the image, its clarity and richness of detail, and depends mainly on the quality of its objective lens. Magnification is of value only when accompanied by high resolution. Objective lenses providing higher magnification are designed to also have higher resolving power. The ocular lens only enlarges the image obtained by the objective and does not improve resolution.

FIGURE 1–3 Components and light path of a bright-field microscope.



Photograph of a bright-field light microscope showing its mechanical components and the pathway of light from the substage lamp to the eye of the observer. The optical system has three sets of lenses:

- The **condenser** collects and focuses a cone of light that illuminates the tissue slide on the stage.
- **Objective** lenses enlarge and project the illuminated image of the object toward the eyepiece. Interchangeable objectives with different magnifications routinely used in histology include ×4 for observing a large area (field) of the tissue at low magnification; ×10 for medium magnification of a smaller field; and ×40 for high magnification of more detailed areas.
- The two **eyepieces** or oculars magnify this image another ×10 and project it to the viewer, yielding a total magnification of ×40, ×100, or ×400.

Virtual microscopy, typically used for the study of bright-field microscopic preparations, involves the conversion of a stained tissue preparation to high-resolution digital images and permits study of tissues using a computer or other digital device, without an actual stained slide or a microscope. In this technique, regions of a glass-mounted specimen are captured digitally in a grid-like pattern at multiple magnifications using a specialized slide-scanning microscope and saved as thousands of consecutive image files. Software then converts this dataset for storage on a server using a format that allows access, visualization, and navigation of the original slide with common web browsers or other devices. With advantages in cost and ease of use, virtual microscopy is rapidly replacing light microscopes and collections of glass slides in histology laboratories for students.

Fluorescence Microscopy

When certain cellular substances are irradiated by light of a proper wavelength, they emit light with a longer wavelength—a phenomenon called **fluorescence**. In **fluorescence microscopy**, tissue sections are usually irradiated with ultraviolet (UV) light and the emission is in the visible portion of the spectrum. The fluorescent substances appear bright on a dark background. For fluorescent microscopy, the instrument has a source of UV or other light and filters that select rays of different wavelengths emitted by the substances to be visualized.

Fluorescent compounds with affinity for specific cell macromolecules may be used as fluorescent stains. The compounds DAPI and Hoechst stain specifically bind DNA and are widely used to stain cell nuclei, emitting a characteristic blue fluorescence under UV. Acridine orange binds both DNA and RNA but emits different colors with these nucleic acids in fluorescent microscopy, allowing them to be localized separately in cells (Figure 1–4a). Another important application of fluorescence microscopy is achieved by coupling compounds such as fluorescein to molecules that will specifically bind to certain cellular components and thus allow the identification of these structures under the microscope (Figure 1–4b). Antibodies labeled with fluorescent compounds are extremely important in immunohistochemistry. (See the section on Visualizing Specific Molecules.)

Phase-Contrast Microscopy

Unstained cells and tissue sections, which are usually transparent and colorless, can be studied with these modified light microscopes. Cellular detail is normally difficult to see in unstained tissues because all parts of the specimen have roughly similar optical densities. **Phase-contrast microscopy**, however, uses a lens system that produces visible images from transparent objects and, importantly, can be used with living cultured cells (Figure 1–5).

Phase-contrast microscopy is based on the principle that light changes its speed when passing through cellular and extracellular structures with different refractive indices. These changes are used by the phase-contrast system to cause the structures to appear lighter or darker in relation to each other. Without the requirements of tissue fixation and staining, phase-contrast microscopes allow study of live cells and are prominent tools in all cell culture laboratories. A modification of phase-contrast microscopy is **differential interference contrast microscopy** with Nomarski optics, which produces an image of cells with a more apparent three-dimensional (3D) aspect (Figure 1–5c).

Confocal Microscopy

With a regular bright-field microscope, the beam of light is relatively large and fills the specimen. Stray (excess) light reduces contrast within the image and compromises the resolving power of the objective lens. Confocal microscopy (Figure 1–6) avoids these problems and achieves high resolution and sharp