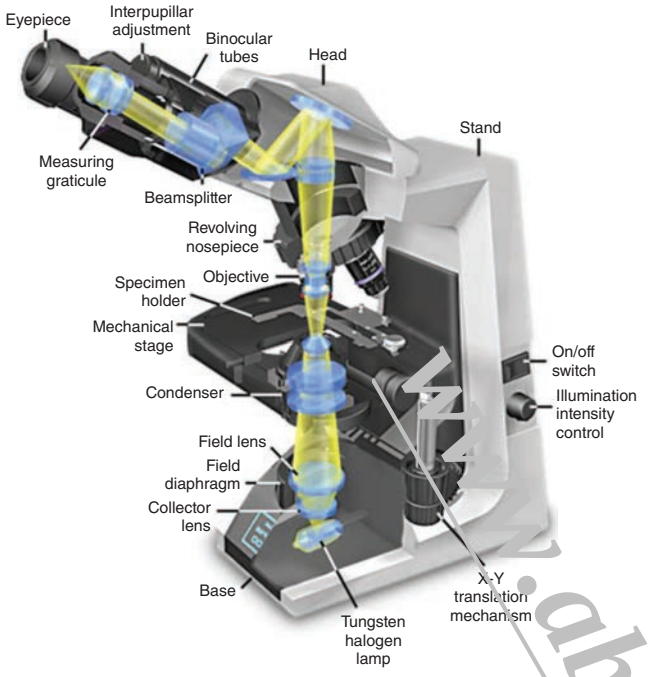
Contents

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

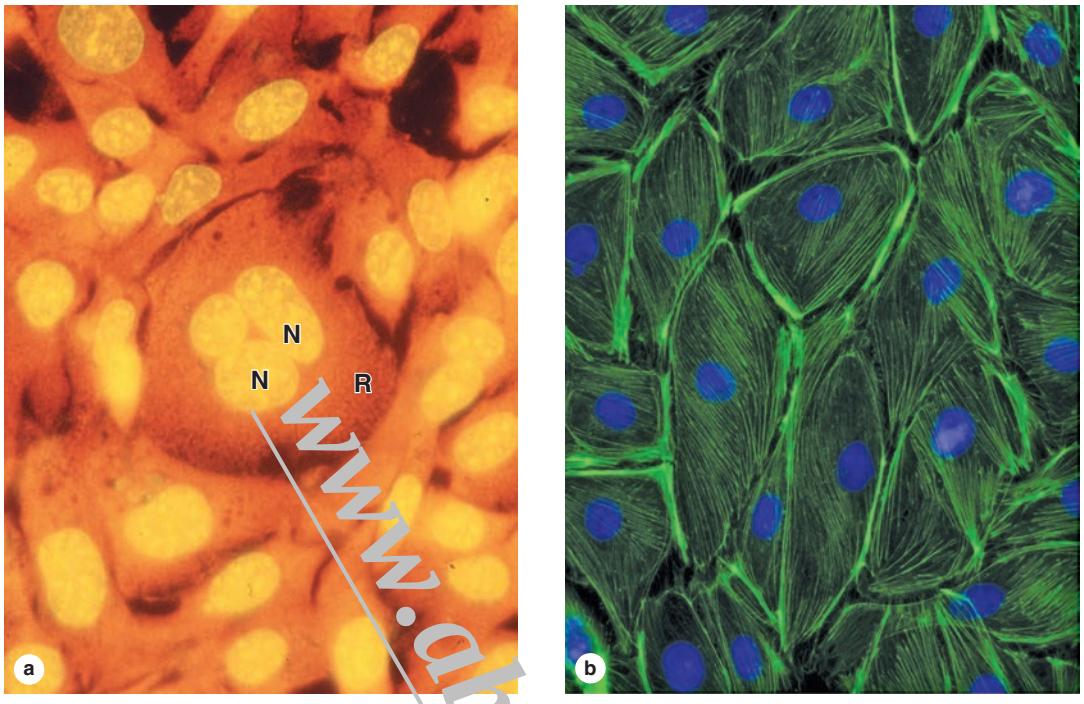
Unstained cells and tissue sections, which are usually transparent and colorless, can be studied with these modified light microscopes. Cellular detail is normally difficult to see in unstained tissues because all parts of the specimen have roughly similar optical densities. **Phase-contrast microscopy**, 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

FIGURE 1–4 Appearance of cells with fluorescent microscopy.



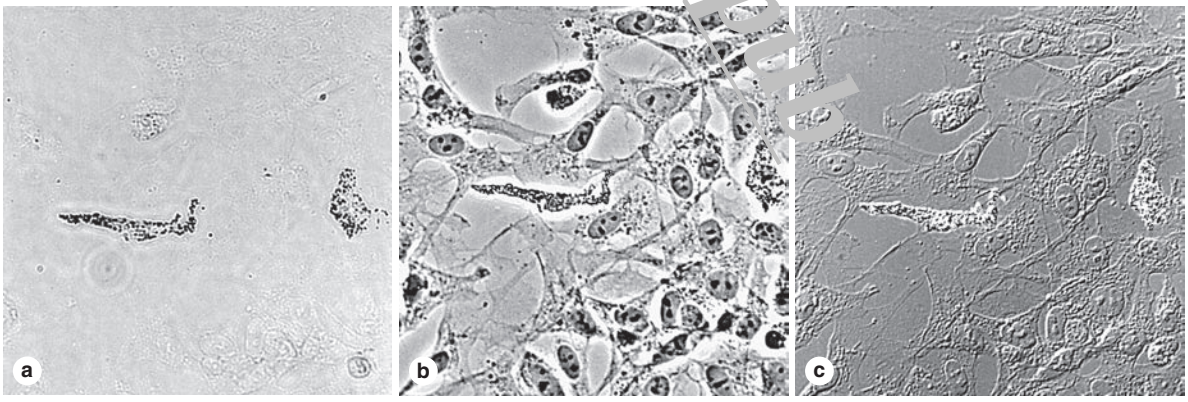
Components of cells are often stained with compounds visible by fluorescence microscopy.

(a) Acridine orange binds nucleic acids and causes DNA in cell nuclei (**N**) to emit yellow light and the RNA-rich cytoplasm (**R**) to appear orange in these cells of a kidney tubule.

(b) Cultured cells stained with DAPI (4′,6-diamino-2-phenylindole) that binds DNA and with fluorescein phalloidin that binds actin filaments show nuclei with blue fluorescence and actin filaments stained green. Important information such as the greater density of microfilaments at the cell periphery is readily apparent. (Both ×500)

(Figure 1–4b, reproduced with permission from Drs Claire E. Walczak and Rania Rizk, Indiana University School of Medicine, Bloomington.)

FIGURE 1–5 Unstained cells' appearance in three types of light microscopies.



Living neural crest cells growing in culture appear differently with various techniques of light microscopy. Here the *same field* of unstained cells, including two differentiating pigment cells, is shown using three different methods (all ×200):

(a) Bright-field microscopy: Without fixation and staining, only the two pigment cells can be seen.

(b) Phase-contrast microscopy: Cell boundaries, nuclei, and cytoplasmic structures with different refractive indices affect in-phase light differently and produce an image of these features in *all* the cells.

(c) Differential interference contrast microscopy: Cellular details are highlighted in a different manner using Nomarski optics. Phase-contrast microscopy, with or without differential interference, is widely used to observe live cells grown in tissue culture.

(Reproduced with permission from Dr Sherry Rogers, Department of Cell Biology and Physiology, University of New Mexico, Albuquerque, NM.)

The utility of all light microscopic methods is greatly extended with digital cameras. Many features of digitized histologic images can be analyzed quantitatively using appropriate software. Such images can also be enhanced to allow objects not directly visible through the eyepieces to be examined on a monitor.

ELECTRON MICROSCOPY

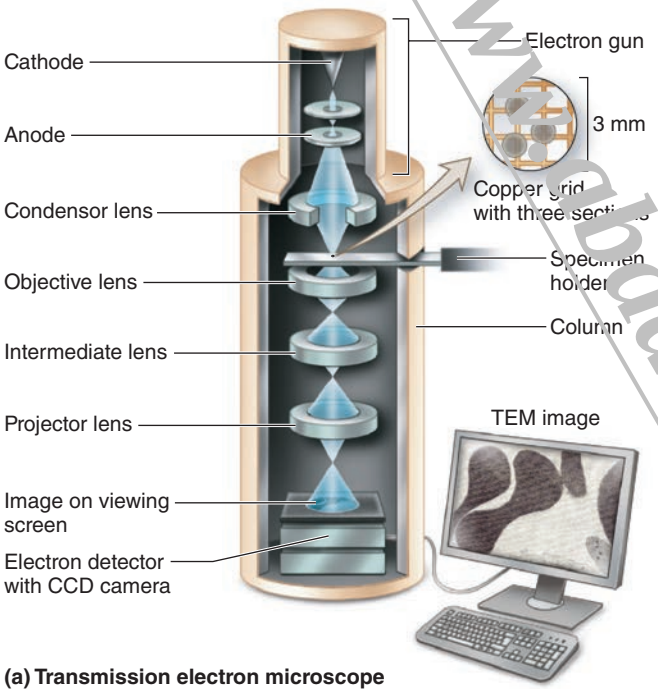
Transmission and scanning electron microscopes are based on the interaction of tissue components with beams of electrons. The wavelength in an electron beam is much shorter than that of light, allowing a 1000-fold increase in resolution.

Transmission Electron Microscopy

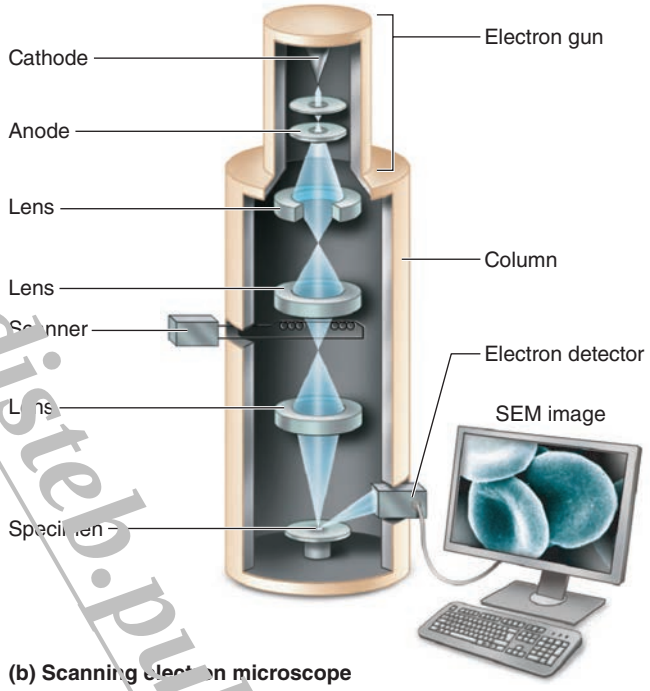
The **transmission electron microscope (TEM)** is an imaging system that permits resolution around 3 nm. This high resolution allows isolated particles magnified as much as 400,000 times to be viewed in detail. Very thin (40–90 nm), resin-embedded tissue sections are typically studied by TEM at magnifications up to approximately 120,000 times.

Figure 1–8a indicates the components of a TEM and the basic principles of its operation: a beam of electrons focused using electromagnetic "lenses" passes through the tissue section to produce an image with black, white, and intermediate shades of gray regions. These regions of an electron micrograph correspond to tissue areas through which electrons

FIGURE **1–8** **Electron microscope**



(a) Transmission electron microscope



(b) Scanning electron microscope

Electron microscopes are large instruments generally housed in a specialized EM facility.

(a) Schematic view of the major components of a **transmission electron microscope (TEM)**, which is configured rather like an upside-down light microscope. With the microscope column in a vacuum, a metallic (usually tungsten) filament (cathode) at the top emits electrons that travel to an anode with an accelerating voltage between 60 and 120 kV. Electrons passing through a hole in the anode form a beam that is **focused electromagnetically** by circular electric coils in a manner analogous to the effect of optical lenses on light.

The first lens is a condenser focusing the beam on the section. Some electrons interact with atoms in the section, being absorbed or scattered to different extents, while others are simply transmitted through the specimen with no interaction. Electrons reaching the objective lens form an image that is then magnified and finally projected on a fluorescent screen or a charge-coupled device (CCD) monitor and camera.

In a TEM image, areas of the specimen through which electrons passed appear bright (electron lucent), while denser areas or those that bind heavy metal ions during specimen preparation absorb or deflect electrons and appear darker (electron dense). Such images are therefore always black, white, and shades of gray, although TEM images are sometimes colorized later.

(b) With the **scanning electron microscope (SEM)** the focused electron beam does not pass through the specimen, but rather is moved sequentially (scanned) from point to point across its surface. For SEM, specimens are coated with metal atoms with which the electron beam interacts, producing reflected electrons and newly emitted secondary electrons. All of these are captured by a detector, transmitted to amplifiers, and processed to produce a black-and-white image on the monitor. The SEM shows only surface views of the coated specimen but with a striking 3D, shadowed quality. The inside of organs or cells can be analyzed after sectioning to expose their internal surfaces.

passed readily (appearing brighter or electron-lucent) and areas where electrons were absorbed or deflected (appearing darker or more electron-dense). To improve contrast and resolution in TEM, compounds with **heavy metal ions** are often added to the fixative or dehydrating solutions used for tissue preparation. These include osmium tetroxide, lead citrate, and uranyl compounds, which bind cellular macromolecules, increasing their electron density and visibility.

Cryofracture and **freeze etching** are techniques that allow TEM study of cells without fixation or embedding and have been particularly useful in the study of membrane structure. In these methods, very small tissue specimens are rapidly frozen in liquid nitrogen and then cut or fractured with a knife. A replica of the frozen exposed surface is produced in a vacuum by applying thin coats of vaporized platinum or other metal atoms. After removal of the organic material, the replica of the cut surface can be examined by TEM. With membranes, the random fracture planes often split the lipid bilayers, exposing protein components whose size, shape, and distribution are difficult to study by other methods.

Scanning Electron Microscopy

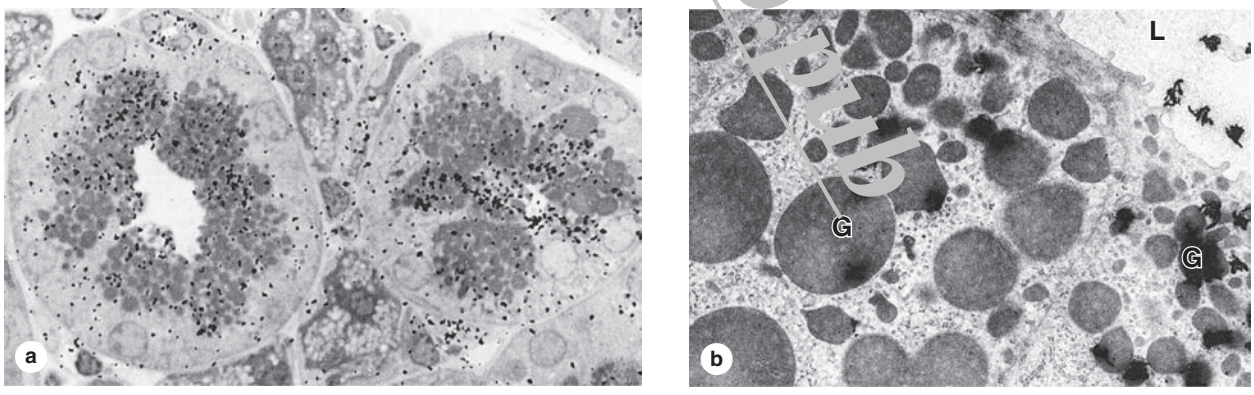
Scanning electron microscopy (SEM) provides a high-resolution view of the surfaces of cells, tissues, and organs. Like the TEM, this microscope produces and focuses a very narrow beam of electrons, but in this instrument, the beam does not pass through the specimen (Figure 1–8b). Instead, the surface of the specimen is first dried and spray-coated with a very thin layer of heavy metal (often gold) that reflects electrons in a beam scanning the specimen. The reflected electrons are captured by a detector, producing signals that are processed to produce a black-and-white image. SEM images are usually easy to interpret because they present a 3D view that appears to be illuminated in the same way that large objects are seen with highlights and shadows caused by light.

AUTORADIOGRAPHY

Microscopic **autoradiography** is a method of localizing newly synthesized macromolecules in cells or tissue sections. Radioactively labeled metabolites (nucleotides, amino acids, sugars) provided to living cells or experimental animals are incorporated into specific macromolecules (DNA, RNA, protein, glycoproteins, and polysaccharides). After tissue fixation, processing, and sectioning only the new, labeled macromolecules continue to emit weak and localized radiation, unincorporated isotope having been washed out during the processing steps. Slides or TEM grids with radiolabeled cells or tissue sections are coated in a darkroom with photographic emulsion in which silver bromide crystals act as microdetectors of the radiation in the same way that they respond to light in photographic film. After an adequate exposure time in lightproof boxes, the slides are developed photographically. Silver bromide crystals reduced by the radiation produce small black grains of metallic silver, which under either the light microscope or TEM indicate the locations of radiolabeled macromolecules in the tissue (Figure 1–9).

Much histological information becomes available by autoradiography. If a radioactive precursor of DNA (such as tritium-labeled thymidine) is used, it is possible to know which

FIGURE **1–9** **Microscopic autoradiography.**



Autoradiographs are tissue preparations in which particles called **silver grains** indicate the cells or regions of cells in which specific macromolecules were synthesized just prior to fixation. Shown here are autoradiographs from the salivary gland of a mouse injected with ^{3}H-fucose 8 hours before tissue fixation. Fucose was incorporated into oligosaccharides, and the free ^{3}H-fucose was removed during fixation and sectioning of the gland. Autoradiographic processing and microscopy reveal locations of newly synthesized glycoproteins containing that sugar.

(a) Black grains of silver from the light-sensitive material coating the specimen are visible over cell regions with secretory granules and the duct indicating glycoprotein locations. (×1500)

(b) The same tissue prepared for TEM autoradiography shows silver grains with a coiled or amorphous appearance again localized mainly over the granules (**G**) and in the gland lumen (**L**). (×7500)

(Figure 1–9b, reproduced with permission from Drs Ticiano G. Lima and A. Antonio Haddad, School of Medicine, Ribeirão Preto, Brazil.)