Introduction

Histology is the study of the tissues of the body and how these tissues are arranged to constitute organs.

-The skin is an organ made up of tissues.

- The outer part of the skin that we can touch is the epithelial tissue.

- underneath the epithelium: there's connective tissue (tissues: groups of cells and the materials surrounding them that work together to perform a particular function)

- The body is composed of several systems.

- Each system has a number of organs.

- The organs in each system share the same function and share some of the tissues together but they have different proportions of each tissue.

- So, the organ itself is composed of a lot of tissues.

- Histology studies one tissue at a time of each organ. For example: the stomach's lining is made of epithelium, the middle layer is a different layers of smooth muscles, and the outside part of the stomach is made of connective tissue.

- Tissues have two interacting components: cells and extracellular matrix (ECM).

1) THE CELL is the basic structural and functional units of an organism.

2) The ECM consists of 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. Also, matrix components bind to specific cell surface receptors that span the cell membrane and connect to structural components inside the cells.

What produces the ECM? → Cells produce the ECM locally and are in turn strongly influenced by matrix molecules.

Basic tissues in our body:

1-Epithelial tissue

2-Connective tissue

3-Muscular tissue

4-Nervous Tissue

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. Most tissue organs are too thick for light to pass through, so by tissue preparation we cut them into thin, translucent sections and place on glass slides for microscopic examination of the internal structures.

1-Fixation of the tissue (the most important step):

When the tissue leaves the body the blood supply stops therefore the oxygen supply stops, the cell starts **degrading** itself throughout enzymes, this is known as autolysis. We need to prevent autolysis from happening to avoid destruction of the tissue structure. Therefore, we immediately fix the sample tissue once we take it or we can leave in the **fridge**, the low temperature is not suitable for the enzymes to function.

There are chemical materials called **fixatives** that we use on the tissue sample for fixation.

Fixatives prevent the autolysis by **cross-linking protein;** the fixatives connect the amino acids (NH2) of the proteins in the tissue which protects them from degrading.

Why do we do fixation?

1. This helps us to see the tissue as close as possible to how it actually looks in our body.

- The most widely fixative used in light microscope is formaldehyde added at certain concertation and pH level, usually a buffered isotonic solution of 37% formaldehyde known as (formalin) is used as a fixative.

- Glutaraldehyde, a fixative used for electron microscopy.

Electron microscopy provides much greater magnification تكبير and resolution تمييز of very small cellular structures and fixation must be done very carefully to preserve additional "ultrastructural" detail so we use glutaraldehyde. Also, when using electron microscopy, glutaraldehyde-treated tissue is immersed in buffered osmium tetroxide, why? Because this solution (1) preserves and (2) stains cellular lipids as well as proteins (this is called double-fixation).

2. Tissue Processing: Processing (dehydration, clearing & impregnation (known as infiltration))

It is a serial steps of (1) dehydration done by a machine to wash the water completely out of the tissue by gradually replacing water with alcohol, followed by (2) clearing alcohol out of the specimen, and then (3) impregnating the specimen in a **liquid** paraffin wax (in this step, paraffin is in liquid form because it is easier to control).

A-Dehydration:

The tissue is transferred through a series of increasingly concentrated alcohol (ethanol) solutions, ending in 100%, to remove all water.

B-Clearing: Replacing the dehydrating fluid -which was the alcohol- with an organic fluid that is totally miscible يتغلل أو يذوب with both the dehydrating fluid and the embedding medium (embedding is the step that follows the processing step).

Examples of organic fluids (known as clearing agents) include: Xylene for LM & propylene oxide for EM.

C-Infiltration: Replacing the clearing agent (inside the cell) with a material that can be ready for the next step which is **embedding**. Examples on infiltrating agents include paraffin for LM, and plastic resin for EM.

Note: In infiltrating sample tissue the temperature of melted paraffin is at 52°-60°C.

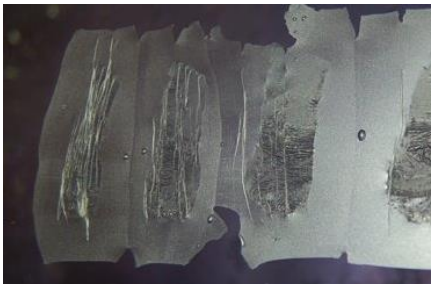
3-Embedding the tissue

Embedding is **molding** يعني وضع العينة في قوالب the tissue sample by **hardening** the material that the tissue was infiltrated with this is important to support biological tissue.

The embedding center (i.e. tool or equipment) consists of a **hot surface** that maintains the paraffin wax in a liquid form and a **cold surface** to solidify the paraffin wax into a mold.

-After tissue processing the tissue sample is placed at the hot surface area and then moved quickly to the cold surface are to thicken and solidify the paraffin into a block that surrounds the tissue sample. This helps to manipulate the sample and easily cut it.

4-Cutting the tissue sample



Tissue sample is sectioned by a device named **microtome**, it cuts the sample to a very thin sections but with a manageable thickness.

Paraffin sections are at 3-10 µm thickness for light microscopy. The microtome forms a paraffin ribbon (see the pic. →) while cutting the sample, that contains 4-5 slices of tissue. The paraffin ribbon is then moved to a warm water bath that helps to soften the paraffin and easily pick up the tissue using a **glass slide**.

5. 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 (1) conspicuous but also (2) distinguishable from one another.

Dyes stain material more or less selectively, often behaving like **acidic** or **basic** compounds and forming electrostatic (salt) linkages (i.e. electrical bonds or connections) with macromolecules in tissues.

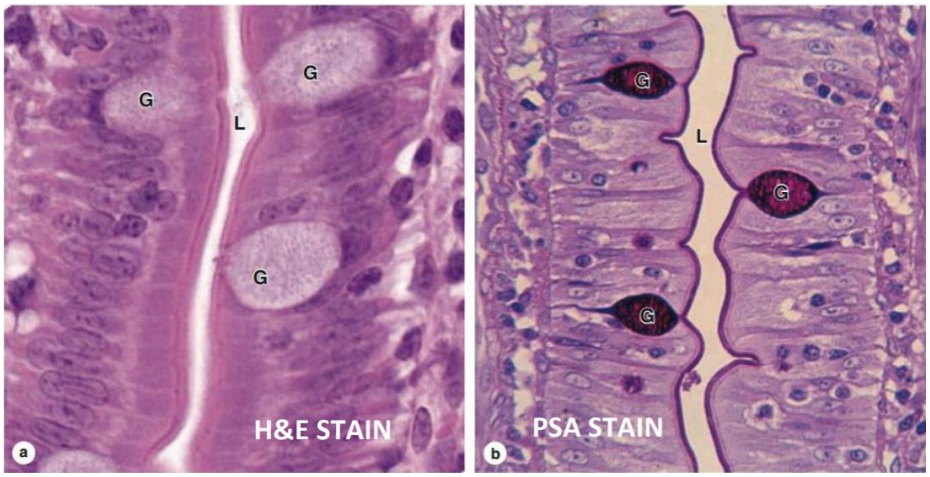
Dyes are classified as BASOPHLIC (have a positive charge) & ACIDOPHILIC (have a negative charge). Staining depends on the components of the cells in the tissue sample. *For general staining we use a **combination** of Hematoxylin & Eosin stain (H&E).*

Hematoxylin (a basic dye) stains DNA & RNA rich components, rER, ribosomes, and GAGs (basophilic components) of the cell and give them a **dark blue** or **purple color**, on the other hand eosin (an acidic dye) stains other cytoplasmic structures, mitochondria, proteins, secretory granules, and collagen pink.

Note: The matrix of cartilage is stained by Hematoxylin (purple).

If we need to distinguish a particular structure in our sample we need to use a special stain.

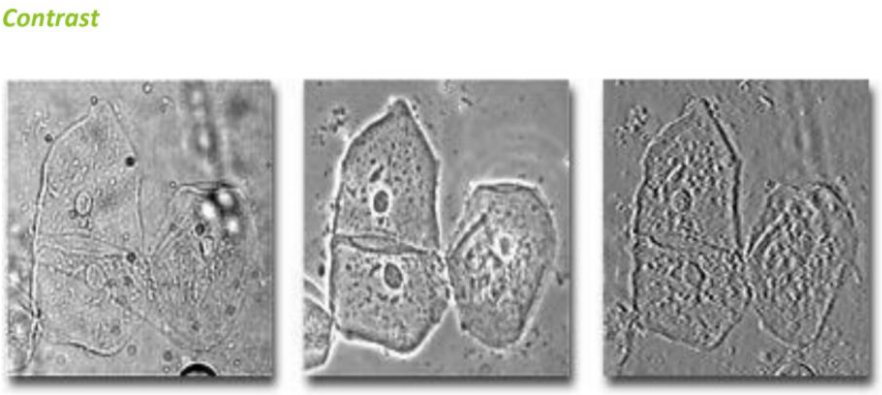
Special staining: used when I am looking at a particular structure like specific type of cell/organelle/blood vessels. For example PAS (periodic acid-schiff stain reaction) is used to distinguish carbohydrates in the cell; it stains polysaccharides and carbohydrate-rich tissue structure with purple or magenta.



An alternate way to prepare tissue sections is to submit the tissues to rapid freezing. In this process, the tissues are fixed by freezing (physical, not chemical fixation) and at the same time become hard and thus ready to be sectioned. A freezing microtome— the cryostat—is then used to section the frozen block with tissue. This rapid method is routinely used in hospitals to study specimens during surgical procedures.

Freezing of tissues is also effective in the study of very sensitive enzymes or small molecules, since freezing, unlike fixation, does not inactivate most enzymes. Frozen sections are also useful when structures containing lipids are to be studied (since xylene solvent dissolves lipids).

Microscopy



-**Magnification:** is the degree to which a lens, mirror, or other device can enlarge an object, or the degree to which the object is enlarged. (X2, X3, X4…X1000 etc.)

-**Resolution** distance (or limit): is the shortest distance between two points on a specimen that can still be distinguished as separate entities. (high resolution **power** = short resolution distance (or limit)).

Resolution depends on:

1. Wave energy (and thus, wave wavelength "λ")
2. The quality of the objective lens (NOT the ocular lens)
3. The distance between the specimen and objective lens)

-**Contrast**: is the difference in light intensity between the image and the adjacent background relative to the overall background intensity.

-Techniques used in histology

1-Light microscope

2-Electron microscope

3-Immunohistochemistry , which depends on antigen-antibody reaction.

1- Light Microscopy, LM applications:

-**Bright**-field microscopy (the **conventional** one)

-Fluorescence microscopy

-Confocal microscopy

-Phase-contrast microscopy

-Polarizing microscopy

They are all based on the interaction of light with tissue components and are used to reveal and study tissue features.

I) Bright-Field Microscopy (the conventional one)

With the bright-field microscope stained tissue is examined with ordinary light passing through the preparation. the microscope includes an **optical system** which includes:

1. **Condenser** focusing light on the object to be studied.
2. **The objective lens** enlarging and projecting the image of the object toward the observer.
3. **The eyepiece (or ocular lens)** further magnifying this image and projecting it onto the viewer's eyes.

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 (appx. (200-300 nm)) 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 (light microscope). 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.

Objective lenses providing higher magnification are designed to also have higher resolving power. The eyepiece lens *only* enlarges the image obtained by the objective and *does not improve resolution*.

P.S. Histology is 2D study of 3D structures in histological sections.

The optical system (i.e. the basic part of the microscope) 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.

Objectives with different magnifications routinely used in histology include:

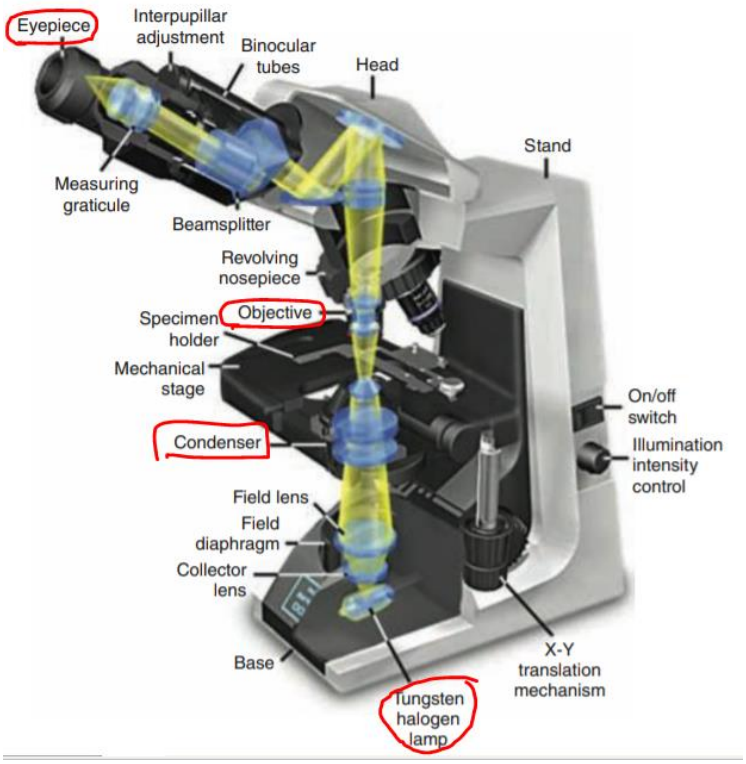
1. X4 for observing a large area (field) of the tissue at low magnification
2. X10 for medium magnification of a smaller field
3. X40 for high magnification of more detailed areas.

■ The two eyepieces or oculars magnify this image another X10 (always ten) and project it to the viewer, yielding a total magnification of X40, X100, or X400.

The distance between the specimen and objective lens also effects on the resolution of the image.

X-Y translation mechanism : are handles used to scan all area of glass light. (x, y ; in both directions)

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

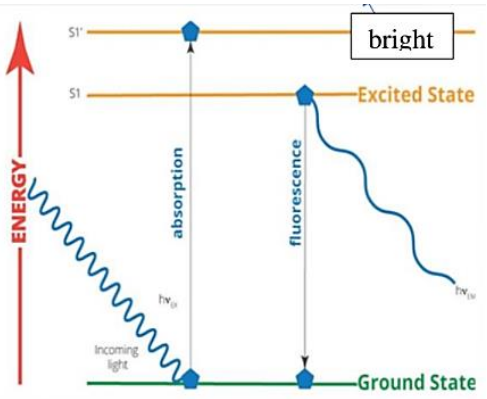


II) Fluorescence Microscopy

When certain cellular substances are irradiated by light of a proper wavelength, they emit light with a longer wavelength (lower energy)—a phenomenon called **fluorescence**. In fluorescence microscopy, tissue sections are usually irradiated with ultraviolet (UV) light (high energy, short wavelength) and the emission is in the visible portion of the spectrum.

The ideas is that, fluorescence occurs when an excited molecule (excited by being targeted with light) relaxes from a higher energy state to a lower energy state through **emission of a photon**.

It may have been directly excited from the ground state S0 to a higher energy state S2 -by absorption of a photon of energy- and subsequently emits a photon of a lower energy it relaxes to state S1 (energy level that's between S0 and S2).

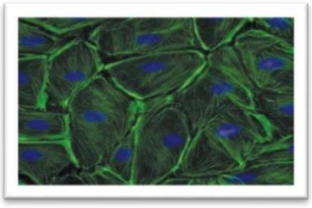


The **fluorescent substances** appear bright on a dark background. For fluorescent microscopy the instrument has a source of UV or other light and filters that select rays of different wavelengths emitted by the substances to be visualized.

Fluorescent compounds (called fluorophores) with affinity for (bind to) specific cell macromolecules may be used as fluorescent stains.

Examples (of fluorophores) include:

1. **Acridine orange binds both DNA and RNA.**
2. **DAPI specifically binds DNA (blue).**
3. **Fluorescein-phalloidin that binds actin filaments (green).**



-this photo was made by immune fluorescence.
-Immuno means antibody (part of immune system).
-Here we have blue and green parts:
BLUE : is the nuclei of the cell
GREEN : let us assume it as "microtubules".

Antibodies labeled with fluorescent compounds (**fluorophores; like these mentioned above**) are extremely important in immunohistologic staining.

VISUALIZING SPECIFIC **MOLECULES**

A specific macromolecule present in a tissue section may also be identified by using tagged (i.e. labeled) compounds or macromolecules that bind **specifically** with the molecule of interest. The compounds that interact with the molecule must be visible with the light or electron microscope, often by being tagged with a detectible label. The most commonly used labels are **fluorescent compounds (fluorophore), radioactive atoms, enzymes, and metal (usually gold) particles that can be seen with light and electron microscopy.** These methods can be used to detect and localize specific sugars, proteins, and nucleic acids.

Immunohistochemistry (using antibodies)

A highly specific interaction between macromolecules is that between an antigen and its antibody. For this reason labeled antibodies are routinely used in immunohistochemistry to identify and localize many specific molecules.

The body's immune cells interact with and produce **antibodies** against other macromolecules—called **antigens**—that are recognized as "foreign," not a normal part of the organism, and potentially dangerous. Antibodies normally bind specifically to their antigens and help eliminate them.

In immunohistochemistry a tissue section that one believes contains the protein of interest is incubated in a solution containing **antibody** against this protein. The antibody binds **specifically** to the protein and the protein's location in the tissue or cells can be seen with either the light or electron microscope by ***visualizing the antibody.*** Antibodies are *commonly* tagged with **fluorescent compounds (known as fluorophores).**

To produce antibodies against protein x of a certain animal species (eg, a human or rat), the isolated protein is injected into an animal of **another** species (eg, a rabbit or a goat). If the protein's amino acid sequence is sufficiently different for this animal to recognize it as **foreign**—that is, as an **antigen**—the animal will produce antibodies against the protein.

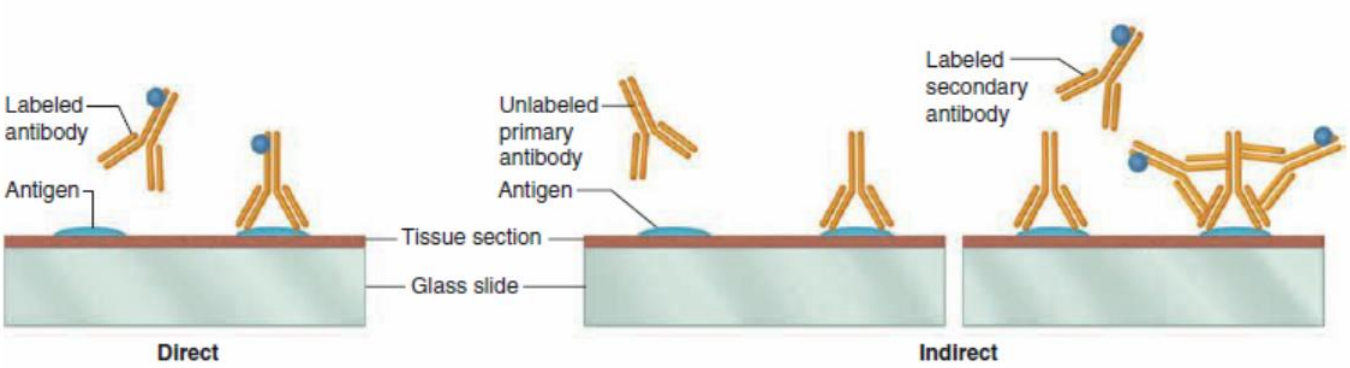
Different groups (clones) of lymphocytes in the injected animal recognize different parts (called **epitopes**; part of antigen) of protein x and each clone produces an antibody against that part. These antibodies are collected from the animal's plasma and constitute a mixture of **polyclonal antibodies**, each capable of binding a different region (epitope) of protein x.

The different antibodies from each clone (group of lymphocytes) against protein x can be collected separately. Each of these antibodies is a **monoclonal antibody.**

An advantage to using a monoclonal antibody rather than polyclonal antibodies is that it can be selected to be highly specific and to bind **strongly** to the protein to be detected, with less nonspecific binding to other proteins that are similar to the one of interest.

There are direct and indirect methods of immunocytochemistry**:**

1. The direct method just involves a labeled antibody that binds the protein of interest.
2. Indirect immunohistochemistry involves sequential application of two antibodies and additional washing steps. The (primary) antibody specifically binding the protein of interest is **not** labeled. The detectible tag is conjugated to a secondary antibody made in an animal species **different** ("foreign") from that which made the primary antibody. For example, primary antibodies made by mouse lymphocytes (such as most monoclonal antibodies) are specifically recognized and bound by antibodies made in a rabbit or goat injected with **mouse antibody immunoglobulin (the primary one).**



Why the indirect method?

With mitochondria, microfilaments or cytoskeleton present more in cytoplasm primary antibody is enough to get good image.

Sometimes with very tiny and rare protein in the cell; this signal won't be enough to get a good image that's why we need **more fluorophore (using high levels of indirect method)**

Another method of immunohistochemistry is by using enzymes as labels, if there is a reaction to convert a substance to product and the product is colorful then whenever there is enzyme the substance will be colorful and whenever there is no enzyme , the substance will be colorless.

Back to microscopy.

III) Phase contrast microscopy

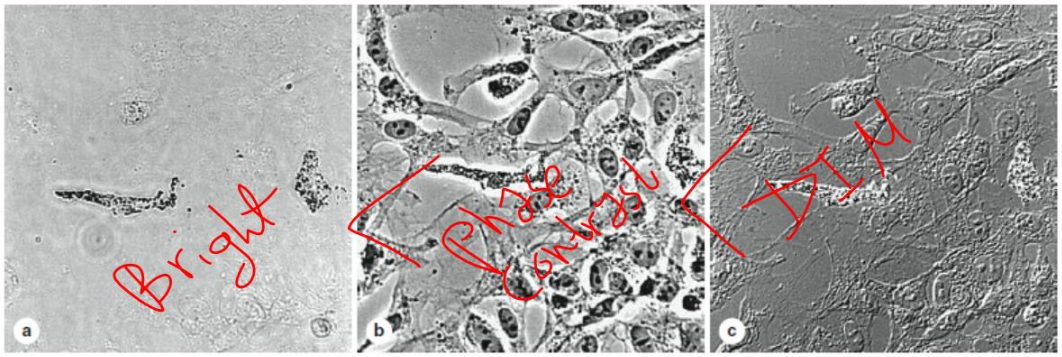
Unstained cells and tissue sections, which are usually transparent and colorless, can be studied with these modified light microscopes. Cellular detail is difficult to see in unstained tissues because all parts of the specimen have similar roughly densities. Phase-contrast microscopy, however, uses a lens system that produces **visible** images from transparent objects and, importantly, can be used with **living**, cultured cells (no fixation or staining!).

Phase-contrast microscopy is based on the principle that light changes its speed when passing through cellular and extracellular structures with different **refractive indices** (ability to diffract/ bend light).

These changes are used by the phase-contrast system to cause the structures to appear lighter or darker in relation to each other. Because they allow the examination of cells **without fixation** or **staining**, phase-contrast microscopes are prominent tools in all cell culture laboratories.

A modification of phase-contrast microscopy is **differential interference microscopy**, which produces an image of living cells with a more apparent three-dimensional (3D) aspect.

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



2- Electron Microscopy

In electron microscope, we don`t use glass slides because the beam is composed of **electrons**, so we use should use **conductive** slide (not a glassy one), like metals for example, preferably, **copper**.

Note: we use **gold** in electron microscopic immunohistochemistry.

Transmission and **scanning** electron microscopes are based on the interaction of tissue components with beams of electrons.

Beam: light vs. electrons
Lenses: glass vs. electromagnetic
Slides: glass vs. metallic (e.g., copper)

The wavelength in an electron beam is much shorter than that of light, allowing a 1000-fold increase in resolution.

- Major parts of electron microscope :

1. **Electron gun:** the source of electrons. It shoots electron beam.
   The electron beam should -then- hit an **anode**, then transmission or scanning takes place.
2. **Condenser** lenses **focus** the electrons as a one beam to the specimen.

I) Transmission Electron Microscopy

The transmission electron microscope (TEM) is an imaging system that permits resolution around 3 nm (very high). 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.

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 passed readily (appearing brighter or electron-lucent) and areas where electrons were absorbed or deflected (appearing darker or more electron-dense).

Stated differently, when a specimen is hit by an electron beam, electrons interact with atoms in the section, being *absorbed* , *scattered,* or *transmitted* through the specimen with no interaction. When electrons reaches the objective lens, they form an image that is then magnified and projected on a **fluorescent screen** or a **charge-coupled device (CCD) monitor** and **camera**.

II) Scanning Electron Microscopy

Scanning electron microscopy (SEM) provides a high resolution view of the **surfaces** (topography from outside of cells), tissues, and organs. In this instrument the electron beam does not pass through the specimen. Instead, the surface of the specimen is first **dried** and spray-coated with a very thin layer of heavy metal (often gold) which reflects electrons in a beam scanning the specimen. The reflected (bent) 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 **three-dimensional** view.

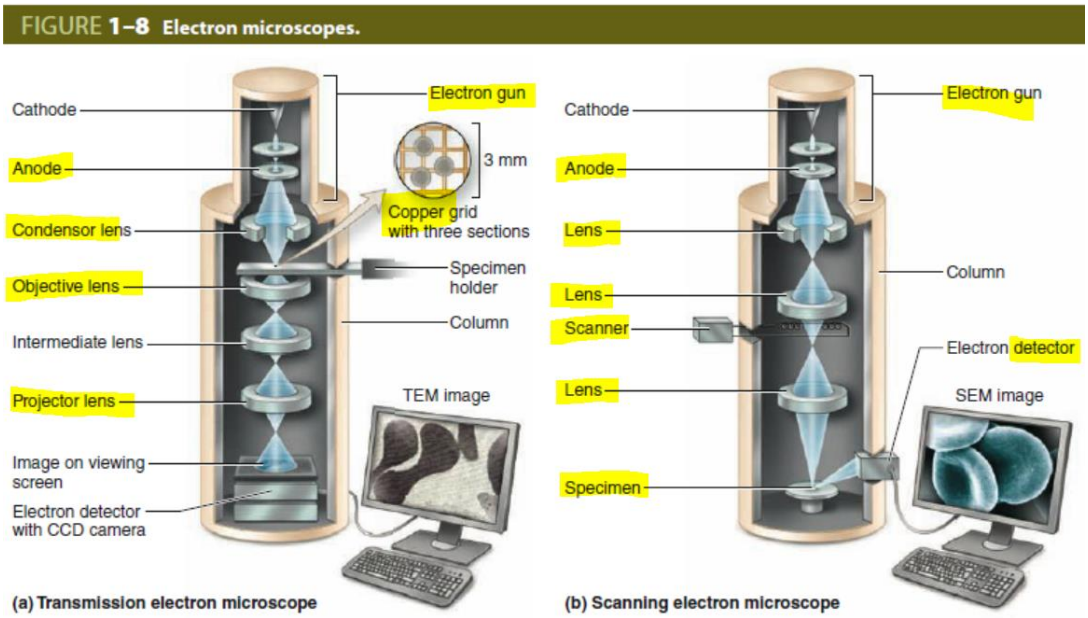
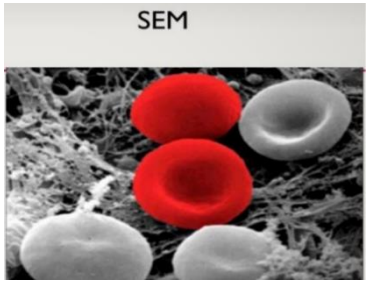
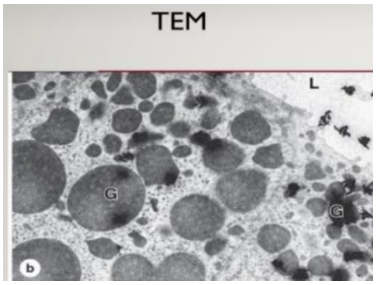


FIGURE 1–8 Electron microscopes.

(a) Transmission electron microscope (b) Scanning electron microscope





Practice Questions:

1. In preparing tissue for routine light microscopic study, which procedure immediately precedes clearing the specimen with an organic solvent?

a. Dehydration
b. Fixation
c. Staining
d. Clearing
e. Embedding

2. Which of the following staining procedures relies on the cationic and anionic properties of the material to be stained?

a. Enzyme histochemistry
b. Periodic acid-Schiff reaction
c. Hematoxylin & eosin staining
d. Immunohistochemistry
e. Metal impregnation techniques

3. In a light microscope used for histology, resolution and magnification of cells are largely dependent on which component?

a. Condenser

b. Objective lens

c. Eyepieces or ocular lenses

d. Specimen slide

e. The control for illumination intensity

4. Cellular storage deposits of glycogen, a free polysaccharide, could best be detected histologically using what procedure?

a. Autoradiography

b. Electron microscopy

c. Enzyme histochemistry

d. Hematoxylin & eosin staining

e. Periodic acid-Schiff reaction

5. Adding heavy metal compounds to the fixative and ultrathin sectioning of the embedded tissue with a glass knife are techniques used for which histological procedure?

a. Scanning electron microscopy

b. Fluorescent microscopy

c. Enzyme histochemistry

d. Confocal microscopy

e. Transmission electron microscopy

6. Resolution in electron microscopy greatly exceeds that of light microscopy due to which of the following?

a. The wavelength of the electrons in the microscope beam is shorter than that of a beam of light.

b. The lenses of an electron microscope are of greatly improved
quality.

c. For electron microscopy the tissue specimen does not require
staining.

d. The electron microscope allows much greater magnification of a projected image than a light microscope provides.

e. An electron microscope can be much more finely controlled than a light microscope.

7. To identify and localize a specific protein within cells or the extracellular matrix one would best use what approach?

a. Autoradiography

b. Enzyme histochemistry

c. Immunohistochemistry

d. Transmission electron microscopy

e. Polarizing microscopy

8. Hospital laboratories frequently use unfixed, frozen tissue specimens sectioned with a cryostat for rapid staining, microscopic examination, and diagnosis of pathological conditions. Besides saving much time by avoiding fixation and procedures required for paraffin embedding, frozen sections retain and allow study of what macromolecules normally lost in the paraffin procedure?

a. Carbohydrates

b. Small mRNA

c. Basic proteins

d. Acidic proteins

e. Lipids