

"Methods in Histology"

Objectives:

- Understand the uses of the most important types of light microscopes
- Understand the basic operation and uses of electron microscopes
- Understand "resolution" and some basic units of size
- Understand basic steps in tissue preparation for light and electron microscopy
- Know major staining methods used in histology and what cell components are visualized with the different stains
- Understand basic principles and uses for other specific histological techniques, including enzyme histochemistry, immunohistochemistry, in situ hybridization, and autoradiography.

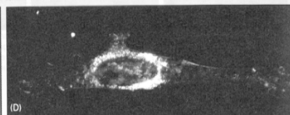
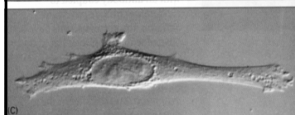
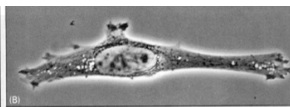
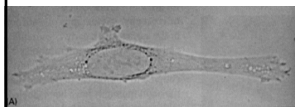
Major types of Light Microscopy

- *Brightfield*: uses light focused on the specimen by a condenser lens, then brought to the eye via objective and ocular lenses; usually used with stains
- *Phase Contrast*: uses a condenser lens system to visualize differences of refractive index within cells and tissues; no stain needed on the specimen
- *Fluorescence*: uses light of a specific wavelength (e.g. UV), usually to visualize very specific stains that emit light at another specific wavelength
- *Confocal*: uses a scanning laser beam to make a series of sharp images on a photomultiplier tube, computers to record, then display these as a combined high resolution image

Microscopy of living (nonfixed) cells can employ various optical methods:

Brightfield

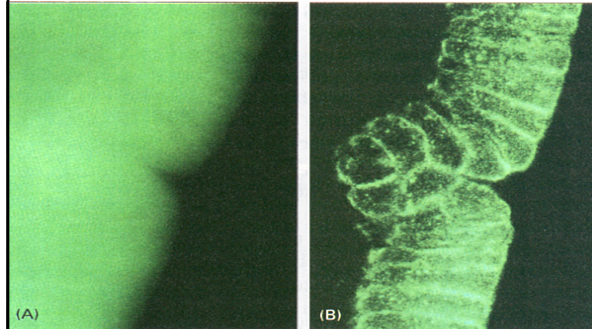
Phase Contrast (changes in index of refraction)



Nomarski optics -DIC
(differential
interference contrast)

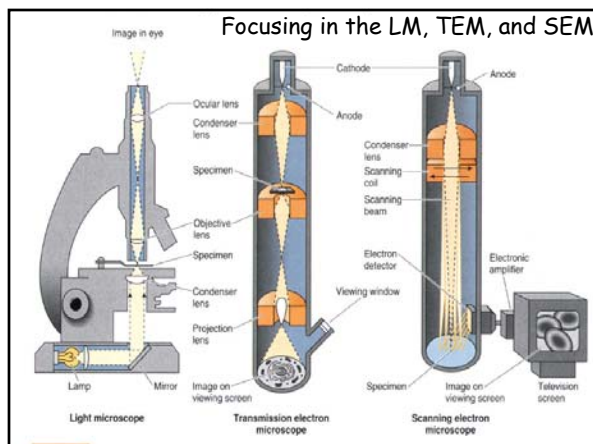
Darkfield (scattered
light is imaged)

Structures with fluorescent components (or stained with such molecules) can be seen using fluorescent microscopy (A) with specific wavelengths. Confocal microscopy (B) provides optimal resolution.



Types of Electron Microscopy

- **Transmission Electron Microscopy (TEM):** electrons pass through specimen stained with heavy metal salts to reveal "electron-dense" areas within cells of a sectioned (thinly sliced) specimen
- **Scanning Electron Microscopy (SEM):** electrons reflect off the surface of a specimen coated with an evaporated gold-carbon film and are then collected by detectors for processing to produce a 3-dimensional-like image



Microscopic lenses allow both *magnification* and *resolution* of details within the specimen.

"Resolution" is the ability to distinguish two close but distinct points. The best "resolving power" of various instruments is:

- human eye $\sim 200\ \mu\text{m}$ (0.2 mm)
- light microscope $\sim 0.2\ \mu\text{m}$
- transmission electron microscope $\sim 1\ \text{nm}$ ($0.001\ \mu\text{m}$) in tissue section.
- scanning electron microscope $\sim 2\ \text{nm}$ on a biological sample

Dimensions used in microscopy:

" μm " = micrometer (or "micron")

nm = nanometer

(1000 μm per mm; 1000 nanometers per μm)

Sizes of various structures in microns:

- red blood cell (human) $7.0\ \mu\text{m}$ diameter
- mature oocyte (a large cell) $100\ \mu\text{m}$ diameter
- paraffin section usually $5\text{--}12\ \mu\text{m}$ thick
- virus $0.02\text{--}0.10\ \mu\text{m}$ diameter
- thin section for TEM $0.05\text{--}0.09\ \mu\text{m}$ thick
- cell membrane $0.007\ \mu\text{m}$ (7 nm) thick

Specimen Preparation for Light Microscopy

- **Fixation**, e.g. 10% neutral buffered **formalin**
- **Dehydration** with alcohol, rinsing with xylene or chloroform & **infiltration with paraffin**
- "**Sectioning**" of paraffin blocks with a microtome at $5\text{--}10\ \mu\text{m}$, mounting on glass slide, clearing of paraffin, **staining**
- Most common stain combination - **Hematoxylin** (blue, basophilic) and **Eosin** (red, acidophilic): **H&E**
- Hematoxylin stains acidic components (DNA, RNA)
- Eosin stains more alkaline or basic cell components
- **Biopsies** - Tissue often frozen and sections cut on cryostat and then stained (often with fluorescent tagged antibody)

Transmission Electron Microscopy

An electron beam is transmitted through a thin specimen (50-90 nm) in a manner similar to the way in which visible light is transmitted through a tissue section for the LM. However, the EM uses magnetic lenses to focus electrons & the LM uses glass lenses to focus photons.

Specimen Preparation for TEM

- **Glutaraldehyde** $O=C-CH_2-CH_2-CH_2-C=O$ (5 Carbon aldehyde) most common fixative.
- **Crosslinks proteins** by forming methylene bridges between polypeptides at reactive side groups
- **Preserves** proteins & nucleoproteins excellently. Slight reaction with lipids.
- **Post-fixation** in **osmium tetroxide** to preserve membranes and other lipid components
- **Dehydration** in alcohol & acetone; infiltration with epoxy (plastic-like) resin
- Sectioning of 50-90 nm sections on **ultramicrotome**
- **Staining** with lead or uranium salts for contrast based on electron density ("black & white staining" only)

Scanning Electron Microscopy

- Microscope uses a beam of electrons (primary beam) to scan the pre-coated specimen surface.
- As the probe scans across the specimen, by-products of secondary electrons, backscatter electrons, x-rays, & photons are produced.
- Secondary electrons are low energy electrons (< 50 ev) emitted from the surface of the specimen (up to a depth of 20 \AA). These electrons contain the surface detail information.
- The electrons and other by-products are collected and amplified by photomultiplier tubes, then used to produce an image on a cathode ray tube (or TV screen).

Specimen Preparation for SEM

- For SEM, fixation is the same as TEM.
- After fixation the specimen (2-10 mm in size) is dehydrated in alcohol and "critical point dried".
- After drying the sample is attached to a specimen stub and given an electron reflective coating (gold or gold-palladium).
- The specimen mount is then placed in the SEM and the primary electron beam turned on.

Acidophilic and Basophilic Stains (e.g., H&E)

- Most common stains are either basic or acidic
- Acid-containing structures such as cell nuclei and ribosomes have affinity for basic dyes like hematoxylin, which stains nuclei purple.
- Such cell components are "basophilic."
- Structures rich in alkaline proteins have affinity for acidic dyes like eosin, which stains such cell components pink or orange.
- These structures are said to be "acidophilic."

Other Common Staining Methods

- Cell/tissue components that are neither acidic nor basic may stain with metal salts, such as silver, osmium, or chromium. **Silver stain** is often used for reticular fibers (type III collagen), which are said to be "argyrophilic."
- "**Trichromes**" are combinations of dyes that stain cell nuclei, **collagen**, and other tissue components in one reaction step.

Enzyme Histochemistry

- uses enzymes in specific cell organelles to localize those components specifically
- reaction of enzyme + an added substrate = metal salt (colored or dark for light microscopy; electron dense product for EM).
- The periodic acid-Schiff (**PAS**) or Feulgen reaction is a common example of histochemistry. This stains **glycogen** & various **carbohydrate**-containing molecules (e.g., glycoproteins on cell surfaces or in ECM).

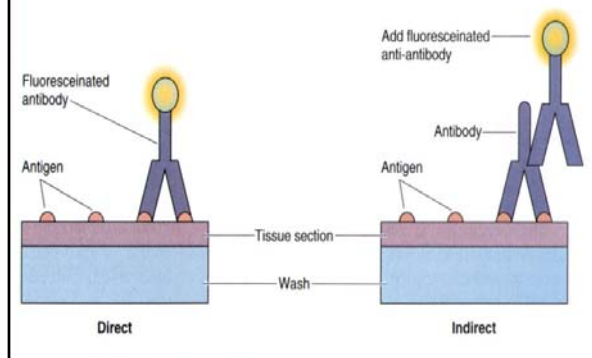
Immunohistochemistry (IHC)

- extremely important research technique
- can be used with LM or TEM
- goal is to visualize some component (antigen) in a tissue section by means of a labeled antibody
- label for LM is usually a fluorescent molecule or enzyme such as peroxidase or alkaline phosphatase bound to antibody
- label for TEM can be colloidal gold or ferritin iron bound to antibody

Immunohistochemistry (cont.)

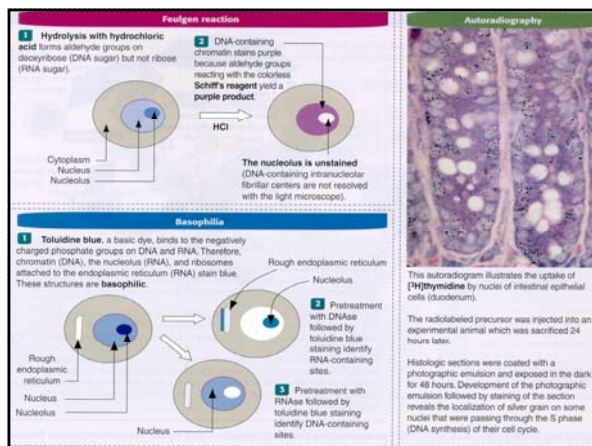
- may use polyclonal or monoclonal antibodies: Polyclonal may have more sensitivity by being directed against many antigenic determinants. Monoclonal are specific for one antigen, but may have lower sensitivity.
- may be direct or indirect, with label directly attached to the primary antibody (which binds the molecule to be localized), or with the primary antibody being unlabeled and visualized by a labeled secondary antibody that binds specifically to the primary antibody.

Direct vs. Indirect Immunofluorescence



Autoradiography (ARG)

- Research method involving LM or TEM
- **Goal:** Localize in a tissue section a radioactive substance (drug, enzyme, etc.) that the living cells metabolized.
- After fixation and sectioning, the specimen is covered with a thin coat of photographic emulsion.
- Radiation from the isotope exposes and produces elemental silver after photographic development.
- The specific points in the emulsion (silver grains) indicate exactly where the radiolabeled metabolite is located in the cell.
- Isotopes that are low energy β -emitters are usually best. H^3 (tritium) and C^{14} are commonly used.



***In situ* Hybridization (ISH)**

- Research technique using LM or TEM
- Uses a radio- or antigen-labeled antisense RNA or cDNA probe to localize cells containing specific mRNAs
- Methods: lightly fixed cells are made permeable, then the labeled probe is added, allowed to hybridize with complementary mRNAs in cells with them, and then those cells are visualized microscopically by ARG or IHC
