Microscopy I
Light and Electron Microscopy

Replica of van Leeuwenhoek’s (1632-1723) microscope constructed c. 1670. Moody Medical Library, Univ. Texas Medical Branch, Galveston, TX
Use the information in this tutorial to supplement the visuals in lab and the information in Chapters 1, 8 and 9 in your lab manual.

Replica of Culpepper tripod microscope built c. 1725 by Edmund Culpepper (1670-1738). Collection of Moody Medical Library, Univ. Tex., Galveston, TX. (Replica by Replica Rara Ltd. Antique Microscopes)
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   A. Light Microscopy
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Microscopes have long been essential tools of cell biologists. This tutorial provides a brief overview of types of microscopes commonly used in biological studies and general techniques for preparing specimens for the various types of microscopy. The two broad categories of microscopy we are concerned with are:

**Light Microscopy (LM)**

and

**Electron Microscopy (EM)**
Light Microscopy

Bright field Microscopes--the most common general use microscopes. Bright field microscopes are named because the microscopic “field” is bright, while the object being viewed is dark.

- Simple design
- Light directed at specimen is absorbed to form image
- Unstained specimens have poor contrast
- Stained specimens show excellent contrast
- Ideal for stained bacteria, cells, tissues
- High N.A., good resolution
- Bright background, dark specimen
- tungsten or halogen light source

Binocular, bright field microscope with movable stage, dioptic adjustment, condenser and iris diaphragm, and built-in light source. These are used as clinical, research and student microscopes.
Bright field images—

Stained blood cells in peripheral blood smear

Section of gut tissue containing ciliated parasites

Flagellate — *Trichomonas*
The basic design of bright field microscopes has been modified for special uses. **Inverted microscopes** (right) allow viewing of cells in flasks, welled-plates, or other deep containers that do not fit between the objectives and stage of standard BF microscopes.
Dark field Microscopes (DF)
The dark field microscope creates a dark background to allow viewing of small unstained objects, such as motile bacteria, that would be difficult to view in a bright field. The central portion of the light is blocked so that only oblique light strikes the specimen, scattering light rays that then enter the objective to form the image.

- A method from the 19th century
- Bright specimen, dark background
- Light not scattered by the specimen bypasses the objective, therefore making the “field” dark.
- Can see very small objects but resolution is variable
- High contrast, good for unstained, live, and motile specimens
Darkfield Images--

The algae *Hydrodictyon reticulatum* viewed with darkfield microscopy. Photo by Dr. D. Folkerts, AU.

*Leptospira*, a spirochete, viewed with darkfield microscopy.
Phase Contrast—Converts differences in refractive index in a specimen to differences in image brightness.

The central portion of the light source is blocked, creating a ring of light from the condenser that illuminates the specimen. The light waves refracted by the specimen are slowed by a phase retardation plate (in phase objectives) increasing the difference in wavelength between refracted and unrefracted rays (which do not pass through the phase plate). When the refracted and unrefracted waves are focused, they produce an interference due to the difference in wavelengths—this is seen as differences in brightness in the specimen.

- technology from 1940’s
- provides high contrast, good resolution
- good for bacteria, flagella, cilia, organelles such as mitochondria
- good for unstained or live mounts
- phase halos (artifacts) occur
Phase Contrast Image (left) compared with DIC image (right)

Unstained squamous epithelial cells observed with phase contrast microscopy (above) and DIC microscopy (right). Differences in refractive index in various regions of the cells account for contrast in the images. (From Becker et al., *The World of the Cell*, Benjamin/Cummings Publ. Co., 2000.)
Polarization Microscopy

Detects specimens that are birefringent (have the characteristic of double refraction, i.e. the velocity of light refracted by a substance is not the same in all directions). The specimen is placed between two polarizers crossed at 90° to each other (one in condenser and one in objective).

- bright image, dark background
- used for substances with highly organized molecular structure, such as crystals, minerals
- can be quantitative
Polarizing Microscopy images—many crystals and minerals display characteristic patterns in polarizing light.

Left, portion of Martian meteorite that has been ground to 0.3 mm depth and viewed under polarizing microscopy (From Calvin J. Hamilton). Top right, BF view of renal epithelial cells containing fat globules; bottom right, high magnification view of same cells viewed with polarizing microscope and showing typical “maltese cross” formation displayed by fat in polarized light. (From R. Hyduke, U. Iowa Hospitals & Clinics.)
Epi-fluorescence Microscopy

Allows the detection of molecules and ions within cells. Fluorescent dyes absorb short wavelengths of light and emit longer wavelengths. Barrier filters and a dichroic prism select the excitation wavelength that strikes the specimen and exclude the excitation wavelength from the detector, allowing only emitted light to reach the detector (oculars).

- uses uv light source = mercury or xenon arc lamp.
- high contrast, high resolution image
- special fluorescent dyes used to locate “molecules” in a specimen
- black background, bright-stained specimen
- no condenser required, light comes from above (“epi”) specimen
- multiple fluorescent probes available
- detects small quantities, molecules; can use antibody staining techniques
Epi-fluorescent images--The locations of specific molecules can be identified using fluorescent probes. In immunofluorescence techniques, antibody probes that bind to the molecules and secondary antibody labeled with a fluorescent dye are used.

Above--Immunofluorescence of *Tetrahymena*, a ciliated protozoan, using the fluorescent dye, fluorescein isothiocyanate, which emits “green light.” Above left, cell is stained with anti-basal body antibody; above right, cell is stained with antibody against cell membrane surface antigen. Compare the information obtained using the fluorescent technique with the BF view of *Tetrahymena* (far right) stained with hematoxylin.
Epi-fluorescent image showing metaphase in newt lung cell. Three fluorescent probes—specific for DNA, keratin, and tubulin—were used. Blue, metaphase chromosomes (DNA); red, keratin filaments; yellow, spindle apparatus made of microtubules. From ASCB, Bethesda, MD; image by Rieder and Hughes, NY State Dept. Health, Albany, NY.
Differential Interference Contrast (DIC) Microscopy—also called Normarski optics

Resembles phase-contrast but more sensitive—gives higher resolution. Uses polarizing lenses like the polarizing microscope, and therefore can be quantitative. The halogen light beam is polarized, split by a beam splitter (Wollaston prism), and passed through the specimen. The split beams are recombined by a second prism in the objective. Any change in a light wave due to passage through a specimen causes interference as the beams recombine, producing differences in image brightness corresponding to the refractive index of specimen structures.

- produces 3-D images
- excellent resolution, high NA, high contrast
- good for unstained specimens, live mounts; can see membranes within cells
- detects changes in refractive index of specimen

Hoffman modulation contrast microscopy (HMCM)

is the poor man’s DIC. Contrast is not as good as in DIC.

Microscope having both epi-fluorescence and DIC capabilities.
DIC image--
comparison of DIC and fluorescent images

Top, DIC image of cricket ovariole showing oocytes (O), oocyte nuclei (N), and Balbiani bodies (B), also called mitochondrial clouds. Note three-dimensional appearance.

Bottom, epi-fluorescent image of Balbiani bodies stained with rhodamine 123, a fluorescent stain specific for mitochondria.
Confocal laser scanning microscopy

A laser is focused at a plane in the specimen and scans the specimen in a horizontal (XY) plane. Only light from the plane of focus reaches the detector. The scanned image (an optical section) is digitally recorded. Images from consecutive focal planes can be recorded, and composite or 3-D images can be digitally created.

- krypton/argon laser
- high resolution, sharp image
- high sensitivity
- Can be used in reflectance or fluorescence mode
- eliminates background interference

Bio-Rad confocal microscope showing fluorescent-labeled cells on monitor
Confocal image--

Left, image composed from 13 optical sections of *Trichomonas* immunostained with anti-tubulin and fluorescein isothiocyanate (FITC). The green structures consist of microtubules. Pf, posterior flagellum; Af, anterior flagella; C, costa; and axostyle (red arrow). Compare with BF image (right). (Photo by B. Estridge)
Electron Microscopy

Scanning Electron Microscopy (SEM)
Transmission Electron Microscopy (TEM)
**Scanning Electron Microscopy (SEM)**

Fixed, dehydrated specimens are mounted on stubs and surface-coated with gold, palladium or rhodium. The specimen is placed in a vacuum and an electron beam scans back and forth over it. Electrons that bounce off the metal-coated specimen surface are collected, converted to a digital image and displayed on a TV-like monitor.

- Electron beam is focused using a magnetic field

- SEM provides a 3-D image

- Gives information about external topography of specimen

- Much higher resolution and magnification than possible in LM
SEM images--comparison of SEM images (top) with images obtained using DIC microscopy (bottom).

Both DIC microscopy and SEM reveal specimen surface topography. Much greater detail is obtained using SEM because of greater resolution and magnification capability. Top, SEM of *Trichomonas* (left) and *Tetrahymena* (right); bottom, DIC images of *Trichomonas* (left) and *Tetrahymena* (right).
Transmission Electron Microscopy (TEM)

Fixed, dehydrated specimens are embedded in a resin, hardened, sectioned, stained with heavy metals such as uranium and lead, and inserted into the electron column in the microscope. The electron beam is absorbed or deflected by the heavy metal stains and shadows are cast onto film or a phosphorescent plate (image is a shadow) at the bottom of the column.

- 2-D image
- reveals internal cell structure
- high resolution, high magnification
- electron beam is focused by magnetic field
Comparison of TEM (right) and SEM (left) images

*Trichomonas* as viewed with SEM (left) and TEM (right). Note that with SEM details of external structure are visible while in TEM internal structures are revealed. N, nucleus; G, Golgi; H, hydrogenosome (equivalent of a mitochondrion); F, flagellum; Rf, recumbent flagellum; B, cross-sections of basal bodies of three anterior flagella.
TEM photos of rat liver cell (left) and pancreatic acinar cells (right) revealing internal cell structure. N, nucleus; Nu, nucleolus, RER, rough endoplasmic reticulum; CB, cell boundary; Z, zymogen granules; M, mitochondrion.
Specimen Preparation

Light Microscopy
Scanning Electron Microscopy
Transmission Electron Microscopy
General Schematic for Preparing Specimens for Light Microscopy

Live mounts

1. View
2. Vital stain
3. View

Fixed (preserved) Specimens (Histology)

1. Fix
2. Dehydrate
3. Infiltrate and Embed
4. Section
5. Mount
6. Stain
7. View
Live Mounts

To view organisms, tissues, or cells in as close to the natural state as possible, unstained live mounts are used. Viewing time of live mounts is limited. Unstained specimens have low contrast.

Supravital stains may be applied to provide more contrast or identify certain components--these are stains that are not harmful to living cells.
Fixed (Preserved) Specimens for Histology--the Study of Tissue

1) Specimens may be preserved using chemicals such as formalin, acetic acid, ethanol, and methanol. Fixation immobilizes molecules such as proteins and lipids.

2) Fixed specimens are dehydrated by serial transfer through an ascending alcohol series, to 100% alcohol.

3) Specimens are infiltrated with melted paraffin, paraffin substitute, or plastic and placed in a mold to harden.
4) Specimens are cut into 5-10 um thick sections using a **steel knife** or **razor** on an instrument called a **microtome**.

Vibratome—uses a vibrating knife to cut sections
5) Sections are then mounted on slides, 
6) Stained to achieve contrast or identify cell structures or components, and 
7) Viewed microscopically

Many variations in technique are used to prepare specimens for light microscopy. Some omit the dehydration, infiltration, embedding and sectioning steps and use aqueous staining systems for viewing whole mounts (unsectioned tissues or cells).

Freezing may be used instead of chemicals to fix tissues that need to be examined quickly or that have components damaged by the chemicals. Examples are frozen biopsies and tissues in which heat-labile structures are to be stained. Frozen specimens are sectioned using a cryotome, a microtome encased in a freezing chamber. Permanent slides may be made from paraffin sections but not from frozen sections.
Schematic for Preparing Specimens for Scanning Electron Microscopy (SEM)

1) Fix
2) Dehydrate
3) Mount on stubs
4) Sputter coat
5) Observe
1) Fixation—fixatives used are glutaraldehyde, paraformaldehyde, osmium tetroxide.

2) Dehydration is accomplished by carrying the specimens through an ascending alcohol series, to 100% alcohol (i.e., no water), then to an organic solvent such as acetone or propylene oxide. Specimens for SEM may also be processed in a critical point drying apparatus.
3) Specimens are mounted on **aluminum stubs using sticky tape.**

4) A **sputter coater** coats the specimen with gold, palladium or rhodium in a special chamber to cover the specimen with a 10-20 nm thick metal layer.
5) The stub is inserted into the SEM, scanned and observed on a video display
Schematic for Preparing Specimens for Transmission Electron Microscopy (TEM)

1) Fix
2) Dehydrate
3) Infiltrate -- Embed
4) Section
5) Apply sections to grids
6) Stain
7) Observe
1) Fixation—specimens are fixed in glutaraldehyde, or paraformaldehyde-glutaraldehyde mixtures, followed by osmium tetroxide.

2) Dehydration is accomplished by carrying the specimens through an ascending alcohol series, to 100% alcohol (i.e., no water), then to an organic solvent such as acetone or propylene oxide.

3) Specimens are then infiltrated with an epoxy or plastic resin and placed in plastic molds to harden.
4) An instrument called an **ultramicrotome** is used to section the specimen.
Glass or diamond knives are used to cut the ultrathin sections.
5) Sections are transferred to tiny metal grids for support (the equivalent of the function of the glass slide in LM).

6) Heavy metal stains such as uranyl acetate and lead citrate are applied to make certain structures electron dense.
7) Grids are then inserted into the transmission electron microscope and observed.