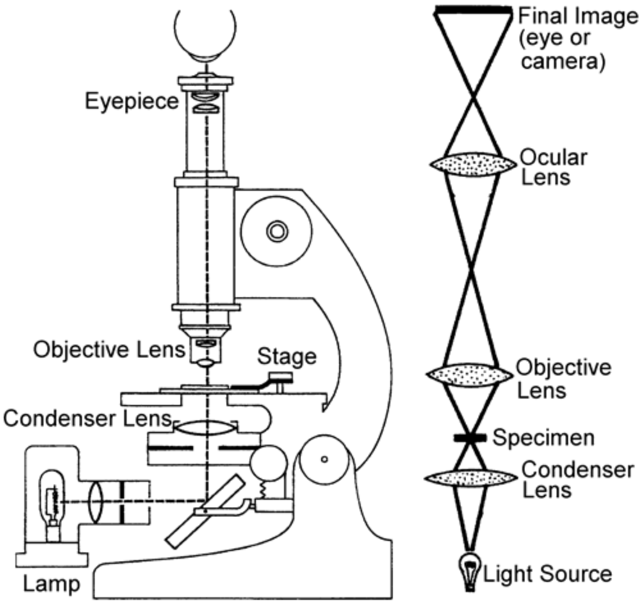
MICROSCOPY

Cells are small and in almost all situations a microscope is needed to observe them and their subcellular components. In fact the invention of the microscope led to the discovery and description of cells (Hooke, 1655). The microscope is still an extremely important tool that is often overlooked in conducting research. The light microscope has a limited capability in regards to the size of a particle that can be examined. The electron microscope provides additional resolution that allows for the examination of subcellular structures and even molecules.

LIGHT MICROSCOPY



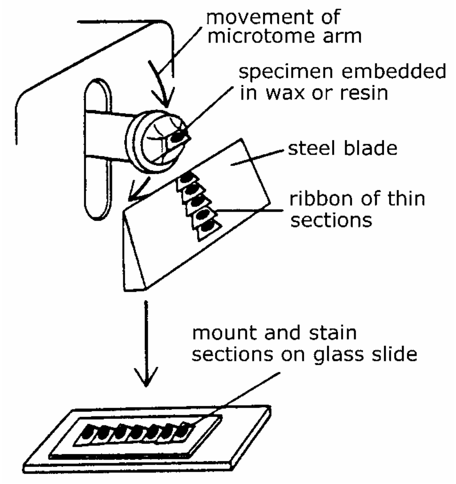
Major components of a light microscope

The principal of light microscopy is to shine light through a specimen and examine it under magnification. The major optical parts of a microscope are the objective lens, the eyepiece, the condenser and the light source. The **objective lens** functions to magnify the object. The high degree of magnification of the objective lens results in a small focal length and the magnified image actually appears directly behind the objective. The **eyepiece** functions to deliver this image to the eye or camera. Eyepieces also magnify the image, but it is an empty magnification. In other words, it enlarges the image but does not increase the resolution. The **condenser** functions to focus the light source on the specimen. It also eliminates stray light and provides an uniform illumination. An iris diaphragm is associated with the condenser lens. The iris is important for controlling resolution vs. the contrast and depth of field. Resolution and contrast are antagonistic in that improving one results in a loss of the other. The specimen is illuminated from a lamp or other **light source**. The best light source is one in which the light intensity is controlled by adjusting the voltage. Optimal contrast vs. resolution is obtained by adjusting both the voltage (i.e., intensity or brightness) of the lamp and the iris diaphragm. It is also important that all of these optical components be centered on an optical axis for the best resolution. Realigning the optical components is usually simple (see instructions manual for the particular microscope) and needs to be done relatively often. Using a microscope (Box) involves focusing the objective lens on the specimen, as well as focusing the condenser and adjusting the illumination as described above.

1. Center light source and all components on optic axis.
2. Focus objective.
3. Focus condenser.
4. Adjust illumination.

Sample Preparation

Specimens can be examined by simply placing them on a glass microscope slide under a glass cover slip. However, it is usually necessary to prepare and stain the samples before examination by microscopy. **Fixation** is a process by which cells are preserved and stabilized. Common fixatives include: acids, organic solvents, formaldehyde and glutaraldehyde (see Appendix for more discussion about aldehyde fixatives). These treatments affix macromolecules in position. For example, glutaraldehyde chemically cross-links the primary amines of neighboring proteins and organic solvents precipitate proteins and other macromolecules.



Thick samples, such as tissues, will need to cut into thin sections. Following fixation the sample or cells are embedded into a supporting medium. Paraffin is a common embedding medium for light microscopy as well as various plastic resins. **Sectioning** is carried out with a microtome (Figure). The microtome makes successive sections of a specified thickness.

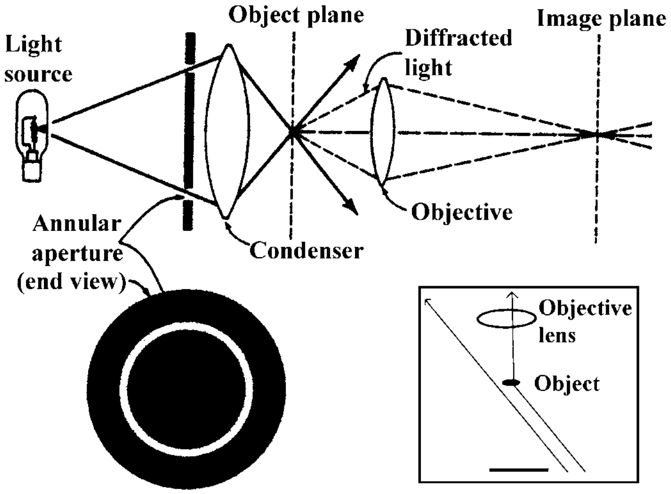
The image generated by microscopy depends upon different components in the sample interacting with and impeding the light waves differentially. Biological samples are fairly homogeneous (i.e., carbon-based polymers) and do not greatly impede light. Therefore, it is often necessary to **stain** cells with dyes. Different dyes have different affinities for different subcellular components. For example, many dyes specifically interact with nucleic acids (i.e., DNA and RNA) and will differentially stain the cytoplasm and nucleus. The stained subcellular components will differentially impede (i.e., absorb) the light waves and provide more contrast than unstained specimens.

Variations to bright field (transmission) microscopy

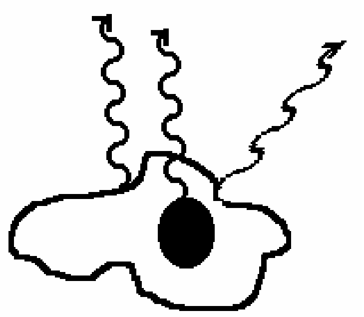
- Dark Field
- Phase Contrast
- Differential Interference Contrast (or Normarski)
- Confocal Scanning
- Fluorescence
- Image Enhancement

Many modifications of light microscopy that have specialized applications have been developed (Box). In **dark-field** microscopy the specimen is illuminated from the side and only scattered light enters the objective lens which results in bright objects against dark background. This is accomplished throught the use of an annular aperture that will produce a hollow cone of light that does not enter the objective lens (see Figure). Some of the light hitting objects within the specimen will be diffracted into the objective lens (see Figure Inset). The images produced by dark-field microscopy are low resolution and details cannot be seen. Dark-field microscopy is especially useful for visualization of small particles such as bacteria.

Both **phase contrast** microscopy and **differential-interference-contrast** allow objects that differ slightly in refractive index or thickness to be distinguished within unstained or living cells. Differences in the thickness or refractive index of the specimen result in a differential retardation of light which shifts the phase (Figure). During phase contrast microscopy the phase differences are converted to intensity differences by special objectives and condensers. Normarski optics use special condensers and objectives to recombine incident and refracted light waves from a single source at the plane of the image. The interference effects between the incident and refracted light enhance small differences in the refractive index or thickness of the specimen and leads to an increased resolution without staining.



Dark Field Microscopy Optics



Phase Shift vs Diffraction

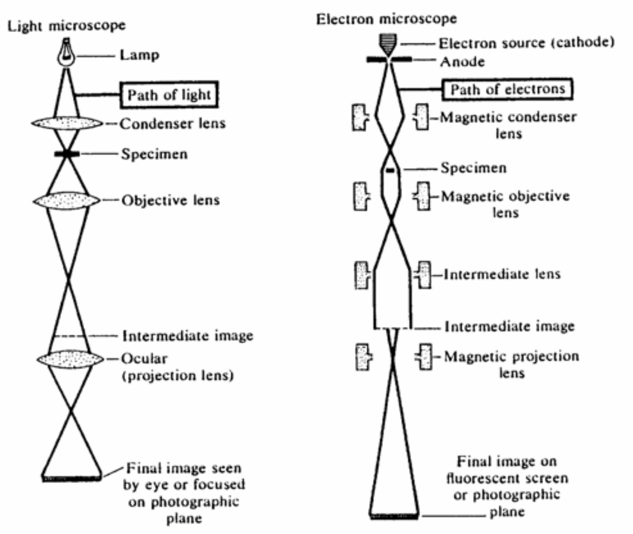
In **fluorescence microscopy** a fluorochrome is excited with ultraviolet light and the resulting visible fluorescence is viewed. This produces a bright image in a dark background. **Confocal microscopy** uses the objective lens as both the objective and the condenser. This allows the illuminating light to be focused on a relatively thin plane. In addition, a ‘pin-hole’ is used to further minimize the light coming from other planes. Minimizing the interference from other planes increases apparent resolution. (Fluorescence and confocal microscopy will be discussed in greater detail in the section of fluorescence.)

Video cameras and image processing have had a major impact on microscopy. Images are digitized and can be manipulated electronically. This can correct imperfections in optical systems and can overcome limitations of human eye. In particular, the human eye is not very effective in dim light and cannot distinguish small differences in intensity against a bright background. Image enhancement can remedy both of these limitations.

ELECTRON MICROSCOPY

Light microscopy exhibits a **limit of resolution** which is generally defined as $0.61\lambda/NA$, where NA (numerical aperture) is a property of the objective lens determined by its magnification, diameter and refractive index. Typical ranges for the NA are 0.25-1.32. Visible light has an average wavelength of approximately 0.5 μm making the maximum limit of resolution approximately 0.2 μm. Mitochondria are about the smallest subcellular structures that theoretically can be seen. No amount of refinement of the optical systems can overcome this physical barrier, even though the image can be enlarged indefinitely.

Comparison of Microscope Optics



The relationship between the limit of resolution and the wavelength of the illumination holds true for any form of radiation. Particles, such as electrons, travelling near the speed of light behave as a wave and their effective wavelength is inversely proportional to electron's velocity. Therefore increased resolution can be achieved by examining a specimen with high velocity electrons.

The principal of the electron microscope is similar to the light microscope (Figure). The illumination source is a white-hot tungsten filament, which emits electrons. The electron beam is focused by a condenser lens onto the specimen. The condenser lens, however, is an electromagnet instead of a glass. The electrons are differentially impeded by the specimen. The resulting electrons are focused with a series of magnetic objective lens on either a photographic plate or a fluorescent screen. The image is formed by the substractive action of the sample. Some of the electrons are scattered or absorbed by the atoms of the specimen. The loss of electrons generates an image in much the same way as the absorption of light creates an image in light microscopy.

Sample preparation

1. Fixation
2. Dehydration
3. Embedding
4. Sectioning
5. Staining

It is not possible to view living material with an electron microscope. Biological samples are usually fixed with glutaraldehyde, which cross-links proteins (see Appendix), and treated with osmium tetroxide, which stabilizes lipid bilayers and proteins. Osmium tetroxide is reduced by many organic compounds, especially lipids, which results in cross-linking. Since electrons have very little penetrating power, samples must be embedded in special plastic resins and cut into thin sections of 0.05-0.1 μm. Removing all water from the specimen is necessary for the proper polymerization of the plastic resin. Following fixation the samples are dehydrated by exposing them to series of increasing alcohol concentrations until reaching 100%. The dehydrated sample is then put into a solution containing monomers of the embedding resin and polymerization is induced. This 'block' containing the sample is sectioned with the ultramicrotome and the ultrathin sections are place onto copper or nickel grids coated with a thin carbon or plastic film for support.

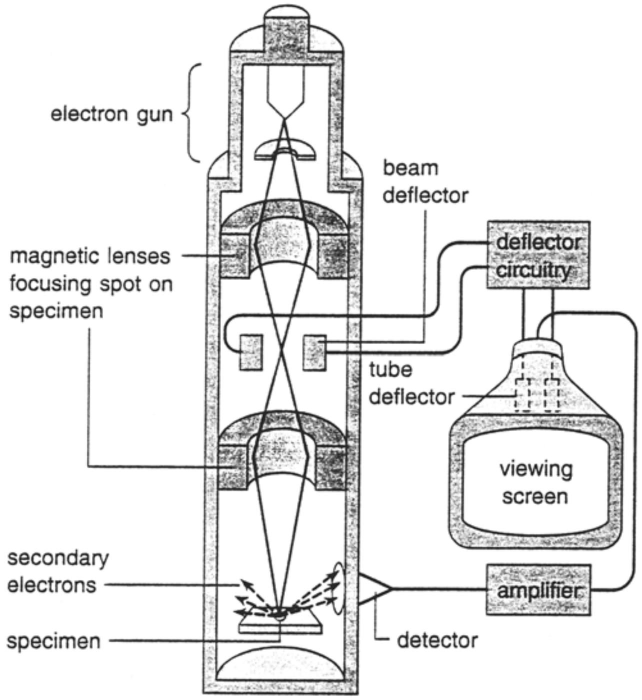
Contrast in electron microscopy is dependent upon atomic number of the atoms in the sample. Biological materials, primarily made of carbon, exhibit low atomic number and exhibit a similar electron scattering as the carbon films on the support grid. To obtain more contrast, samples are stained with salts of heavy metals, such as osmium, uranium and lead. Staining can be carried out before the dehydration and embedding or after sectioning. Different cellular compartments and structures stain differently with the heavy metals.

Electron microscopes are expensive instruments and require substantial training to operate. Electron microscopy will usually require collaboration with someone having expertise in electron microscopy. Many universities have shared instrument facilities in which users pay a fee that includes use of the instrument and technical assistance. Typically fixed samples are provided to the electron microscopy service for further processing and analysis.

- Transmission (TEM)
- Scanning (SEM)
- Shadow-casting
- Freeze-fracture
- Freeze-etching
- Negative staining
- cryoEM

Variations in Electron Microscopy

The standard form of electron microscopy involves shooting an electron beam through the sample. This is refereed to as **transmission electron microscopy**, often abbreviated TEM. **Scanning electron microscopy** (SEM) detects the electrons that are scattered by the specimen to form a 3-dimensional image. The sample is fixed and coated with a thin layer of a heavy metal such as platinum to form a replica of the specimen. This replica is then scanned with a thin beam of electrons and the quanitity of electrons scattered along each successive point of the specimen is measured by detectors which surround the sample (see Figure). Since the amount of electron scattering depends on the angle and depth of the surface relative to the beam, the image has highlights and shadows that give it a three dimensional appearance. The resolution of SEM is not very high (approximately 10 nm with an effective magnification of up to 20,000 times) and only suface features can be examined. Therefore, the technique is generally used to study whole cells or tissues.



Scanning Electron Microscope

A 3-dimensional appearance with higher resolutions than SEM can be obtained by TEM by **shadowing**. In this case the metal coating is applied at an angle resulting in a replica reflects the height and depth of the specimen. Shadowing is often used in conjunction with other techniques. For example, in **freeze-fracture** and **freeze-etching** cells are frozen in cryoprotectant and cut with a knife. Freeze-fracture will often split the lipid bilayer membranes which are then shadowed with platinum. Alternatively, in freeze-etching, the water is sublimated and replicas formed.

Negative staining can be used to visualize macromolecules and supramolecular structures such as virus particles or cytoskeletal filaments. The samples are placed on the electron transparent carbon grids and stained with heavy metals. Areas with biological structures appear more electron transparent.

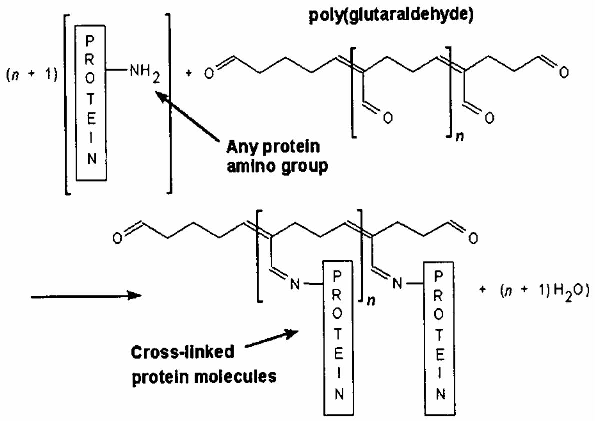
The fixation and manipulation of the specimen will often distort cells. **Cryo-electron microscopy** is used to overcome this problem. Special holders, which keep hydrated specimen at -160^{o}C, allows viewing without fixation, staining or dehydration.

A good overview on light and electron microscopy can be found at:
http://nsm1.fullerton.edu/~skarl/EM/Instruction.html.

MICROSCOPY APPENDIX. ALDEHYDE FIXATIVES

Formaldehyde and glutaraldehyde are a commonly used fixatives for both light and electron microscopy. Formaldehyde is a small molecule (HCHO). The formaldehyde monomers form polymers in aqueous solutions. The liquid known as formalin is 37-40% formaldehyde by weight and most of the polymers are 2-8 units long. Higher polymers (n up to 100) are insoluble in water and sold as a white powder called paraformaldehyde. To be useful as a fixative, the solution must contain monomeric formaldehyde. Dilution of formalin with a buffer at physiological pH results in an almost instantaneous formation of monomers. Conversion of paraformaldehyde to monomers requires heat (typically 60°C) and the addition of hydroxide ions. Commercial formalin also contains about 10% methanol and small amounts of formate ions, whereas a formaldehyde solution prepared from paraformaldehye initially does contain any methanol or formate.

Formaldehyde's mechanism of action is based on the reaction of the aldehyde group with primary amines in proteins. A cross-link between neighboring proteins can also be formed if the primary amines are close enough together. The initial reaction of formaldehyde with protein is complete within 24 hours, but the formation of cross-links, called methylene bridges, proceeds much more slowly (several weeks). Lipids, nucleic acids and carbohydrates are trapped in a matrix of insoluble and cross-linked proteins. In practical terms, formaldehyde penetrates tissues rapidly (small size), buts its reactions with proteins, especially the cross-linking, is slow.



Glutaraldehyde contains two aldehyde groups separated by three methylene bridges ($HCO-[CH_2]_3-CHO$). These two aldehyde groups and the flexible methylene bride greatly increases the cross-linking potential of glutaraldehyde over formaldehyde. In solution glutaraldehyde exists as polymers of various sizes which exhibit an enormous potential for cross-linking proteins (Figure). In contrast with formaldehyde, the chemical reaction of glutaraldehyde with protein is fast, but the penetration of tissue is slower, especially for the larger oligomers. Therefore, an 'EM grade' glutaraldehyde, which contains low polymers, should be used for fixation. In addition, fixation with glutaraldehyde results many leftover free aldehyde groups which cannot be washed out of the tissue. For many applications these free aldehyde groups needs need to be removed or blocked. A common blocking method is to treat with glycine or another small primary amine.

The combination of formaldehyde and glutaraldehyde is also used as a fixative for electron microscopy. This takes advantage of the rapid penetration of formaldehyde molecules, which initiate a structural stabilization of the tissue. A thorough cross-linking is mediated by the more slowly penetrating glutaraldehyde.