Light Microscopy

Leeuwenhoek developed the first powerful microscope

Chromatic aberration

Chromatic aberration is caused by a lens having a different refractive index for different wavelengths of light (the dispersion of the lens).Longitudinal and lateral chromatic aberration of a lens is seen as "fringes" of color around the image, because each color in the optical spectrum cannot be focused at a single common point on the optical axis.

Numerical aperture

Numerical aperture (**NA**) is the light gathering ability of the Lens

The numerical aperture of an optical system such as an objective lens is defined by

$$\mathrm{NA} = n \sin \theta$$

where n is the index of refraction of the medium in which the lens is working (1.0 for air, 1.33 for pure water, and up to 1.56 for oils)

θ is the half-angle of the maximum cone of light that can enter or exit the lens.

In microscopy, NA is important because it indicates the resolving power of a lens.

The size of the finest detail that can be resolved is proportional to λ/NA, where λ is the wavelength of the light.

A lens with a larger numerical aperture will be able to visualize finer details than a lens with a smaller numerical aperture. Lenses with larger numerical apertures also collect more light and will generally provide a brighter image.

NA of the condenser lens control resolution of the objective

Lm = λ / NA of objective + NA of condenser

NA of oil immersion objective is 1 to 1.35

Lm = 0.61 λ / 1.3 = 0.5 λ

In air NA is less than 1.

The practical way to increase NA is to increase refractive index of the medium

Refractive index of Air is 1. Optical lenses less than 1.6. Oil 1.4

Resolution

Optical resolution describes the ability of an imaging system to resolve detail in the object that is being imaged. Lens resolution is the ability of a lens to resolve detail is usually determined by the quality of the lens but is ultimately limited by diffraction.

A measure of the resolving power of a lens is given by its numerical aperture, NA:

$$\delta = \frac{\lambda}{NA}$$

where λ is the wavelength of light. From this it is clear that a good resolution (small δ) is connected with a high numerical aperture.

The ability to distinguish two close points as distinct points is called Resolving power or Limit of resolution

Limit of resolution of light microscope Lm = 0.16 / NA

Limit of resolution of unaided human eye is 100 micrometer

Limit of resolution of optical microscope is 0.25 micrometer

Limit of resolution is inversely proportional to the wavelength of light.

 Lm is determined using the Abbe equation Lm = 0.61λ / NA

Lm is the limit of resolution; λ is the wavelength of light and NA numerical aperture

Lm can be improved by

 1. Using shorter wavelength of light

 2. Increasing refractive index of the medium (n) or Sine of semi angle of light (Sin α)

 Sin α is the sine of angle of light passing into the objective from the specimen

It can be higher than 1. Because Sin 90 degree is equal to 1, while Sine 70 degree = 0.94

For best objective lens semi angle is 70 degree

Resolution of a microscope is roughly ½ the wavelength of the illumination light

Average wavelength of light is 500 – 600 nm which gives a resolution of 250 – 300 nm or 0.25 – 0.3 micrometer.

The visible light of shortest wavelength is around 426 nm giving an Lm of 200 nm or 0.2 micrometer

So superior microscopes uses blue light or blue filters

Magnification

Enlargement or Ratio of size of an object obtained under the microscope and its actual size seen with the naked eye

Magnification equals to the magnification of the objective lens multiplied by the magnification of the eye piece

Working Distance

Distance between the object (or cover glass) and the surface of the front lens of objective

Low power objectives (10X, 45X) have large working distance and smaller NA

High power objectives (100X) have short working distance and large NA

Magnification is also a number describing by which factor an object was magnified. When this number is less than one it refers to a reduction in size, sometimes called **Minification**.

Depth of Field

In optics, particularly as it relates to film and photography, the depth of field (DOF) is the portion of a scene that appears sharp in the image.

The DOF is determined by the subject distance the lens focal length, and the lens *f*-number (relative aperture)

Real Image

A real image is a representation of an actual object (source) formed by rays of light passing through the image. If a screen is placed in the plane of a real image the image will generally become visible.

Virtual Image

A virtual image is an image in which the outgoing rays from a point on the object never actually intersect at a point.

Wavelength

Wavelength is the distance between repeating units of a propagating wave of a given frequency.

It is commonly designated by the Greek letter *lambda* (λ). Examples of wave-like phenomena are light, water waves, and sound waves.

The wavelength is related to the frequency by the formula: wavelength = wave speed / frequency.

Wavelength is therefore inversely proportional to frequency.

Higher frequencies have shorter wavelengths. Lower frequencies have longer wavelengths, assuming the speed of the wave is the same.
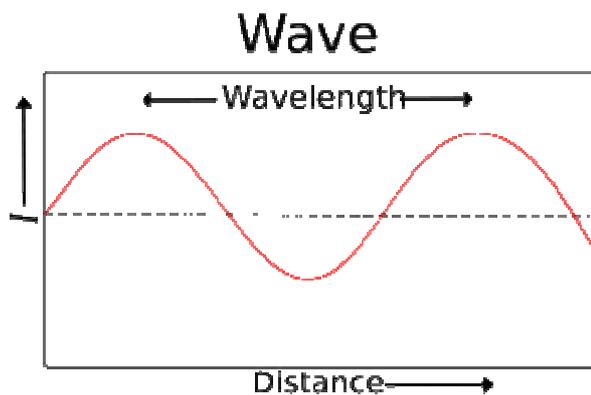
Oil immersion Objective

In light microscopy, **oil immersion** is a technique used to increase the resolution of a microscope. This is achieved by immersing both the objective lens and the specimen in a transparent oil of high refractive index, thereby increasing the numerical aperture of the objective lens.

Immersion oils (Cedarwood oil**)** are transparent oils that have specific optical and viscosity characteristics necessary for use in microscopy. An **oil immersion objective** is an objective lens specially designed to be used in this way

Electron microscope

An electron microscope is a type of microscope that uses a particle beam of electrons to illuminate a specimen and create a highly-magnified image.

Electron microscopes have much greater resolving power than light microscopes that use electromagnetic radiation and can obtain much higher magnifications of up to 2 million times, while the best light microscopes are limited to magnifications of 2000 times.

Transmission Electron Microscope (TEM)

Knoll and Ruska 1932

The original form of electron microscope, the transmission electron microscope (TEM) uses a high voltage electron beam to create an image.

The electrons are emitted by an electron gun, commonly fitted with a tungsten filament cathode as the electron source.

The electron beam is accelerated by an anode typically at +100keV (40 to 400 keV)

Magnification 1 to 3 Lakhs. Resolution 0.25 Angstrom theoretically but 2-10 Angstrom

Resolution depends on the wavelength of electrons (typical 0.5 Å) which in turn depends on the applied voltage

λ (in Å) = 12.2 / $\sqrt{V}$ = If V is 50000 Volt, λ will be 0.5 Å

This wavelength is less than the diameter of the smallest atom – Hydrogen – 1.06 Å

Wavelength of 0.5 Å gives resolution of 0.25 Å in TEM Transmission Electron Microscope (TEM)

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Scanning Electron Microscope (SEM)

Knoll 1935

The Scanning Electron Microscope (SEM) is a type of electron microscope that images the sample surface by scanning it with a high-energy beam of electrons in a raster scan pattern.

The electrons interact with the atoms that make up the sample producing signals that contain information about the sample's surface topography, composition and other properties such as electrical conductivity.

The types of signals made by an SEM can include secondary electrons, back scattered electrons, characteristic x-rays and light (cathodoluminescence). These signals come from the beam of electrons striking the surface of the specimen and interacting with the sample at or near its surface

In a typical SEM, electrons are thermionically emitted from a tungsten filament cathode and are accelerated towards an anode. Tungsten is normally used in thermionic electron guns because it has the highest melting point and lowest vapour pressure of all metals Other electron sources include lanthanum hexaboride (LaB_6) cathodes

The electron beam, which typically has an energy ranging from a few hundred eV to 40 keV, is focused by one or two condenser lenses into a beam with a very fine focal spot sized 0.4 nm to 5 nm

The beam passes through pairs of scanning coils or pairs of deflector plates in the electron column, typically in the final lens, which deflect the beam horizontally and vertically so that it scans in a raster fashion over a rectangular area of the sample surface.

Magnification

15 – 20,000 times. Unlike optical and transmission electron microscopes, image magnification in the SEM is not a function of the power of the objective lens

Resolution

In its primary detection mode, secondary electron imaging, the SEM can produce very high-resolution images of a sample surface, revealing details about 1 to 5 nm in siz

Environmental SEM - ESEM

Conventional SEM requires samples to be imaged under vacuum, because a gas atmosphere rapidly spreads and attenuates electron beams. Consequently, samples that produce a significant amount of vapour, e.g. wet biological samples or oil-bearing rock need to be either dried or cryogenically frozen.

Processes involving phase transitions, such as the drying of adhesives or melting of alloys, liquid transport, chemical reactions, solid-air-gas systems and living organisms in general cannot be observed.

The first commercial development of the Environmental SEM (ESEM) in the late 1980s allowed samples to be observed in low-pressure gaseous environments (e.g. 1-50 Torr) and high relative humidity (up to 100%).

The first commercial ESEMs were produced by the ElectroScan Corporation in USA in 1988. ElectroScan were later taken over by Philips (now FEI Company) in 1996.

ESEM is especially useful for non-metallic and biological materials because coating with carbon or gold is unnecessary.

Uncoated Plastics and Elastomers can be routinely examined, as can uncoated biological samples.

ESEM may be the preferred for electron microscopy of unique samples from criminal or civil actions, where forensic analysis may need to be repeated by several different experts.

Scanning Transmission Electron Microscope (STEM)
Scanning Probe Microscope

The STEM rasters a focused incident probe across a specimen that (as with the TEM) has been thinned to facilitate detection of electrons scattered through the specimen.

The focusing action (and aberrations) occurs before the electrons hit the specimen in the STEM, but afterward in the TEM.

Magnification – up to 100 million times. A single atom can be imaged

Scanning Tunneling Microscope

Small tungsten probe move across the surface of the specimen and causes jumping of electrons

The energy is transformed into corresponding current and the computer builds the image

Detects defects in electronic chips and electric conductors

Magnification – up to 100 million times

Atomic Force Microscope (AFM)

Uses diamond probe consists of a single atom

Probe move over the specimen just like the stylet on a record player.

Oscillations are converted into image by the computer

Used to study DNA, Proteins etc which do not possess electrical conductivity

Reflection Electron Microscope (REM)

In the Reflection Electron Microscope (REM) as in the TEM, an electron beam is incident on a surface

Instead of using the transmission (TEM) or secondary electrons (SEM), the reflected beam of elastically scattered electrons is detected.

This technique is typically coupled with Reflection High Energy Electron Diffraction and Reflection high-energy loss spectrum (RHELS).

Another variation is Spin-Polarized Low-Energy Electron Microscopy (SPLEEM), which is used for looking at the microstructure of magnetic domains.

Sample preparation in Electron Microscopy

Materials to be viewed under an electron microscope may require processing to produce a suitable sample. The technique required varies depending on the specimen and the analysis required

Chemical Fixation for biological specimens aims to stabilize the specimen's mobile macromolecular structure by chemical cross linking of proteins with aldehydes such as formaldehyde and glutaraldehyde, and lipids with osmium tetroxide.

Cryofixation – freezing a specimen so rapidly, to liquid nitrogen or even liquid helium temperatures, that the water forms vitreous (non-crystalline) ice. This preserves the specimen in a snapshot of its solution state.

Dehydration – freeze drying, or replacement of water with organic solvents such as ethanol or acetone, followed by critical point drying or infiltration with embedding resins.

Embedding, biological specimens – after dehydration, tissue for observation in the transmission electron microscope is embedded so it can be sectioned ready for viewing.

To do this the tissue is passed through a 'transition solvent' such as epoxy propane and then infiltrated with a resin such as Araldite epoxy resin; tissues may also be embedded directly in water-miscible acrylic resin.

After the resin has been polymerized (hardened) the sample is thin sectioned (ultra thin sections 0.1 nm or 100 Å) and stained

Staining – uses heavy metals such as lead, uranium or tungsten to scatter imaging electrons and thus give contrast between different structures, since many (especially biological) materials are nearly "transparent" to electrons (weak phase objects).

Typically thin sections are stained for several minutes with an aqueous or alcoholic solution of uranyl acetate followed by aqueous lead citrate.

Negative Staining

In electron microscopy, staining is usually done with heavy metal salts commonly derived from molybdenum, uranium, or tungsten.

Heavy ions are used since they will readily interact with the electron beam and produce phase contrast.

A small drop of the sample is deposited on the carbon coated grid, allowed to settle for approximately one minute, blotted dry if necessary, and then covered with a small drop of the stain (for example 2% uranyl acetate).

After a few seconds, this drop is also blotted dry, and the sample is ready for viewing.

Freeze-fracture or freeze-etch or **freeze&break**

A preparation method particularly useful for examining lipid membranes and their incorporated proteins in "face on" view.

The fresh tissue or cell suspension is frozen rapidly (cryofixed), then fractured by simply breaking or by using a microtome while maintained at liquid nitrogen temperature.

The cold fractured surface (sometimes "etched" by increasing the temperature to about -100°C for several minutes to let some ice sublime) is then shadowed with evaporated platinum or gold at an average angle of 45° in a high vacuum evaporator.

A second coat of carbon, evaporated perpendicular to the average surface plane is often performed to improve stability of the replica coating.

The specimen is returned to room temperature and pressure, and then the extremely fragile "pre-shadowed" metal replica of the fracture surface is released from the underlying biological material by careful chemical digestion with acids, hypochlorite solution or SDS detergent.

The still-floating replica is thoroughly washed from residual chemicals, carefully fished up on EM grids, dried then viewed in the TEM.

Ion Beam Milling – thins samples until they are transparent to electrons by firing ions (typically argon) at the surface from an angle and sputtering material from the surface.

Conductive Coating – An ultra thin coating of electrically-conducting material, deposited either by high vacuum evaporation or by low vacuum sputter coating of the sample.

This is done to prevent the accumulation of static electric fields at the specimen due to the electron irradiation required during imaging.

Such coatings include gold, gold/palladium, platinum, tungsten, graphite etc. and are especially important for the study of specimens with the scanning electron microscope.

Fluorescence microscopy

The absorption and subsequent re-radiation of light by organic and inorganic specimens is typically the result of well-established physical phenomena described as being either **fluorescence or phosphorescence.**

The emission of light through the fluorescence process is nearly simultaneous with the absorption of the excitation light due to a relatively short time delay between photon absorption and emission, ranging usually less than a microsecond in duration.

When emission persists longer after the excitation light has been extinguished, the phenomenon is referred to as phosphorescence.

The technique has made it possible to identify cells and cellular components with a high degree of specificity. For example, certain antibodies and disease conditions or impurities in inorganic material can be studied with the fluorescence microscopy.

British scientist Sir George G. Stokes first described fluorescence in 1852

Fluorescein isothiocyanate, a reactive derivative of fluorescein, has been one of the most common fluorophores chemically attached to other, non-fluorescent molecules to create new and fluorescent molecules for a variety of applications.

Other historically common fluorophores are derivatives of Rhoda mine, coumarin and cyanine.

The basic function of a fluorescence microscope is to irradiate the specimen with a desired and specific band of wavelengths, and then to separate the much weaker emitted fluorescence from the excitation light.

In order to generate sufficient excitation light intensity to produce detectable emission, powerful compact light sources, such as high-energy short arc-discharge lamps, are necessary.

The most common lamps are *mercury burners*, ranging in wattage from 50 to 200 Watts, and the *xenon burners* that range from 75 to 150 Watts.

These light sources are usually powered by an external direct current supply, furnishing enough start-up power to ignite the burner through ionization of the gaseous vapor and to keep it burning with a minimum of flicker.

Confocal microscopy

Confocal microscopy is an optical imaging technique used to increase micrograph contrast and/or to reconstruct three-dimensional images by using a spatial pinhole to eliminate out-of-focus light or flare in specimens that are thicker than the focal plane.

Typical applications include life sciences and semiconductor inspection.

The principle of confocal imaging was patented by **Marvin Minsky in 1957**.

In a conventional (i.e., wide-field) fluorescence microscope, the entire specimen is flooded in light from a light source.

All parts of the specimen throughout the optical path will be excited and the fluorescence detected by a photodetector or a camera

Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM or LSCM) is a technique for obtaining high-resolution optical images.

The key feature of confocal microscopy is its ability to produce in-focus images of thick specimens, a process known as optical sectioning. Images are acquired point-by-point and reconstructed with a computer, allowing three-dimensional reconstructions of topologically-complex objects.

Atomic de Broglie microscope

The atomic de Broglie microscope is an imaging system which is expected to provide resolution at the nanometer scale using neutral

He atoms as probe particles.

Such a device could provide the resolution at nanometer scale and be absolutely non-destructive

It is not developed so well as optical microscope or an electron microscope.

Dark field microscopy

Dark field microscopy is a technique for improving the contrast of unstained, transparent specimens.

Dark field illumination uses a carefully aligned light source to minimize the quantity of directly-transmitted (unscattered) light entering the image plane, collecting only the light scattered by the sample.

Darkfield can dramatically improve image contrast—especially of transparent objects while requiring little equipment setup or sample preparation.

Infrared microscopy

The term infrared microscope covers two main types of diffraction-limited microscopy.

The first provides optical visualization plus IR spectroscopic data collection.

The second (more recent and more advanced) technique employs focal plane array detection for infrared chemical imaging, where the image contrast is determined by the response of individual sample regions to particular IR wavelengths selected by the user.

Ultrasonic force Microscopy

Ultrasonic Force Microscopy (UFM) has been developed in order to improve the details and image contrast on "flat" areas of interest where the AFM images are limited in contrast.

The combination of AFM-UFM allows a near field acoustic microscopic image to be generated.

Ultrasonic force microscopy allows the local mapping of elasticity in atomic force microscopy by the application of ultrasonic vibration to the sample.

Digital Pathology (virtual microscopy)

Digital Pathology is an image-based information environment enabled by computer technology that allows for the management of information generated from a digital slide.

Digital pathology is enabled in part by virtual microscopy, which is the practice of converting glass slides into digital slides that can be viewed, managed, and analyzed.

Ultraviolet Microscopy

Used to view unstained living cells because of strong absorption of ultraviolet by nucleoproteins

Quantitatively measures the amount of nucleoproteins

Uses mercury vapour lamp for illumination

Uses fused Quartz lenses since glass is opaque to ultraviolet

Image is viewed on a fluorescent screen