Microscopy

(greek *mikros* = small; *skopein* = to observe)

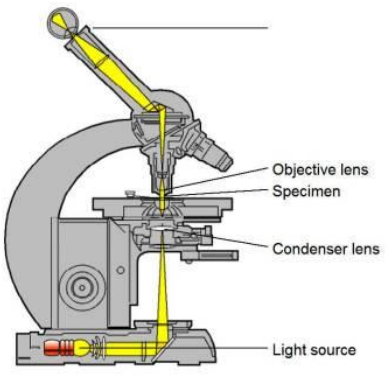
Zacharias Jansen put several lenses in a tube (first compound microscope) and the object near the end of tube appeared to be greatly enlarged, much larger than any simple magnifying glass could achieve. The first microscope was more of a novelty than a scientific tool since maximum magnification was only around 9X and the images were somewhat blurry. No Jansen microscopes survived.

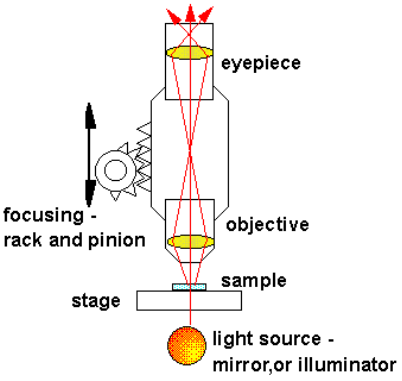
Antony van Leeuwenhoek (1632-1723), Father of Microscopy, a Dutch draper and scientist, and one of the pioneers of microscopy who in the late 17th century became the first man to make and use a real microscope. Van Leeuwenhoek achieved greater success than his contemporaries did by developing ways to make superior lenses, grinding and polishing five hundred and fifty lenses to make his new lens tube that had a magnifying power of 270X and could view objects one millionth of a meter (other microscopes of the time were lucky to achieve 50X magnification). Van Leeuwenhoek made many biological discoveries using his microscopes. He was the first to see and describe bacteria, yeast plantsjotted down as '*Little Animalcules*' although the microscopes were invented quite before.

Ernst Ruska, was conferred on **Nobel Prize in Physics in 1986**, for his fundamental work in electron optics, and for the design of the first electron microscope.

Microorganisms cannot be visualized with naked eyes, as limit of resolution with the naked eye of human being is 200 um. The size of microorganisms generally ranges between 0.2-8u (most of the bacteria and fungi). Viruses are ultramicroscopic, pathogenic viruses of veterinary and medical importance ranges from 17 nanometer to 300 nanometer in size (nanometer- i.e., 10^{-9} meter).

The bacteria and fungi can be visualized by compound microscope, whereas viruses can be visualized only with the help of electron microscope.The limit of resolution of compound microscope is200nm and that of electron microscope is more than 1 nm. The magnification achieved with the help of compound microscope is 1500X and that of electron microscope is more than 1,000,000X





Depending upon the principle on which magnification is based the microscopy is categorized into:

1. Light (Optical) Microscopy &
2. Electron Microscopy
3. Scanning Tunneling and Atomic Force Microscopy

Light microscopy or optical microscopy the magnification is based on a system of optical lenses using light waves. The light microscopy includes:

a. Bright field Microscopy,
b. Dark Field Microscopy,
c. Phase contrast Microscopy
d. Fluorescence Microscopy and
e. Confocal Microscopy

In **electron microscope** beam of electrons is used instead of light waves to produce the image. There are two types:

a. Transmission electron microscopy and
b. Scanning Electron Microscopy

a. Bright field Microscopy: The microscopic field is brightly lighted/illuminated and the microorganisms appear dark as they absorb some of the light.Staining with dyes increases the light absorbing ability that results into good contrast and colordifferentiation. Microscopes of this type produces generally 1000X and 2000X magnification, above which the image becomes blur with bad resolution.

Resolving power:
The ability to distinguish two closely placed spots as two separate spots. Mere greater magnification without ability to distinguish fine details is not useful. Large image without greater resolution may be fuzzy or unclear.

Resolving power is expressed quantitatively as limit of resolution.The limit of resolution depends upon wavelength of light used and numerical aperture (NA) of the lens system.Resolution increases with the decreasing wavelength of light. e.g., Violet color light gives more resolution than red colored light. Electron beams do have very low wavelength, offers maximum resolution.

The limit of resolution of human unaided eye is 200um. i.e., humans cannot see objects smaller than 200um. The resolving power of compound microscope is 0.2 um. And that of electron microscope is 1-10nm.

The limit of resolution can be worked out as follows:

The greatest resolution in optical microscopy is obtained with shortest wavelength of visible light and an objective with maximum NA.

Resolution d= lambda/2NA (NA is 1.3 ,0.55 u –wavelength of green light)

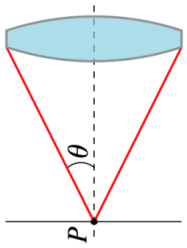
Resolution can be calculated as 0.55/2x1.30=0.21u i.e. smallest details that can be seen with the light microscope are those having 0.2um.

Numerical aperture:

NA of a microscope objective is a measure of its ability to gather light. The more light (higher NA) the better the resolving power of the lens. Numerical aperture of the objective lens is an important consideration in optics as it dictates the angle at which light enters it. Light gathering ability of microscope objective is quantitatively expressed in terms of the numerical aperture (NA). High NA produces image of high resolution.

$$NA = n\sin\theta$$

n-is the refractive index of the medium between the object and objective
Refractive index of oil is 1.56 and that of air is 1.003,Water -1.33 Glycerol -1.47
θ- Half aperture angle formed by the two most divergent rays of light which enter the objective.



Therefore,
NA of dry objective: 1.003 x sin 32° = <1, (Highest practical numerical aperture of a dry lens is 0.95.)

NA of oil immersion objective: 1.56x sin 58° = 1.56X0.85=1.33
Immersion media increase the NA of an objective or a condenser by bringing the beams with higher incidence angle into the light path.

Magnification:

Magnification means number of times the image of a specimen is amplified. 10x i.e., the size of the image is increased by ten times.

Magnifying power of an objective is determined by the dividing the optical tube length by the focal length of lens. Optical tube length is the length of the microscope body tube between the nosepiece opening, where the objective is mounted, and the top edge of the observation tubes where the eyepieces are inserted. In most microscopes, it is fixed i.e., 160mm.
Low power dry objective: 160/16 = 10x
High power dry objective: 160/4 = 40x
Oil immersion objective: 160/1.7 = 94x or approximately 100x

Eye pieces of magnifying power of 5x, 10X & 20X are available.Generally an eye piece having magnification of 10X is used. The optical microscopes are equipped with three objective lens, capable of a different degree of magnification referred as Low power (10X), High power(40X) and Oil immersion(100X).The magnification of an objective is engraved on barrel.

The total magnification of the system is determined by multiplying the magnifying power of an objective by that magnifying power of aneye piece.

In optical microscopy with the best optics, the highest useful magnification achieved is 1400x .

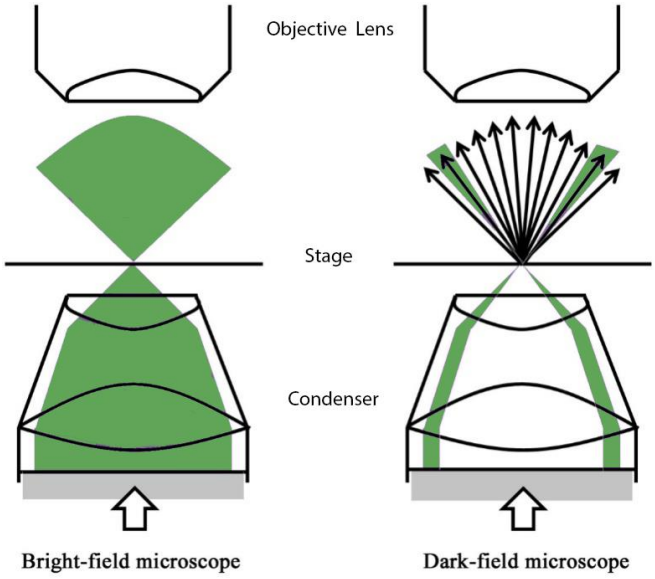
M_{obj}	$M_{eyepiece}$	NA_{obj}	M_{tot}	M_{useful}	Magnification
10x	10x	0.35	100	175-350	low
40x	10x	0.70	400	350-700	ok
100x	10x	1.40	1000	700-1400	ok
100x	15x	1.40	1500	700-1400	empty

Magnification beyond a good resolution is of no value. NA and wavelength of light limit microscope resolution. Mere enlargement of image does not necessarily resolve new features. The magnifying power of the lens is limited. After a certain point, the magnification results in a blurred image and is termed empty magnification. (The airy disk on retina/camera should not exceed twocell/pixel sizes).

Useful magnification = 500 to1000 (Total Magnification) x NA of objective= 1400.

b. Dark field microscopy

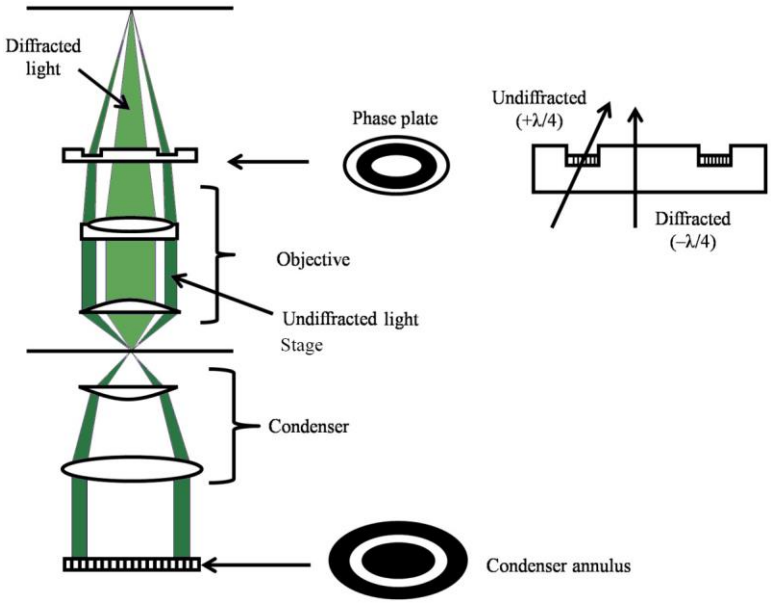
Dark field microscopy is accomplished by equipping the light microscopy with a special kind of condenser that transmits a hallow cone of light from the source of illumination. Not all the light rays directed through the condenser enters the objective. The microscopic field is essentially dark, if the transparent medium contains microbes; the diffracted light enters the objective and reach the eye. The object is illuminated against dark background i.e., microbes appears bright against dark background. Application includes examination of unstained microorganisms (Spirochetes) suspended in a fluid specially wet mounts and hanging drop preparations for bacterial motility assessment.



c. Phase contrast microscopy

Phase contrast microscopy is accomplished by equipping the light microscopy with a phase contrast objective and a phase contrast condenser. Such type of special arrangement of optical systems makes it possible to distinguish unstained structures within the cell, which differs slightly in their refractive indices.

Light passing through one material and into another material with slightly different refractive index and thickness will undergo a change in phase. The differences in phases are translated into variations in brightness of the structure and can be detected by eye in unstained preparation.

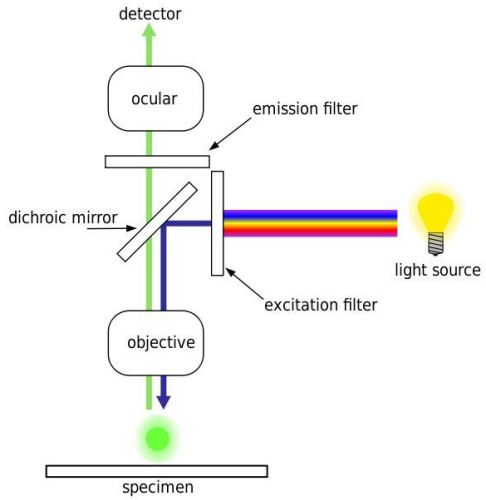


d. Fluorescence microscopy

Fluorescent dye used are the chemical substances, which absorbs light of a particular wavelength, and energy, emits light of longer wavelength and lesser energy. This phenomenon is known as fluorescence and application of this phenomenon is the basis for fluorescence microscopy.
In this microscopy, the microorganisms are stained with a fluorescent ye and then illuminated with blue light; the blue light is absorbed by the fluorescent dye and green light is emitted by the dye used.
After focusing with blue light,the exciter filter removes all but blue light, where as the barrier filters blocks the blue light and allows green light or other light emitted by fluorescing specimen, to pass through and reach eye.

Barrier filters are selected based on dye used.

It is possible to chemically combine fluorescent dyes with antibodies i.e., Labeled antibodies. When specimen stained with labeled antibodies and observed under fluorescence microscopy, such type of microscopy is termed as Immuno-fluorescence.



e. Confocal Microscopy

Laser beam is used to illuminate a variety of planes in the specimen and computer compiles a 3D master image. With confocal microscopy, the living cells can be viewed.

Limitations of light microscopy:

1. Limited useful magnification i.e., 1400X.
2. Limit of resolution is 0.21 um.

Electron Microscopy

Historical aspects in development of electron microscope:

- 1931: German scientists **Max Knoll** (1897–1969) and his pupil **Ernst Ruska** (1906–1988) build the first experimental TEM in Berlin.
- 1933: Ernst Ruska builds the first electron microscope that is more powerful than an optical microscope.
- 1935: Max Knoll builds the first crude SEM.
- 1941: German electrical engineers Manfred Von Ardenne and Bodo von Borries patent an "electron scanning microscope" (SEM).

Examination of viruses and the ultrastructure of microbial cells is only possible with electron microscope. Instead of light source and optics for illumination and creation of image, electron beam and magnetic fields are used. The electron microscope provides tremendous magnification with much higher resolution using very short wavelength of the electron beam to magnify the specimen. By employing 60-80 Kv electrons the wavelength is only 0.05 A It is possible to resolve object as small as 10A The resolution power of electron microscope is 100 times more than optical microscope. It produces useful magnification of 4,00,000X to 10,00,000X.

A high voltage electron gun is used to produce a beam of electrons. Electrons are emitted from a tungsten filament. Electrons are accelerated with an electric field (80 kV or 200 kV, for example) towards the specimenin vacuum chamber (Air can scatter electrons).

Electrons do have poor penetrative power hence, the specimen for electron microscopy should be extremely thin and dry. The specimenis introduced into the instrument at a point between the magnetic condenser and the magnetic objective (Comparable with stage in light microscopy). The magnified image will be seen on fluorescent screen through an airtight window or can be recorded on photo plate with the help of inbuilt camera.
Various techniques such as shadow casting, negative staining, freeze etching, ultrathin sectioning, autoradiography are available for use with electron microscopy for characterizing cellular structures.

a. Scanning electron microscopy

In SE, the specimen is subjected to a narrow electron beam scan over the surface of specimen resulting into shower of secondary electrons and other radiations from the specimen surface. The intensity of the secondary electrons depends upon the shape and chemical composition of irradiated specimen. The secondary electrons collected by a detector which generates electronic signals which are scanned to produce a striking and sharp 3D image (compared to the flat images produced by TEMs) on a screen, similar to a cathode-ray TV screen. Surface topography can be well studied with SE.

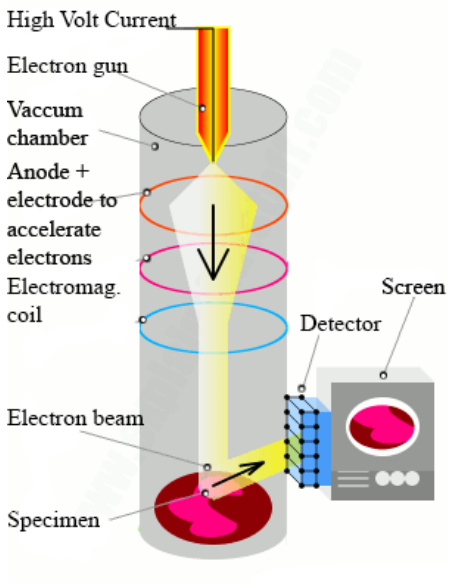
Samples must be fixed, dried and coated with metal.

Advantages:

Almost for all kinds of samples, conducting and non-conducting (stain coating needed);
Based on surface interaction. No requirement of electron-transparent sample.
Produces very sharp, 3D images. Imaging at all directions through **x-y-z (3D)** rotation of sample is possible.

Disadvantages:

Low resolution, SEMs are generally about 10 times less powerful than TEMs (so can use them to see things about 10 nanometers in size).
Usually required surface stain-coating with metals for electron conducting.



Scanning electron microscopy

b. Transmission electron microscopy

In transmission electron microscopy (TEM), a beam of highly focused electrons are directed toward a thinned sample (<200 nm). These highly energetic incident electrons interact with the atoms in the sample producing characteristic radiation and particles providing information for materials characterization. Information is obtained from both deflected and non-deflected transmitted electrons, backscattered and secondary electrons, and emitted photons are focused with the help of coils to form an image on screen or on a photographic plate (for making a permanent record of the image).

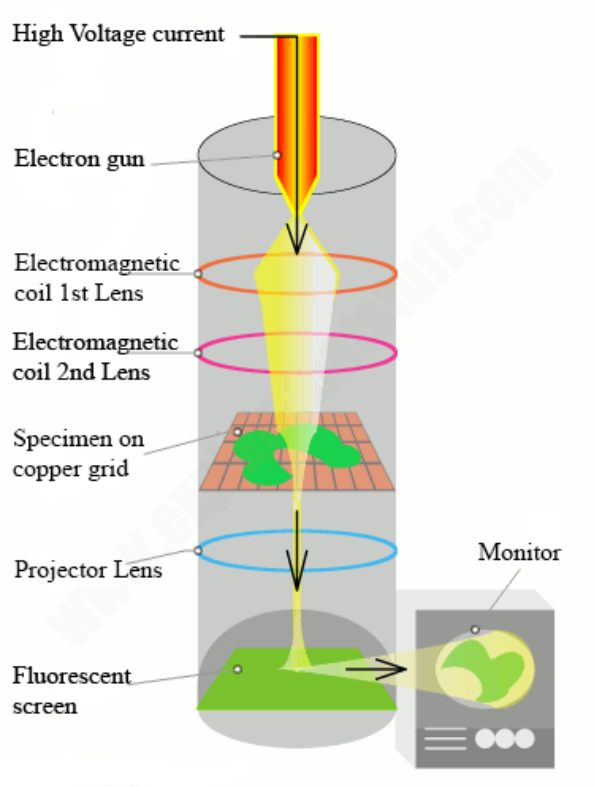
In TEM, a giant electron gun uses electromagnetic coils and high voltages (typically from 50,000 to several million volts) to accelerate the electrons to very high speeds. The faster the electrons travel, the smaller the waves they form and the more detailed the images they show up.

Advantages:

High resolution, as small as 0.2 nm.
No metallic stain-coating needed, thus convenient for structural imaging of organic materials.

Disadvantages:

To prepare an electron-transparent sample is difficult.



Transmission electron microscopy

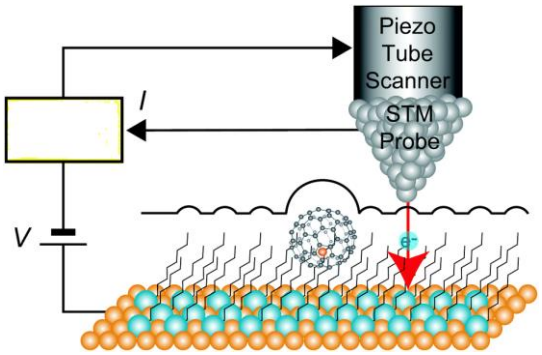
Limitations of Electron Microscopy:

1. Live microbes cannot be examined, as dry specimens are required, as Vacuum has to be maintained to avoid scattering of electron in the presence of air and low penetration power of electrons.

2. Thin sections are required as penetration power of electron is very low.

III. Scanning Force Tunneling and Atomic Force Microscopy

Scanning tunneling microscopes (STMs)



Scanning Tunneling microscopes, STMs were invented by Gerd Binnig and Heinrich Rohrer in 1981. invent the STM and produce detailed images of atoms on the surface of a crystal of gold.

STMs are designed to make detailed images of the atoms or molecules on the surface of something like a crystal. STMs have an extremely sharp metallic probe that scans back and forth across the surface of the specimen. As it does so, electrons try to wriggle out of the specimen and jump across the gap, into the probe, by an unusual phenomenon called "tunneling". The closer the probe is to the surface, the easier it is for electrons to tunnel into it, the more electrons escape, and the greater the tunneling current. The microscope constantly moves the probe up or down by tiny amounts to keep the tunneling current constant. By recording how much the probe has to move, it effectively measures the peaks and troughs of the specimen's surface. A computer turns this information into a map of the specimen that shows up its detailed atomic structure.

Steady tunneling current is maintained between microscope probe and specimen. Up and down movements of probe as it maintains constant current and is used to create image of the surface.

Advantage:
One of the major limitations with ordinary electron microscopes is that they produce amazing detail using high-energy beams of electrons, which tend to damage the objects they are imaging. STMs avoid this problem by using much lower energies.

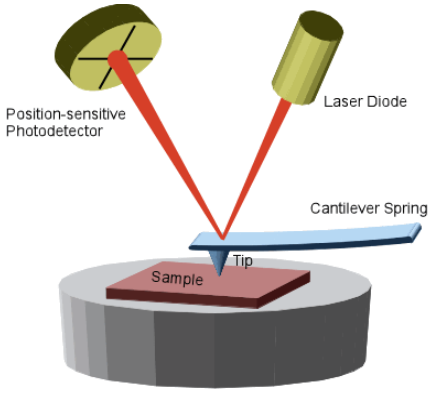
Limitations:
Scan only conductors of electricity, can make images of conductors.

Atomic force microscopes (AFMs)

In 1985, G. Binnig, C. Quate and Christoph Gerber invented the first atomic force microscope (AFM) by attaching a diamond to a piece of gold foil.

AFMs (atomic force microscopes), overcomes the limitations of STMs is that, they rely on electrical currents (flows of electrons) passing through materials, so they can only make images of conductors. AFMs don't suffer from this problem because, although they use still tunneling, they don't rely on a current flowing between the specimen and a probe, can make atomic scale images of non conducting materials such as plastics.

An AFM is a microscope is equipped with a little arm called a cantilever with a tip on the end that scans across the surface of a specimen. As the tip sweeps across the surface, the force between the atoms from which it's made and the atoms on the surface constantly changes, causing the cantilever to bend by minute amounts. The amount by which the cantilever bends is detected by bouncing a laser beam off its surface. By measuring how far the laser beam travels, we can measure how much the cantilever bends and the forces acting on it from moment to moment, and that information can be used to figure out and plot the contours of the surface.



In AFM, probe moves over specimen surface at constant distance. Up and down movement as probe maintains constant distance. AFMs can make images of things at the atomic level and they can also be used to manipulate individual atoms and molecules—one of the key ideas in nanotechnology.

Comparison between AFM and Electronic Microscopes

Optical and electron microscopes can easily generate two-dimensional images of a sample surface, with a magnification as large as 1000X for an optical microscope, and a few hundred thousands ~100,000X for an electron microscope. However, these microscopes cannot measure the vertical dimension (z-direction) of the sample, the height (e.g. particles) or depth (e.g. holes, pits) of the surface features.

AFM, which uses a sharp tip to probe the surface features by raster scanning, scan image, the surface topography with extremely high magnifications, up to 1,000,000X, comparable or even better than electronic microscopes. The measurement of an AFM is made in three dimensions, the horizontal X-Y plane and the vertical Z dimension. Resolution (magnification) at Z-direction is normally higher than X-Y.

Technique	Image Formed By	Lowest Resolvable Unit	Approx Lower Limit
Optical Microscopy	Light Rays	Microns (µm)	1 µm (monochromatic light)
Transmission Electron Microscopy	Electrons	Angstroms (Å)	2 Å (high resolution TEM)
Scanning Electron Microscopy	Electrons	Nanometers (nm) to Angstroms (Å)	10 nm(100 Å)
Atomic Force & Scanning Tunneling Microscopies	Molecular Mechanical Probes	Angstroms (Å)	40 Å(theoretical)

Reference:

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Microbiology, Michael J Pelczar, Jr., E.C.S. Chan, Noel R Krieg, The Microscopic Examination of Microorganisms 5th Edition, Pg.50-69

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