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PG AND RESEARCH DEPARTMENT OF BIOCHEMISTRY
E-NOTES

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UNIT-I: Microscopy

Principles,application,light,contrast microscope,fluorescence,scanning,transmission electron micropes.

MICROSCOPY-PRINCIPLES

Microorganisms are too small to be seen by our unaided eyes and the microscopes are of crucial importance as they help to view the microbes. A microscope is an optical instrument consisting of one or more lenses in order to magnify images of minute objects. Thus it is important to gain a preliminary knowledge about the principles of microscope and its types. This chapter gives a brief introduction to microscopy.

PROPERTIES OF LIGHT

To understand how a light microscope operates, one must know something about the way in which lenses bend and focus light to form images.

When a ray of light passes from one medium to another, **refraction** occurs, i.e., the ray is bent at the interface. The **refractive index** is a measure of how greatly a substance slows the velocity of light, and the direction and magnitude of bending is determined by the refractive indexes of the two media forming the interface.

When light passes from air into glass, a medium with a greater refractive index, it is slowed and bent toward the normal, a line perpendicular to the surface. As light leaves glass and returns to air, a medium with a lower refractive index, it accelerates and is bent away from the normal. Thus a prism bends light because glass has a different refractive index from air, and the light strikes its surface at an angle. Lenses act like a collection of prisms operating as a unit. When the light source is distant so that parallel rays of light strike the lens, a convex lens will focus these rays at a specific point, the **focal point**. The distance between the center of the lens and the focal point is called the **focal length**. Our eyes cannot focus on objects nearer than about 25 cm or 10 inches. This limitation may be overcome by using a convex lens as a simple magnifier (or microscope) and holding it close to an object. A magnifying glass provides a clear image at much closer range, and the object appears larger. Lens strength is related to focal length; a lens with a short focal length will magnify an object more than a weaker lens having a longer focal length.

PRINCIPLES OF LIGHT MICROSCOPY

The light is the primary source on which magnification is based in light microscopes. The magnification is obtained by a system of optical lenses using light waves. Magnification refers the number of times a specimen is appeared to be larger than its original size.

BASIC UNITS FOR MICROSCOPE

1 meter = 1000 millimeter

1 millimeter = 1000 micrometer (μm) = 10^{-6} meter

1 micrometer = 1000 nanometer (nm) = 10^{-9} meter

1 Angstrom (1 A) = 10^{-10} meter

1 nanometer = 10 Angstrom

Relative size of the microorganisms and their visibility. Man can see about 0.5 mm sized object whereas the light microscopes can be used to visualize upto 1 μm and EM (electron microscopes) can be used to view 1 nm objects.

BASIC QUALITY PARAMETERS OF MICROSCOPIC IMAGES

The microscopic images should have four basic quality parameters, through which the microscopes can be graded.

1. **Focus:** It refers whether the image is well defined or blurry (out of focus). The focus can be adjusted through course and fine adjustment knobs of the microscope which will adjust the focal length to get clear image. The thickness of specimen, slide and coverslip also decide the focus of the image. (Thin specimens will have good focus).
2. **Brightness:** It refers how light or the dark the image is. Brightness of the image is depends on the illumination system and can be adjusted by changing the voltage of the lamp and by condenser diaphragm.
3. **Contrast:** It refers how best the specimen is differentiated from the background or the adjacent area of microscopic field. More the contrast will give good images. It depends on the brightness of illumination and colour of the specimen. The contrast can be achieved by adjusting illumination and diaphragm and by adding colour to the specimen. The phase contrast microscopes are designed in such a way that the contrast can be achieved with out colouring the specimen.
4. **Resolution:** It refers the ability to distinguish two objects close to each other. The resolution depends on the resolving power, which refers minimum distance between the two objects which can be distinguishable.

MAGNIFICATION AND RESOLUTION

The total **magnification** of compound microscope is the product of the magnifications of objective lens and eyepiece. Magnification of about 1500x is the upper limit of compound microscopes. This limit is set because of the resolution.

Resolution refers the ability of microscopes to distinguish two objects close to each other, it depends on resolving power, which refers the minimum distance. Ex : Man has the resolving power of 0.2 mm (meaning that he can distinguish two objects with a distance of 0.2 mm close to each other) If he want to see beyond the limit of his resolving power, further magnification is necessary.

$$\textbf{Resolving power} = \frac{\mu}{n(\sin\theta)}$$

where, μ is the wave length of light source and n (sin θ) is the numerical aperture (NA).

For compound microscopes, resolving power is μ/2NA. The resolving power of an microscope can be improved either by reducing the wave length of light or by increasing the n(sin θ) value.

Numerical aperture (n sin$^{\theta}$) measures how much light cone spreads out between condenser & specimen. More spread of light gives less resolving power means better resolution. The numerical aperture depends on the objective lens of the microscope. There are two types of objective lenses are available in any compound microscope.

THE LIMIT OF RESOLUTION

The limit of resolution refers the smallest distance by which two objects can be separated and still be distinguishable or visible as two separate objects.

Optical Instrument	Resolving Power	RP in Angstroms
Human eye	0.2 millimeters (mm)	2,000,000 A^{o}
Light microscope	0.20 micrometers (µm)	2000 A^{o}
Scanning electron microscope (SEM)	5-10 nanometers (nm)	50-100 A^{o}
Transmission electron microscope (TEM)	0.5 nanometers (nm)	5 A^{o}

TYPES OF MICROSCOPE

Microbiologists use a variety of microscopes, each with specific advantages and limitations. Microscopes are of two categories.

a. Light Microscope: Magnification is obtained by a system of optical lenses using light waves. It includes (i) Bright field (ii) Dark field (iii) Fluorescence (iv) Phase contrast and (v) UV Microscope.

b. Electron Microscope: A system of electromagnetic lenses and a short beam of electrons are used to obtain magnification. It is of two types: (I) Transmission electron microscope (TEM) (ii) Scanning electron microscope (SEM).

LIGHT MICROSCOPE

Light microscopy is the corner stone of microbiology for it is through the microscope that most scientists first become acquainted with microorganisms. Light microscopes can be broadly grouped into two categories.

1. **Simple microscope:** It consists of only one bi-convex lens along with a stage to keep the specimen.

2. **Compound microscope:** It employs two separate lens systems namely, (i) objective and (ii) ocular (eye piece).

BRIGHT FIELD MICROSCOPE

The compound student microscope is a bright field microscope. It consists of mechanical and optical parts.

1. Mechanical parts

These are secondary but are necessary for working of a microscope. A 'Base', which is horsehoe, shaped supports the entire framework for all parts. From the base, a 'Pillar' arises. At the top of the pillar through an 'Inclination Joint' arm or limb is attached. At the top of the pillar, a stage with a central circular opening called 'Stage aperture' is fixed, with a stage clip to fix the microscopic slide. Beneath the stage, there is one stage called 'sub stage' which carries the condenser. At the top of the arm, a hollow cylindrical tube of standard diameter is attached in-line with the stage aperture, called 'body tube'. The body tube moves up and down by two separate arrangements called 'coarse adjustment' worked with pinion head and 'fine adjustment' worked with micrometer head. At the bottom of the body tube an arrangement called 'revolving nose-piece' is present for screwing different objectives. At the top of the body tube eye- piece is fixed.

2. Optical parts

It includes mirror, condenser, objective and ocular lenses. All the optical parts should be kept in perfect optical axis.

a. Objectives : Usually 3 types of magnifying lenses (i) Low power objective (10x) (ii) High dry objective (45x) and (iii) Oil immersion objective (100x)

b. Eye-piece : Mostly have standard dimensions and made with different power lenses. (5x, 10x, 15x, 20x). A compound microscope with a single eyepiece is said to be monocular, and one with two eyepieces is said to be binocular.

c. Condenser : Condenses the light waves into a pencil shaped cone thereby preventing the escape of light waves. Also raising or lowering the condenser can control light intensity. To the condenser, iris diaphragm is attached which helps in regulating the light.

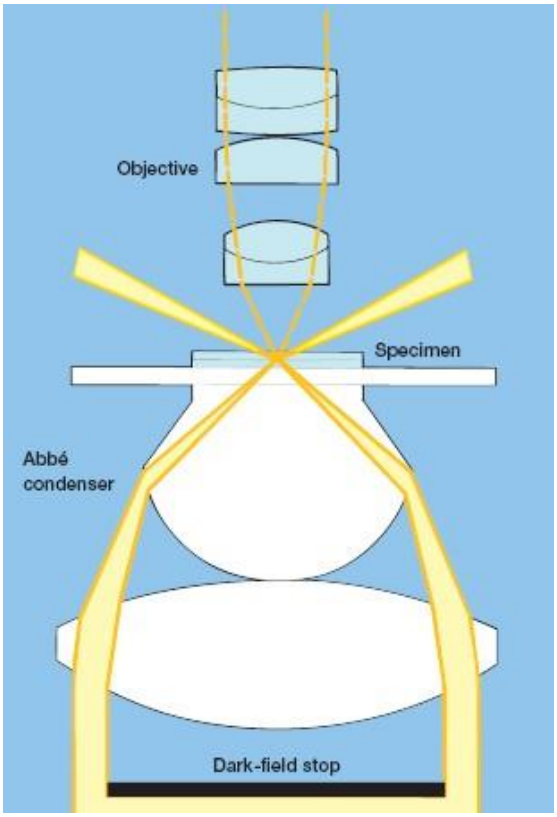
d. Mirror : It is mounted on a frame and attached to the pillar in a manner that it can be focused in three different directions. The mirror is made of a lens with one plane surface and another concave surface. Plane surface is used, when the microscope is with a condenser.

In case of microscopes with oil immersion, when light passes from a material of one refractive index to material of another, as from glass to air or from air to glass, it bends. The refractive index of air is 1.0, which is less than that glass slide (1.56). So, when light passes from glass (dense medium) to air (lighter medium), the rays get refracted, which led to loss of resolution of image. Light of different wavelengths bends at different angles, so that as objects are magnified the images become less and less distinct. This loss of resolution becomes very apparent at magnifications of above 400x or so. Even at 400x the images of very small objects are badly distorted. Placing a drop of oil (Cedar wood oil) with the same refractive index (1.51) as glass between the cover slip and objective lens eliminates two refractive surfaces and considerably enhances resolution, so that magnifications of 1000x or greater can be achieved. Oil immersion **is essential for viewing individual bacterial cell**. A disadvantage of oil immersion viewing is that the oil must stay in contact, and oil should be viscous.

DARK-FIELD MICROSCOPE

In dark-field microscopy, specimen is brightly illuminated against a dark background. This type of microscope possesses a special type of condenser, which prevents the parallel and the

oblique rays entering in to the objective and thus making the microscopic field dark. In the absence of specimen the entire field will appear as dark. In the presence of specimen, which differs in refractive index, the oblique rays are scattered by reflection and refraction and the scattered rays enter the objective making the specimen brightly illuminated.



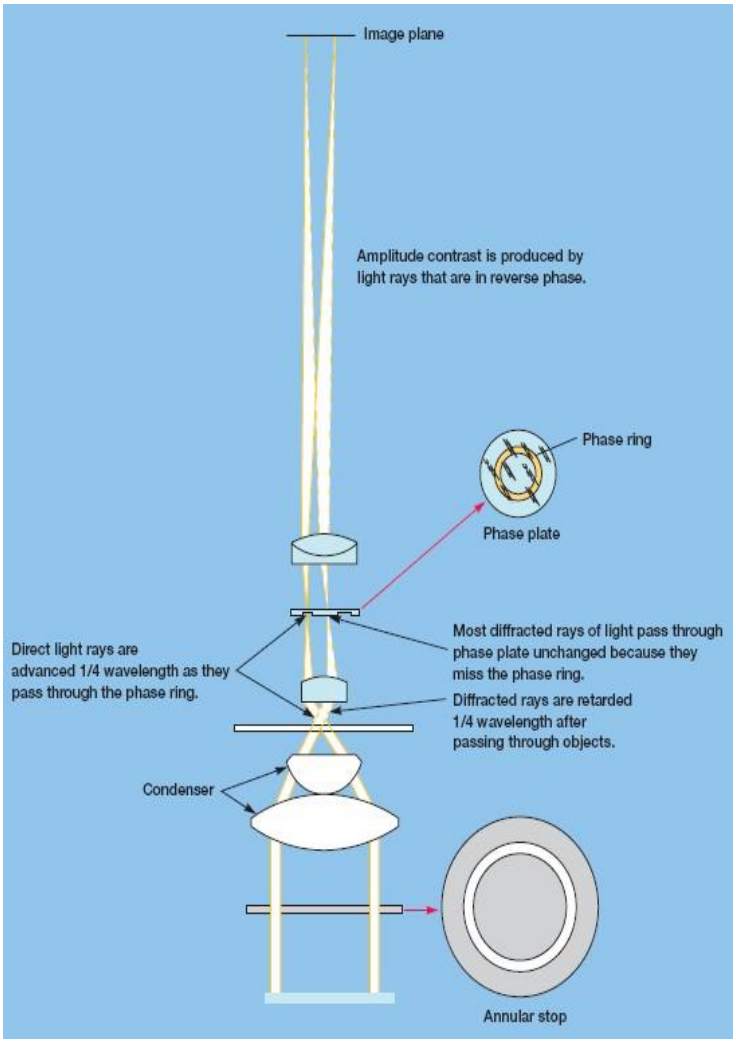
Dark-Field microscope

Maximum magnification of 1500x and resolution of 0.1 – 0.2 μm can be obtained. It is useful in studying the morphology and motility of microorganisms. Dark field is especially useful for finding cells in suspension. Dark field makes it easy to obtain the correct focal plane at low magnification for small, low contrast specimens. The following are its uses:

a. Initial examination of suspensions of cells such as yeast, bacteria, small protists, or cell and tissue fractions including cheek epithelial cells, chloroplasts, mitochondria, even blood cells (small diameter of pigmented cells makes it tricky to find them sometimes despite the color).
b. Initial survey and observation at low powers of pond water samples, hay or soil infusions, purchased protist or metazoan cultures.
c. Examination of lightly stained prepared slides. Initial location of any specimen of very small size for later viewing at higher power.
d. Determination of motility in cultures

PHASE CONTRAST MICROSCOPE

In the 1930's Frederick Zernike devised a method of converting phase changes into differences in light intensity. This invention leads to the development of phase contrast microscope.



Phase contrast microscope

It has a special type of condenser, objective and a special magnifier. Light passing from one material into another of slightly different refractive index will undergo a change in the phase. This change in the phase of the light wills in-turn increase the contrast. A system of rings in the condenser and objective separate the diffracted rays from the specimen. These diffracted rays from the specimen and the undiffracted rays combine and the phase difference is converted into difference in light intensity. In phase-contrast microscopy, unstained/living organisms can be examined. As a result, the internal contrast of various parts of a specimen against its surroundings is increased. It has a resolving power of 0.1 – 0.2 μm.

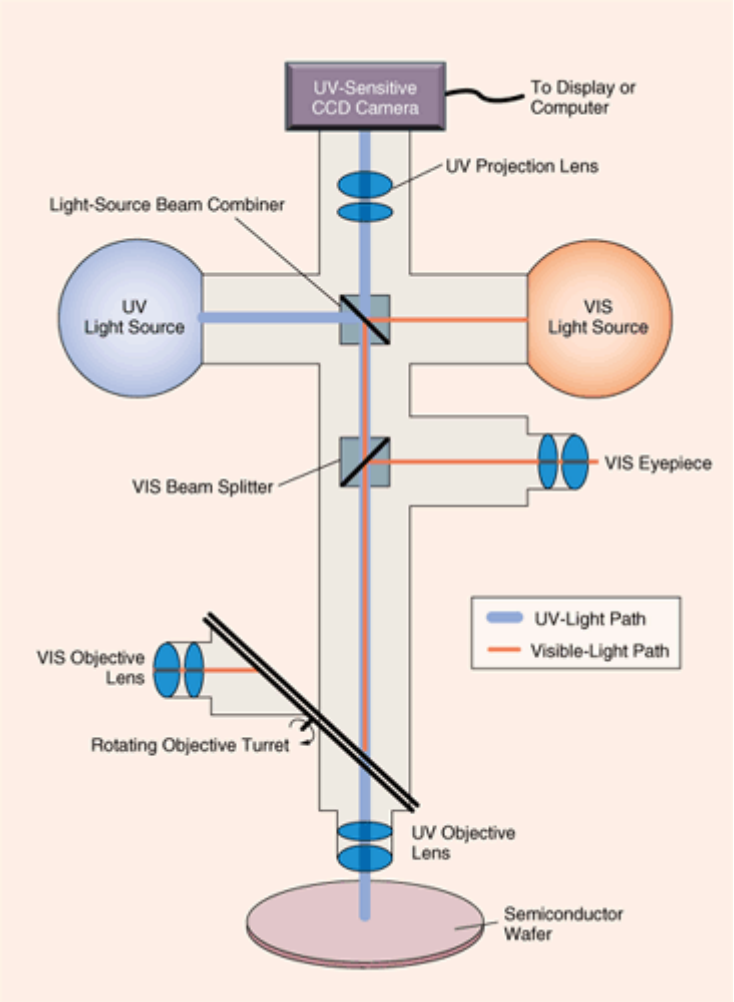
To the left: Arrangement of the ring-shaped mask below the objective and of the phase-ring within the objective

To the right: The path of light rays within a phase-contrast microscope. 1. ring-shaped mask, 2. Condenser, 3. Specimen, 4. Objective, 5. Phase plate, 6. Focal plane of the objective. The wave character of the light is indicated by the change of light and dark areas.

UV MICROSCOPE

Resolution of a microscope depends upon the wavelength of light used. If, longer the wavelength of light used, lower will be the resolving power while shorter the wavelength,

more will be the resolution. With this principle, UV rays of shorter wavelength are used as light source. Since UV rays can't penetrate the glass, quartz lenses are used. Since the UV rays are invisible, photographic plates should be used to record the image or special type of filters should be used to eliminate the UV rays from reaching the eyepiece. This is used in conjunction with fluorescent microscopy. Upon illumination with UV light certain fluorescent dyes emit light in visible range, which can be directly viewed.

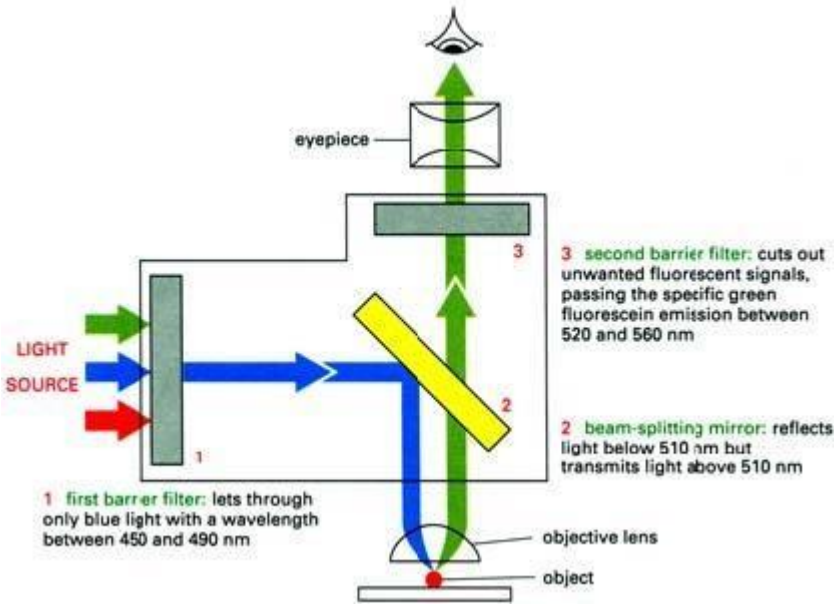


Working principles of UV microscope

FLUORESCENCE MICROSCOPE

Certain chemical compounds absorb light and reemit part of the radiant energy as light of longer wavelength. Such substances are called fluorescent and the phenomenon is termed as fluorescence. In fluorescence microscopy, a high intensity mercury lamp is used as the light source, which emits white light. The exciter filter transmits only blue light to the specimen and blocks out all the colours. The blue light is reflected downward to the specimen by the dichroic mirror. The specimen is stained with fluorescent dye (acrydine orange). Only certain portions of the specimen retain the dye, others do not. The stained portion of the specimen absorb blue light and emit green light, which passes upwards, penetrate the dichroic mirror and reaches the barrier filter. This filter allows only green light to pass through and the eye receives only green light emitted from the specimen against the black background whereas unstained portions are invisible. Also, ultraviolet light is used to excite molecules so that they release light of a different wavelength.

This technique is especially important in **immunology** in which the reactions of antigens and antibodies are studied in great detail. Fluorescent antibody staining is now widely used in diagnostic procedures to determine whether an antigen is present. Not all bacteria get stained with fluorescent chemicals. *Excitation and fluorescence with chromatic beam splitters.* Similar to the interference filters these are specially coated mirrors used under 45° to the illuminating beam. They reflect certain spectral ranges, while others are completely transmitted. The separating line between reflection and transmission may be set at any point of the spectrum. 1. Exciting radiation, 2. Fluorescence emission



Working Principles of Fluorescence Microscope

Confocal Laser Scanning Microscope (CLSM)

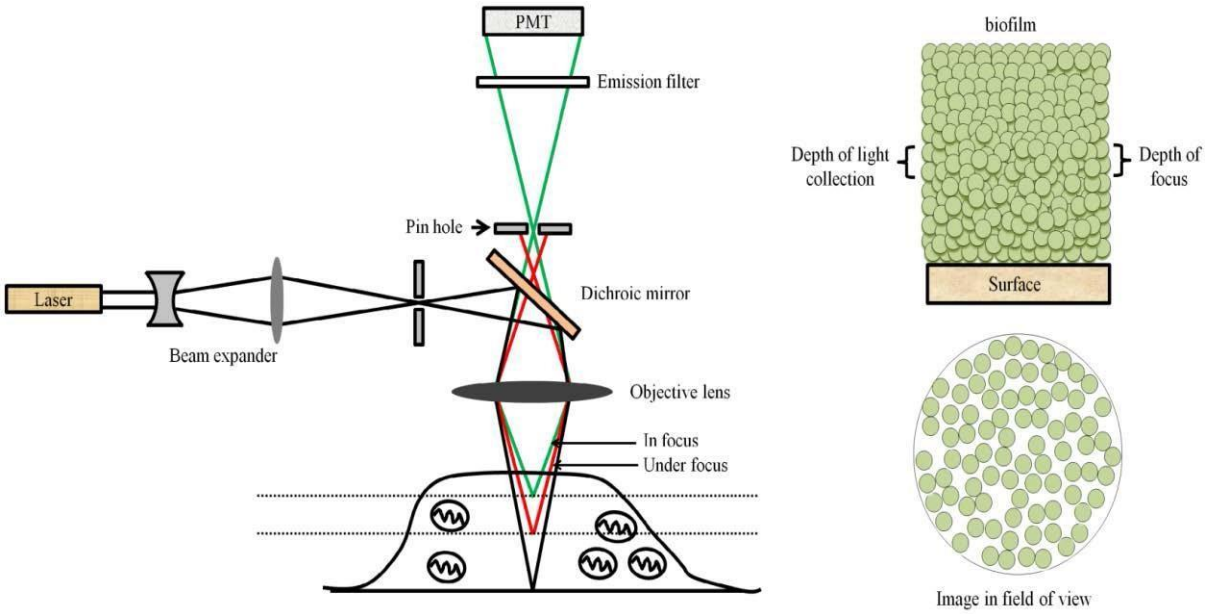
A schematic diagram of a confocal laser scanning microscope is shown in figure 16.2A. Let us see how exactly a CLSM works:

i. Light source and illumination: Light sources used in confocal microscopes are lasers. The microscope works in epi-illumination mode. The laser beam is spread by a diverging lens so as to fill the back aperture of the objective lens which functions as condenser as well. The expanded laser light is reflected by the dichroic mirror on the objective that focuses the light as an intense diffraction-limited spot on the sample. The fluorescence from the illuminated spot is collected by the objective and sent to the eyepiece/camera/detector through a pinhole aperture.

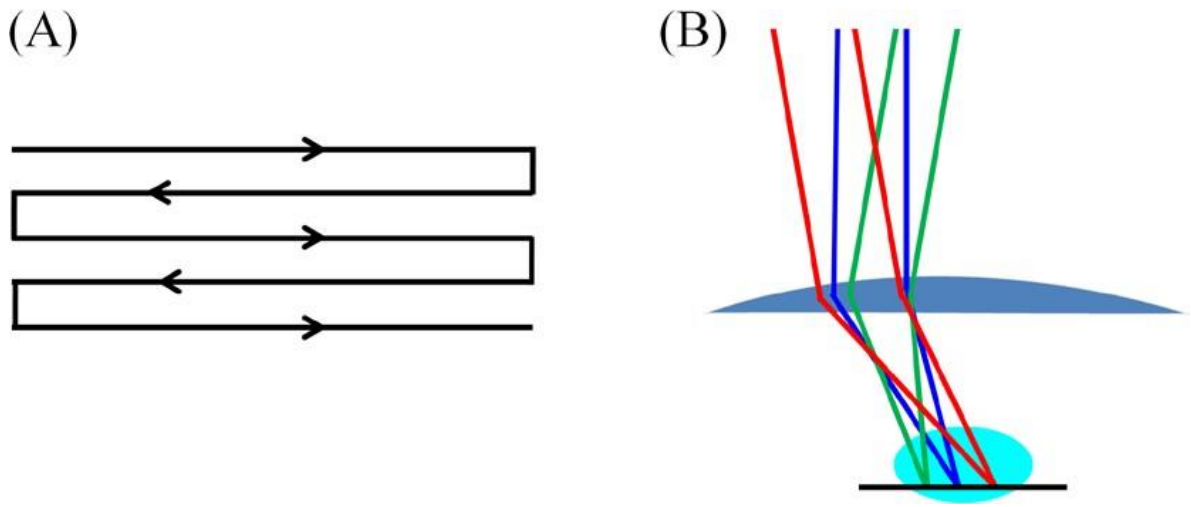
ii. Pinhole aperture: The fluorescence light emitted by the illuminated sample is focused as the confocal point at the pinhole. Any light coming from below or above the focal plane is blocked by the pinhole plate.

iii. Raster scanning: As the fluorescence is detected from a diffraction limited spot, the focused laser spot is scanned over the sample in a raster fashion collecting light from the entire focal plane (Figure 16.3A). The laser spot is scanned over the sample by changing the direction of the incident radiation as shown in Figure 16.3B. As the position of the illuminating spot changes, the pinhole moves so as to be confocal with the illuminated spot of the same focal plane.

iv. Emission filter: The light that passes through the pinhole is filtered by the emission filter before it reaches the detector.



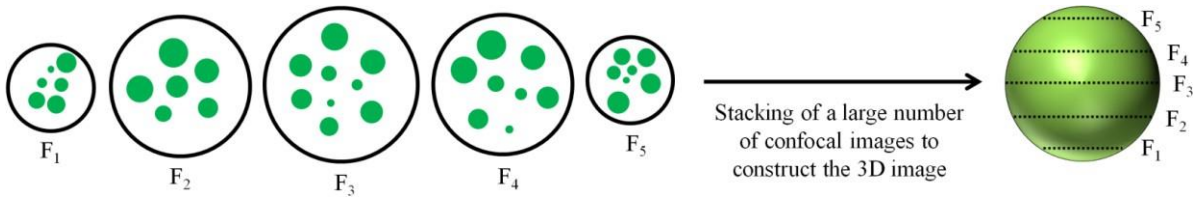
Optical diagram of a confocal laser scanning microscope; the pinhole rejects the light coming from non-confocal planes (A); a hypothetical image generated from the light coming from the focal plane.



A raster scan (A); raster scanning by changing the direction of the exciting radiation (B).

Optical sectioning and three-dimensional reconstruction

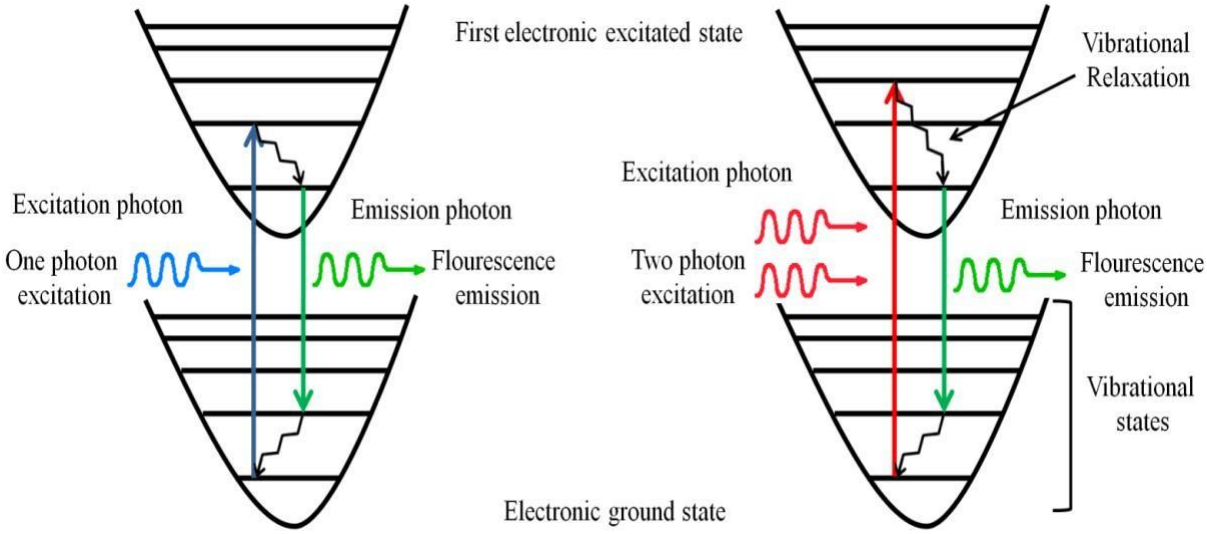
A confocal microscope records the intensity of all the diffraction-limited spots in a focal plane, essentially providing an optical section of the sample. This can be understood as a plot of intensity in a two-dimensional coordinate system. Obtaining such plots for closely spaced focal planes allows three-dimensional reconstruction of the sample by stacking the images



A diagram showing images recorded from five different focal planes and three-dimensional reconstruction of the object by stacking a large number of images from different focal planes.

Two photon and multiphoton laser scanning microscopy

If a fluorophore absorbs the light of energy, $E = \frac{hc}{\lambda}$, where λ is the wavelength of the absorbed radiation; it is possible to excite the fluorophore with the light of wavelength 2λ if two photons are simultaneously absorbed by the molecule



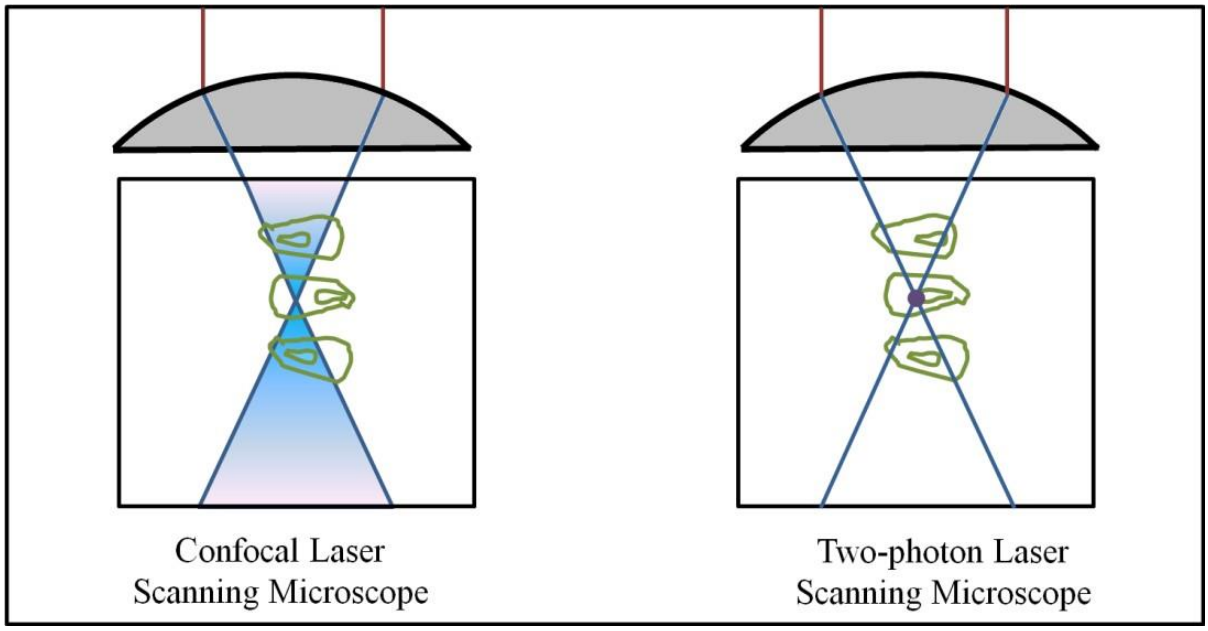
A simplified Jablonski diagram showing single-photon and two-photon excitation of a fluorophore

The probability of simultaneous absorption of two photons is very small; multiphoton microscopes therefore need very intense light sources. Pulsed infrared lasers, however, have realized the multiphoton microscopy. Titanium:sapphire lasers operating at 800 nm can cause excitation of the fluorophores with $\lambda_{max} \sim 400$ nm through two photon absorption. Multiphoton fluorescence microscopy offers following advantages over single photon microscopy:

i. Biological specimens absorb the near-IR radiation very poorly as compared to the UV and blue green radiation, the electromagenetic region commonly used for fluorescence microscopy; this implies that a thicker specimen can be studied using multiphoton microscopy.

ii. As the fluorophores are excited at $\sim 2\lambda$ in a two photon fluorescence imaging experiment, the incident and the emitted radiations are well separated; this separation allows detection of the emitted radiation clear of the excitation radiation and the Raman scattering.

iii. The probability of simultaneous absorption of two photons depends on the square of the light intensity. The laser light in a two-photon set up does not excite the fluorophores along its path due to insufficient photon density to cause two-photon absorption. A photon density high enough to cause excitation is achieved only at the focus, thereby exciting the molecules only in the focal plane. A multiphoton microscope therefore does not require a pinhole for recording confocal images.



A comparison of the excitation region in a confocal laser scanning microscope and a two photon laser scanning microscope.

ELECTRON MICROSCOPE

In electron microscope, short beam of electrons and magnetic condenser lenses are employed to produce the image. The electrons have short wavelength, which helps in better resolution. It is possible to resolve objects as small as 10°A, which is 100 times more than that of light microscope. It can magnify object up to 200,000X.

In electron microscope, a hot tungsten filament forms the source of electrons. The object is placed in the path of moving electrons. Since electrons move only in the vacuum, the entire path of electrons should be kept under vacuum. The magnetic condenser lens causes the primary magnification. A second magnetic lens amplifies the primary image and this image is viewed on a fluorescent screen or captured on photographic plates. There are two types of electron microscope.

a. Transmission electron microscope (TEM)
b. Scanning electron microscope (SEM)

TRANSMISSION ELECTRON MICROSCOPE

A high voltage established between the filament and the anode accelerates the electrons from the hot tungsten filament. The electron beam is focused on the specimen with an electromagnetic condenser. Ultra thin sections of the specimen must be prepared since electrons can penetrate matter only a short distance. This is done by embedding or freezing the specimen and sectioning it with a diamond or a glass Knife. The sections are floated in water and collected in a copper grid. They are stained with heavy metals such as gold or palladium and kept within the evacuated column of the electron microscope.

TEM has a projector lens that project the image onto a fluorescent viewing screen or film plate, because the beam cannot be viewed directly. With TEM greater resolution and higher magnifications than light and Scanning Electron Microscope can be obtained. In TEM, the differential scattering of electrons by the specimen makes the contrast. Since most of the atoms of the biological material are of low mass, the contrast of the specimen is low. Staining with heavy metals such as platinum, uranium or tungsten can increase the contrast.

They are of various types such as,

a. **Positive staining:** The heavy metals are fixed on the specimen.

b. **Negative staining:** It is used to increase the electron opacity of the surrounding area.

Two techniques commonly employed for the observation of biological specimen are:

a. **Metal shadowing:** The dried specimen is exposed at an acute angle to a stream of heavy metals like platinum, palladium or gold and thereby producing an image, that reveals the three-dimensional structure of the object.

b. **Freeze fracturing:** The frozen specimen is fractured with a knife, and the exposed surface is coated with a heavy metal (Gold) at an acute angle. A supporting layer of carbon is evaporated on the metal surface. Then the specimen is destroyed and the replica is examined. This method is used for studying cell wall and cell membrane. The penetrating power of electrons is low; hence the ultra thin sections of the specimens should be used.

SCANNING ELECTRON MICROSCOPE

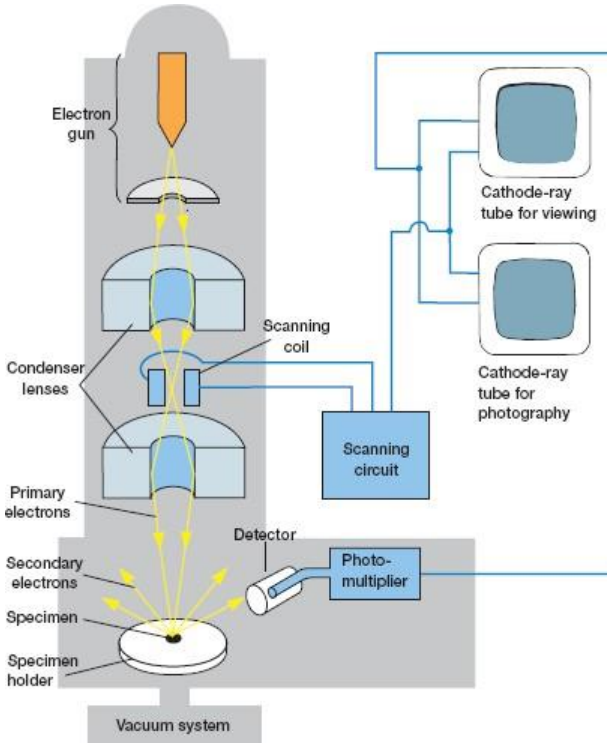
The specimen is coated with a thin layer of heavy metal and the specimen is subjected to a narrow beam of electrons, which rapidly moves and scans the surface of the specimen. The irradiated specimen depending upon its physical and chemical composition will release secondary electrons. These secondary electrons are then collected by anode detector, which generates an electronic signal. Then the electronic signal is scanned in TV system to produce an image on a cathode ray tube. Magnification on SEM is about 75,000 to 1,00,000 times.

Limitations

a. Specimen is kept under high vacuum on the path of electron beam. So, living cells can’t be examined.

b. Electrons have low penetration capacity, hence ultra thin section and staining should be done which is time consuming and also sometimes alter or distort the structures of microorganisms.

c. High cost and specialized techniques prevent its use in all microbiology labs in spite of greater magnification and resolution.



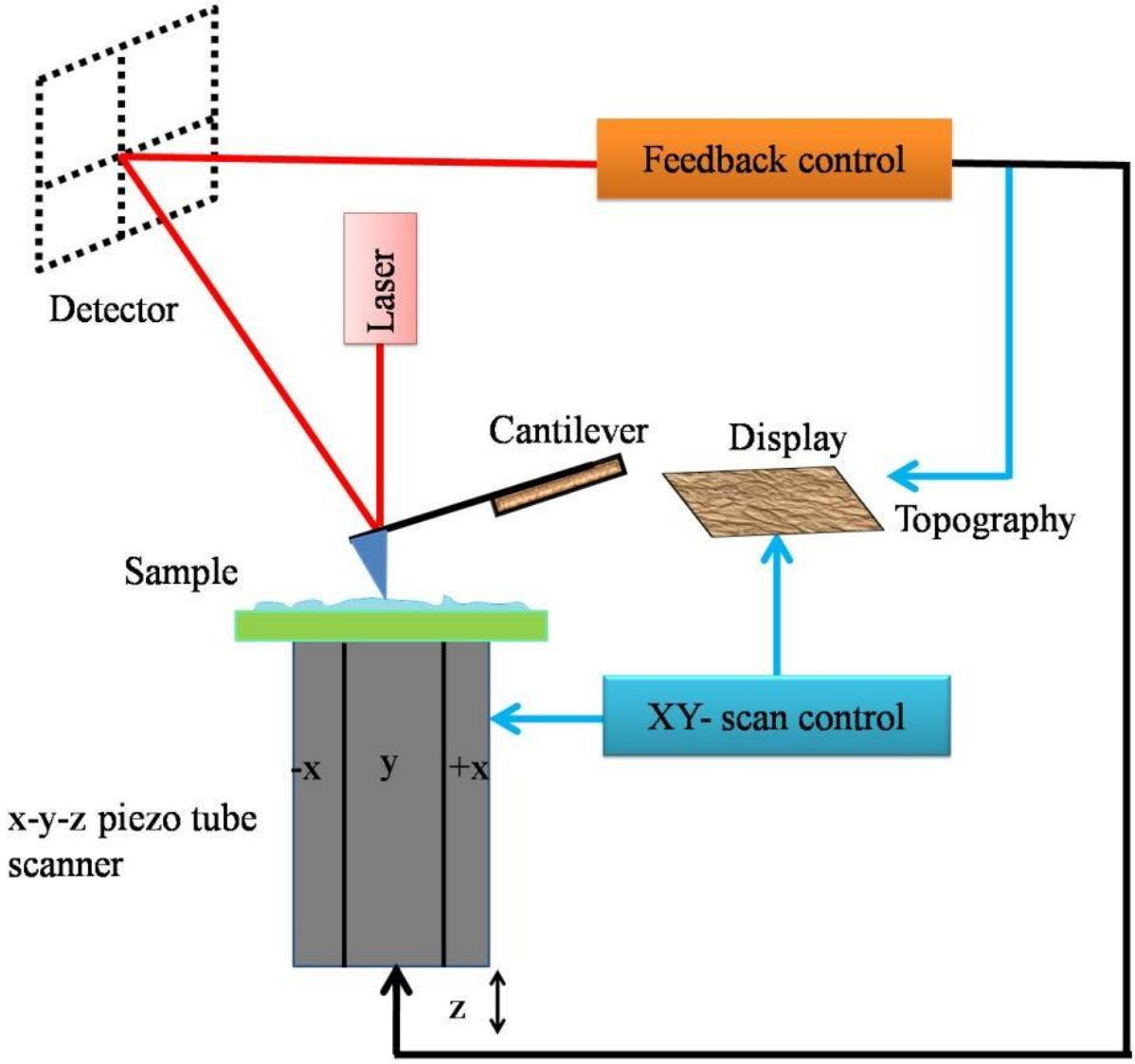
ATOMIC FORCE MICROSCOPE

Atomic force microscope is a type of scanning probe microscope that records the force between the probe and the specimen. The working principle of an AFM can be understood like this: Consider yourself to be in a dark room in front of a table. The table has a book, a pen, a wristwatch, a spoon, a fork, and a screw driver. Will you be able to selectively lift the spoon if asked to do so? The answer for most people is yes. You can distinguish two distinct objects by touching them with your fingers. In this example, your fingers act as the probes, your arm acts as the positioner of your fingers, and your brain works as the processing unit. An AFM works exactly the same way; it has three basic components: a probe, a positioner, and a processing unit.

Modes of operation

An AFM experiment can be recorded in both attractive and repulsive regimes of the Lennard-Jones potential. There are three basic modes of AFM imaging. Another mode, called force spectroscopy is not used for imaging but for characterizing physico-chemical properties of the

specimen as discussed later in this section.



A schematic diagram of an atomic force microscope.

Contact mode AFM: In contact mode AFM, the tip is brought in close contact with the specimen (in the repulsive regime) and scanned over the surface. As the tip is in contact with the sample throughout the scan, the frictional forces are very high. This mode of operation therefore may not be suitable for soft samples including biological samples.
Non-contact mode AFM: In non-contact mode AFM, a cantilever with very high spring constant is oscillated very close to the sample (in the attractive regime). The quantities that are measured are changes in the oscillation amplitude and the phase. The forces between the tip and the sample are very small, of the order of piconewtons. This mode is therefore well-suited for very soft samples but resolution is compromised. *Intermittent mode or tapping mode AFM:* A stiff cantilever is oscillated so close to the specimen that a small part of oscillation lies in the repulsive regime of the Lennard-Jones potential. The tip therefore intermittently touches the sample while scanning. This mode of imaging allows imaging with very high resolution and has become the method of choice for scanning the soft biological samples.
Force mode AFM/Force spectroscopy: Force mode of AFM is not an imaging mode. Briefly, the sample is brought close to the cantilever, pushed against it causing deflections in it, and then

withdrawn. A plot of force (depends on the spring constant of the cantilever) against the distance is called a force spectrum. Force spectroscopy mode is often used to study the interactions of the tip with the sample and to determine the mechanical properties of the specimen.

Resolution

Atomic force microscopes can provide resolutions comparable to that obtained with electron microscopes. As neither light nor particles are used to generate the images, resolution of atomic force microscopes does not depend on any wavelength. The resolution of an AFM is determined by the shape and the diameter of the tip. It is also evident that the resolution in the X-Y plane is poorer as compared to that in the Z-direction. A Z-resolution of ~0.2 nm or better is often achieved using AFM

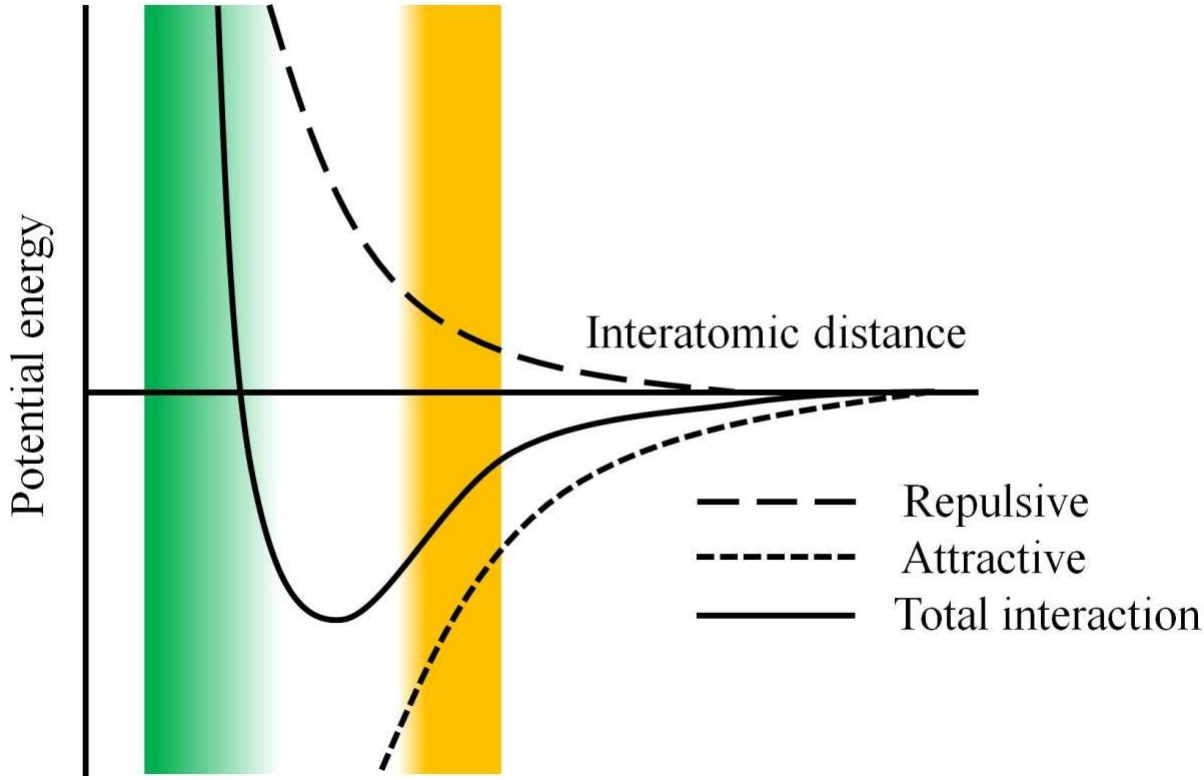
Advantages of AFM

Both AFM and EM provide very high resolution images but AFM has few distinct advantages over EM:

i. Easy sample preparation: AFM does not involve a tedious sample preparation. A sample to be analyzed can simply be placed on a smooth surface and scanned.

ii. Imaging in solution: Unlike EM; it is possible, in fact routine; to record AFM images in solution. No other microscopic method, except the scanning probe microscopes, provides a sub-nanometer resolution in solution.

iii. Manipulation: An AFM tip can be used to mechanically manipulate the specimen at very high spatial resolution.



Lennard-Jones potential and the regions of attraction (orange) and repulsion (green).