

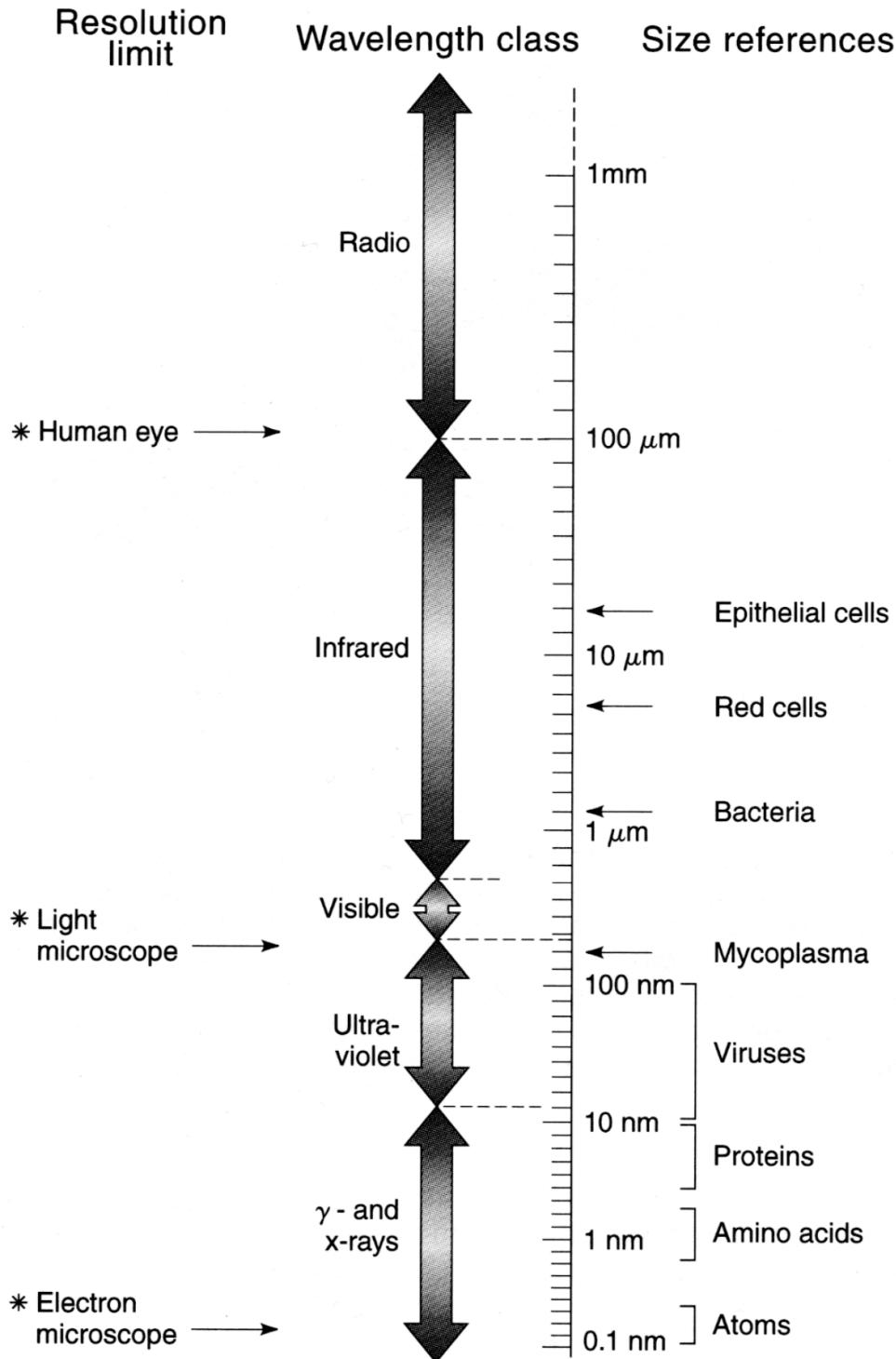
2. Microscopy

Introduction to Microscopy

Microscopy is the study of small objects or structures that cannot be seen with the naked eye, hence a powerful tool used to explore the microscopic world. The image formation in the light microscope is entirely based on the interactions between light and matter. Thus, firstly, a reminder on waves is presented.

Waves Reminder

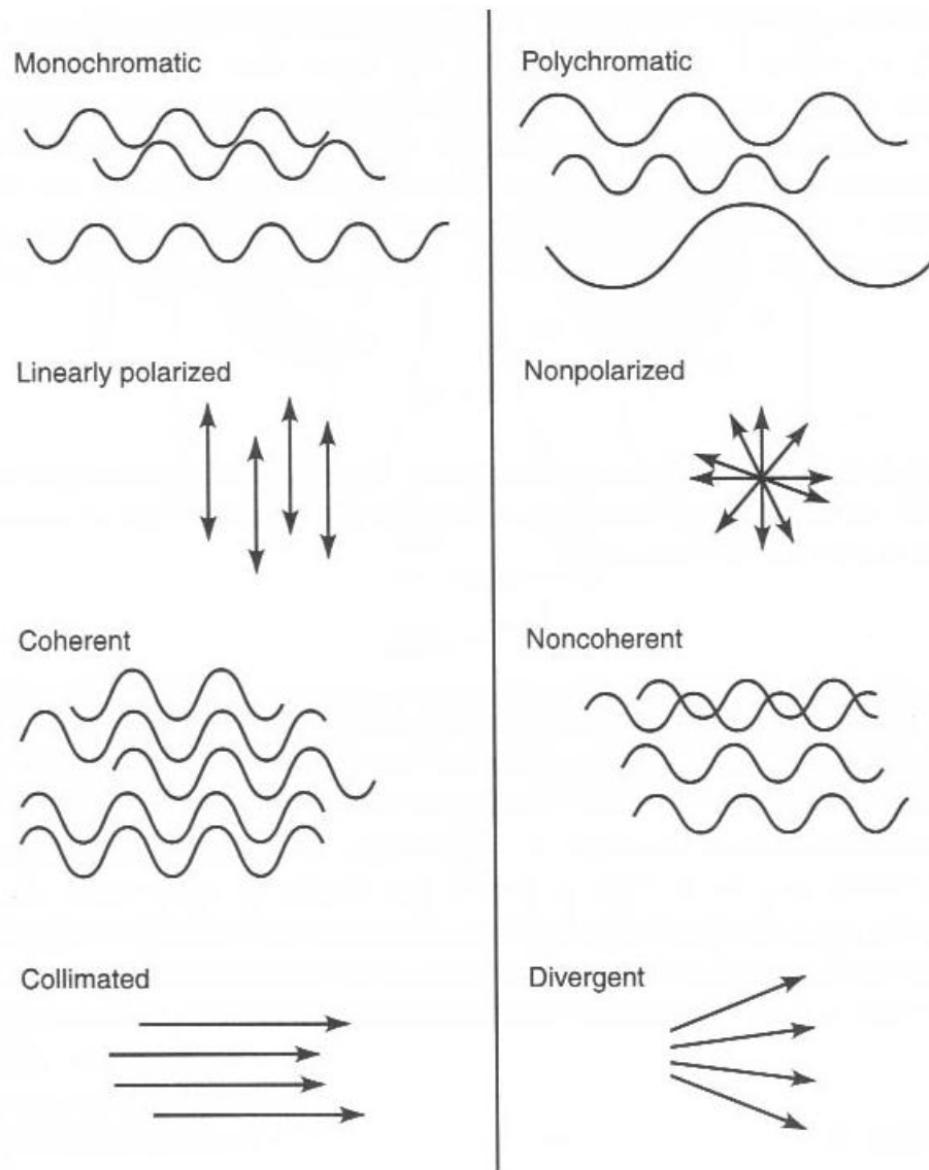
Light is a fundamental aspect of the physical world that can be described in a variety of ways, depending on the context and level of analysis. In this regard, light is a form of electromagnetic radiation, and the electromagnetic spectrum is illustrated in the accompanying figure, which presents a logarithmic scale of distances ranging from 1 mm to 0.1 nm. One side of the scale delineates the wavelength ranges of common categories of electromagnetic radiation, while the other side depicts the sizes of various cells and macromolecules. For example, a red blood cell with a size of 7.5 μm is 15 times larger than the wavelength of visible green light with a size of 500 nm. The figure also highlights the resolution limits of the eye, light microscope, and electron microscope. In the case of the eye, the resolution limit of 0.1 mm is defined as the minimum interval in an alternating pattern of black and white bars on a sheet of paper held 25 cm in front of the eye under conditions of bright illumination. Notably, the visible wavelength range represents only a small fraction of the entire electromagnetic spectrum.



Waves can also be classified in accordance with various criterion:

- ▶ Monochromatic: electromagnetic radiation that consists of a single wavelength or frequency.
- ▶ Polychromatic: light made up of multiple wavelengths or frequencies.
- ▶ Linearly polarised: light waves that oscillate in a single plane, which is called the polarisation plane.
- ▶ Non-polarised: composed of light waves oscillating in many different planes, and thus has no single polarisation plane.
- ▶ Coherent: waves with a constant phase relationship with one another, meaning that the peaks and troughs of the waves align as they propagate.

- ▶ Noncoherent: waves have random phase relationships with one another, meaning that the peaks and troughs of the waves do not necessarily align.
- ▶ Collimated: waves are parallel to one another, meaning that they do not diverge or converge as they propagate.
- ▶ Divergent: waves spread out as they propagate, meaning that their angle of incidence increases over time.

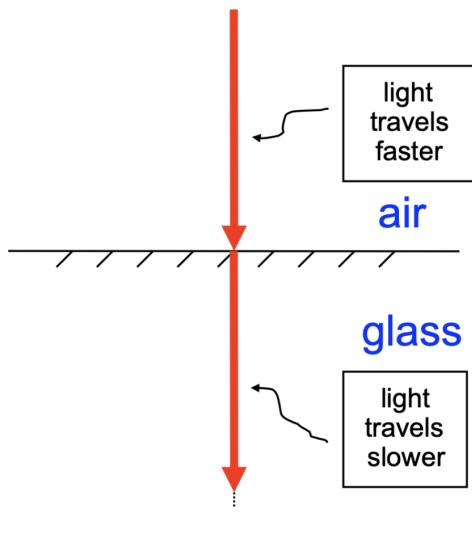


Optical Microscopy

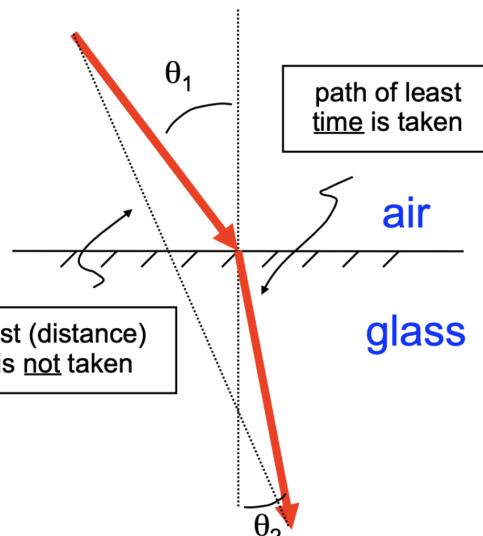
Principle of Lensing

Two essential concepts in the interaction between light and matter are diffraction and refraction. Diffraction refers to the scattering of the incident illuminating light by the detailed substructure within the specimen. Refraction, on the other hand, is the "bending" of light caused by a lens, which converges scattered light to form an image. Refraction occurs when light rays travelling through one type of medium meet an interface with another type of medium. The extent of refraction depends on the angle of incidence, according to Snell's law.

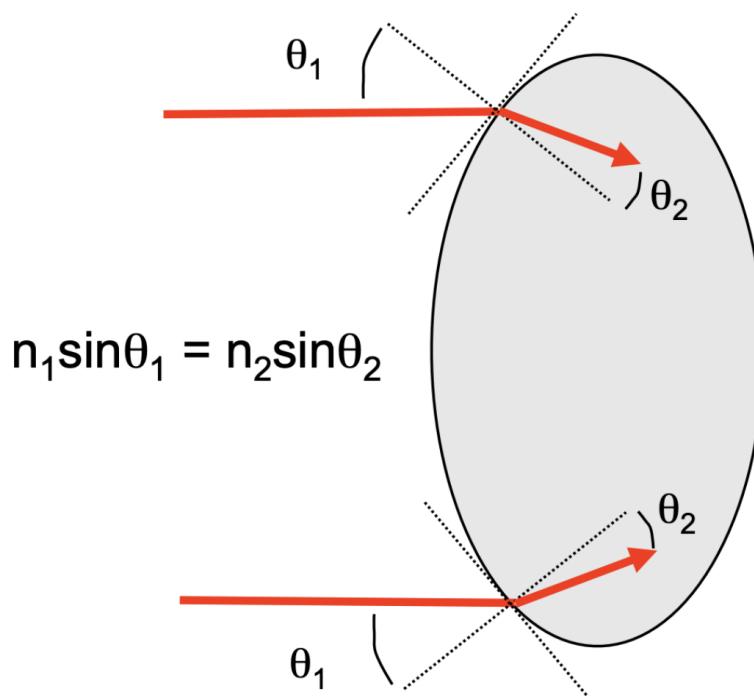
Here the light ray is
orthogonal to the interface



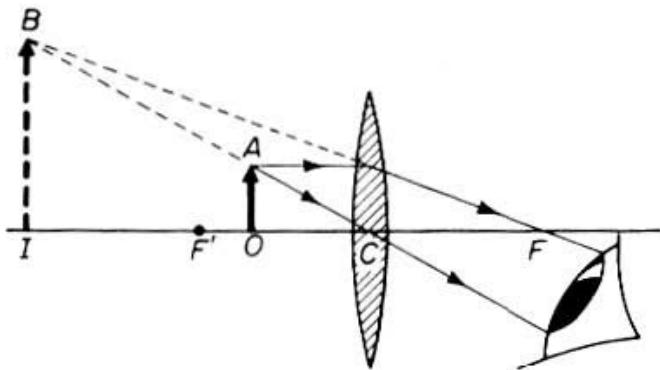
Here the light ray is
oblique to the interface



Lensing occurs when the interface is curved. Positive lenses, also called convex lenses, converge light rays, allowing rays that would otherwise never meet, such as parallel or diverging rays, to do so. Negative lenses, known as concave lenses, diverge light rays.

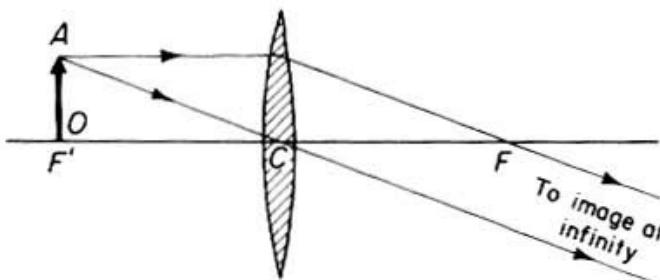
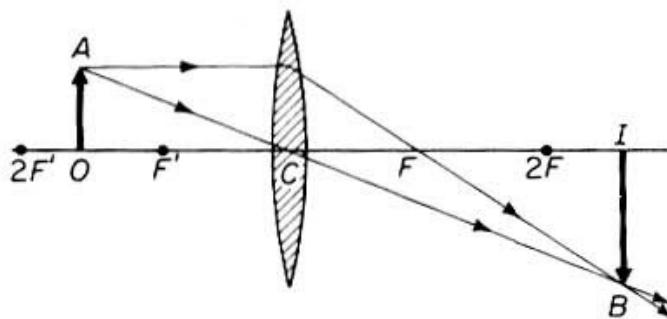


For a simple lens, the incident light rays can pass through the lens and converge at a point on the opposite side, creating a real image. In addition, lenses can also create virtual images, where the light rays appear to diverge from a point behind the lens. The position and magnification of an image are determined by the curvature (focal length) of the lens and the physical distance between the object and the lens. The following diagram illustrates the geometrical optics of a simple lens, highlighting the focal length f , focal point F , object-lens distance a , and lens-image distance b .

OBJECT BETWEEN LENS and F'

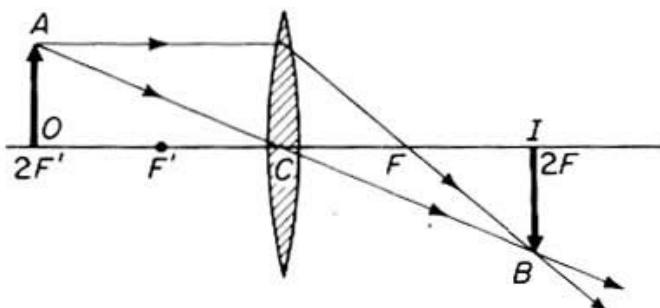
the image is,

- (1) Behind the object
- (2) Virtual
- (3) Erect
- (4) Larger than object

OBJECT AT F' the image is
at infinityOBJECT BETWEEN F' and $2F'$

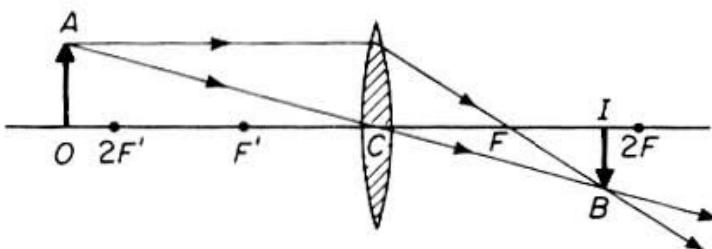
the image is,

- (1) Beyond $2F$
- (2) Real
- (3) Inverted
- (4) Larger than object

OBJECT AT $2F'$

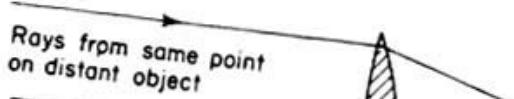
the image is,

- (1) At $2F$
- (2) Real
- (3) Inverted
- (4) Same size as object

OBJECT BEYOND $2F'$

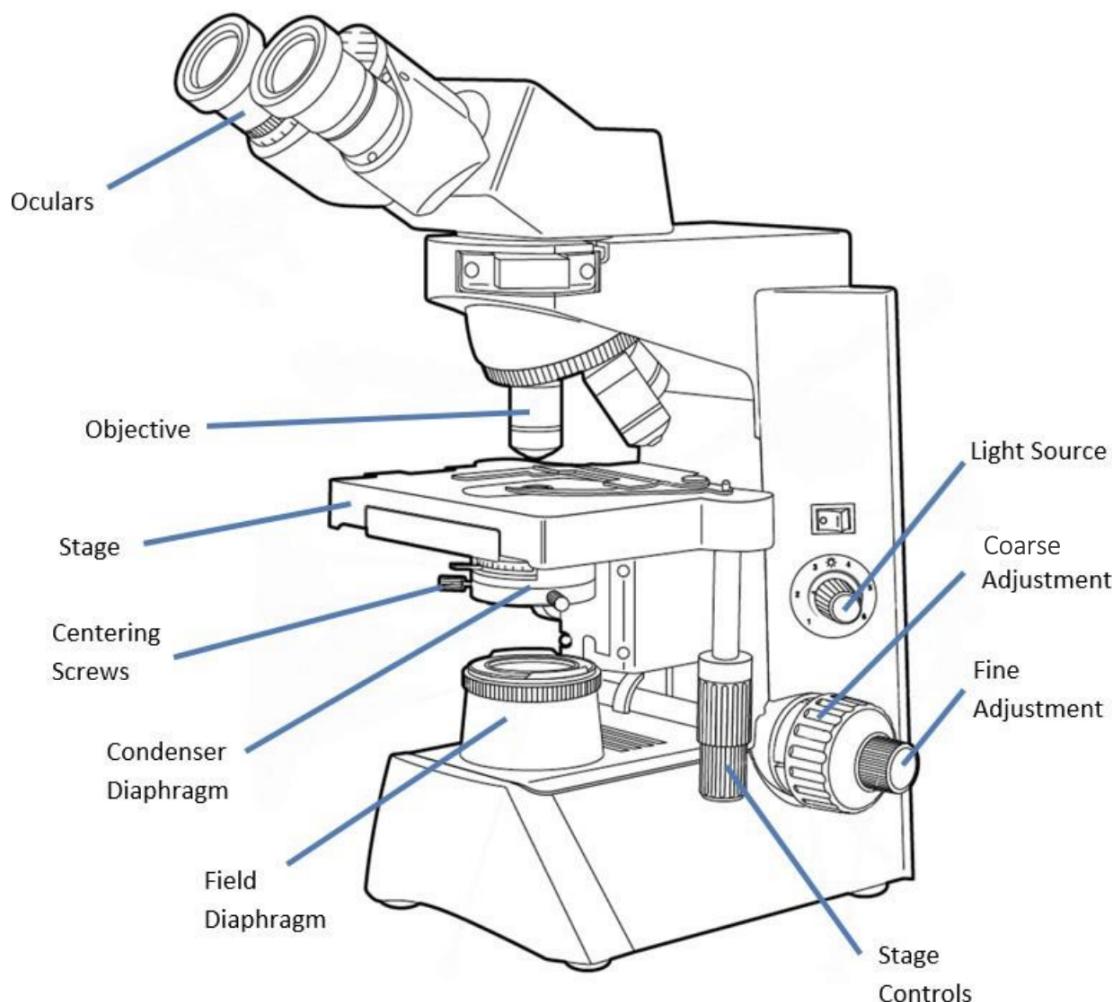
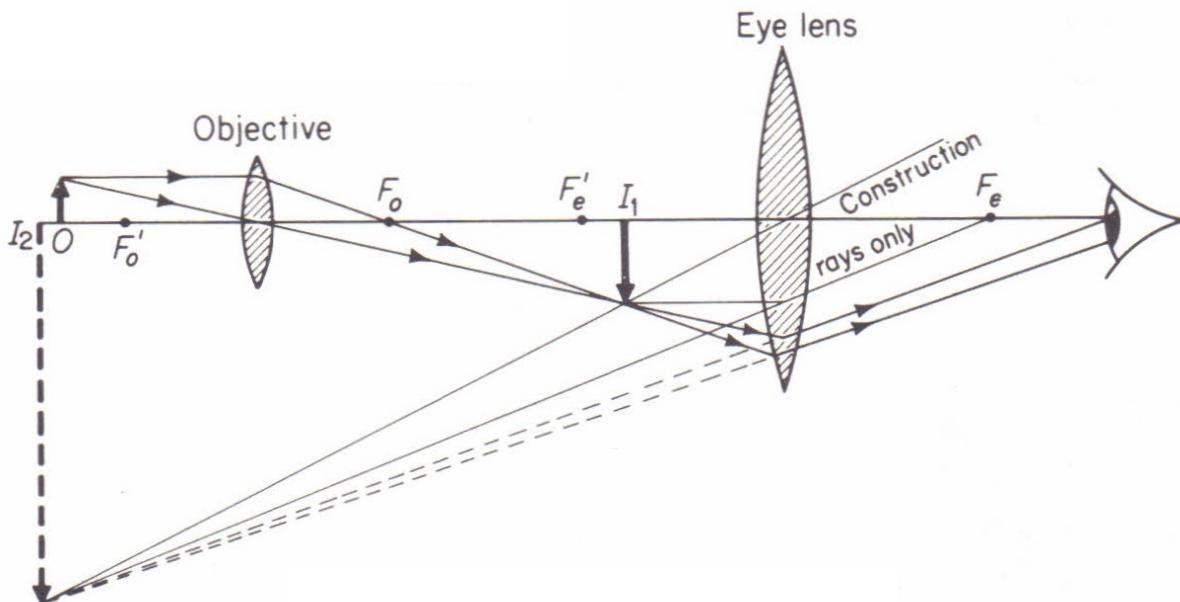
the image is,

- (1) Between F and $2F$
- (2) Real
- (3) Inverted
- (4) Smaller than object

OBJECT AT INFINITY

the image is,

The use of multiple lenses allows for much higher magnification than what can be achieved with a single lens. A double lens system, also known as a compound microscope, uses two lenses (an objective lens and an eyepiece lens) to magnify a specimen. The objective lens is placed close to the object, and the eyepiece lens is positioned near the observer's eye. When light from the object passes through the objective lens, it forms a real image. This image is then magnified by the eyepiece lens, resulting in a larger virtual image that can be observed by the human eye.

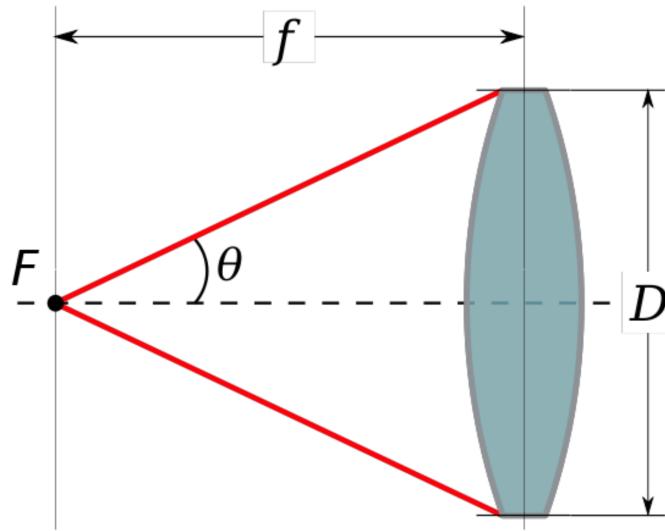


Objective

The objective lens is typically spring-loaded and parfocalled, meaning it maintains focus when switching between objectives. The magnification of the objective lens is determined by multiplying its magnification by the magnification of the eyepiece lens. For instance, $40 \times 10 = 400$. The objective lens is usually mounted on a rotating nosepiece or turret that allows for easy switching between lenses. Objective lenses come in various types, such as dry, water-immersion, or oil-immersion, depending on the type of sample being observed.

Numerical aperture (NA) is a measure of the ability of an objective lens to gather and focus light:

$$NA = n \sin \theta$$



where n is the refractive index of the medium in which the lens is working (usually air, water, or oil), and θ is half of the maximum angle of light that can enter the lens. The higher the NA of an objective lens, the better its ability to resolve fine details in the specimen being observed. This is because a higher NA allows for a narrower cone of light to be focused on the specimen, resulting in better resolution. However, there are some trade-offs associated with a higher NA , including decreased light transmission (a decrease in the amount of light that is able to pass through the objective lens), decreased working distance (the distance between the objective lens and the object being viewed; difficult to manipulate the object or place other instruments near the sample), and decreased depth of field (the range of distance over which an object can be viewed in sharp focus; objects at different distances from the objective lens may not be in focus simultaneously and it is thus difficult to obtain a clear image of the entire object).

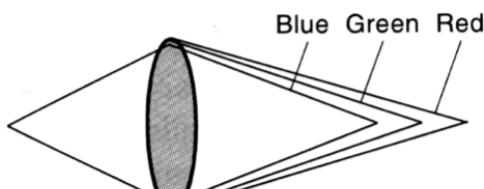
Abbreviation

Various aberrations can occur on a simple lens:

- ▶ Chromatic aberration: parallel incident rays of different wavelength are focused at different locations.
- ▶ Spherical aberration: incident rays parallel to the optic axis and reaching the centre and the periphery of the lens are focused at different locations.
- ▶ Coma: off-axis rays passing through the centre and periphery of the lens are focused at different locations.
- ▶ Astigmatism: an off-axis aberration causes waves passing through the vertical and horizontal diameters to focus an object point as a streak.

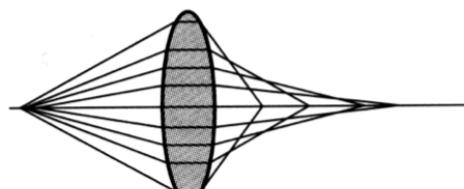
- Distortion and field curvature: the image plane is curved and not planar. So-called barrel and pincushion distortions produce images that are not high in fidelity compared to the object.

On-axis aberrations



Chromatic

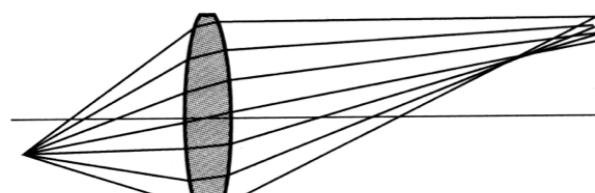
(a)



Spherical

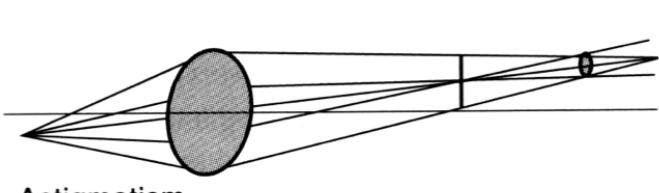
(b)

Off-axis aberrations



Coma

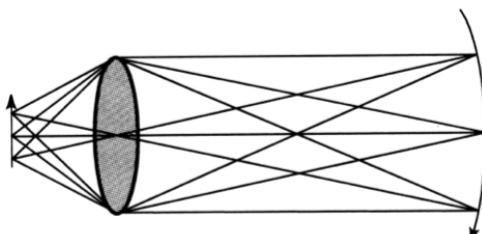
(c)



Astigmatism

(d)

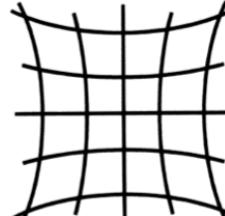
Distortion



Field curvature



Barrel distortion

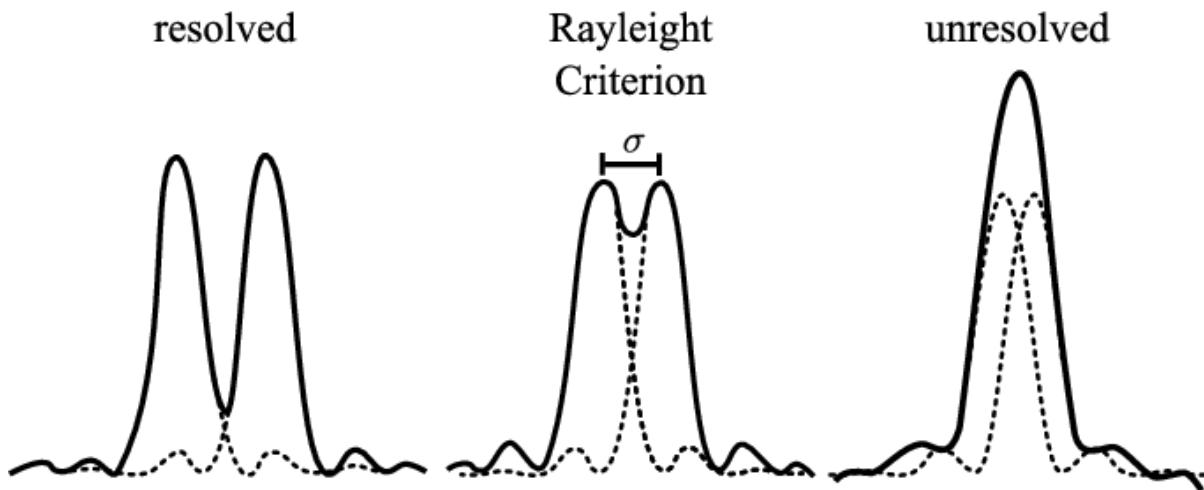


Pincushion distortion

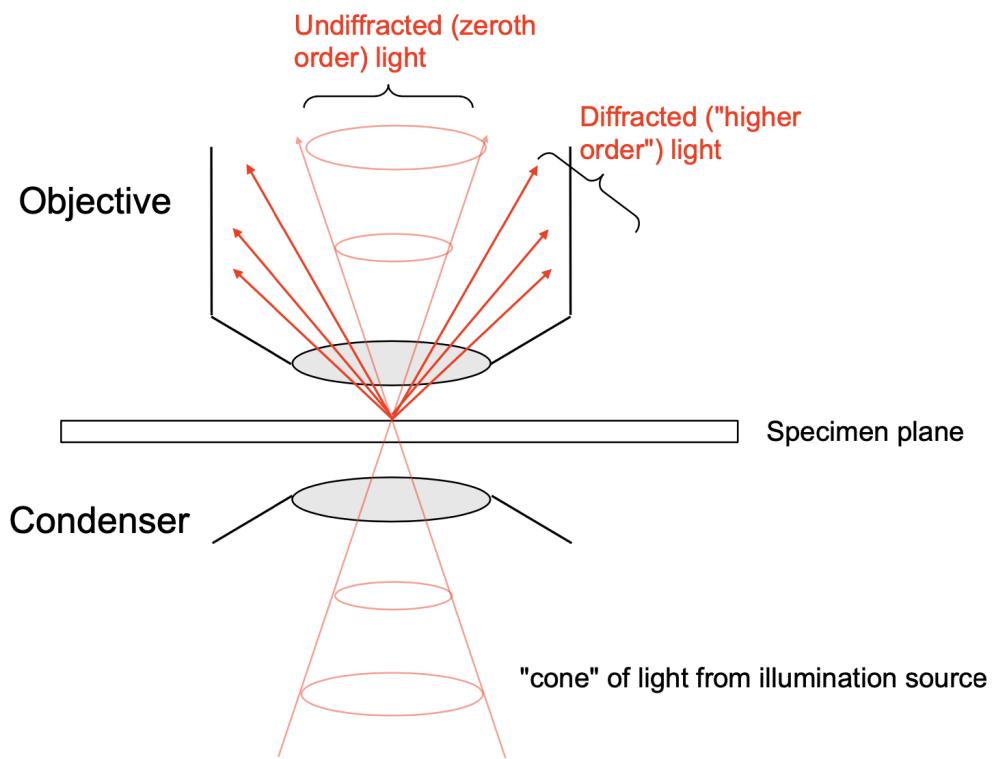
(e)

Resolution

Resolution refers to the ability of a microscope to produce separate and distinguishable images of two adjacent points in a specimen.

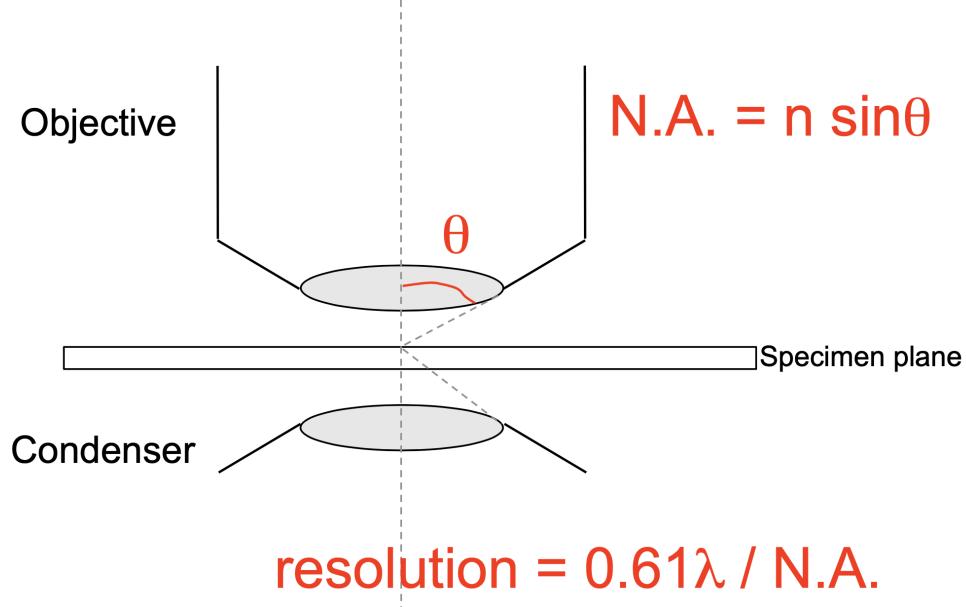


When light passes through the objective lens, it is diffracted, and the diffracted waves interfere with each other. Abbe's theory suggests that the microscope image is the interference effect of a diffraction phenomenon, and the resolution of an optical microscope is limited by the diffraction of light. Experiments have demonstrated that getting the highest resolution image depends on capturing the largest angle of scattered light:

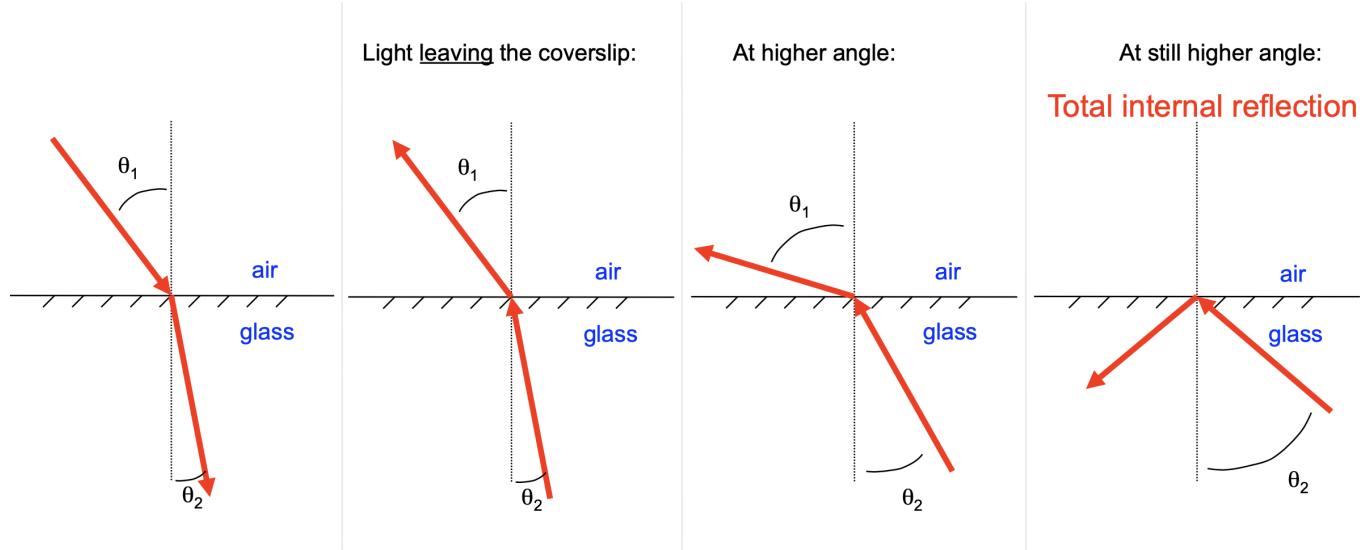


and removing higher-orders of the diffraction pattern reduces the resolution of the resulting image. The extent of the diffraction depends on the numerical aperture, as well as the wavelength of the light. The maximum resolution of an optical microscope is therefore limited by the diffraction limit, which is defined as the smallest distance between two points that can be distinguished as separate entities. According to the Rayleigh criterion,

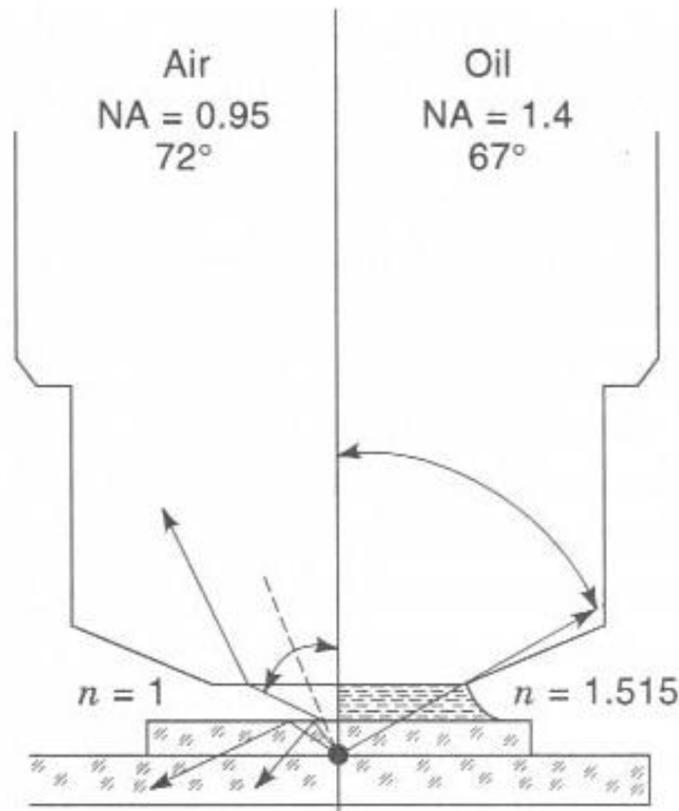
$$\sigma = \frac{0.61\lambda}{NA}$$



where λ is the wavelength of the light. The higher the numerical aperture and the shorter the wavelength, the better the resolution. Therefore, immersion oil is used with high-power objectives in optical microscopy to increase the NA of the objective lens and thereby improve the resolution of the microscope. This is because the refractive index of air ($n = 1$) is much lower than that of glass ($n \approx 1.5$) or other transparent materials typically used in microscopy, and this difference in refractive index can cause a significant amount of light to be lost or scattered as it passes through the interface between the specimen and the air, sometimes even completely lost due to total internal reflection:

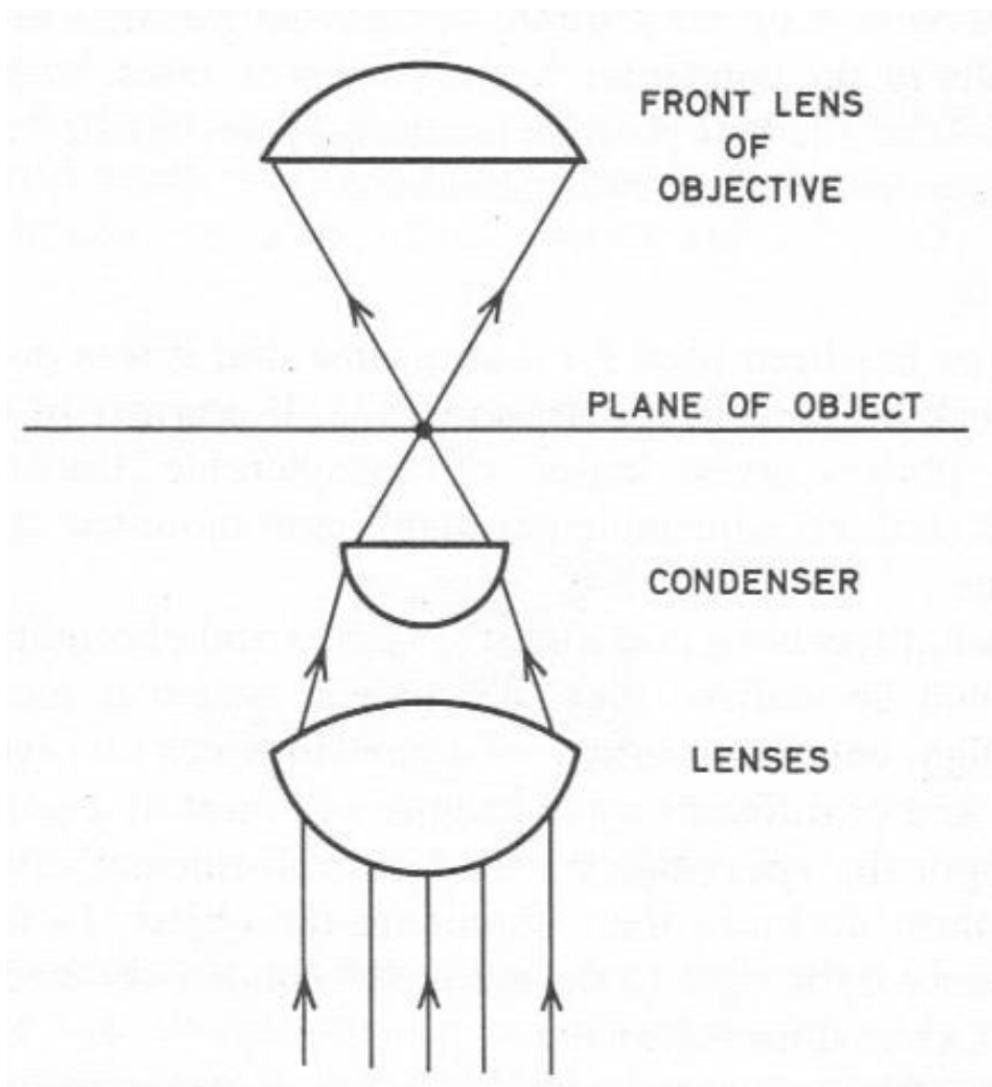


By using immersion oil with a refractive index ($n = 1.515$) that closely matches that of the glass coverslip and the lens, the light can be refracted in a way that reduces the amount of light loss and increases the NA:

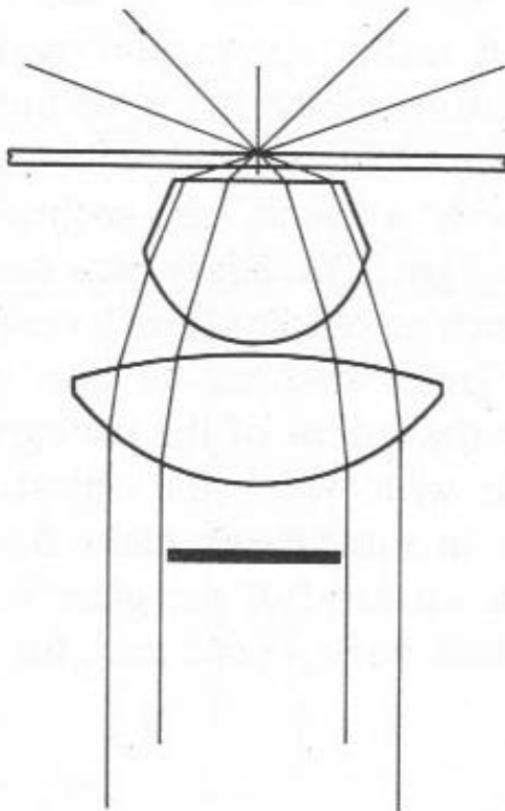


For dry lenses, NA is limited, because rays subtending angles of 41° or greater are lost by total internal reflection and never enter the lens (dotted line). By adding high-refractive index immersion oil matching that of the glass coverslip, an oil immersion objective can collect light diffracted up to 67° , which corresponds to $NA = 1.4$.

Furthermore, the role off the condenser should not be ignored when considering resolution. This is an optical component that is used to focus and direct a beam of light onto the specimen being observed, typically located beneath the microscope stage and above the objective lens. The primary function of the condenser is to collect light from the microscope's illumination source, typically a lamp or bulb, and focus it into a concentrated cone-shaped beam that passes through the specimen and into the objective lens.



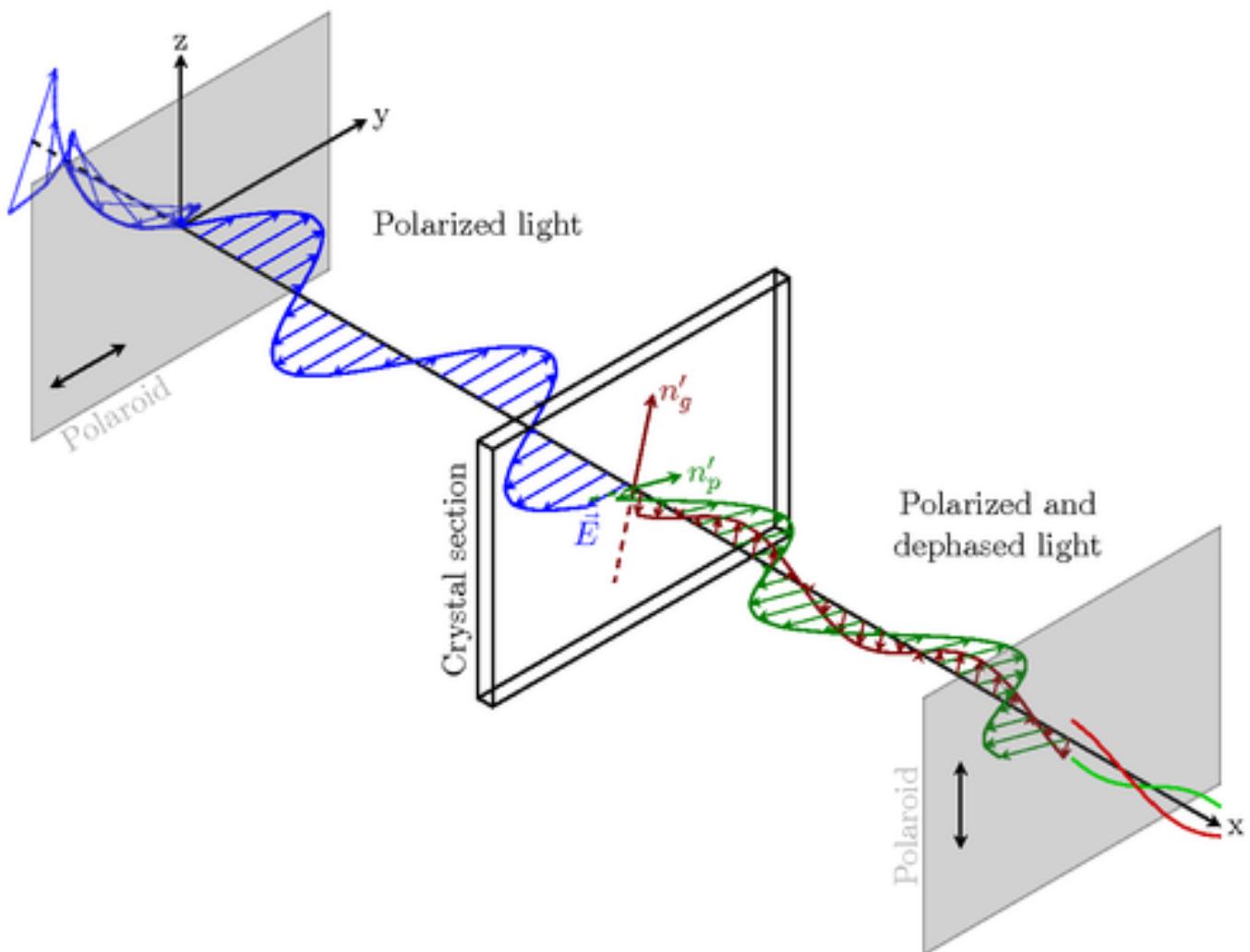
Bright field illumination is the most commonly used illumination technique in microscopy, where a light source is positioned below the specimen, and the light passes through the specimen and enters the objective lens. The specimen appears bright against a dark background, and this technique is useful for observing samples that have a strong contrast with their surroundings. Dark field illumination, on the other hand, is where the specimen is illuminated from the side at an oblique angle, so that the light does not directly enter the objective lens. Instead, the light that is scattered by the specimen enters the objective lens and is detected by the observer. This creates a bright specimen against a dark background, and is useful for observing samples that are transparent or have a low contrast with their surroundings.



Polarisation Microscopy

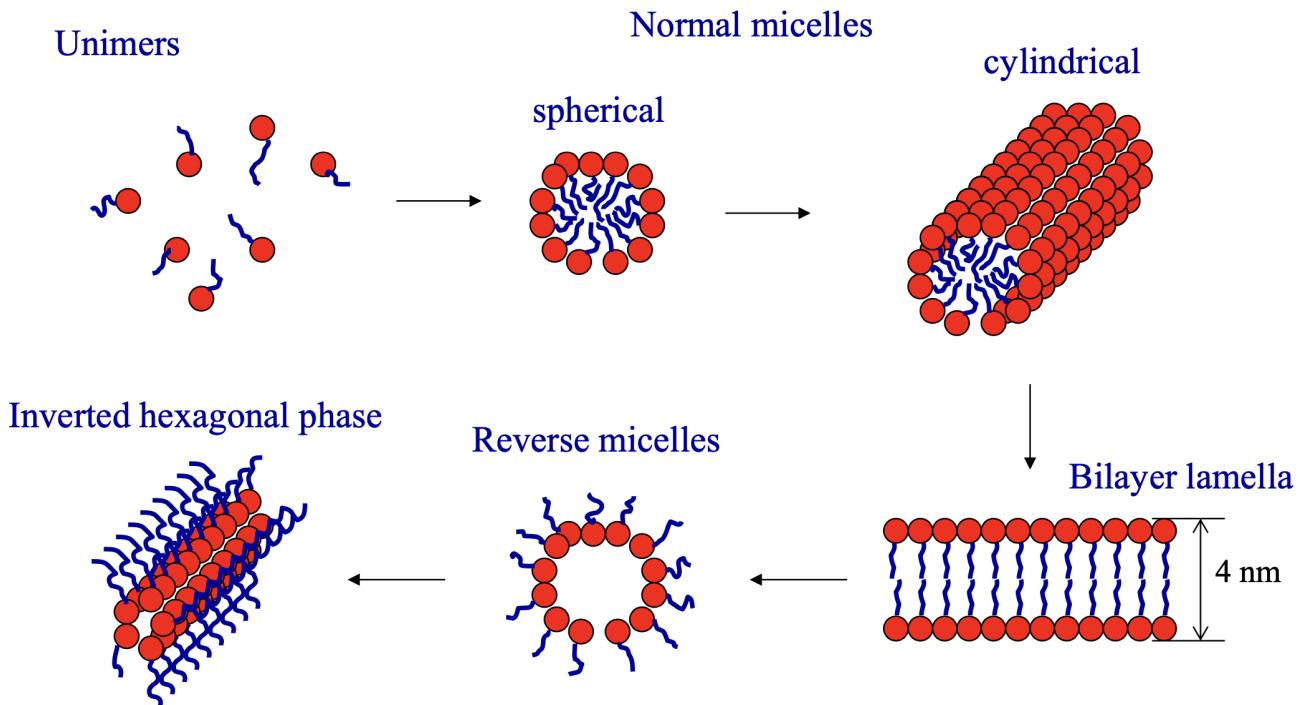
Polarisation microscopy is a type of optical microscopy that uses polarised light to examine and analyse the optical properties of materials. It is a type of optical microscopy that is used to investigate the birefringence or anisotropy of samples. Birefringence, also known as double refraction, is the ability of a material to split a beam of light into two components with different refractive indices, meaning the two components travel at different speeds through the material. This splitting occurs because the material has different optical properties in different directions, and the polarization of the light is affected differently depending on the orientation of the material. Anisotropy refers to the property of a material that exhibits different physical or optical properties when measured in different directions. Anisotropy is related to the birefringence of the material, and it is caused by the material having different optical properties in different directions.

In polarisation microscopy, polarised light is passed through a polariser, which produces light waves that vibrate in only one plane. The polarised light is then passed through the sample, which may be a thin section of a material or a small crystal, and the light waves are altered in various ways depending on the optical properties of the sample. The polarised light is then passed through an analyser, which is set at a specific angle relative to the polariser, and the resulting image is captured by a camera or viewed through an eyepiece.

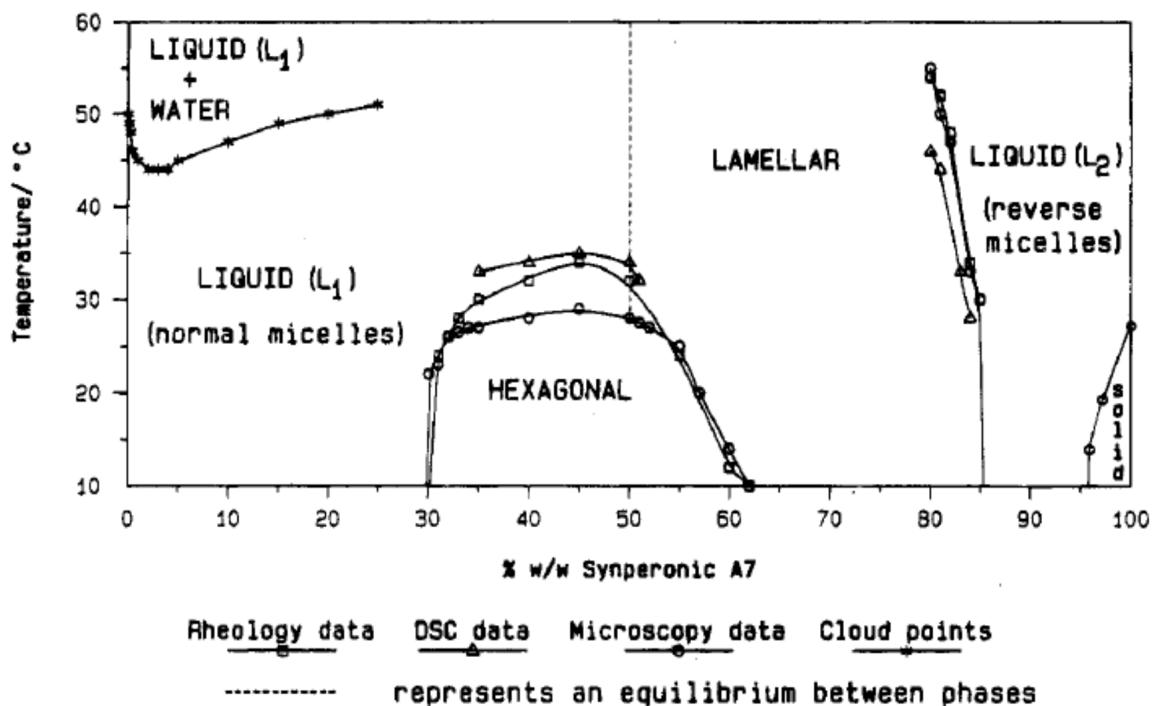


Light behavior in a petrographic microscope with light polarizing device. Only one incident wavelength is shown (monochromatic light). The magnetic field, perpendicular to the electric one, is not drawn.

Polarisation microscopy is useful for studying surfactant phases as surfactants often exhibit liquid crystal behaviour, which can be easily observed using polarised light. Surfactants are molecules that have both hydrophilic (water-loving) and hydrophobic (water-repelling) properties, and when they are dispersed in water, they can form various phases or structures such as micelles, lamellar phases, and hexagonal phases:



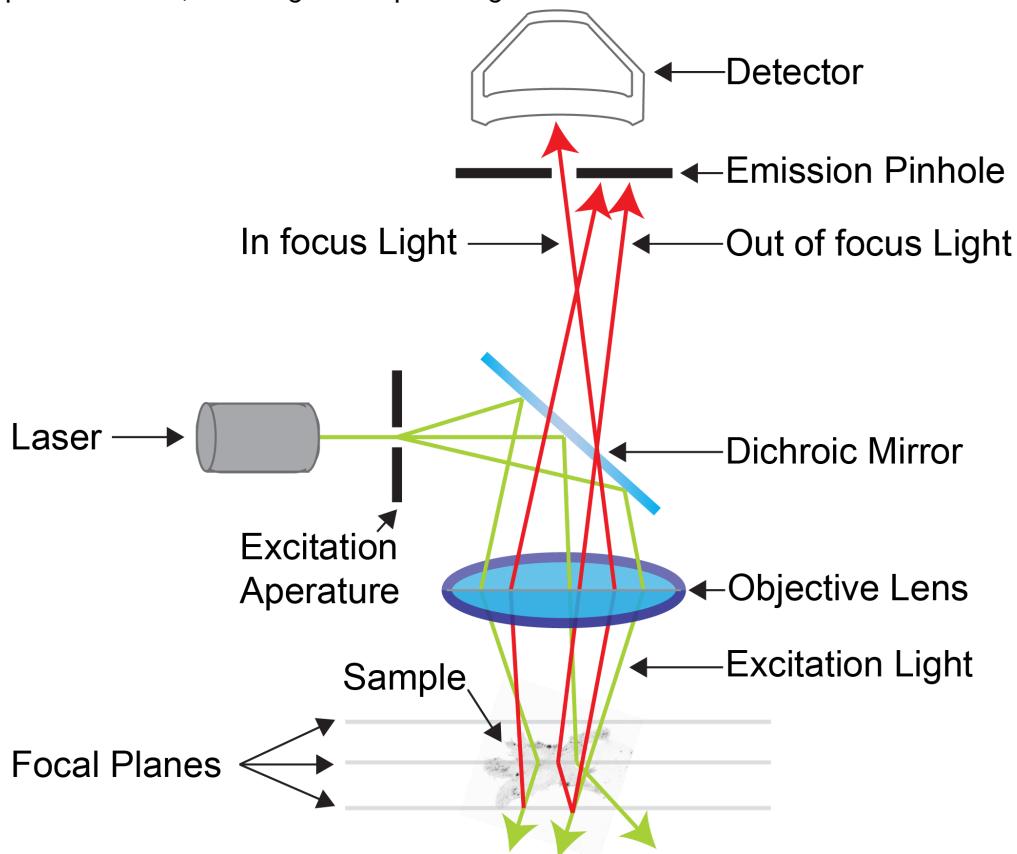
By using polarisation microscopy, it is possible to observe the birefringence of surfactant phases. This can provide information about the orientation and structure of the surfactant phase, which can be useful in understanding the properties and behaviour of the phase. For example, the type of surfactant phase that is present can affect the stability, viscosity, and other physical properties of the system.



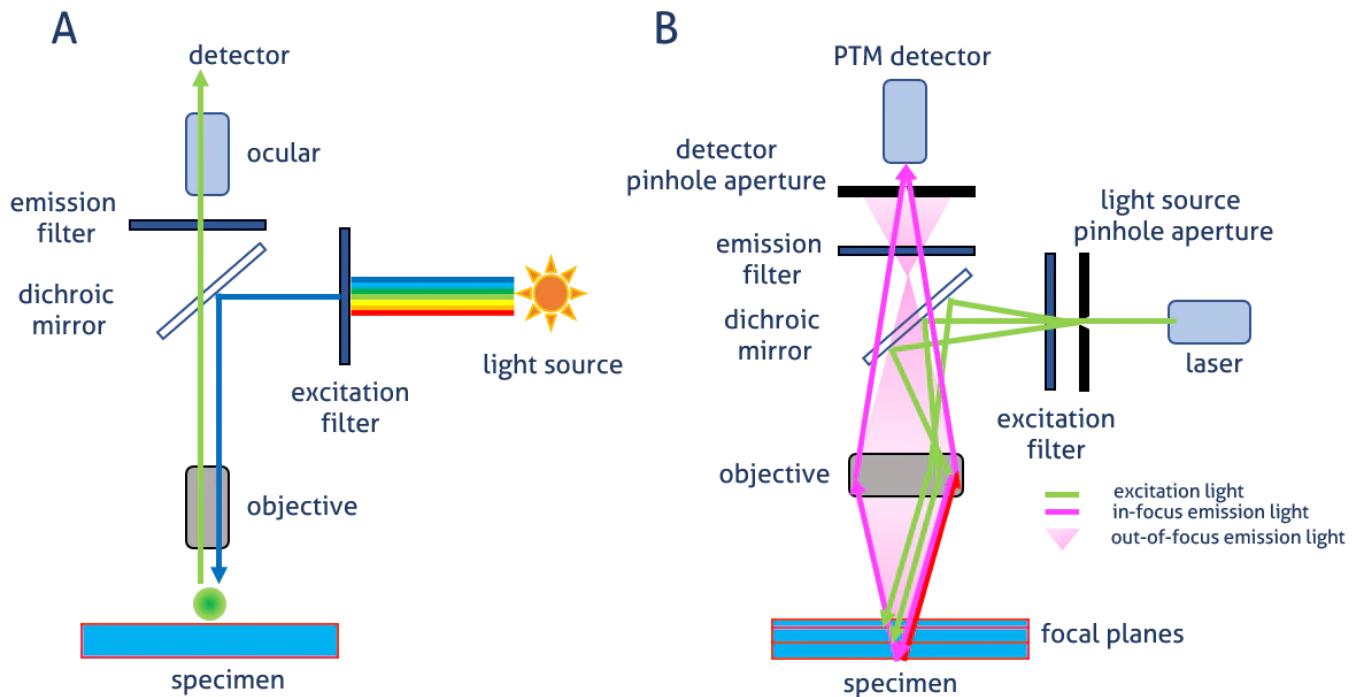
Confocal Microscopy

The fundamental principle of confocal microscopy involves the use of a pinhole and a spatial filter to eliminate out-of-focus light, resulting in improved contrast and resolution. Unlike conventional widefield microscopy, which captures all the emitted light from the sample, confocal microscopy uses a point illumination and a

pinhole aperture to only detect light coming from a specific focal plane. This eliminates the light from above and below the plane of focus, resulting in sharper images.



In conventional widefield microscopy, when fluorescent specimens are imaged, the secondary fluorescence emitted by the specimen that is located outside the region of interest (the specific area being observed) can interfere with the resolution of the features that are in focus. This means that the clarity and sharpness of the features of interest may be compromised due to the presence of unwanted fluorescence signals from other areas of the sample. This issue becomes particularly problematic when dealing with specimens that have a thickness greater than approximately 2 mm. In thicker specimens, the interference from out-of-focus fluorescence is more pronounced, leading to decreased image quality and difficulty in distinguishing the desired features. While confocal microscopy provides only a marginal improvement in both axial (depth) and lateral (horizontal) resolution compared to conventional microscopy, its key advantage lies in its ability to exclude the out-of-focus fluorescence from the final image. By using a pinhole aperture to block the light emitted from areas outside the focal plane, confocal microscopy effectively eliminates the unwanted "out-of-focus" flare that occurs in thick fluorescently labelled specimens.



Various advantages also include:

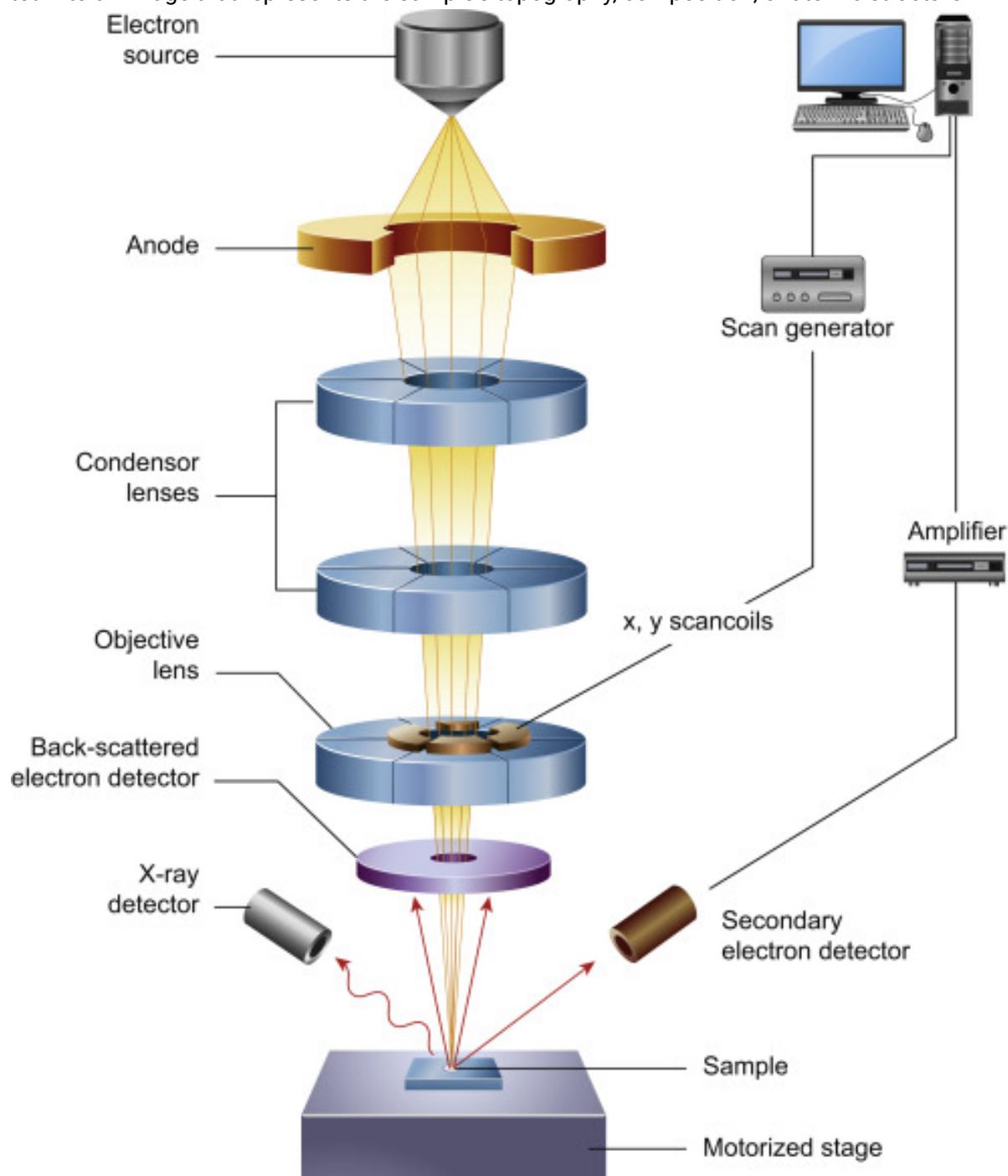
- ▶ Shallow depth of field: only a thin slice of the specimen is in focus at any given time, which allows for precise localisation and imaging of specific structures within a sample, even in complex and thick specimens. By selectively capturing the in-focus plane, confocal microscopy enhances the clarity and detail of the imaged features.
- ▶ Serial optical sectioning: confocal microscopy allows for the collection of serial optical sections from thick specimens. By sequentially scanning through multiple focal planes, a three-dimensional representation of the specimen can be reconstructed. This capability is particularly useful in studying complex structures, such as cellular organelles, tissues, and even whole organisms, and this enables detailed analysis of the internal structures and spatial relationships within the specimen.
- ▶ Fluorescent labelling: confocal microscopy is commonly used to image cells and tissues that have been labelled with fluorescent probes. These probes selectively bind to specific molecules or structures within the sample, enabling visualisation of specific cellular components or biochemical processes. The high sensitivity of confocal microscopy to fluorescent signals allows for the detection and localisation of even low-abundance targets within the specimen.

Electron Microscopy

Electron microscopy is a powerful imaging technique that uses a beam of electrons to examine the structure and morphology of samples at very high magnification and resolution (magnifications up to several million times and reveal details at the nanoscale level). The wavelength of electrons depends on their energy. At an energy level of 200 kiloelectron volts (KeV), the electron wavelength is approximately 2×10^{-12} m, which is much smaller than that of visible light and enables electron microscopes to achieve higher resolution. The achievable resolution of an electron microscope is approximately 2 Å m.

In electron microscopy, a beam of electrons is generated in an electron gun. Once the electrons are emitted from the gun, an electric field is applied to accelerate them. The electric field provides the necessary energy to

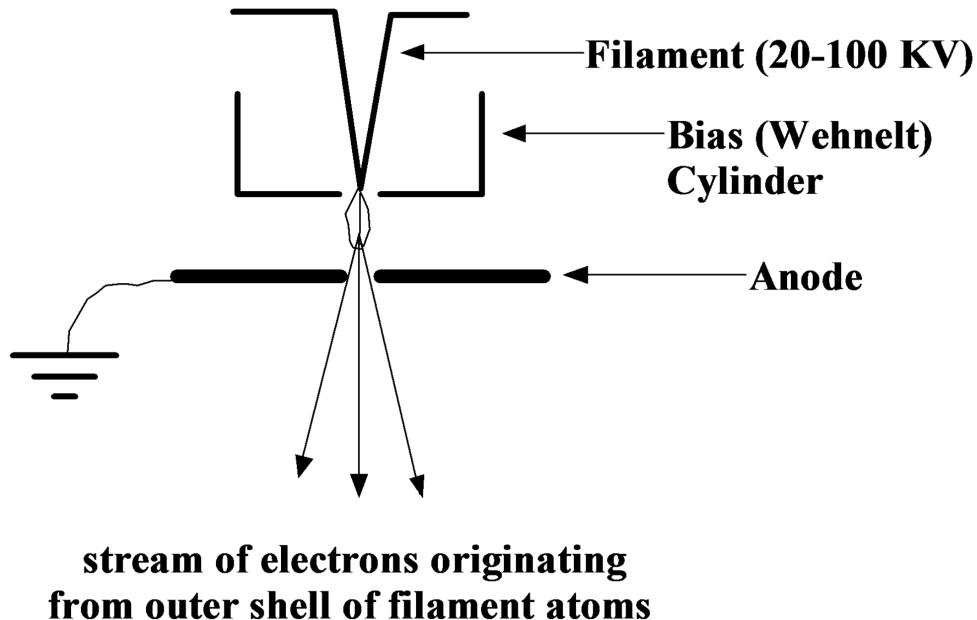
increase the speed and kinetic energy of the electrons, allowing them to have sufficient energy to interact with the sample. To control the path of the accelerated electrons, the accelerated beam is then focused onto the sample using electromagnetic lenses, similar to how glass lenses focus light in a traditional microscope. The interaction between the electrons and the sample produces various signals, such as backscattered electrons, secondary electrons, transmitted electrons, and characteristic X-rays. These signals are detected and converted into an image that represents the sample's topography, composition, or atomic structure.



The electron gun consists of several key elements:

- Cathode: the cathode is a filament or a heated source of electrons, typically made of a material such as tungsten or lanthanum hexaboride (LaB₆). When the cathode is heated, it emits electrons through a process called thermionic emission. These emitted electrons form the initial electron beam.

- ▶ Wehnelt cylinder: The Wehnelt cylinder, also known as the control electrode or electron gun electrode, surrounds the cathode. It acts as a negatively charged electrode that helps focus and shape the electron beam. By controlling the voltage applied to the Wehnelt cylinder, the intensity and size of the electron beam can be adjusted.
- ▶ Accelerating anode: after the electrons are emitted from the cathode, they pass through the accelerating anode. The accelerating anode is a positively charged electrode that provides the necessary electric field for accelerating the electrons.



The wavelength (λ) of electrons is determined by the accelerating voltage (V) on the filament from which they are emitted:

$$\lambda = 0.1 \left(\frac{150}{V} \right)^{0.5}$$

Thus very high voltages (up to 100 kV) are used to produce small values of $\lambda < 0.005$ nm.

Electron microscopes also require a high vacuum environment (at least 10^{-5} mbar), which prevents electron scattering and interference from gas molecules that could degrade the quality and resolution of the images. A high vacuum environment also reduces the chance of contamination and damage to the delicate electron optics within the microscope, as the cathodes or filaments used in electron guns, particularly those made of tungsten, are susceptible to oxidation and burning when exposed to air. A high vacuum environment also helps eliminate airborne particles (dust) and ensures a clean operating environment for the complex column assemblies within the microscope. To achieve the necessary high vacuum, a combination of pumping techniques is commonly used:

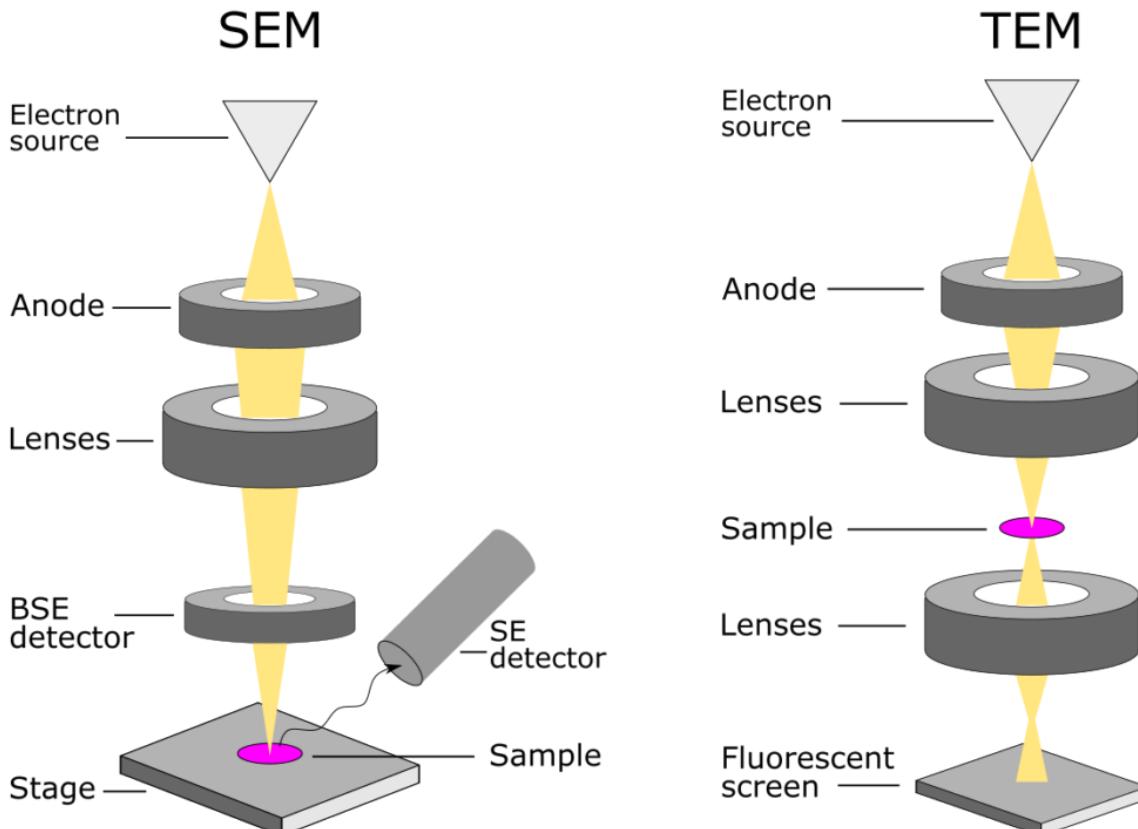
- ▶ Rotary (mechanical) pump: relies on rotating blades to create a vacuum. It removes gas molecules from the sample chamber by trapping them between the blades and the walls of the pump, gradually reducing the pressure.
- ▶ Diffusion pump: a high-speed vacuum pump that operates by creating a vapor jet. Vapor from a heated oil or mercury source is directed into the pump, and as the vapor molecules collide with gas molecules, they transfer momentum and drag the gas molecules out of the system, thus reducing the pressure.

- ▶ Turbo pump: a high-speed rotational vacuum pump that uses a rapidly spinning rotor to create a pressure gradient and evacuate the chamber. The spinning rotor blades impart momentum to gas molecules, propelling them towards the outlet and effectively reducing the pressure.

Finally, the electromagnetic lens is responsible for focusing and shaping the electron beam. It functions similarly to a lens in optical systems, such as the human eye, but operates based on the principles of electromagnetism rather than light optics:

- ▶ Fixed focal distance and variable focal length: there is a specific distance between the lens and the focal point where the beam converges. However, the focal length of the lens can be varied, which determines the extent to which the beam is converged or diverged.
- ▶ Variation of lens power through current control: the power or strength of an electromagnetic lens can be adjusted by varying the current passing through the lens. This is typically done using a rheostat, which is a variable resistor that regulates the current. By changing the current passing through the lens, the lens's magnetic field strength is altered, which, in turn, modifies its focusing power.
- ▶ Electron spiralling through the lens: as electrons pass through the electromagnetic lens, they experience the influence of the lens's magnetic field. Due to the magnetic field's configuration, the electrons are deflected and guided in a spiral path through the lens. This spiralling effect can be visually observed and is a consequence of the interaction between the electrons and the lens's magnetic field.

As the signals from interactions between the electrons and the sample are detected, different imaging modes, such as transmission electron microscopy (TEM) and scanning electron microscopy (SEM), are employed to capture different types of information about the sample.



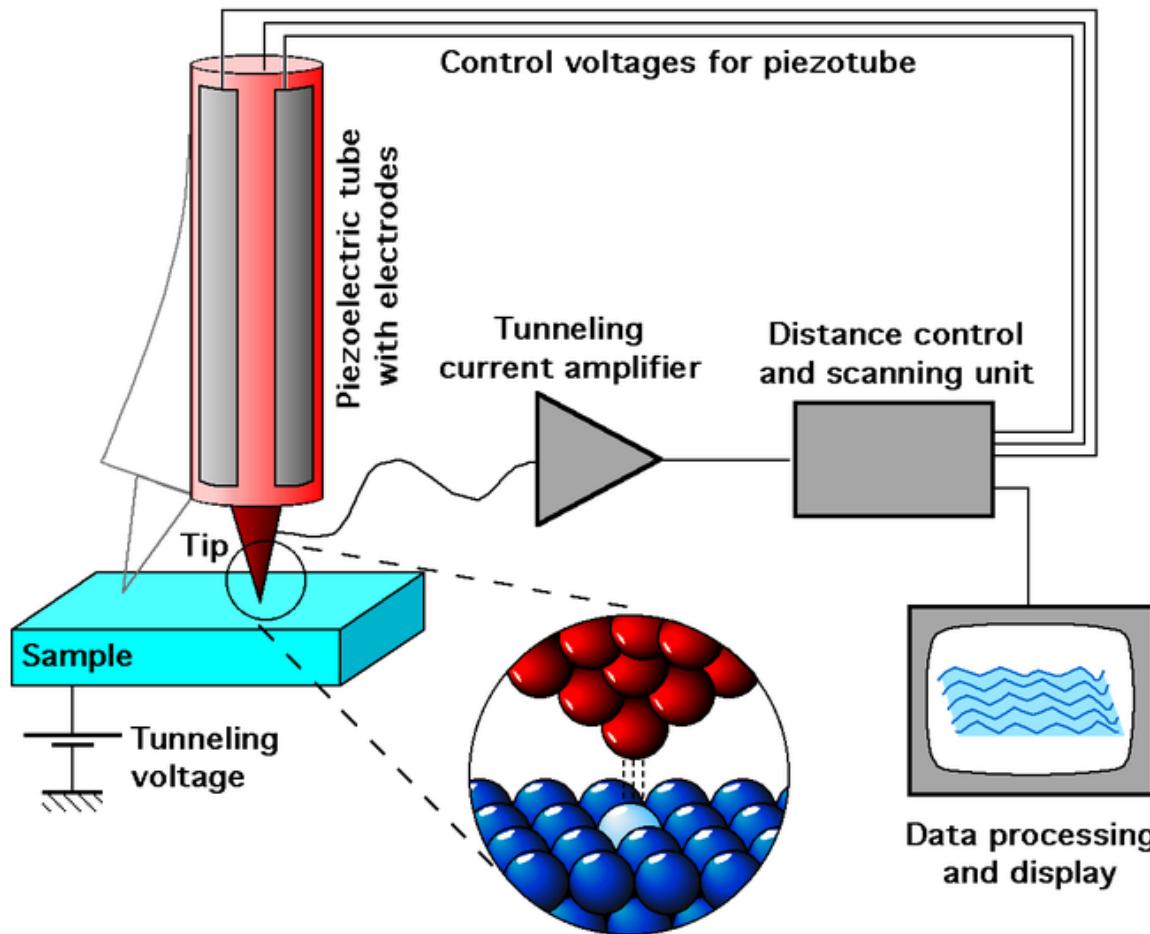
- ▶ In TEM, a thin specimen is placed in the path of an electron beam. The beam passes through the specimen, and the transmitted electrons are then focused by a series of electromagnetic lenses to create an image. The image reveals details about the sample's internal structure, such as the arrangement of atoms, lattice defects, and the thickness of individual layers. TEM can achieve extremely high magnification, allowing for the visualisation of fine details at the atomic and nanoscale levels. TEM is particularly useful for studying the microstructure of materials, investigating crystalline structures, analysing nanoparticles, and examining biological samples.
- ▶ In SEM, a focused electron beam is scanned across the surface of the specimen in a raster pattern. As the beam interacts with the specimen, secondary electrons, backscattered electrons, and characteristic X-rays are emitted. These emitted signals are detected and used to generate an image. The image produced in SEM provides surface topography information and can also reveal composition variations. SEM offers a three-dimensional view of the sample surface, allowing for the observation of surface features, textures, and roughness.
SEM also offers significantly better depth of focus compared to an optical microscope, making it suitable for studying rough surfaces. The depth of focus refers to the thickness of a specimen that can be in focus at a given magnification level. In microscopy, there is an inverse relationship between magnification and depth of focus. As the magnification increases, the depth of focus decreases. This is due to the inherent optical properties and limitations of the microscope's imaging system. At higher magnifications, the depth of focus becomes narrower, meaning that only a thin slice of the specimen will be sharply focused, while the rest will appear blurred or out of focus. SEM's superior depth of focus enables it to capture a wider range of heights and surface irregularities in focus, providing detailed information about the roughness, texture, and topography of the specimen.

Scanning Probe Microscopy

Scanning probe microscopy (SPM) is a branch of microscopy that allows the imaging, manipulation, and characterization of surfaces at the nanoscale. SPM techniques rely on the use of a sharp probe, typically a small tip or needle, which scans the surface of a sample in a controlled manner.

Scanning Tunnelling Microscopy

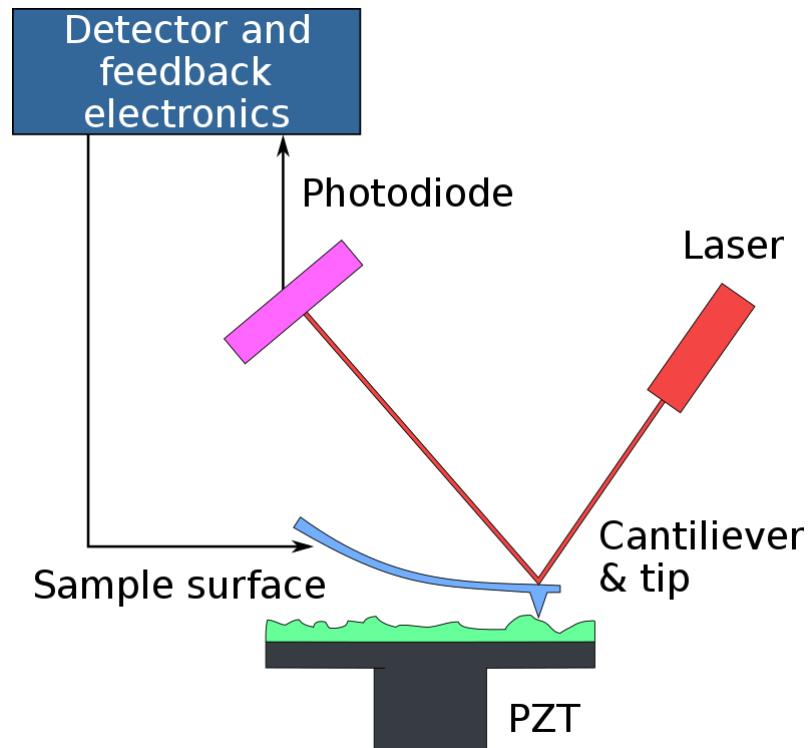
Scanning tunnelling microscopy (STM) relies on the quantum tunnelling effect. When a conducting tip is brought close to the surface being examined, a voltage difference is applied between them. This voltage allows electrons to tunnel through the vacuum between the tip and the surface. The resulting tunnelling current depends on the position of the tip, the applied voltage, and other factors. By monitoring this current as the tip scans across the surface, information about the surface's characteristics is obtained.



However, using an STM can be challenging. It requires exceptionally clean and stable surfaces to ensure accurate measurements. The tip used in the STM must be sharp and carefully controlled. Vibration control is crucial to minimise any unwanted movement or disturbances during the scanning process. Additionally, sophisticated electronics are necessary to measure and interpret the tunnelling current accurately.

Atomic Force Microscopy

Atomic force microscopy (AFM) is a technique used to scan a sample's surface using a probe attached to a cantilever. It allows for the determination of surface topography at the atomic level. The probe is a sharp tip attached to a flexible cantilever. The cantilever serves as a sensitive mechanical spring that responds to interactions between the probe and the sample surface. When the tip approaches the sample surface, the probe-sample interactions are primarily repulsive at short distances. These interactions cause the cantilever to flex, which is detected by the instrument. The topography of the sample's surface is determined by measuring the deflection of laser light on the probe. The deflection is detected by a position-sensitive detector, allowing for the mapping of surface features.



An extremely precise piezo-electric system is used to position the tip or the sample. This system enables high-resolution scanning in the x , y , and z directions, with Ångstrom-level (\AA) resolution. In contact mode, the tip is in permanent contact with the sample surface. This mode is suitable for imaging rigid surfaces. In tapping mode, the tip oscillates at its resonance frequency (around 100 kHz) and briefly taps the sample surface. This tapping action minimises lateral forces and is commonly used for imaging soft or delicate samples. Additionally, to maintain a constant oscillating amplitude in tapping mode, a feedback mechanism is employed. The system continuously adjusts the oscillation parameters based on the detected deflection, ensuring consistent imaging conditions.