UNIT II

Microscopy

With the advent of high resolution microscopes modern microbiologists have access to microscope that produces images with high clarity and magnification. The Leeuwenhoek's single lens microscope has been transformed into a high resolution multi-lens combination with magnification upto two thousand times. Further, electron microscopes with magnification upto one lakh times can be used to study fine structure of cells and sub-cellular components. Broadly, microscopes are categorized in two categories: Light (optical) microscopes and Electron microscopes. Light microscopy involves use of optical lenses and light radiations. It can further be categorized as: (1) Bright field microscopy, (2) Dark field microscopy, (3) Phase contrast microscopy, and (4) Fluorescence microscopy. Electron microscopy is of two types (1) Transmission microscopy and (2) Scanning electron microscopy. Light travels as a wave in crests and troughs. The amplitude of the crests and troughs determine the brightness of the light. The number of times complete wave occur per unit time is called as frequency and the distance between two consecutive crests is called as wavelength (λ) of the light. Wavelengths in the range of 400–700 nm make up visible spectrum while the UV region consists of wavelengths ranging from 100–385 nm. Visualizing any object directly by human eye involves incidence and reflection of light in the visual range. Microscopes use day light or light emitted by incandescent bulb. Fluorescent and UV microscope employ UV radiations. Electron microscopes use beam of electrons in place of light and thus object cannot be perceived by our eyes directly. The image produced by electron microscopes is perceived by CRT or X-ray plates.

MAGNIFICATION

The limit of an unaided human eye to visualize any tiny object is 0.1 mm. To see microorganisms, which are much smaller than 0.1 mm, a system of convex lenses is required. When parallel rays pass through the convex lens, they get converged at a point, called as focal length (f) of the lens. The magnification obtainable with a particular lens depends upon its focal length. Therefore, a lens with shorter focal

length will have higher magnification power. In a compound microscope the real image is formed by the objective (10X, f = 16 mm; 40X, f = 4 mm; 100X, f = 1.8 mm). The image produced by objective lens falls on the eyepiece lens (10X; 15X) and serves as object for it. The image formed in the eyepiece is perceived by one eye and is the product of magnifications of two lenses. **Figure 2.1** shows a modern compound microscope and its various parts.

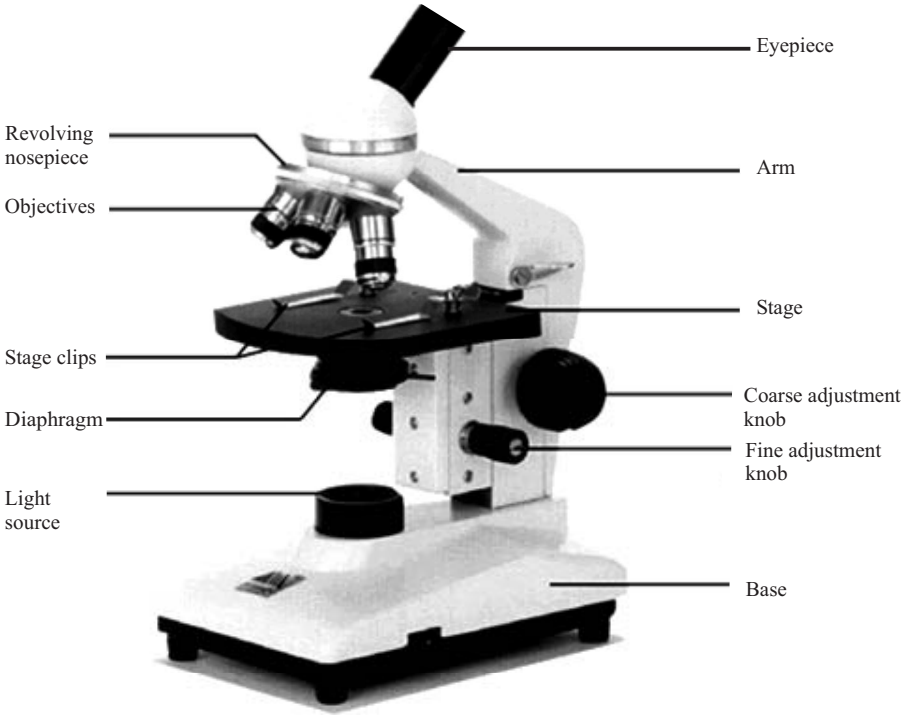

Figure 2.1: Parts of a compound microscope.

RESOLUTION

The ability to see two close objects as two distinct objects is called as resolution. The limit of resolution is the closest distance between two points at which the points still can be distinguished as separate entities. Magnification should be coupled with good resolution to visualize small microorganisms; else magnification alone will produce an inconclusive or blurred image. Resolution is defined as the distance at which two objects can be viewed as separate or distinct objects. The resolution (or limit of resolution) can be calculated as:

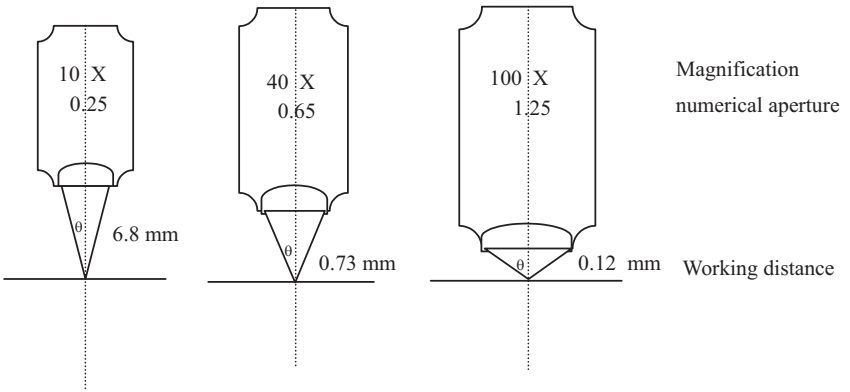

Figure 2.2(a): Numerical aperture and working distance of the 10X, 40X, and 100X (oil immersion) objectives of a compound microscope.

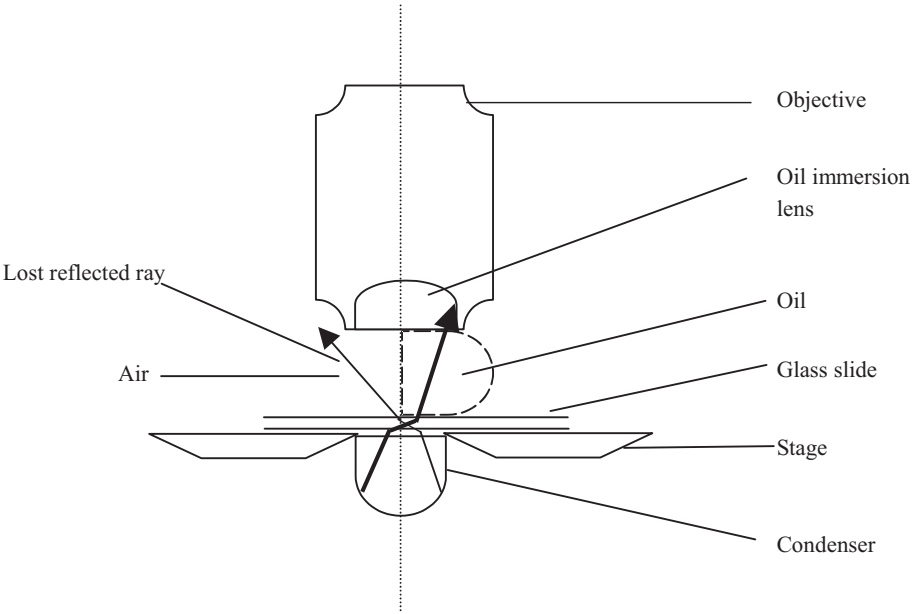

Figure 2.2(b): Ray diagram showing effect of immersion oil on cone of light (numerical aperture).

by one million (10^{-6}). A nanometer (millimicron) is equal to 10^{-9} meters. An angstrom (Å) is equal to 10^{-10} meters. The choice of microscopic unit is grossly dictated by the specimen size. However, all these above mentioned units are used in scientific literature and hence one should be acquainted with all these.

and 100X (oil immersion). Most ocular lenses have magnifying power of 10X or 15X. As mentioned earlier, the resulting magnifying power will be obtained by multiplying the magnification powers of objective and ocular lenses. For an ocular lens of 10X, magnification produced by low power objective will be 10X × 10X = 100X and similarly 400X and 1000X for high power and oil immersion objectives, respectively.

Next to magnification a very important parameter in microscopy is resolution (resolving power). It is defined as the ability of the microscopic lens setup to distinguish between two close points. General principle in case of microscopes is that shorter the wavelength of illuminating light, the greater will be the resolution. The wavelength of commonly used white light is long and can effect resolution up to 2 nm only. Considering this and other practical constraints, the limit of magnification of the best compound microscope can be upto 2000X only.

For a sharp and clear image in compound microscope the specimen should sharply contrast with the medium (air). In order to attain such contrast, the refractive index of the specimen should be changed from that of its own medium. The refractive index is defined as the measure of relative velocity with which light passes through any material. The refractive index of any specimen can be changed by staining it. In normal course, light rays move in a straight line through a single medium. After staining, the incident light has to pass through two different media, i.e. air and the stained specimen. Due to difference in refractive indices the rays adapt a bending and refracting pattern at the edges of specimen and hence the contrast between medium and specimen is increased. The rays that scatter away from the specimen are spread out resulting in separation of close points, i.e. better resolution.

Objectives with high magnification (100X) have small lenses (small diameter) which are not sufficient enough to gather refracted rays. Light rays emerging out from the slide are refracted as they enter the air and the high power lens being small is not able to capture them resulting in a fuzzy image with low resolution. Thus, to maintain the direction of light rays at 100X, immersion oil is put between glass slide and objective lens. Immersion oil has similar refractive index to that of the glass and becomes part of optical path **(Figure 2.3)** and does not allow the light rays emerging from specimen to get away.

IMPROVISATIONS IN COMPOUND LIGHT MICROSCOPES

Bright Field Microscope

In normal course the field of a compound light microscope is brightly illuminated by focused natural sunlight or white light (electrical illuminator) resulting in a bright field image of the stained specimen. Such commonly used microscopes are called as bright field microscopes. However, certain improvisations in conventional light microscope produce special images that are used to draw important scientific conclusions.

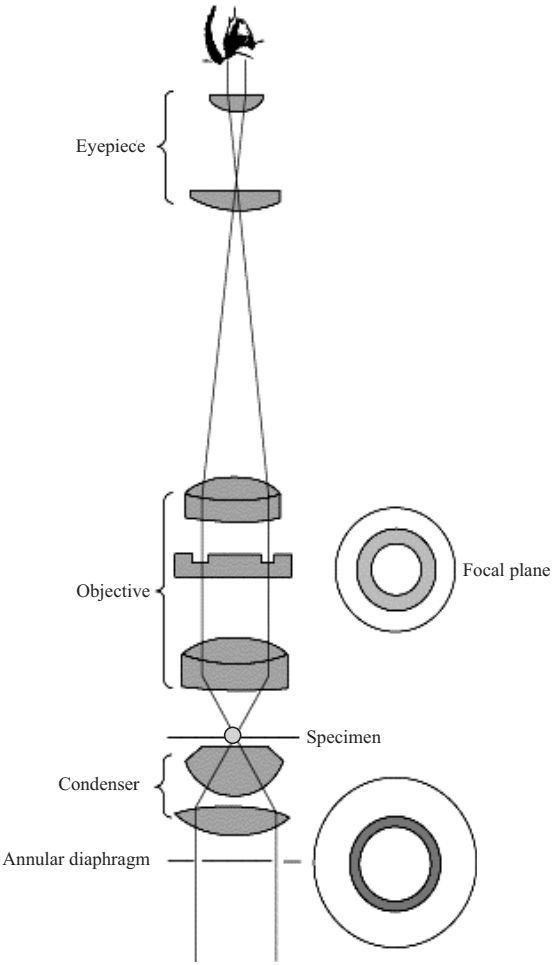

Figure 2.4: Diagram showing working of phase contrast microscope.

fluorescent light excepting blue. The specimen appears as a luminescent bright object against a dark background. Fluorescent microscopy is also used effectively for diagnostic or identification purposes. For example, flourescein isothiocyanate (FITC) selectively stains *Bacillus anthracis* that appear green while auramine 'O' is selective for *Mycobacterium tuberculosis* that appear yellow when fluoresce. The most important use of this technique is made in immunodiagnostics and is referred as immunofluorescence. In this technique, the fluorescent group is attached to homologous antibodies raised against the pathogen. Suspected clinical sample such as sputum, blood, etc. is mixed with labeled antibody on a slide. If the sample contains the pathogen, fluorescent antibodies bind firmly to the antigenic determinants of the pathogen making it fluoresce under UV light **(Figure 2.5b)**. Fluorescent treponemal antibody absorption (FTA-ABS) is a good example of

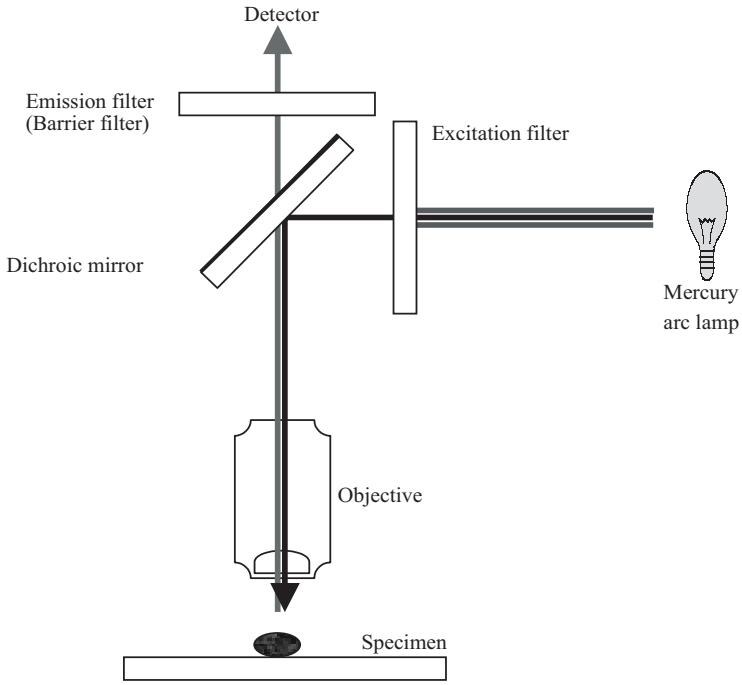

Figure 2.5(a): Line diagram of working of a fluorescent microscope.

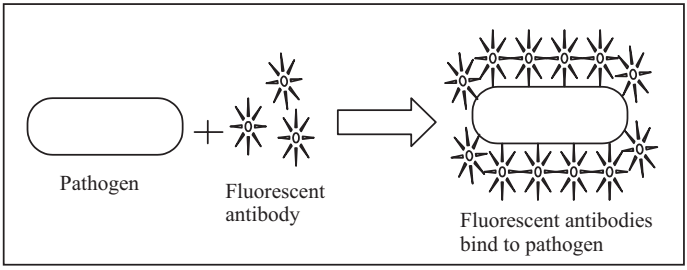

Figure 2.5(b): Fluorescent treponemal antibody absorption (FTA-ABS) for diagnosis of syphilis.

diagnosis of treponemal infection (Syphilis). When excitation light is transmitted from underneath or below the specimen, the phenomenon is called as transmitted fluorescence while if the excitation light is made to fall upon the specimen through objective lens, the phenomenon is referred as epi-fluorescence.

Confocal Microscopy

Confocal microscopy is a technique applied to enhance the contrast of photomicrograph for developing 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. It was described and patented by Marvin Minsky in 1957. A confocal microscope uses point illumination and a pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus information. Thus, only the light within the focal plane can be detected, so the undesired flare of light is avoided and image quality is enhanced than that of normal optical images. Since a single point is illuminated at a time in confocal microscopy, 2D or 3D images are developed by scanning over a regular raster on the object.

MICROMETRY

A compound microscope is a combination of lenses resulting in formation of a virtual and magnified image of the microorganism or any specimen. For the purpose of characterization and identification of any species it is important to measure the dimensions micrometrically. **Figure 2.6** shows a comparison of dimensions of a variety of microorganisms. In this technique, firstly, the magnification of the lenses is calculated using a standard stage micrometer. For this an ocular micrometer (OM) having 100 equal spaced divisions without any standard unit is placed in the eyepiece and stage micrometer (SM) is placed on the stage. The value of ocular micrometer is calibrated with stage micrometer by focusing the scale of stage micrometer (having divisions spaced at 10 μm) through objective and superimposing its graduations with those of ocular micrometer **(Figure 2.7)**.
The value of one division of ocular micrometer (OM) is calculated as:

1 division of O.M. = (No. of divisions of S.M. × 10 μm) / No. of divisions of O.M.

Stage micrometer is removed after calibration of ocular micrometer, and the slide of specimen is placed on the stage and examined. The dimensions of desired microscopic structures are measured using calibrated ocular micrometer. In case of fungi, length and breadth of spores, sporangiophores and other structures of taxonomic interest are recorded and compared with their taxonomic or monographic descriptions for identification.

Camera Lucida

This is an accessory instrument that allows us to draw the exact drawings of microscopic images seen through a microscope. The principle of camera lucida is based on simple optics. The image formed by the microscope in the eyepiece is reflected on the paper by a prism and reflecting mirror. The observer then moves the pencil around the desired structure of the microorganism visible against the white background of paper and draws the exact figure of the microorganism. There are three main parts of a camera lucida—the attachment ring, the prism and the mirror. The attachment ring attaches the camera lucida on the body tube around eyepiece. The prism is placed above the eyepiece when the instrument is attached with the microscope. The image of the specimen is reflected by the prism horizontally onto the plain mirror. The mirror is set at an angle of 45° with reference

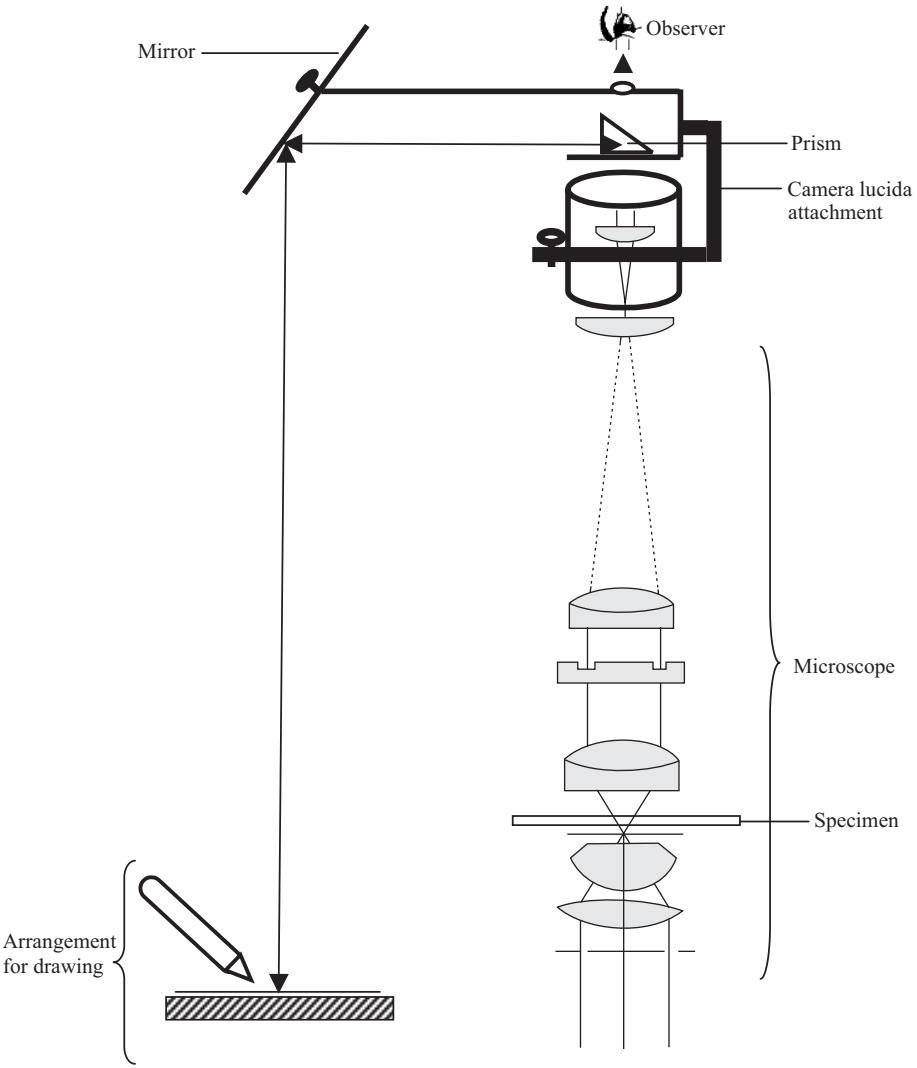

Figure 2.8: Schematic showing working of a camera lucida.

Å) which is almost 100 times than that of a light microscope. The magnified image can be viewed on a fluorescent screen (CRT- cathode ray tube or TV) or recorded on a photographic plate.

The specimen to be studied is prepared as a very thin dry film on small screens and is put in the instrument at a point (equivalent to stage of a light microscope) between the objective and condenser. The resulting magnified image can be viewed on a fluorescent screen through an airtight window or can be printed on a photographic plate with the help of an inbuilt camera.

Several techniques such as staining, shadow casting, freeze fracturing, etc. when used with electron microscope make it a highly useful instrument for study of finest details of cellular structure. *Shadow casting* involves deposition of a thin layer of metal (e.g. gold, platinum) at an oblique angle on the organism so that the organism produces a shadow on the uncoated side. This produces an image that provides topographical information, e.g. rough surface or echinulations of spore surface. Compounds that do not allow electrons to pass through (electron dense materials) such as phosphotungstic acid can be used to stain the outline of the object (*negative staining*). The deposition of such electron dense materials on the cell surface highlights its clefts and crevices and helps to derive fine details of the cell surface and appendages (flagella, pili, etc.)

Visualizing intracellular structures requires sectioning of the specimen cells. The cells are fixed in a block and then *ultrathin sections* (60 nm) are cut with ultramicrotome using fine glass knives. The resulting ribbon of thin slices is then examined and as one can imagine, different slices reveal different level and angle of intracellular structure. The contrast among the intracellular structures can be enhanced by using stains, e.g. uranium salts. Thin sectioning of specimen under frozen condition is called as *freeze etching*. Carbon replicas of the structures thus exposed reveal internal structures of the cell. Chemical fixation and staining procedures that may produce distortions or artifacts are avoided. Intracellular components of a cell can also be localized using immunochemical techniques. Thin sections can be treated with antibody labeled with electron dense substance such as Ferritin. The combination of such labeled antibody with specific cell antigens produces an image that highlights the ferritin in sharp contrast. Similarly, enzymes and other molecules can also be localized using homologous antibodies raised against a particular enzyme or molecule. The antibodies are labeled with colloidal gold or electron dense materials for providing a sharp contrast to the enzyme-antibody complex in electron microscopic image.

In transmission electron microscope (TEM) electron beams traveling through magnetic condenser lens fall upon specimen and create an image having high resolution at the final aperture **(Figure 2.9 a)**. In scanning electron microscopy (SEM) the specimen is scanned or rapidly moved over by a narrow beam of electrons resulting in release of secondary electrons and other radiations from the specimen surface **(Figure 2.9 b)**. The intensity of these secondary electrons varies with the shape and chemical composition of the specimen surface. These secondary electrons and radiations are collected by a detector which generates an electronic signal. These signals are then accumulated and scanned to produce an image at the cathode ray tube (similar to that of television). In this case the resolution is not as good as TEM but the pictures reveal three-dimensional topography of the specimen along with the depth of the field.

In electron microscope, specimens are examined in a chamber that is under a very high vacuum and hence these can not be examined in living state. Due to low

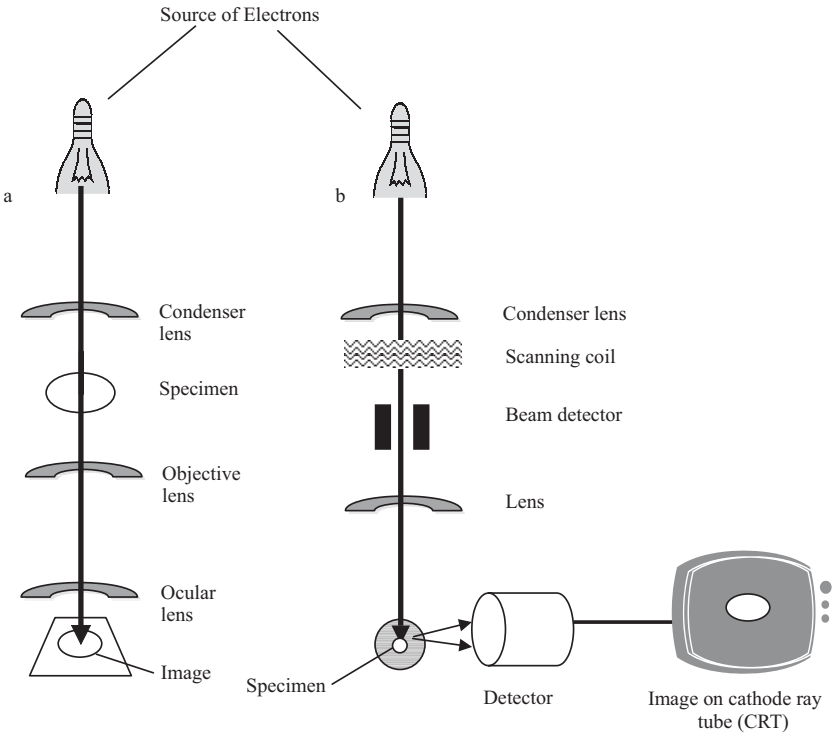

Figure 2.9: Diagram showing working of
(a) Transmission electron and (b) Scanning electron microscope.

penetration power of the electron beams, the specimen is to be prepared as a thin film and has to undergo processing, e.g. drying, freezing, staining, etc. which can lead to misleading morphological alterations, distortions or artifacts in it.

Scanning Tunnelling Microscopy

Scanning tunnelling microscopy (STM) is specifically meant for the study of the three-dimensional structure of the proteins and nucleic acids. A normal SEM picture of the macromolecule will lack desired vertical resolution while direct hit of the electrons during TEM distorts these molecules. In STM, images of the molecules are obtained only by scanning its surface under ultra-high vacuum. By using STM, we can determine intramolecular distances in materials with surfaces that can conduct electrons. Gerd Binning and Heinrich Rohrer were awarded Nobel prize in Physics in 1986 for inventing *scanning tunneling microscope.*

Immunoelectron Microscopy

This microscopy technique utilizes the power of an electron microscope to analyze cellular structural and functional details based on antigen and antibody reactions. Immunoglobulins (Igs) are labeled with electron dense materials, e.g. ferritin,

violet binds firmly to their cell wall and resists decolourization with alcohol. In bacteria that appear red (Gram negative), crystal violet gets washed away with the decolourizing agent and hence they take up the counter stain colour.

The most plausible explanation of this differential behaviour lies in the chemistry and structure of cell wall. Gram-positive bacteria have a thick peptidogylcan cell wall while Gram-negative bacteria have thin layers of peptidoglycan and lipids. During the treatment with alcohol, the lipids of Gram-negative cell wall get washed away leading to increased porosity through which CV-Iodine complex escapes and the cells get decolourized. When treated with safranin they get stained to appear red. On the other hand, the porosity of thick peptidoglycan cell wall of Gram-positive bacteria is reduced due to dehydration by alcohol and CV-Iodine complex is retained with it providing a deep violet colour to the cells.

The behaviour can also be explained on the basis of structure and porosity of the cell walls. Gram-positive cell walls are made up of highly cross-linked peptidoglycan and bear very small pores whereas Gram-negative bacteria have very thin and less cross-linked peptidoglycan with larger pores. In Gram-positive bacteria the pore size is reduced after the alcohol treatment and CV-I complex is entrapped. Due to less peptidoglycan, there is not much diminution in the pore size of Gram-negative cell wall after the alcohol treatment and the CV-I is able to escape with decolourizing agent. Also, the fact that protoplasts of Gram-positive bacteria do not retain CV-I complex supports the idea that the retention of this stain depends upon their characteristic cell wall.

Old Gram-positive cells exhibit Gram variable behaviour, i.e. they many lose CV and get stained red with safranin. This alteration is attributed to changes in the cell wall with age and environment. The cell wall of Archaebacteria have entirely different structure and composition and some members of the group are Gram positive while some are Gram negative. The walls of Gram-negative archaebacteria are thinner than Gram-positive archaebacteria. Thus, the Gram behaviour is an important parameter for bacterial classification and identification. Gram staining does not apply to other microbial groups, e.g. protozoa and fungi, however, yeasts, unicellular ascomycetous fungi, are always Gram positive.

Staining in which the acidic dyes are repelled by the cell and thus background appears coloured while the specimen is light is called as negative staining, e.g. staining with Nigrosine. Usually, positive staining is used in which stain binds and imparts colour to the specimen. Acid fast staining or Zeihl-Nelson staining is also a differential staining techinique for differentiating acid fast bacteria from non-acid fast bacteria. Also bacterial appendages (flagella), capsules, endospores and intracellular deposits of starch, glycogen, poly-hydroxybutyrate, and lipids can be specially stained and visualized under light microscope.