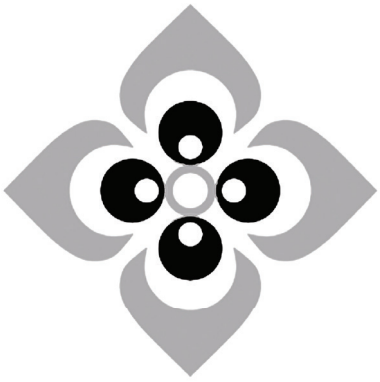

Consortium for Educational Communication

Module on

MICROSCOPY AND ITS TYPES

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INTRODUCTION

1. History of Microscopy

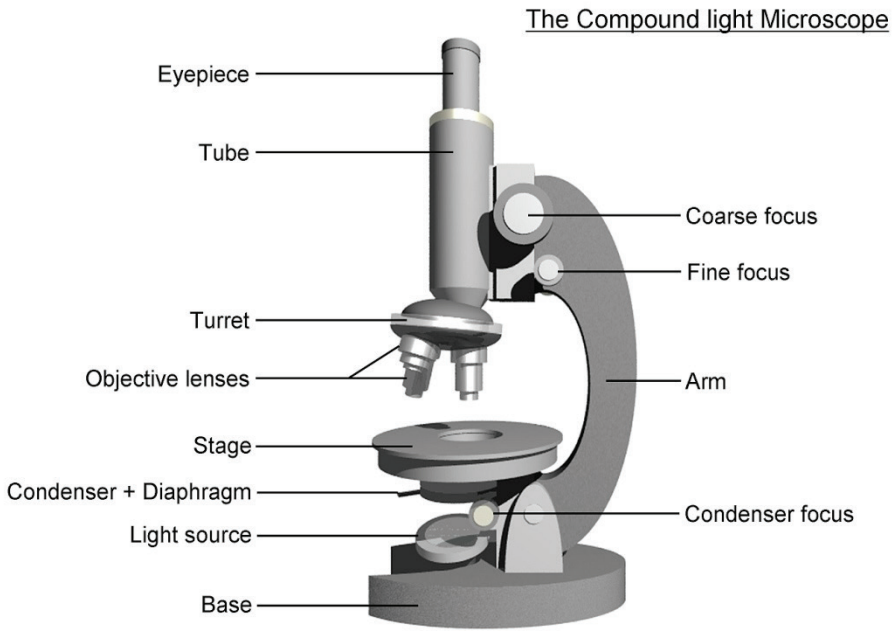
The early simple "microscopes" which were only magnifying glasses had one power, usually about 6x - 10x. It was Antony Van Leeuwenhoek (1632-1723), a Dutch scientist, and one of the pioneers of microscopy who in the late 17th century became the first man to make and use a real microscope. He made his own simple microscopes, which had a single lens and were hand-held. Leeuwenhoek's microscope used a single convex glass lens attached to a metal holder and was focused using screws. Leeuwenhoek became more involved in science and with his new improved microscope was able to see things that no man had ever seen before. He saw bacteria, yeast, blood cells and many tiny animals swimming about in a drop of water. People did not realize that magnification might reveal structures that had never been seen before - the idea that all life might be made up of tiny components unseen by the unaided eye was simply not even considered.

Later on Englishman Robert Hooke is credited with the microscopic milestone of discovering the basic unit of all life, the cell. In the mid 17th century, Hooke saw a structural mesh while studying a sample of cork that reminded him of the small monastic rooms called cells (Micrographia). Hooke is also credited with being the first to use the basic three-lens configuration that is still used in microscopes today. Compound microscope system was invented in the 17th century. This type of microscope incorporates more than one lens so that the image magnified by one lens can be further magnified by another. Today, the term "microscope" is generally used to refer to this type of compound microscope. In the compound microscope, the lens closer to the object to be viewed is refers to as the "objective", while the lens closer to the eye is called the "eyepiece".

In recent times, the development of the microscope has achieved its heights, since optical principles are well understood and to an extent, the optical limits have been reached. The majority of microscopes follow the same structural principles that describe monocular, mono-binocular and stereo-binocular microscopes. While the technical limits of design have been reached, Vision Engineering has taken the approach of developing the practical day-to-day user friendliness of the microscope.

2. Basic components of Microscope

The compound microscope uses lenses and light to enlarge the image and is also called an optical or light microscope. The simplest optical microscope is the magnifying glass and is good to about ten times (10X) magnification. The compound microscope has two systems of lenses for greater magnification. 1) The ocular, or eyepiece lens that one looks into and 2) The objective lens, or the lens closest to the object. The microscope and its basic parts are defined below:



1) **Eyepiece Lens:** The lens is at the top that, through which we look is an eye piece. They are usually 10X or 15X power.

2) **Tube:** Tube connects the eyepiece to the objective lenses.

3) **Arm:** Supports the tube and connects it to the base

4) **Base:** The bottom of the microscope, used for support

5) **Illuminator or light source:** A steady light source used in place of a mirror. If microscope has a mirror, it is used to reflect light from an external light source up through the bottom of the stage.

6) **Stage:** The flat platform where slides are placed. Stage clips hold the slides in place. If microscope has a mechanical stage, than it will be able to move the slide around by turning two knobs. One moves it left and right, the other moves it up and down.

7) **Revolving Nosepiece or Turret:** This is the part that holds

two or more objective lenses and can be rotated to easily change power.

8) **Objective Lenses:** Usually there are 3 or 4 objective lenses on a microscope. They almost always consist of 4X, 10X, 40X and 100X powers. When coupled with a 10X (most common) eyepiece lens, we obtain total magnifications of 40X (4X times 10X), 100X, 400X and 1000X. To have good resolution at 1000X, it is needed a relatively sophisticated microscope with a condenser.

9) **Condenser Lens:** The purpose of the condenser lens is to focus the light onto the specimen. Condenser lenses are most useful at the highest powers (400X and above).

10) **Diaphragm or Iris**: Many microscopes have a rotating disk under the stage. This diaphragm has different sized holes and is used to vary the intensity and size of the cone of light that is projected upward into the slide.

Proper procedure to use microscope and some precautions:

1. Always carry microscope with both hands. Grasp the arm with one hand and place the other hand under the base for support.

2. Turn the revolving nosepiece so that the lowest power objective lens is "clicked" into position.

3. Place the microscope slide on the stage and fasten it with the stage clips. You can push down on the back end of the stage clip to open it.

4. Using the coarse adjustment, lower the objective lens down as far as it will go without touching the slide.

5. Look through the eyepiece and adjust the illuminator (or mirror) and diaphragm for the greatest amount of light.

6. Slowly turn the coarse adjustment so that the objective lens goes *up* (away from the slide). Continue until the image comes into focus. Use the fine adjustment, if available, for fine focusing.

7. Move the microscope slide around so that the image is in the center of the field of view and readjust the mirror, illuminator or diaphragm for the clearest image.

8. The proper way to use a monocular microscope is to look through the eyepiece with one eye and keep the other eye open (this helps avoid eye strain). If you have to close one eye when looking into the microscope, it's ok. Remember, everything is upside down and backwards. When you move the slide to the right, the image goes to the left.

9. Do not touch the glass part of the lenses with your fingers. Use only special lens paper to clean the lenses.

10. When finished, raise the tube, click the low power lens into position and remove the slide.

3. MICROSCOPE AND ITS TYPES

The invention of the microscope has brought about great inventions that have transformed the human race. This device is effective and very important in science because it provides opportunity for scientist to study natural elements that are not visible to the naked eye.

There are different types of microscopes depending on the purpose for which it is intended. Microscopes can also be classified based on their image making physical principles, area of application and versatility. Below is a list of the major types of microscopes and their uses.

A) **Light or Compound Microscopes**

These types of microscopes are based on a simple principle of light and lens. A light source illuminates the object while the lens magnifies it so that it can be visible to the human eye for the purpose of studying or evaluating. Under this category are the simple microscope, compound microscope and the stereo microscope.

i) **Simple Microscope**

This is one of the oldest microscopes that use a single lens for magnifying any samples. However, this microscope has been regarded as primitive because of its less relevance in serious scientific work.

ii) **Compound Microscope**

This type of microscope operates on the same principle as the simple microscope. But the difference is that it makes use of two different optical parts for the magnifying of objects. The compound microscopes are the most commonly used in many laboratories because they are efficient, inexpensive, and can magnify objects as much as 2000 times the original size. They are used mainly for the study of cells, chromosomes and the DNA.

iii) **Dissection or Stereo Microscope**

This is another member of the optical microscopes that makes use of light and lens. This microscope is different from other types of microscopes because it allows you to view objects in three dimensional (3D). It contains lens in different angles that provides a three dimensional viewing of objects for complete diagnosis.

However, the stereo microscope doesn't have very strong magnifying power like the compound microscope, but can be very useful in studying of dissection parts of living organisms. It is used mainly in the field of medical science including forensics, fine

repair, sorting, and microsurgery.

Other types of optical microscopes that are not very common but still well used includes the UV microscope that makes use of UV light to observe objects, the inverted microscope that is used for viewing thick or large objects upside down, and the metallurgic microscope used by engineers and scientists for viewing the structure of metals, ceramic and plastic. There are also digital microscopes that make use of optical lens and Charge-coupled device sensors to magnify objects to about 1000 times. Digital microscopes are also good because they have a 2 million pixel camera that provides high quality recording of the objects in view, and is connected to a TV monitor for high resolution viewing or observation.

B) **Electron Microscope**

These are the most advanced types of microscopes used in modern science. The electron microscopes are powered by a beam of electron that strikes any objects that comes to its path to magnify it. Electron microscopes are used for studying cells and small particles of matter, as wells as large objects. Types of electron microscopes include transmission and reflection electron microscopes.

i) **Transmission Microscope**

Transmission microscopes are used for studying cells and tiny slices of microorganisms like viruses, after they have been stained with palladium and gold and placed upon a wire grid. The transmission electron microscope (TEM) operates on the same basic principles as the light microscope but uses electrons instead of light. TEM use electrons as "light source" and their much lower wavelength makes it possible to get a resolution a thousand times better than with a light microscope. TEMs work by using a tungsten

filament to produce an electron beam in a vacuum chamber. The emitted electrons are accelerated through an electromagnetic field that also narrowly focuses the beam. The beam is then passed through the sample material. The specially prepared sample is a very thin (less than 100nm) slice of material. The electrons that pass through the sample hit a phosphor screen or film and produce an image. Where the sample has less density, more electrons get through and the image is brighter. A darker image is produced in areas where the sample is denser and therefore less electrons pass through.

ii) **Reflection Electron Microscope**

Reflection electron microscopes also uses electron beams but is different from transmission and scanning electron microscopes being that it is build to detect electrons that have been scattered elastically.

All types of microscopes are used based on their purpose and the results that the scientist or the observer is trying to achieve. There are other microscopes designed for specific use in different types of field or based on their source like the X-ray microscope that uses X-ray beams to create images of an object. And the scanning acoustic microscope that makes use of sound waves to detect images. This type of microscope is used material science and biological science for detecting cracks in material and to uncover elasticity and stress in biological structures respectively.

4. MICROSCOPY

Microscopy is the technique used to view objects that cannot be seen by the naked eye but can be seen with the help of microscopes. There are 3 main microscopic techniques that are used which include Optical microscopy, Scanning probe microscopy and Electron microscopy.

I) .OPTICAL MICROSCOPY OR LIGHT MICROSCOPY

Optical microscopy is also known as light microscopy. It involves the use of visible light and one or more lens to produce an enlarged image of an object that is placed in the focal plane of the lens. This can either branch off into transmission, where the beam of light passes through the sample or reflection where the beam reflects off the sample surface, i.e. reflected light microscope. There are many applications of Optical microscopy such as in nanophysics and biotechnology but in medicine it is mostly known as being used in diagnosis when we are dealing with tissues or tests on free cells known as a smear test.

Types of Optical Microscopy

1) 6. Transmitted Light Microscopy

Transmitted light microscopy is the general term used for any type of microscopy where the light is transmitted from a source on the opposite side of the specimen to the objective lens. Usually, the light is passed through a condenser to focus it on the specimen to get maximum illumination. After the light passes through the specimen it goes through the objective lens to magnify the image of the sample and then to the oculars, where the enlarged image is viewed.

In order to get a usable image in the microscope, the specimen must be properly illuminated. The light path of the microscope must be correctly set up for each optical method and the components used for image generation. The condenser was invented to concentrate the light on the specimen in order to obtain a bright enough image to be useful. The optimum set-up for specimen illumination and image generation is known as Kohler illumination after the man

who invented it. It is used for most of the optical configuration.

The microscope techniques requiring a transmitted light path include bright field, dark field, phase contrast, polarisation and differential interference contrast optics which are described below:

a) **Bright Field Microscopy**

This is "normal" microscopy when no optical contrast technique is employed. It uses transmitted light to view a specimen that contains inherent contrast/colour or is stained. In order to get the best image possible from any transmitted light form of microscopy, and it is crucial that the light path be set up properly. The method for doing this is called illumination. It is also known as double diaphragm illumination because it employs both a field and an aperture iris diaphragm to set up the illumination. It simply works by allowing the light rays to pass directly through the eye without being deflected by an intervening opaque plate in the condenser.

The condenser is used to focus parallel rays of light on the specimen, as if coming from infinity, thereby giving the advantages of an evenly illuminated field, a bright image without glare and minimum heating of the specimen. As most cells and tissues have insufficient contrast in themselves, staining techniques are generally used. Properly stained, specimens may be magnified to 1200x; utilizing an oil immersion objective will increase resolution at this high magnification. Common stains include Papanicolaou's stain, which is used for cervical smears, and toluidine blue, a general stain used for semi-thin sections of all tissue types.

b) **Dark Field Microscopy**

The specimen is illuminated obliquely, with no direct light entering the objective. Features in the specimen plane which

scatter light can clearly be seen against a dark background. Dark field illumination is provided by either a simple patch stop, a dark field element in a phase contrast condenser or purpose-built dark field condenser. The latter is required for high-resolution objectives to prevent the oblique rays entering the wide aperture of the objective. Applications include detection of micro-organisms in unstained smear preparations and classical diatom studies.

c) **Phase Contrast**

Phase contrast microscopy is devised by Frederick Zernike, this technique exploits the fact that light slows slightly when passing through biological specimens. The specimen is illuminated by a hollow cone of light coming through a phase annulus in the condenser. Phase contrast objectives must be used, which have a corresponding phase plate. Light rays passing through the specimen are slightly retarded, and further retardation takes place in the phase plate. When these rays combine with rays which have not taken this path, degrees of constructive and destructive interference occur which produce the characteristic light and dark features in the image.

Applications of Phase Contrast Microscopy

Phase contrast is preferable to bright field microscopy when high magnifications (400x, 1000x) are needed and the specimen is colourless or the details are so fine that colour does not show up well. Cilia and flagella, for example, are nearly invisible in bright field but show up in sharp contrast in phase contrast microscopy. Amoebae look like vague outlines in bright field, but show a great deal of detail in phase contrast. Most living microscopic organisms are much more obvious in phase contrast microscopy.

d) **Polarised Light Microscopy**

Polarised light microscopy uses plane-polarised light to analyse

structures that are birefringent; structures that have two different refractive indices at right angles to one another (e.g. cellulose micro fibrils). Normal, un-polarised, light can be thought of as many sine waves, each oscillating at any one of an infinite number of orientations (planes) around the central axis. Plane-polarised light, produced by a polar, only oscillates in one plane because the polar only transmits light in that plane. The polarised light microscope must be equipped with both a polarizer, positioned in the light path somewhere before the specimen, and an analyser (a second polarizer), and placed in the optical pathway after the objective rear aperture. Image contrast arises from the interaction of plane-polarized light with a birefringent (or doubly-refracting) specimen to produce two individual wave components that are each polarized in mutually perpendicular planes. The velocities of these components are different and vary with the propagation direction through the specimen. After exiting the specimen, the light components become out of phase, but are recombined with constructive and destructive interference when they pass through the analyzer. Polarised light microscopy can be used to measure the amount of retardation that occurs in each direction and so give information about the molecular structure of the birefringent object (e.g. orientation).

e) **Differential Interference Contrast**

In this complex form of polarised light microscopy two slightly separate, plane polarised beams of light are used to create a 3D-like image with shades of grey. Wollaston prisms situated in the condenser and above the objective produce the effect, and additional elements add colour to the image. Care must be taken to interpreting Differential interference contrast (DIC) images as the apparent hills and valleys in the specimen can be misleading. The height of a "hill" (e.g. the nucleus) is a product of both the actual thickness of the feature (i.e. ray path length)

and its refractive index. Variations of the DIC system are named after their originators, Nomarski and de Senarmont. Options can be selected to maximise either resolution or contrast.

2) 7. Fluorescence Microscopy

Fluorescence can be used as a label or tag when preparing specific biological probes. Some biological substances, such as chlorophyll and some oils and waxes, have primary fluorescence (auto-fluorescence). However, most biological molecules do not fluoresce on their own, so they must be linked with fluorescent molecules (fluorochromes) in order to create specific fluorescent probes. The fluorochromes emit light of a given wavelength when excited by incident light of a different (shorter) wavelength. Specimens labelled with a fluorochrome such as fluorescein or green fluorescent protein (GFP) are illuminated with the relevant wavelength of light (blue in these examples) and emit the energy as a longer wavelength (green).

The key feature of fluorescence microscopy is that it employs reflected rather than transmitted light, which means transmitted light techniques such as phase contrast and DIC can be combined with fluorescence microscopy. To view the fluorescence in the microscope, several light filtering components are needed. At the heart of the fluorescence microscope is the dichroic mirror cube which comprises three components: a dichroic beam splitter (partial mirror), an excitation filter and a barrier filter. Specific filters are used to isolate the excitation and emission wavelengths for each fluorochrome. The dichroic mirror reflects shorter wavelengths of light and allows longer wavelengths to pass and is required because the objective acts as both the condenser lens (excitation light) and objective lens (emission light); therefore the beam splitter isolates the emitted light from the excitation wavelength. This epi-illumination type of light path is required to create a dark background

so that the fluorescence can be easily seen. The wavelength at which a beam splitter allows the longer wavelengths to pass must be set between the excitation and emission wavelengths of any given fluorochrome so that excitation light is reflected and emission light is allowed to pass through it. A bright light source producing the correct wavelengths for excitation is also required for fluorescence microscopy, normally a mercury arc lamp. When using confocal microscopy to view fluorescence, where up to 95% of the emission light is filtered out, specific wavelength lasers are used, as these are extremely bright and monochromatic.

3) 8. Confocal microscopy

Although conventional light and fluorescence microscopy allow the examination of both living and fixed specimens, certain problems exist with these techniques. One of the main problems is out-of-focus blur degrading the image by obscuring important structures of interest, particularly in thick specimens. In conventional microscopy, not only is the plane of focus illuminated, but much of the specimen above and below this point is also illuminated resulting in out-of-focus blur from these areas. This out of focus light leads to a reduction in image contrast and a decrease in resolution. In the confocal microscope all out-of-focus structures are suppressed at image formation. This is obtained by an arrangement of diaphragms, which, at optically conjugated points of the path of rays, act as a point source and as a point detector respectively. The detection pinhole does not permit rays of light from out-of-focus points to pass through it (as shown in figure 2). The wavelength of light, the numerical aperture of the objective and the diameter of the diaphragm (wider detection pinhole reduces the confocal effect) affect the depth of the focal plane. To obtain a full image, the point of light is moved across the specimen by scanning mirrors. The emitted/reflected light passing through the detector pinhole is transformed into electrical

signals by a photomultiplier and displayed on a computer monitor.

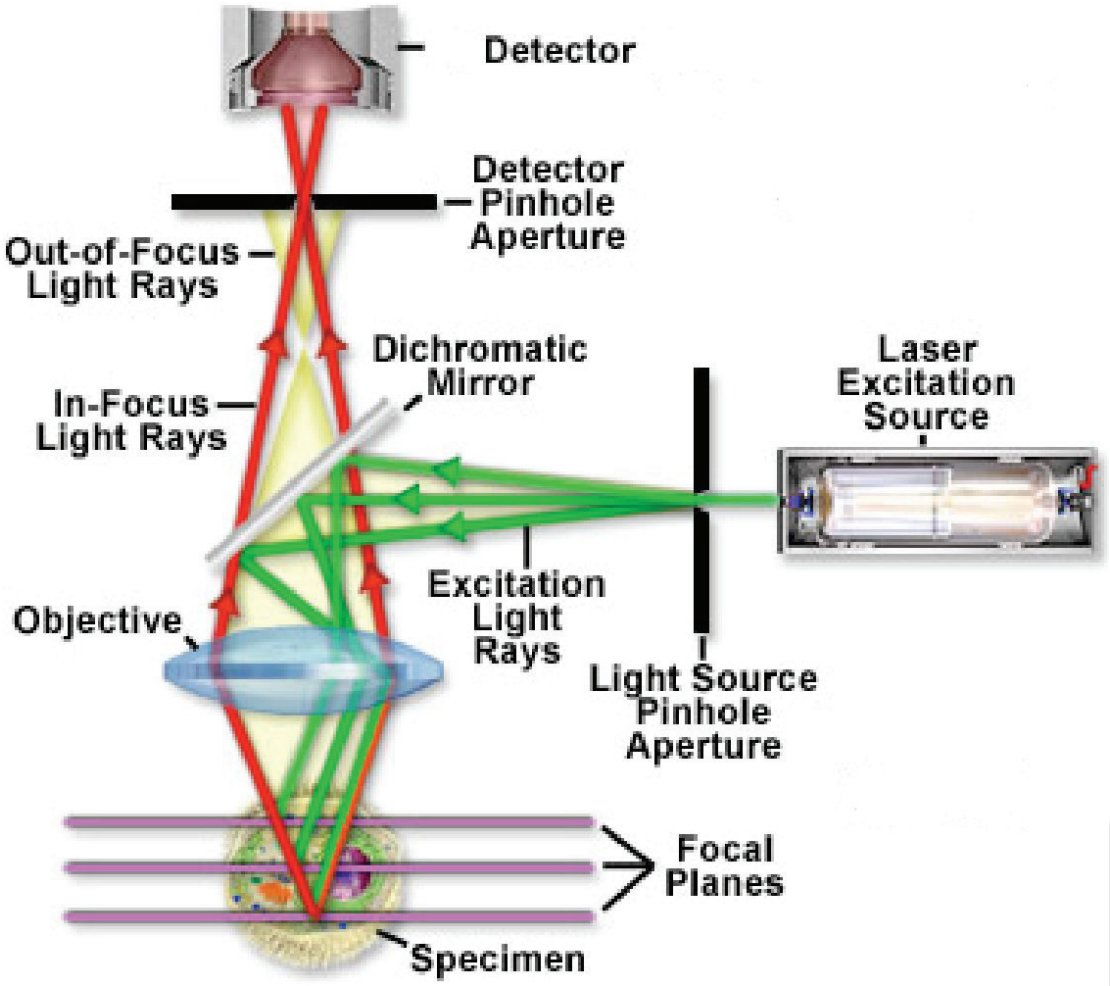


Figure 2: Principle behind Confocal microscopy

Source: staff.science.uva.nl

II) 9. SCANNING PROBE MICROSCOPY

This is another branch of microscopy that involves using a probe to scan the object. Basically it works by being moved around in a rectangular pattern known as raster scanning. A type of scanning probe microscopy is called STM (scanning tunneling microscopy) this is when a very sharp conducting tip is brought to the surface and a voltage is applied between them and we are able to find out the tunnel current and if this is maintained we can trace the elevation of the surface and thus produce it on an x-ray. There are

advantages and disadvantages to SPM however, for example the major advantages being that small structures can be created as the interaction can be modified from the probe and unlike electron microscopy we do not need a vacuum. The disadvantages being that the maximum image is generally small and the detailed shape can be hard to distinguish of the scanning tip.

Types of electron microscopy

All electron microscopes use electromagnetic or electrostatic lenses to control the path of electrons. The basic design of an electromagnetic lens is a solenoid (a coil of wire around the outside of a tube) through which one can pass a current, thereby inducing an electromagnetic field. The electron beam passes through the centre of such solenoids on its way down the column of the electron microscope towards the sample. Electrons are very sensitive to magnetic fields and can therefore be controlled by changing the current through the lenses.

The faster the electrons travel, the shorter their wavelength. The resolving power of a microscope is directly related to the wavelength of the irradiation used to form an image. Reducing wavelength increases resolution. Therefore, the resolution of the microscope is increased if the accelerating voltage of the electron beam is increased. The accelerating voltage of the beam is quoted in kilovolts (kV).

Although modern electron microscopes can magnify objects up to about two million times, they are still based upon Ruska's prototype and the correlation between wavelength and resolution. The electron microscope is an integral part of many laboratories. Researchers can use it to examine biological materials (such as microorganisms and cells), a variety of large molecules, medical biopsy samples, metals and crystalline structures, and the

characteristics of various surfaces. Nowadays, electron microscopes have many other uses outside research. They can be used as part of a production line, such as in the fabrication of silicon chips, or within forensics laboratories for looking at samples such as gunshot residues. In the arena of fault diagnosis and quality control, they can be used to look for stress lines in engine parts or simply to check the ratio of air to solids in ice cream.

1) Transmission Electron Microscope (TEM)

The original form of electron microscopy is Transmission electron microscopy (TEM) which involves a high voltage electron beam emitted by a cathode and formed by magnetic lenses. The electron beam has been partially transmitted through the very thin (and so semitransparent for electrons) specimen which carries information about the structure of the specimen. The spatial variation is then magnified by a series of magnetic lenses until it is recorded by hitting a fluorescent screen, photographic plate, or light sensitive sensor such as a CCD (charge-coupled device) camera. The image detected by the CCD may be displayed in real time on a monitor or computer.

Transmission electron microscopes produce two-dimensional, black and white images.

2) Scanning Electron Microscope (SEM)

Unlike the TEM, where the electrons in the primary beam are transmitted through the sample, the Scanning Electron Microscope (SEM) produces images by detecting secondary electrons which are emitted from the surface due to excitation by the primary electron beam. In the SEM, the electron beam is scanned across the surface of the sample in a raster pattern with detectors building up an image by mapping the detected signals with beam position. TEM resolution is about an order of magnitude better than the

SEM resolution. TEM can easily resolve details of 0.2nm. SEM image relies on electron interactions at the surface rather than transmission it is able to image bulk samples and has a much greater depth of view, and so can produce images that are a good representation of the (3 dimensional) 3D structure of the sample. SEM images are therefore considered to provide us with 3D, topographical information about the sample surface but will still always be only in black and white.

3) **Reflection Electron Microscope (REM)**

In terms of structure the reflection electron microscope works much similar to the SEMs. Here, the reflected electrons are detected and gathered to study the surface of the specimen object. The REMs are usually grouped with spin polarized and low energy electron microscopes to image the specimen structure.

4) **Scanning Transmission Electron Microscope (STEM)**

Just like the traditional TEMs, the scanning transmission electron microscopes pass an electron beam through a very thin slice of an object. The STEM focuses on the beam that passes beforehand and constructs an image through raster scanning, instead of focusing on the beam after passing through the object. It is a combination of high magnification of TEM and better surface detail of SEM. STEM is usually used to perform very complex analysis of objects and specimens that is not possible by just using the TEM.