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DEPARTMENT OF BIOTECHNOLOGY
FACULTY OFENGINEERING & TECHNOLOGY

LT.3 Flourescence microscope, Transmission & Scanning Electron Microscope

Content Outline

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Flourescence microscopy

Fluorescence microscopy

An object also can be seen because it actually emits light, and this is the basis of fluorescence microscopy. When some molecules absorb radiant energy, they become excited and later release much of their trapped energy as light. Any light emitted by an excited molecule will have a longer wavelength (or be of lower energy) than the radiation originally absorbed. **Fluorescent light** is emitted very quickly by the excited molecule as it gives up its trapped energy and returns to a more stable state.

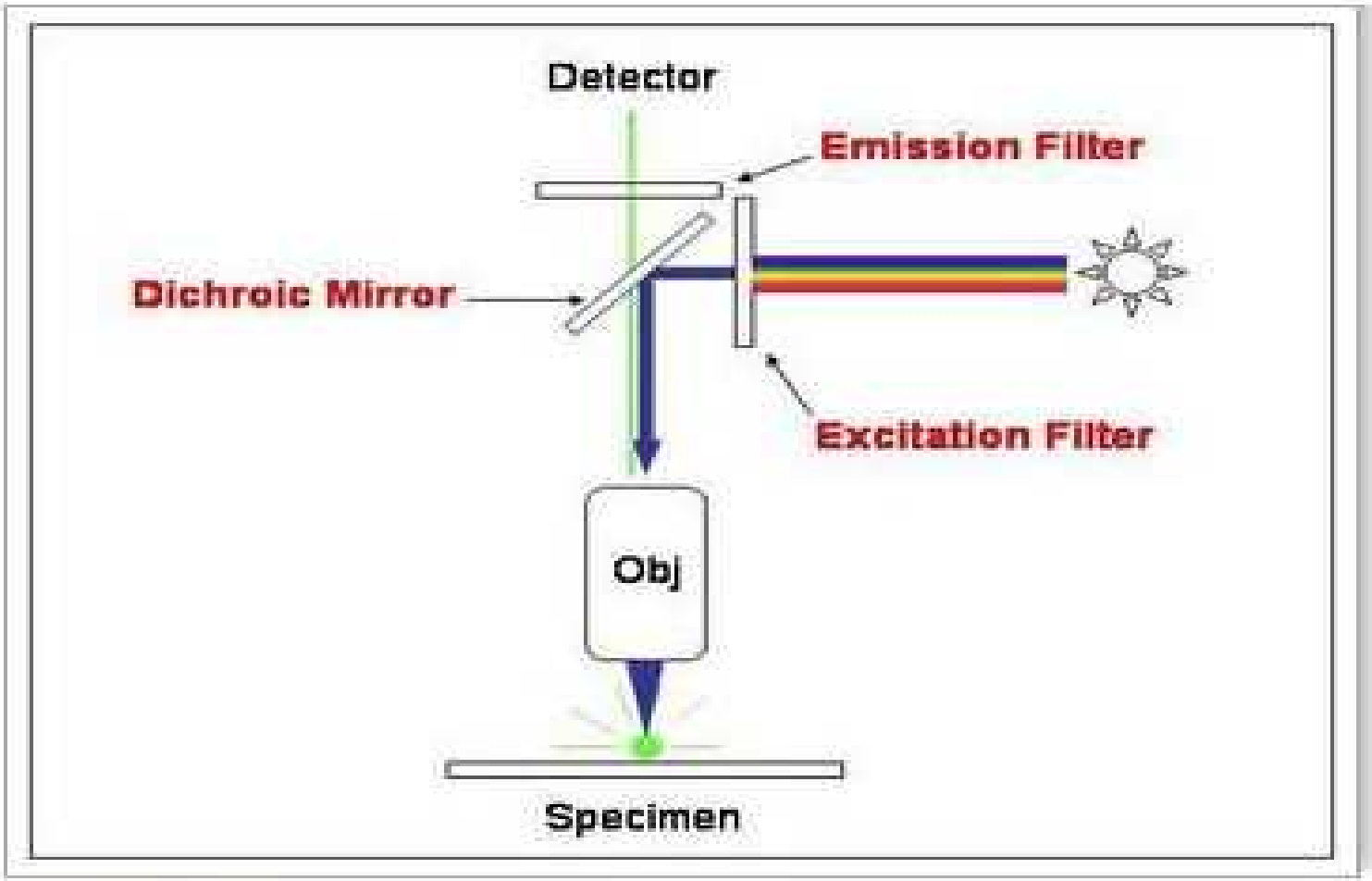
- British scientist Sir George G. Stokes first described fluorescence in 1852 and was responsible for coining the term when he observed that the mineral fluorspar emitted red light when it was illuminated by ultraviolet excitation.

Principle

•The basic function of a fluorescence microscope is to irradiate the specimen with a desired and specific band of wavelengths, and then to separate the much weaker emitted fluorescence from the excitation light. In a properly configured microscope, only the emission light should reach the eye or detector so that the resulting fluorescent structures are superimposed with high contrast against a very dark (or black) background. The limits of detection are generally governed by the darkness of the background, and the excitation light is typically several hundred thousand to a million times brighter than the emitted fluorescence.

•The specimen to be analyzed should exhibit autofluorescence. Some microorganisms exhibit autofluoresciong. However, the study of animal tissues and pathogens is often complicated with either extremely faint or bright, nonspecific autofluorescence. If specimen lacks autofluorescing, then it can be labeled with fluorochromes (also termed **fluorophores**), which are excited by specific wavelengths of irradiating light and emit light of defined and useful intensity. Fluorochromes are stains that attach themselves to visible or sub-visible structures, are often highly specific in their attachment targeting, and have a significant quantum yield (the ratio of photon absorption to emission).

Ray Diagram



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APplication of Flourescence microscope

•The refinement of epi-fluorescent microscopes and advent of more powerful focused light sources, such as lasers, has led to more technically advanced scopes such as the confocal laser scanning microscopes (CLSM) and total internal reflection fluorescence microscopes (TIRF).

➢CLSM's are invaluable tools for producing high resolution 3-D images of subsurfaces in specimens such as microbes. Their advantage is that they are able to produce sharp images of thick samples at various depths by taking images point by point and reconstructing them with a computer rather than viewing whole images through an eyepiece.

These microscopes are often used for -

•Imaging structural components of small specimens, such as cells

•Conducting viability studies on cell populations (are they alive or dead?)

•Imaging the genetic material within a cell (DNA and RNA)

•Viewing specific cells within a larger population with techniques such as FISH

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Specific application

•Bacterial pathogens (e.g., *Mycobacterium tuberculosis,* the cause of tuberculosis) can be identified after staining them with fluorochromes or specifically labeling them with fluorescent antibodies using immunofluorescence procedures.

•In ecological studies the fluorescence microscope is used to observe microorganisms stained with fluorochrome-labeled probes or fluorochromes such as acridine orange and DAPI (diamidino-2-phenylindole, a DNA-specific stain). The stained organisms will fluoresce orange or green and can be detected even in the midst of other particulate material.

•Live and dead cell staining with calcein AM ethidium homodimer-1. Green (live) and Red (Dead).

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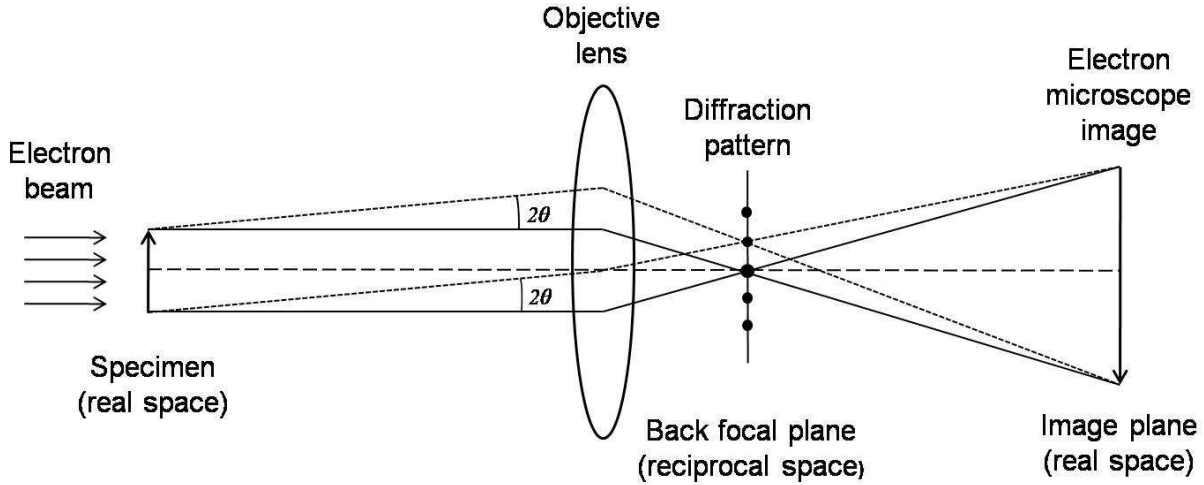
Transmission Electron Microscope

•The detailed internal structure of larger microorganisms also cannot be effectively studied by light microscopy. These limitations arise from the nature of visible light waves, not from any inadequacy of the light microscope itself. To obtain high magnification and to study internal structure TEM is used. Because the wavelength of electrons is much smaller than that of light, the optimal resolution attainable for TEM images is many orders of magnitude better than that from a light microscope. The transmission electron microscope (TEM) operates on many of the same optical principles as the light microscope. The TEM has the added advantage of greater resolution. This increased resolution allows us to study ultrastucture of organelles, viruses and macromolecules. Specially prepared materials samples may also be viewed in the TEM.



Principle & working of TEM

•A heated tungsten filament in the electron gun generates a beam of electrons that is then focused on the specimen by the condenser. Since electrons cannot pass through a glass lens, doughnut-shaped electromagnets called magnetic lenses are used to focus the beam. The column containing the lenses and specimen must be under high vacuum to obtain a clear image because electrons are deflected by collisions with air molecules. The specimen scatters electrons passing through it, and the beam is focused by magnetic lenses to form an enlarged, visible image of the specimen on a fluorescent screen. A denser region in the specimen scatters more electrons and therefore appears darker in the image since fewer electrons strike that area of the screen. In contrast, electron-transparent regions are brighter. The screen can also be moved aside and the image captured on photographic film as a permanent record.

Components & Their Function : TEM

•Since electrons are very small and easily deflected by hydrocarbons or gas molecules, it is necessary to use the electron beam in a vacuum environment. A series of pumps are used to accomplish an adequate vacuum for this purpose. Rotary Pumps are the first in the series. They are also called the "roughing pumps" as they are used to initially lower the pressure within the column through which the electron must travel to 10 -3 mm of Hg range. Diffusion Pumps may achieve higher vacuums (in the 10-5 mm Hg range) but must be backed by the rotary pump.

•Glass lenses of course, would impede electrons, therefore electron microscope (EM) lenses are electromagnetic converging lenses. A tightly wound wrapping of copper wire makes up the magnetic field that is the essence of the lens.

•The condenser lenses in the TEM serve much the same function as that of the condenser in the light microscope. They gather the electrons of the first crossover image and focus them onto the specimen to illuminate only the area being examined

•. A condenser aperture is used to reduce spherical aberration.

•The Objective lens is used primarily to focus and initially magnify the image. The specimen stage is inserted into the objective lens for imaging purposes. the microscope is used

•A cold finger or anti-contaminator also sits near the objective lens. It consists of a thin copper rod at liquid nitrogen temperatures, so that contaminants are attracted to it. The cold finger reservoir must be filled with liquid nitrogen before.

•Contaminants sometimes cause a phenomenon known as drift. Drift is the apparent "movement" of the specimen across the screen. It is caused by poor contact between the grid and the specimen holder causing a buildup of heat and static charges. An objective aperture is used to enhance specimen contrast. Intermediate lenses magnify the image coming from the objective lens. Finally, projector lenses further magnify the image coming from the intermediate lens and projects it on to the phosphorescent screen.

•The final image is viewed by projection onto a phosphorescent screen which gives off photons when irradiated by the electron beam. A film camera is located beneath the phosphorescent screen. The screen is raised in order to expose a special photographic film with a thicker emulsion layer than light film. An alternative to photographic film is digital capture with a computer digitizing and archiving (CCD) camera.

Preparation of specimen for TEM

•Specimens must be very thin so that electrons are able to pass through the tissue. This may be done by cutting very thin slices of a specimen's tissue using an ultramicrotome.

•The specimen must be around 20 to 100 nm thick, about 1⁄50 to 1⁄10 the diameter of a typical bacterium, and able to maintain its structure when bombarded with electrons under a high vacuum.

•The tissue must first be put in a chemical solution to preserve the cell structure.

•Specimens are prepared for observation by soaking thin sections with solutions of heavy metal salts like lead citrate and uranyl acetate.

•Heavy osmium atoms from the osmium tetroxide fixative also "stain" cells and increase their contrast.

•The stained thin sections are then mounted on tiny copper grids and viewed. The tissue must also be completely dehydrated (all water removed). Once preserved and dehydrated, tissue samples are placed in hard, clean plastic. The plastic supports the tissue while it is being thinly cut with the ultramicrotome.

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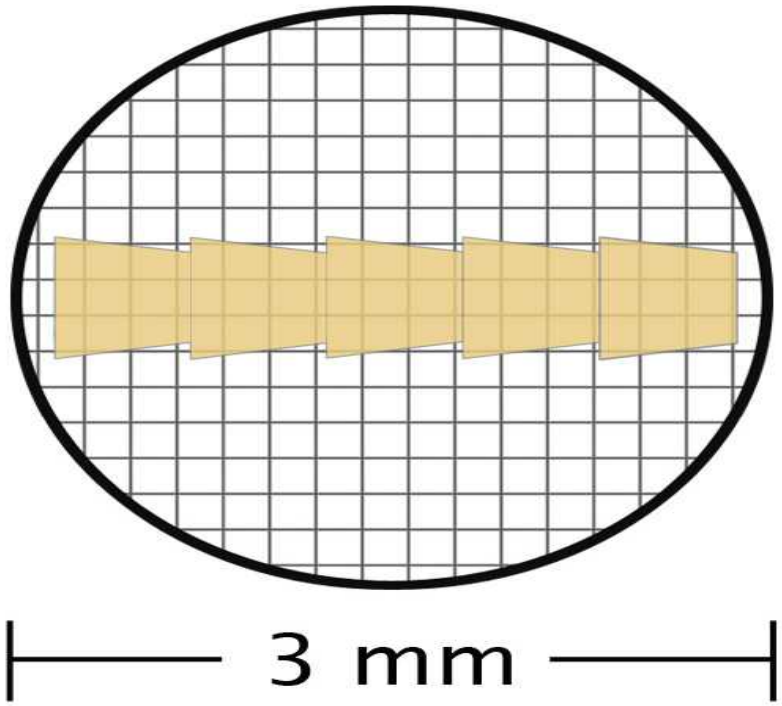
Copper grid used for TEM

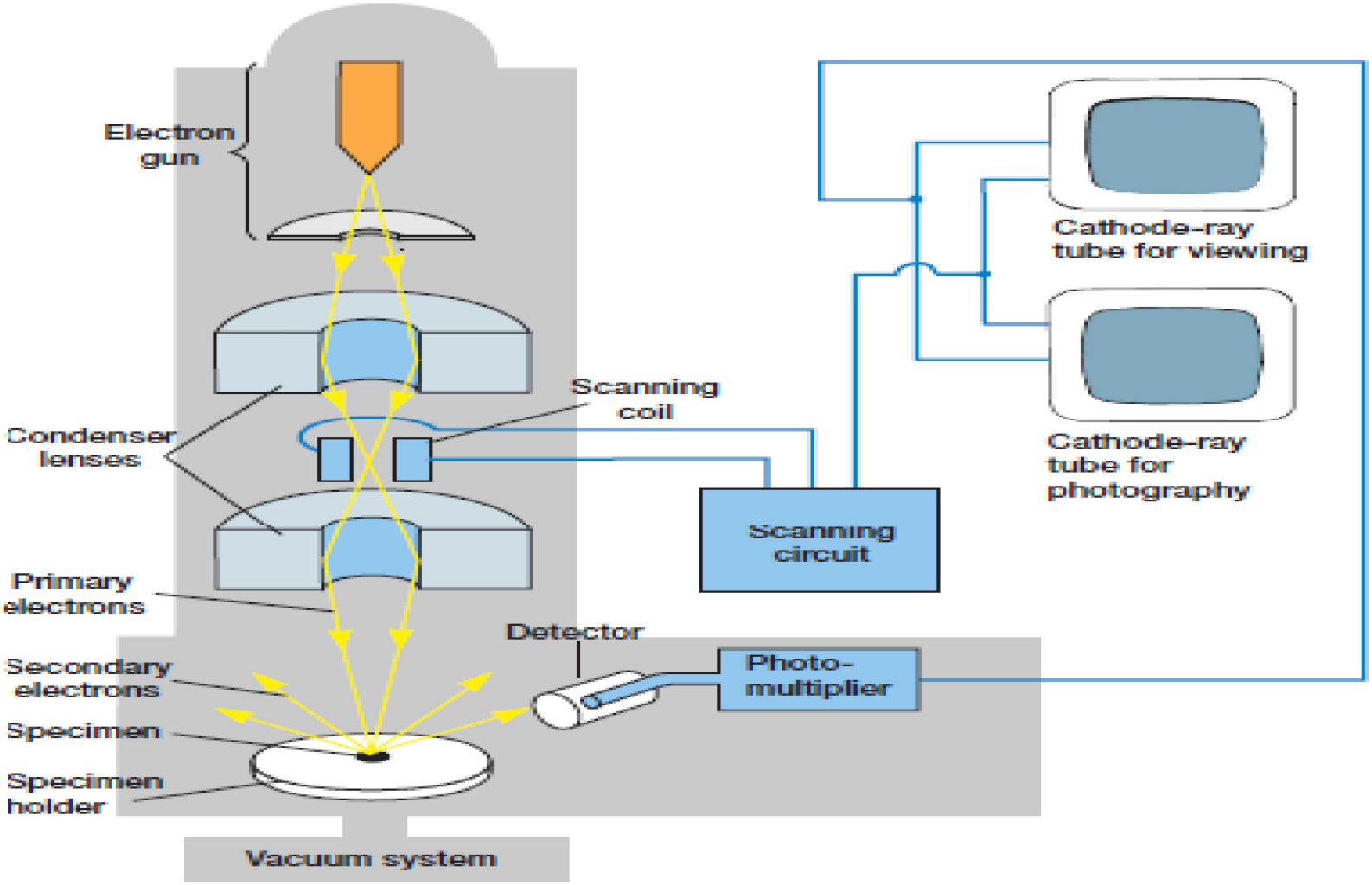


Scanning Electron Microscope (SEM)

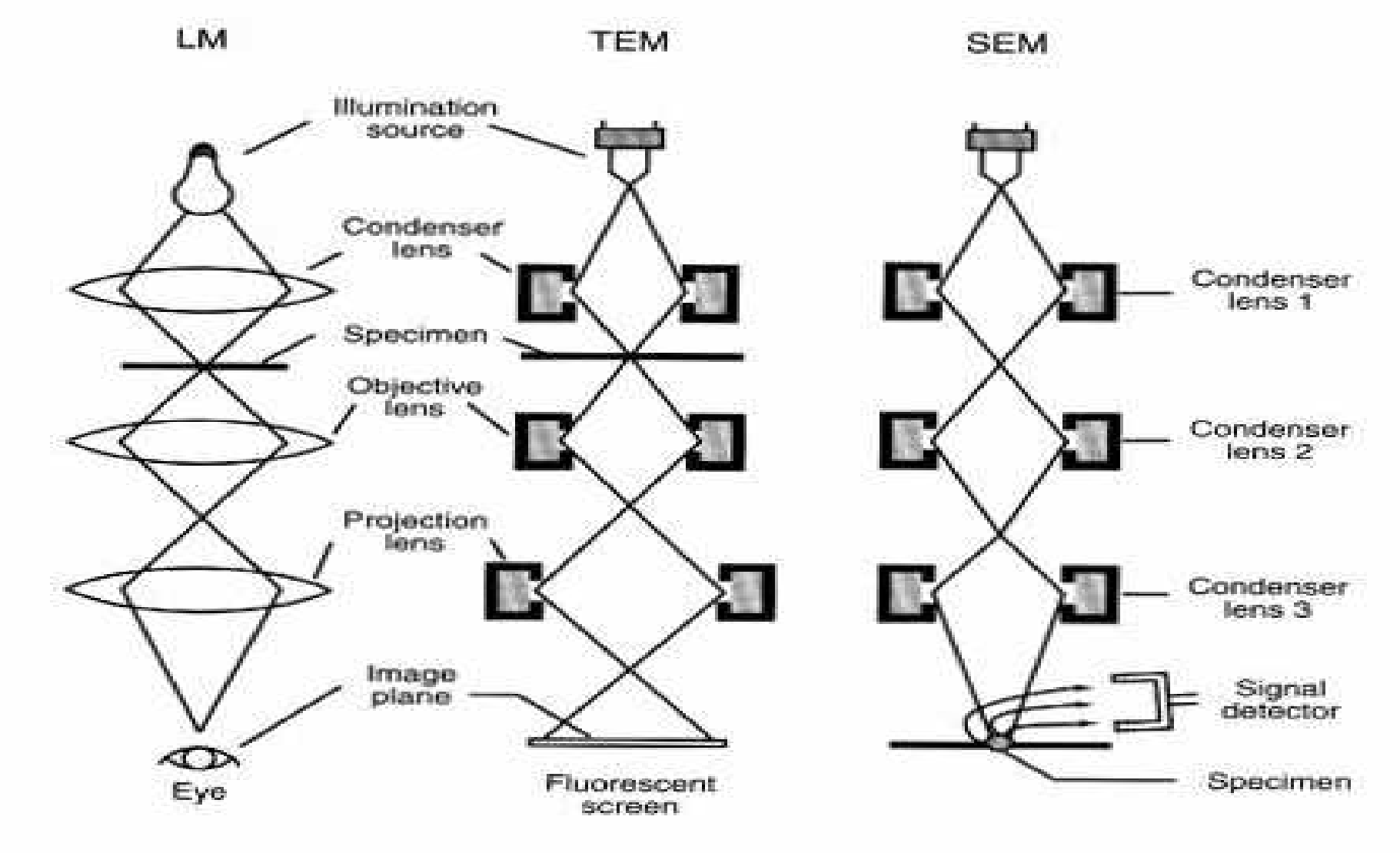
The scanning electron microscope (SEM) uses a focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimens. The signals that derive from electron-sample interactions reveal information about the sample including external morphology (texture), chemical composition, and crystalline structure and orientation of materials making up the sample. The SEM differs from other electron microscopes in producing an image from electrons emitted by an object's surface rather than from transmitted electrons.

➢In most applications, data are collected over a selected area of the surface of the sample, and a 2-dimensional image is generated that displays spatial variations in these properties. Areas ranging from approximately 1 cm to 5 microns in width can be imaged in a scanning mode using conventional SEM techniques (magnification ranging from 20X to approximately 30,000X, spatial resolution of 50 to 100 nm). The SEM is also capable of performing analyses of selected point locations on the sample; this approach is especially useful in qualitatively or semi-quantitatively determining chemical compositions (using EDS), crystalline structure, and crystal orientations (using EBSD). The design and function of the SEM is very similar to the EPMA and considerable overlap in capabilities exists between

Scanning Electron Microscope (SEM) configuration



Ray diagram: Difference between Light, Transmission & Scanning microscope



Scanning Electron Microscope (SEM) : Components & Infrastructure

Essential components of all SEMs include the following:

Electron Source ("Gun")

Electron Lenses

Sample Stage

Detectors for all signals of interest

Display / Data output devices

Infrastructure Requirements:

- Power Supply
- Vacuum System
- Cooling system
- Vibration-free floor
- Room free of ambient magnetic and electric fields

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•SEMs always have at least one detector (usually a secondary electron detector), and most have additional detectors. The specific capabilities of a particular instrument are critically dependent on which detectors it accommodates, Computer and display to view the images

•Electrons are produced at the top of the column, accelerated down and passed through a combination of lenses and apertures to produce a focused beam of electrons which hits the surface of the sample. The sample is mounted on a stage in the chamber area and, unless the microscope is designed to operate at low vacuums, both the column and the chamber are evacuated by a combination of pumps. The level of the vacuum will depend on the design of the microscope.

•The position of the electron beam on the sample is controlled by scan coils situated above the objective lens. These coils allow the beam to be scanned over the surface of the sample. This beam rastering or scanning, as the name of the microscope suggests, enables information about a defined area on the sample to be collected. As a result of the electron-sample interaction, a number of signals are produced. These signals are then detected by appropriate detectors.

Scanning Electron Microscope (SEM) : Working

The SEM scans a narrow, tapered electron beam back and forth over the specimen. When the beam strikes a particular area, surface atoms discharge a tiny shower of electrons called secondary electrons, and these are trapped by a special detector. Secondary electrons entering the detector strike a scintillator causing it to emit light flashes that a photomultiplier converts to an electrical current and amplifies. The signal is sent to a cathode-ray tube and produces an image like a television picture, which can be viewed or photographed. The number of secondary electrons reaching the detector depends on the nature of the specimen's surface. When the electron beam strikes a raised area, a large number of secondary electrons enter the detector; in contrast, fewer electrons escape a depression in the surface and reach the detector. Thus raised areas appear lighter on the screen and depressions are darker. A realistic three-dimensional image of the microorganism's surface with great depth of focus results.

Test your understanding

Reason for using electron instead of light waves in Transmission Electron Microscope for imaging

a. Wavelength of electrons is much smaller than that of light

b. Wavelength of electrons is much larger than that of light

c. Wavelength of electrons equals than that of light

d. None of these

Citrate and Uranyl acetate treatment of sample is required for Transmission Electron Microscopy

a. To fix the sample

b. To permeabilize the sample

c. To lighten the sample

d. To stain and increase the contrast of sample To stain and increase the contrast of sample

What is function of photomultiplier in Scanning Electron Microscope?

a. Converts and amplifies flashes of light emitting from scintillator into magnetic signal

b. Converts and amplifies flashes of light emitting from scintillator into electrical signal

c. Converts and amplifies flashes of light emitting from scintillator into force field

d. None of these

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