BHARATHIDASAN UNIVERSITY

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Tamil Nadu, India.

Programme: M.Sc., Botany

Course Title : CELL BIOLOGY AND BIOINSTRUMENTATION
Course Code : 22PGBOT104

Unit – IV
BIOINSTRUMENTATION

Topic: Spectrophotometry & Microscopy

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Spectrophotometer

Definition

A spectrophotometer is an instrument that measures the amount of light absorbed by a sample.

- Spectrophotometer is an instrument which measures the amount of light absorbed by a sample.
- Arnold J Beckman and his colleagues (National Technologies Laboratory) invented Spectrophotometer in 1940 – Beckman
- Result – 99.9% accuracy.
- Calorimeter – 1789 – Antonie Lavoisier in collaboration with Pierre Simon

Light → Lens → Slit → Monochromator → Slits

→ Sample → Detector → Quantitative Analysis

- X ray : 0.1 – 10 nm
- UV : 200 – 400 nm
- Visible : 400- 750 nm
- IR: 750 nm – 1 mm
- Microwave: 1mm – 10 cm

Visible

- Violet; 380–450
- Blue: 450–485
- Cyan:485–500
- Green: 500–565
- Yellow: 565–590
- Orange: 590–625
- Red: 625–750

- Spectrophotometer - an instrument used to measure optical density of a sample at any wave length.
- Fluorometer - measures the intensity of fluorescent light emitted by a sample exposed to UV light under specific conditions.

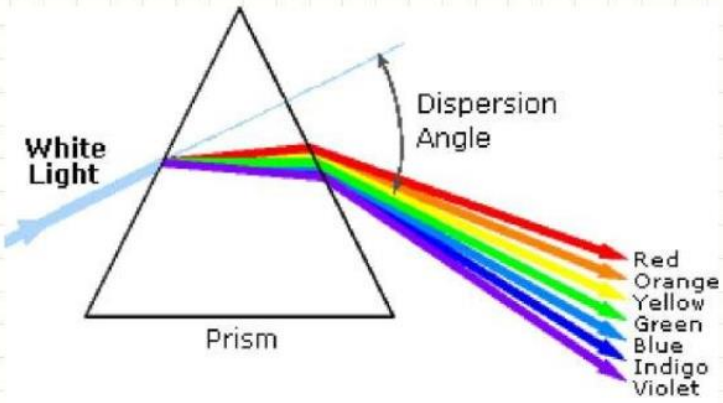
Beer and Lamberts law

- Beer's: Parallel beam of monochromatic light passes through an isotropic, light absorbing medium, the amount of light that is absorbed is directly proportional to the number of light absorbing molecules in that medium or in other words the concentration of the substance in that medium.
- A (absorbance) α C (concentration of light absorbing substance) in the medium.

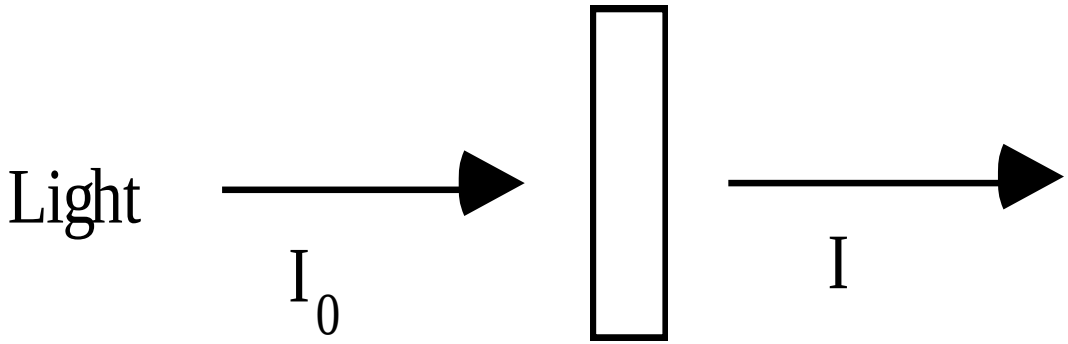
- Lambert's: Parallel beam of monochromatic light passes through an isotropic, light absorbing medium, the amount of light that is absorbed is directly proportional to the length of the medium through which the light passes.
- $A \alpha L$ (length of the medium).
- Since the measurement of light absorption depends on both the laws, it is popularly known as Beer-Lambert's Law.
- Thus $A \alpha C \times L$

- A = log Ii/It = log 100/T = log100-logT= 2-logT.
- Where Ii is the intensity of incident light
- It is the intensity of transmitted light.
- It/Ii is the Transmittance (T).

Electromagnetic Radiation



Violet	400 - 420 nm	Yellow	570 - 585 nm
Indigo	420 - 440 nm	Orange	585 - 620 nm
Blue	440 - 490 nm	Red	620 - 780 nm
Green	490 - 570 nm		

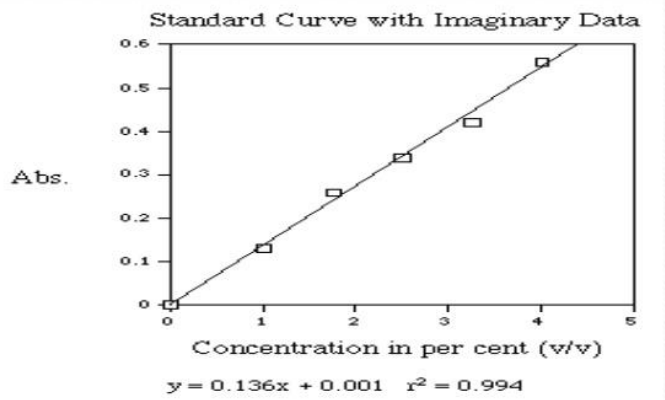


Glass cell filled with
concentration of solution (C)

- Spectrophotometer measures light absorption as a function of wavelength (UV, visible). Light is a form of energy and is propagated in the form of waves.
- These light waves are considered to be a packet of energy or photons.
- - the photons have a defined energy which depends on the wave length of particular radiation.

INTRODUCTION

- Compounds absorbs light radiation of a specific wavelength.
- The light absorbed by the sample is directly proportional to the concentration of sample in the solution.
- As concentration increases , absorption increases exponentially.

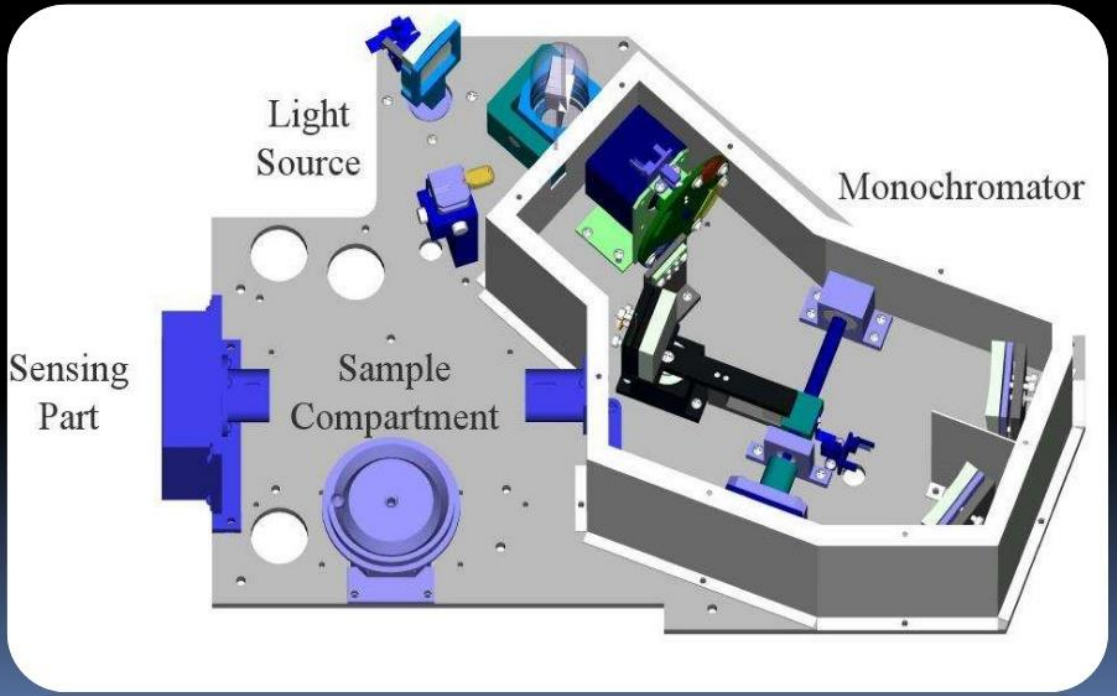


- Each compound absorbs or transmits light over a range of wavelength.

parts

- Light source
- Condensing lense
- Monochromator
- Sample holder
- Detector/PMT
- Reader

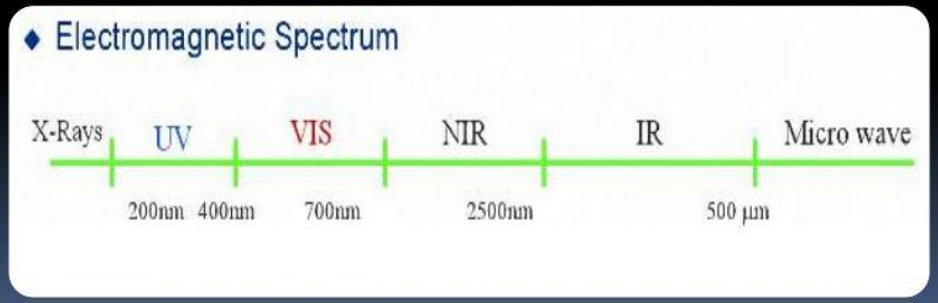
Components of Spectrophotometer



- Light:
- Tungsten lamp (commonly used, range: 330 - 900 nm)
- Hydrogen/ Deuterium lamp (for the ultra violet region, range: 200 – 450nm)
- Xenon lamp (emit both UV, Visible, range: 190- 1000nm)

Light Source

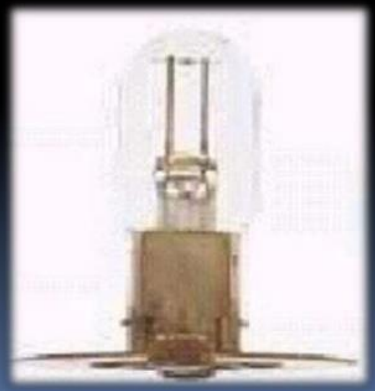
- to provide a sufficient of light which is suitable for marking a measurement.
- Tungsten Lamp
- Hydrogen Lamp
- Xenon Lam



I) Tungsten Lamp

- It is the most common light source used in spectrophotometer wavelength
- range of about 330 to 900 nm
- It has long life

about 1200h.

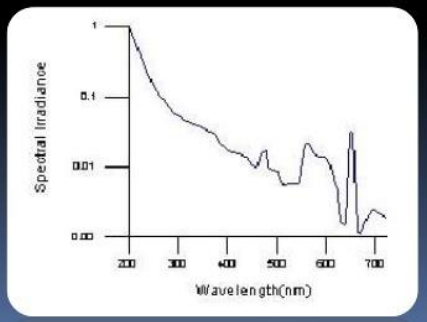


II)Hydrogen / Deuterium Lamps

For the ultraviolet region, hydrogen or deuterium lamps are frequently used.

their range is approximately 200 to 450 nm.

Deuterium lamps are generally more stable and has long life about 500h.This lamp generates continuous or discontinuous spectral.



III) Xenon flash lamps

Xenon flash lamps have several advantages as the following :

1)Their range between (190nm - 1000 nm)

2) Emit both UV and visible wavelengths

3) Long life

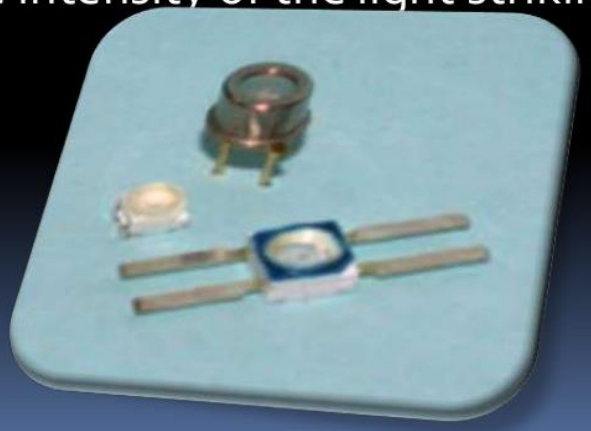
4) Do not heat up the instrument

5) Reduce warm up time

- Cuvette: is a kind of cell made of plastic, glass or quartz and designed to hold sample.
- UV: Quartz
- Glass/Plastic: Visible

Detectors

Any photosensitive device can be used as a detector of radiant energy .The photocell and phototube are the simplest photodetectors, producing current proportional to the intensity of the light striking them.



Display devices

The data from a detector are displayed by a readout device, such as an analog meter, a light beam reflected on a scale, or a digital display , Or liquid crystal display(LCD) .The output can also be transmitted to a computer.

A

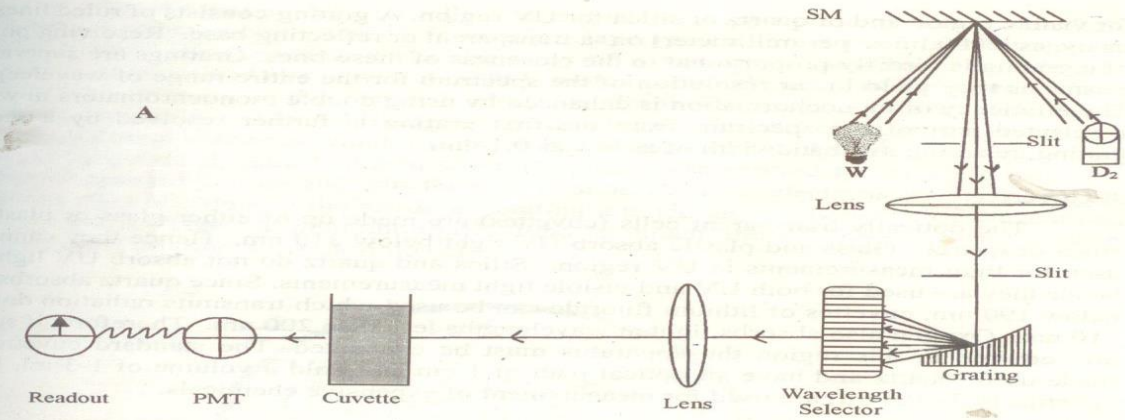


FIG. A. SCHEMATIC DIAGRAM OF A SINGLE- BEAM SPECTROPHOTOMETER

B

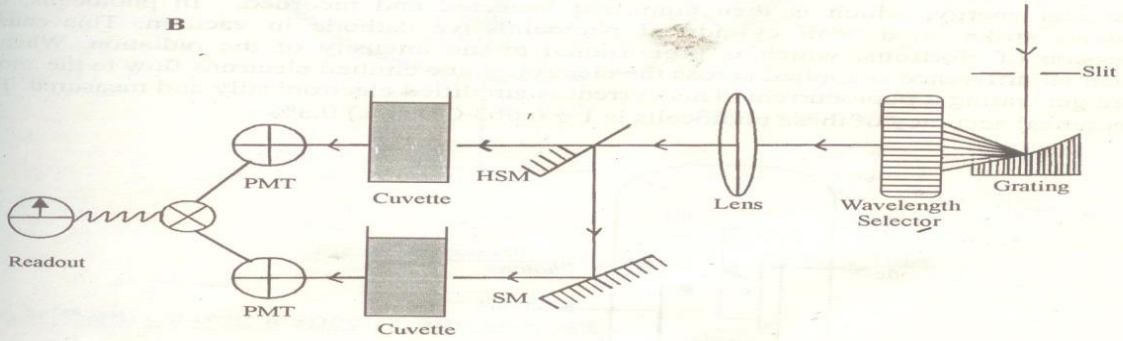
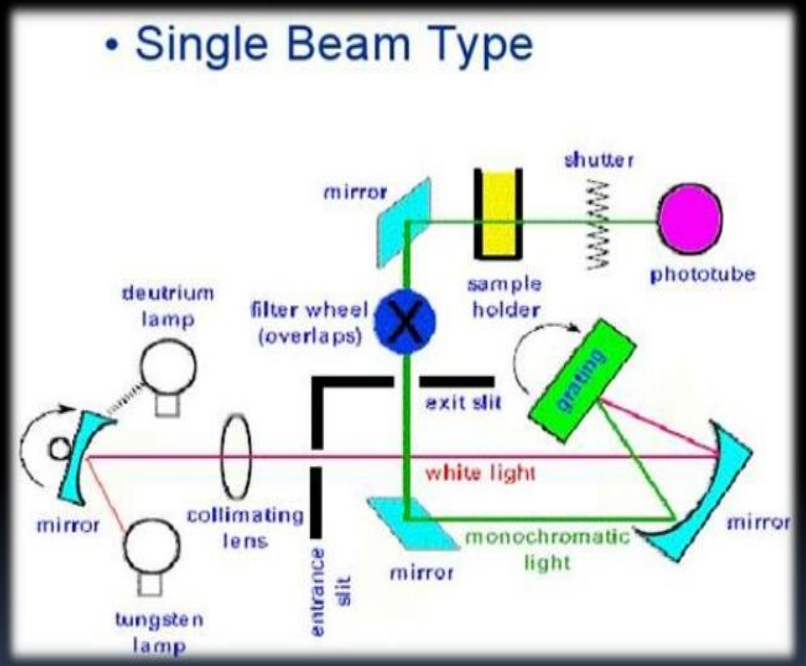


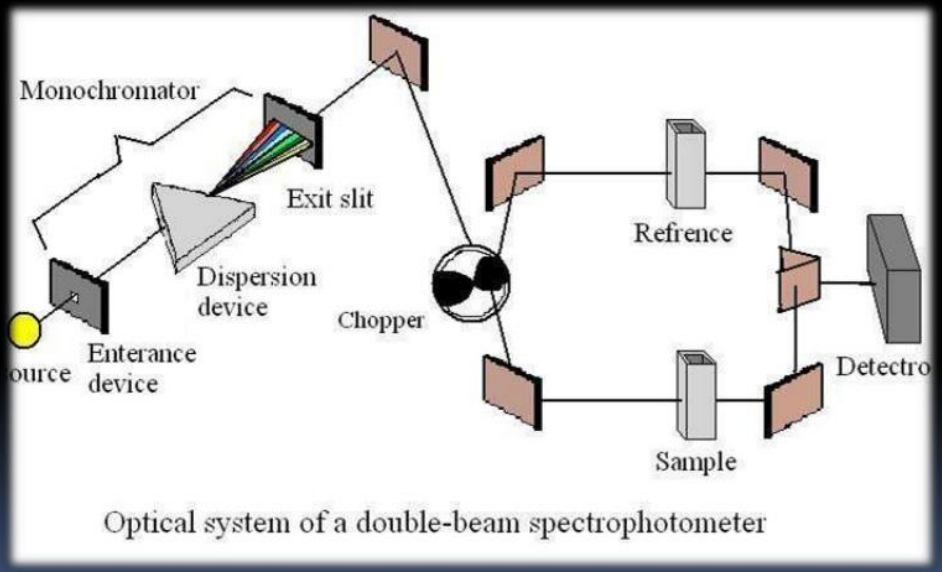
FIG.B. SCHEMATIC DIAGRAM OF A DOUBLE -BEAM SPECTROPHOTOMETER

Note: In single beam spectrophotometers, the variations in source intensities are not compensated whereas in double beam spectrophotometers, the variations in source intensity are corrected automatically by equally dividing the monochromatic light between reference and sample at any given time.

Single Beam SPM



Double Beam SPM



Optical system of a double-beam spectrophotometer

- A double beam spectrophotometer has two light beams, one of which passes through the sample while other passes through a reference cell.
- This allows more reproducible measurements as any fluctuation in the light source/ instrument electronics appears in both reference and the sample and therefore can easily be removed from the sample spectrum by subtracting the reference spectrum.
- Modern instruments can perform this subtraction automatically.

- Spectrophotometer- two light sources:
- UV (200-400)
- and Visible (400-900)
- This can be selected based on our requirements (fall on the silver mirror SM)
- The reflected light from the mirror passes through an entrance slit and a condensing lens.

- The lens renders the light rays into parallel beams and the parallel beams of light now fall on a monochromator (grating).
- The monochromator disperses the light into its component wavelengths.
- Now the selected beam of monochromatic light passes again through a lens to a light tight compartment where the sample is kept ina cuvette.

- After passing through the sample, transmitted light falls on a photomultiplier (PMT).
- PMT converts the light energy into electrical energy, which is amplified, measured and recorded on the digital readout.

- In double beam spectrophotometer: the monochromatic light coming out from the lens split into two halves by placing a half-slivered mirror (HSM) on its path.
- 50% of the light passes directly through the mirror and falls on the reference cuvette
- 50% of the light reflected into a second silvered mirror and then allowed to fall on the sample cuvette.

Light sources

- UV Spectrophotometer
- 1. Hydrogen Gas Lamp/ Deutrium lamp
- 2. Mercury Lamp/Xenon
- Visible Spectrophotometer
- 1. Tungsten Lamp
- IR Spectrophotometer
- 1. Carborundum (SIC)

Monochromator

- Produce radiations of single wavelength, based either upon refraction by a prism or diffraction by a grating.

Cuvettes (optically transparant)

- UV Spectrophotometer
- Quartz
- Visible Spectrophotometer
- Glass
- IR Spectrophotometer
- NaCl

Photocell/PMT

- Photocell is a photoelectric device, which converts light energy into electrical energy, amplified, detected.

Absorption spectrum

- Spectrum is a graph – intensity of absorbed or emitted radiation by sample verses wavelength.
- Spectrometer is designed to measure the spectrum of a compound.

- Absorption verses wavelength
- quantification

- Used to estimate the concentration of both coloured as well as colourless solutions which absorb light.
- Does not degrade or modify the materials studied- recovered
- Find out absorbtion maxima
- Enzyme kinetics
- Growth of the culture

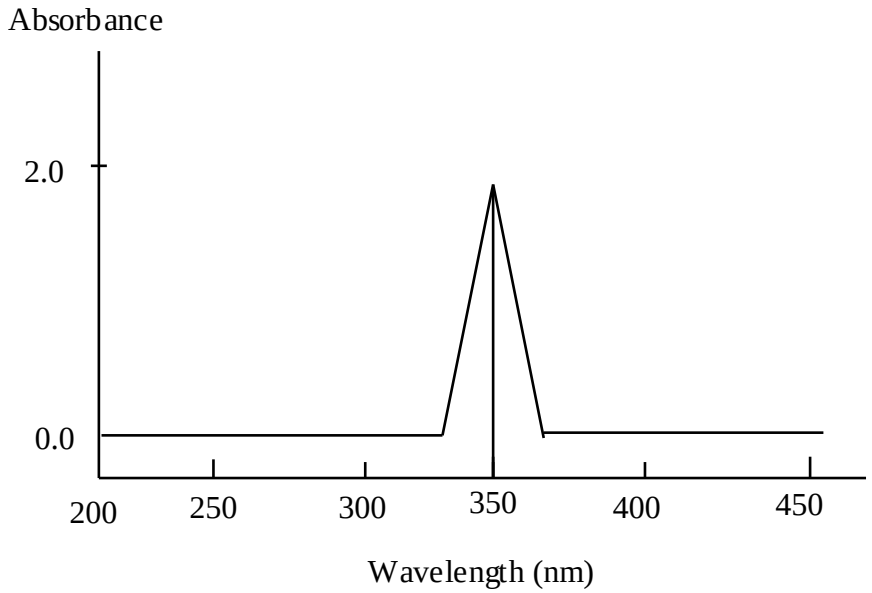
Absorption maxima

- Light absorbing molecules absorb light maximally at a particular wavelength. This wavelength is called λmax of the compound.
- Estimation of compound, λmax should be known.
- Identify the λmax of BSA.

1. Run the sample for spectrum

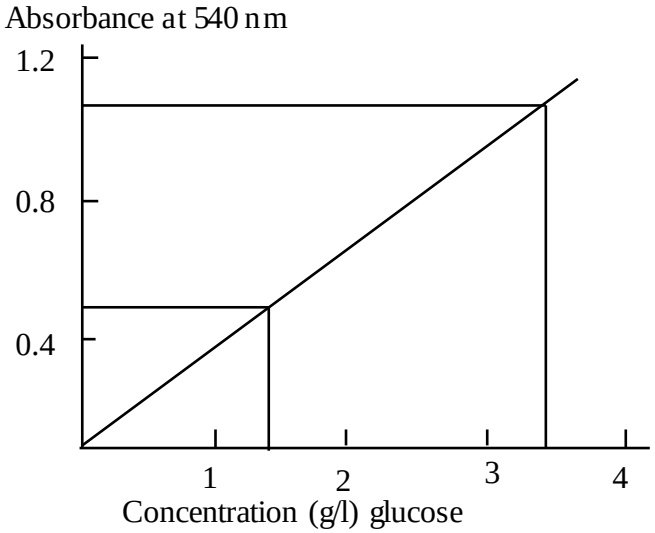
- 2. Obtain a monochromatic wavelength for the maximum absorption wavelength.
- 3. Calculate the concentration of your sample

Absorption maxima



- To identify the concentration of the given sample.

Standard curve



- Thank You

spectrofluorometer

- A large numbers of substances are known which can absorb UV or Visible light radiation. But these substances lose excess energy as heat through collisions with neighboring atoms or molecules.
- However, a large numbers of important substances are also known which lose only part of this excess energy as heat and emit the remaining energy as electromagnetic radiation of a wavelength longer than that absorbed. This process of emitting radiation is collectively known as luminescence. Fluorescence Spectroscopy

- Luminescence is the emission of light by a substance. It occurs when an electron returns to the electronic ground state from an excited state and loses it's excess energy as a photon.
- In luminescence, light is produced at low temperature; therefore the light produced by this process is regarded as “ light without heat” or “cold light”.
- Luminescence spectroscopy is a collective name given to three related spectroscopic techniques. They are: Molecular fluorescence spectroscopy Molecular phosphorescence spectroscopy Chemiluminescence spectroscopy Fluorescence Spectroscopy

Principle of fluorescence and phosphorescence : (photoluminescence)

- The electronic states of most organic molecules can be divided into single t states and triplet states:
- Singlet state: All electrons in the molecule are spin-paired Symbol: ⍰⍰
- Triplet state: Unpaired electrons of same spin present Symbol: ⍰ ⍰
- Singlet excited state: Unpaired electrons of opposite spin present Symbol: ⍰ ⍰ Fluorescence Spectroscopy

Fluorescence

- Fluorescence is the phenomenon of emission of radiation when electrons undergo transition from singlet excited state to singlet ground state . A part of energy is lost due to vibrational transitions & remaining energy is emitted as UV/Visible radiation of longer wavelength than Incident light Energy of emitted radiation is less than that of absorbed radiation because a part of energy is lost due to vibrational or collisional processes. Hence the emitted radiation has longer wavelength (less energy) than the absorbed radiation.

- wavelength of absorbed radiation is called excitation wavelength (□ ex) and that of emission radiation is called as emission wavelength (□ em).
- These two wavelengths are specific or characteristic for a given substance under ideal conditions.

Property of luminescence spectrum

1. Phosphorescence is always at longer wavelength compared with fluorescence
2. Phosphorescence is narrower compared with fluorescence
3. Phosphorescence is weaker compared with fluorescence

1. absorption is mirrored relative to emission
2. Absorption is always on the shorter wavelength compared to emission
3. Absorption vibrational progression reflects vibrational level in the electronic excited states, while the emission vibrational progression reflects vibrational level in the electronic ground states
4. λ_0 transition of absorption is not overlap with the λ_0 of emission

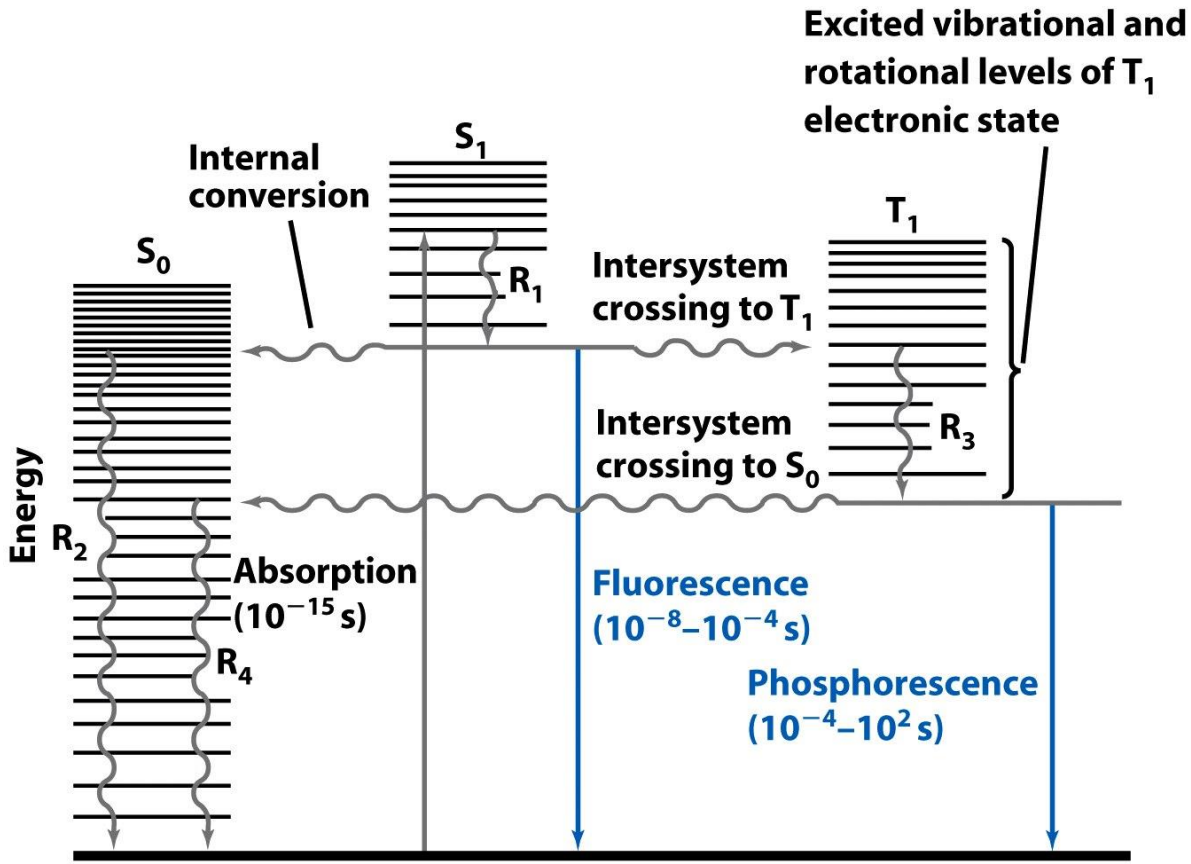


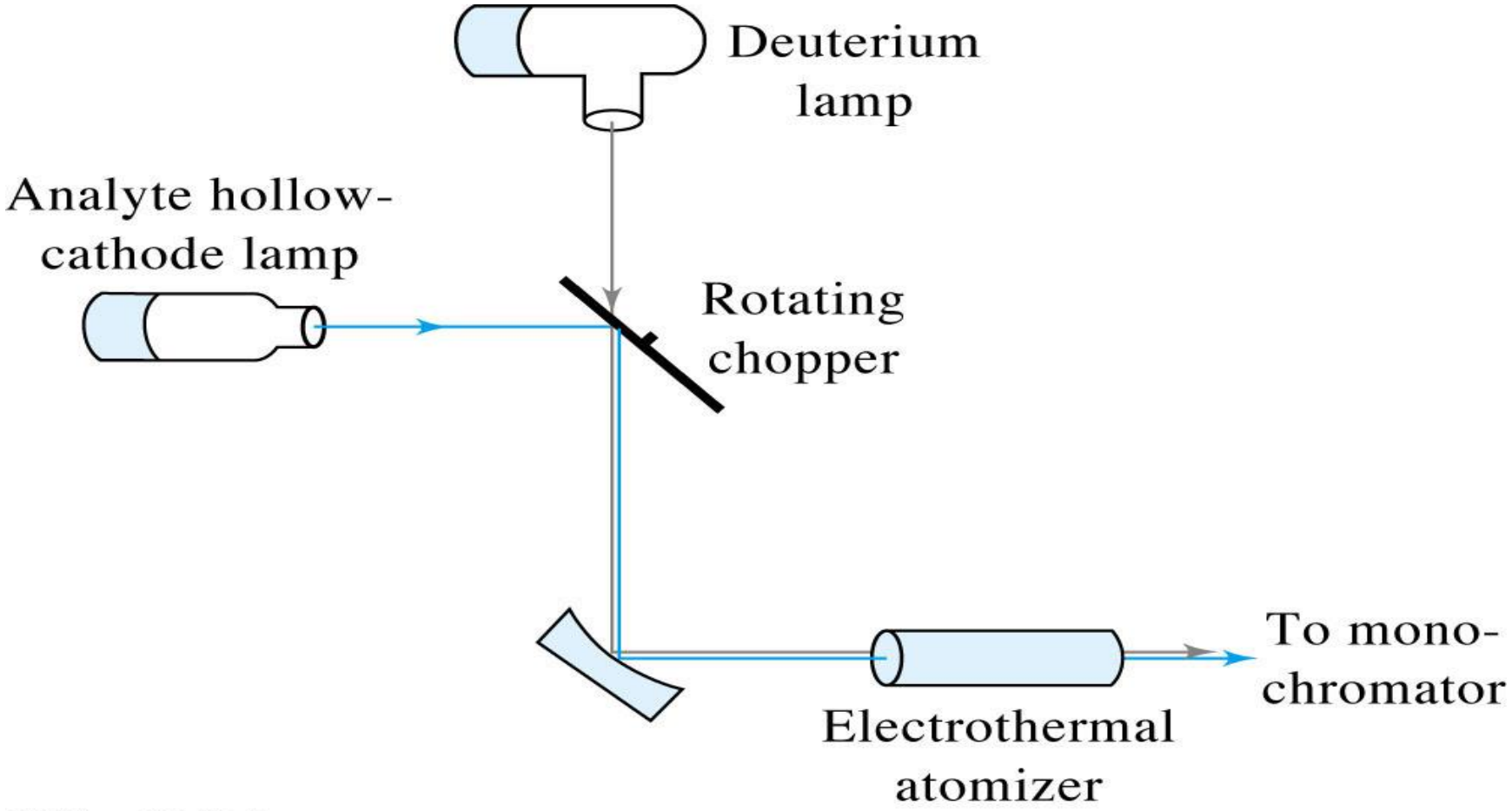
Figure 18-13
Quantitative Chemical Analysis, Seventh Edition

Atomic Absorption Process

- A neutral atom in the gaseous state can absorb radiation and transfer an electron to an excited state.
- Simple electronic transitions possible with no vibrational and rotational energy levels possible. Bandwidth much narrower!
- Occur at discreet λ
- Na(g) 3s $\rightarrow$ 3p and 3p $\rightarrow$ 5s as well as other transitions are possible at the correct photon energy a transition.

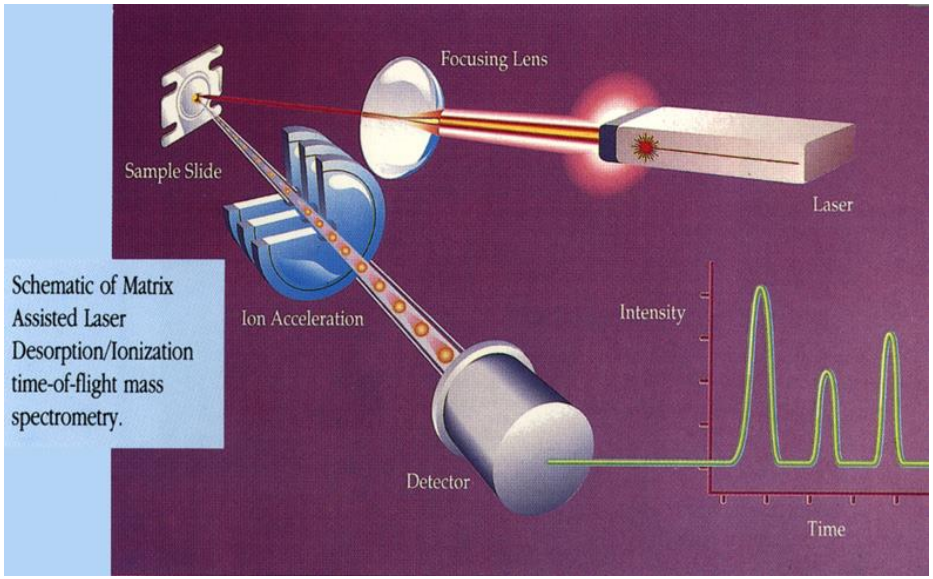
MEASURING ATOMIC ABSORPTION

- Recall Beer's Law ($A = \log \quad = \varepsilon bC$) is obeyed when line width
  small compared to absorption band.
- Atoms or molecules absorb radiation at discrete wavelengths.
- Broadband radiation contains photons of several wavelengths, some of which may be useful but many of which will not. This will make P_o ($= P_{usable} + P_{useless}$) larger and the absorbance smaller than would be expected with only the usable portion of the light available for absorption.
- Besides the P_{usable} can be composed of wavelengths with different absorptivities i.e. the sample does not absorb all radiation to the same degree.
- Non-linear behavior observed when λ range of excitation source is greater than λ range of absorber; bandwidth of excitation source must be narrower than bandwidth of absorber.

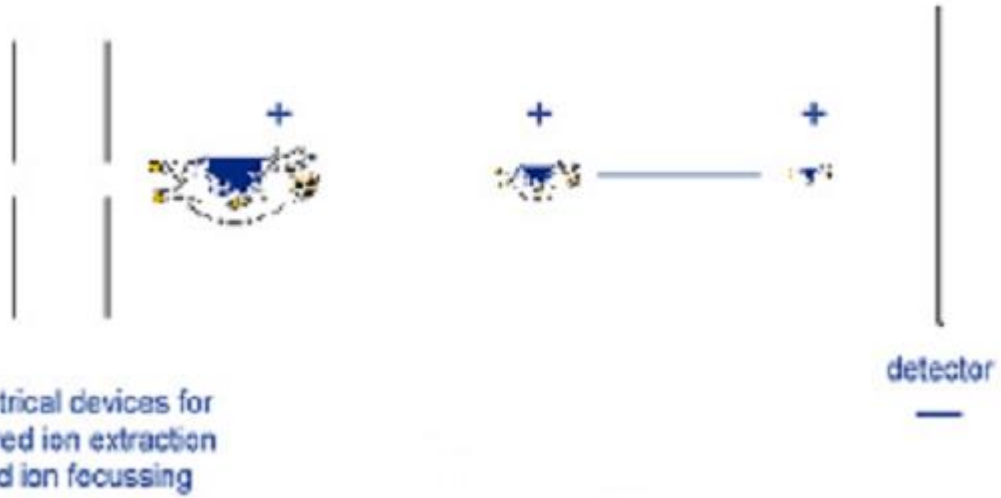


MALDI-TOF

- Matrix-assisted laser desorption/ionization time of flight mass spectrometry
  - Ionizes molecules via laser pulses
  - Separates molecules according to mass to charge ratio
- Mainly used for detection of large biomolecules



Dissolve compound and add matrix
Evacuate air from sample chamber
Laser shoots pulses of light at sample


detector
Electrical devices for
delayed ion extraction
and ion focussing

- Used to characterize and identify large molecules
- Used in pharmaceutical for QC, monitoring of enzyme reactions
- Used in DNA sequencing for forensics
- Used to identify different strains of viruses to help develop vaccines

Microscopy

- 1655 – Robert Hooke used a compound microscope to observe pores in cork
  - –He called them “cells”

Microscope Vocabulary

- Magnification: increase of an object's apparent size
- Resolution: power to show details clearly
- Both are needed to see a clear image

Types of Microscopes

- 1. Compound Light Microscope
  - –1st type of microscope, most widely used
  - –light passes through 2 lenses
  - –Can magnify up to 2000x

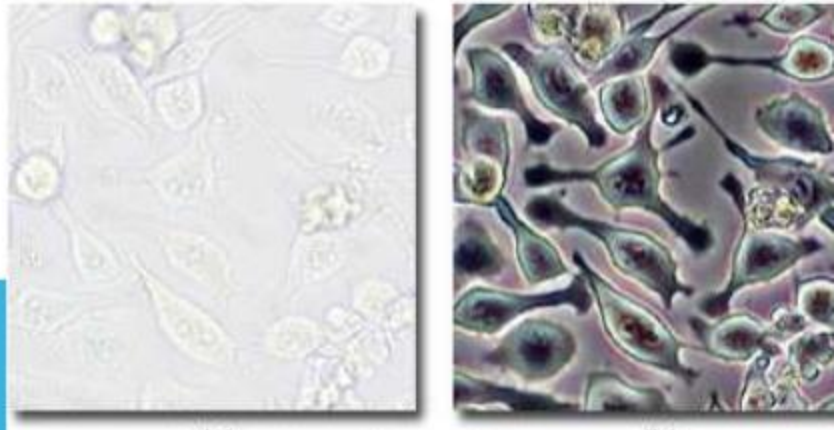
Ocular lens

Objective lenses

INTRODUCTION

- The microscope is commonly described as an instrument used for seeing small objects.
- The human eye sees because of two properties of the light entering the eye from the objects seen.
- The eye recognizes only differences in brightness and differences in color.
- Differences in brightness of different objects or their component parts give rise to brightness contrast; differences in color cause color contrast.
- Brightness = Amplitude
- Color = Wavelenght

Living Cells in Brightfield and Phase Contrast



(a) (b)

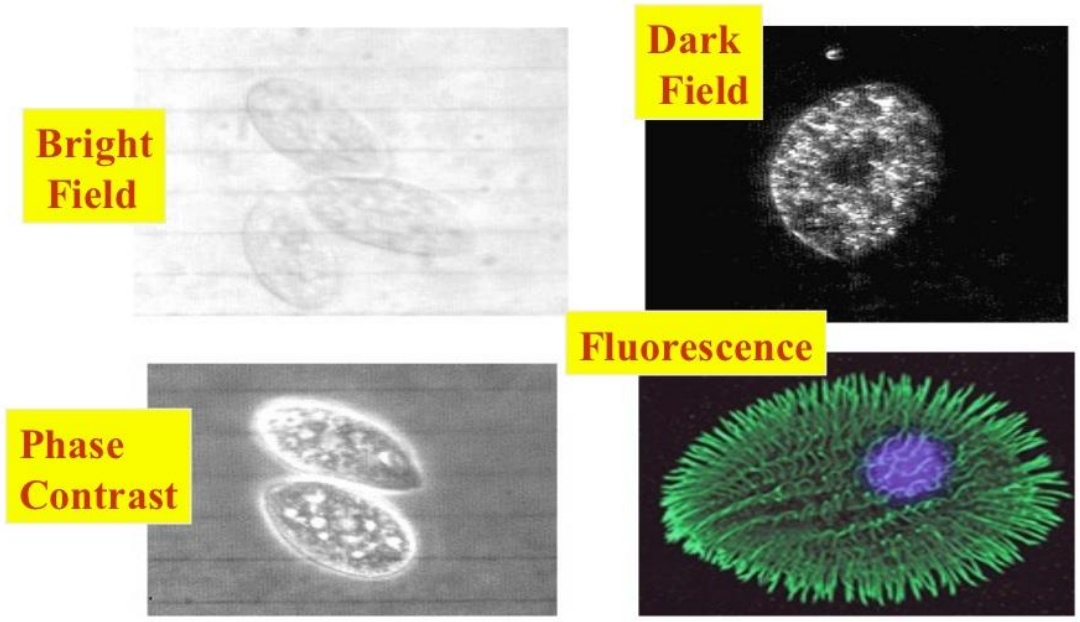
Figure 2

The Light Microscope

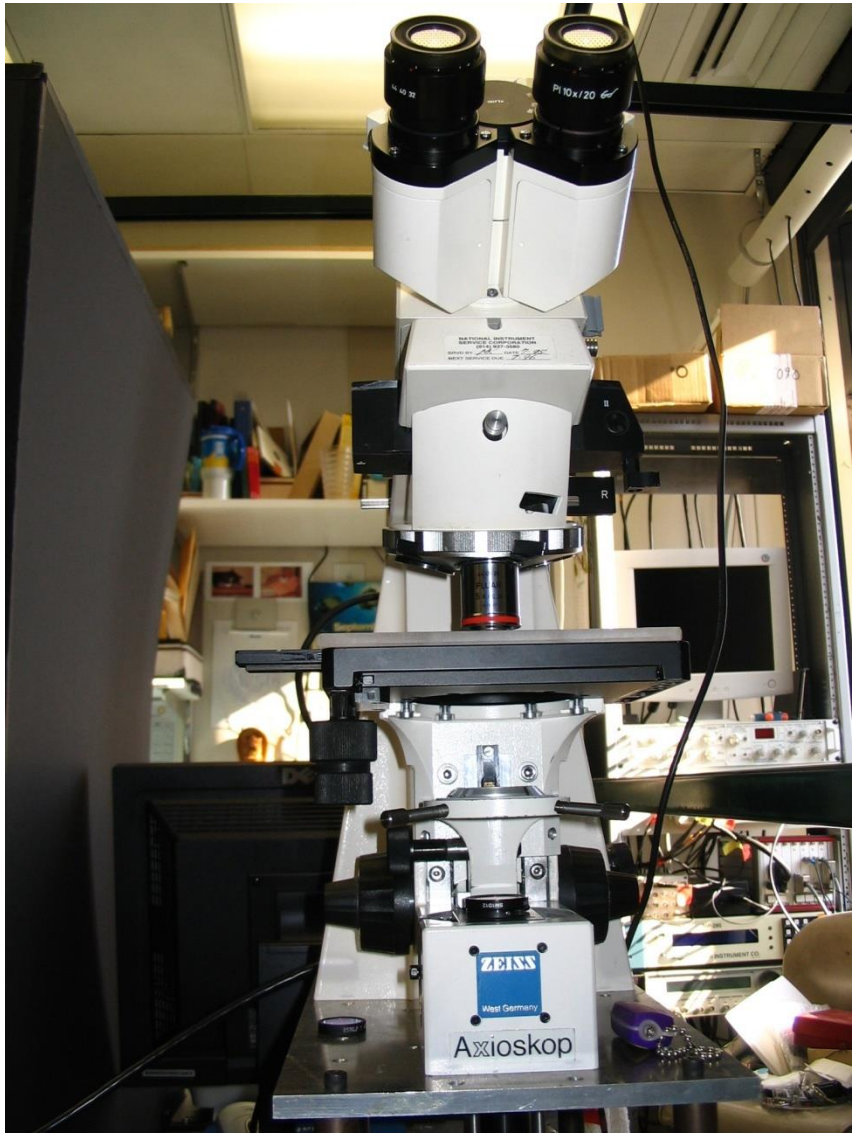
- many types
  - bright-field microscope
  - dark-field microscope
  - phase-contrast microscope
  - fluorescence microscopes
- are compound microscopes
  - image formed by action of $\geq$2 lenses

"Advanced" Light Microscopic Methods

Single Cell Organism (*Tetrahymena*) observed with:



(Dr. Gorovksy)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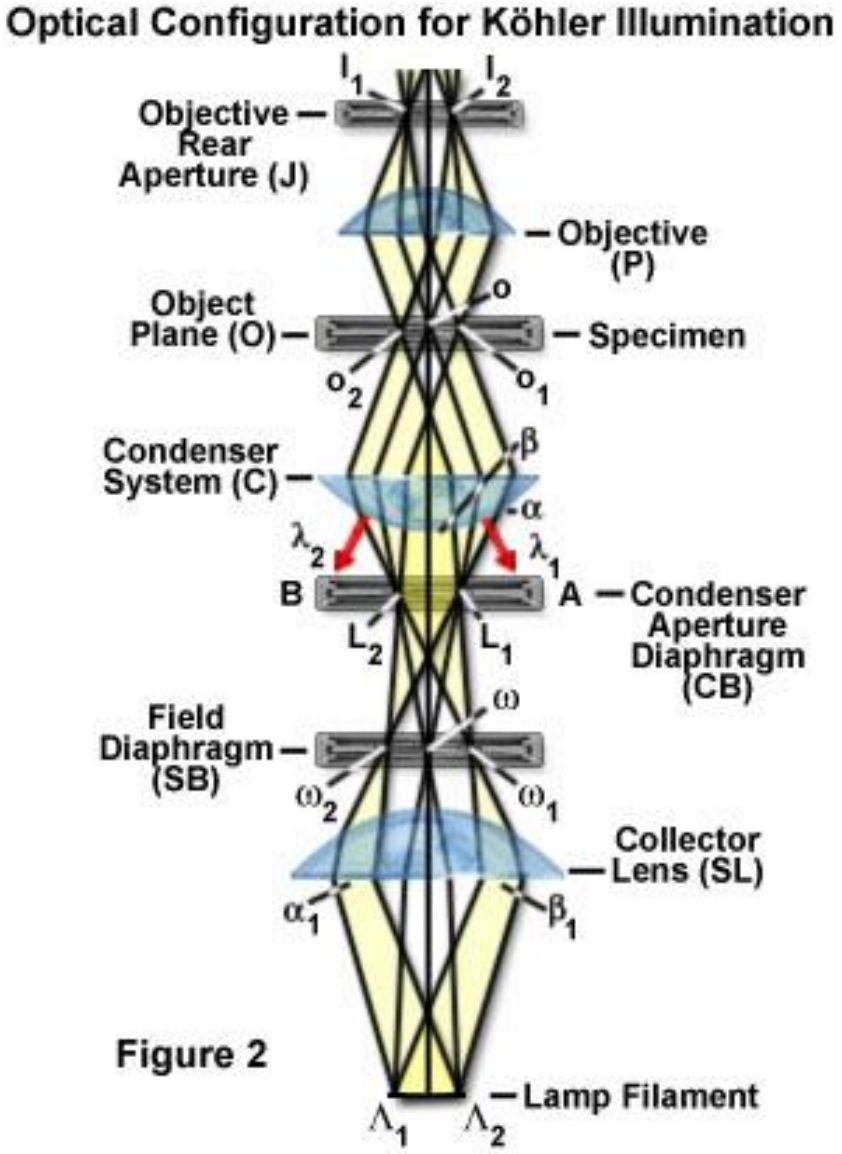

Optical Configuration for Köhler Illumination
I1
I2
Objective Rear Aperture (J)
Objective (P)
Object Plane (O)
o
Specimen
o2
o1
Condenser System (C)
β
α
λ2
λ1
B
A
Condenser Aperture Diaphragm (CB)
L2
L1
ω
Field Diaphragm (SB)
ω2
ω1
Collector Lens (SL)
α1
β1
Λ1
Λ2
Lamp Filament

Figure 2

The Bright-Field Microscope

- produces a dark image against a brighter background
- has several objective lenses
  - parfocal microscopes remain in focus when objectives are changed
- total magnification
  - product of the magnifications of the ocular lens and the objective lens

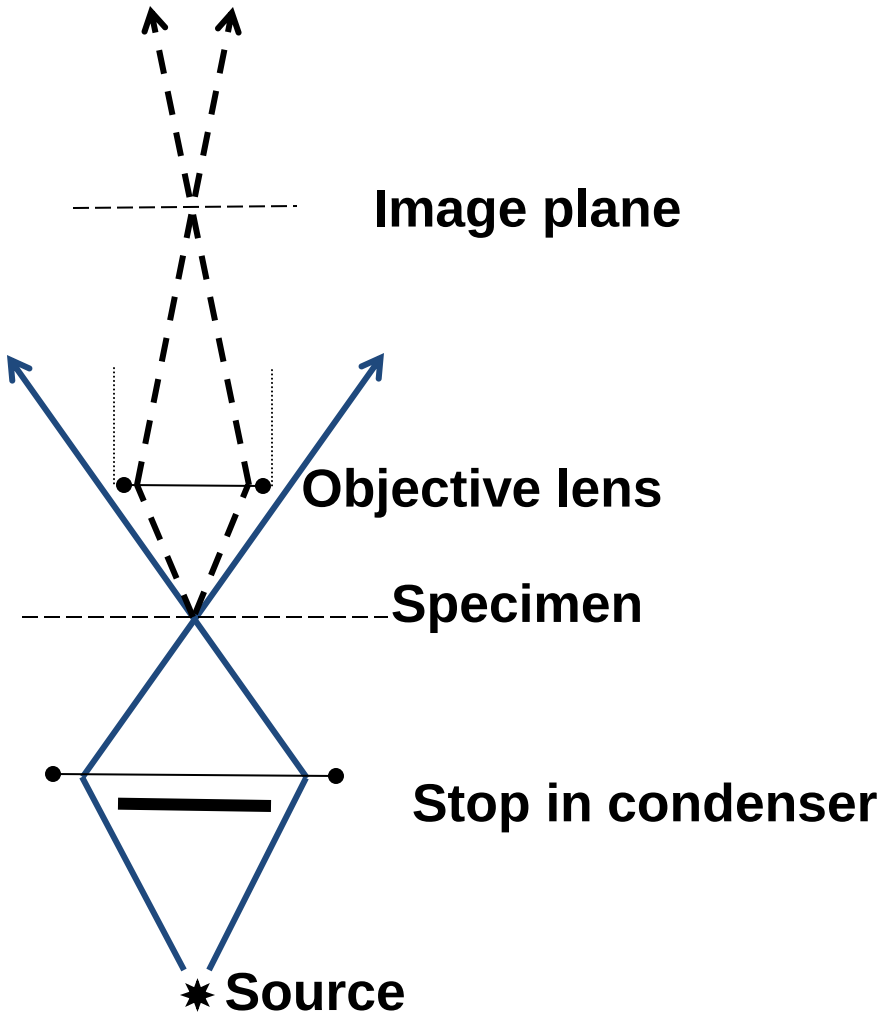
The Dark-Field Microscope

- produces a bright image of the object against a dark background
- used to observe living, unstained preparations

Darkfield Microscopy

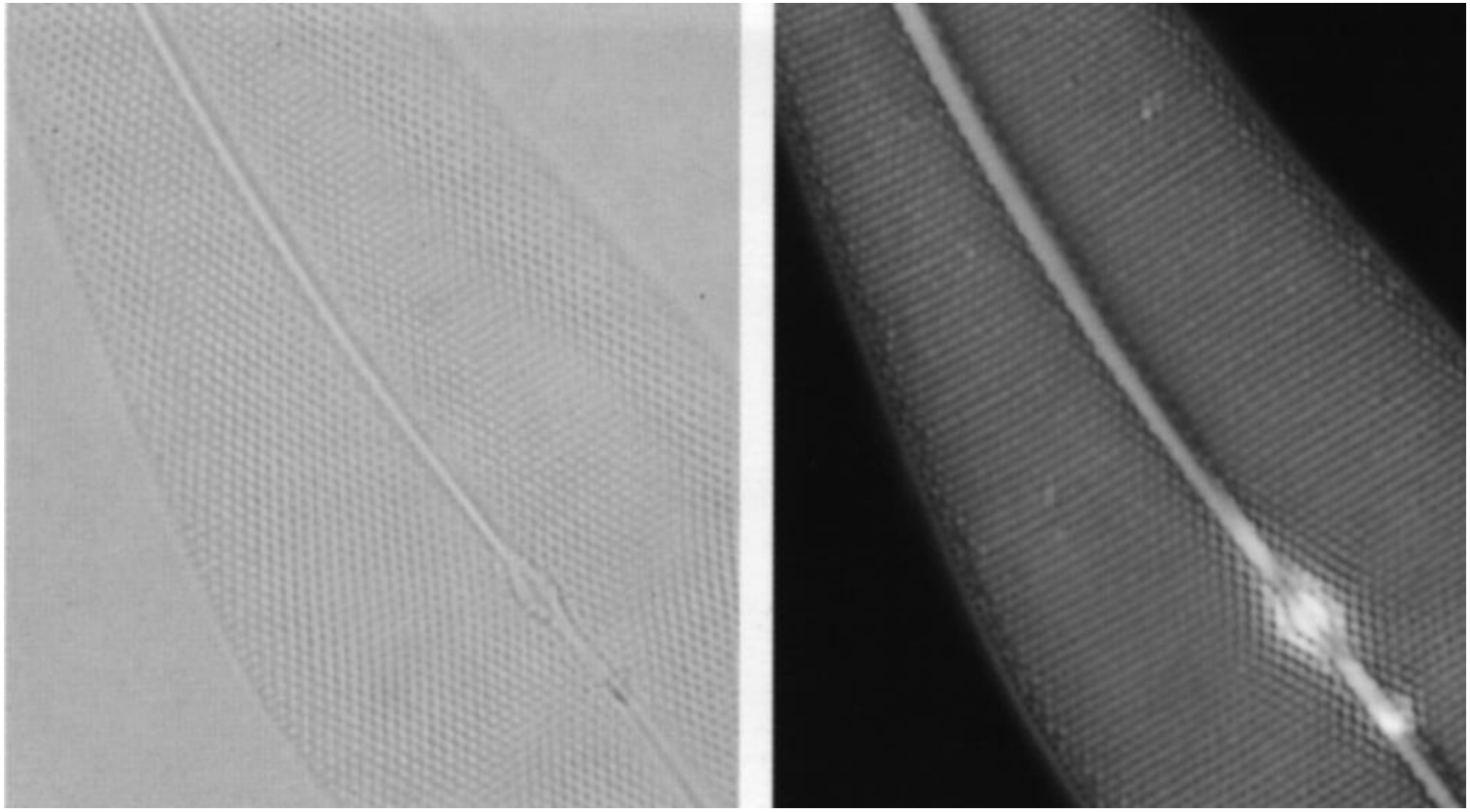
Objective collects
Only diffracted light

Condensor allows only
high angle rays



Comparison of bright and darkfield

Brightfield Microscopy **Darkfield Microscopy**

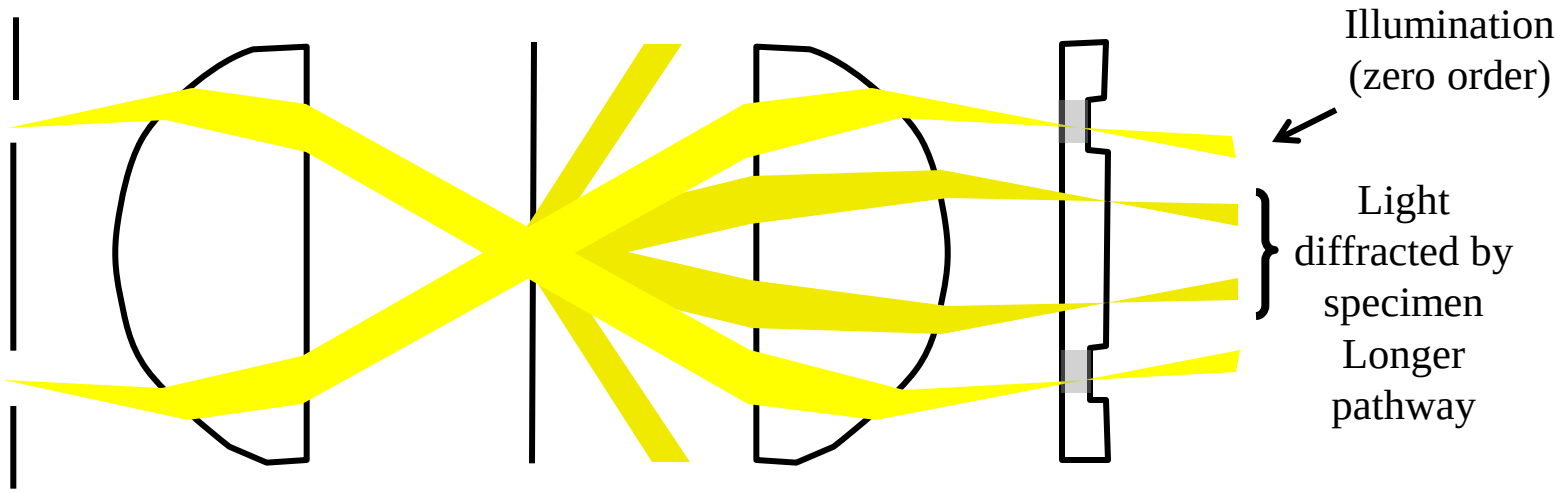


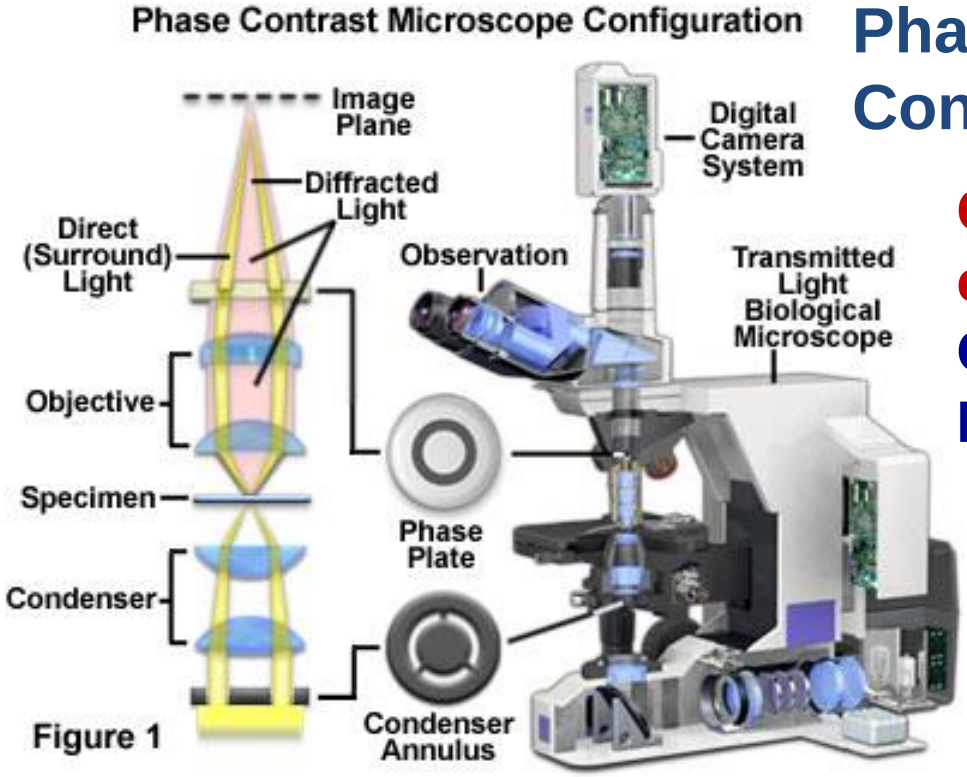
Contrast is reversed in these modalities

The Phase-Contrast Microscope

- enhances the contrast between intracellular structures having slight differences in refractive index
- excellent way to observe living cells

Phase Contrast Microscopy





Phase contrast: Kohler-same Conjugate planes as brightfield

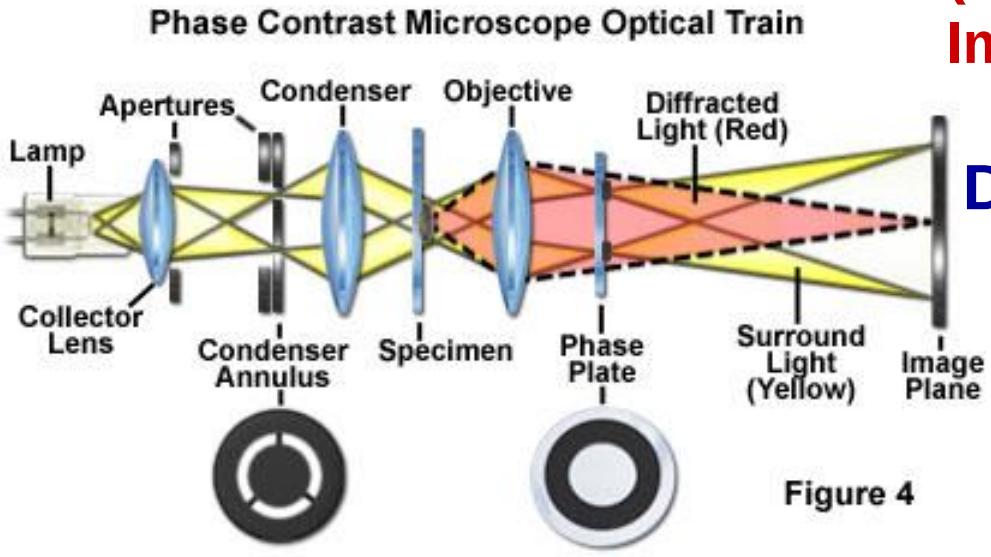
Condenser annulus replaces condenser variable diaphragm

Objective has phase ring in Backfocal plane

S wave projects bright image of annulus onto back aperture (Kohler illumination, conjugate Image planes)

Diffracted waves traverse whole back aperture

Surround waves un-deviated



Types of Microscopes

- 2. Electron Microscope
  - –Used to observe VERY small objects: viruses, DNA, parts of cells
  - –Uses beams of electrons rather than light
  - –Much more powerful

Types of Microscopes

- Transmission Electron Microscope (TEM)
  - –Can magnify up to 250,000x

The Transmission Electron Microscope

- electrons scatter when they pass through thin sections of a specimen
- transmitted electrons (those that do not scatter) are used to produce image
- denser regions in specimen, scatter more electrons and appear darker

EM

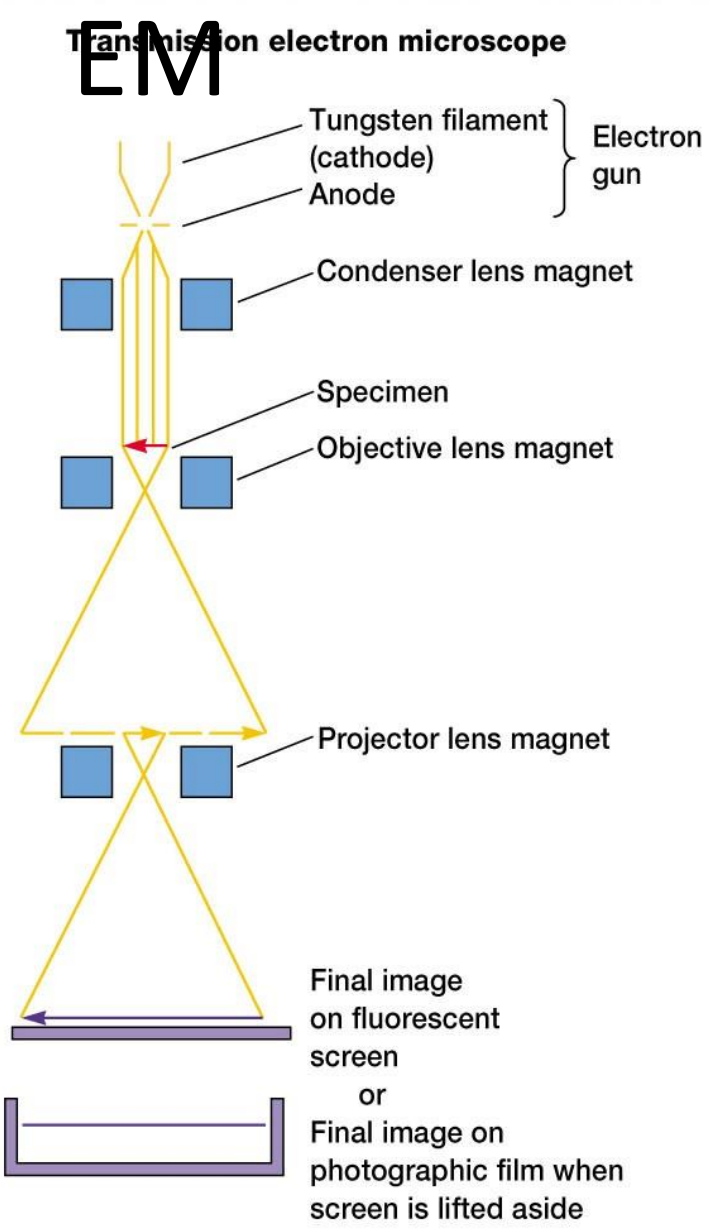
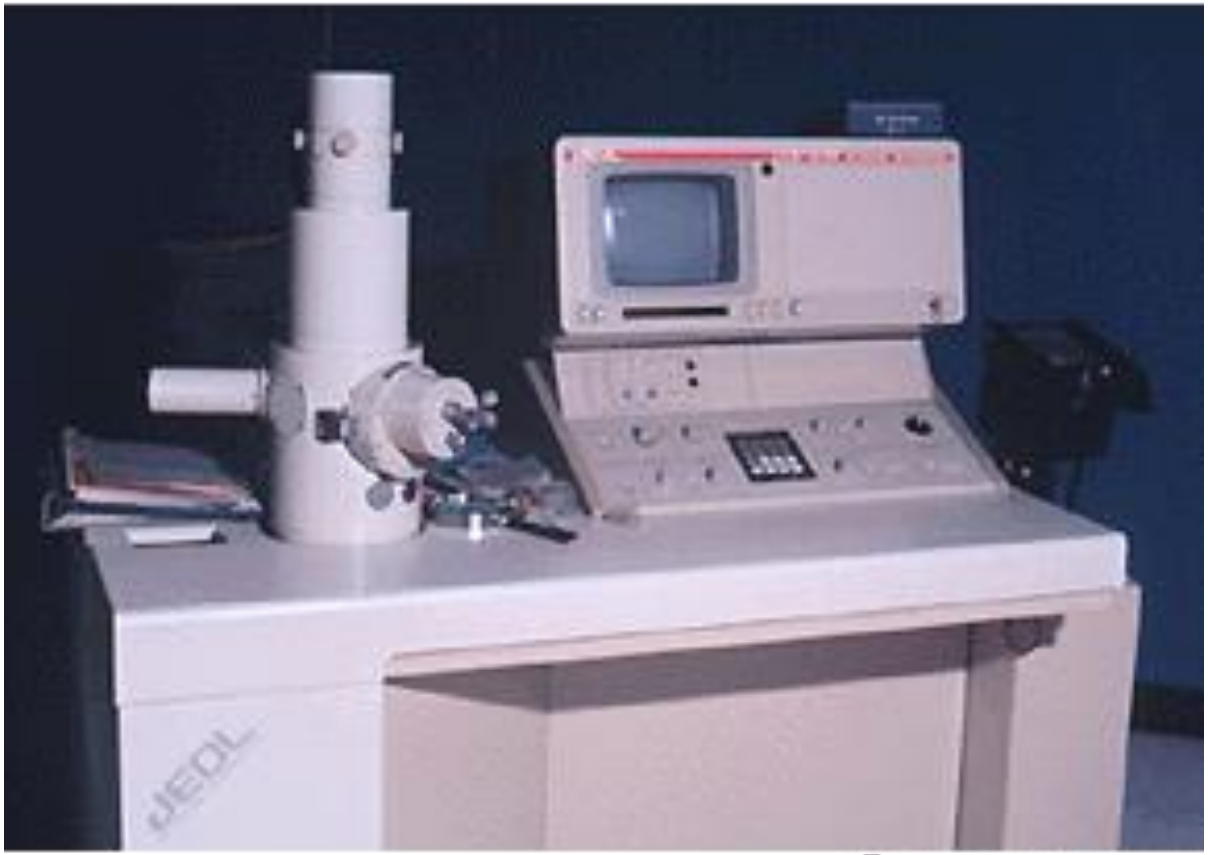


Figure 2.23

Types of Microscopes

- Scanning Electron Microscope (SEM)
  - –Can magnify up to 100,000x



Scanning Electron Microscopes

Scanning electron microscopes use electrons to probe the sample. Note that there are no lenses or eyepieces on this SEM. Images are created from measurements of how the electrons scatter off the sample.

Image: Grant Institute of Earth Science, University of Edinburgh

The Scanning Electron Microscope

- uses electrons reflected from the surface of a specimen to create image
- produces a 3-dimensional image of specimen's surface features

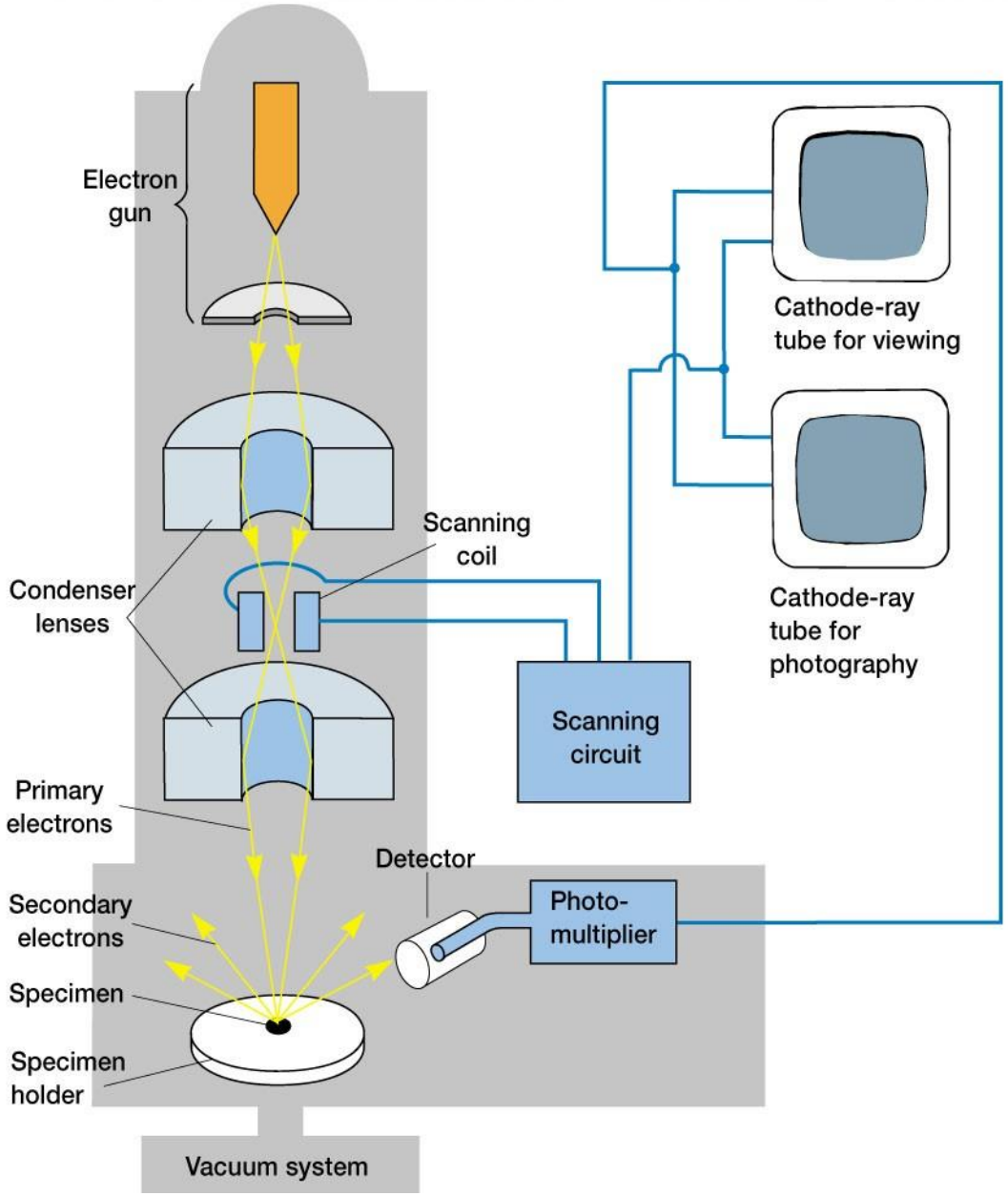

Electron gun
Cathode-ray tube for viewing
Cathode-ray tube for photography
Scanning coil
Condenser lenses
Scanning circuit
Primary electrons
Detector
Photo-multiplier
Secondary electrons
Specimen
Specimen holder
Vacuum system

Figure 2.27