DIPARTIMENTO di ELETTRONICA, INOFROMAZIONE e BIOINGEGNERIA

POLITECNICO DI MILANO

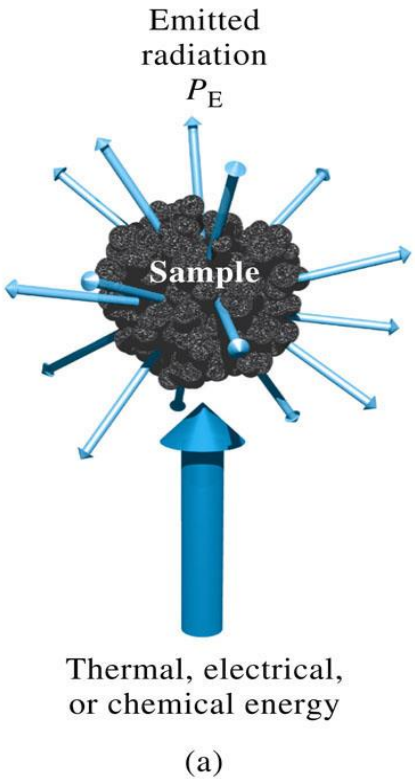




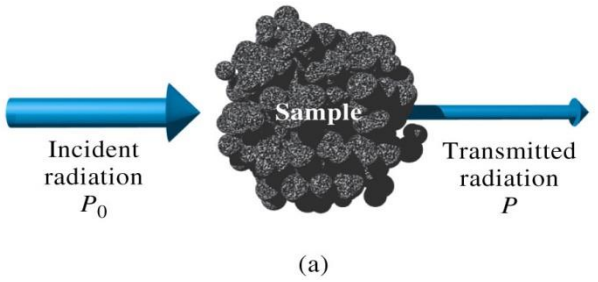
Lecture 15
Basic principles and Instruments for Clinical Laboratory Analysis

Interaction phenomena between electromagnetic radiation and matter

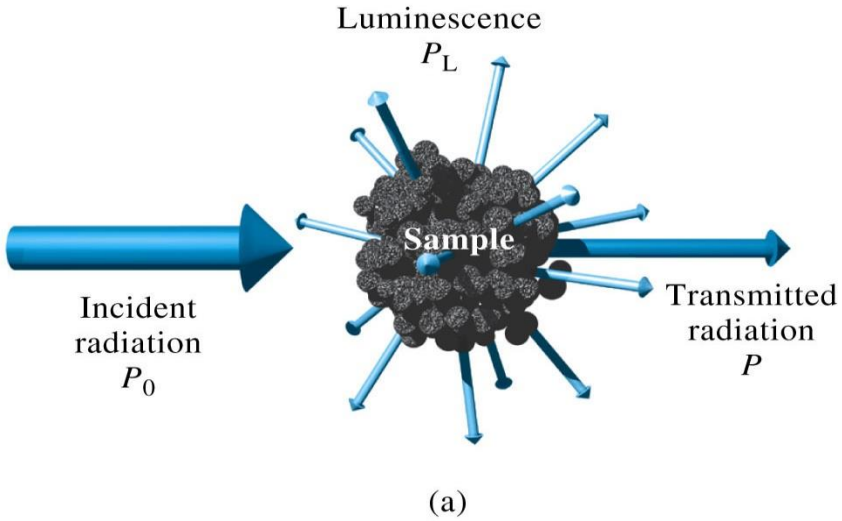
EMISSION



ABSORPTION



LUMINESCENCE



Luminescence

- Luminescence
  - Cathodo-luminescence
  - Electro-luminescence
  - Chemi-luminescence → Bio-luminescence
  - Sono-luminescence
  - Photo-luminescence
    - Fluorescence
    - Phosphorescence
  - Thermo-luminescence
  - Radio-luminescence
  - …

Luminescence

Cathodoluminescence: electrons impacting on a luminescent material such as a phosphor, cause the emission of photons which may have wavelengths in the visible spectrum.

Electroluminescence: a material emits light in response to the passage of an electric current or to a strong electric field

Sonoluminescence: emission of short bursts of light from imploding bubbles in a liquid when excited by sound

Chemiluminescence: excitation is obtained by an exergonic reaction, i.e. a chemical reaction where the change in the free energy is negative, indicating a spontaneous reaction

Bioluminescence: a particular form of chemiluminescence occurring in living organisms and that can be reproduced in vitro by using substances extracted by micro-organisms (e.g. luciferine)

Thermoluminescence: a form of luminescence that is exhibited by certain crystalline materials, such as some minerals, when previously absorbed energy from electromagnetic radiation or other ionizing radiation is re-emitted as light upon heating of the material.

Radioluminescence: light is produced in a material by bombardment with ionizing radiation such as beta particles.

Photoluminescence: fluorescence and phosphorescence

Fluorescence:

"an effect in which a substance releases electromagnetic radiation while absorbing another form of energy, but ceases to emit the radiation immediately upon cessation of the input energy"; "the light emission of a given wavelength by a substance that is activated by light of a different wavelength."

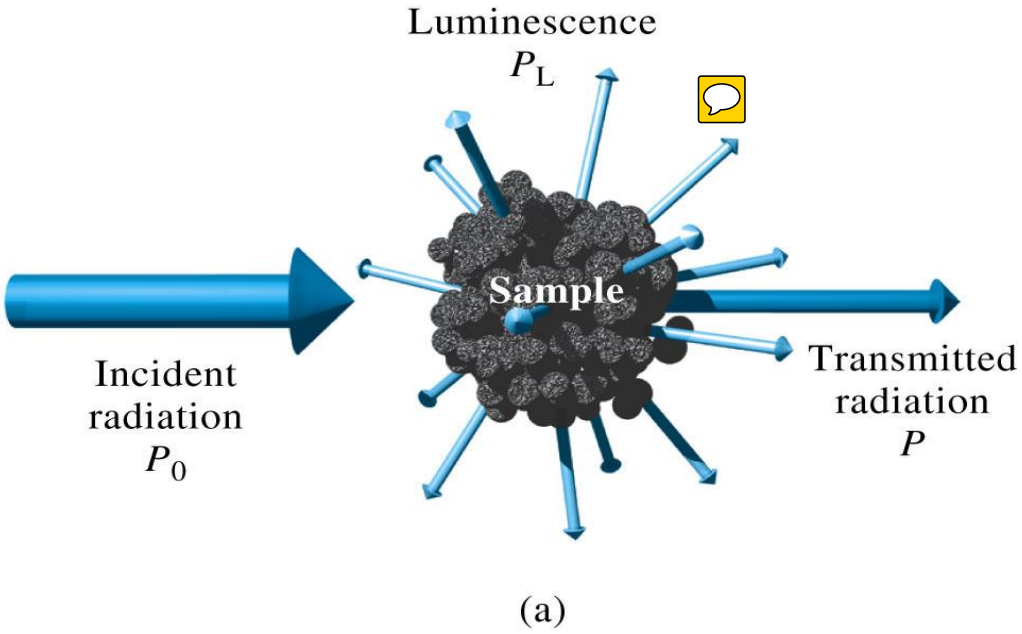
Academic Press Dictionary of Science and Technology, 1992.

"The emission of a longer wavelength radiation by a substance as a consequence of absorption of energy from a shorter wavelength radiation, continuing only as long as the stimulus is present;"

"distinguished from **phosphorescence** in that, in the latter, emission persists for a perceptible period of time after the stimulus has been removed."

Stedman's Medical Dictionary, 24th ed., 1982.

Luminescence



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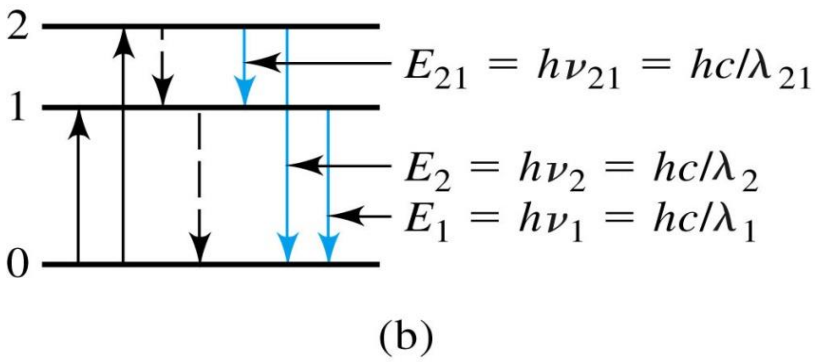

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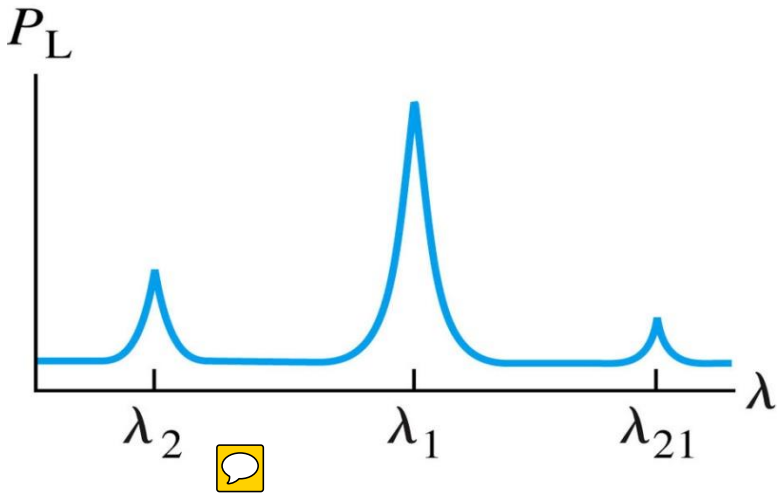


(c)

Luminescence = emission of photons from electronically excited states

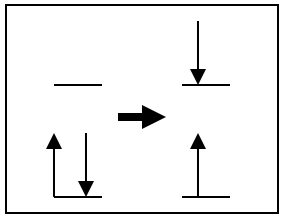
Two types of luminescence:
- relaxation from singlet excited state
- relaxation from triplet excited state

Singlet and triplet states

- Ground state – two electrons per orbital; electrons have opposite spin and are paired

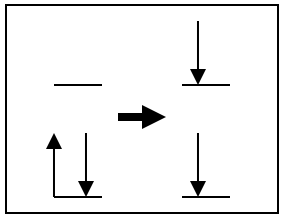
- Singlet excited state

  Electron in higher energy orbital has the opposite spin orientation relative to electron in the lower orbital



- Triplet excited state

  The excited valence electron may spontaneously reverse its spin (spin flip). This process is called intersystem crossing. Electrons in both orbitals now have same spin orientation



Types of emission

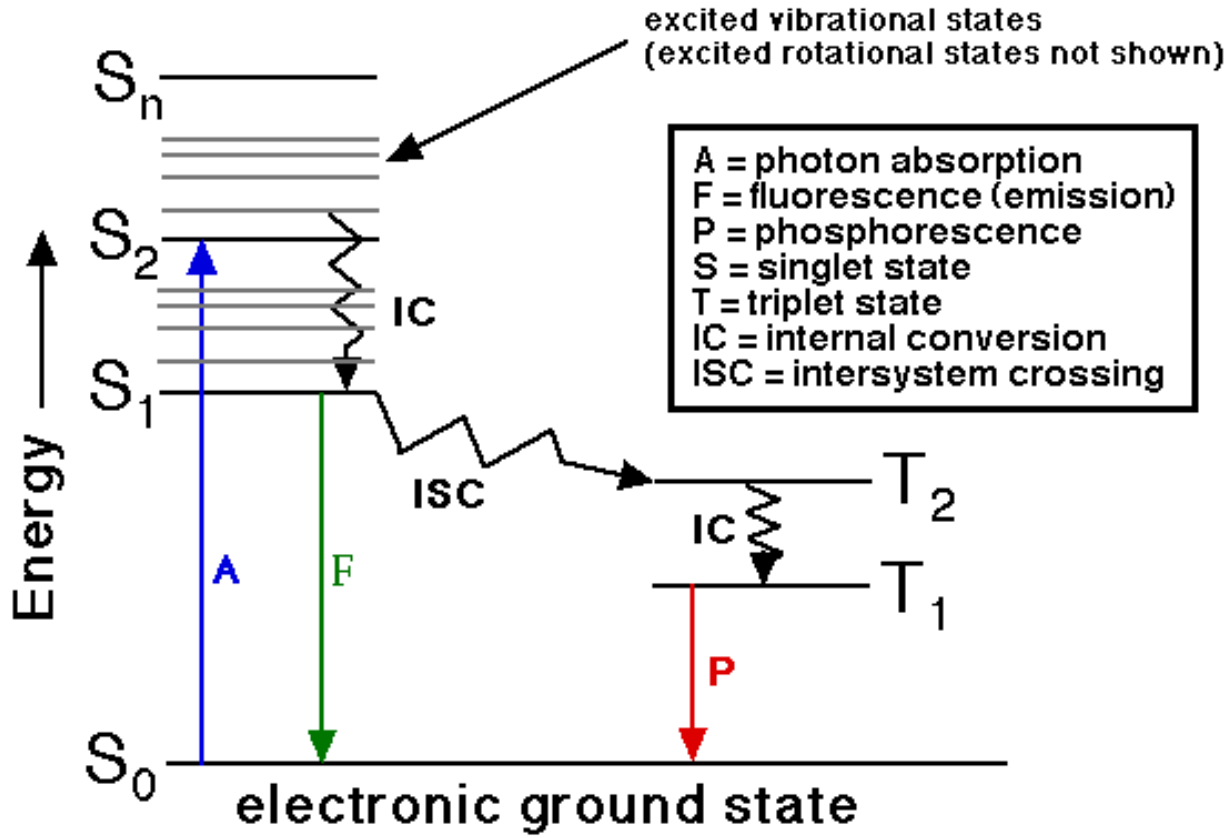
- Fluorescence – return from excited singlet state to ground state; does not require change in spin orientation (more common of relaxation)

- Phosphoresence – return from a triplet excited state to a ground state; electron requires change in spin orientation

- Emissive rates of fluorescence are several orders of magnitude faster than that of phosphorescence

Jablonski energy diagram

It illustrates the electronic states of a **molecule** and the transitions between them. The states are arranged vertically by energy and grouped horizontally by spin multiplicity. Nonradiative transitions are indicated by squiggly arrows and radiative transitions by straight arrows. The vibrational ground states of each electronic state are indicated with thick lines, the higher vibrational states with thinner lines.



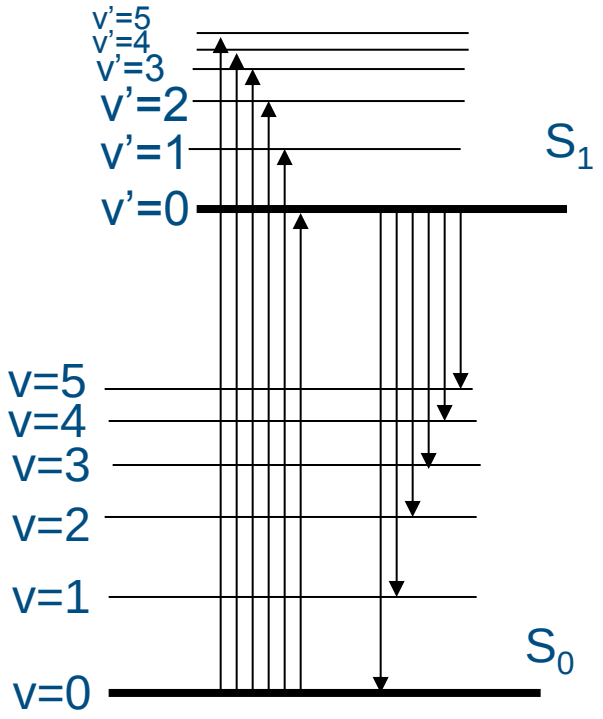
Mirror image rule

Vibrational levels in the excited states and ground states are similar

An absorption spectrum reflects the vibrational levels of the electronically excited state

An emission spectrum reflects the vibrational levels of the electronic ground state

Fluorescence emission spectrum is mirror image of absorption spectrum



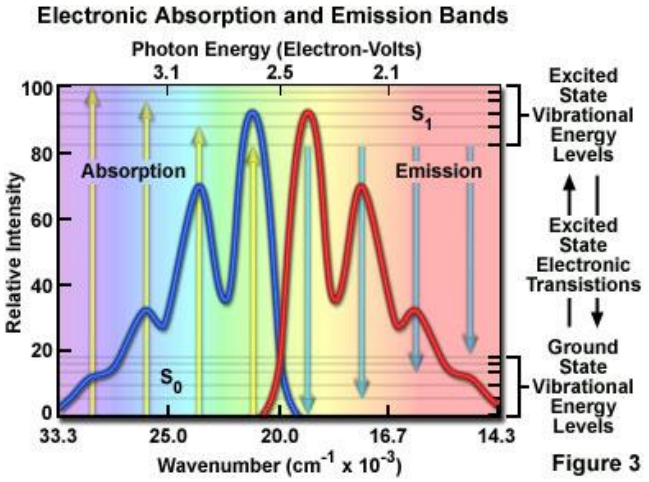
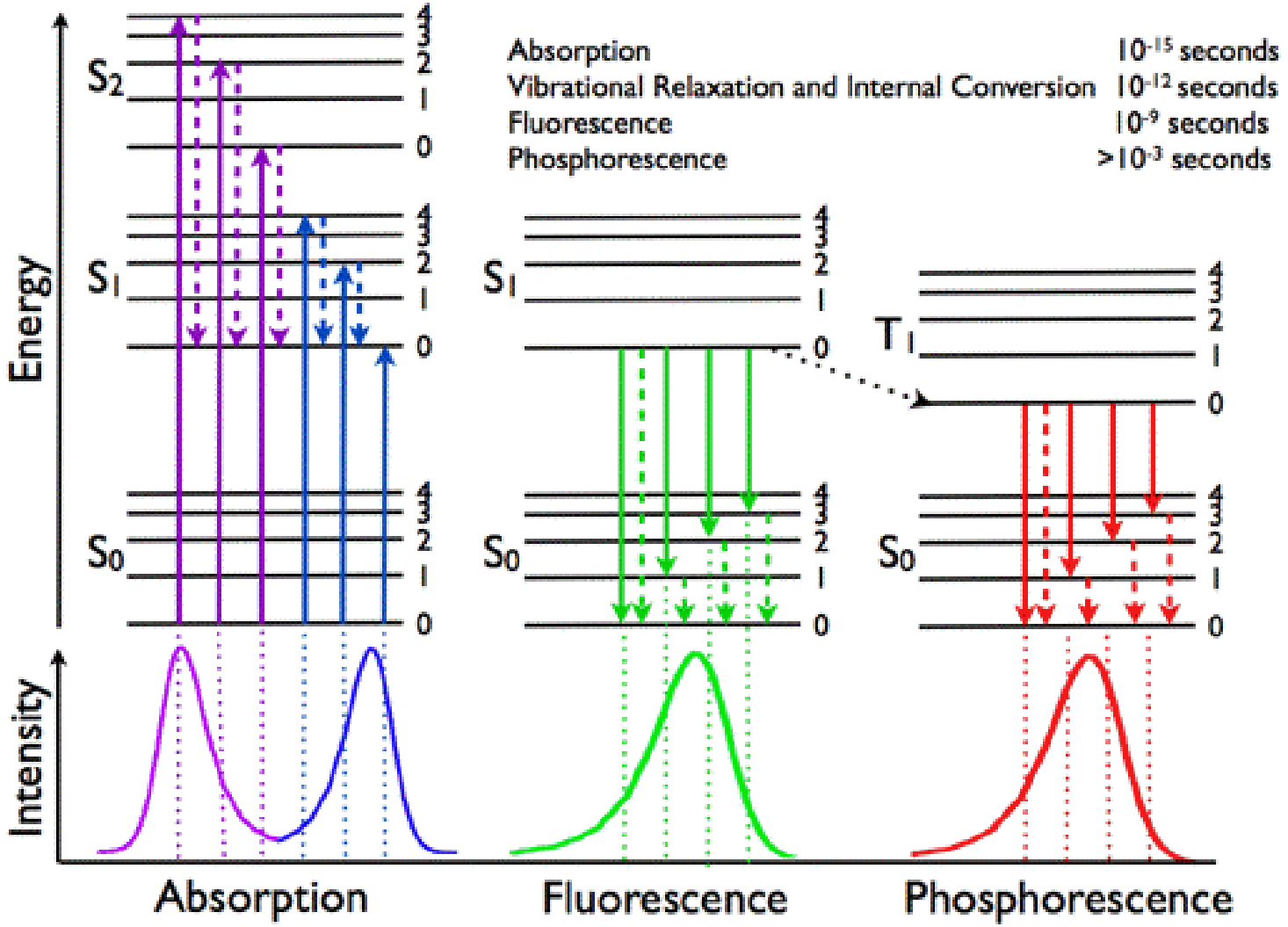


Figure 3

Absorption, fluorescence, phosphorescence



Fluorometry

Fluorescence indicates effective absorbance and re-emission of electromagnetic radiation (often in the visible range, ~300 - 800 nm, but may involve other ranges, e.g., x – ray).

If the endpoint is presence of fluorescence, e.g. qualitative staining of a microscopic specimen, it can be directly observed or recorded on film or by digital means. When a quantitative test is done, color is usually measured with a **spectrofluorimeter**.

Fluorometry is used in the analysis of clinical samples, pharmaceuticals, natural products, and environmental samples.

There are fluorescence methods for steroids, lipids, proteins, amino acids, enzymes, drugs, inorganic electrolytes, chlorophylls, natural and synthetic pigments, vitamins, and many other types of analytes.

Biological Fluorophores

Endogenous Fluorophores →

- amino acids
- structural proteins
- enzymes and co-enzymes
- vitamins
- lipids
- porphyrins

Exogenous Fluorophores

- Cyanine dyes
- Photosensitizers
- Molecular markers, etc.

Endogenous fluorophores	Excitation maxima (nm)	Emission maxima (nm)
Amino acids		
Tryptophan	280	350
Tyrosine	275	300
Phenylalanine	260	280
Structural proteins		
Collagen	325	400, 405
Elastin	290, 325	340, 400
Enzymes and coenzymes		
FAD, flavins	450	535
NADH	290, 351	440, 460
NADPH	336	464
Vitamins		
Vitamin A	327	510
Vitamin K	335	480
Vitamin D	390	480
Vitamin B_6 compounds		
Pyridoxine	332, 340	400
Pyridoxamine	335	400
Pyridoxal	330	385
Pyridoxic acid	315	425
Pyridoxal 5′-phosphate	330	400
Vitamin B_{12}	275	305
Lipids		
Phospholipids	436	540, 560
Lipofuscin	340–395	540, 430–460
Ceroid	340–395	430–460, 540
Porphyrins	400–450	630, 690

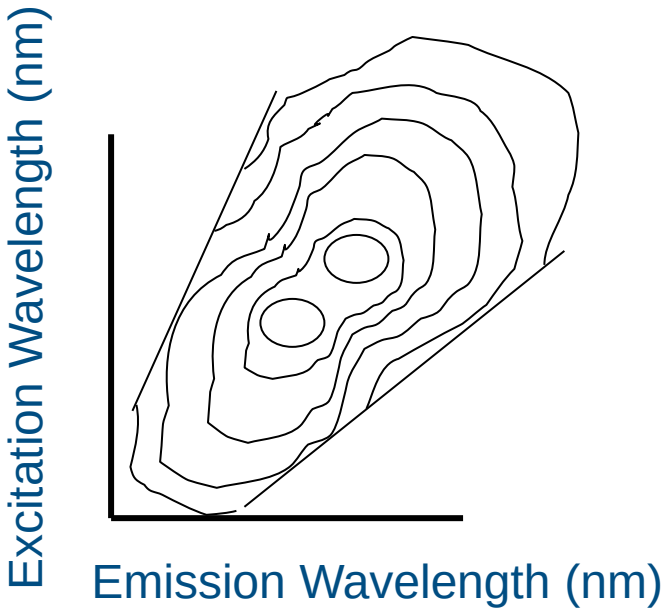
FAD, flavin adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; AND(P)H, reduced nicotinamide adenine dinucleotide phosphate.

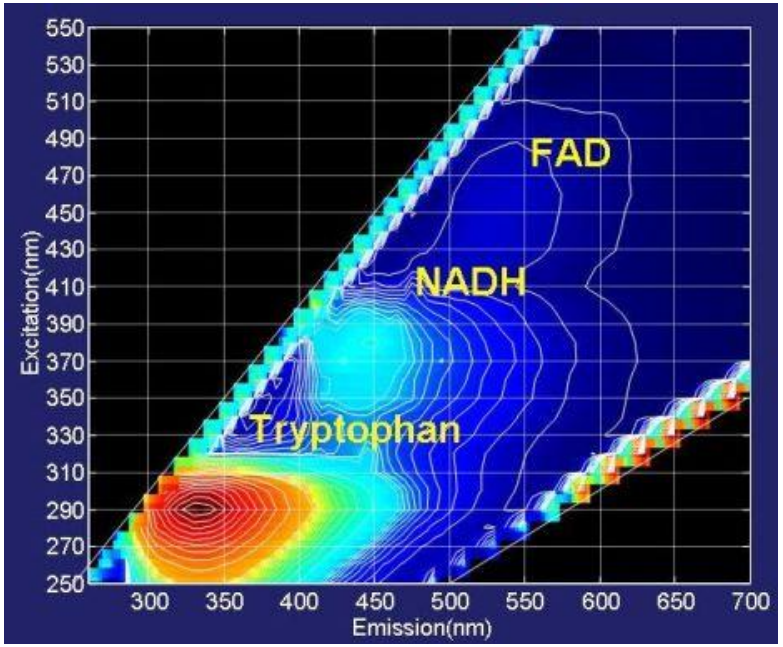
Biological Fluorophores

Nicotinamide adenine dinucleotide (NAD) is coenzyme found in all living cells. NAD exists in two forms, an oxidized (NAD+) and reduced (NADH). In metabolism, NAD is involved in **redox reactions**, carrying electrons from one reaction to another. NAD+ is an oxidizing agent (it accepts electrons from other molecules and becomes reduced). This reaction forms NADH, which can then be used as a reducing agent to donate electrons. NAD is also used in other cellular processes, the most notable one being a substrate of enzymes that add or remove chemical groups from proteins. Because of the importance of these functions, the enzymes involved in NAD metabolism are **targets for drug discovery**.

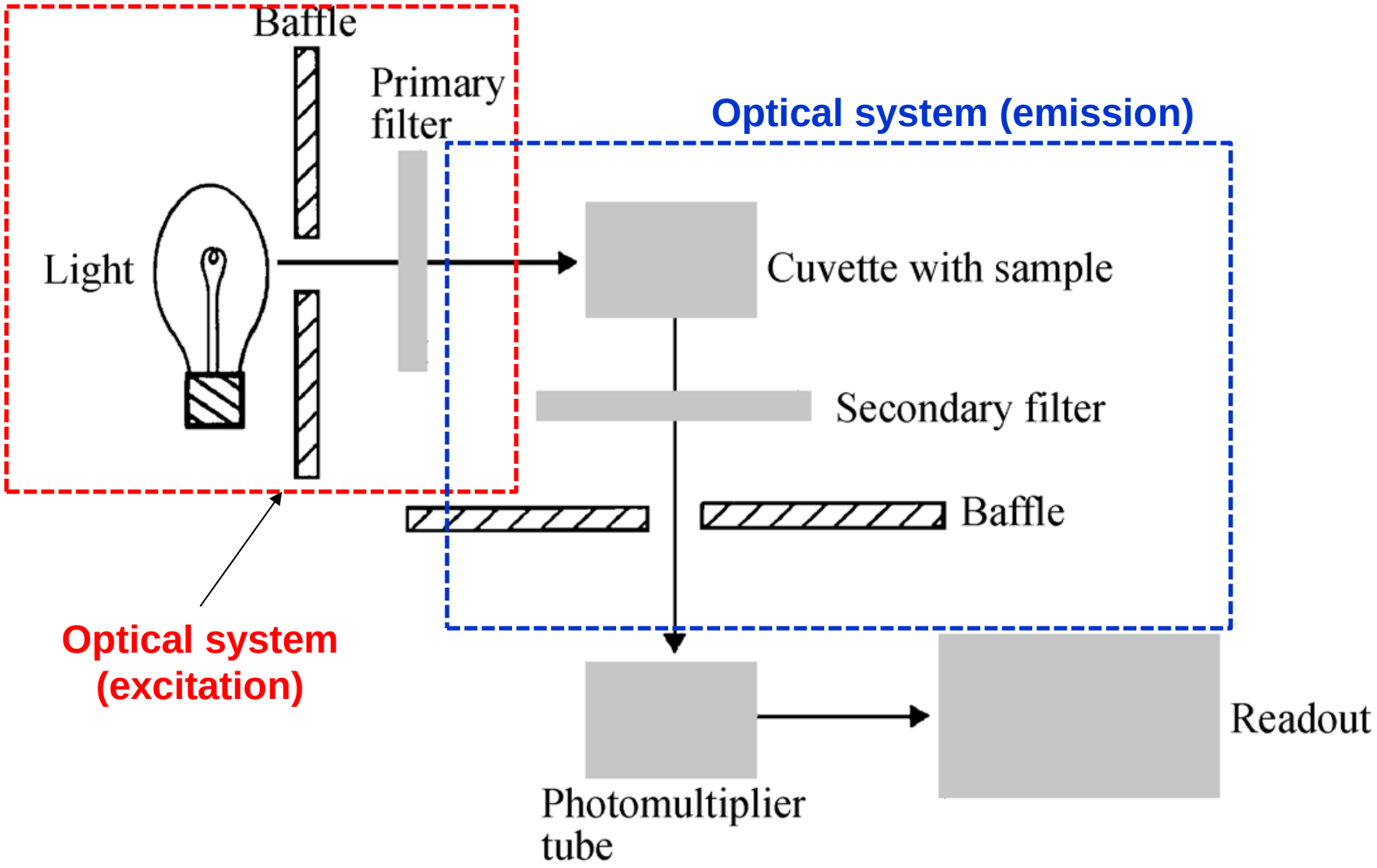
Collagen (the major extracellular matrix component, which is present to some extent in nearly all organs and serves to hold cells together in discrete units) fluorescence in tissues is associated with cross-links (hydroxylysyl pyridoline, HP, and lysyl pyridinoline, LP). Collagen crosslinks are altered with **age** and with invasion of **cancer** into the extracellular matrix

Excitation-emission matrix

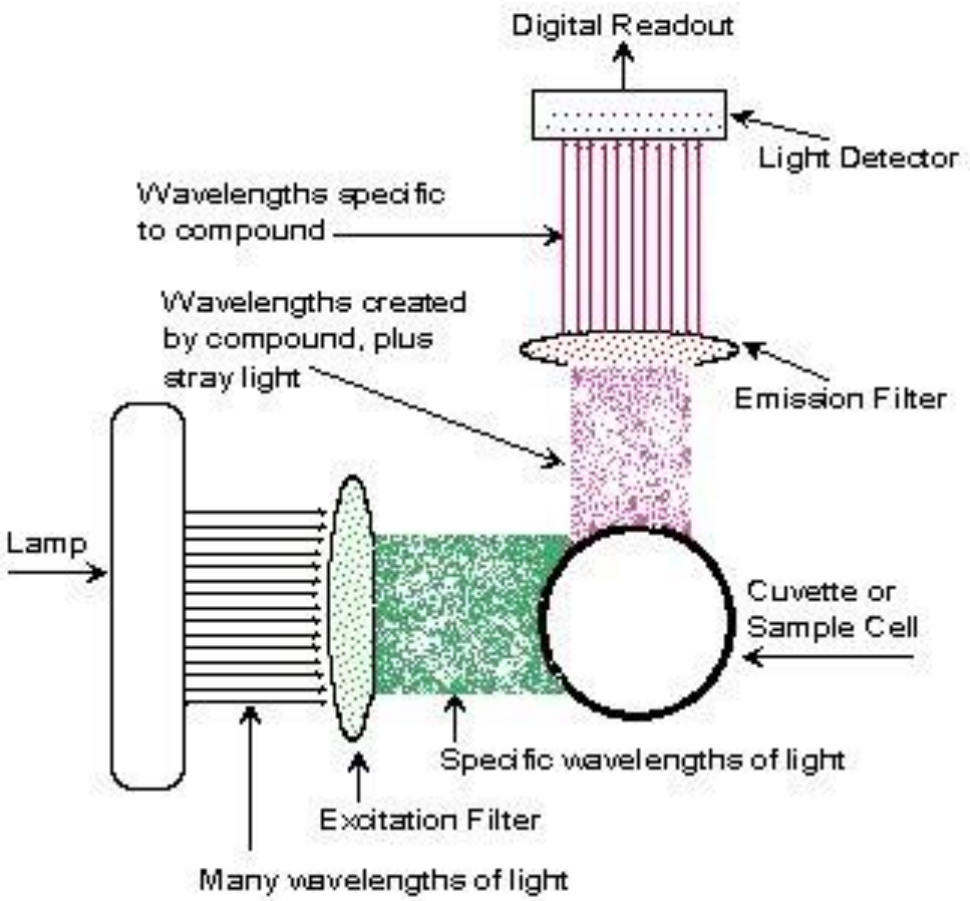


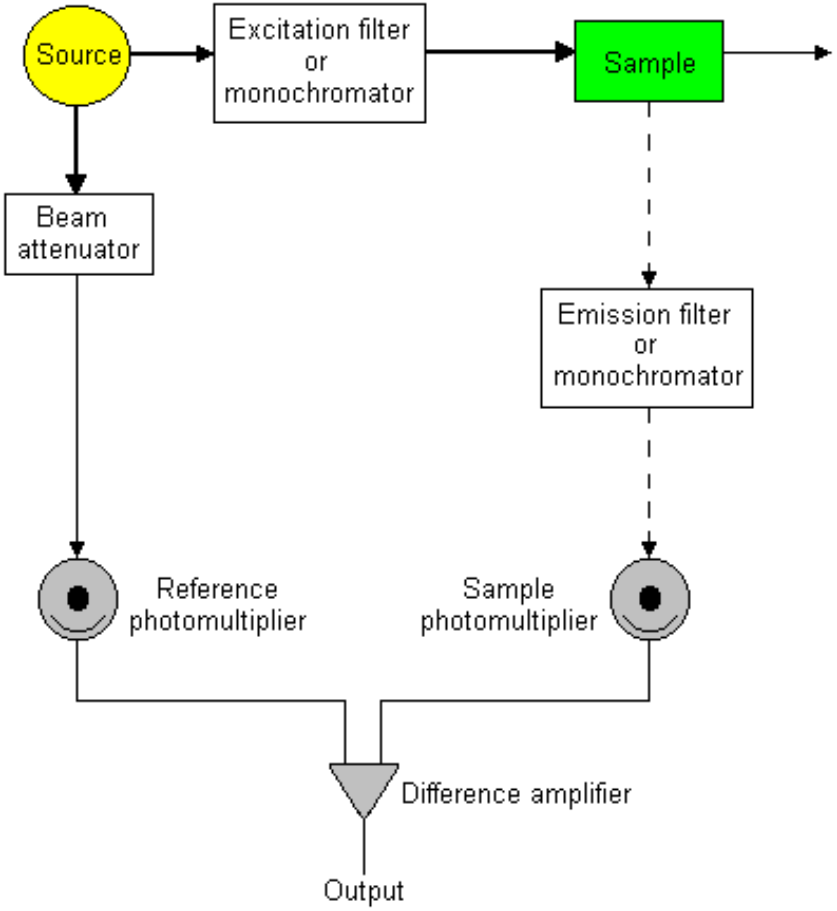


Spectrofluorimeter: schematic diagrams



Spectrofluorimeter: schematic diagrams

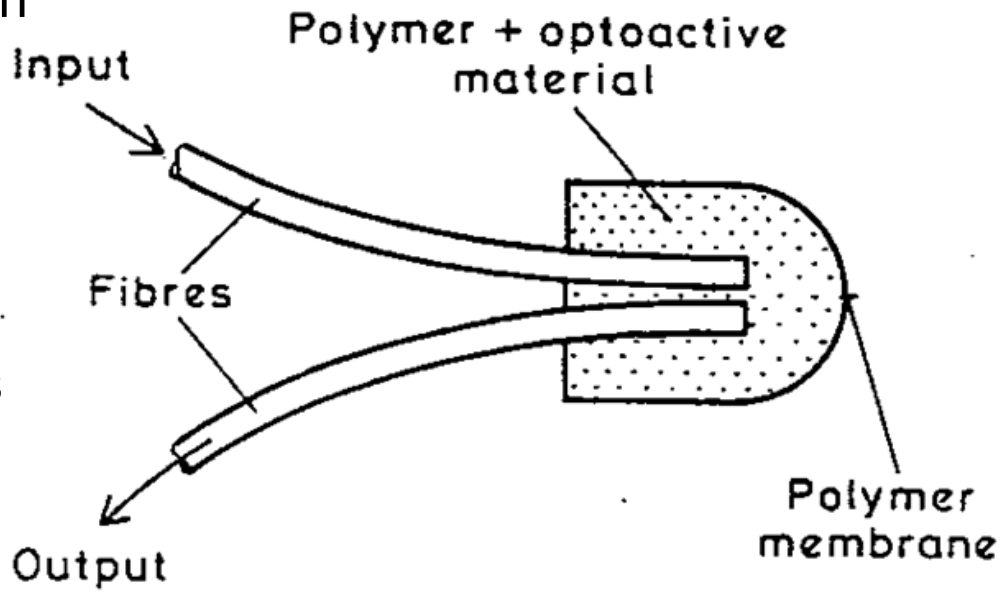




Optrode

A device used to measure gases, ions, enzymatic substrates, macro-molecules. The active material, placed on the tip, includes chemical indicators, fluorescent dyes, chemi- or bio-luminescent enzymes, depending on the application.
The use of reagents or selective membranes can increase sensitivity and selectivity.

Optrode measurements are based on
- **Colorimetric assessment** (color changes of the indicator trapped in the tip, or directly of the substance under exam)
- **Fluorimetric assessment** (the intensity of fluorescent light changes in response to variations of the substance under examination)
- **Phosphorescence**



Indicator-mediated fiber optic sensor tips

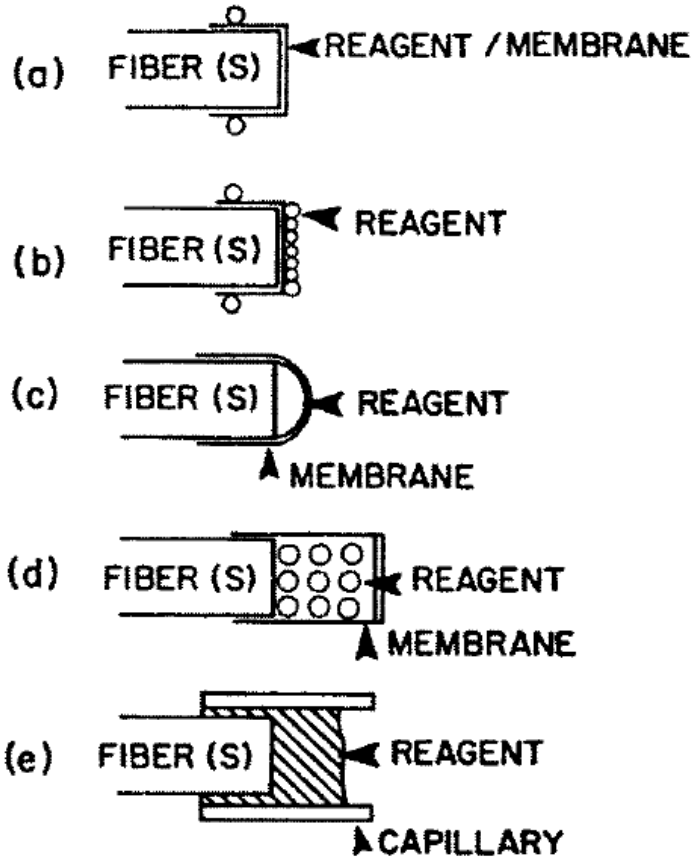
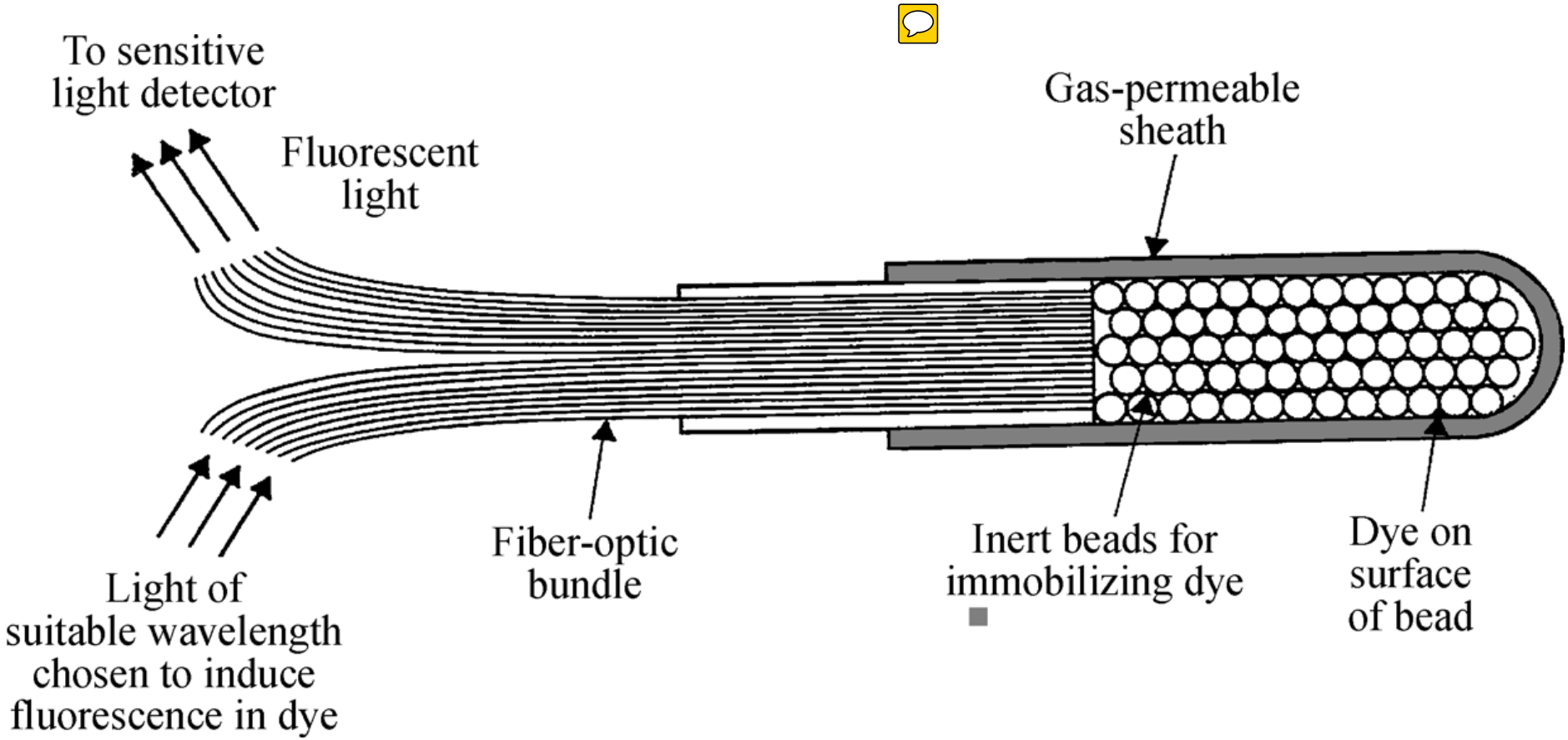


FIGURE 50.2 Typical configuration of different indicator-mediated fiber optic sensor tips (from Otto S. Wolfbeis, Fiber Optic Chemical Sensors and Biosensors, vol. 1, CRC Press, Boca Raton, 1990).

Fiber optic oxygen sensor



In a fiber-optic oxygen sensor, irradiation of dyes causes fluorescence that decreases with PO_2

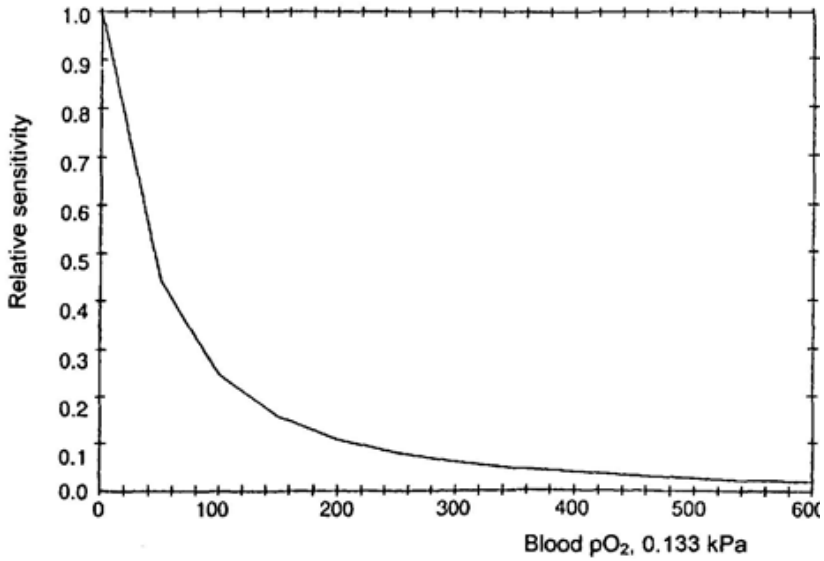
Fiber optic oxygen sensor

In a fiber-optic oxygen sensor, irradiation of dyes (e.g., perylene-dibutirate) causes fluorescence that decreases with PO_2
According to the following relationship:

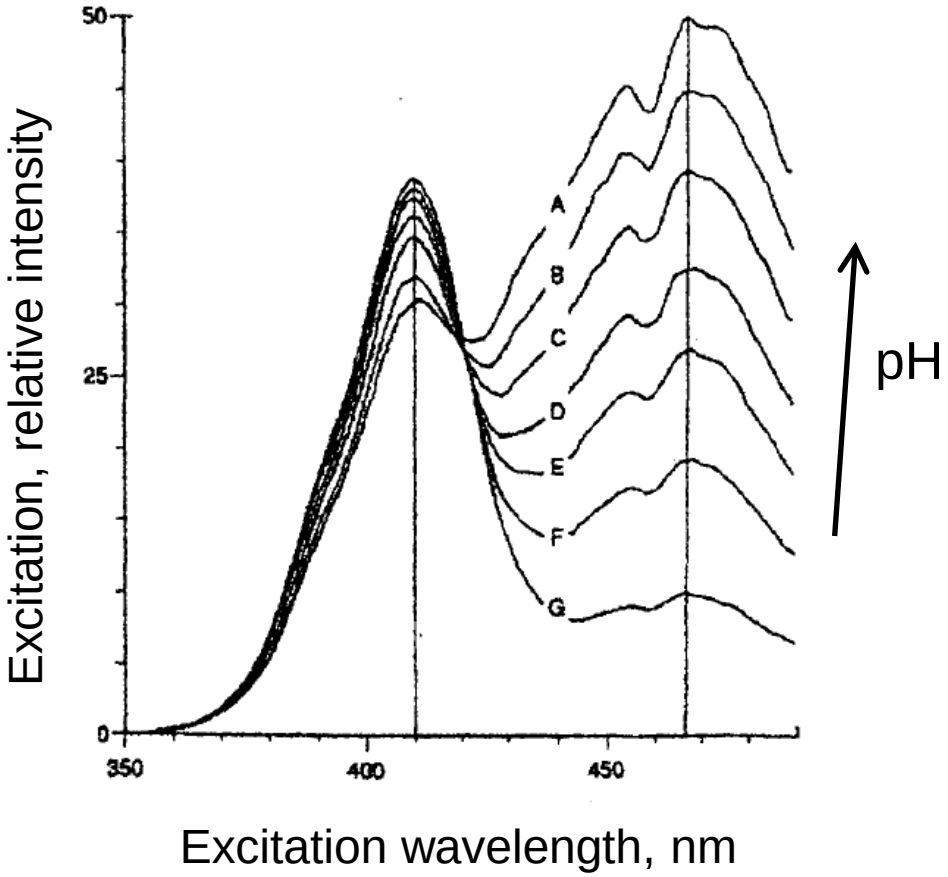
$$I = \frac{I_0}{(1 + k \cdot pO_2)}$$

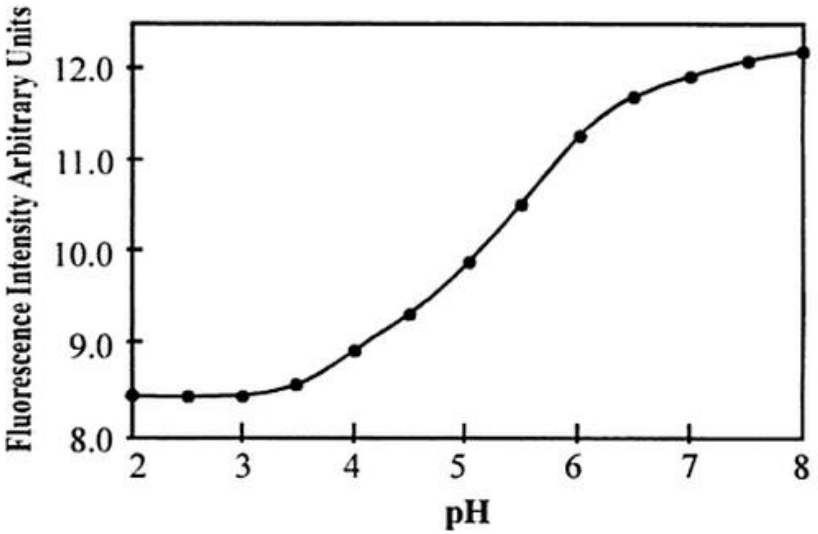
where
I = intensity of emitted fluorescence light
I_0 = intensity of emitted fluorescence light without dye

Sensitivity is not constant →

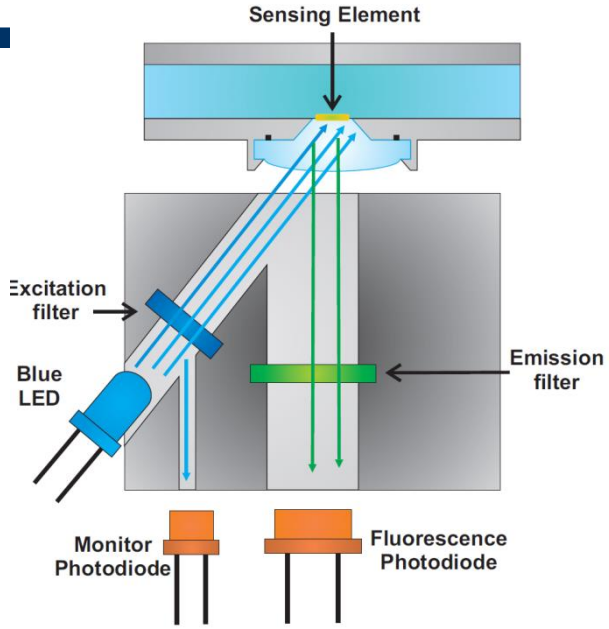


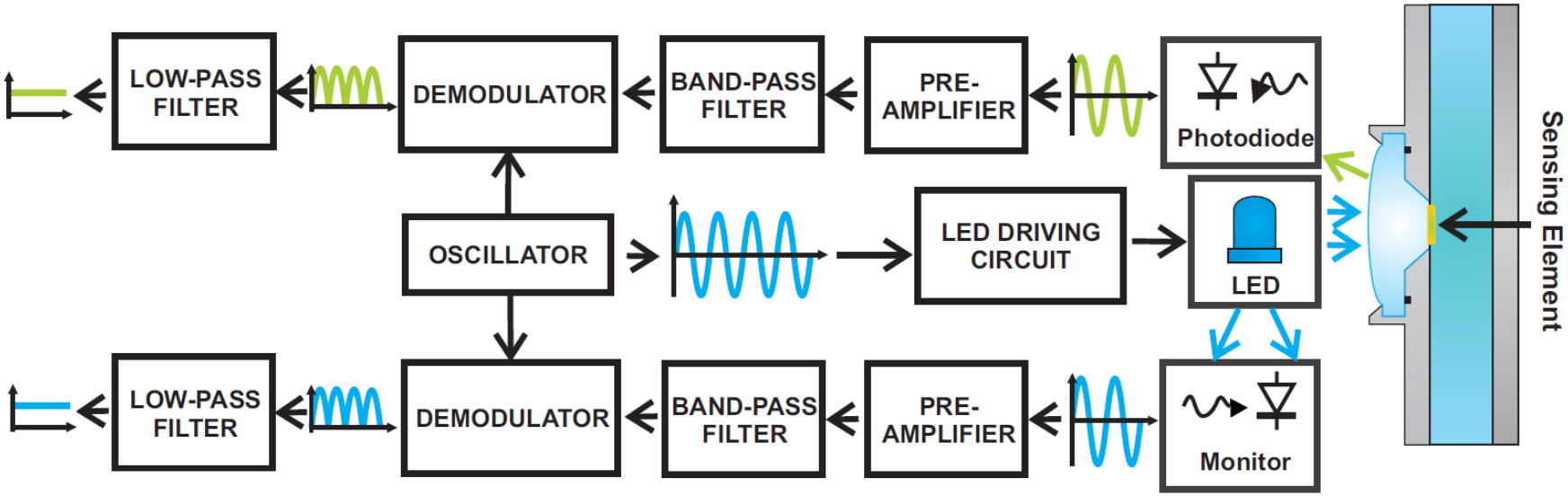
Fluorescence Fiber Optic Sensor for blood pH monitoring



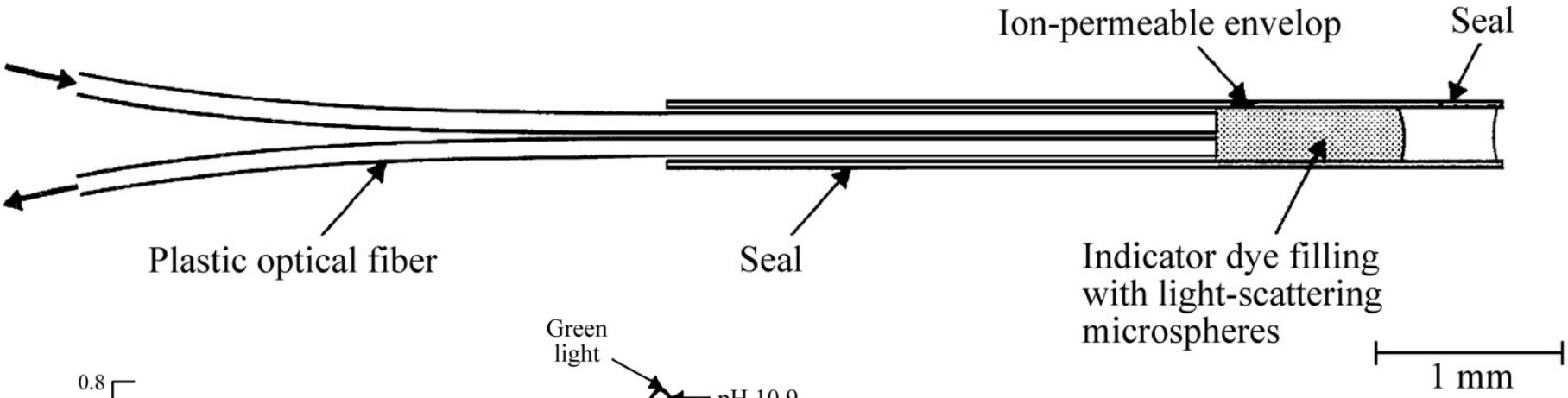


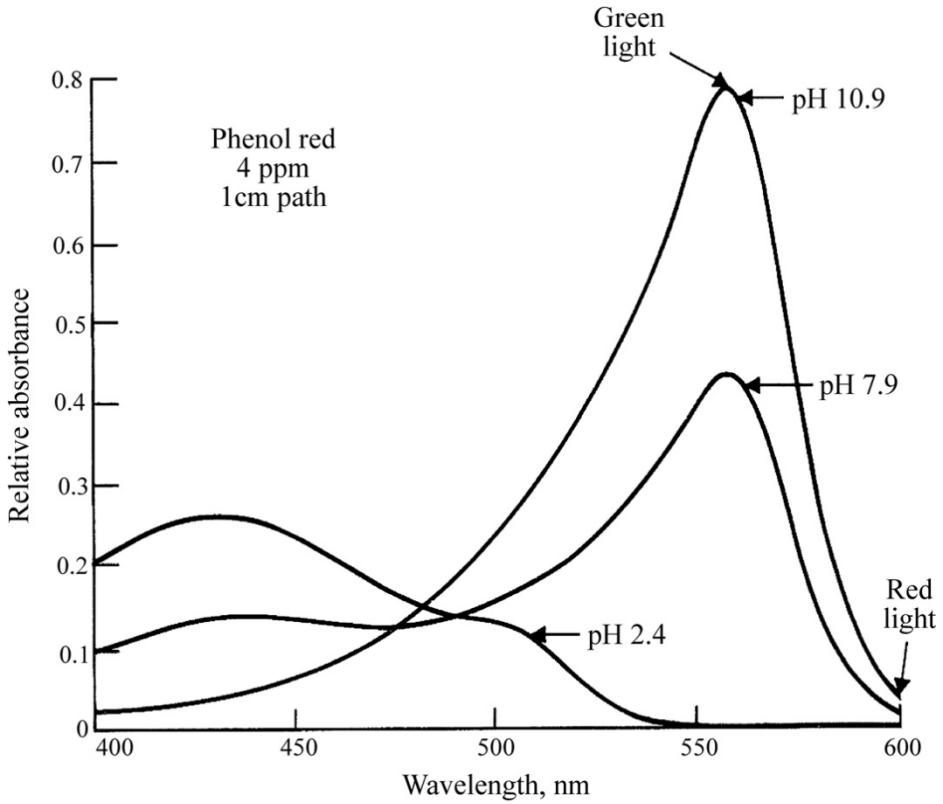
Fluorescence Fiber Optic Sensor for blood pH monitoring





Reversible-dye (phenol red) Optical Absorbance measurement of pH





The plot of absorbance against wavelength of phenol red (base form) increases with pH for green light but is constant for red light.

Absorption, emission and fluorescence spectroscopy

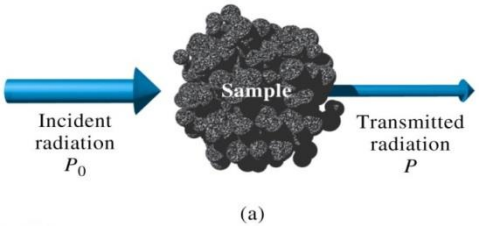
1. Absorption spectroscopy:
   Source → Dispersive device → Sample → Detector → Data output
2. Emission spectroscopy:
   Sample → Dispersive device → Detector → Data output
3. Fluorescence, phosphorescence, and scattering spectroscopy:
   Sample → Dispersive device → Detector → Data output

↑

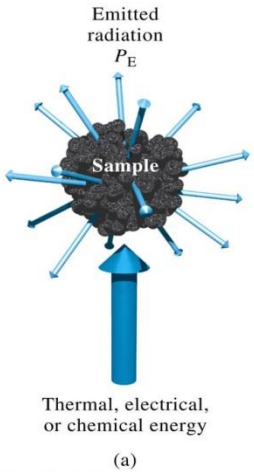
Dispersive device

↑

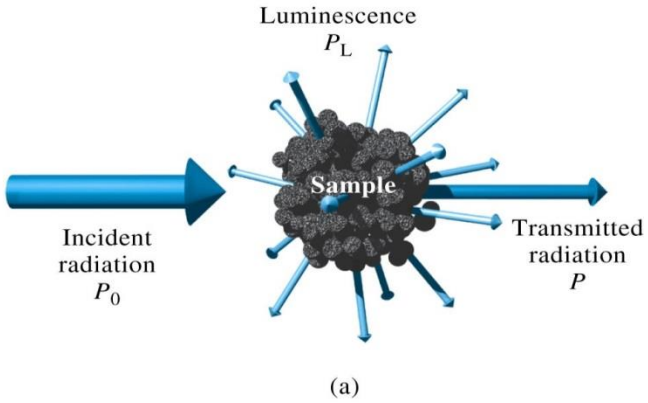
Source



ABSORPTION

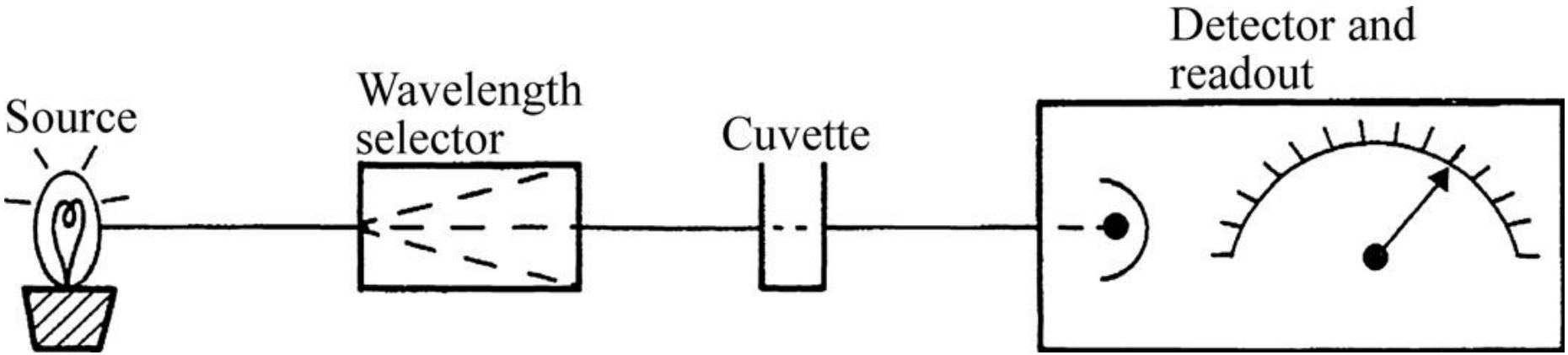


EMISSION

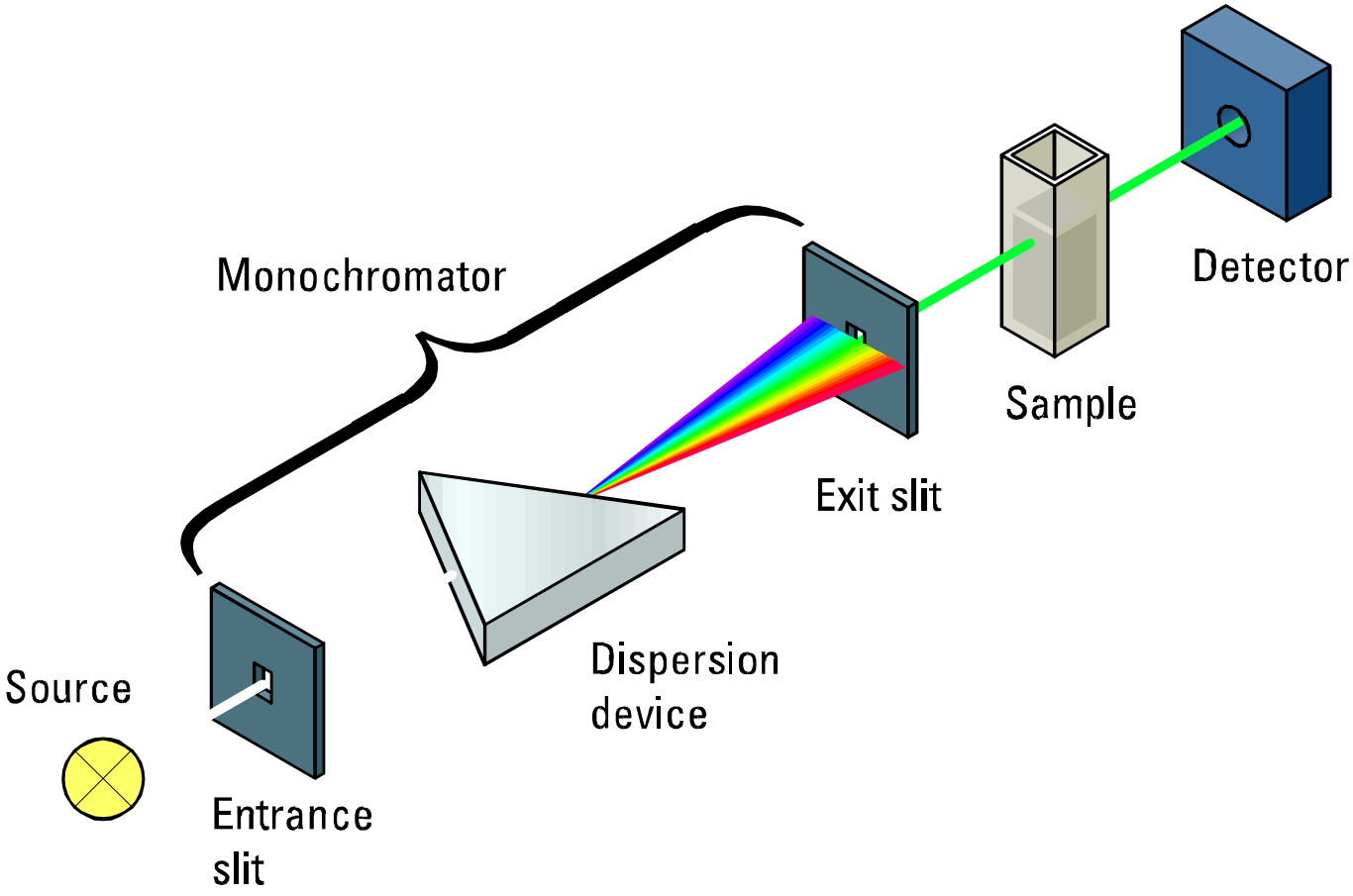


LUMINESCENCE

Absorption Spectrometry



Conventional Spectrophotometer



Schematic of a conventional single-beam spectrophotometer

Drift

- When single-beam optics are used, any variation in the intensity of the source while measurements are being made may lead to analytical errors.
- Slow variation in the average signal (not noise) with time is called drift
- Drift can cause a direct error in the results obtained.

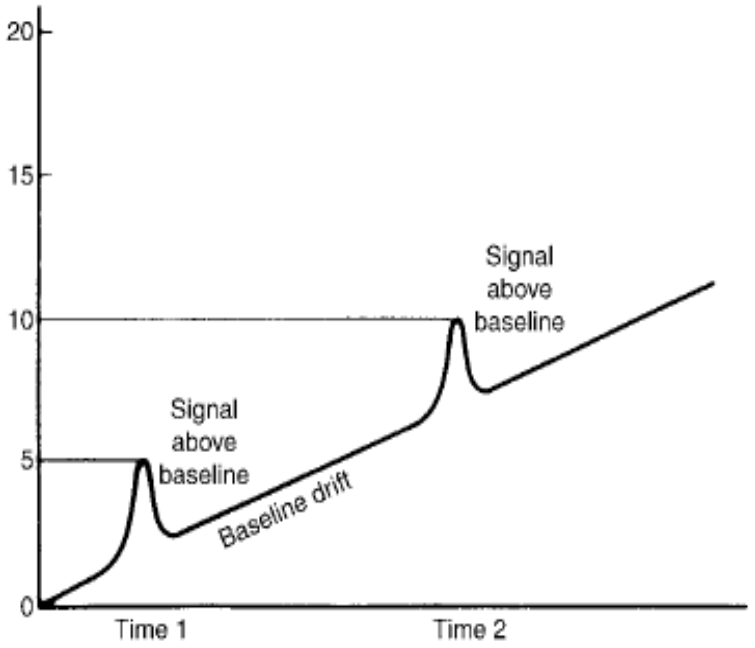


Figure 2.27 Error caused by baseline drift in a spectroscopic measurement.

Drift

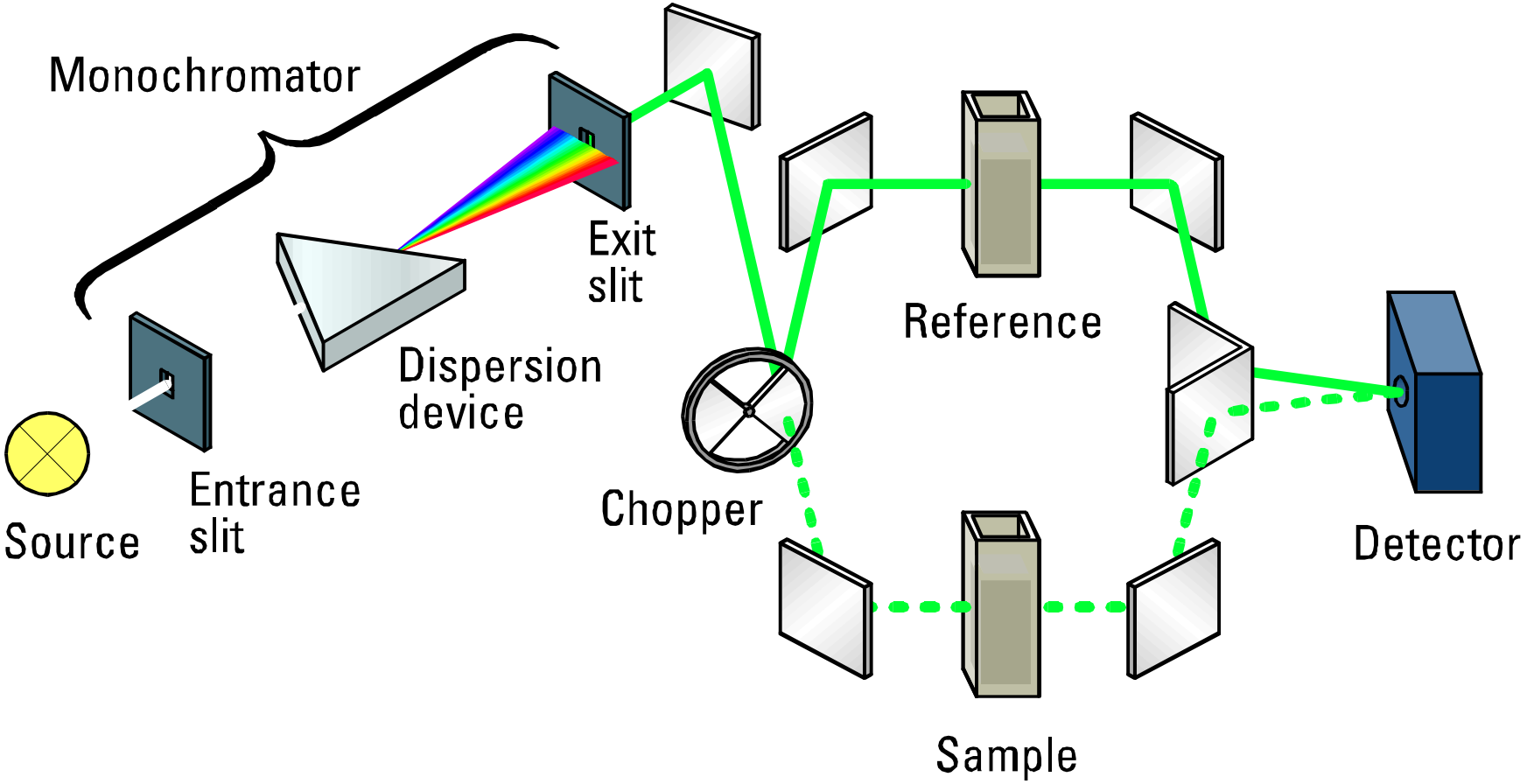
The radiation source intensity may change because of line voltage changes, the source warming up after being recently turned on, or the source deteriorating with time.

The monochromator may shift position as a result of vibration or heating and cooling causing expansion and contraction.

The line voltage to the detector may change, or the detector may deteriorate with time and cause a change in response.

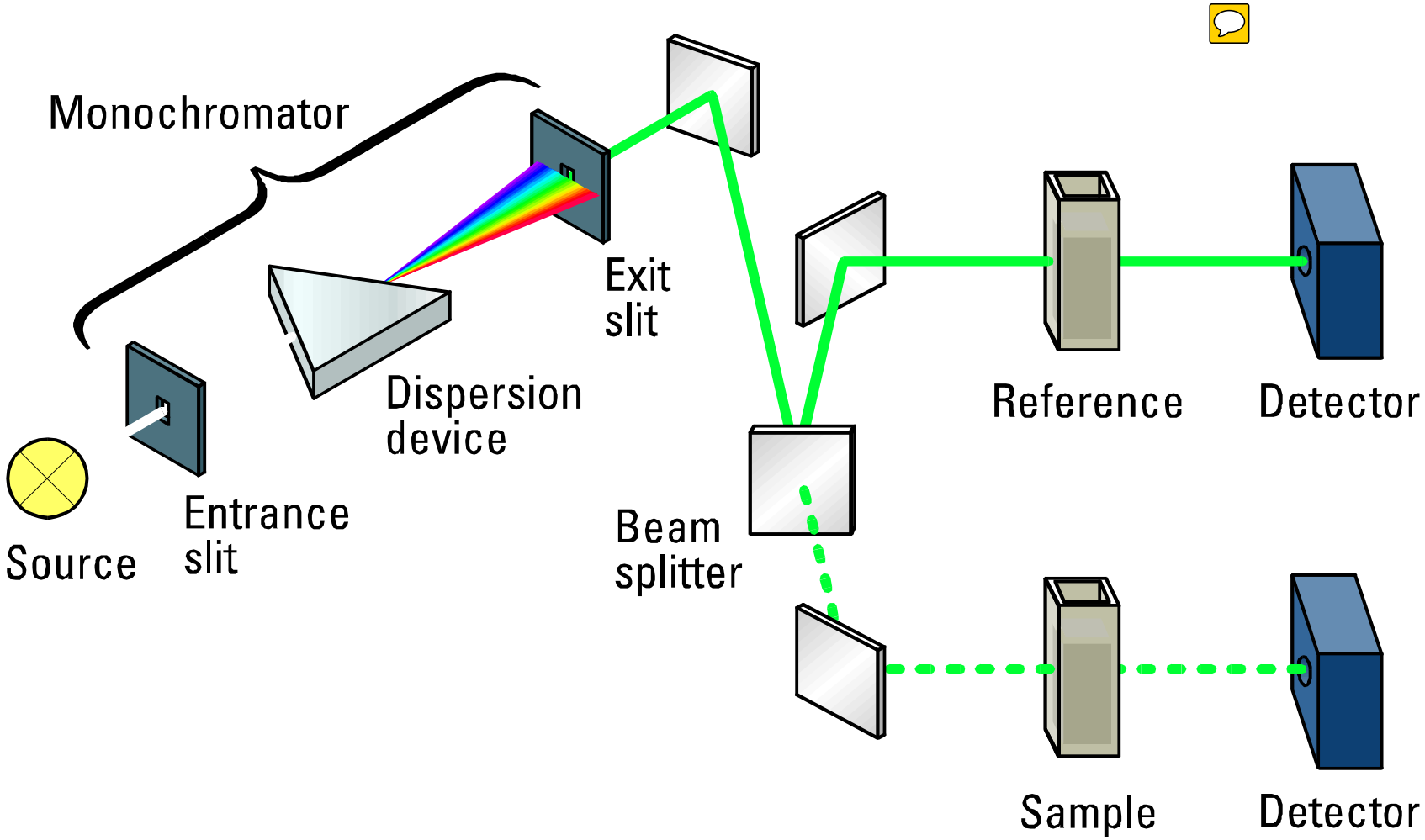
Solution $\Rightarrow$ optical system based on a **double-beam spectrophotometer**

Double beam spectrophotometer (with chopper)



Optical system of a double-beam spectrophotometer

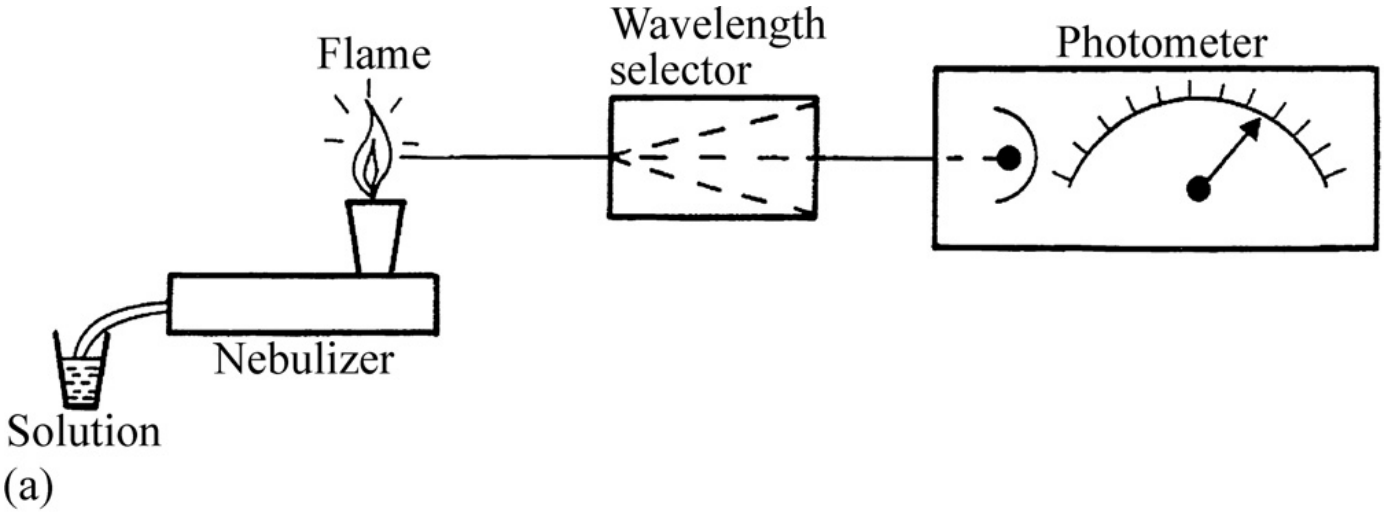
Double beam spectrophotometer (with beam splitter)



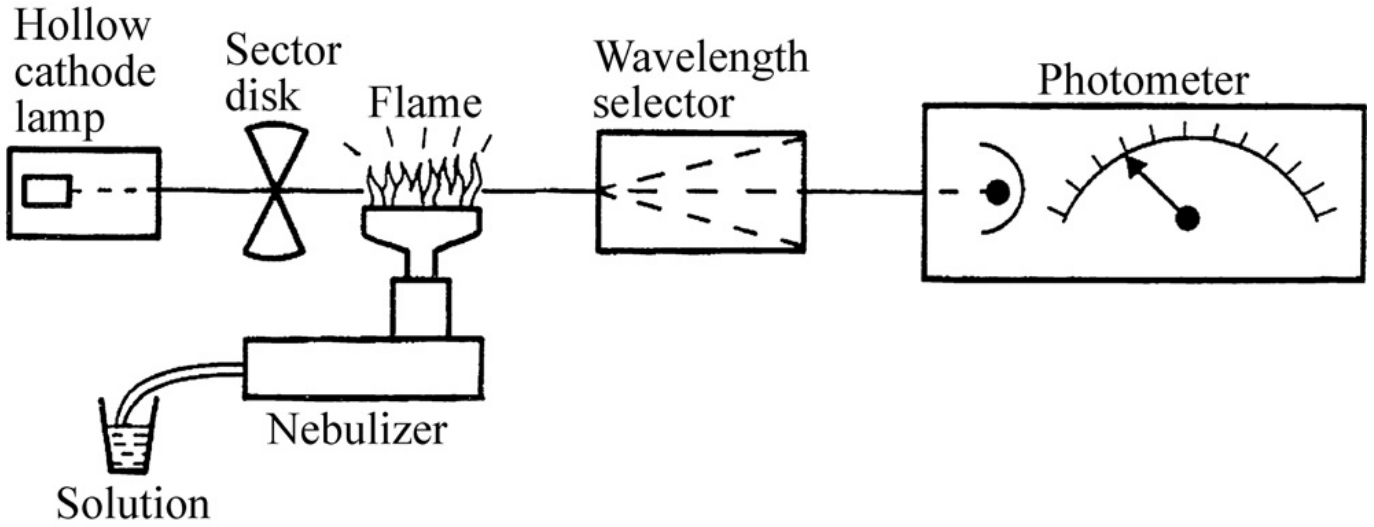
Atomic Absorption Spectrometry (AAS)

- AAS is an elemental analysis technique capable of providing quantitative information on 70 elements in almost any type of sample.
- AAS are that no information is obtained on the chemical form of the analyte (no “speciation”) and that often only one element can be determined at a time.
- This last disadvantage makes AAS of very limited use for qualitative analysis.
- AAS is used almost exclusively for quantitative analysis of elements, hence the use of the term “spectrometry” in the name of the technique instead of “spectroscopy”.

Emission spectrometry: flame photometry



(a)



(b)

Flame atomic emission spectrometry is particularly useful for the determination of the elements in the first two groups of the periodic table, including sodium, potassium, lithium, calcium, magnesium, strontium, and barium.

The determination of these elements is often called for in medicine, agriculture, and animal science.

Flame photometry is used for the quantitative determination of alkaline metals and alkaline-earth metals in blood, serum, and urine in clinical laboratories. It provides much simpler spectra than those found in other types of atomic emission spectrometry, but its sensitivity is much reduced.
- sodium, potassium, magnesium and calcium in blood

Emission spectrometry: Electrical discharge excitation

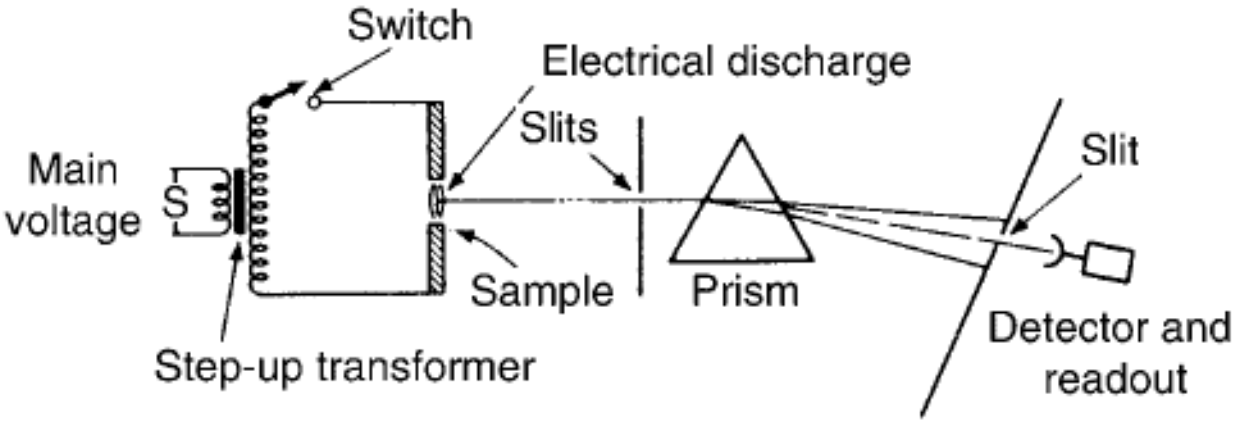


Figure 7.6 Schematic diagram of an emission spectrometer with an electrical discharge excitation source. The prism is meant only to illustrate a dispersive device; a diffraction grating is used in all modern spectrometers with a single dispersive device. Echelle spectrometers use two dispersive devices, either a prism and a grating or two gratings.

Spectroscopy using UV and visible light

- In the medical field, it is used for the determination of enzymes, vitamins, hormones, steroids, alkaloids, and barbiturates.

- These measurements are used in the diagnosis of diabetes, kidney damage, and myocardial infarction, among other ailments. In the pharmaceutical industry, it can be used to measure the purity of drugs during manufacture and the purity of the final product. For example, aspirin, ibuprofen, and caffeine, common ingredients in pain relief tablets, all absorb in the UV and can be determined easily by spectrophotometry.

Radiation Sources

UV Spectrophotometer

- Hydrogen Gas Lamp
- Mercury Lamp

Visible Spectrophotometer

- Tungsten Lamp

InfraRed (IR) Spectrophotometer

- Carborundum (SiC)

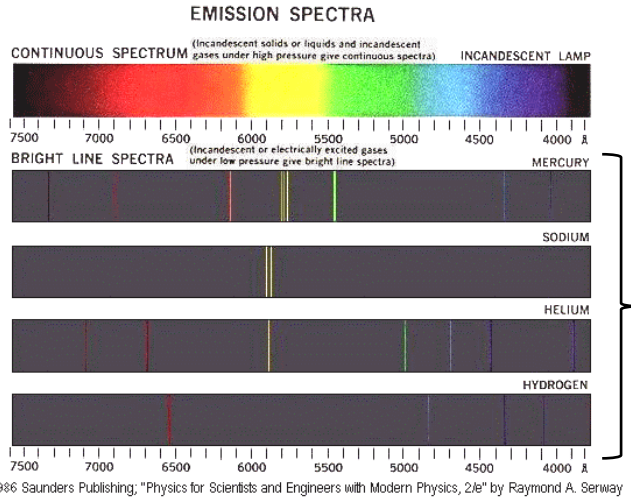
Radiation Sources for spectroscopy: characteristics

- The source must emit radiation over the entire wavelength range to be studied.
- The intensity of radiation over the entire wavelength range must be high enough so that extensive amplification of the signal from the detector can be avoided.
- The intensity of the source should not vary significantly at different wavelengths.
- The intensity of the source should not fluctuate over long time intervals.
- The intensity of the source should not fluctuate over short time intervals. Short time fluctuation in source intensity is called “flicker”.

Radiation sources: voltage regulators

Most sources will have their intensities change exponentially with changes in voltage, so in all cases a reliable, steady power supply to the radiation source is required. Voltage regulators (also called line conditioners) are available to compensate for variations in incoming voltage.

Radiation Sources/ Detectors



Wavelength, nm	100	200	400	700	1000	2000	4000	7000	10,000	20,000	40,000
Spectral region	VAC	UV	Visible	NEAR IR		IR				FAR IR	
(a) Sources											
Continuum: Ar lamp											
Continuum: Xe lamp											
Continuum: H_2 or D_2 lamp											
Continuum: Tungsten lamp											
Continuum: Nernst glower ($ZrO_2 + Y_2O_3$)											
Continuum: Nichrome wire (Ni + Cr)											
Continuum: Globar (SiC)											
Line: Hollow cathode lamps											
Line: Lasers											
(b) Detector											
Photon detectors: Photographic plate											
Photon detectors: Photomultiplier tube											
Photon detectors: Phototube											
Photon detectors: Photocell											
Photon detectors: Silicon diode											
Photon detectors: Charge-transfer detector											
Photon detectors: Photoconductor											
Thermal detectors: Thermocouple (voltage) or bolometer (resistance)											
Thermal detectors: Golay pneumatic cell											
Thermal detectors: Pyroelectric cell (capacitance)											

Continuum sources

Continuum sources are used for most molecular absorption and fluorescence spectrometric instruments.

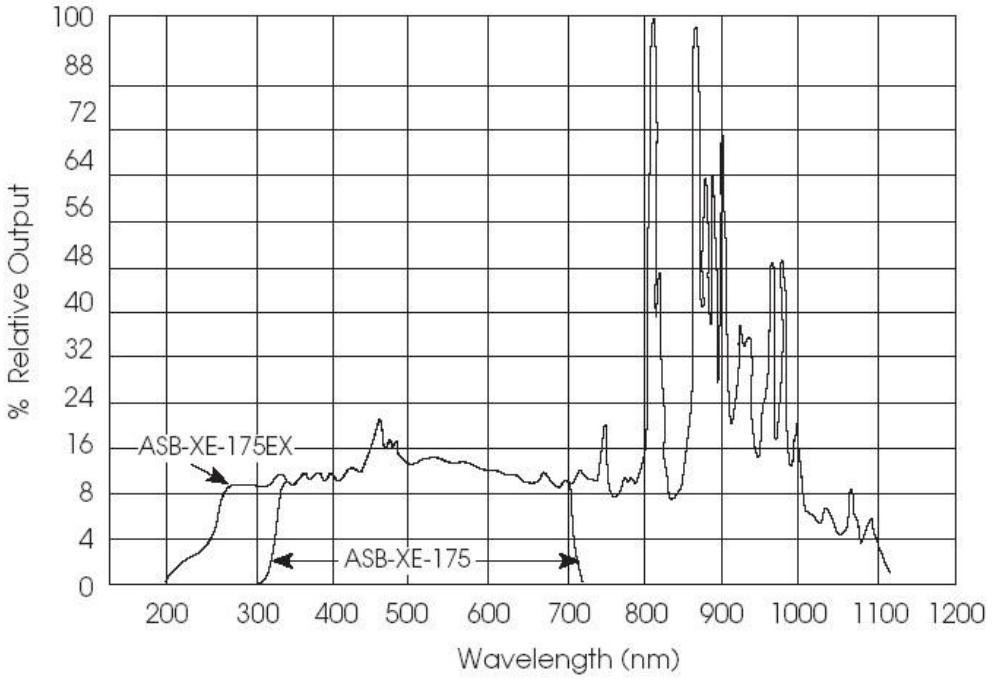
Continuum sources emit radiation over a **wide range of wavelengths** and the intensity of emission varies slowly as a function of wavelength.

Typical continuum sources include:

- the tungsten filament lamp which produces visible radiation (white light);
- the deuterium lamp for the UV region,
- high pressure mercury or xenon arc lamps for the UV region,
- heated solid ceramics or heated wires for the IR region of the spectrum.

(Xenon arc lamps are also used for the visible region)

Xenon lamp - spectrum



Line sources

Line sources are used as sources in molecular and atomic fluorescence spectroscopy and Raman spectroscopy.

Line sources emit only a **few discrete wavelengths** of light, and the intensity is a strong function of the wavelength.

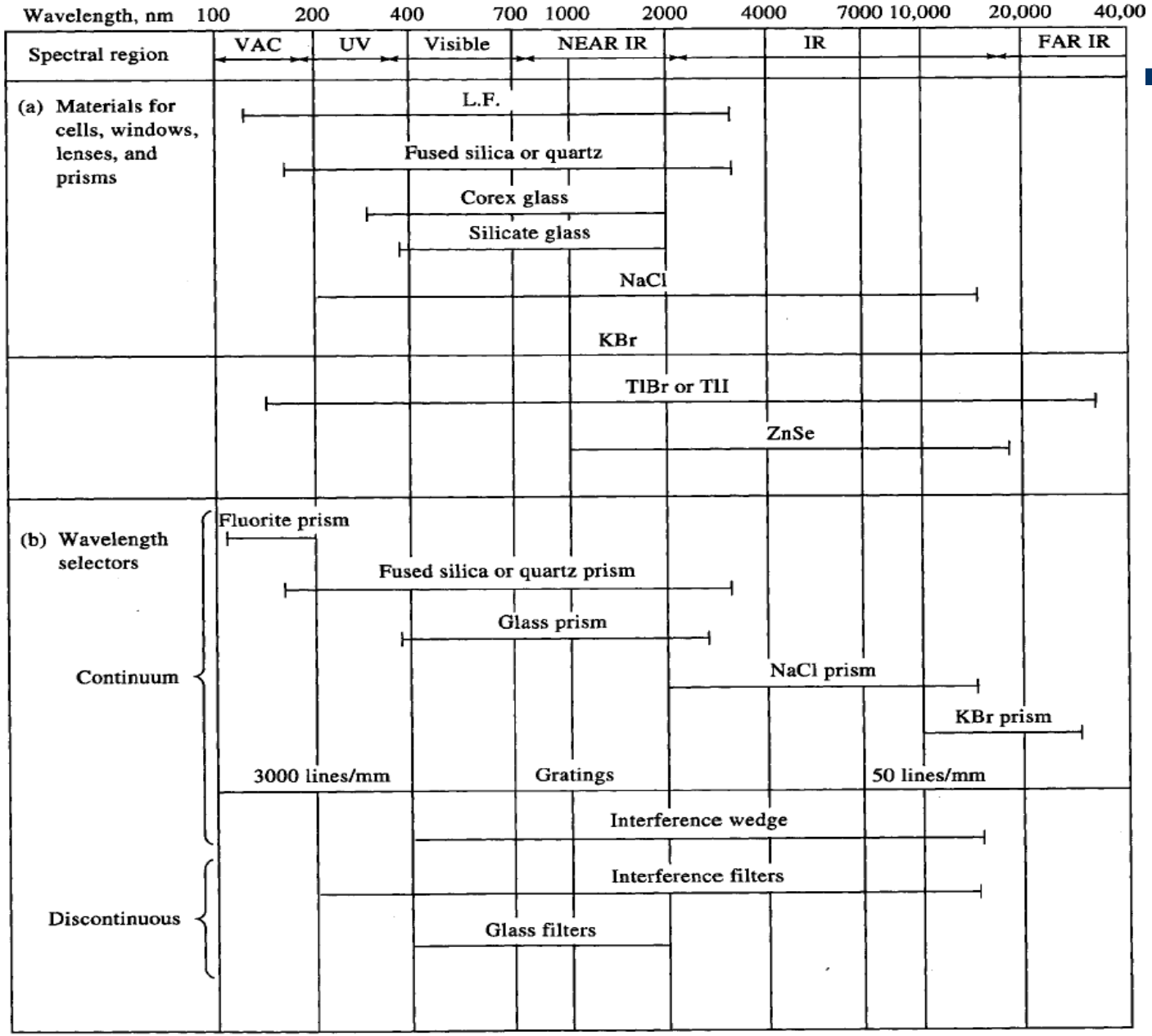
Typical line sources include:

- hollow cathode lamps and
- electrodeless discharge lamps, used in the UV and visible regions for AAS and atomic fluorescence spectrometry,
- sodium or mercury vapor lamps (similar to the lamps now used in street lamps) for lines in the UV and visible regions, and lasers.

Wavelength Selection Devices

- Filters
  - absorption filters colored glass
  - interference filter

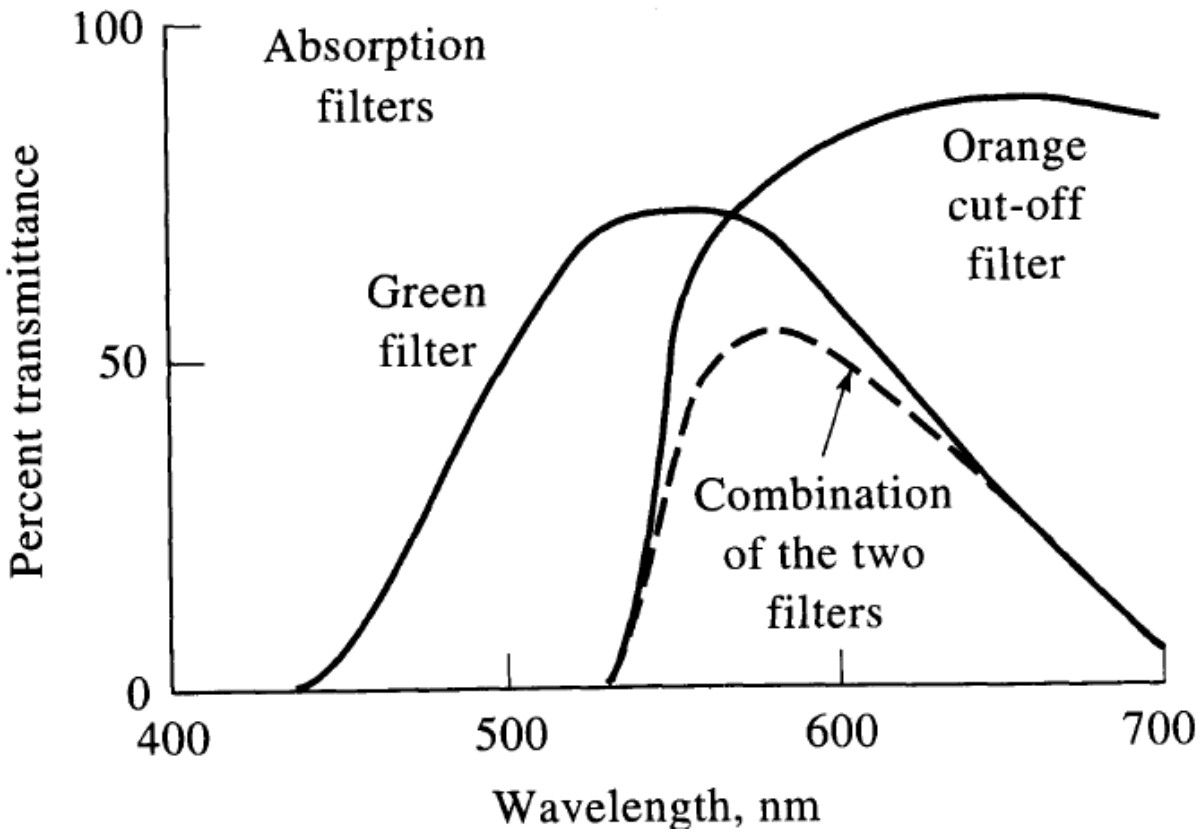
- Monochromators
  - entrance slit
  - prisms
  - diffraction gratings



Wavelength selector

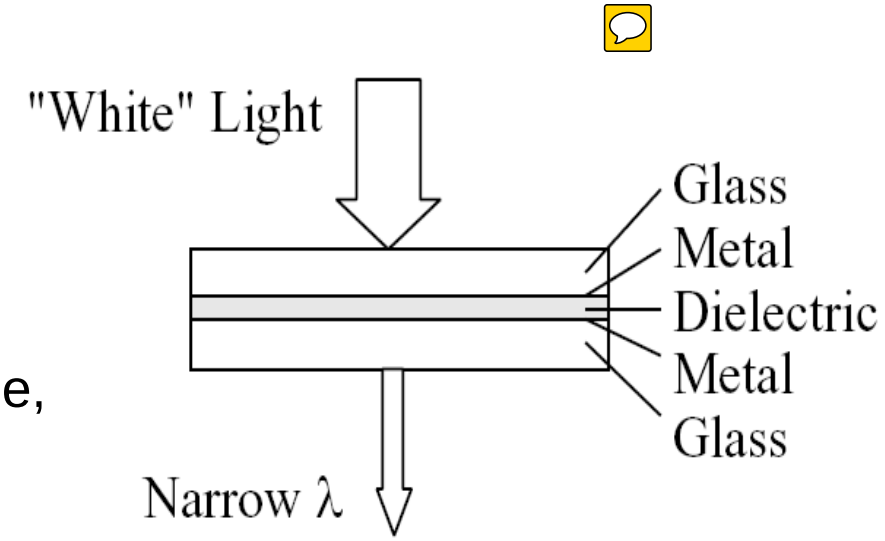
Filters: colored glass

- stable, simple, and cheap,
- blue glass transmits blue wavelengths of the visible spectrum but absorbs red and yellow wavelengths.
- the range of wavelengths transmitted is broad compared with prisms and gratings which are also devices used to select a narrow wavelength range from a broad band polychromatic source. The transmission range may be 50–300 nm for typical absorption filters.



Interference Filters

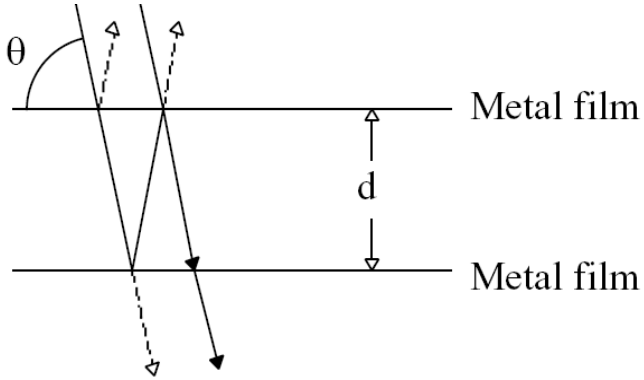
- two thin sheets of metal sandwiched between glass plates, separated by transparent material.
- interference filters can be constructed for transmission of light in the IR, visible, and UV regions of the spectrum.
- the wavelength ranges transmitted are much smaller than for absorption filters, generally 1–10 nm, and the amount of light transmitted is generally higher than for absorption filters.



Interference Filter

- The filter operates on the principle of constructive interference to transmit selected wavelength ranges. The wavelengths transmitted are controlled by the thickness and refractive index of the center layer of material.

- Interference for transmitted wave through 1st layer and reflected from 2nd layer



Constructive interference when

$$n\lambda = 2d\sin\theta$$

when $\theta \to 90°$, $\sin\theta \to 1$

$$n\lambda = 2d$$

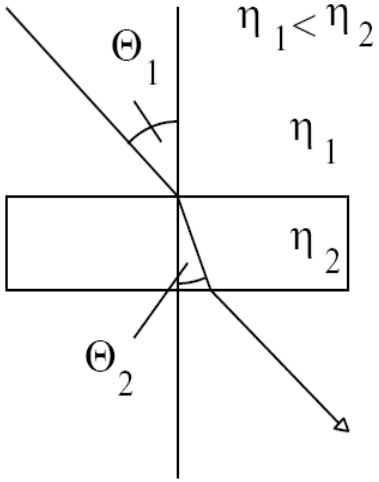
wavelength in glass!

$$\lambda_{air} = \lambda_{glass} \cdot \eta$$

Therefore λ transmitted through filter is

$$\lambda = \frac{2d \cdot \eta}{n}$$

Prisms



$$\frac{\sin\Theta_2}{\sin\Theta_1} = \frac{\eta_1}{\eta_2} = \frac{v_2}{v_1} = \frac{\upsilon_2 \cdot \lambda_2}{\upsilon_1 \cdot \lambda_1}$$

Snell's Law

Short wavelengths refracted more!

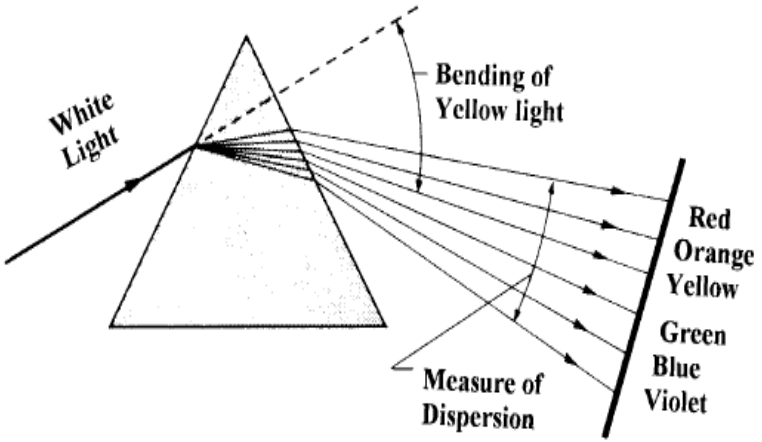
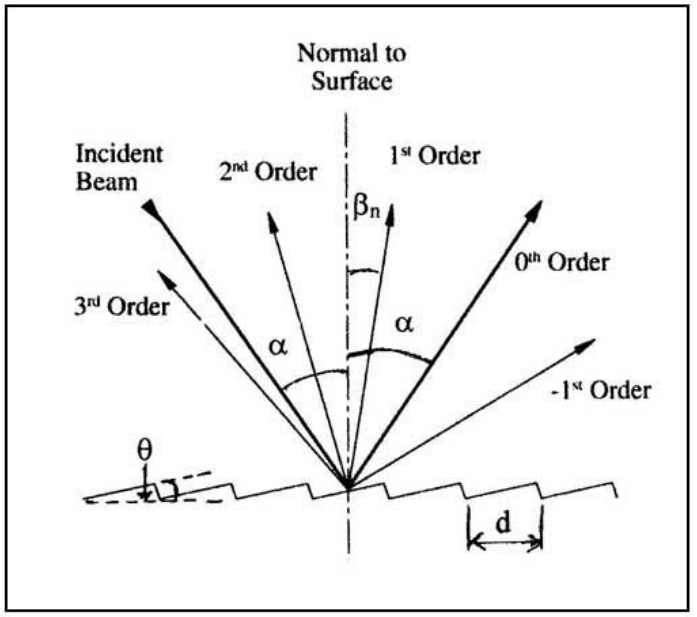


Figure 2.18 Dispersion of visible light by a prism.

Prisms are used to disperse IR, visible, and UV radiation.
The most common prisms are constructed of quartz for the UV region, silicate glass for the visible and near-IR region, and NaCl or KBr for the IR region.

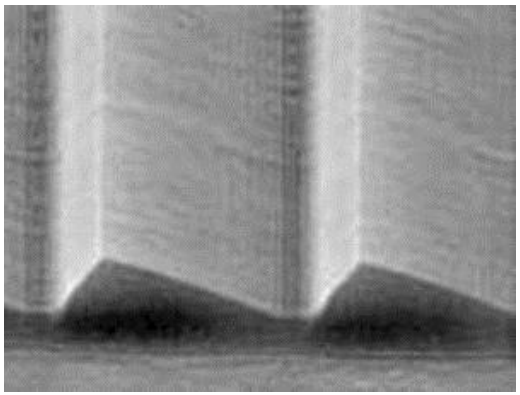
Reflective diffraction Gratings

The polychromatic light incident on the grating is dispersed so that each wavelength is reflected from the grating at a slightly different angle. The dispersion arises from the wavefront division and interference of the incident radiation from the periodic structure of the grating. The dispersed light is then re-imaged by the spectrograph and the required wavelength range is directed to a detection system.



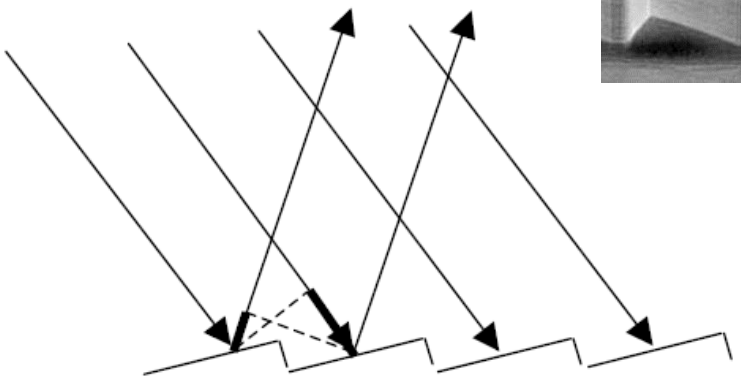
Reflection Grating Diffracted Orders

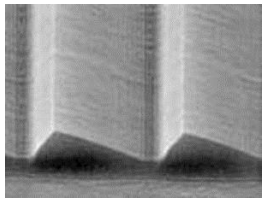
The dispersion and efficiency of a grating are dependent on the distance between adjacent grooves and the groove angle.

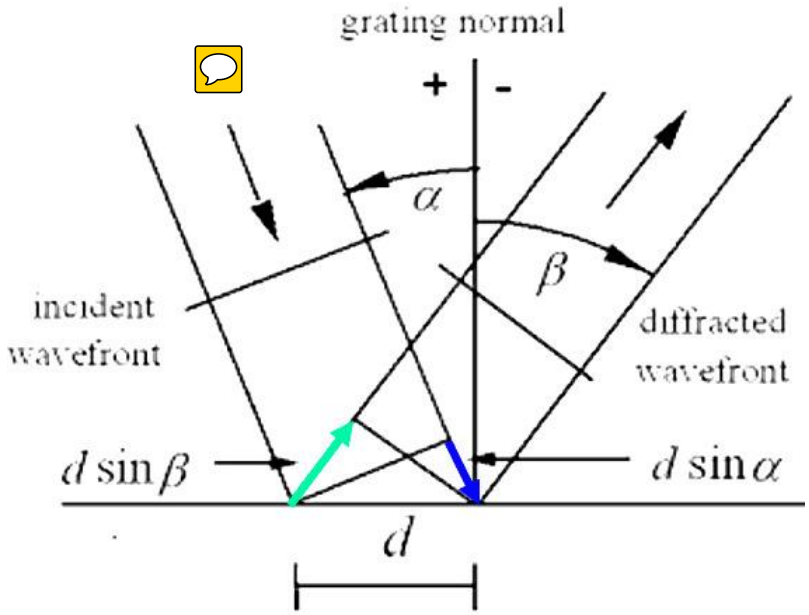


Gratings consist of equally spaced parallel grooves, formed on a reflective coating and deposited on a substrate.

Reflective diffraction Gratings







The general grating equation which enables selection to separate polychromatic radiation into its constituent wavelengths is usually written as:

$$n\lambda = d(\sin\alpha + \sin\beta_n)$$

where n = order number of diffraction, λ = diffracted wavelength, d = distance between successive grooves or fringes, α = angle of incidence measured from the grating normal and β_n = angle of diffraction of the n-th order measured from the grating normal. θ = brazed angle.

Reflective diffraction gratings

Gratings are generally better than prisms, they are more efficient, they provide a linear dispersion of wavelengths and do not suffer from the absorption effects that prisms have which limits their useful wavelength range.

Typical densites of the grooves 600, 1200, 2400 mm^{-1},
i.e. d=1.667 μm, 0.833 μm, 0.417 μm
Because usually the first order line (n=1) is the most intense, it means that these densities are ‘specialized’ for IR, visible and UV light.

Typical lateral dimensions of the grating are few cm, e.g. 5 cm:
→ total number of lines N = 30000, 60000, 120000.

Resolution Power

- Resolution Power= resolution required to separate two lines of different wavelength.

- Example: in order to observe an absorption band at 599.9 nm without interference from an absorption band at 600.1 nm, we must be able to resolve, or separate, the two bands.

- The resolving power R of a monochromator is equal to λ/dλ, where λ is the average of the wavelengths of the two lines to be resolved and dλ is the difference in wavelength between these lines.

$$R = \frac{\text{average of 599.9 and 600.1}}{\text{absolute difference between 599.9 and 600.1}} = \frac{600}{0.2} = 3000$$

Detectors

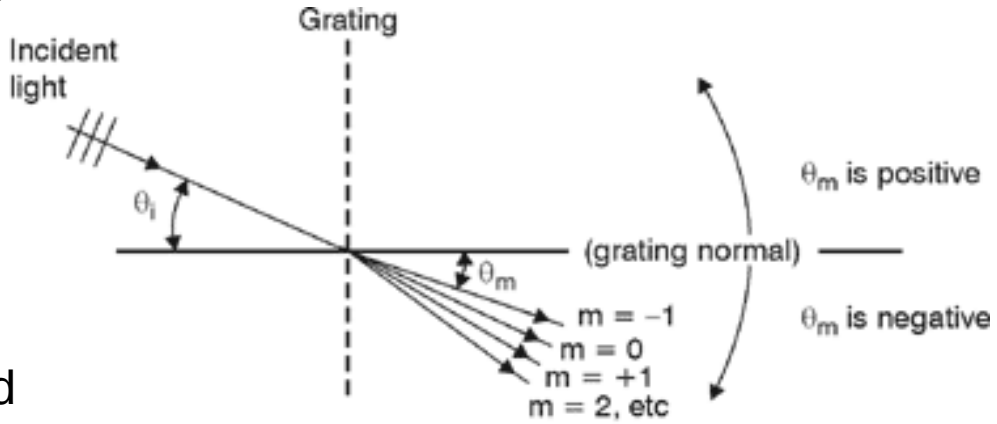
Different types of photon detectors are used in the UV/VIS and IR regions for both atomic and molecular spectroscopy

- photomultiplier tube
- silicon photodiode
- photovoltaic cell
- charge transfer devices (photodiode arrays, charge-coupled devices (CCDs), and charge-injection devices (CIDs).

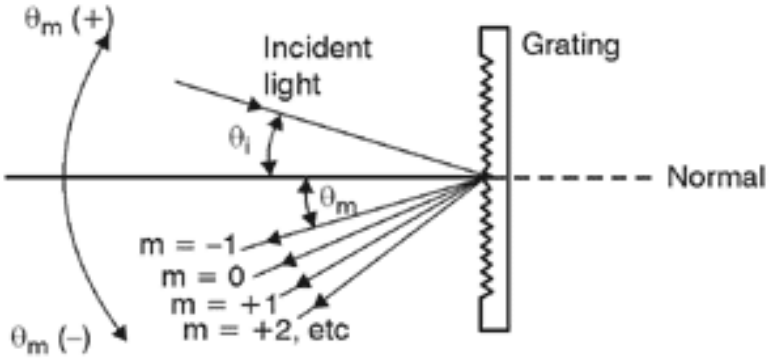
Transmission and reflection gratings

The difference between the two gratings is that the transparent slits are considered to be individual "point sources" of light in the transmission grating while small reflecting surfaces are considered to be the individual "point sources" in a reflection grating.

Transmission gratings seldom are used today. One reason for this is that light must pass through the glass or other material that forms the grating. So the light is subject to absorption and distortion caused by that material. Another reason is that reflection gratings yield more compact systems, because of the "folding" of the optical path through reflection.



a. Transmission grating.



b. Reflection grating.

Photomultiplier tube (PMT)

Photomultiplier tube (PMT) - irradiation of cathode produces electrons, series of anodes (dynodes) increases gain to 10^5-10^7 electrons per photon. Low incident fluxes only!

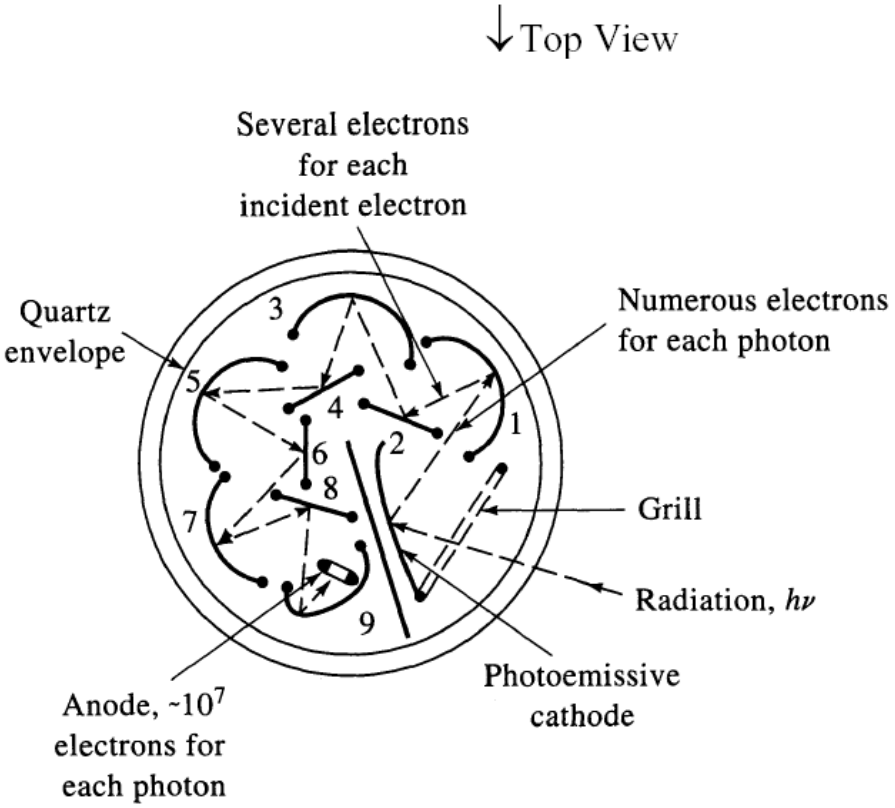


Fig. 7-29 (a)

Photomultiplier tube (PMT)

A PMT consists of a **photocathode** and a series of dynodes in an evacuated glass enclosure. When a photon of sufficient energy strikes the photocathode, it ejects a photoelectron due to the photoelectric effect.

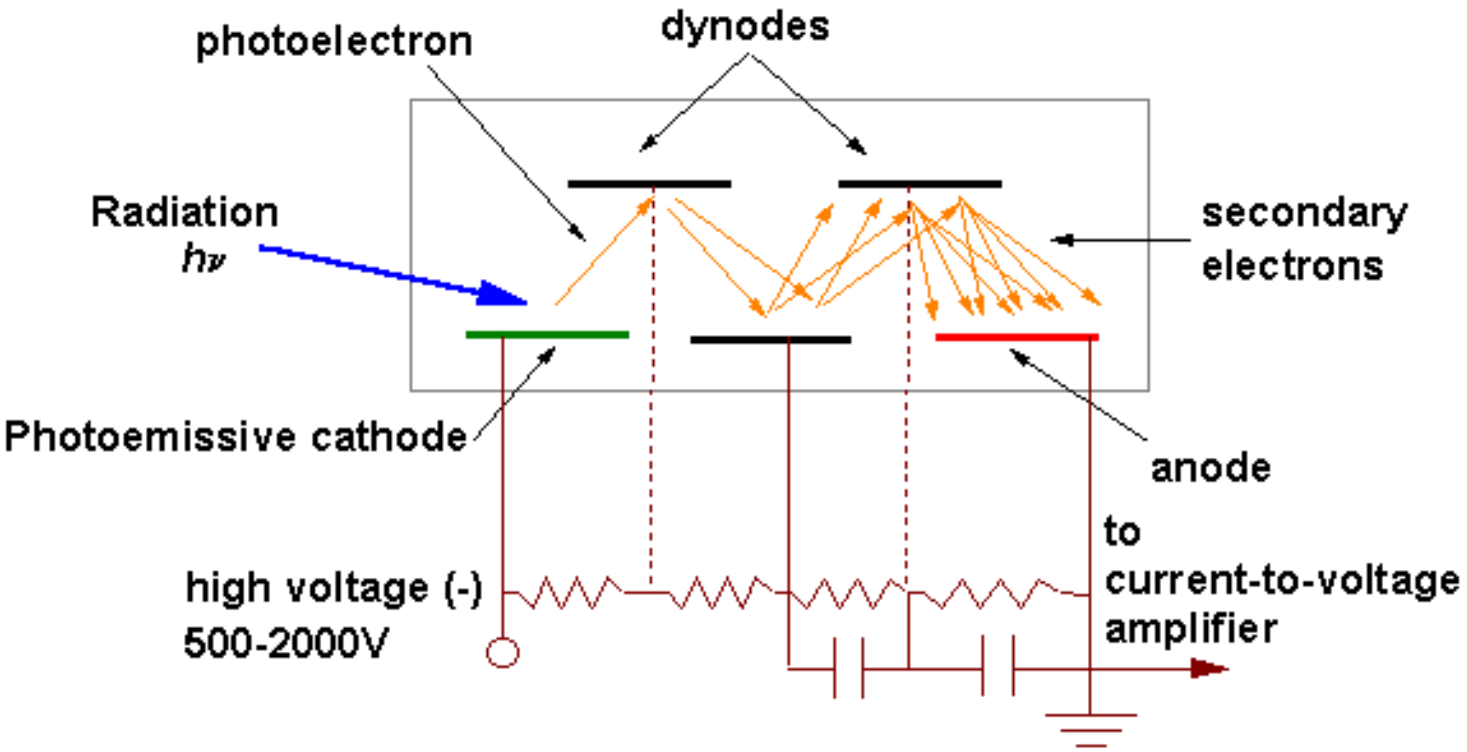
The photocathode material is usually a mixture of alkali metals, which make the PMT sensitive to photons throughout the visible region of the electromagnetic spectrum. The photocathode is at a **high negative voltage**, typically -500 to -1500 volts.

Photomultiplier tube (PMT)

The photoelectron is accelerated towards a series of additional electrodes called **dynodes**. These electrodes are each maintained at successively less negative potentials. Additional electrons are generated at each dynode.

This cascading effect creates 10^5 to 10^7 electrons for each photoelectron that is ejected from the photocathode. The amplification depends on the number of dynodes and the accelerating voltage. This amplified electrical signal is collected at an anode at ground potential, which can be measured.

Photomultiplier tube (PMT)

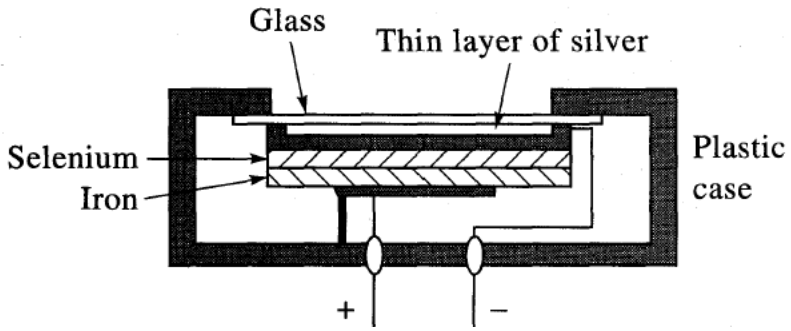


Wavelength range:	**110-1100 nm**
Quantum efficiency Q.E.=(number of electrons ejected by the photocathode / number of incident photons)	**1-10%**
Response time:	**1-20 ns**

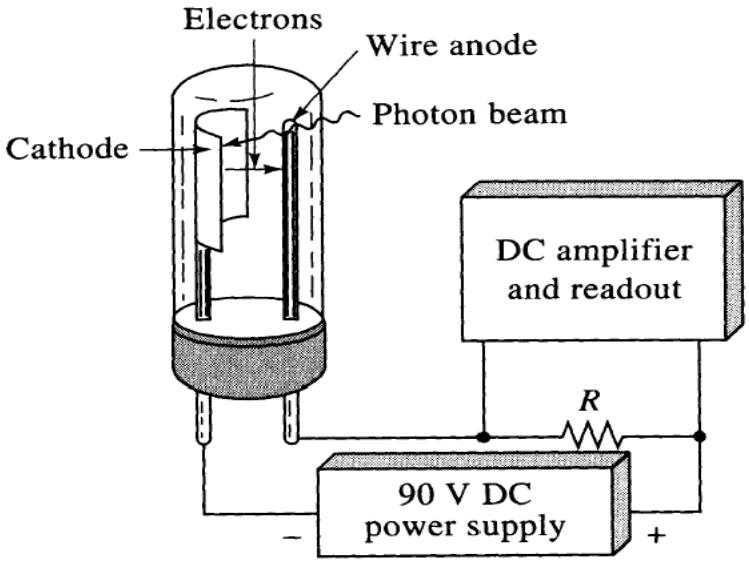
Signal amplification $G = \delta^{N-1}$

(N: no. of dynodes, δ: gain per dynode)

Photovoltaic cells - metal-semiconductor-metal sandwiches that produce voltage when irradiated (350-750 nm)



Phototube - electrons produced by irradiation of cathode travel to anode. λ response depends on cathode material (200-1000 nm)



Phototubes are similar to PMTs, but consist of only a photocathode and anode. Since phototubes do not have a dynode chain to provide internal amplification, they are used in less sensitive applications such as absorption spectrometers.

Clinical Chemistry Laboratory Organization

Core Lab Facility

- found at virtually all hospitals
- operates 24h/day 7 days/wk to provide the essential most requested tests
- Highly automated environment
- Instruments with Multi-analyte capabilities

Special Chemistry

- less frequently ordered tests
- labour intensive and often manual methods
- generally non-stat tests (result not required immediately)

Point of Care Testing (POCT)

- Instruments located outside of chemistry laboratory such as CCU, ER, ICU or satellite centre (clinic)

Core Lab

- High volume tests (many per day). Often require quick turn-around-time (TAT)
- Many tests where abnormal values are incompatible with life and therefore of critical value to the physician
- Electrolytes: sodium (Na), potassium (K), Chloride (Cl)
- Blood gases: pO_2, pCO_2, pH, HCO3, oxygen saturation
- Endocrine: Thyroid hormones

  Prolactin

  Testosterone
- Lipids: Total Cholesterol, Low Density Lipoprotein Cholesterol (LDLc), High Density Lipoprotein Cholesterol (HDLc), Triglyceride (Fats)
- Proteins: Total Protein
  - Specific proteins such as Albumin, immunoglobulins,
- Glucose
- Tumour Markers: Prostate Specific Antigen (PSA)
- Vitamins (Vit B12) and minerals (Calcium, (Ca))
- Toxicology

  Ethanol, methanol

  Drugs of abuse generally conducted as a screen

Core Lab Instrumentation

Core Lab Instrumentation

- Bar-coded test tubes are loaded onto to the instrument.
- Menu Driven Test selection
- All pipetting, mixing and measurements are automatic
- Random Access (can perform specific tests on a specific sample)
- Analyzer is interfaced with Laboratory Information System (LIS).
- Once resulted are verified, they can be broadcast (sent out)
- All reagents for specific tests such as control calibrators, buffers come as kits that a loaded directly onto the instruments.
- Instruments constantly monitors amount of consumables on-board and volume of liquid and solid waste generated

Type of analytical techniques found on Multi analyte analyzers

- Many are immunoassays based with colorimetric detection
- Ion-selective electrodes. Designed with a membrane that is specific for a particular analyte (Na, K, Cl). Change in potential is measured when electrode is placed in sample.

Special Chemistry Laboratory

- Generally lower volume tests
- Not available on auto-analyzer
  - Because the demand is not there
  - The test is not easily automated (extraction steps)
- Instruments are usually batch analyzers. Either research instruments or diagnostic instruments that perform 1 specific test on multiple samples.
- More training involved with test protocols and instrumentation than Core Lab instrumentation. Longer time required to reach competency
- Only larger centres have Special Chemistry Lab because
  - Requires the volume of specimens to justify the test
  - High cost of equipment to relative few specific tests

Special Chemistry Instrumentation and Analytical Methods

- **Electrophoresis**
  - To separate serum proteins into 5 distinct bands
  - To separate Lipoproteins into 4 distinct bands
  - To separate isoforms of enzymes

- **High Performance Liquid Chromatography (HPLC)**
  - To measure vitamins and hemoglobin variants

- **Infrared Spectroscopy**
  - To analyze components of Kidney stones

- **Radioimmunoassay (RIA)**
  - Used less and less but still employed for those analytes present in minute amounts (pmol) in the blood (ie. testosterone)

- **GC-MS (Gas chromatography-mass spectroscopy) and/or LC-MS (liquid chromatography- mass spectroscopy.**
  - quantitative drug measurement

Point of Care Testing (POCT)

- Tests are of urgent importance, and results will affect the immediate management of the patient

- Instruments are available that can perform certain tests at remote locations, such as at the bedside on in a clinical care unit
  - Blood glucose
  - Urinalysis
  - Blood gases
  - Electrolytes
  - Cardiac markers (Troponin I & T)
  - Drug screens

- POC are nearly always more expensive, than the same tests performed in the central laboratory

- Many are immunoassay based. Can be qualitative or quantitative

Biological Specimens

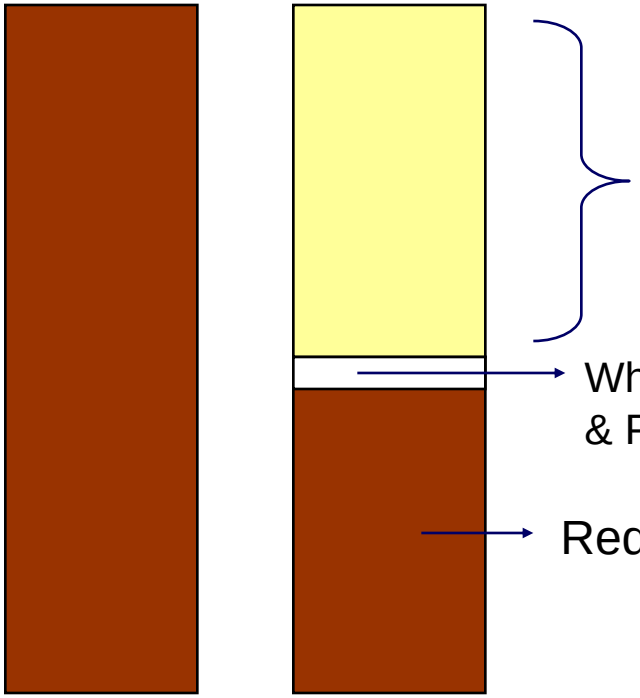
- Blood
- Urine
- Cerebrospinal Fluid

Comprise the majority of all specimens analyzed

- Amniotic Fluid
- Duodenal Aspirate
- Gastric Juice
- Gall stone
- Kidney Stone
- Stools
- Saliva
- Synovial Fluid
- Tissue Specimen

- Choice of specimen type depends on
  - Analyte to be measured
  - Ease of collection

Blood Composition



Plasma

Plasma is fluid component of blood. Comprises ~55% of total volume of whole blood. Contains proteins, sugars, vitamins,minerals, lipids, lipoproteins and clotting factors.
95% of plasma is water

White Blood cells (WBC) & Platelets

Red Blood cells (RBC)

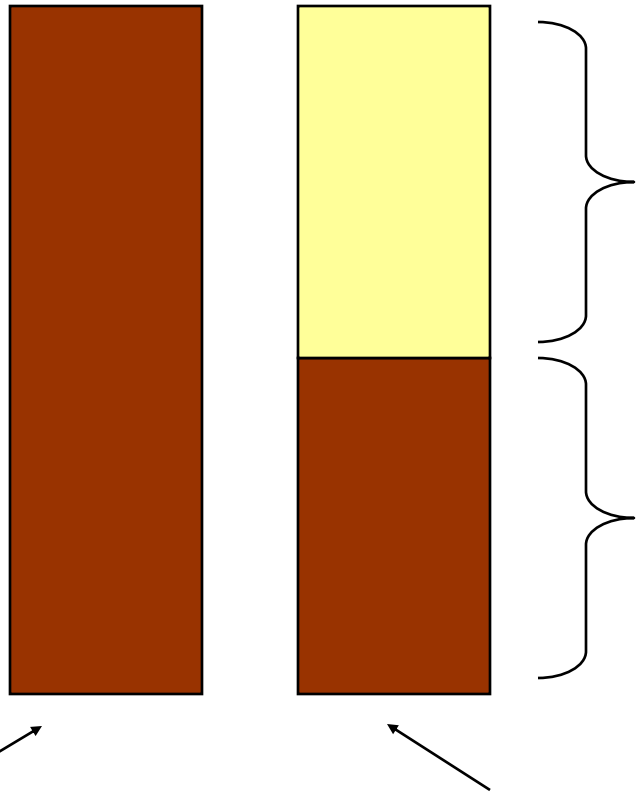
Cellular Components

Whole Blood

Whole Blood after centrifugation (if clotting has been prevented)

Blood Composition

If blood is collected and allowed to stand it will clot. Formation of an insoluble fibrin clot. If blood is then centrifuged the fluid portion is known as SERUM



Serum

Plasma is fluid component of blood. Comprises ~55% of total volume of whole blood. Contains proteins, sugars, vitamins,minerals, lipids, lipoproteins
No clotting factors
95% of plasma is water

Blood Clot

-comprised of clotting factors (Fibrin, platets etc)
-RBCs

Whole Blood

Whole Blood after clotting and centrifugation

Blood Analysis

Source

- Veins
- Arteries
- Skin puncture-capillary blood

Collection Method

- Syringe
- Evacuated tube
  - Additives
  - Separator gel
- Intravenous lines

Factors affecting choice of Blood Source and Collection Method

- Analyte under investigation
- Patient
  - vascular status
  - ease of collection

Blood components

Plasma (liquid)

46%-60% of total volume in men / 53%-63% in women

It contains mostly water (up to 95% by volume), plus dissolved proteins (6–8%) (i.e., serum albumins, globulins, and fibrinogen), glucose, clotting factors, electrolytes (Na+, Ca2+, Mg2+, HCO3−, Cl−, etc.), hormones, and carbon dioxide.

Cellular components (solid) - Ematocrito

40%-54% of total volume in men / 37%-47% in women

- Red blood cells (Erythrocytes)
  - between $4{,}6 \cdot 10^6$ and $6{,}2 \cdot 10^6$ /mm^3 in men
  - between $4{,}2 \cdot 10^6$ and $5{,}4 \cdot 10^6$ /mm^3 in women
- White blood cells
  - between 4.000 and 9.000/mm^3 including

    granulocytes (0,1-1% basophils; 1-3% eosinophils; 55-70% neutrophils)

    agranulocytes (20-30% linfociti; 5-8% monociti)
- platelets
  - between 150.000 and 380.000/mm^3

Blood cells

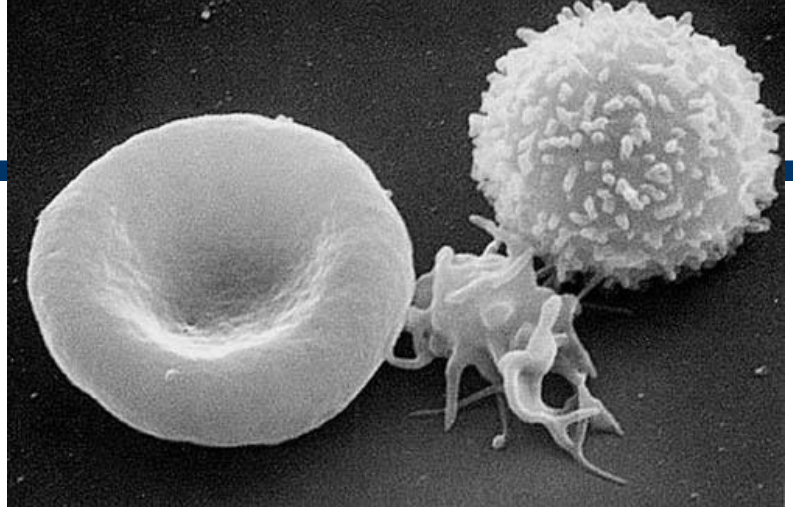


Table 1. Characteristic of Normal Blood Cells[a]

Cell Type	Concentration	Size, μm	Density, g·mL	Shape	Nucleus	Cytoplasm
Platelet(thrombocyte)	$0.15–0.5 \times 10^6 \cdot \mu L^{-1}$	2–3	1.03–1.06	Small disk shape	None	Granular
Erythrocyte (RBC)	$4–6 \times 10^6 \cdot \mu L^{-1}$	6–8	1.09–1.11	Biconcave disk	None	Hemoglobin
Leukocyte (WBC)	$5–10 \times 10^3 \cdot \mu L^{-1}$	8–20	1.05–1.10			
Neutrophil	55–70% of WBC	9–15	1.08–1.10	Various	Lobed	Granular
Eosinophil	2–4% of WBC	9–15	1.08–1.10	Various	Lobed	Granular
Basophil	0.5–1% of WBC	10–16	1.08–1.10	Various	Lobed	Granular
Monocyte	3–8% of WBC	14–20	1.05–1.08	Various	Round	Fine
Lymphocyte	20–40% of WBC	8–16	1.05–1.08	Round	Round	Clear

Blood Analysis in the Chemistry

- Since most tests in the chemistry lab involve analytes that are dissolved in the fluid portion of blood, **serum** or **plasma** are the specimens of choice.

- Important exceptions include
  - Hemoglobin, Red blood cell (RBC) Folate
  - Blood gases

- Protein electrophoresis was developed based on the analysis of serum. Not done on plasma because of the presence of the protein fibrinogen which distorts the electrophoretic pattern.

- Many tests can use either serum or plasma

Electrophoresis

Electrophoresis is a method whereby charged molecules in solution, chiefly proteins and nucleic acids, migrate in response to an electrical field.

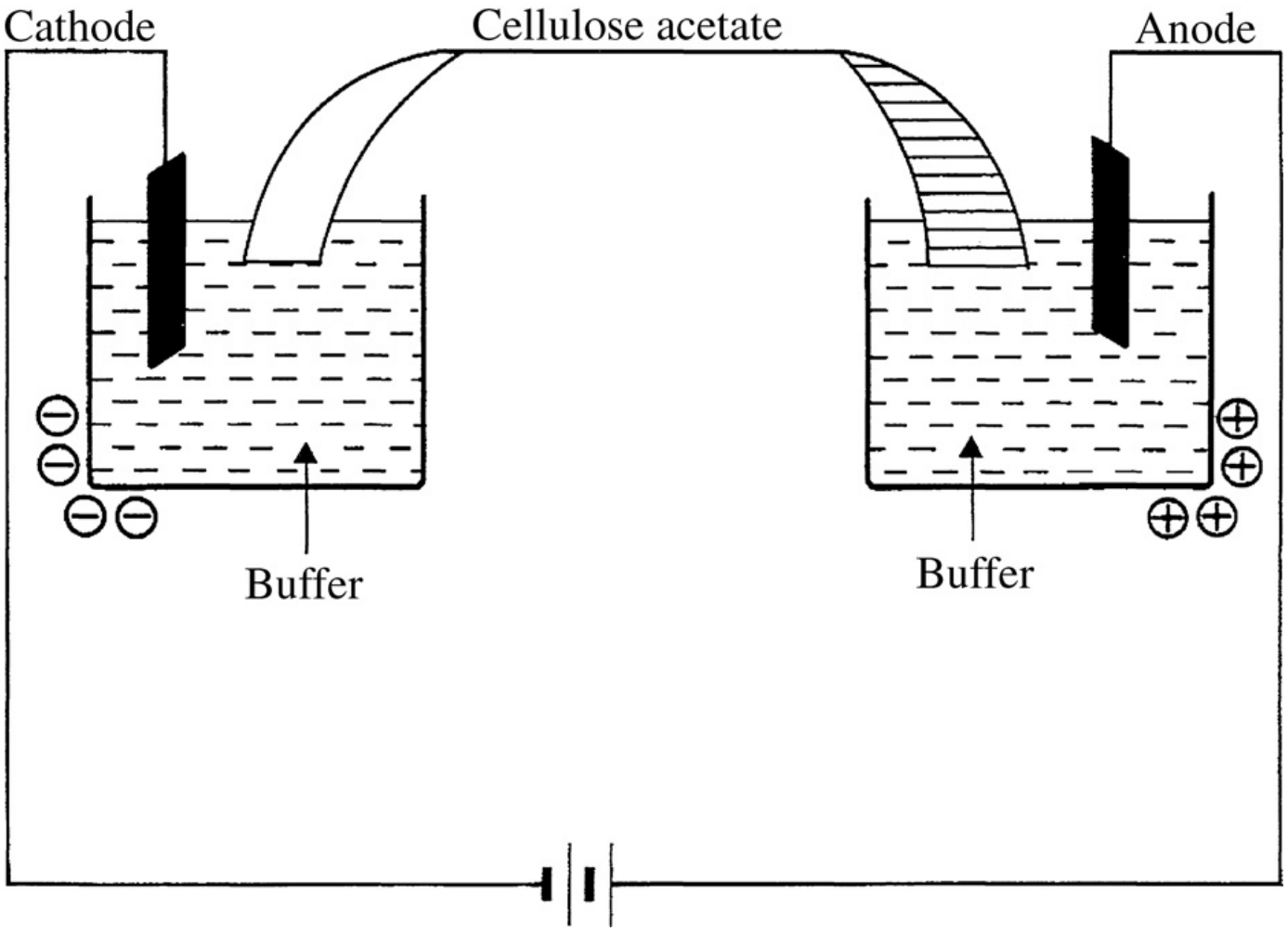
Their rate of migration in the medium through the electrical field, depends on

- strength of the field,
- net charge
- size and shape of the molecules
- ionic strength and viscosity
- temperature of the medium.

As an analytical tool, electrophoresis is simple, rapid and highly sensitive.

It can be used analytically to study the properties of a single charged species or mixtures of molecules. It can also be used preparatively as a separating technique

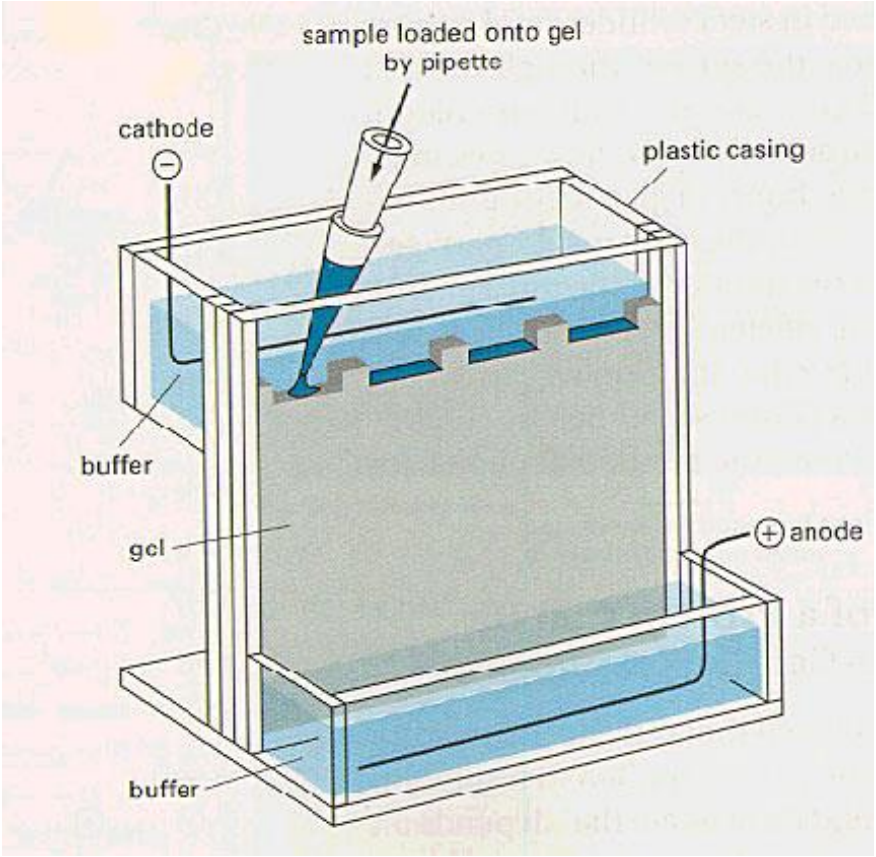
Electrophoresis



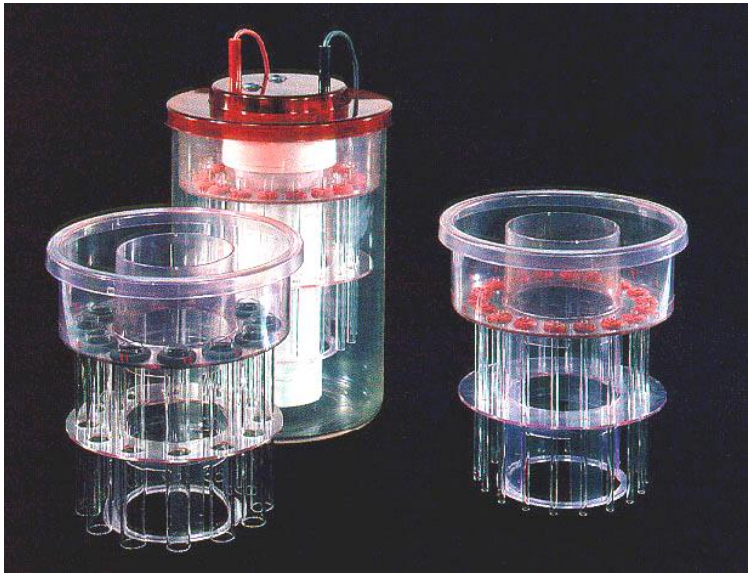
Electrophoresis

- Electrophoresis is usually done with gels formed in tubes, slabs, or on a flat bed.

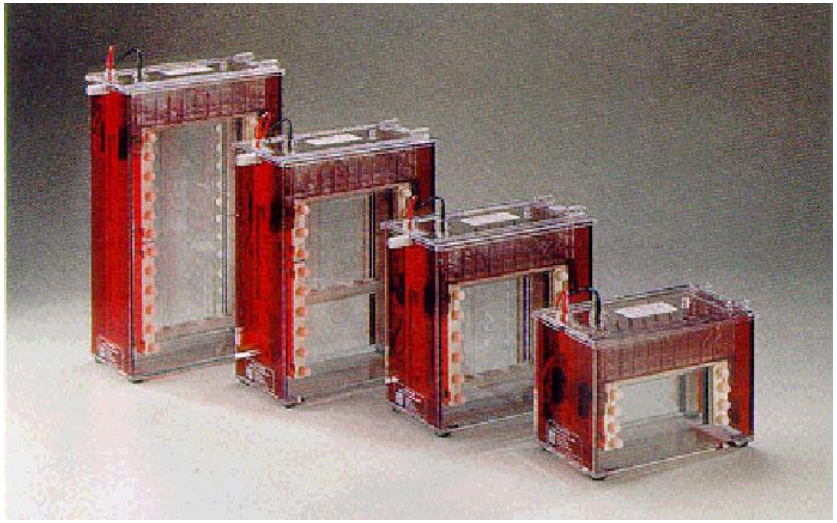
- In many electrophoresis units, the gel is mounted between two buffer chambers containing separate electrodes, so that the only electrical connection between the two chambers is through the gel.

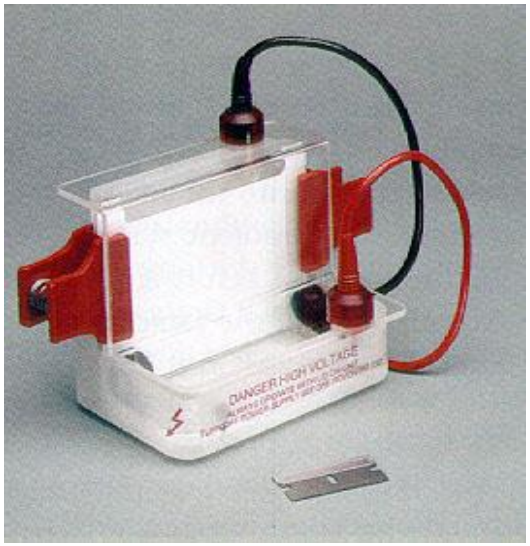


Tube Gel Units

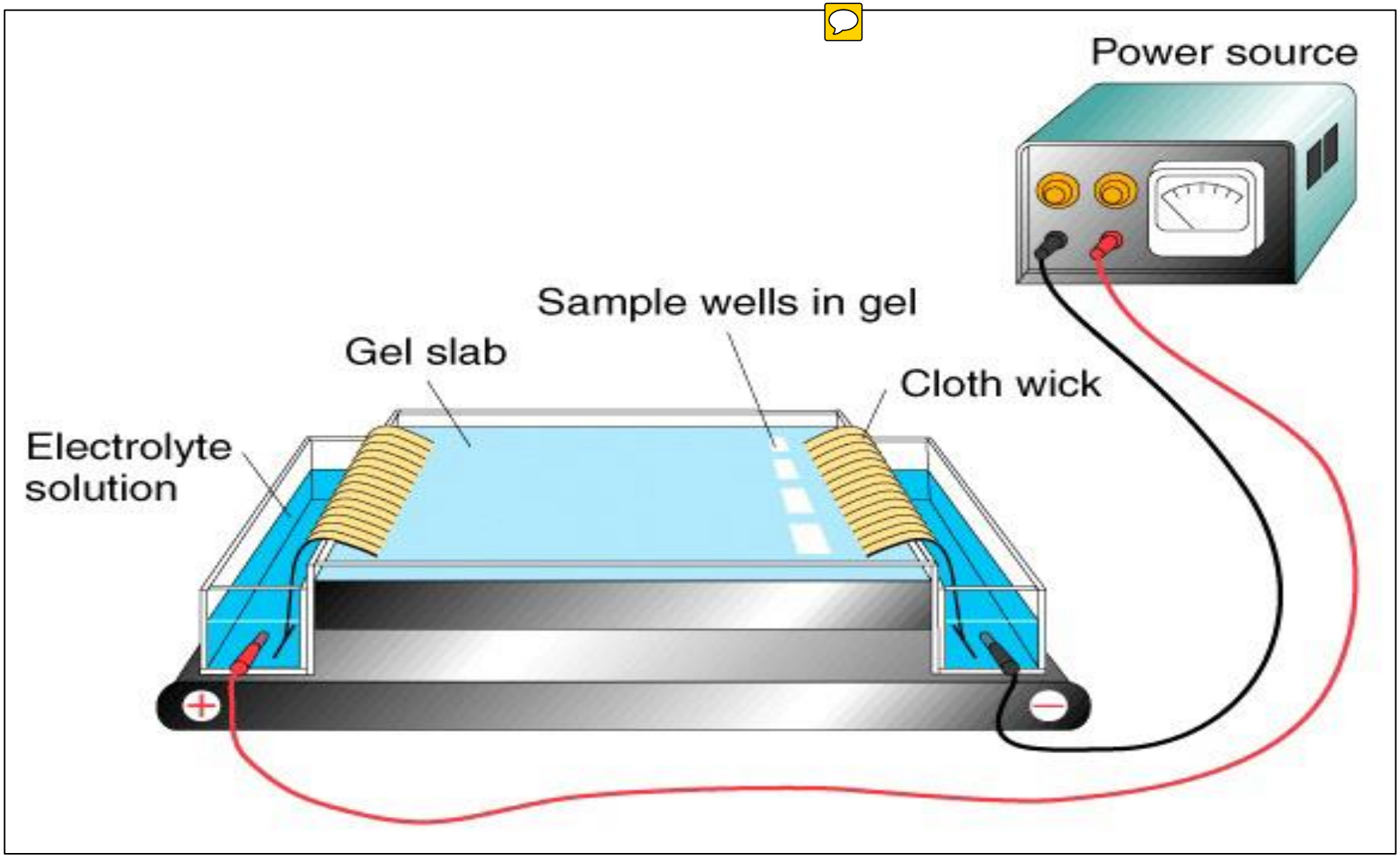


Slab Gel Units

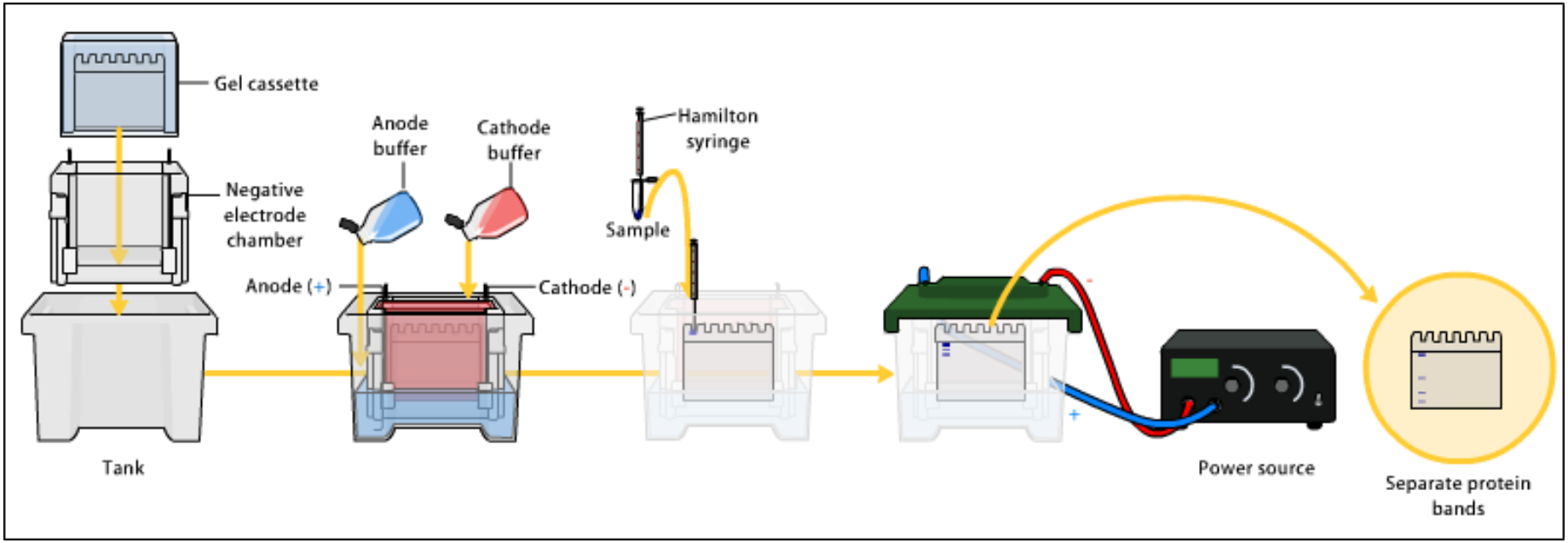




Serum protein electrophoresis on agarose gel (horizontal gel electrophoresis)

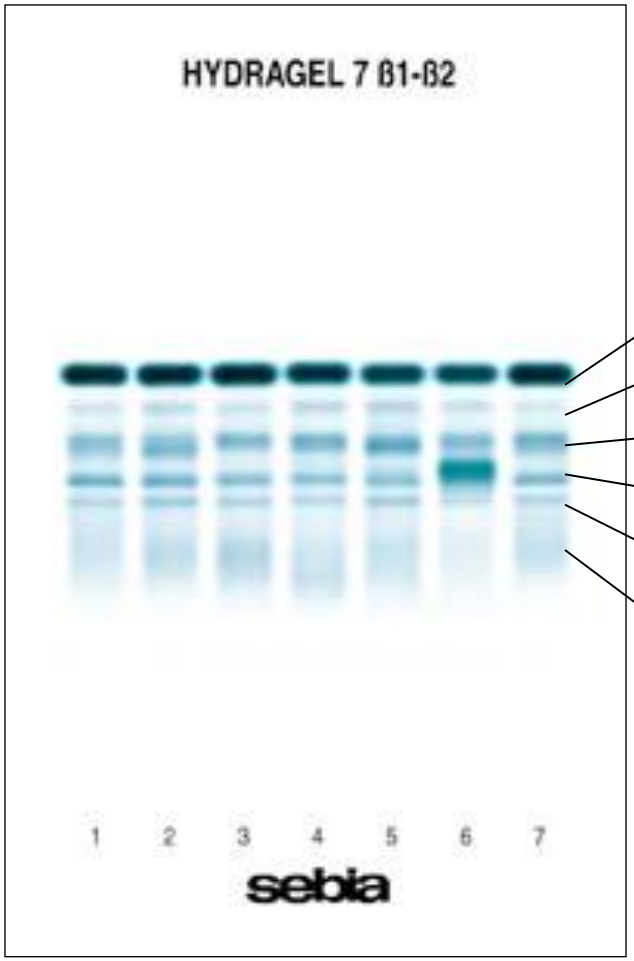


Process of electrophoresis



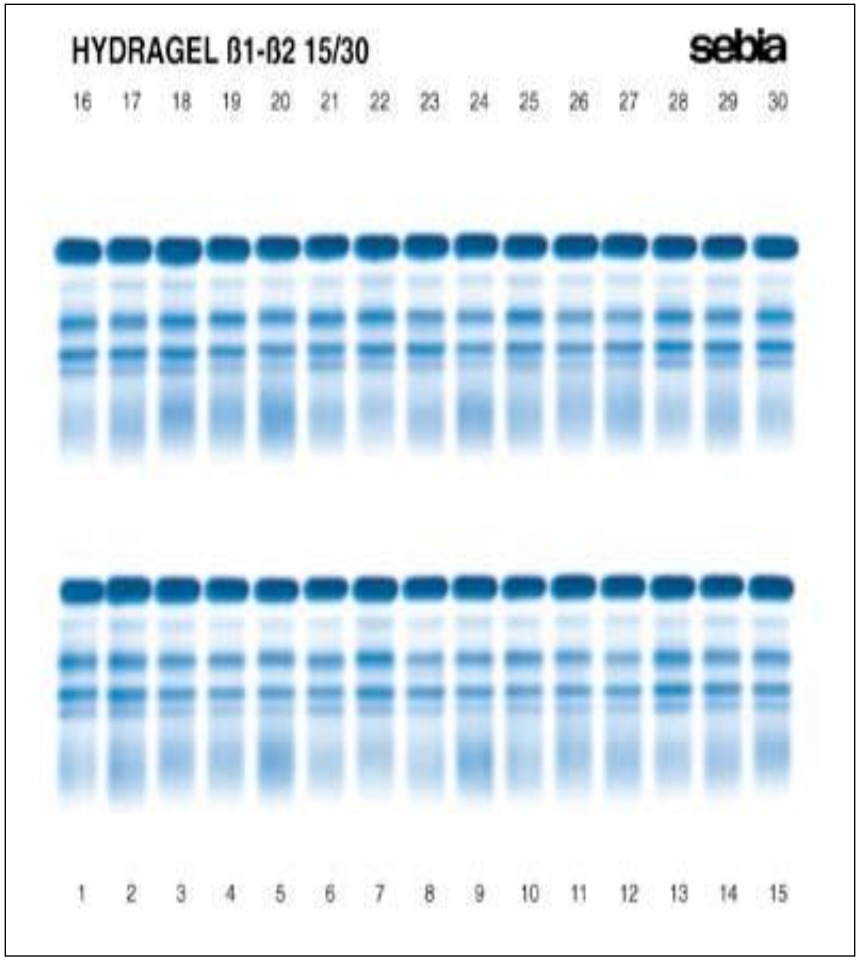
1. **sample application**
2. **adjustment of voltage or current** (gel-electrophoresis about 70 - 100 volts)
3. **separation time:** minutes
   (e.g. gel-electrophoresis of serum proteins 30 min.)
4. electrophoresis in supporting medium: **fixation, staining** and **destaining**
5. **evaluation:**
   - qualitative (standards)
   - quantitative (densitometry)

Serum protein electrophoresis on agarose gel (horizontal gel electrophoresis)



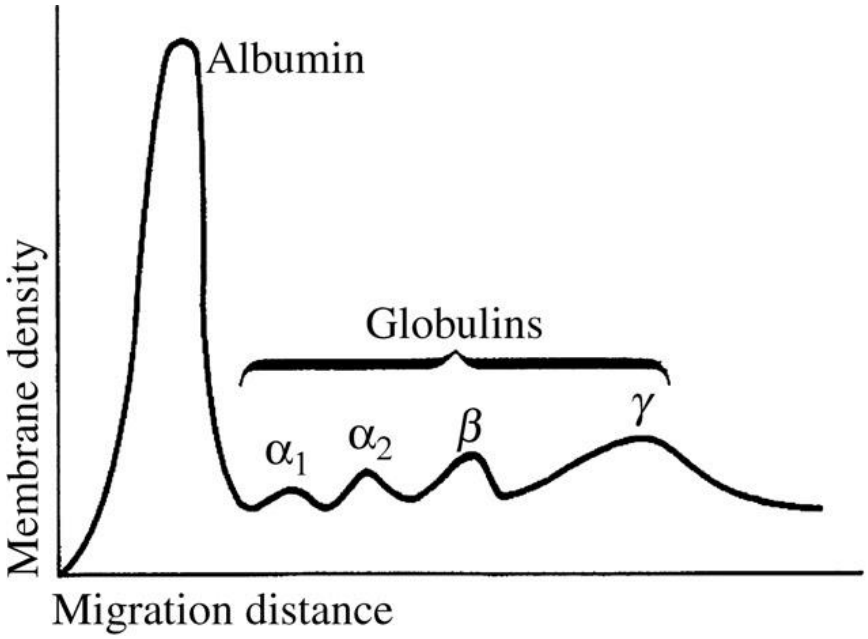
- Serum proteins are separated into 6 groups:
- Albumin
- α1 - globulins
- α2 - globulins
- β1 - globulins
- β2 - globulins
- γ - globulins

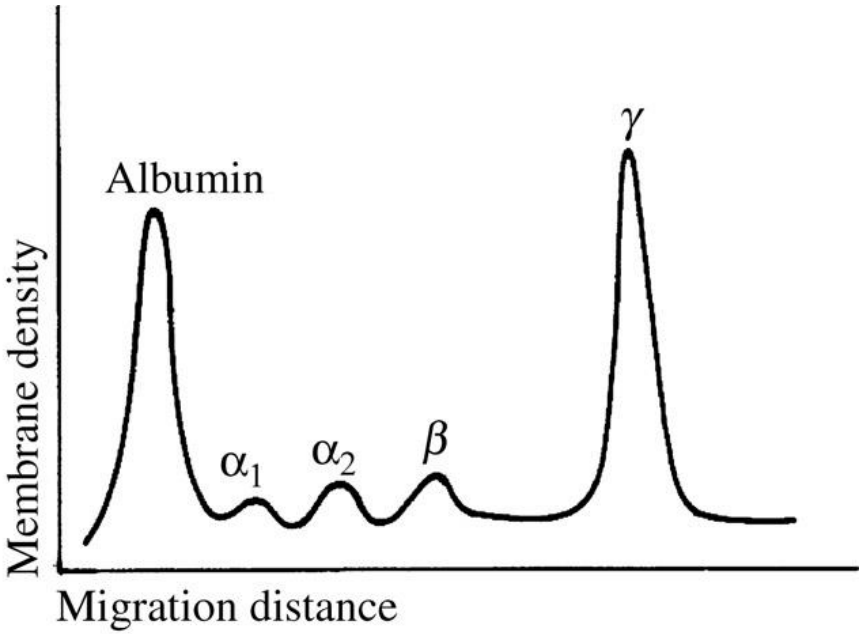
Hydragel 15/30



- Gels with 15 or 30 wells (serum samples) are used in laboratories of clinical biochemistry.
- Electrophoresis is also used for separation of **isoenzymes,nucleic acids** and **immunoglobulins**

Evaluation of separated protein fractions
Densitometry



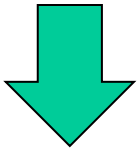


Densitometer is used for scanning of separated proteins in the gel. Scanning the pattern gives a quantitative information about protein fractions.

Use of protein electrophoresis in diagnostics of diseases

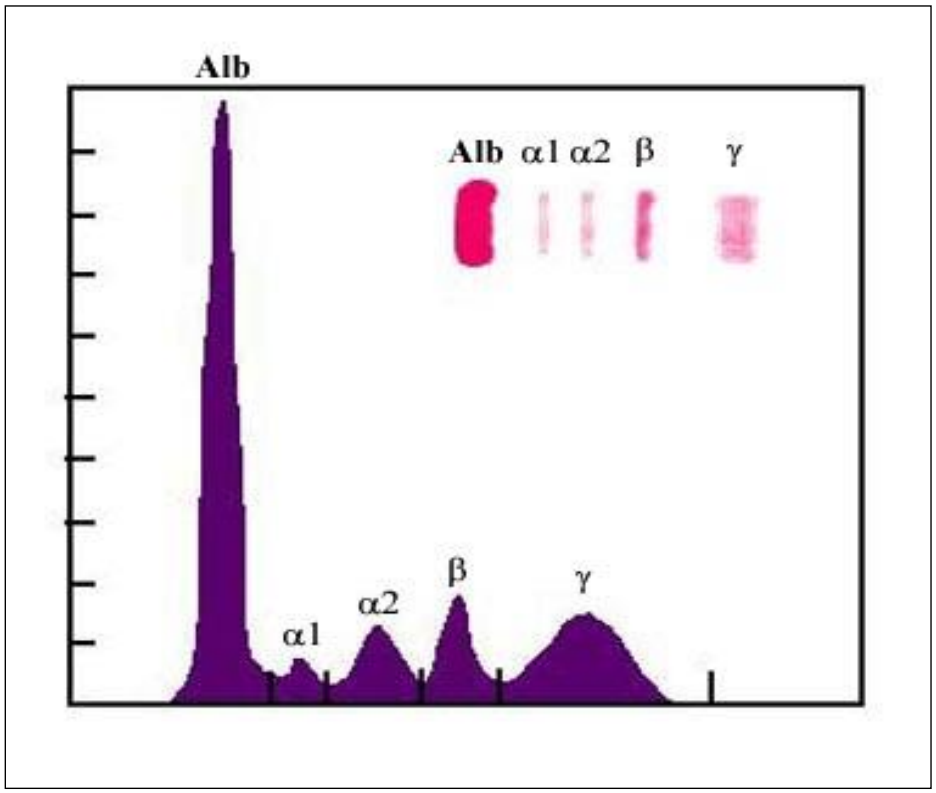
Electrophoretic pattern is **constant** under physiological conditions *(intensity of bands).*

Spectrum of plasma proteins **changes** under various diseases *(their ratio)*



evaluation of electrophoretic pattern
(bands or peaks)

Serum proteins electrophoresis in diagnostics of diseases: Normal pattern

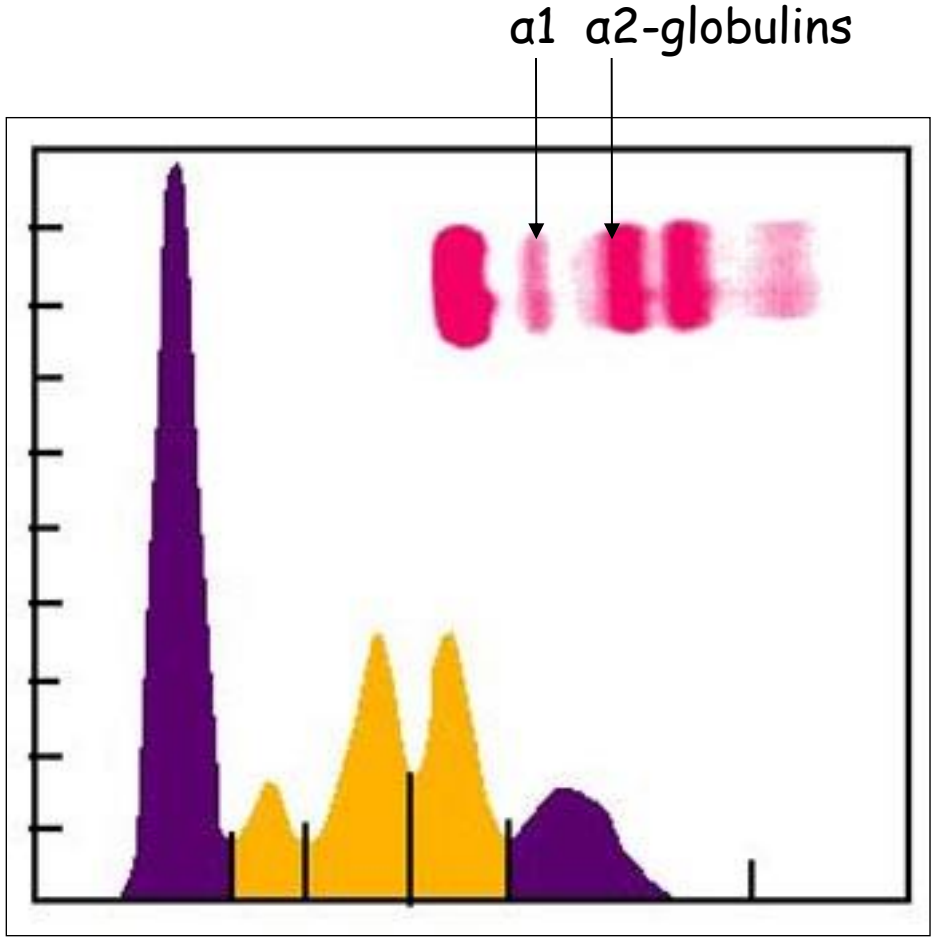


Reference ranges:

Total protein	6.0 – 8.0 g/dL
Albumin	3.5 – 5.0 g/dL
α1-globulins	0.1 – 0.4 g/dL
α2-globulins	0.4 – 1.3 g/dL
β-globulins	0.6 – 1.3 g/dL
γ-globulins	0.6 – 1.5 g/dL

Serum proteins electrophoresis in diagnostics of diseases: Acute inflammatory response

- Immediate response occurs with stress or inflammation caused by infection, injury or surgical trauma
- normal or ↓ albumin
- ↑ α1 and α2 globulins

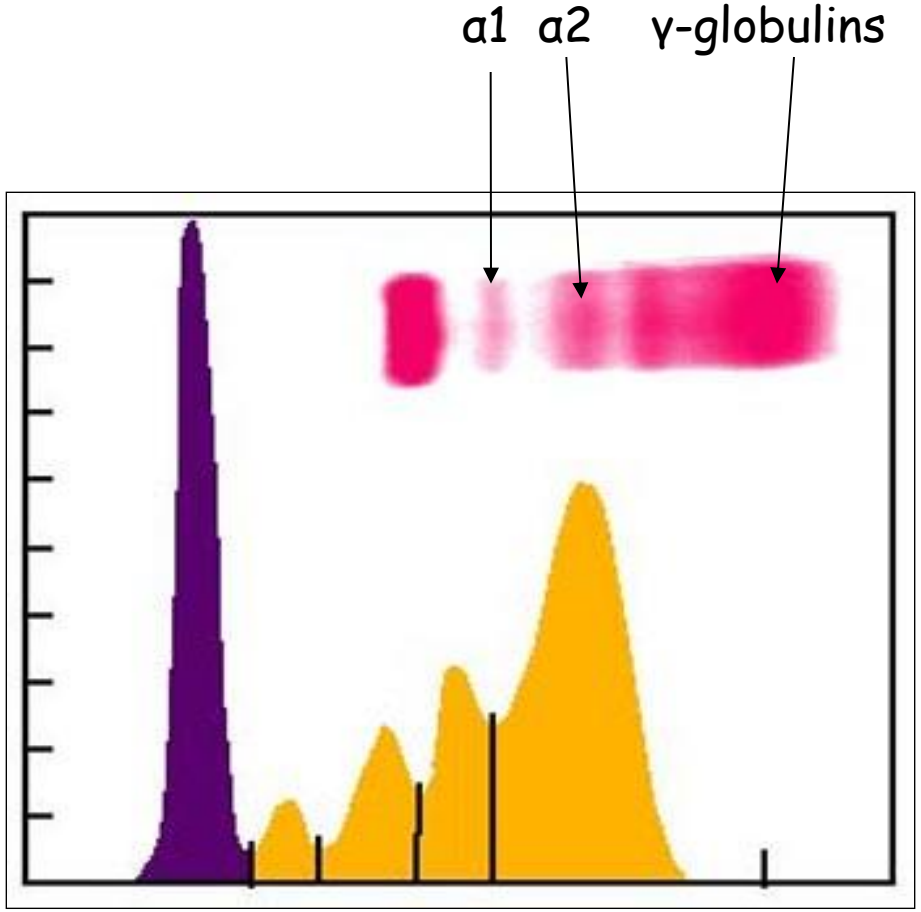


Serum proteins electrophoresis in diagnostics of diseases: Chronic inflammatory response

- Late response is correlated with chronic infection

(autoimmune diseases, chronic liver disease, chronic infection, cancer)

- normal or ↓ albumin
- ↑α1 or α2 globulins
- ↑↑ γ globulins



Blood cell counters

Blood cell counters count the number of RBC or WBC per unit of volume of blood using either of two method:

- Electrical method called **aperture impedance** change
- Optical method called **flow cytometry**

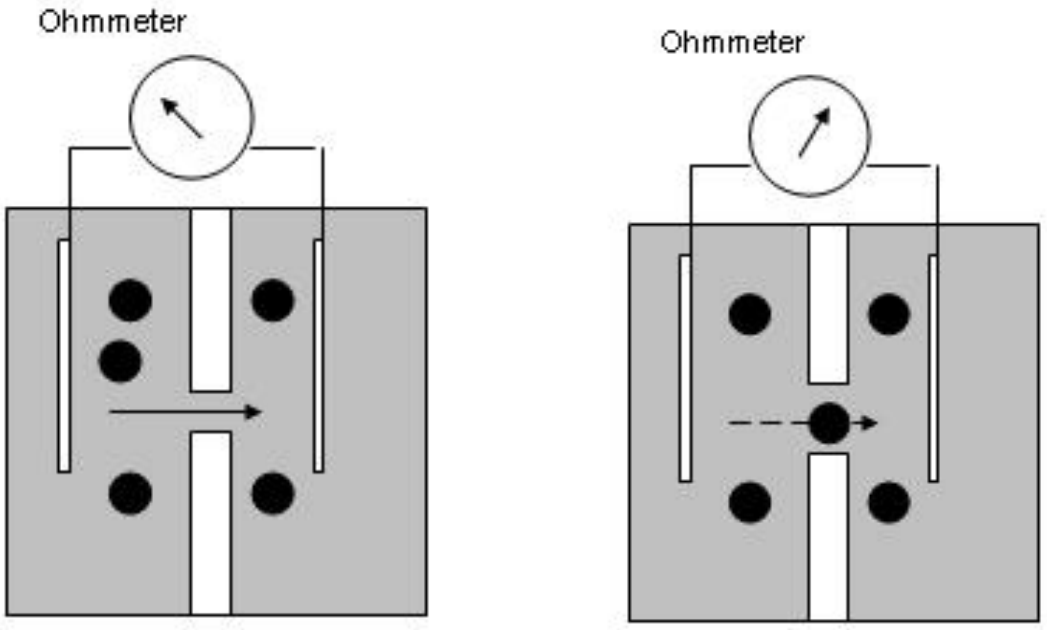
Aperture impedance change

- When blood is diluted in the proper type of solution, the electrical resistivity of blood cells (ρ_c) is higher then the resistivity of the surrounding fluid (ρ_f)

- By contriving a situation in which these resistivities can be differentiated from each other, we can count cells

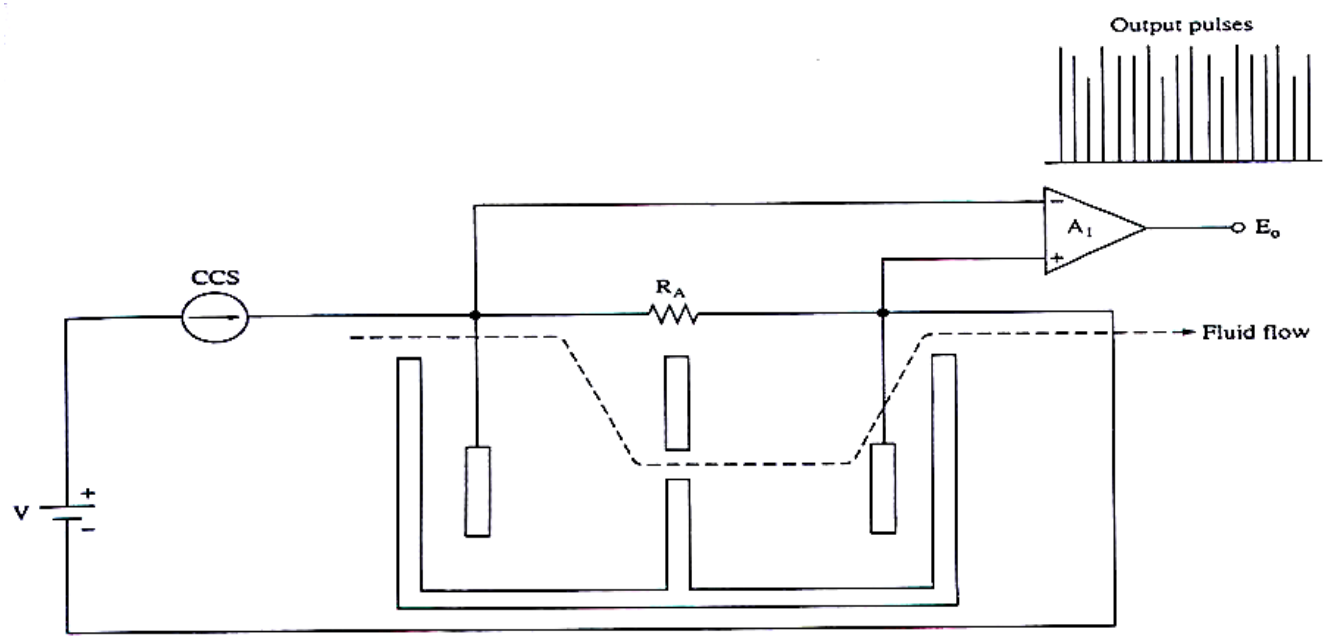
Aperture impedance change

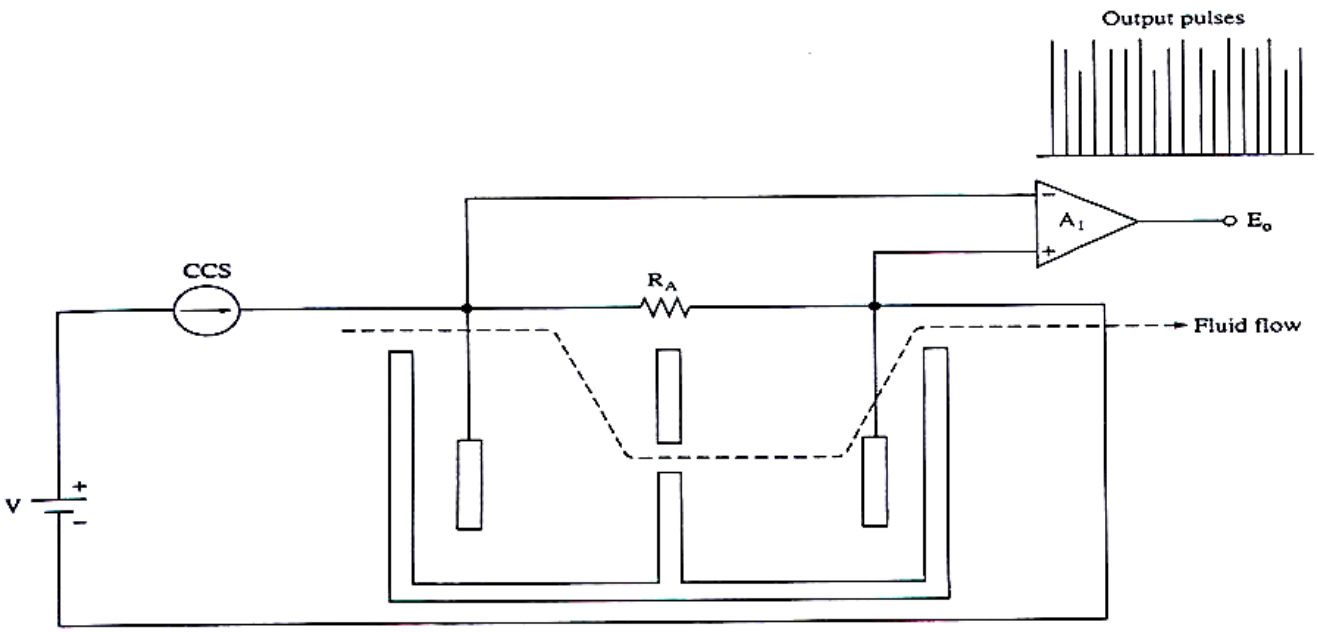
- The sensor consist of a two-chamber vessel in which the dilute incoming blood is on one side of barrier, and the waste blood to be discarded is on the other
- A hole with a small diameter (50μm) is placed in the partition between the tow halves of the cell
- Ohmmeter measure the change on the resistance when the blood cell pass the aperture



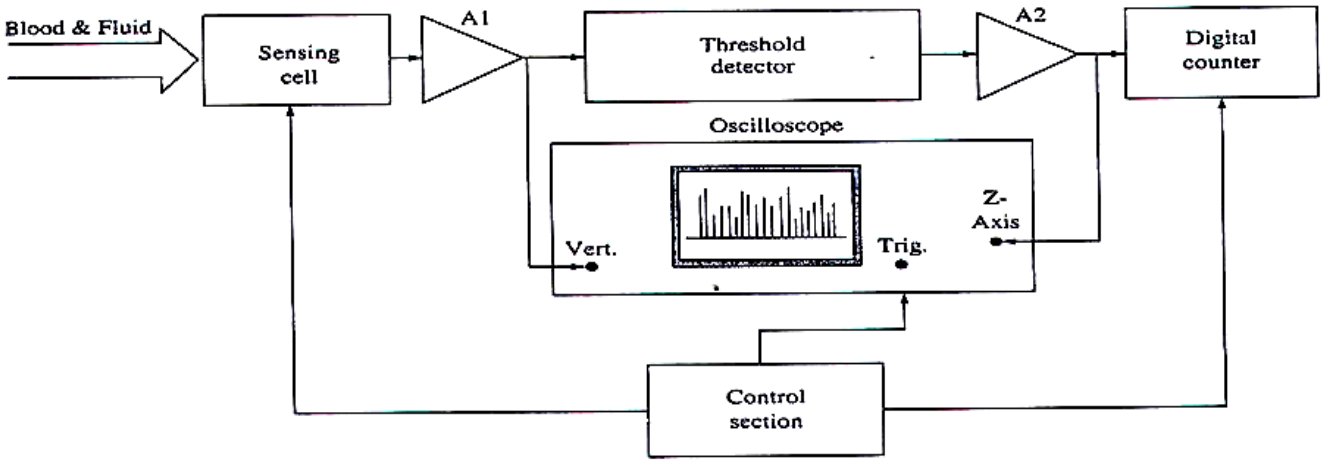
Coulter Counter

- Constant current source (CCS) and voltage amplifier replace the ohmmeter
- R_A is the resistance of the aperture and will be either high or low, depending on whether or not the blood cell is inside the aperture.
- Amplifier convert the current pulse to voltage pulse





Blood cell counters. *(a)* Coulter model F. *(b)* Coulter model senior.



Impedance aperture cell counter.

Coulter counter

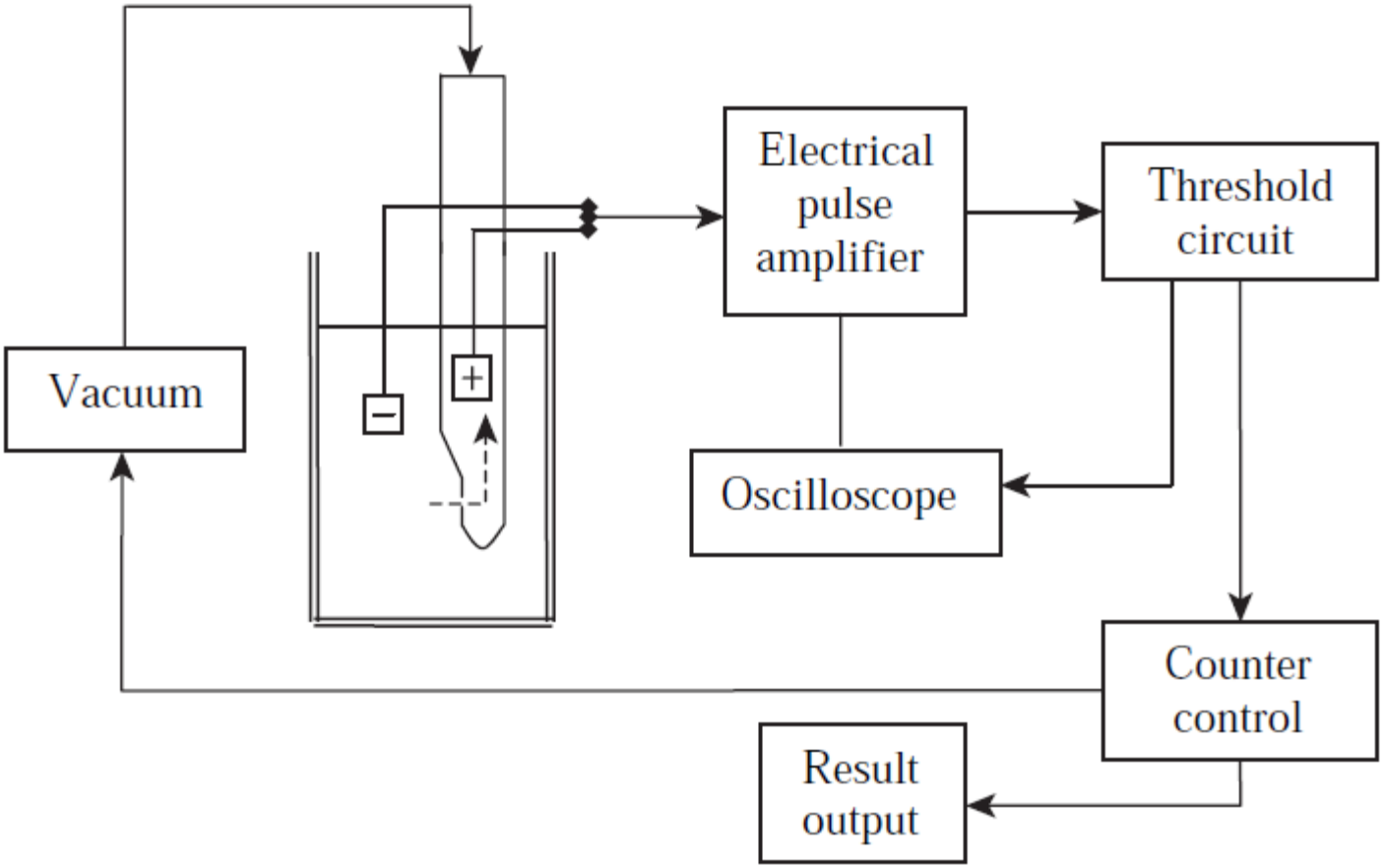
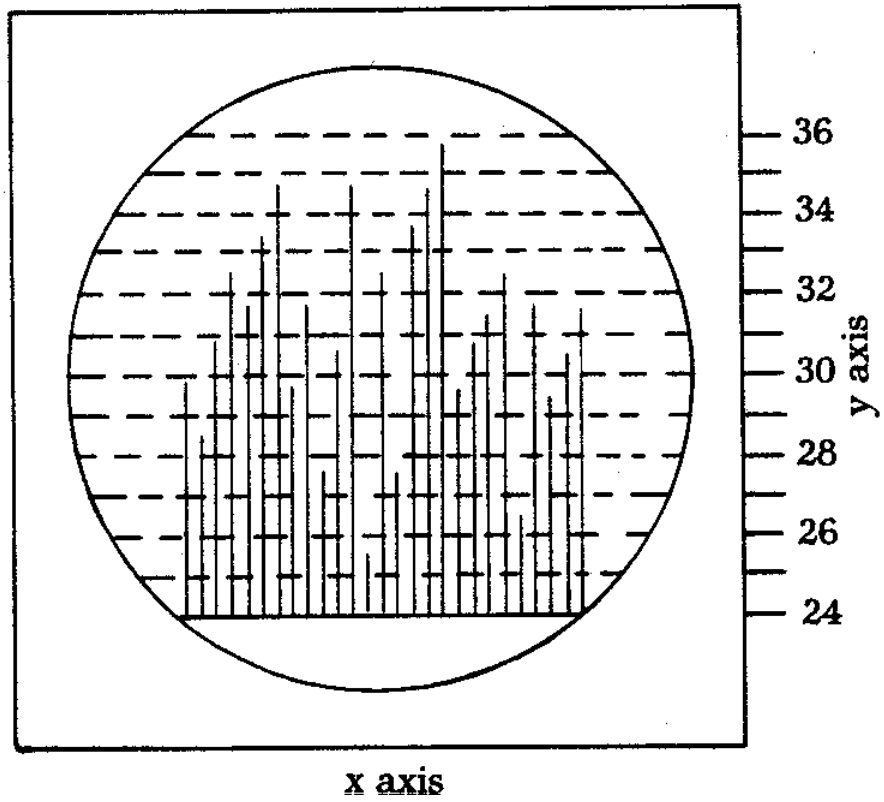
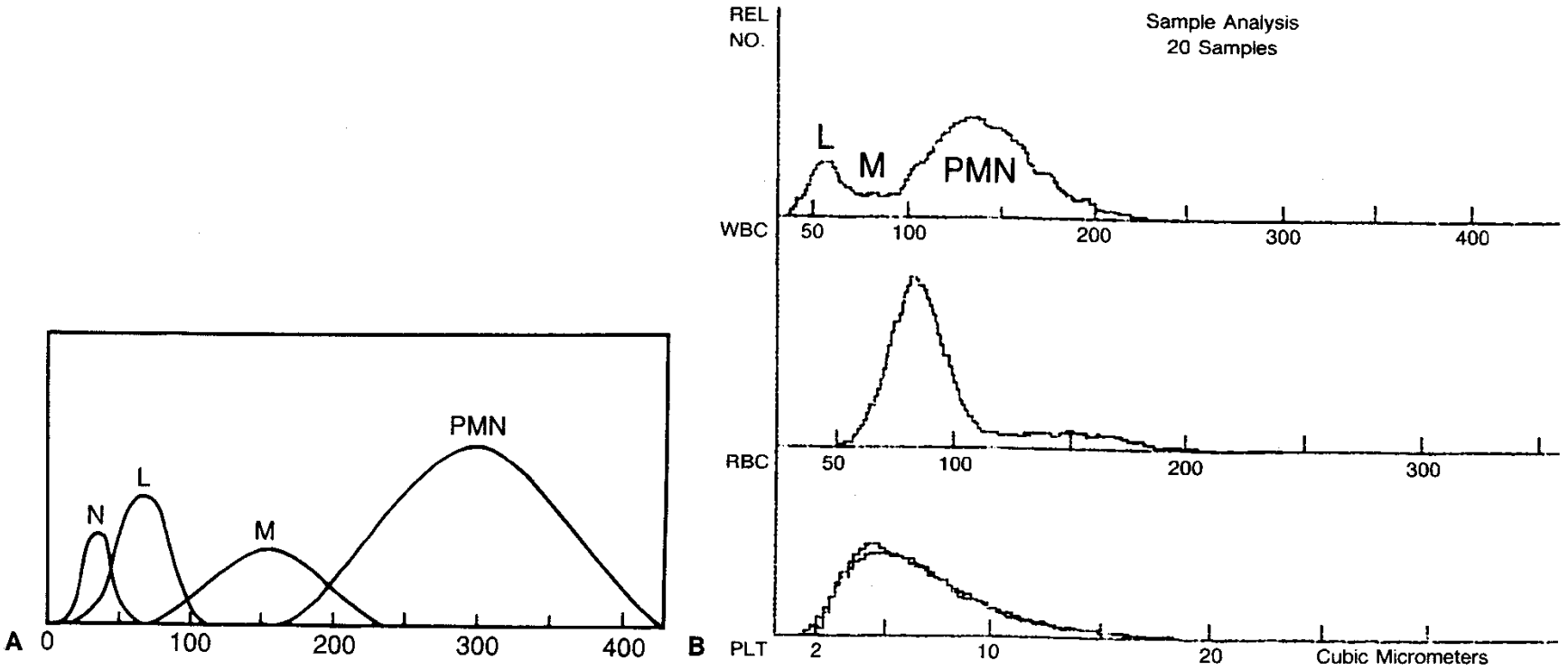


Figure 3. Schematic diagram of electronic cell counter using electric resistance method.

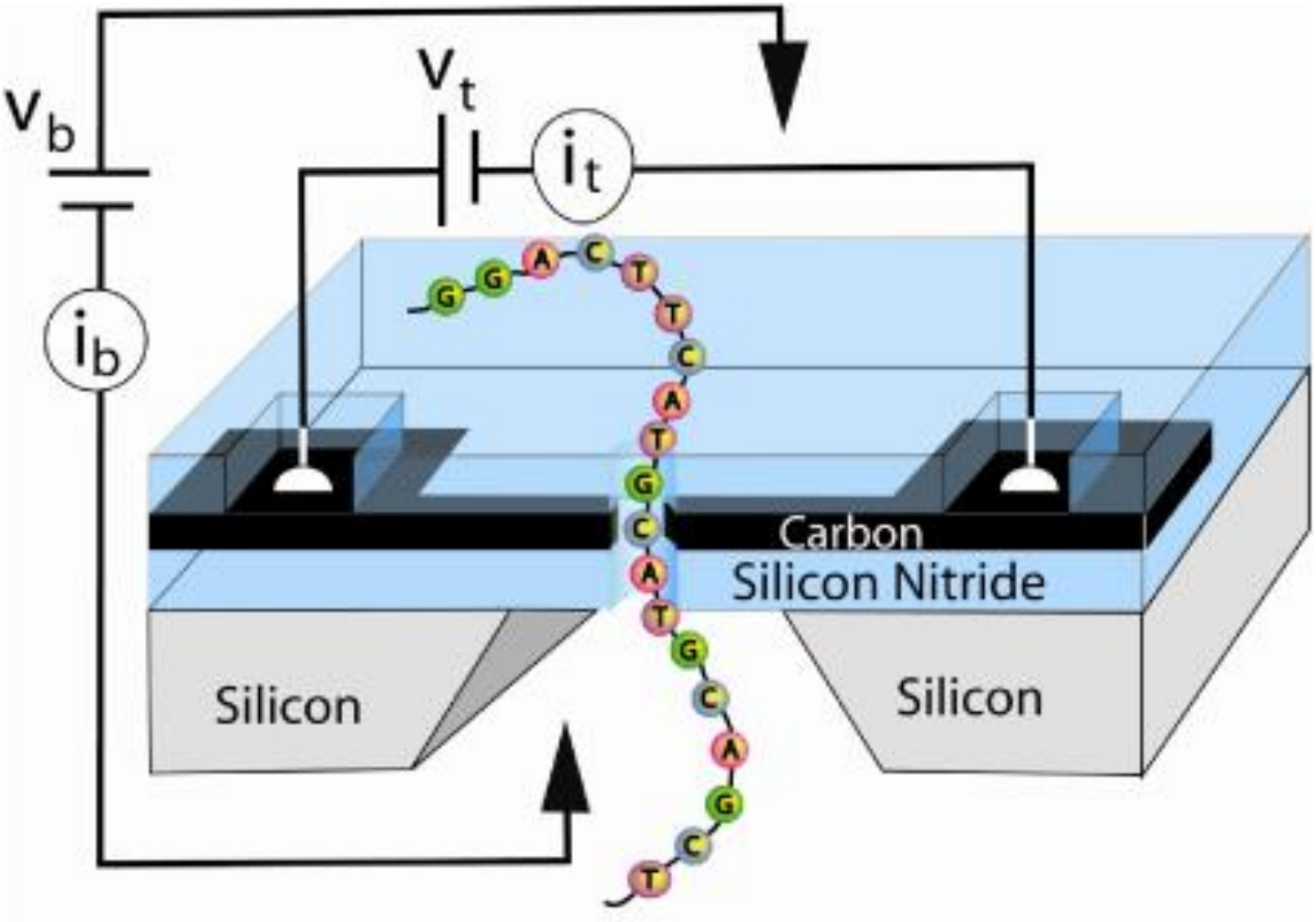


Each cell passing through the Coulter counter aperture causes a resistance change proportional to the cell volume. Thus measuring the height of each voltage spike yields the cell volume.



Number of cells versus cell volume from a Coulter counter. (a) Nucleated RBCs (N), lymphocytes (L), mononuclear cells (M), and polymorphonuclear leukocytes (PMN). (b) Leukocyte differential distribution (WBC), RBC distribution (RBC), and platelet distribution (PLT).

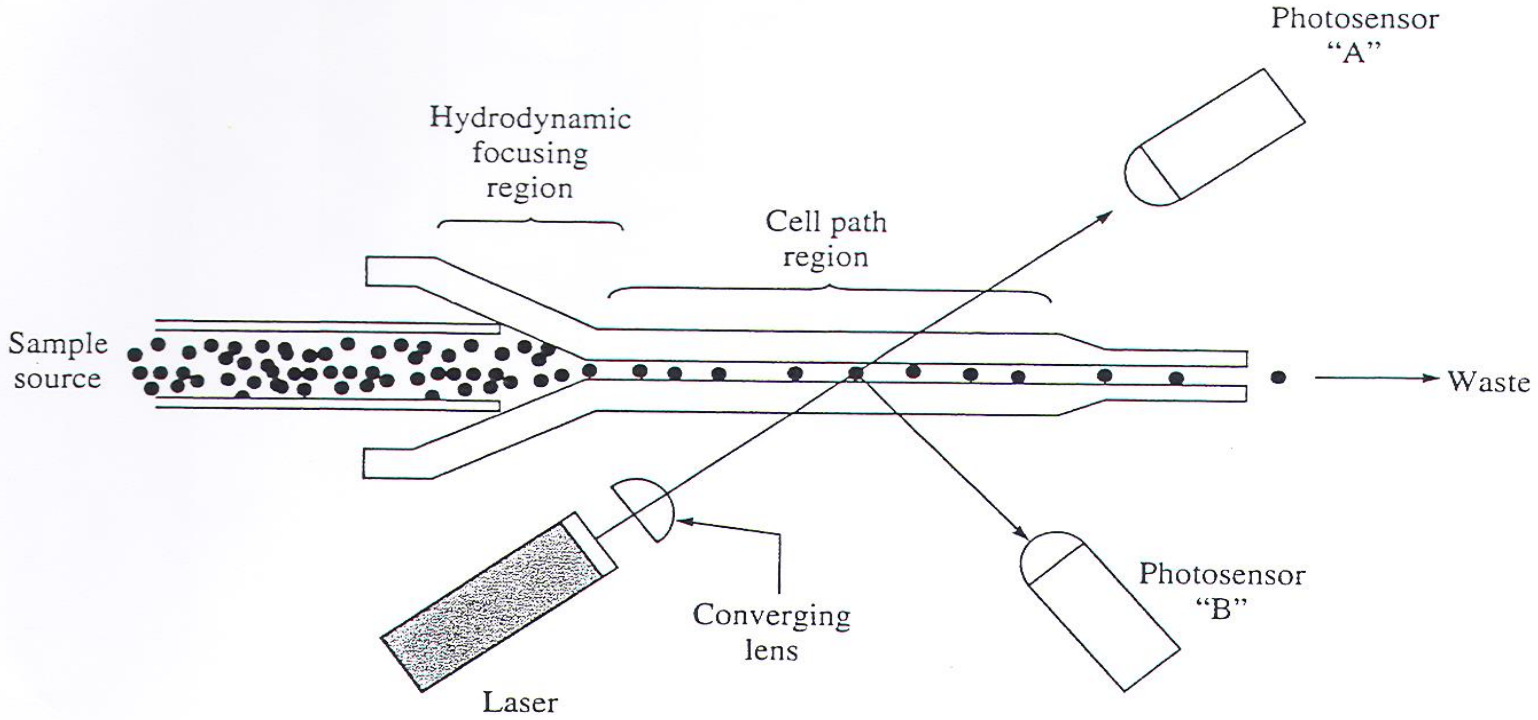
Future trend: molecular sensors based on resistive solid-state nanopore (Spinney et al, Nanotechnology, 2012)



Flow cytometry cell counters

- The optical cytometry sensor consists of a quartz sensing sheath designed with a
  - hydrodynamic focusing region
  - cell path region that passes only a single cell at time.
- Focusing is done by decreasing the diameter of the aperture.
- Light source is (He-Ne) Laser
- Two Photodetectors (photosensors)
  - Photodetector A detects forward scatted light
  - Photodetector B detects orthogonal scatted light
- blood sample enters the analyzer
  - Optical counter → WBC count
  - Colorimeter → hemoglobin
  - Optical flow sensor → RBC count

Schematic



Optical flow cytometry sensor.

Flow Cytometry

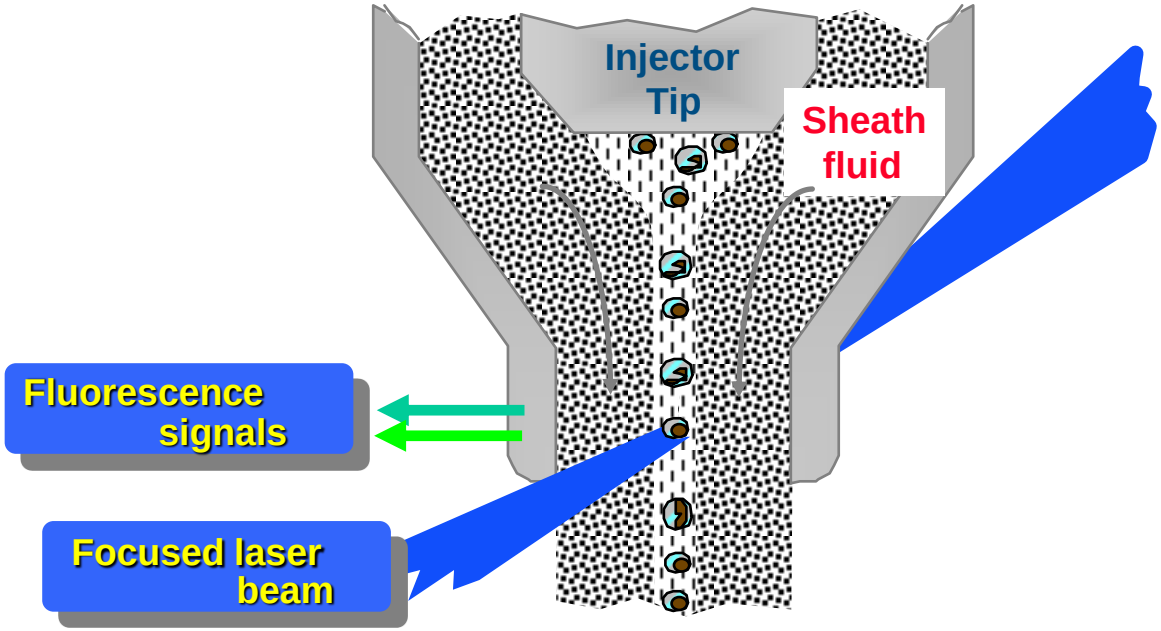
Fluidics Cells in suspension flow in single-file

Optics through an illuminated volume where they scatter light and emit fluorescence

Electronics that is collected, filtered and converted to digital values that are stored on a computer

Flow Cytometry: fluidics

- Need to have cells in suspension flow in single file through an illuminated volume
- In most instruments, accomplished by injecting sample into a sheath fluid as it passes through a small (50-300 µm) orifice

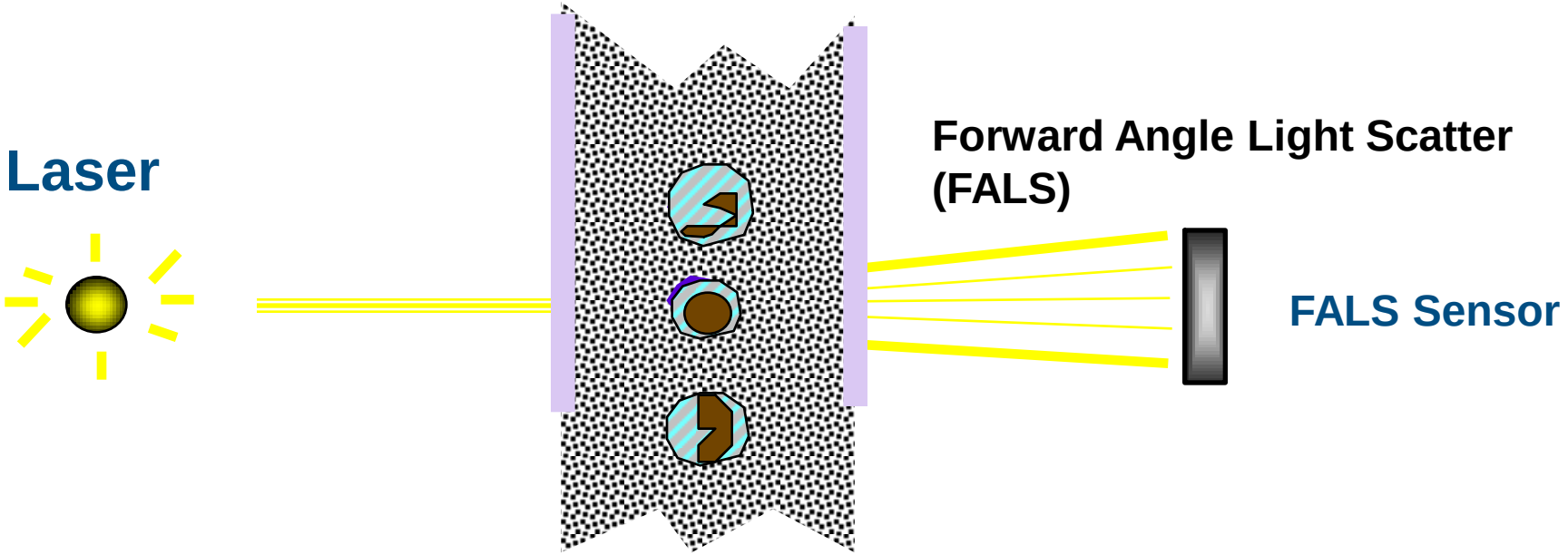


Flow Cytometry: Optics

- Need to have a light source focused on the same point where cells have been focused (the illumination volume)
- Two types of light sources
  - **Lasers**
  - **Arc-lamps**

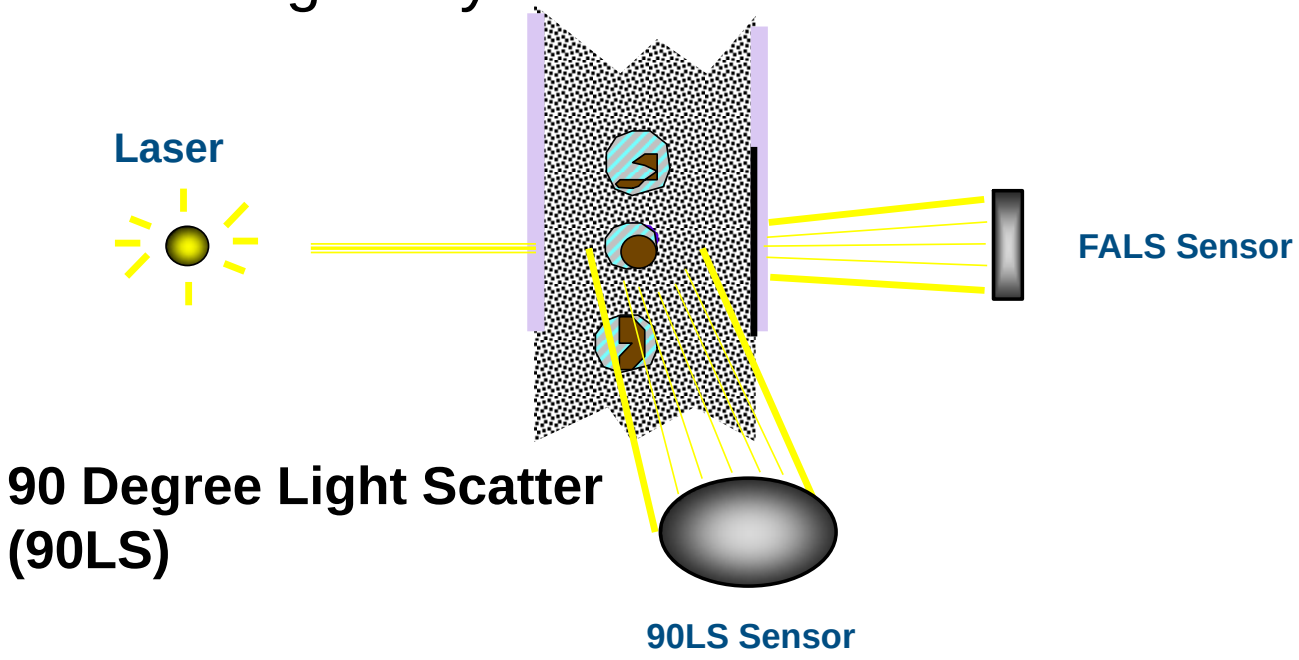
Flow Cytometry
Optics - Forward Scatter Channel

- When a laser light source is used, the amount of light scattered in the forward direction (along the same axis that the laser light is traveling) is detected in the **forward scatter channel**
- The intensity of forward scatter is most influenced by the **size** of cells (or other particles)



Flow Cytometry-
Optics - Side Scatter Channel

- When a laser light source is used, the amount of light scattered to the side (perpendicular to the axis that the laser light is traveling) is detected in the **side or 90º scatter channel**
- The intensity of side scatter is most influenced by the **shape** and optical homogeneity of cells

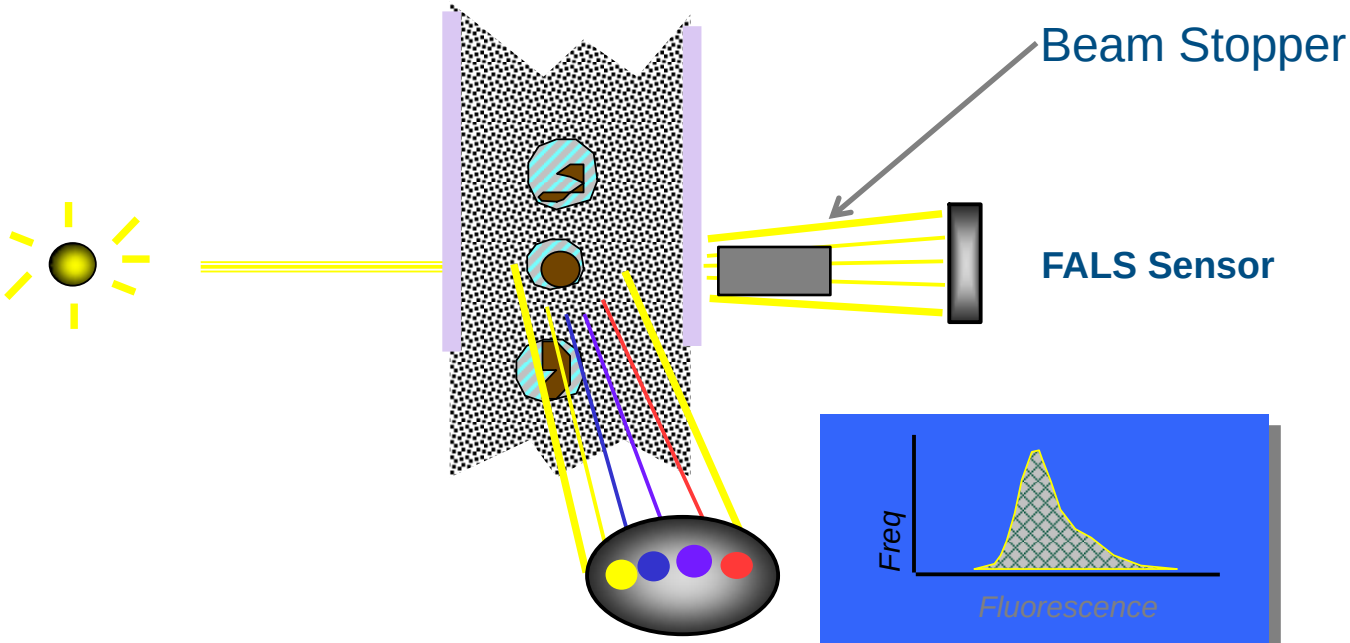


Flow Cytometry
Optics - Light Scatter

- Forward scatter tends to be more sensitive to surface properties of particles (e.g., cell ruffling) than side scatter
  - can be used to distinguish live from dead cells
- Side scatter tends to be more sensitive to inclusions within cells than forward scatter
  - can be used to distinguish granulated cells from non-granulated cells

Flow Cytometry
Optics - Fluorescence Channels

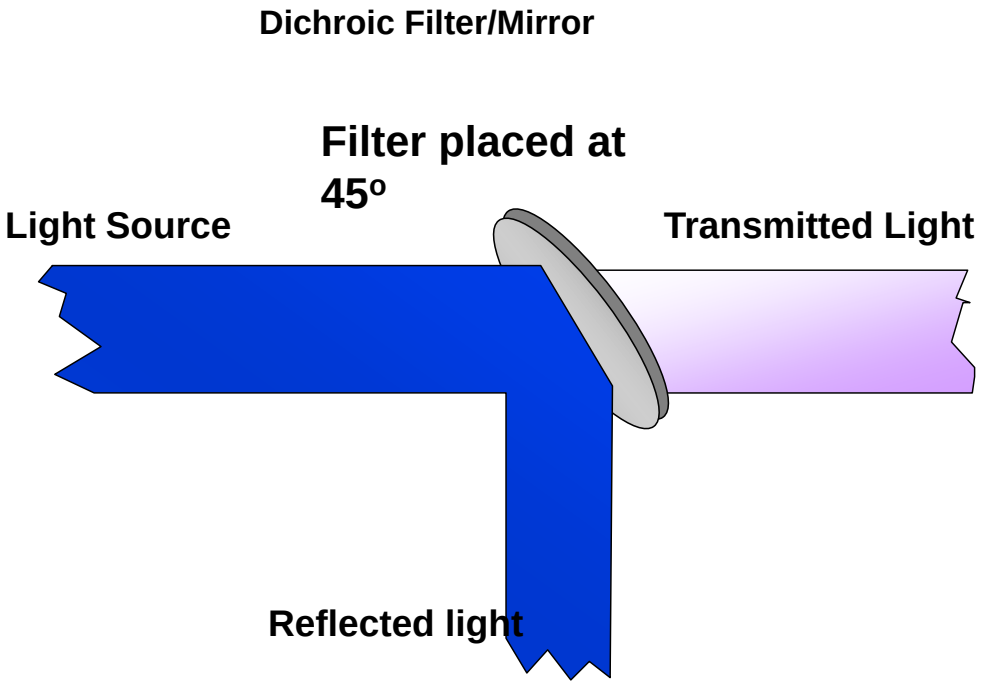
- The fluorescence emitted by each fluorochrome is usually detected in a unique **fluorescence channel**
- The specificity of detection is controlled by the wavelength selectivity of optical filters and mirrors



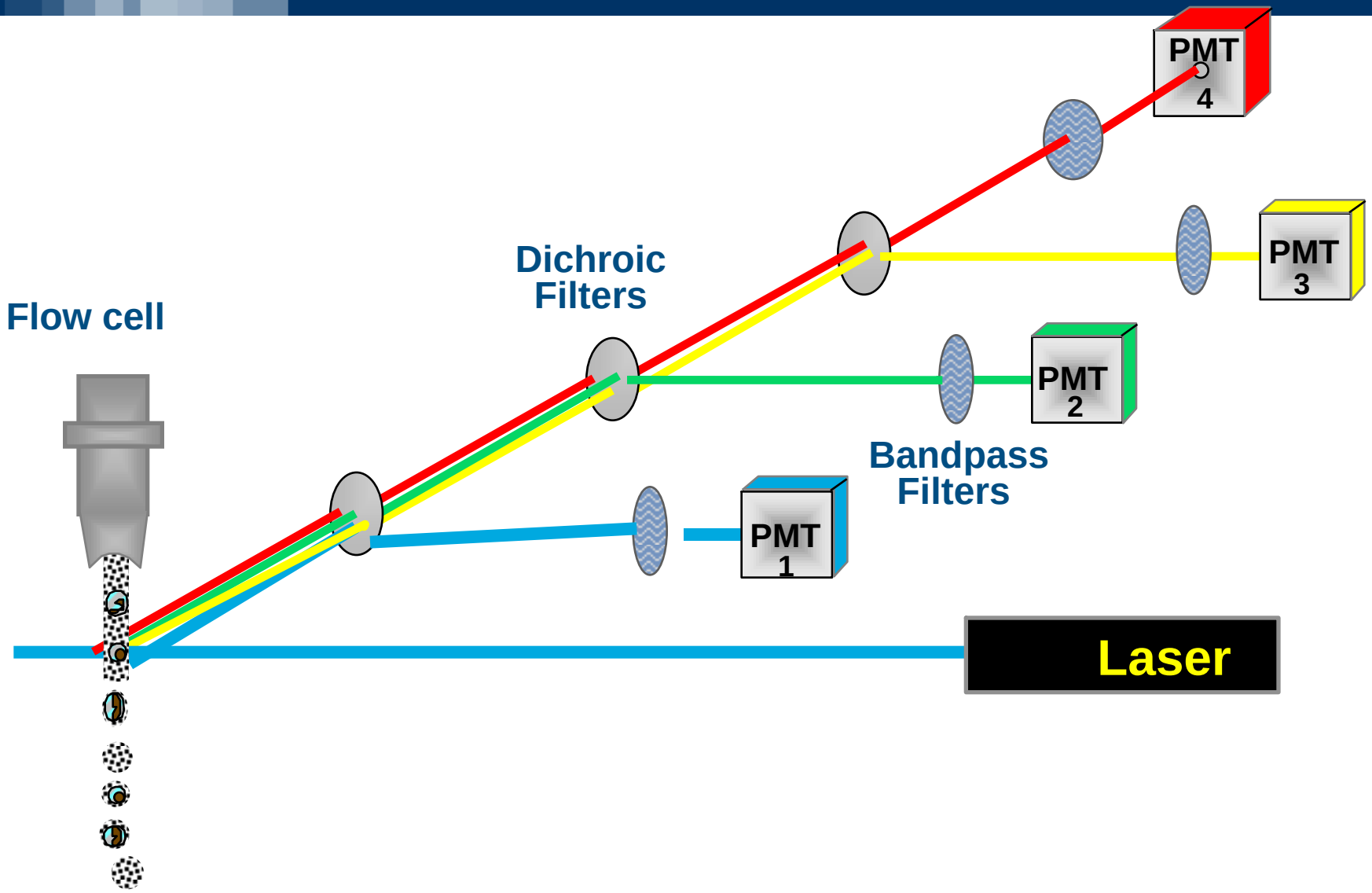
Fluorescence detector (PMT3, PMT4 etc.)

Optics - Filter Properties

- When a filter is placed at a 45^{o} angle to a light source, light which would have been transmitted by that filter is still transmitted but light that would have been blocked is reflected (at a 90^{o} angle)
- Used this way, a filter is called a **dichroic filter** or **dichroic mirror**



Channel Layout for Laser-based Flow Cytometry

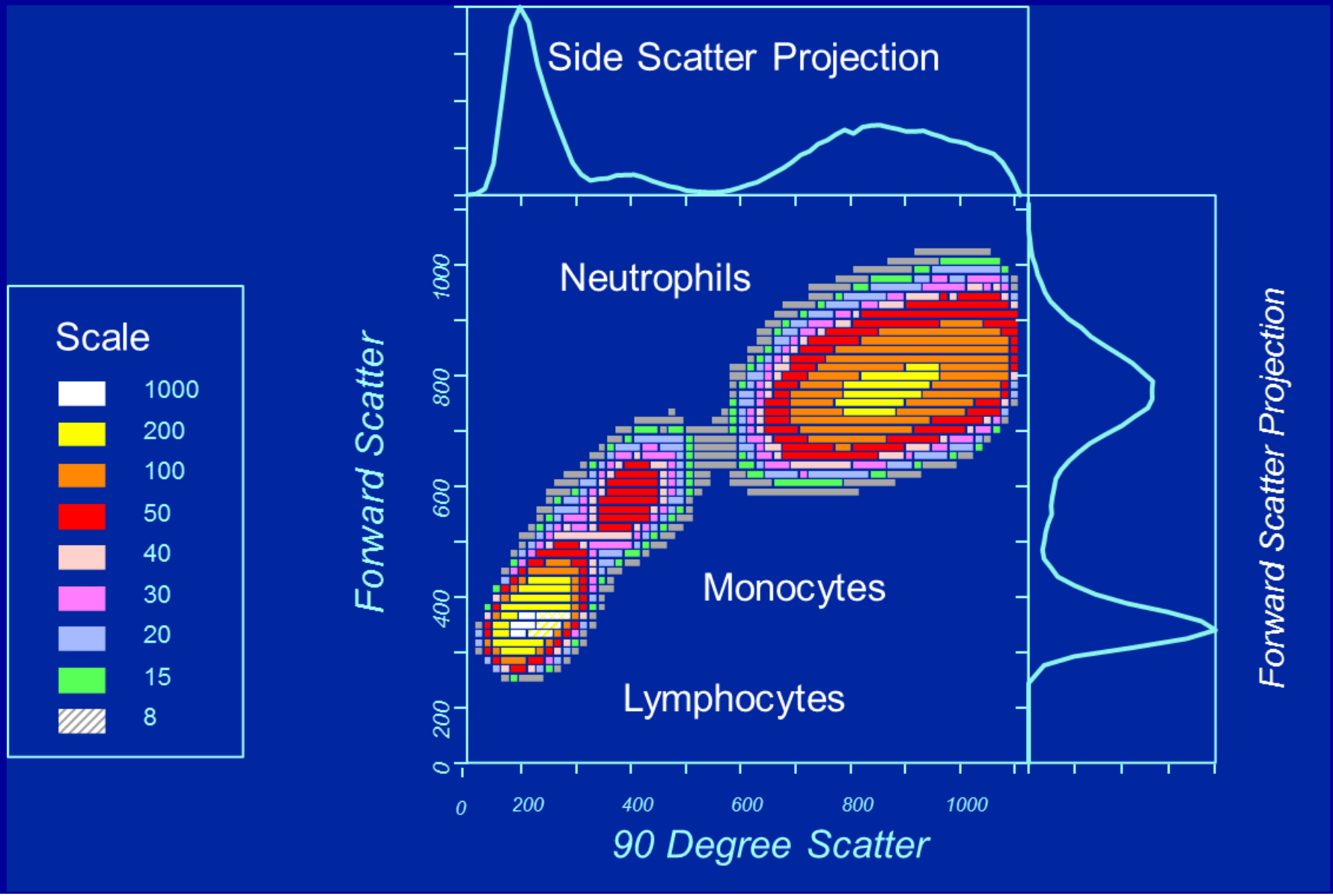


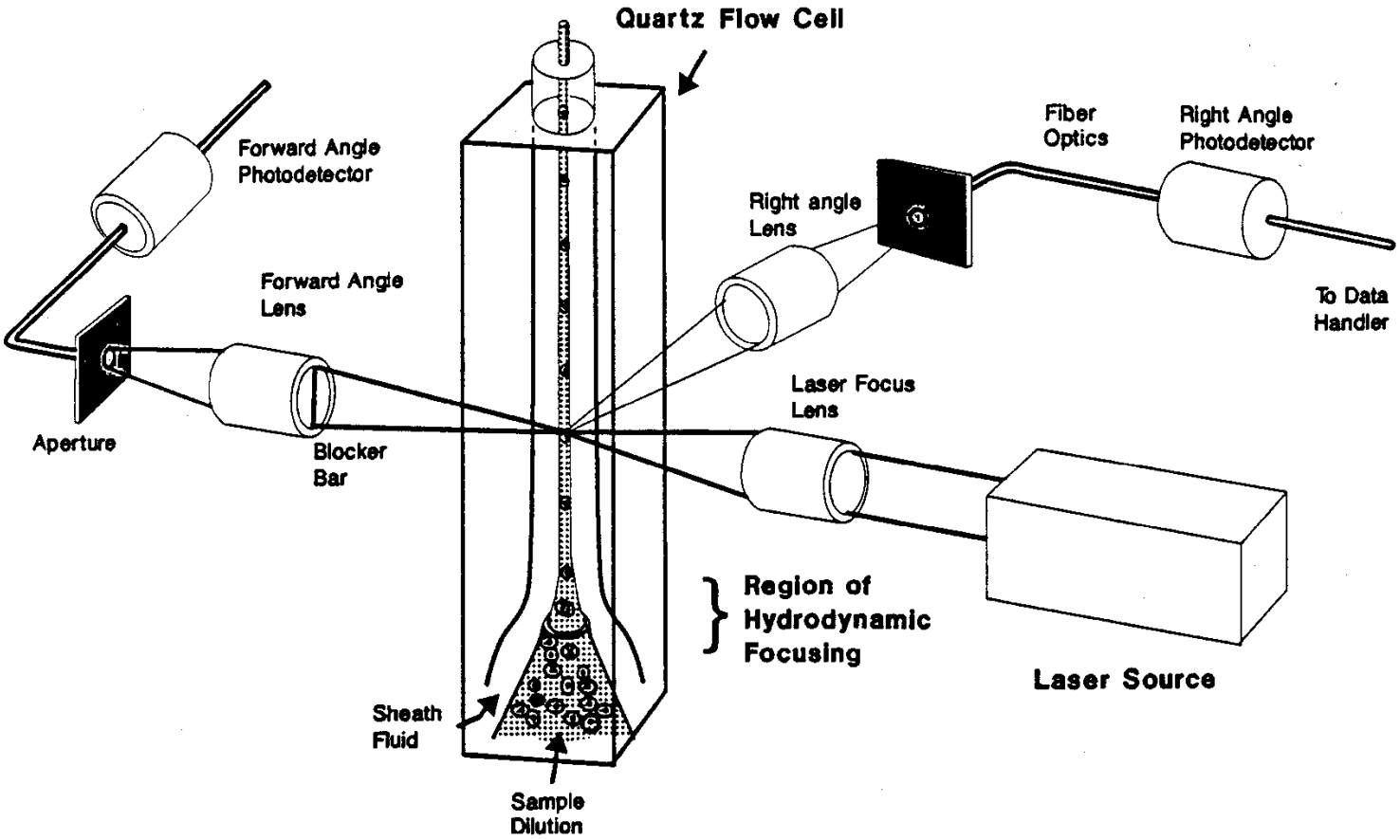
Flow cytometry – Optics - Detectors

- Two common detector types
  - **Photodiode**
    - used for strong signals when saturation is a potential problem (e.g., forward scatter detector)
  - **Photomultiplier tube (PMT)**
    - more sensitive than photodiode but can be destroyed by exposure to too much light

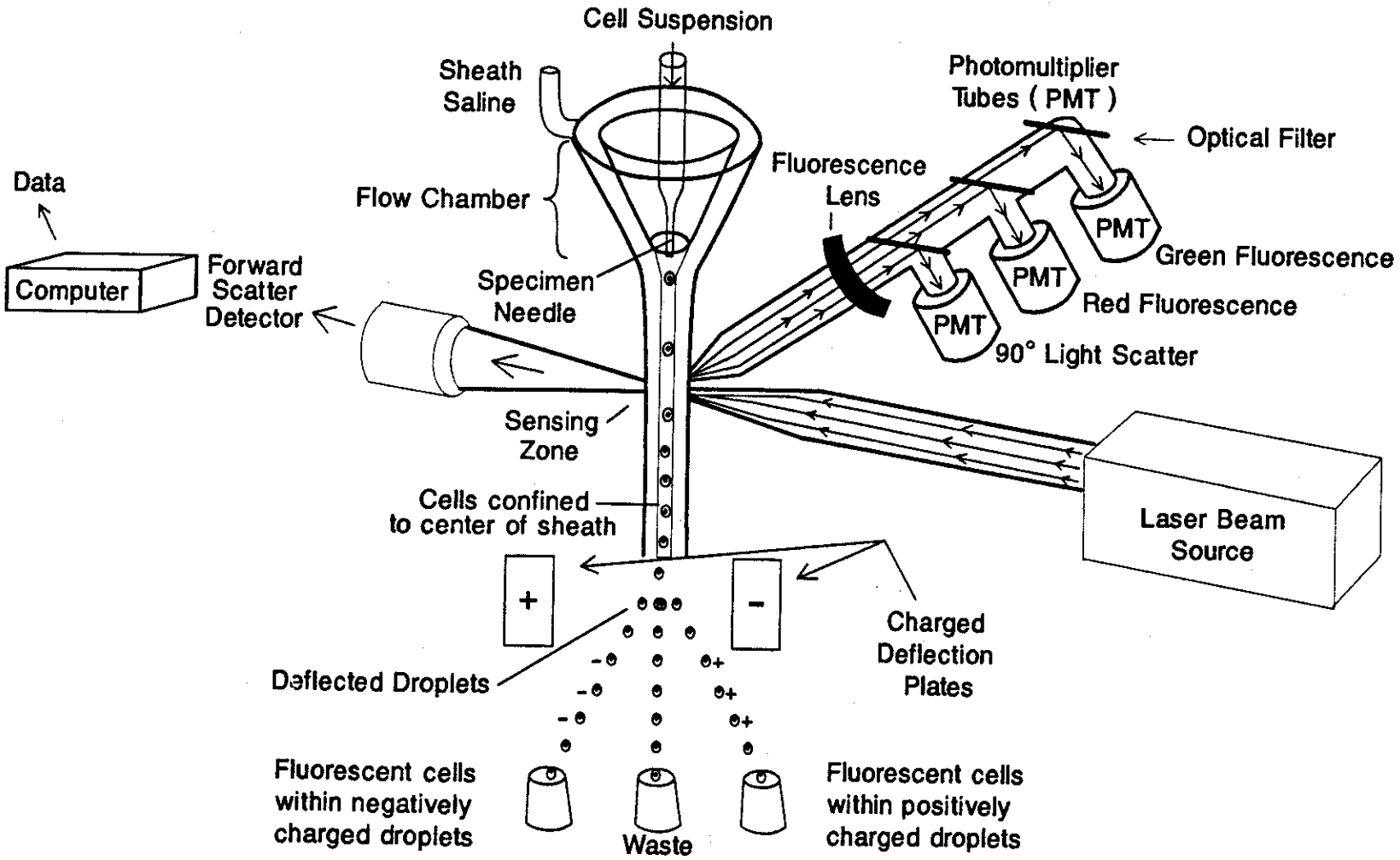
Immunofluorescence Analysis

- A major application of flow cytometry is the analysis (and sorting) of subsets of blood cells using surface markers.
- A useful feature is that the major blood cell types show distinct forward and side scatter profiles.

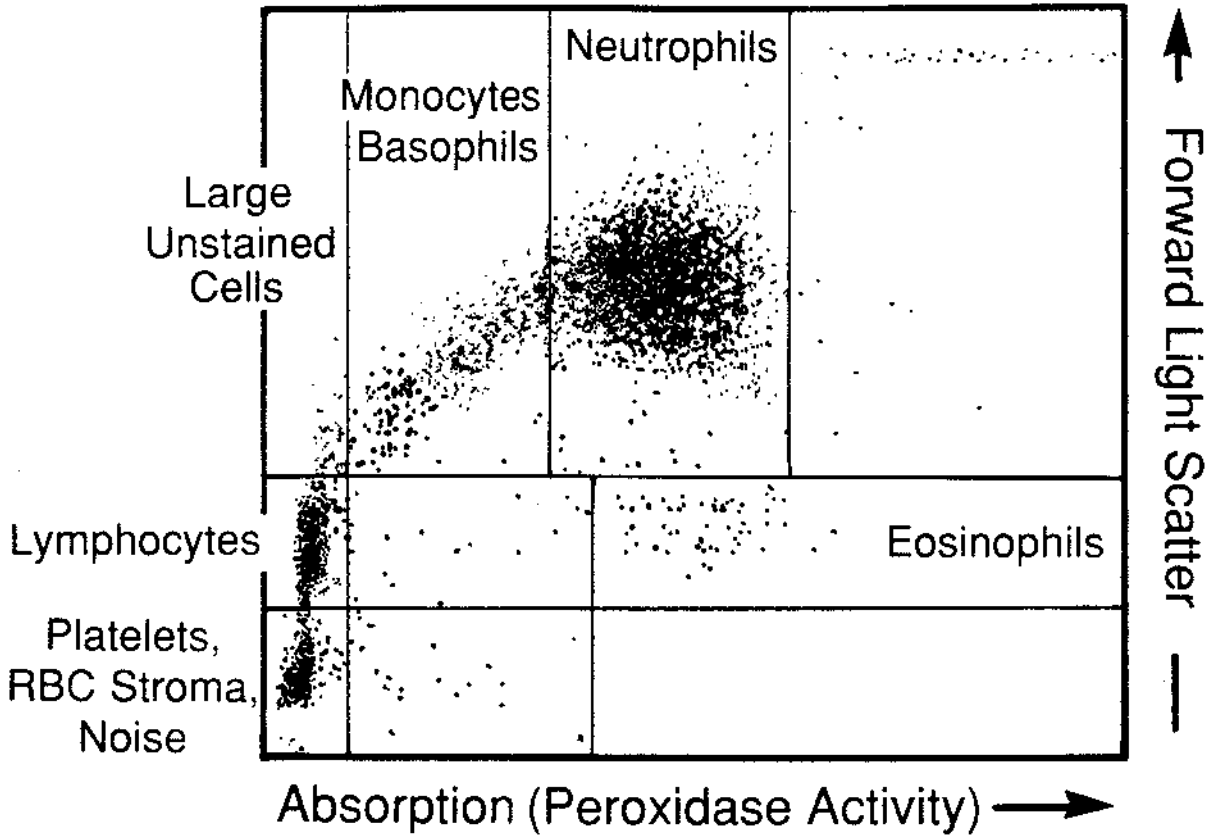

Side Scatter Projection
Neutrophils
Monocytes
Lymphocytes
Scale
1000
200
100
50
40
30
20
15
8
Forward Scatter
0
200
400
600
800
1000
90 Degree Scatter
Forward Scatter Projection



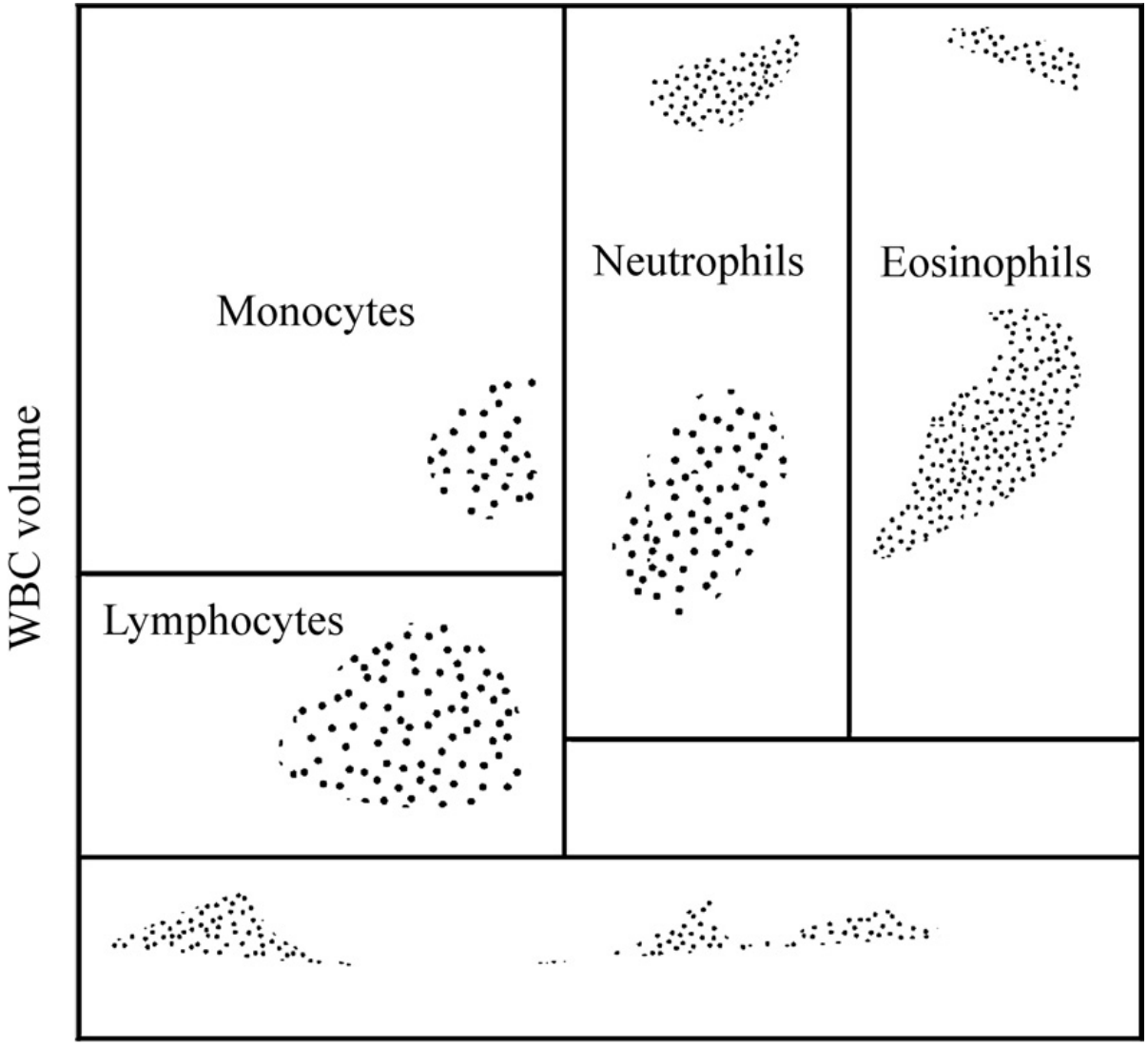
In flow cytometry, a sheath surrounds the sample to hydrodynamically focus the cells to the center, where they are illuminated by a laser. Forward (low) angle scatter measures cell volume. Right (high) angle scatter measures cell type.



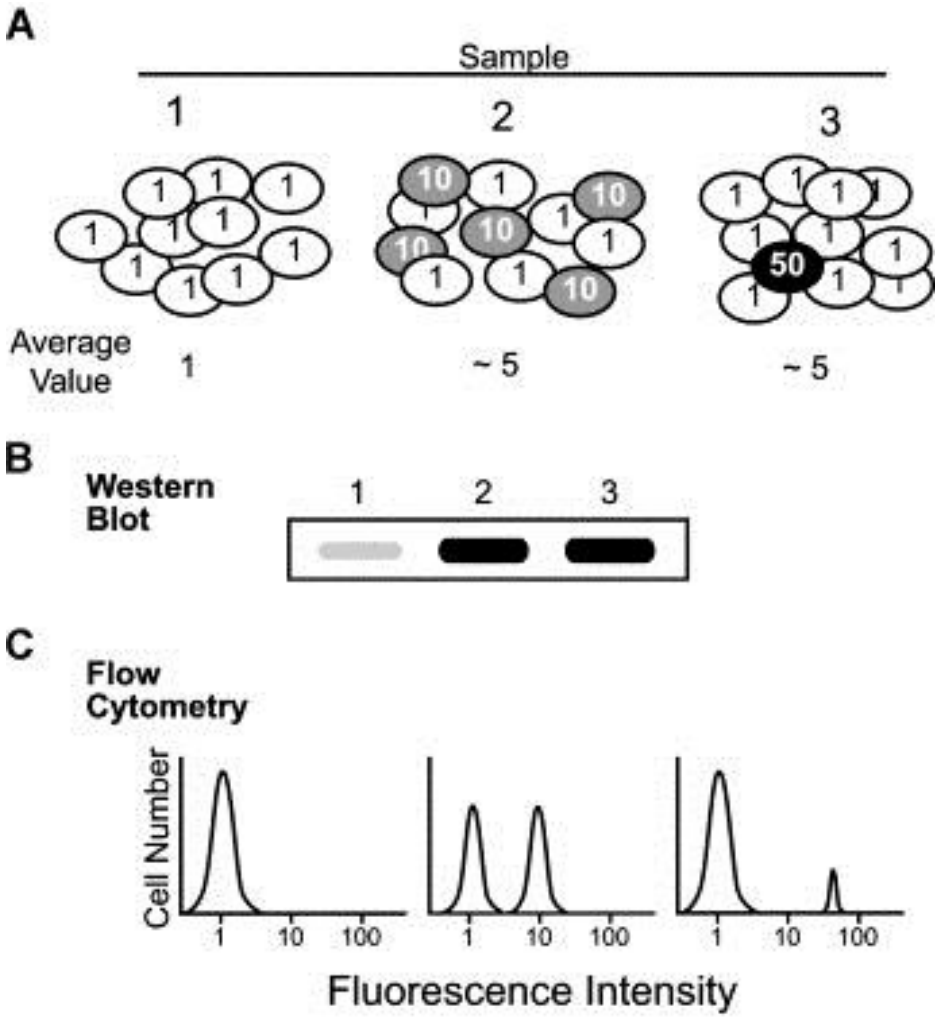
After cell identification by fluorescent light scattering, the cell sorter charges each cell droplet, and electrostatically deflects it to separate cells by type. .



Leukocyte differential classifies the five basic leukocyte classes by forward light scatter versus peroxidase absorption.


Monocytes
Neutrophils
Eosinophils
Lymphocytes
WBC volume
Light scatter

Single cell analysis



(A) three samples are obtained that contain a protein of interest at 1, 10, or 50 copies per cell as indicated. The average number of protein molecules per cell is 1 for sample 1, and 5 for both samples 2 and 3.

(B) when these cell populations are analyzed by **Western blotting**, samples 2 and 3 will show darker bands but will appear identical to one another.

(C) When the samples are stained for the protein with fluorescently labeled antibodies and analyzed by **flow cytometry**, however, one can clearly see that sample 2 contains cells in two distinct populations that are equally represented, while in sample 3, only about 1 in 10 cells has an elevated level of protein. This kind of heterogeneity in the samples could be due to different cell types (i.e., immune cells), or because of all-or-none type signaling responses.

Advantages of flow cytometry

- The advantage of any cytometric measurement (image or flow) is that it records data from single cells.
- The flow cytometer can also make measurements on large numbers of cells; consequently, it is possible to obtain a good statistical analysis on cell populations
- It identifies small sub-populations of cells. If a suitable label (for example, an antibody) is available, as few as 1 cell in 10000 can be enumerated.
- Finally, using one of the more sophisticated flow cytometers, cells can be physically sorted. Sub-populations can be purified for further study.

Measurements on single cells by a cytometric technique can be contrasted to a biochemical measurement, which records an average value for all cells in a sample

Disadvantages of flow cytometry

- It needs single **particles** (cells, nuclei, chromosomes) for analysis. The strength of flow cytometry is also its major disadvantage.

- The tissue architecture is lost when single cells or nuclei are prepared. In a tumor, using a tissue section, a pathologist can gain information from the relationship between different cells.

- Flow cytometry does not give information about intra-cellular distributions of an entity (such as a protein). To determine the distribution of an entity within a cell, image cytometry must be used (for example, confocal microscopy).