UNITE

CENTRIFUGATION TECHNIQUES

I.C. **LABORATORY OF BIOCHEMICAL METHODOLOGIES AND EXPERIMENTAL MODELS**

- MODULE: **BIOCHEMICAL METHODOLOGIES**

BIOS-07/A

5 ECTS (CFU)



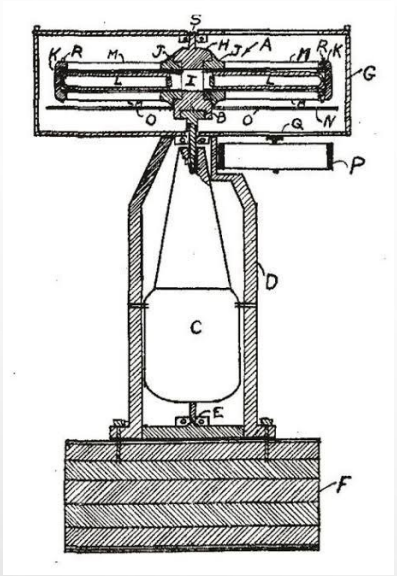
Centrifugation techniques

BY THE END OF THE LESSON, THE STUDENT WILL BE ABLE TO:

- Describe the physical principle of sedimentation in a centrifugal field and the relevant parameters (centrifugal force, friction, sedimentation coefficient).
- Distinguish between preparative and analytical centrifugation and outline their purposes.
- Explain and compare differential centrifugation and density gradient centrifugation (zonal and isopycnic).
- Identify the main types of rotors and centrifuges (microcentrifuges, high-speed centrifuges, ultracentrifuges) and relate them to specific applications.
- Interpret the concept of the sedimentation coefficient (svedberg) and its significance for the separation of biological particles.

Centrifugation techniques





Theodore Svedberg (1920)
First Analytical Ultracentrifuge,
designed to monitor and record the sedimentation process

- **Biological centrifugation** is a process that uses centrifugal force to separate and purify mixtures of biological particles in a liquid medium - based on their sedimentation properties (**particle size, density, and shape**);
- A centrifuge operates using the principle of sedimentation: under the influence of **gravitational force (g-force)**, substances separate based on their size, shape, density, the viscosity of the medium, and the rotor speed.
- Key technique **to isolate and analyse** cells, subcellular fractions, supramolecular complexes and, isolated macromolecules such as proteins or nucleic acids;
- Today, centrifugation techniques represent a critical tool for modern biochemistry and are employed in all invasive subcellular studies, we distinguish:
  - **Analytical ultracentrifugation** is mainly concerned with the study of purified macromolecules or isolated supramolecular assemblies;
  - **Preparative centrifugation** is devoted to actual separation of tissues, cells, subcellular structures, membrane vesicles and other;

The purpose of centrifugal techniques is to exert a force on the particles that is greater than the force exerted by the Earth's gravitational field, thereby increasing their sedimentation velocity.

Centrifugation techniques - Principles of Sedimentation

Sedimentation due to Earth gravitational field	vs	Sedimentation increase rate in a centrifugal field
$G = g = 9.81 m\ s^{-2}$		$G > 9.81 m\ s^{-2}$

The centrifugal field: G, rpm and RCF

Spinning creates a centrifugal field that drives sedimentation against a viscous drag.

Considerations to design centrifugation protocols:	
Sediment in a centrifugal field	
Faster	**Slower**
denser biological structure	denser biological buffer
massive biological particle	great frictional coefficient
greater centrifugal force	
=0 when the density of the particle = density of surrounding medium	

CENTRIFUGAL FIELD ($cm\ s^{-2}$), fundamental equation describing the sedimentation velocity of a particle

$$G = \omega^2 \cdot r$$

G: centrifugal field; ω: Angular speed (rad/s); r: radial distance of the particle (cm)

FROM ROTOR SPEED: Angular velocity is often expressed in rpm, remembering that 360°= 2π

$$\omega = 2\pi \cdot rpm / 60$$

Therefore, centrifugal field can be expressed as:

$$G = 4\pi^2\ rad^2 \cdot rpm^2 \cdot r$$

Centrifugation techniques - Principles of Sedimentation

The centrifugal field is generally expressed in multiples of the earth's gravitational field, g (9.81 m s-2). The **RELATIVE CENTRIGUGAL FIELD · g-FORCE** is the ratio of the centrifugal acceleration at a specified radius and the speed to the standard acceleration of gravity.

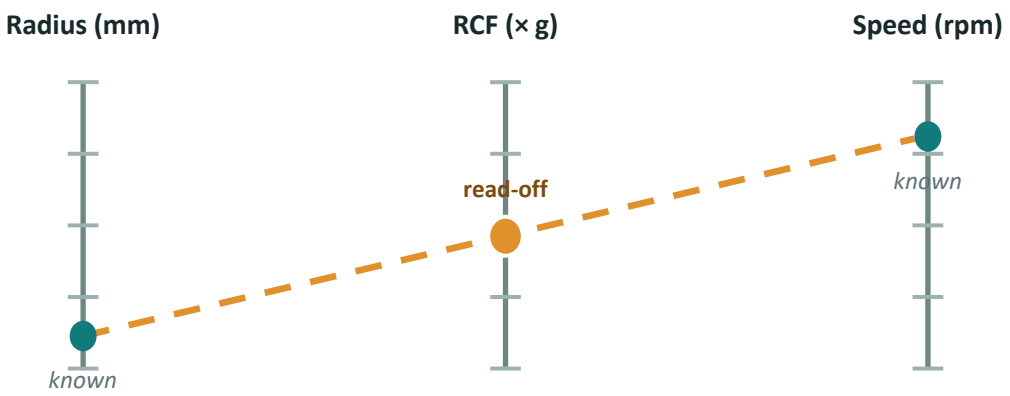
$$RCF = G/g = \frac{4\pi^2\, rad^2 \cdot rpm^2 \cdot r}{g}$$

Grouping numerical constant:

$$RCF \approx 1.12\times10^{-5} \cdot rpm^2 \cdot r$$

(dimensionless, in × g; r in cm)

NOMOGRAPH — a graphical rpm ⇄ RCF converter



How to use it

Three scales connect radius, g-force and rotor speed. Lay a straight edge across the two values you know — here radius and speed — and read the answer where it crosses the middle scale.

Handy when the instrument shows rpm but the protocol specifies × g.

Centrifugation techniques - Sedimentation

How do particles behave under the action of the centrifugal field?

The sedimentation of particles, assuming they are spherical, can be explained **by Stokes' equation,** which describes the motion of a sphere in a gravitational field

$$v = 2 \cdot Rh^2 \cdot (\rho_p - \rho_m) \cdot g \,/\, 9\eta$$

$\rho_p - \rho_m$ *: density difference (*drives sedimentation — denser particles fall faster*),* Rh: Hydrodynamic radius (set by particle size & shape; v scales with Rh^2); η: medium viscosity a thicker medium slows sedimentation down).

When $\rho_p = \rho_m$ the particle does not sediment (v = 0).Elongated particles sediment more slowly.

Particles inside the solution move with uniformly accelerated motion; however, they generate friction as they migrate through the solution, owing to the viscosity of the medium and their velocity.

Sedimentation (centrifugal field G) ⇄ opposed by **frictional drag F = f · v** — the coefficient f depends on particle size & shape.

F: force in Newton (N); f: coefficient (proportionality constant combining fluid viscosity, surface area, and characteristic length); v: velocity of the object moving relative to the fluid (m/s).

$$f_0 = 6\pi\eta r_p$$

η = viscosity coefficient of the medium; $r_{p=}$ radius of the spherical particle

if the particle is not spherical, the frictional ratio (f/f0) must be considered — i.e. the ratio between the frictional coefficient f of the 'real' particle and that of a perfectly spherical, non-hydrated particle, f0

Centrifugation techniques - Sedimentation

The sedimentation velocity can also be expressed as the sedimentation velocity per unit of applied centrifugal field, commonly called the **"sedimentation coefficient" (s)**:

$$s = v / G = v/ \omega^2 r$$

$$s = 2r^2_p(\rho_p - \rho_m)/ 9\eta$$

- The sedimentation coefficient depends on temperature, density, the viscosity of the solution, and the shape and mass of the particle.
- For this reason it is corrected to standard conditions (water at 20 °C): **the standard sedimentation coefficient (s20,w).**

- *the sedimentation coefficients of most biological structures are very small, so by convention a specific unit is adopted for biological particles, the* ***Svedberg (S)****, which equals* 10^{-13} *seconds (****1 S =*** 10^{-13} ***s.).***

Sedimentation time: ***t = distance sedimented / v***

Centrifugation techniques - Sedimentation

SVEDBERG SEDIMENTATION COEFFICIENTS (S)

Biological structures	Svedberg (S)
Enzymes – Hormones – Soluble proteins	2–25
Nucleic acids	3–100
Polysomes	20–220
Viruses	40–1,000
Lysosomes	4,000
Membranes	100–100,000
Mitochondria	20,000–70,000
Nuclei	4×10^{5}–4×10^{7}

- In general, a larger particle has a higher Svedberg value and therefore a higher sedimentation velocity.
- Centrifugation can be used to separate molecules with different sedimentation coefficients, as reported in the *table*

Centrifugation techniques - Types of centrifuge

What each one is used for, ordered by increasing g-force — from collecting whole cells to isolating and characterising single macromolecules.

Bench-top / clinical

low speed

Collect cells & coarse precipitates

e.g. blood cells; refrigerated to protect proteins.

Microfuge

~10,000 × g

Quick pelleting of small samples

Eppendorf tubes in minutes; also concentrates dilute proteins.

Preparative bench-top

3,000–7,000 × g

Batch separations at scale

whole cells & coarse precipitates; 5–250 mL tubes or 96-well plates.

High-speed refrigerated

~100,000 × g

Sediment organelles & microbes

nuclei, mitochondria, chloroplasts, debris; continuous-flow harvesting.

Preparative ultracentrifuge

up to 900,000 × g

Isolate the smallest particles

microsomes, ribosomes & density-gradient separations.

Analytical ultracentrifuge

live optics

Characterise macromolecules

purity, s-value, molar mass & ligand binding (live optics).

The two ultracentrifuge tiers are detailed on the next slide.

Centrifugation techniques - Types of centrifuge

Ultracentrifuges in detail

Preparative ultracentrifuge

Purpose: isolate & purify particles — collected and used after the run.

USED FOR

- **Pellet subcellular fractions** — microsomes, ribosomes, membranes.
- **Purify biomolecules** — viruses, nucleic acids, proteins.
- **Gradient separations** — differential, density-gradient / zonal, isopycnic.
- **Bulk preparation** — handles large volumes; no optical readout.

Sealed, evacuated & refrigerated · up to ~900,000 × g

Analytical ultracentrifuge

Purpose: measure macromolecule properties in solution — watched during the run.

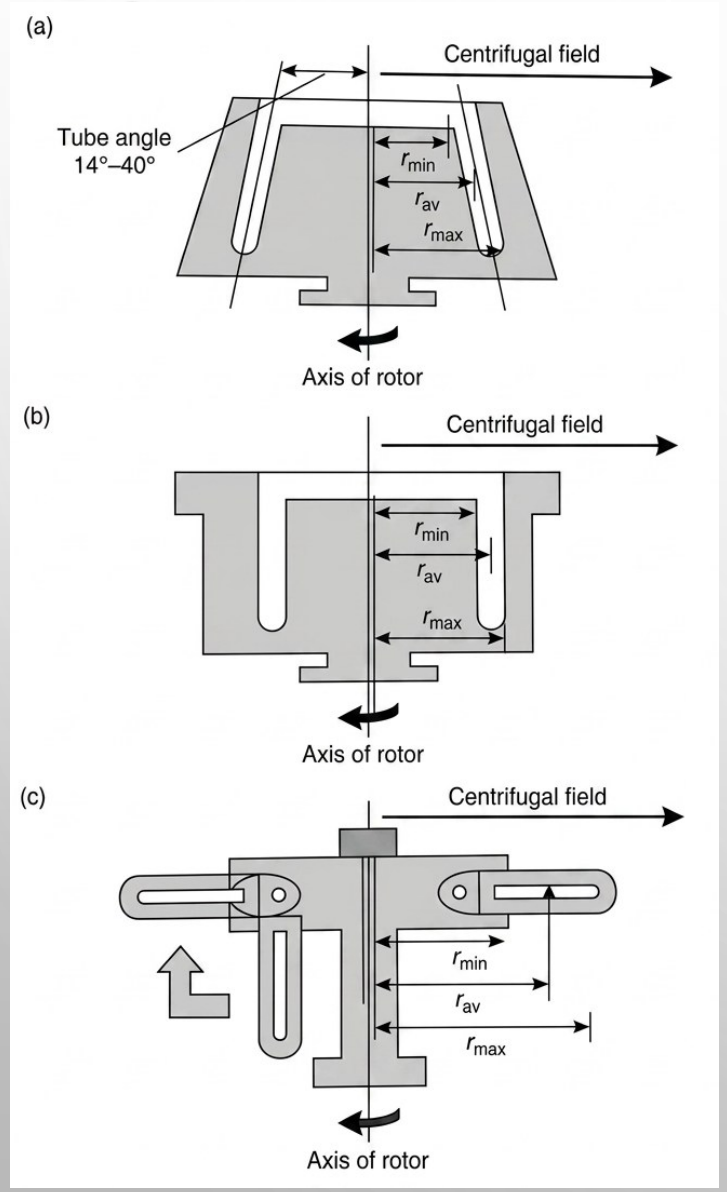
USED FOR

- **Purity & heterogeneity** of the sample.
- **Size & mass** — sedimentation coefficient (s), molar mass & distribution.
- **Molecular behaviour** — conformational changes & ligand-binding studies.
- **Real-time optics** — UV/Vis absorption or Rayleigh interference.

Solid rotor: 1 counterbalance + 3 or 7 cells · tiny volumes

Centrifugation techniques - Types of rotor

Three geometries differ in tube angle, path length and band resolution.



a) Fixed-angle

14°–40° to vertical

Short radial path to the tube wall. Ideal for pelleting in differential separation (nuclei, mitochondria, microsomes) and for isopycnic banding.

b) Vertical tube

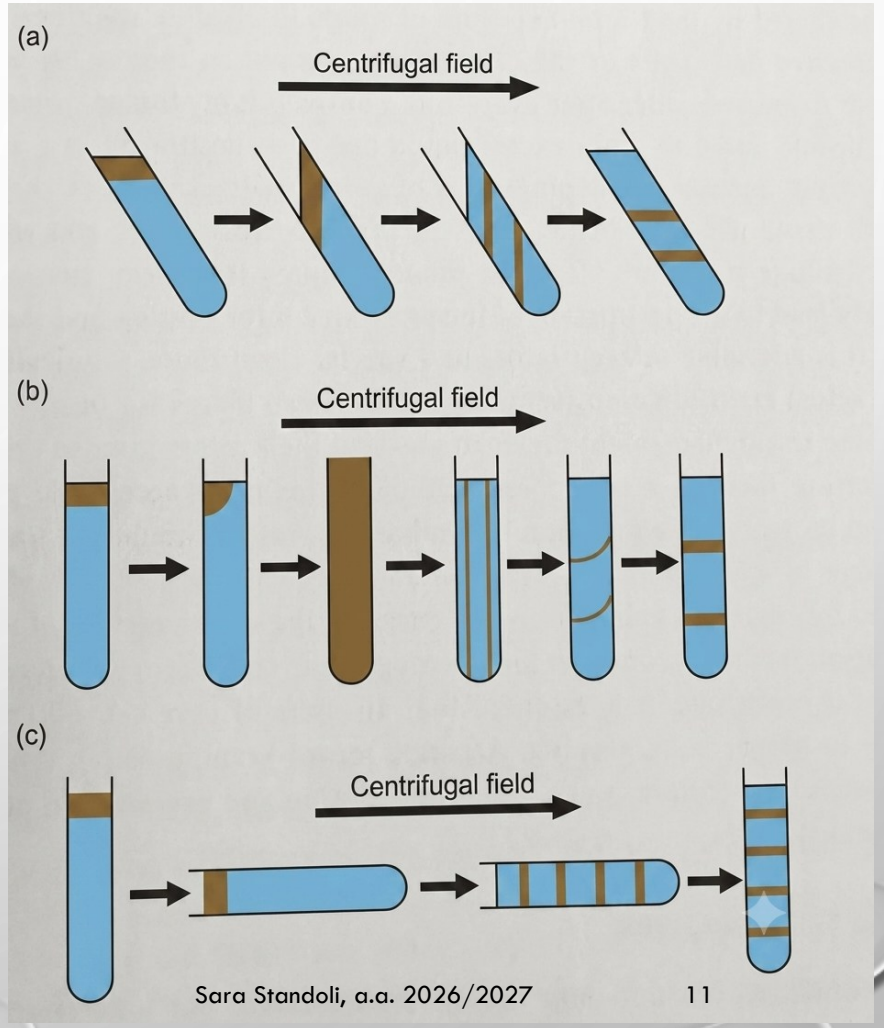
‖ to axis (0°)

Tubes parallel to the axis; separation runs across the tube diameter → much shorter isopycnic times. Near-vertical (7°–10°, quick-seal) are faster still.

c) Swinging-bucket

swings to horizontal

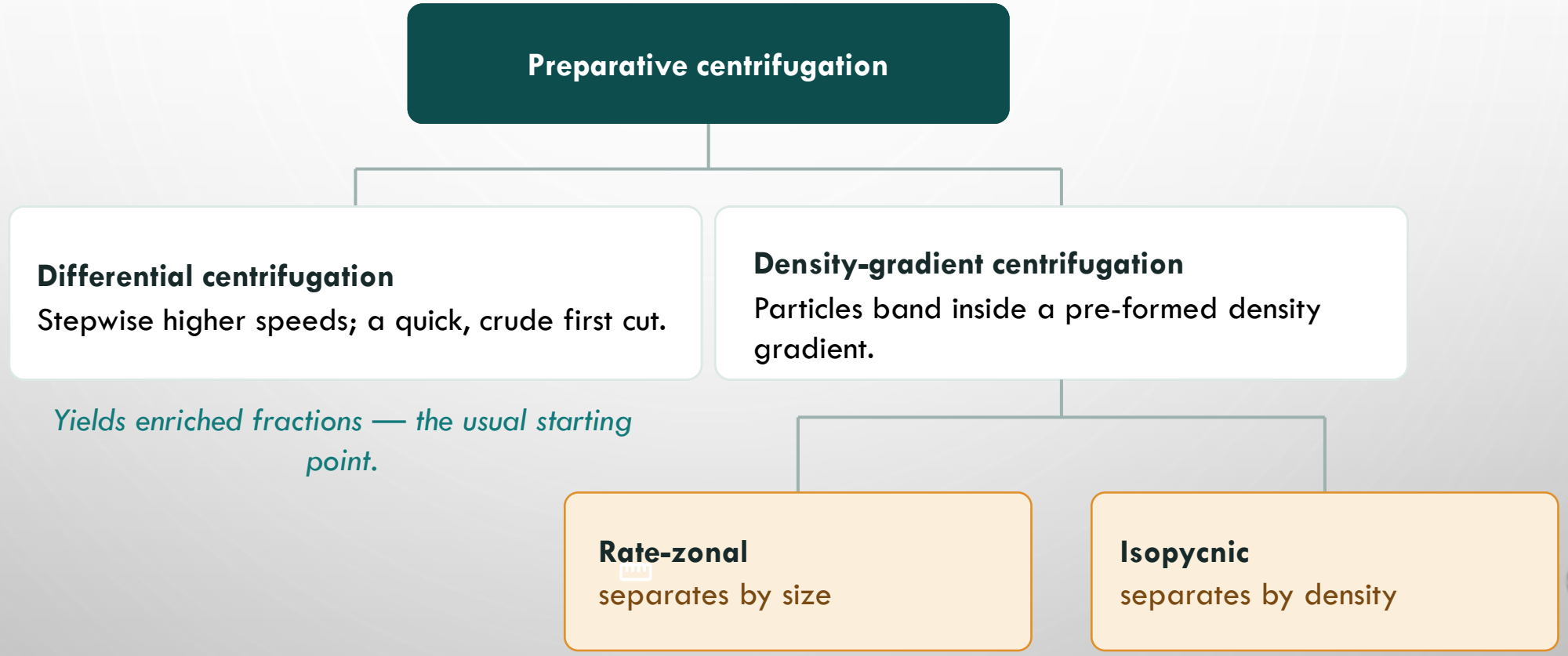
Buckets swing out horizontally when spinning. Best band resolution and widest gradient choice — the method of choice for rate-zonal separation by s-coefficient.



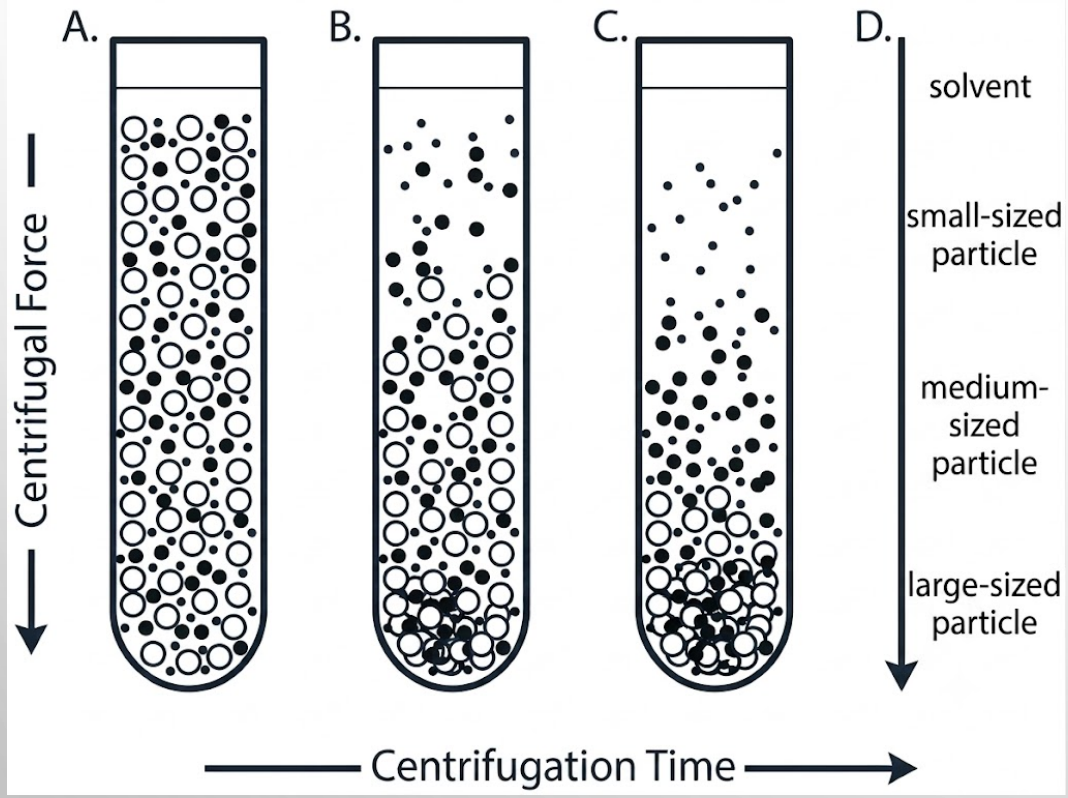
Centrifugation techniques - Preparative Centrifugation

Separating cells and subcellular structures — differential & density-gradient methods

Every method sorts particles by a physical property — their size, their density, or both.



Centrifugation techniques - Differential Centrifugation



- **Sorts by size & density →differential *pellet:*** larger, denser particles sediment faster.
- **Stepwise increasing g:** each step pellets the next-heaviest class.
- **Pellet + supernatant:** the pellet is kept; the supernatant is re-spun.
- **Crude & fast:** ideal for harvesting cells or a first bulk cut.

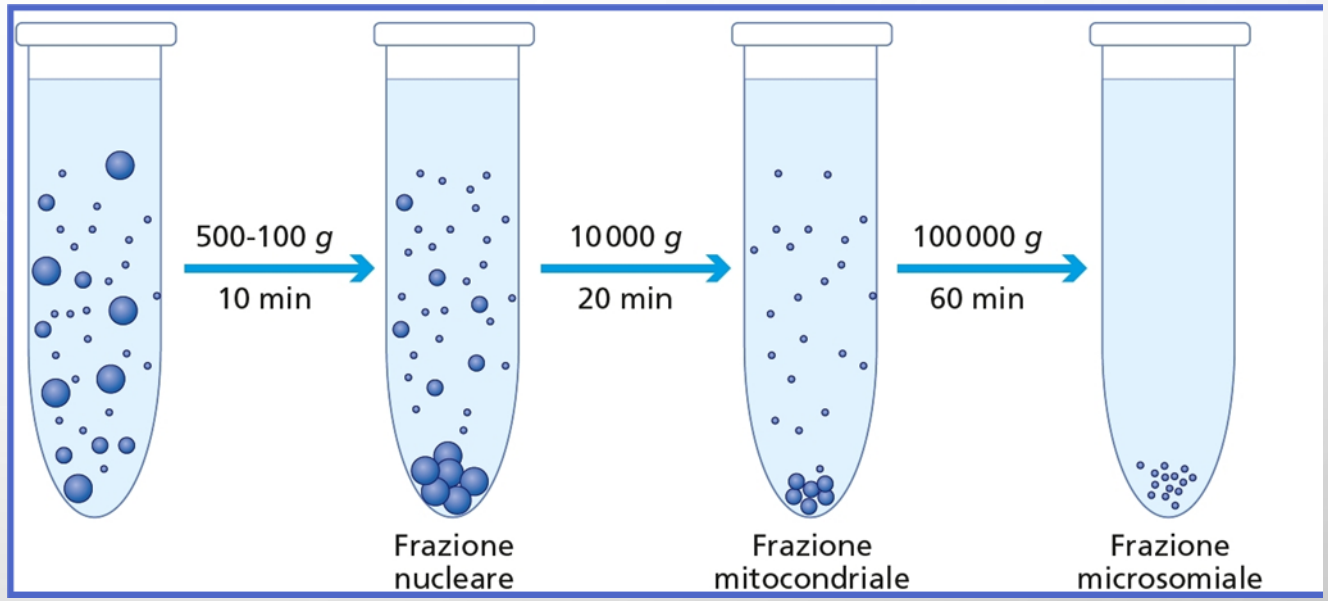
- **Enriched, not purified:** fractions are concentrated in a component, not clean.
- **Contamination & low yield:** small particles get trapped in the pellet.

- **Wash the pellet:** resuspend & re-spin to reduce carry-over (lowers yield).
- **A first step:** usually precedes gradient (zonal / isopycnic) methods.

Centrifugation techniques - Differential Centrifugation

Differential centrifugation produces enriched fractions rather than purified fractions.

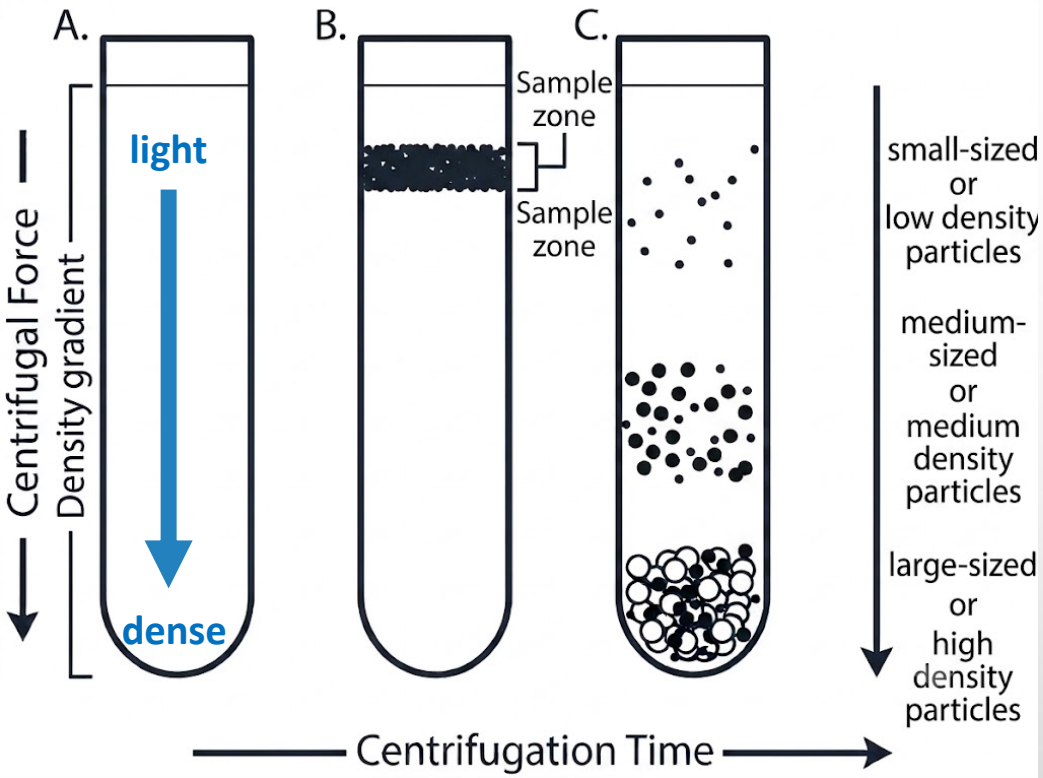
Spin	Pellet — enriched in
1,000 × g · 10 min	Nuclei & cell debris
10,000 × g · 10 min	Contractile apparatus (myosin/actin)
20,000 × g · 20 min	Mitochondria-enriched fraction
100,000 × g · 1 h	Microsomes (pellet) vs cytosol (supernatant)



Progressively higher speed & longer time → each spin pellets a smaller, lighter class of particle.

Centrifugation techniques - Density Gradient Centrifugation

The sample is layered on top of a pre-formed gradient (e.g. sucrose 20 → 70%), then spun.



- **Step or continuous:** stack layers by hand, or use a gradient-maker.
- **Two modes:** rate-zonal (by size) or isopycnic (by density).
- **Gentle handling:** keep cold; move slowly so layers don't mix.

Common gradient media

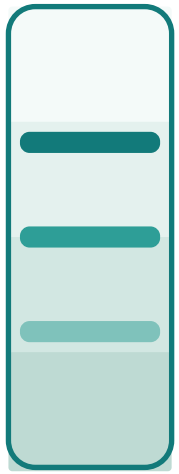
Medium	Typical use
Sucrose	Membrane vesicles & organelles
Caesium chloride	DNA banding, plasmids, viruses
NaBr / NaI	Lipoproteins; DNA / RNA
Percoll, Ficoll, Nycodenz	Whole cells & subcellular particles

Centrifugation techniques - Density Gradient Centrifugation
Rate-Zonal *vs* Isopycnic

Rate-zonal

by size

The sample is layered as a narrow band onto a density gradient to avoid the problem of contamination by particles with different sedimentation velocities.

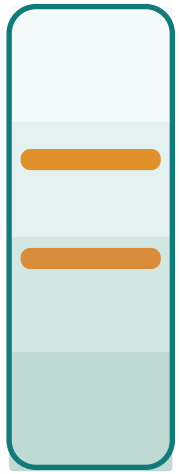


- **Separates by size** (mass & shape), not density.
- **Thin sample band on top** load kept small (~10% of volume).
- **Time-critical** stop before bands hit the bottom.
- **Gradient < particle density** so particles keep moving.
- **Limits.** The loading zone, which is normally narrow, limits the sample volume (typically to 10% of the total) that can be accommodated by the density gradient.

Isopycnic

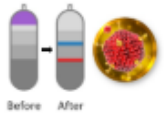
by density

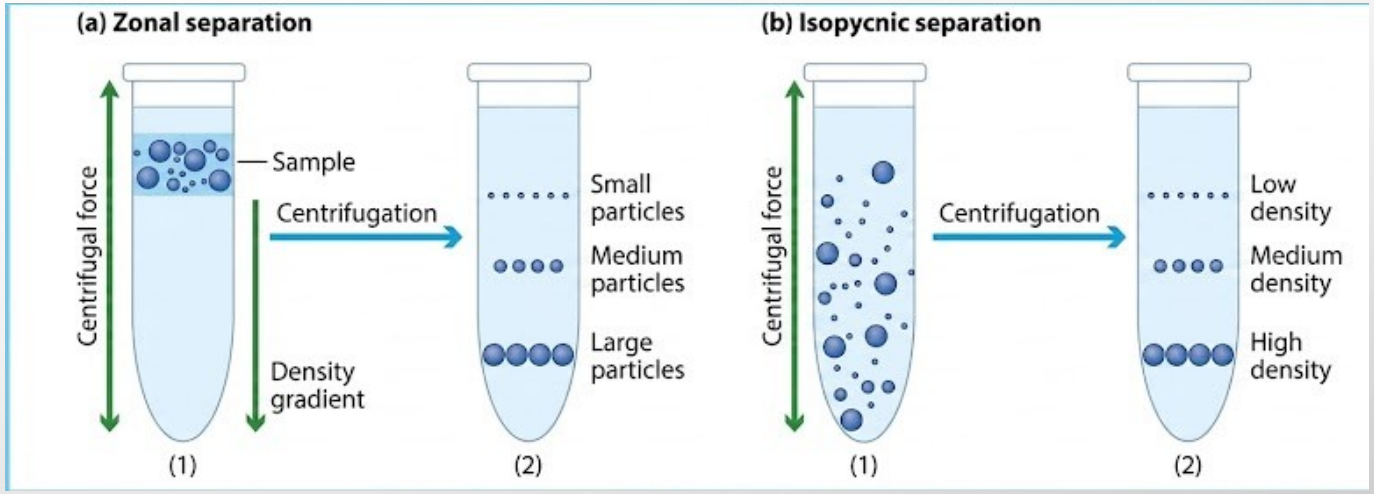
Starting from a uniform mixture of sample and density gradient under centrifugal force, the particles will move until their density equals that of the surrounding medium;



- **Separates by density only** size just sets how fast they get there.
- **Bands at buoyant density** where ρ(particle) = ρ (medium).
- **Never pellets** position is stable at any run time.
- **Medium's max density > densest particle** also called equilibrium / flotation - particles never settle at the bottom of the tube, regardless of the centrifugation time, but instead come to rest at their isopycnic point.

Centrifugation techniques - Density Gradient Centrifugation Rate-Zonal *vs* Isopycnic

	Pelleting	Rate Zonal	Isopycnic
Separation Basis	Materials separate by s-value (size and mass) in any solution	Materials separate by s-value (size and mass) in a pre-formed density gradient	Materials separate by buoyant density in a self-forming (continuous) density gradient
Typical Gradient	No density gradient is used	Continuous gradient (e.g., linear sucrose gradients)	Continuous gradient (e.g., CsCl gradients)
Common Gradient Material(s)	N/A	Sucrose, Iodixanol	CsCl, Iodixanol
Advantage	Fast and simple separation of materials with very different s-values	High resolution rate-based separation of materials with similar s-values	Highest resolution separation by density
			



Centrifugation techniques - Density Gradient Centrifugation

Choosing the gradient medium

An ideal medium spans the density range you need — yet leaves the sample untouched.

It should provide: enough solubility to cover the required density range, forming a stable gradient.

BUT IT MUST NOT

Turn viscous

become too viscous across the working density range.

Shift osmolarity

be hyper- or hypo-osmotic for osmotically sensitive particles.

Perturb biology

alter the biological activity of the sample.

Be toxic

be toxic to, or metabolised by, the cells.

Disturb assays

interfere with downstream assays.

Attack the tubes

react with the centrifuge tubes.

Centrifugation techniques - Density Gradient Centrifugation

How pure is the fraction? Marker enzymes

Because differential fractions are only enriched, purity is judged with organelle-specific markers.

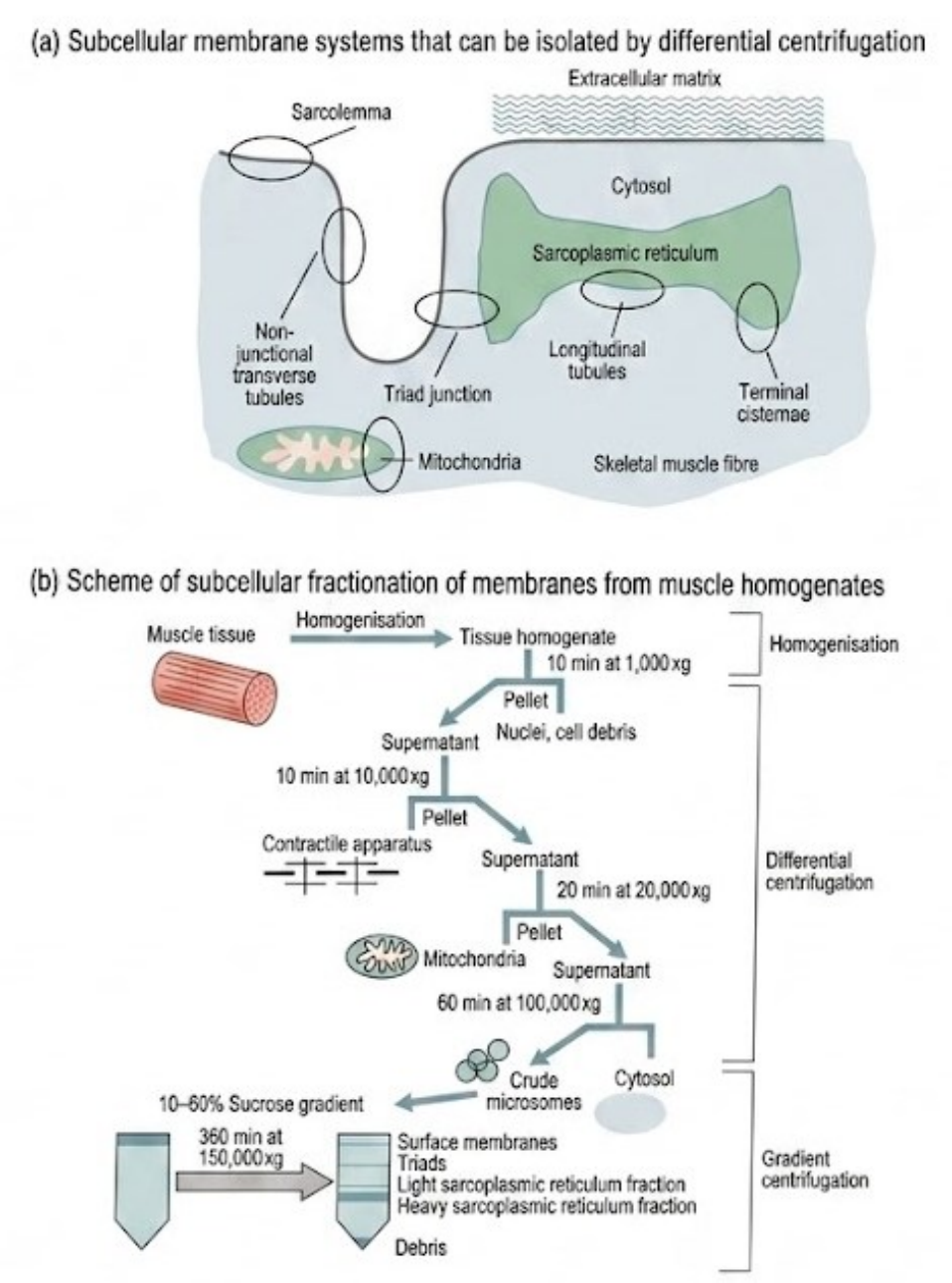
Specific activity = *enzyme units / mg protein.*

A fraction is enriched when its marker's specific activity is higher than in the starting homogenate.

Cross-contamination is unavoidable. For truly pure preparations, follow up with affinity methods (e.g. immuno-affinity).

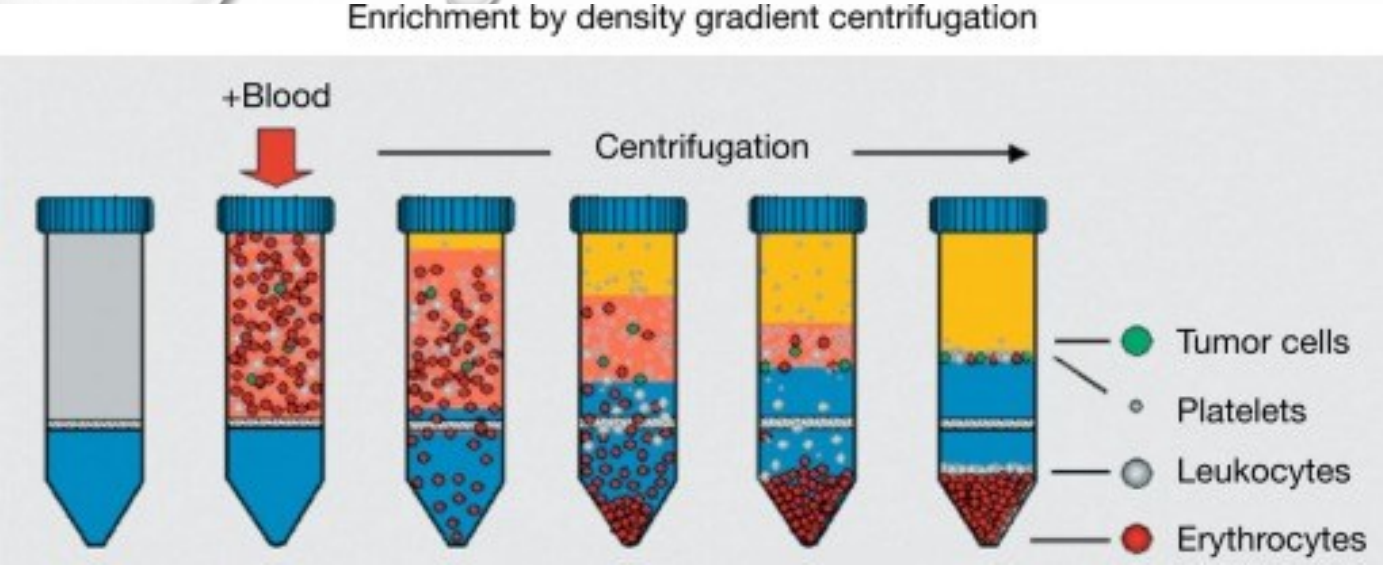
Classic subcellular markers

Fraction	Marker
Plasma membrane	5'-nucleotidase; Na^+/K^+-ATPase
Mitochondria	Succinate dehydrogenase; cytochrome c oxidase
Lysosomes	Acid phosphatase
ER / microsomes	Glucose-6-phosphatase
Cytosol	Lactate dehydrogenase (LDH)
Nucleus	DNA content



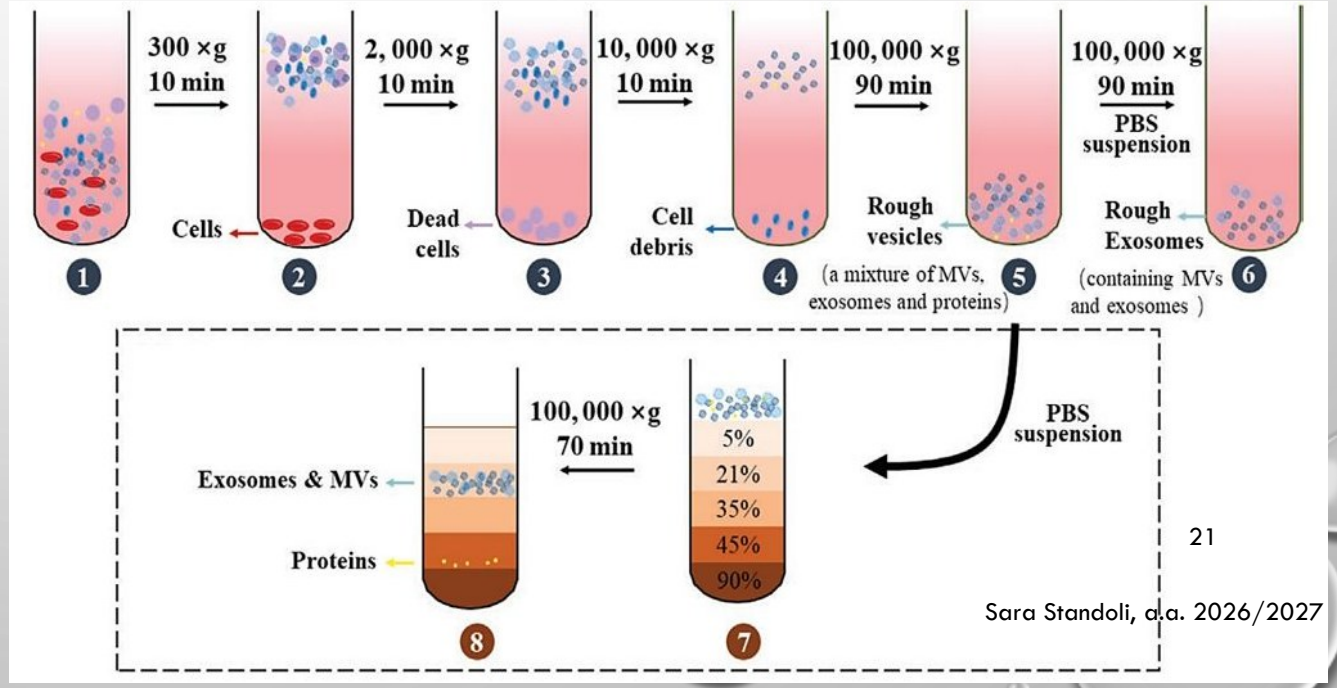
Scheme of the fractionation of skeletal muscle homogenate into various subcellular fractions.
Shown is a diagrammatic presentation of the subcellular membrane system from skeletal muscle fibres (a) and a flow chart of the fractionation protocol of these membranes from tissue homogenates using differential centrifugation and density gradient methodology (b),

Examples of use



Blood cells isolation

Cellular vesicles isolation



Analytical ultracentrifugation: measuring, not collecting

A high-speed ultracentrifuge (up to ~200,000 × g) with optics that record sedimentation in real time — in the native solution.

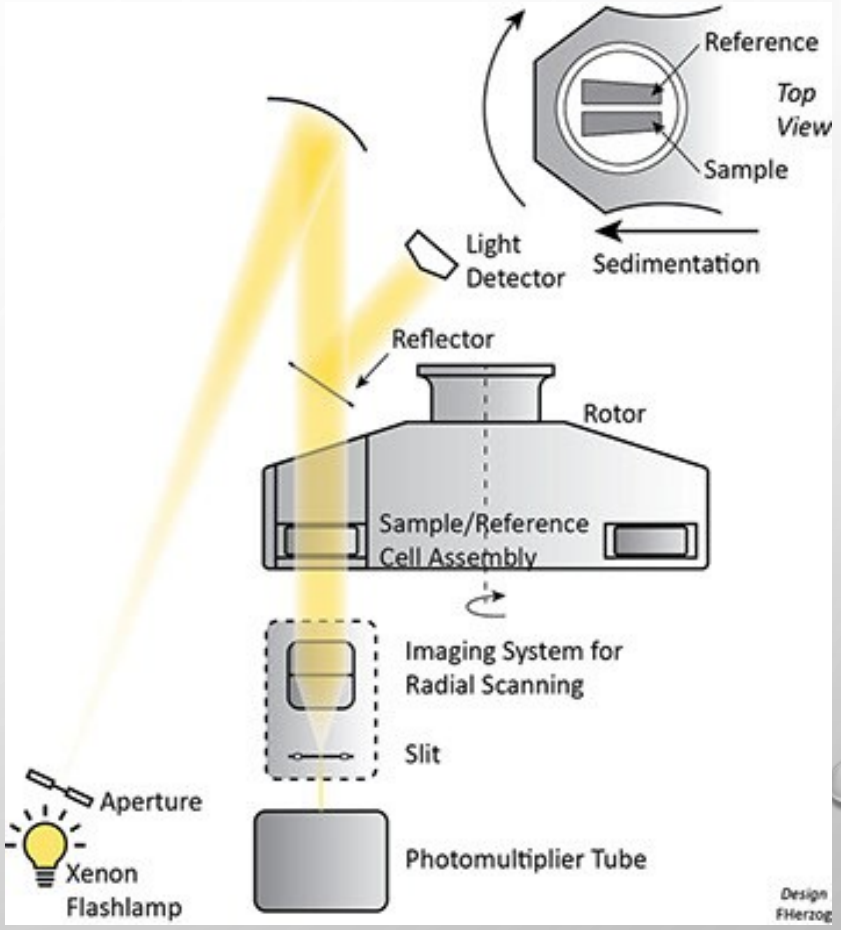
Why so much g: random thermal motion keeps molecules evenly dispersed under gravity — only an intense centrifugal field makes them sediment measurably, while the transparent cell lets light read the concentration.

Absorption optics (UV/Vis)

Reads concentration from **light absorbance**. A xenon flash lamp covers ~190–800 nm and detects very dilute proteins or nucleic acids (even below 230 nm). *e.g. Beckman Optima XL-A.*

Rayleigh interference optics

Reads concentration from the **refractive-index difference** vs the solvent — works for proteins, nucleic acids, polysaccharides, glycoproteins & synthetic polymers. *e.g. Optima XL-I (has both).*



Combine them to study interactions; rarer systems use fluorescence or Schlieren (refractive-gradient) optics.

Analytical ultracentrifugation: measuring, not collecting

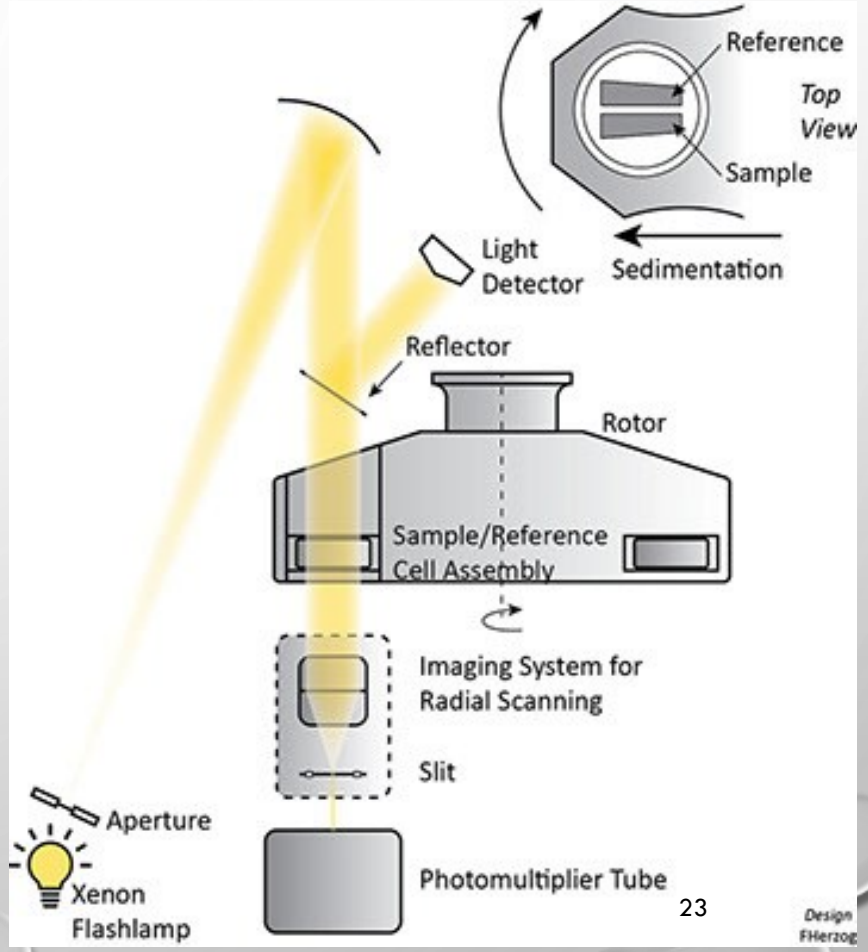
Functioning

- The rotor chamber features a lower lens and an upper lens; the latter is connected to a camera lens that projects light onto a photographic plate. Light from a specific source passes through the bottom of the rotor;

- The monitoring mechanism is based on the principle of ultraviolet absorption: if the solute concentration is uniform, the light passes through the solution without deviation; however, if the solution under examination contains zones of varying density, the light is refracted at the boundaries between these zones.

- By measuring the refractive index difference between the reference solvent and the solution under examination, it is possible to determine the solute concentration at any given point.

- In more recent models, the photographic plate system has been replaced by an electronic scanning system capable of directly measuring and plotting the sample concentration throughout the analytical cell.



Analytical ultracentrifugation: velocity vs equilibrium

Run the same sample two ways for complementary answers — its shape & heterogeneity, or its mass.

Sedimentation velocity

high speed

Reads: shape & heterogeneity

- **A boundary forms** between solution and cleared solvent.
- **Boundary speed → s** the sedimentation coefficient (size + shape).
- **Heterogeneous sample** gives a distribution of s-values.
- **High resolution** aggregation, oligomeric state, conformation.

Sedimentation equilibrium

low speed

Reads: molar mass

- **No boundary** sedimentation is balanced by back-diffusion.
- **Equilibrium after hours** no net motion, so no friction term.
- **Depends only on mass** an "absolute" molar-mass method.
- **Self-association** stoichiometry & strength; interactions.

Complementary: velocity reports shape & heterogeneity; equilibrium reports (average) molar mass.

Analytical ultracentrifugation: what it tells you

From the sedimentation records, a lot can be read about a macromolecule in its native state.

Most common uses:

- **Purity & oligomeric state** from the distribution of s (velocity).
- **Molar mass & distribution** in the native state (equilibrium).
- **Complex assembly** changes in mass of supramolecular complexes.
- **Conformation** and conformational changes (velocity).
- **Ligand binding** stoichiometry & strength of interactions.

Uses in Pharmaceutical Industry:

- **Production of bulk drugs** After crystallization, drugs are separated from the mother liquor by centrifugation (e.g., aspirin crystals);
- **Production of biological material** Most biological products consist of proteins and other macromolecules. During production, they remain as a colloidal dispersion in aqueous solution (e.g., blood cells are separated from plasma via centrifugation);
- **Evaluation of suspensions and emulsions** Sedimentation is a common issue with suspensions, just as creaming is with emulsions. To accelerate sedimentation—and consequently creaming—the suspension or emulsion is subjected to centrifugation at speeds ranging from 200 to 3000 g;
- **Determination of polymer molecular weight** Polymer molecular weights can be determined via ultracentrifugation. Measuring the sedimentation rate allows for the determination of solute molecule size and molecular mass.

Sedimentation coefficient $s^\circ_{20,w}$: rises with size (for near-globular particles $s \propto M^{\wedge 2/3}$). Unit: 1 S = 10^{-13} s.

Typical $s^\circ_{20,w}$ values

Species	s (Svedberg)
Many soluble proteins / nucleic acids	1–20 S
Ribosomes, microsomes, mitochondria	80 – several thousand S

Example: the dystrophin–glycoprotein complex was sized at ~18 S (between β-galactosidase 16 S and thyroglobulin 19 S).