Swayam Course - Analytical Techniques

Week: 2, Module 6 - Differential Centrifugation for Subcellular Fractionation

Content Writer - Dr. Amar Preet Kaur, Senior Resident, Department of Biochemistry, All India Institute of Medical sciences, New Delhi.

Objectives:

a. To study the principle of centrifugation and differential centrifugation
b. To understand the significance of subcellular fractionation
c. To study types of cell fractionation methods
d. Characterization & detection

1. Introduction

Centrifugation is a technique used for the separation of particles from a solution according to their size, shape, density, viscosity of the medium and rotor speed.
The particles are suspended in a liquid medium and placed in a **centrifuge tube**. The tube is then placed in a **rotor** and spun at a definite speed.
Separation through sedimentation could be done naturally with the earth gravity, which would take ages. **Centrifugation** is making that natural process much faster. Rotation of the rotor about a central axis generates a **centrifugal force** upon the particles in the suspension.

2. Factors affecting centrifugation :

- Density of both samples and solution
- Temperature/viscosity
- Distance of particles displacement
- Rotation speed

3. Types of centrifuge procedures:

There are two types of **centrifuge procedures**;

3.1 Preparative, the purpose of which is to isolate specific particles, and

3.2 Analytical, which involves measuring physical properties of the sedimenting particles.

4. Velocity of sedimentation

As a rotor spins in a **centrifuge**, a centrifugal force is applied to each particle in the sample; the particle will then sediment at the rate that is proportional to the centrifugal force applied to it. The viscosity of the sample solution and the physical properties of the particles also affect the sedimentation rate of each particle.

At a fixed centrifugal force and liquid viscosity, **the sedimentation rate of a particle is proportional to its size** (molecular weight) and to the difference between the particle density and the density of the solution.

$$v = \frac{d^2 (p - L)\, 3\, CF}{18\, n}$$

Where,

v = sedimentation rate or velocity

d = diameter

p = particle density

L = medium density

n = viscosity of medium

CF = Centrifugal force

From the above equation following important behaviours of particles can be explained:

1. The rate of particle sedimentation is proportional to the particle size.

2. The sedimentation rate is proportional to the difference in density between the particle and the medium. In a solution, **particles whose density is higher than that of the solvent sink** (sediment), and particles that are lighter than it float to the top.

The greater the difference in density, the faster they move. If there is no difference in density (isopyknic conditions), the particles stay steady.

4. The sedimentation rate decreases as the medium viscosity increases.

5. The sedimentation rate increases as the gravitational force increases.

5. Forces acting on a sedimenting particle:

Two forces counteract the centrifugal force acting on the suspended particles (Figure 1):

- **Buoyant force**: force with which the particles must displace the liquid media into which they sediment.
- **Frictional force**: force generated by the particles as they migrate through the solution.

Particles move away from the axis of rotation in a centrifugal field only when the centrifugal force exceeds the counteracting buoyant and frictional forces resulting in sedimentation of the particles at a constant rate.

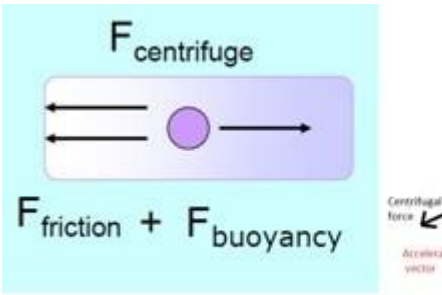

Figure 1: Forces acting on a particle during centrifugation

6. Relative Centrifugal Force:

The force on the particles (compared to gravity) is called Relative Centrifugal Force (RCF). For example, an RCF of 500 x g indicates that the centrifugal force is 500 times greater than earthly gravitational force. RCF is dependent on the speed of rotation in rpm and the distance of the particles from the center of rotation. When the speed of rotation is given in rpm and the distance (r) is expressed in centimeters, RCF can be calculated by using the formula.

$RCF = 1.11 \times 10^{-5} \times (rpm)^2 \times r$

7. Types of Centrifugation Separations

There are two types of centrifugal techniques for separating particles, differential centrifugation and density gradient centrifugation.

Density gradient centrifugation can further be divided into rate-zonal and isopycnic centrifugation.

7.1 Differential Centrifugation The simplest form of separation by centrifugation is differential centrifugation. Particles of different densities or sizes in a suspension will sediment at different rates, with the larger and denser particles sedimenting faster. These sedimentation rates can be increased by using centrifugal force. A suspension of cells subjected to a series of increasing centrifugal force cycles will yield a series of pellets containing cells of decreasing sedimentation rate. Particles of different densities or size will sediment at different rates with the largest and most dense particles sedimenting the fastest followed by less dense and smaller particles. Differential pelleting is commonly used for harvesting cells or producing crude subcellular fractions from tissue homogenate. For example, a rat liver homogenate containing nuclei, mitochondria, lysosomes, and membrane vesicles that is centrifuged at low speed for a short time will pellet mainly the larger and more dense nuclei. Subsequent centrifugation at a higher centrifugal force will pellet particles of the next lower order of size (e.g., mitochondria) and so on. Due to the heterogeneity in biological particles, differential centrifugation suffers from contamination and poor recoveries which can be addressed by resuspension and repeating the centrifugation steps (i.e., washing the pellet).

7.2 Density Gradient Centrifugation Density gradient centrifugation is the preferred method to purify subcellular organelles and macromolecules. Density gradients can be generated by placing layer after layer of gradient media such as sucrose in a tube with the heaviest layer at the bottom and the lightest at the top. The cell fraction to be separated is placed on top of the layer and centrifuged. Density gradient separation can be classified into two categories, rate – zonal (size) separation and isopycnic (density) separation.

7.2.1 Rate-Zonal Centrifugation In rate-zonal centrifugation the problem of cross-contamination of particles of different sedimentation rates may be avoided by layering the sample as a narrow zone on top of a density gradient. In this way the

faster sedimenting particles are not contaminated by the slower particles as occurs in differential centrifugation. However, the narrow load zone limits the volume of sample that can be accommodated on the density gradient. The gradient stabilizes the bands and provides a medium of increasing density and viscosity. Under centrifugal force, particles move at different rates depending on their mass. The speed at which particles sediment depends primarily on their size and mass instead of density. As the particles in the band move down through the density medium, zones containing particles of similar size form as the faster sedimenting particles move ahead of the slower ones. Because the density of the particles is greater than the density of the gradient, all the particles will eventually form a pellet if centrifuged long enough.

7.2.2 Isopycnic Centrifugation In isopycnic separation, also called buoyant or equilibrium separation, particles are separated solely on the basis of their density. Particle size only affects the rate at which particles move until their density is the same as the surrounding gradient medium. The density of the gradient medium must be greater than the density of the particles to be separated. By this method, the particles will never sediment to the bottom of the tube, no matter how long the centrifugation time. Upon centrifugation, particles of a specific density sediment until they reach the point where their density is the same as the gradient media (i.e., the equilibrium position). The gradient is then said to be isopycnic and the particles are separated according to their buoyancy. Since the density of biological particles is sensitive to the osmotic pressure of the gradient, isopycnic separation may vary significantly depending on the gradient medium used.

8. Subcellular Fractionation

Subcellular fractionation is a technique used to fractionate/isolate various organelles or macromolecular complexes from cells. Separation technique uses a rotating machine (centrifuge) to generate centrifugal force to separate solids from liquids (or 2 immiscible liquids) by the application of centrifugal force.

8.1 Sedimentation coefficient (s): The sedimentation coefficient of a particle is used to characterize its behaviour in sedimentation processes. The sedimentation coefficient has the dimensions of a unit of time and is expressed in svedbergs. One

svedberg is defined as exactly 10^{-13} s. It is defined as the ratio of a particle's sedimentation velocity to the acceleration that is applied to it (causing the sedimentation). Sedimentation coefficient is influenced by shape, size, and density of particle and hence is commonly used to characterize a particular molecule or structure (Table 1 and figure 2). Generally, larger the molecule or particle, the larger is the Svedberg unit and hence the faster is the sedimentation rate.

Particles/molecules	Svedberg unit (S)
Enzymes, peptide hormones and soluble proteins	2-25
Nucleic acids	3-100
Ribosomes, polysomes	20-200
viruses	40-1000
Mitochondria	20-70 X 10^3
Nuclei	4 X 10^7

Table 1: Svedberg unit of various cellular organelles

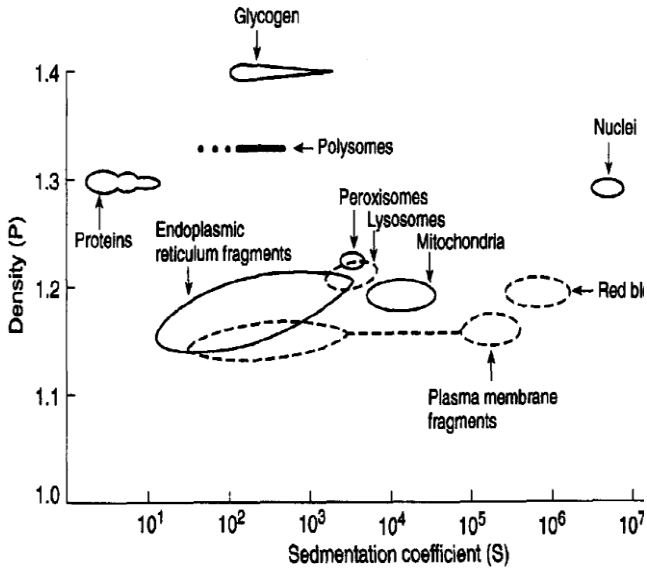

Figure 2: Sedimentation coefficient of various cellular organelles

8.2 Methods for subcellular fractionation

Figure 2 shows the organelles and cellular structures commonly found within higher organisms and plants. The approaches used to isolate target organelles from the rest of the cellular compartments depend on biochemical methods to isolate the diverse organelles found in cells. However, the complete global profiling of organelle proteomes demands robust sample preparation and organelle enrichment methods to ensure the correct annotation of protein distribution. Such classical approaches have the disadvantage of co-purifying contaminants. Recent developments in

methodological and technological advances have the potential to yield organelles of higher purity, and as technologies progress, the effectiveness will improve, albeit at a cost of higher complexity or prohibitive resource requirements. Here we discuss both classical and emerging techniques.

8.2.1 Conventional methods

Specific proteomes of cellular compartments and organelles can be isolated via several approaches, and the most commonly used strategy is subcellular fractionation. The concept of subcellular fractionation was pioneered by De Duve and coworkers in 1955 using rat liver tissue. The procedure for subcellular fractionation first involved disruption of cells and tissues using physical methods such as mechanical or liquid shear or non-physical methods such as detergents or hypo-osmotic shock. The cell disruption step aimed to obtain high protein yield, and maintain structural and functional integrity of intracellular organelles. The efficiency of cell disruption is often monitored with immunochemical and microscopic studies. Conventional methods involved in subcellular fractionation, typically by differential and density-gradient centrifugation, employed a series of centrifugation steps to separate different populations of cellular compartments or organelles from cell homogenates based on their mass and/or density.

8.2.1.1 Differential centrifugation

Differential centrifugation operates via sequential centrifugation of the cell or tissue homogenate to obtain subcellular organelles such as nuclei, mitochondria and lysosomes. This separation method is based on differences in size and density, whereby larger and denser organelles sediment at lower centrifugal forces. However, this method has poor resolving power and thus may result in fractions containing a different organelle type that has similar sedimentation velocities.

8.2.1.2 Density-gradient centrifugation

The more commonly applied density-gradient centrifugation method separates organelles based on continuous or discontinuous gradients using various media, such as sucrose of different osmolarities, viscosities or densities. In general, the cell homogenate or post-nuclear supernatant is first added to the top of the medium and centrifuged. Upon centrifugation, the organelle focuses in the gradient where its

density equals the density of the surrounding medium (isopycnic point). Sucrose is the most commonly used medium for density-gradient centrifugation as sucrose is biologically inert, inexpensive and dialyzable. Other media include Ficoll, Percoll, Nycodenz and Metrizamide

8.2.2 Emerging methods

8.2.2.1 Free-flow electrophoresis

Free-flow electrophoresis (FFE) offers an alternative method to isolate and fractionate organelles such as peroxisomal membranes, mitochondria, secretory vesicles, plasma membrane vesicles and peroxisomes. Organelle isolation by FFE is based on separation by their net global isoelectric charges or electrophoretic mobilities. During FFE, the carrier buffer is flowing continuously and laminarly in a narrow gap between two cooling plates, with an electric field being applied perpendicularly to the laminar flow of the buffer and sample, thus resulting in the differential deflections of the sample molecules (Figure 3).

Advantages:

1. wide choice of pH,
2. high sample recoveries
3. the purified organelles retain their intactness and functionality, which makes FFE an attractive technique not only for proteomic analyses but also complementary functional studies.

   Disadvantages:

1. Comigration of organelles or cellular structures having similar isoelectric points. For example, in the FFE separation of neutrophil secretory vesicles, contaminations with ER and mitochondria are evident, probably as a result of the similarity in isoelectric points. Similarly, mitochondrial and microsomal proteins can be found in peroxisomal preparations and hence other orthogonal approaches are required to resolve them.

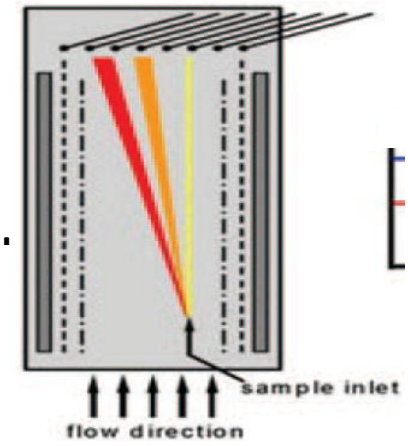

Figure 3: Free-flow electrophoresis

8.2.2.2 Immunoaffinity purification

Immunoaffinity purification is a powerful method to isolate organelles with specificity and in adequate yields. It is based on the principle of binding between ligands (antibodies) immobilized on solid supports and targets (organelle of interest) (Figure 4).

Advantages:

1. High specificity

Disadvantages:

1. Expensive
2. Time consuming.

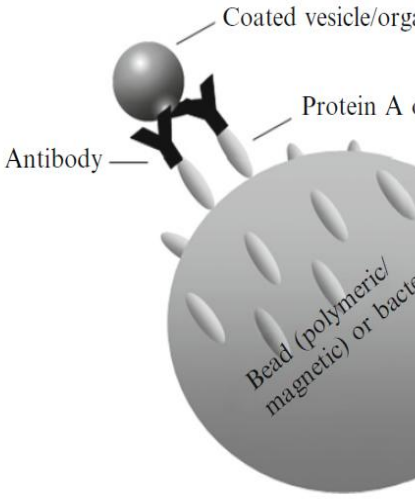

Figure 4: Immunoaffinity purification

8.2.2.3 Fluorescent-assisted organelle sorting (FAOS)

Fuorescent-assisted organelle sorting (FAOS) is used to isolate a number of subcellular organelles such as phagosomes, mitochondria, endocytic vesicles and secretory granules. It uses a flow cytometer to detect and sort organelles with specific fluorescence and scattering characteristics (Figure 5). The technique requires organelle specific fluorescent probes including chemical reagents, fluorescently labeled antibodies and fluorescent proteins (only applicable to cell cultures and animal models in which fluorescently proteins have been expressed). There are a few things to observe when sorting organelles of interest. Intact organelles are important for successful sorting and proteomic analysis. A protein marker that is specific to the organelle of interest will be mandatory for their successful sorting.

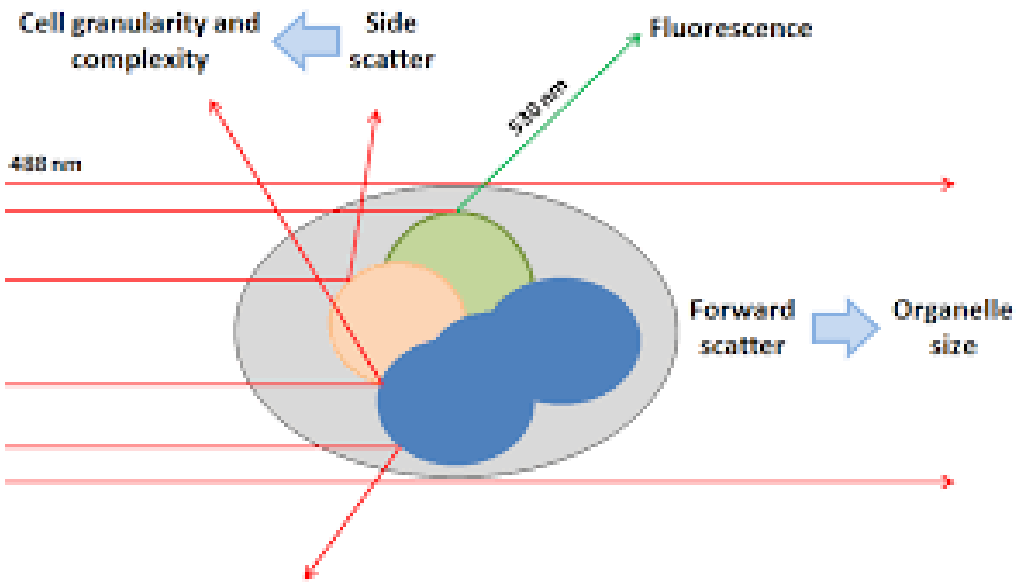

Figure 5: Fluorescent-assisted organelle sorting (FAOS)

9 Characterization & detection

9.1 Classical methods

Classical methods for the characterization of organelles included qualitative methods, such as those based on Western blots and enzymatic marker assays (table 2), and morphological methods such as those based on scanning electron microscopy.

Table 2: Markers for organelles

9.2 Advanced methods

9.2.1 Atomic force microscopy

Atomic force microscopy (AFM) uses a nanometer-size tip to probe and analyze surfaces, thus producing 3D images with sub-molecular resolution. AFM has been successfully applied to study properties of DNA, proteins, bacteria, viruses, cells and organelles.

9.2.2 Optical tweezers

Optical tweezers refers to the use of highly focused laser beams to create force fields capable of capturing and manipulating nanometer- and micrometer-size objects. The Raman spectra of objects that are manipulated with IR laser-based optical tweezers can be obtained without needing a second laser.

9.2.3 Sensors

Quarato et al. (2011) monitored membrane potentials in individual mitochondriam. They deposited isolated mitochondria from mice liver onto a poly-lysine coated glass and incubated the mitochondria with the membrane potential probe tetramethylrhodamine ester (TMRE). The fluorescence signal of each mitochondrion was detected using laser scanning confocal microscopy. By measuring multiple mitochondria, they were able to describe basal heterogeneity of the mitochondria and the effects of substrates and inhibitors on the membrane potential of mitochondria.

9.2.4 Flow cytometry

Flow cytometry is used to detect fluorescent and scattering properties of particles as they are hydrodynamically focused through a laser beam. The technique is high throughput as it can analyze up to thousands particles per second. It has been commonly used to analyze microbes, cells, and organelle.

9.2.5 Capillary electrophoresis techniques

Electrophoretic separations provide the electrophoretic mobilities of organelles, which are determined by their size, surface charge density, and overall morphology as well as the ionic strength of the separation medium. In capillary electrophoresis (CE), the separation takes place in a narrow bore fused silica capillary using high electric fields. CE separations are rapid, have high separation efficiency, and consume small volumes of samples and buffers. Because of its sensitivity, laser induced fluorescence (LIF) has been the detector of choice for CE analysis of fluorescently labeled organelles, making it possible to detect individual organelles.

9.2.6 Microfluidics

Microfluidic technologies have become powerful tools in many fields, such as cell biology, clinical diagnosis and environmental monitoring. Compared to capillary systems, they offer faster separations, higher throughput, and the potential of integration of the whole analysis workflow (i.e. sample preparation, enrichment, separation and detection) on a single platform.

10. Summary

- **Centrifuge:** device for separating particles from a solution according to their size, shape, density, viscosity of the medium and rotor speed
- **Differential centrifugation** is based on the size and density of the particles whereby larger and denser organelles sediment at lower centrifugal forces.
- **Subcellular fractionation** is the process used to separate cellular components while preserving individual functions of each component
- **Steps of subcellular fractionation-** Cell disruption, Organelle fractionation, Characterization and detection
- **Cell disruption methods-** Mechanical and non mechanical
- **Methods of subcellular fractionation-** Differential centrifugation, Density gradient centrifugation, Free flow electrophoresis, Immunoaffinity purification, FAOS (fluorescent-assisted organelle sorting)
- **Characterization & detection**

- **Classical methods-** Enzymatic marker assays, Western blot, Morphological methods.
- **Advanced methods-** Atomic force microscopy, Optical tweezers, Organelle sensors, Flow cytometry, Capillary electrophoresis, Microfluidic approaches.