

Biol 266 - Cell Biology

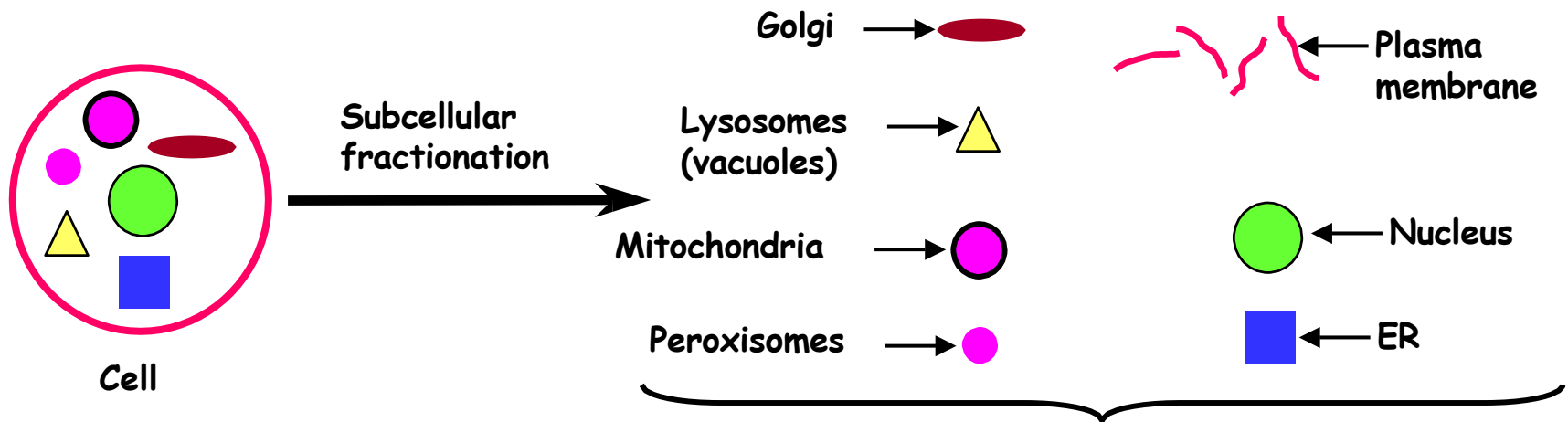
UNIT 2

"How cells are studied - II"

To address many of the questions concerning the function of organelles in the cell, it is necessary to **isolate organelles** of eukaryotic cells in a form that can be used for biochemical studies

Subcellular fractionation

the separation of the cell into its functioning organelles or isolation (purification) of individual organelles from the cell

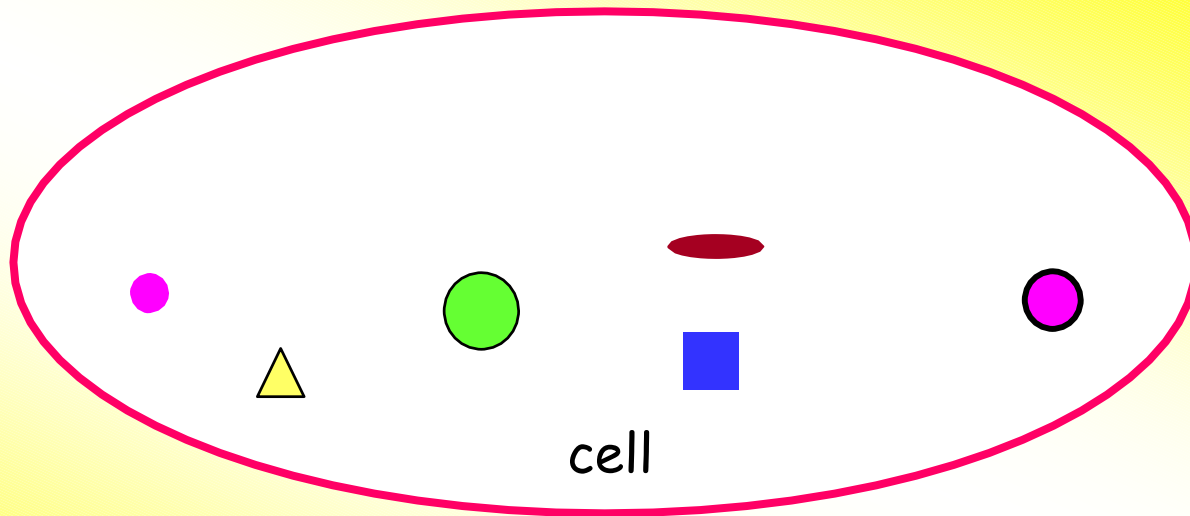


Study:

- 1) protein composition
- 2) lipid composition
- 3) metabolism (biochemical reactions)

- The initial step in subcellular fractionation is the **disruption of the plasma membrane** and the cell wall (if present) under conditions that do not destroy organelles

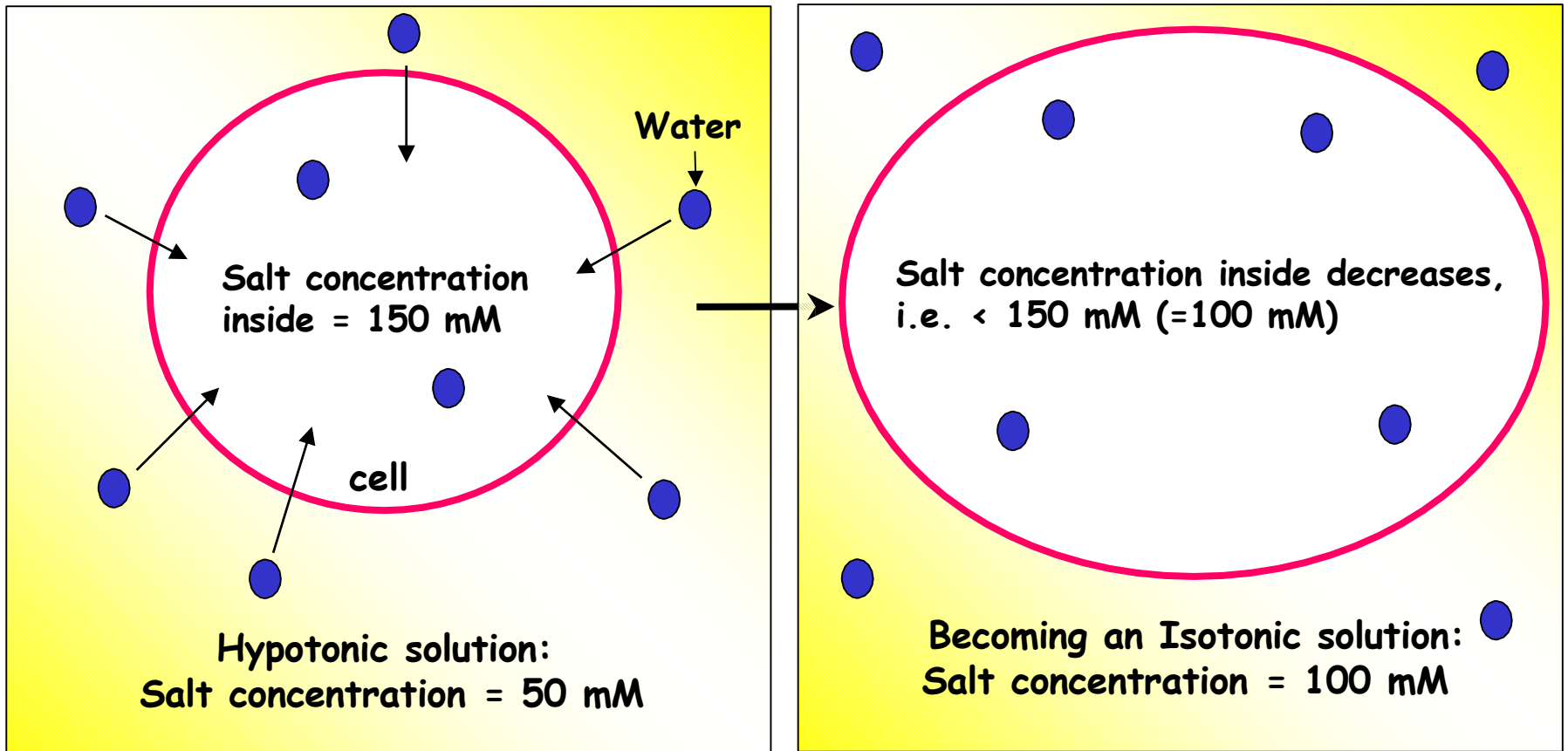
The cells are suspended in an isotonic solution of appropriate pH (pH ~ 5.5) and salt content



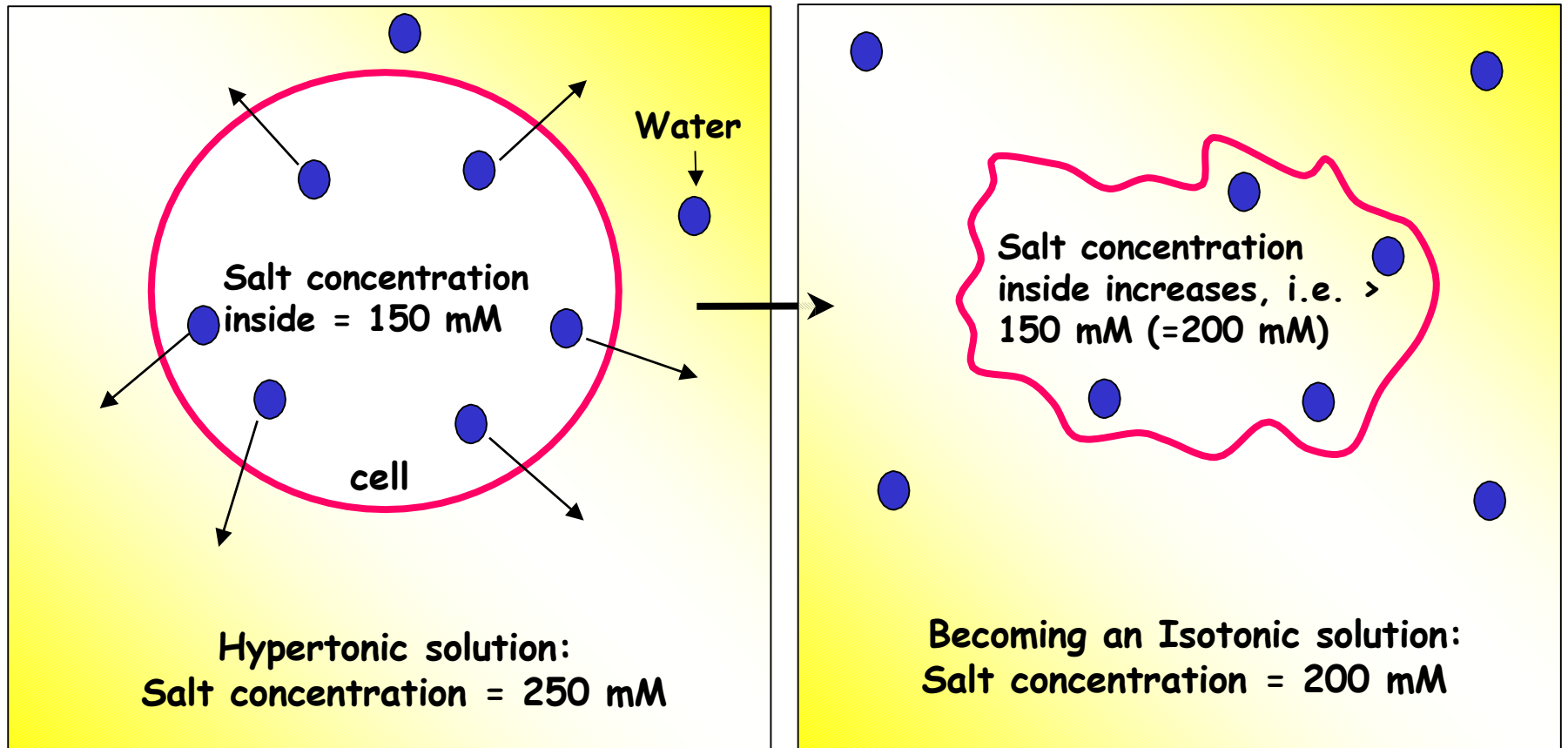
Isotonic solution has a salt concentration (150 mM) and a water concentration equal to that of the cell interior

- Water flows across the plasma membrane from a solution of high water (low salt) to one of low water (high salt) concentration until the water concentration on both sides is equal

When cells are placed in a **hypotonic solution** (i.e. one with a lower salt concentration than that of the cell interior), **water flows into the cells** until the ion concentration inside and outside the cell is the same. Thus, the cells **swell** (the organelles also swell).

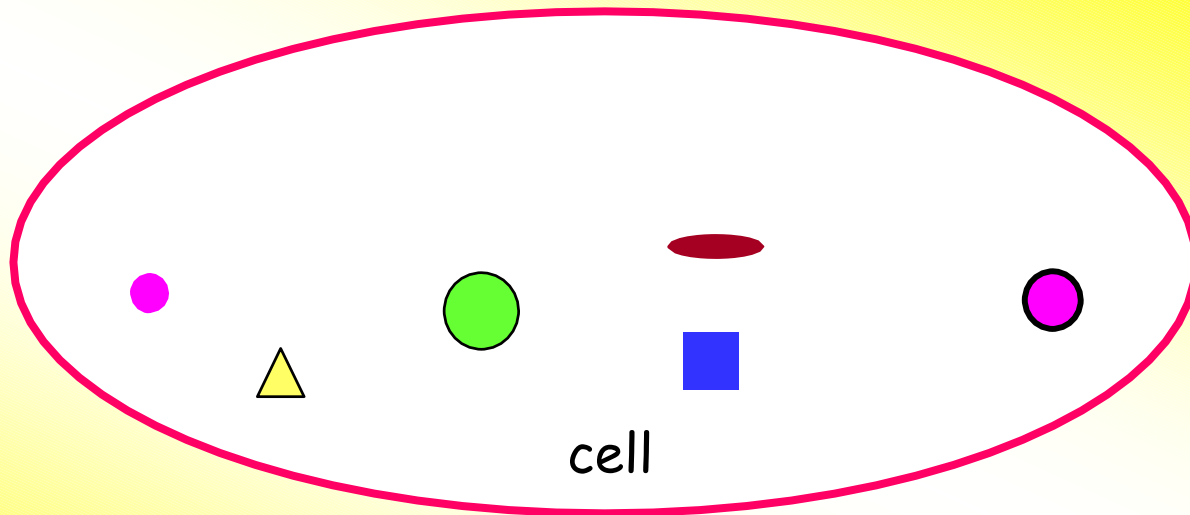


When cells are placed in a **hypertonic solution** (i.e. one with a higher salt concentration than that of the cell interior), **water flows out of cells** until the ion concentration inside and outside the cell is the same. Thus, the cells **shrink** (the organelles also shrink).



When cells are placed in an **isotonic solution**, there is **no net movement of water** in and out of cells. Thus, the cells and organelles do not swell or shrink

For this reason, an isotonic solution is best for preserving the normal structure and function of organelles



Isotonic solution has a salt concentration (150 mM) and a water concentration equal to that of the cell interior

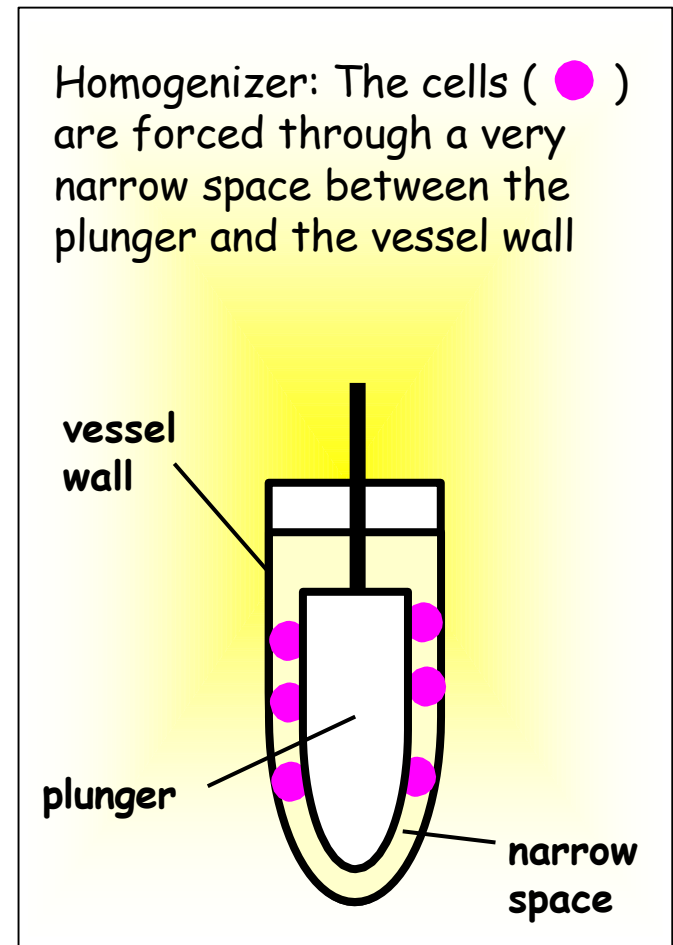
The initial step in subcellular fractionation is the **disruption of the plasma membrane** and the cell wall (if present) under conditions that do not destroy organelles

1. Conditions

- Isotonic solution
- On ice (0°C) to preserve organelles, proteins and other constituents

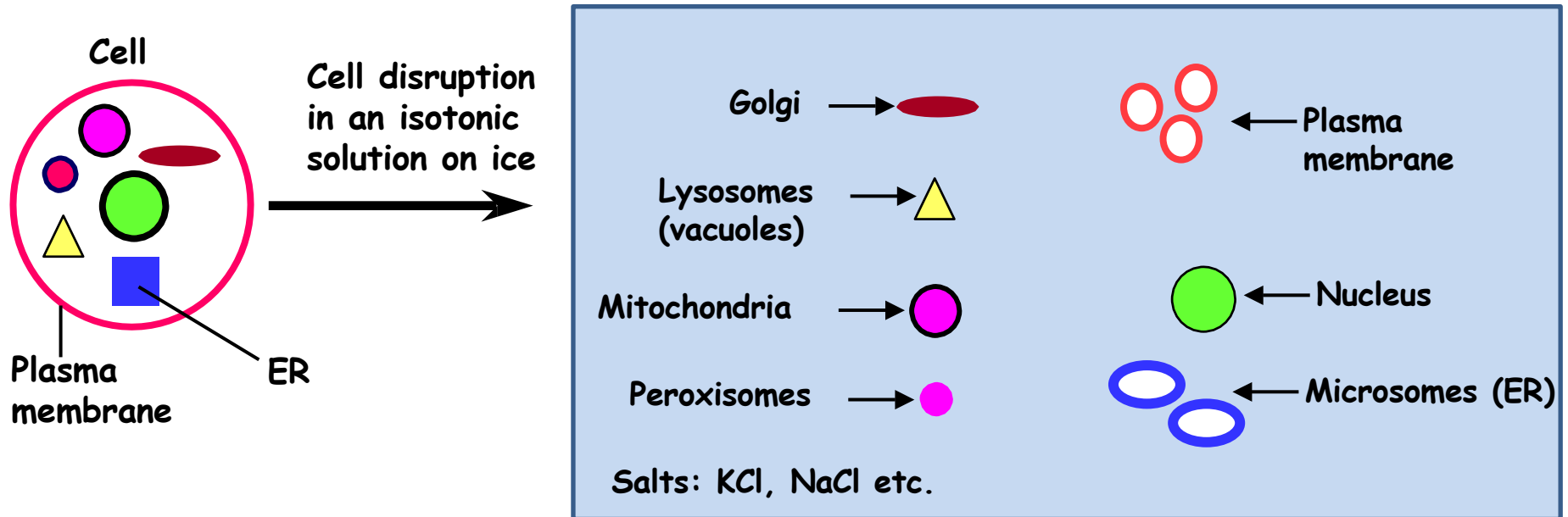
2. Methods

- Stirring the cell suspension in a high-speed **blender**
- **Sonication** (exposure to high-frequency sound)
- Grinding in a mechanical **homogenizer**



The cell disruption procedures:

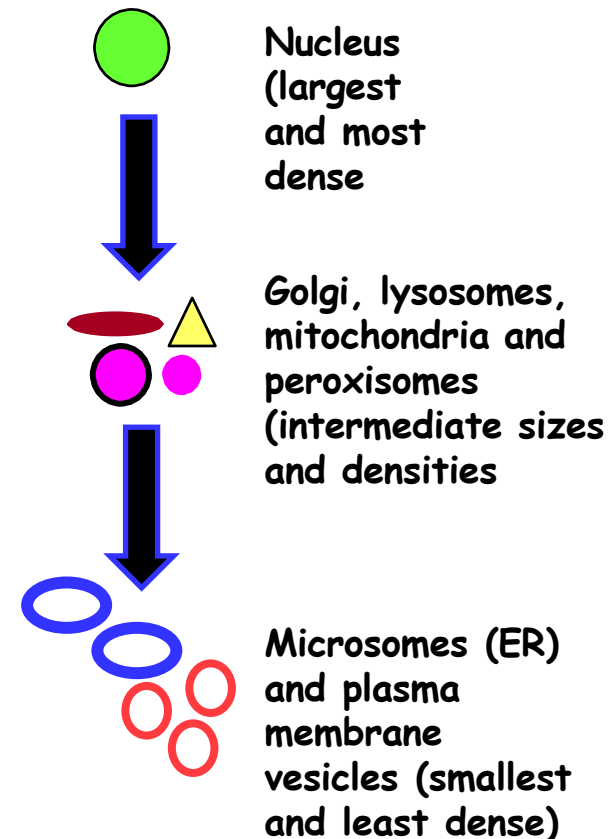
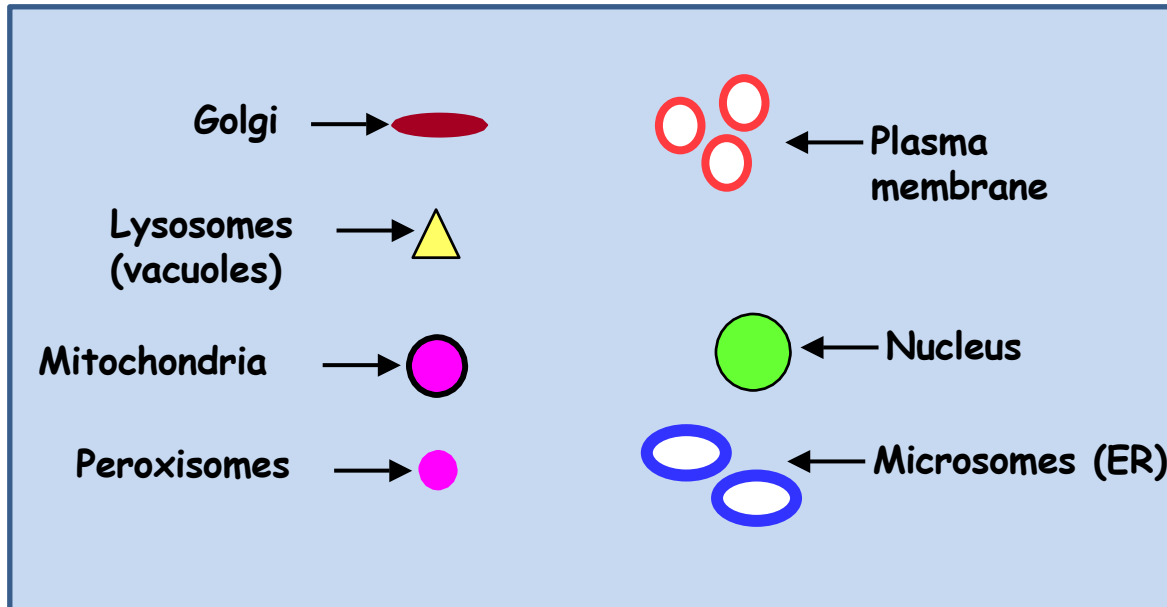
1. Break the plasma membrane and the membrane of the ER into small fragments that immediately reseal to form small closed vesicles (the vesicles derived from the ER are called **microsomes**)
2. Leave other organelles (nuclei, mitochondria, Golgi, chloroplasts, lysosomes and peroxisomes) intact



The resulting suspension of broken cells is called a **homogenate**, **extract** or **lysate**

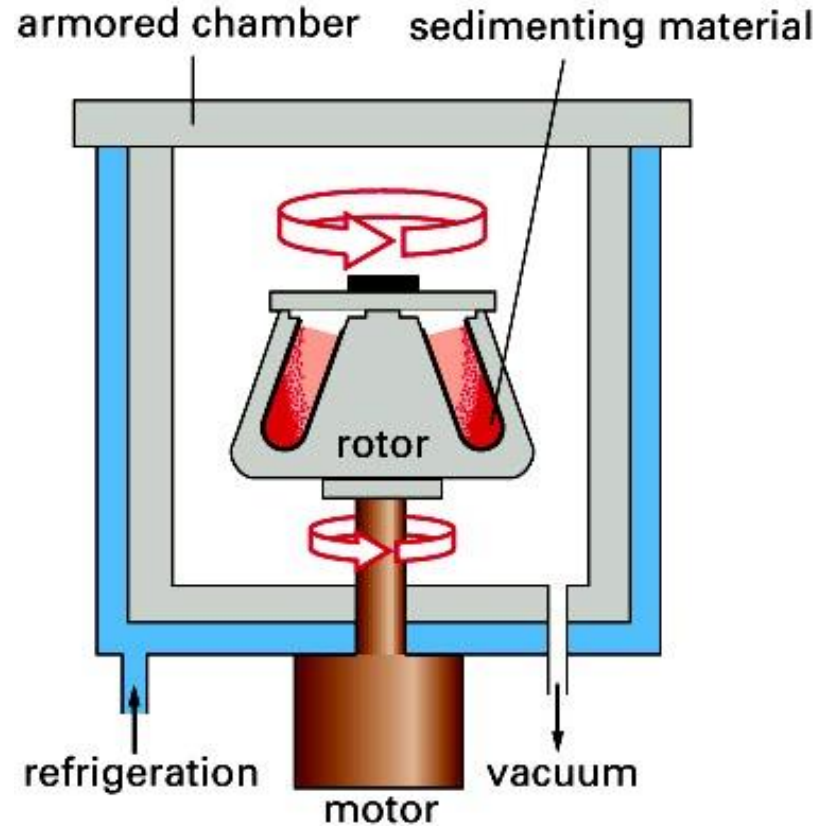
Most subcellular fractionation procedures begin with differential centrifugation at increasingly high speeds

The centrifugal force causes organelles, microsomes and plasma membrane vesicles to move toward the bottom of the centrifuge tube and form a pellet (sedimentation) **at a rate that depends on their size and density**. The largest and the heaviest organelles and vesicles sediment most rapidly.



Ultracentrifuge

1. Consists of a rotor housed in a refrigerated chamber
2. The rotor is rotated at high speed by an electric motor
3. The rotor holds tubes that contain homogenates for fractionation
4. The ultracentrifuge is equipped with a vacuum system to reduce friction between the rotor and air. The vacuum prevents heating of the rotor and allows the refrigeration system to maintain the sample at 4°C
5. Some ultracentrifuges reach speeds over 200,000 rpm, subjecting samples to forces up to 500,000 times greater than gravity (g)
6. The centrifugal force causes organelles to move toward the bottom of the centrifuge tube and form a pellet (a process called sedimentation)

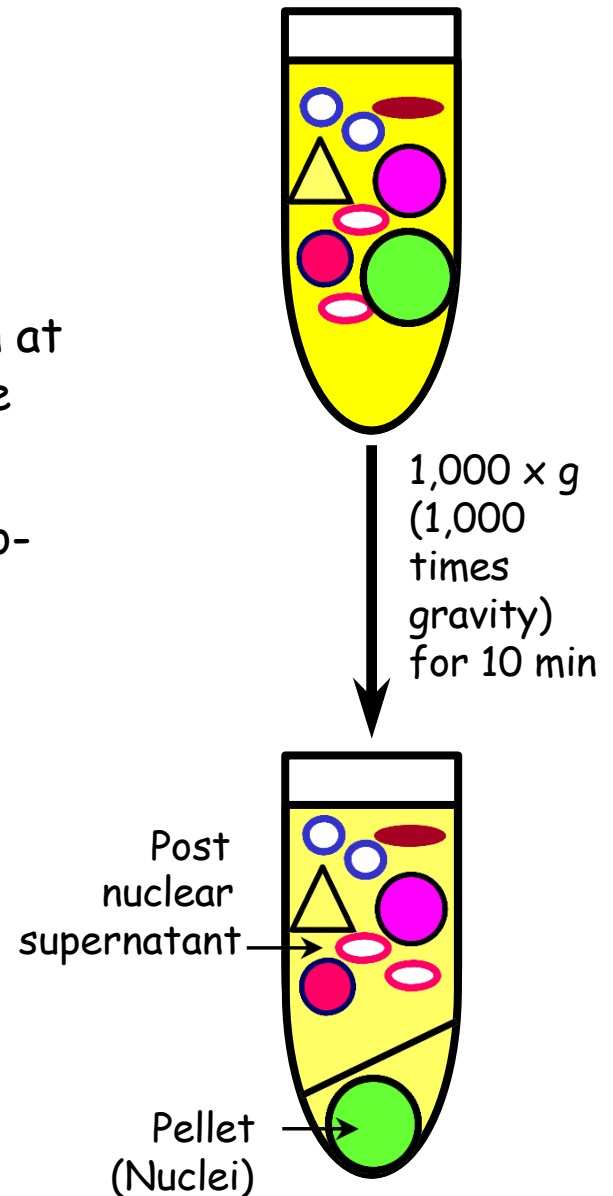


Only **nuclei** sediment during centrifugation for 10 min at $1,000 \times g$, as they are the largest and the most dense organelles

All other organelles remain in the supernatant (the so-called **post-nuclear supernatant**)

The supernatant is poured off into a new centrifuge tube and then returned to the centrifuge and subjected to greater centrifugal force to obtain the next pellet

The pellet (purified nuclei) is used for biochemical studies or electron microscopy



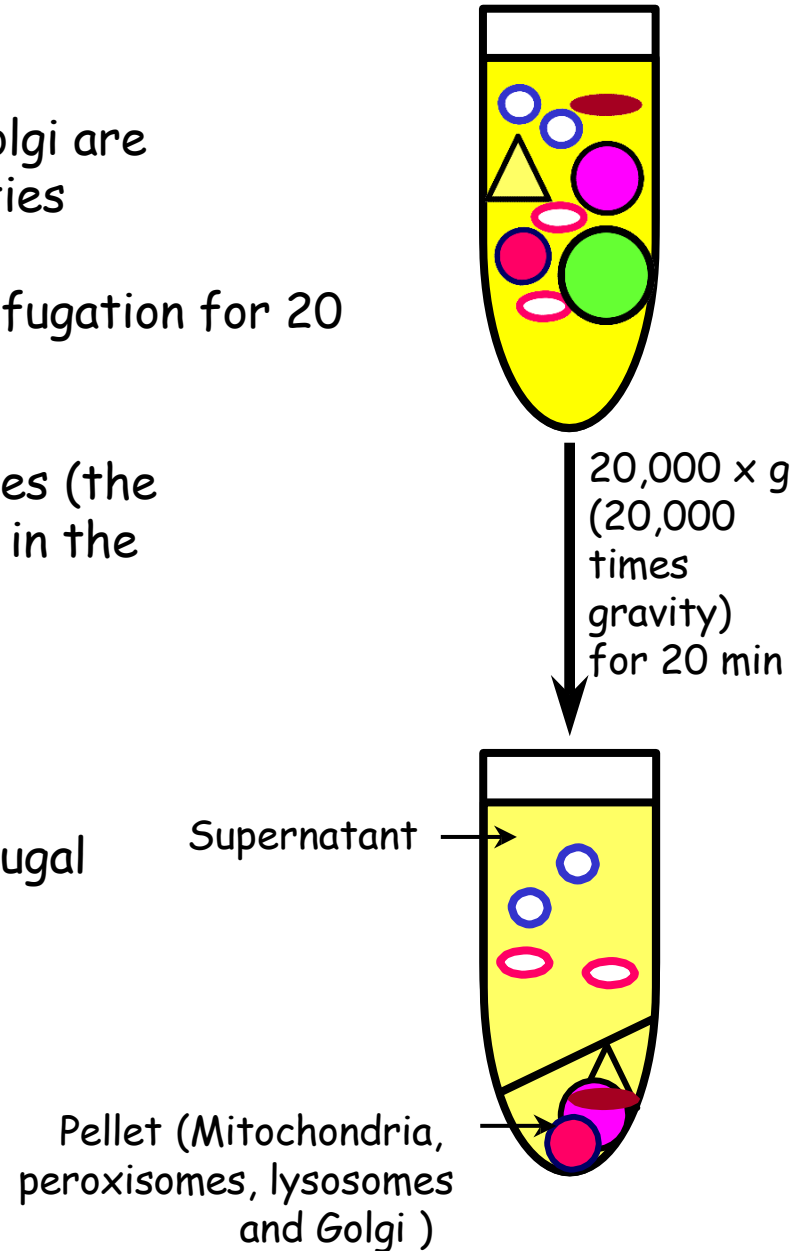
Mitochondria, peroxisomes, lysosomes and Golgi are organelles with intermediate sizes and densities

These four organelles sediment during centrifugation for 20 min at $20,000 \times g$

Microsomes (ER) and plasma membrane vesicles (the smallest and the least dense vesicles) remain in the supernatant

The supernatant is poured off into a new centrifuge tube and then returned to the centrifuge and subjected to greater centrifugal force to obtain the next pellet

The pellet is resuspended in a buffer and subjected to **equilibrium density-gradient centrifugation** to separate mitochondria, peroxisomes, lysosomes and Golgi from each other

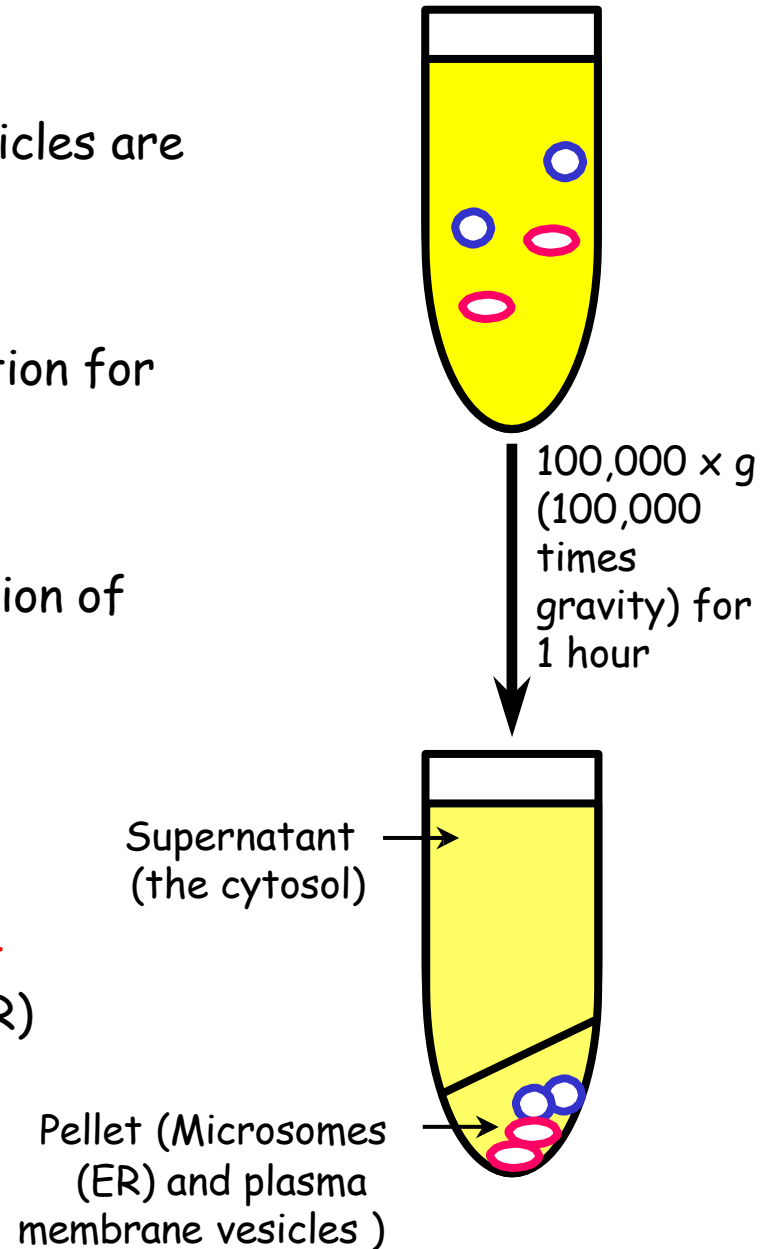


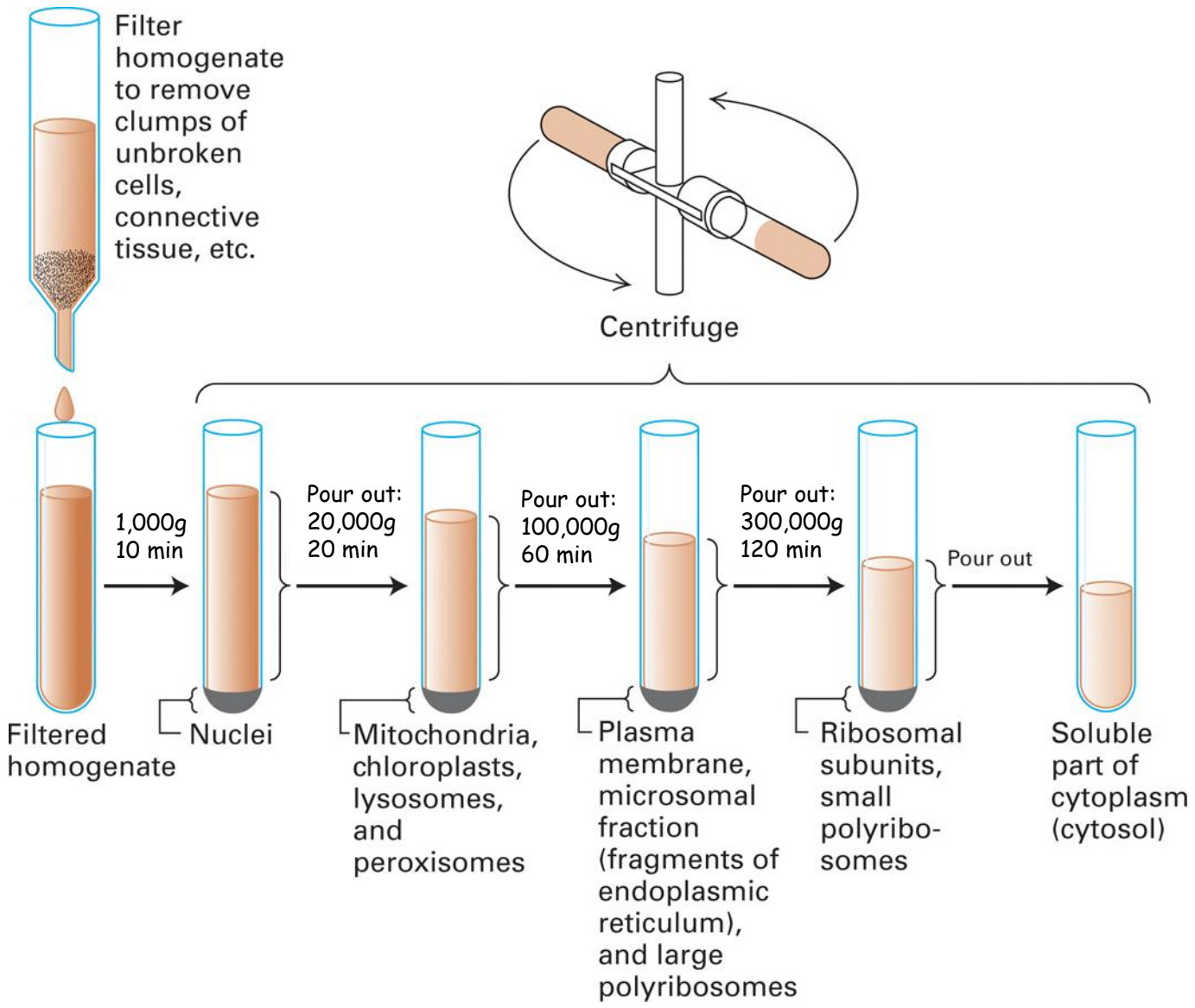
Microsomes (ER) and plasma membrane vesicles are the smallest and the least dense vesicles

These vesicles sediment during centrifugation for 1 hour at $100,000 \times g$

Only the cytosol - the soluble aqueous portion of the cytoplasm - remains undeposited after centrifugation for 1 hour at $100,000 \times g$

The pellet is resuspended in a buffer and subjected to **equilibrium density-gradient centrifugation** to separate microsomes (ER) and plasma membrane vesicles from each other



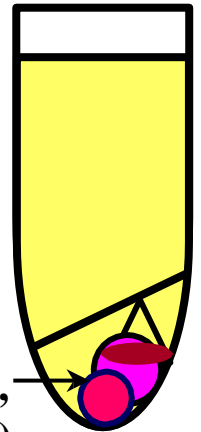


Differential centrifugation **does not yield totally pure organelles**

One method for further purifying organelles is **equilibrium density-gradient centrifugation**

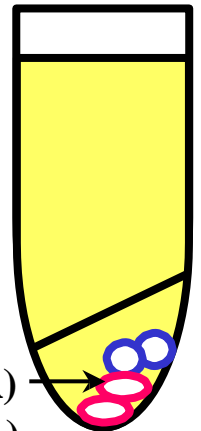
The pellet is resuspended in a buffer and subjected to equilibrium density- gradient centrifugation to separate mitochondria, peroxisomes, lysosomes and Golgi from each other

A 20,000-g pellet (Mitochondria, peroxisomes, lysosomes and Golgi)



The pellet is resuspended in a buffer and subjected to equilibrium density- gradient centrifugation to separate microsomes (ER) and plasma membrane vesicles from each other

A 100,000-g pellet (Microsomes (ER) and plasma membrane vesicles)



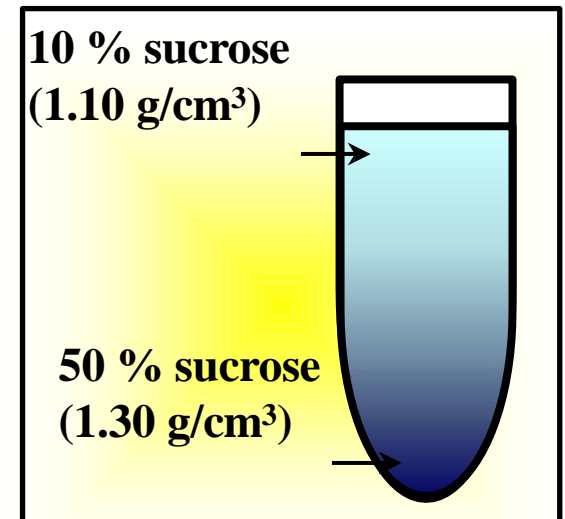
Equilibrium density-gradient centrifugation is used to separate organelles on the basis of their density, independently of their size and shape

Equilibrium density-gradient centrifugation involves a gradient of solute that increases in concentration and density from the top of the tube to the bottom

The density of solute gradient spans the range of densities of the organelles to be separated

For organelles, a gradient of sucrose is used

The density range of sucrose is $1.10 - 1.30 \text{ g/cm}^3$
(= $0.75 - 2.3 \text{ M}$ or $10 - 50 \%$ [w/w] sucrose)



Densities of organelles:

Peroxisomes = 1.23 g/cm^3

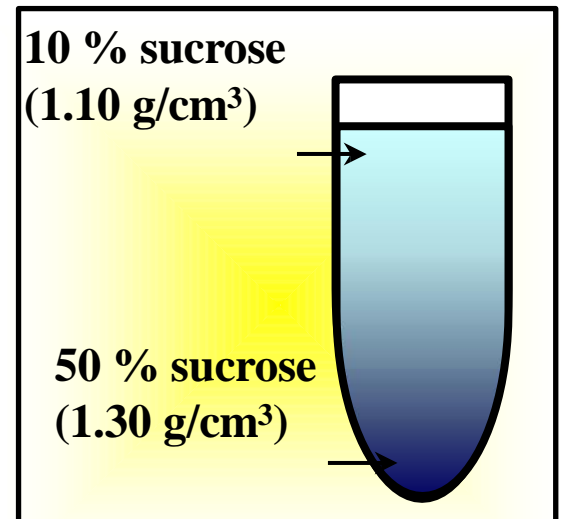
Microsomes of the rough ER = 1.20 g/cm^3

Mitochondria = 1.18 g/cm^3

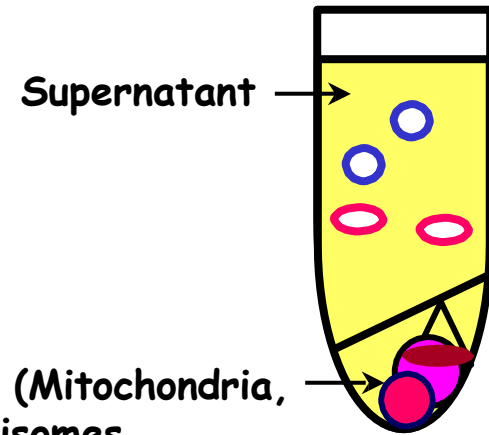
Golgi = 1.14 g/cm^3

Plasma membrane vesicles = 1.12 g/cm^3

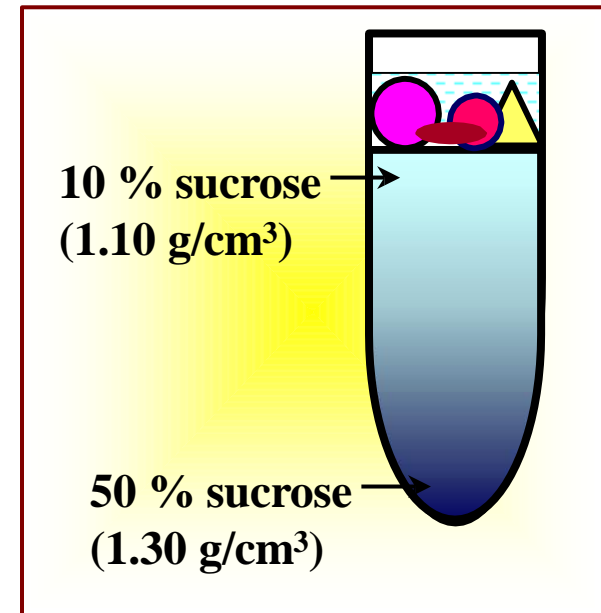
Lysosomes (Vacuoles) = 1.10 g/cm^3



First step of equilibrium density-gradient centrifugation:



The pellet is resuspended in a buffer and layered on a density gradient composed of layers of increasingly more dense sucrose solutions in a centrifuge tube

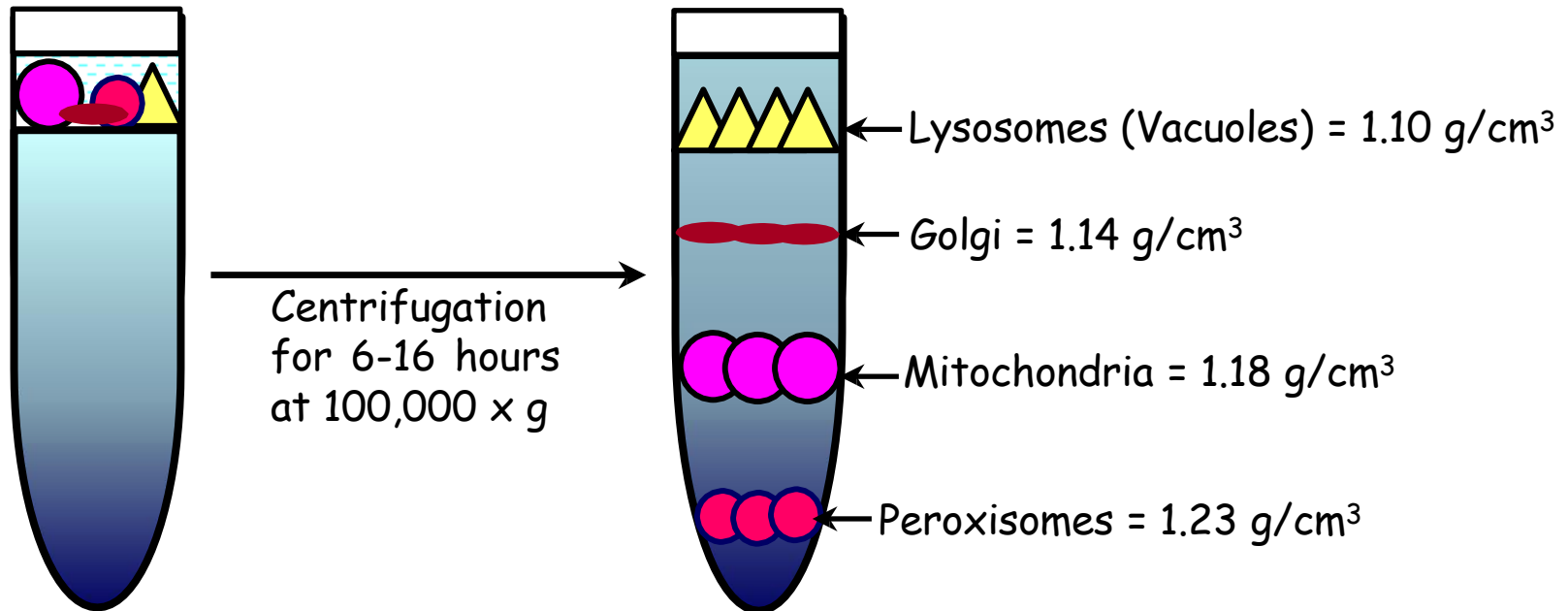


Second step of equilibrium density-gradient centrifugation

The tube is centrifuged for 6-16 hours at $100,000 \times g$

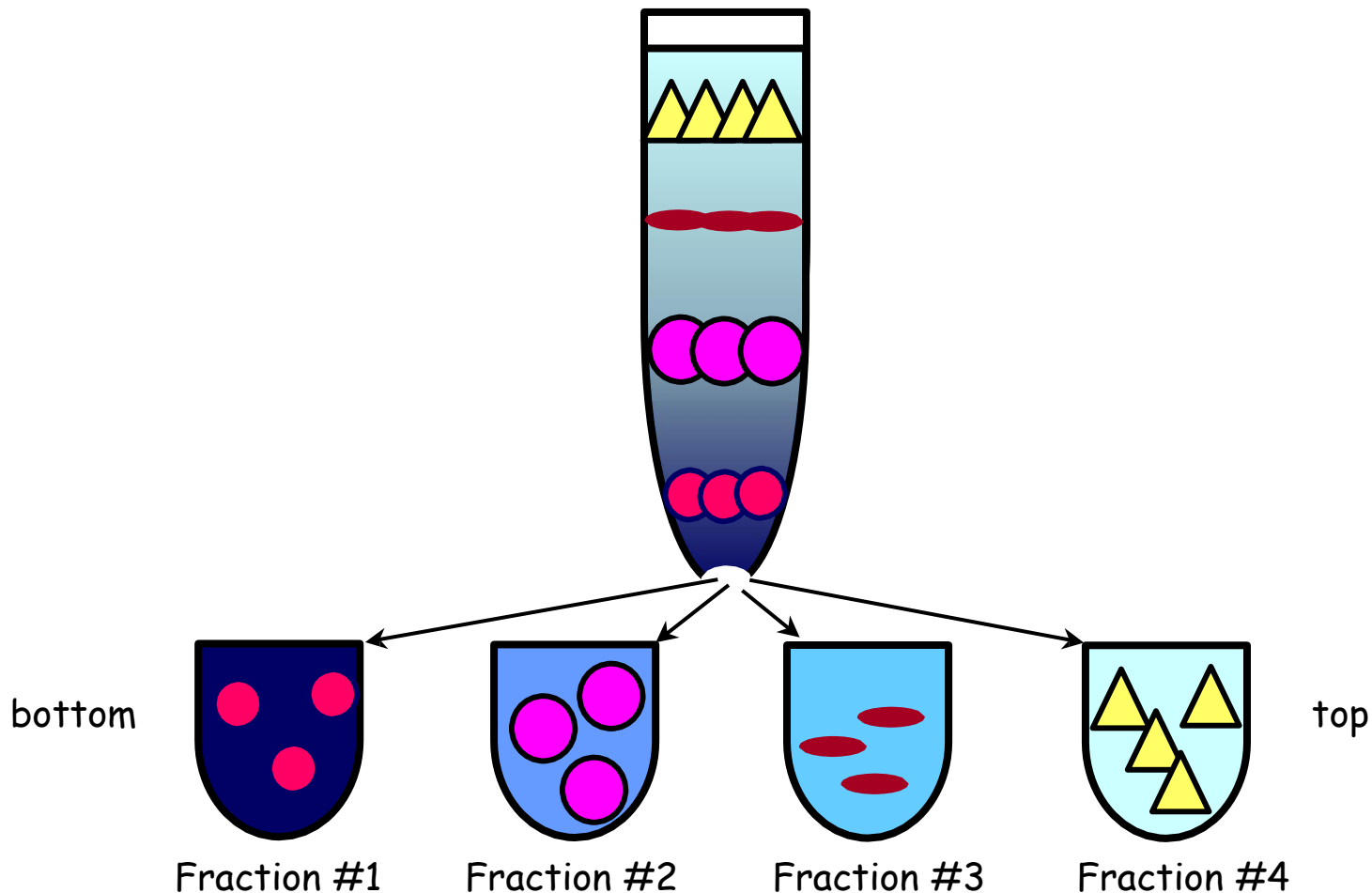
During centrifugation, **each organelle migrates to an equilibrium position** where the density of the surrounding sucrose is exactly equal to the density of the organelle

All the organelles reach their characteristic equilibrium position in the gradient and remain there

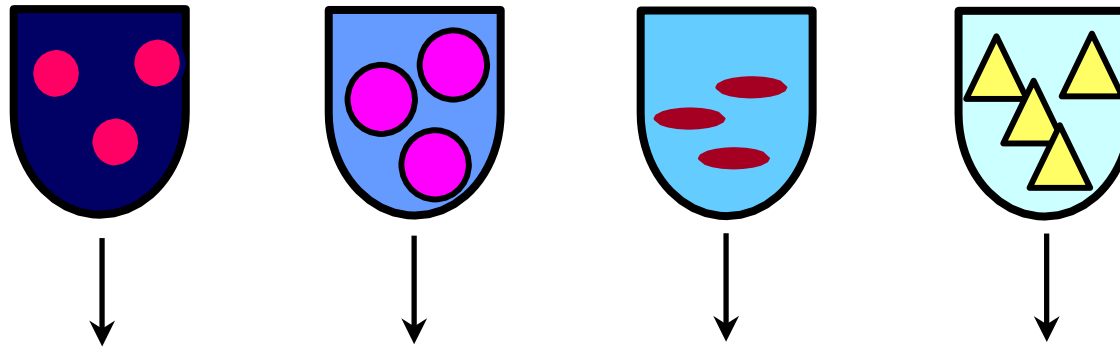


Second step of equilibrium density-gradient centrifugation

When the organelles are at their equilibrium positions, the centrifuge is stopped. The **bottom of the tube is punctured**, and the fractions are collected. Because of their different densities, the four types of organelles are collected in different fractions.



Since each organelle has unique morphological features, the purity of organelle preparations can be assessed by examination in an electron microscope or biochemically for enzymatic activity.



electron microscopy,
biochemistry

Proteins in a cell extract (cytosolic proteins) or from purified organelles can be examined by **enzymatic assays** or a technique called **western blot analysis**.

Enzymatic assays

catalase, uricase - **peroxisomes**

cytochrome c oxidase, succinate dehydrogenase - **mitochondria**

acid phosphatase - **lysosomes**

glucose-6-phosphatase - **endoplasmic reticulum**

UDP-galactosyl transferase - **Golgi**

5' nucleotidase- **plasma membrane**

Some of these assays were used by **Christian de Duve** in his pioneering work on cell fractionation (Nobel Prize in Physiology or Medicine, 1974)

Western analysis

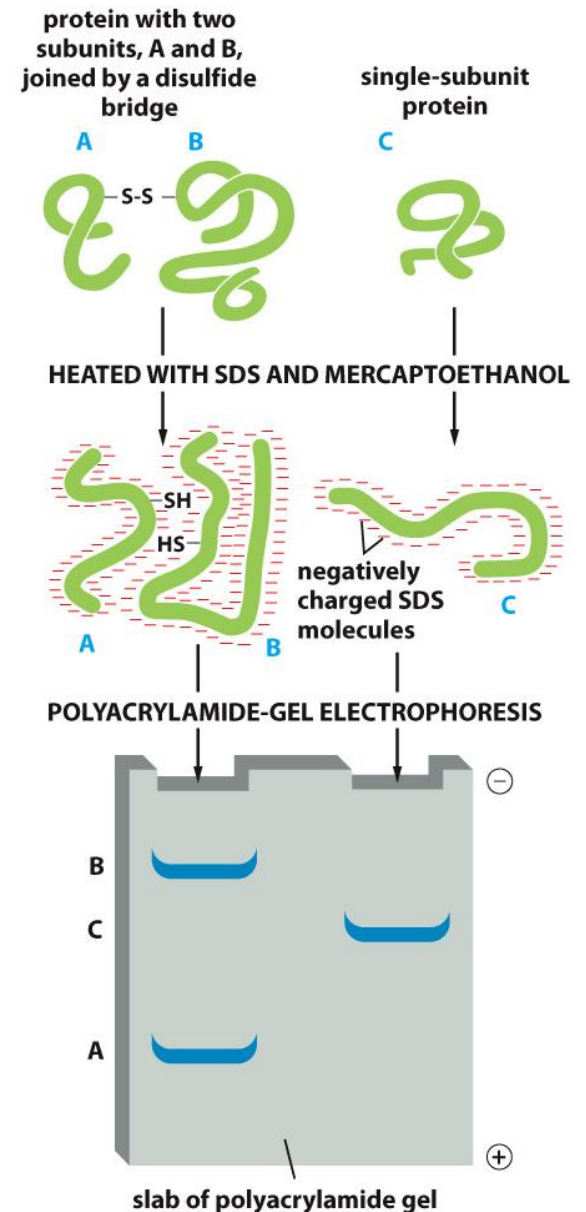
Western analysis (western blotting) is a method used to **detect a specific protein in a sample that contains many proteins**. It can be used, for example, to assess the purity of an organelle following purification.

1. Separate proteins on a polyacrylamide gel
2. Transfer the proteins onto a thin membrane
3. Probe the membrane with a specific antibody
4. Detect the antibody

Polyacrylamide gel electrophoresis

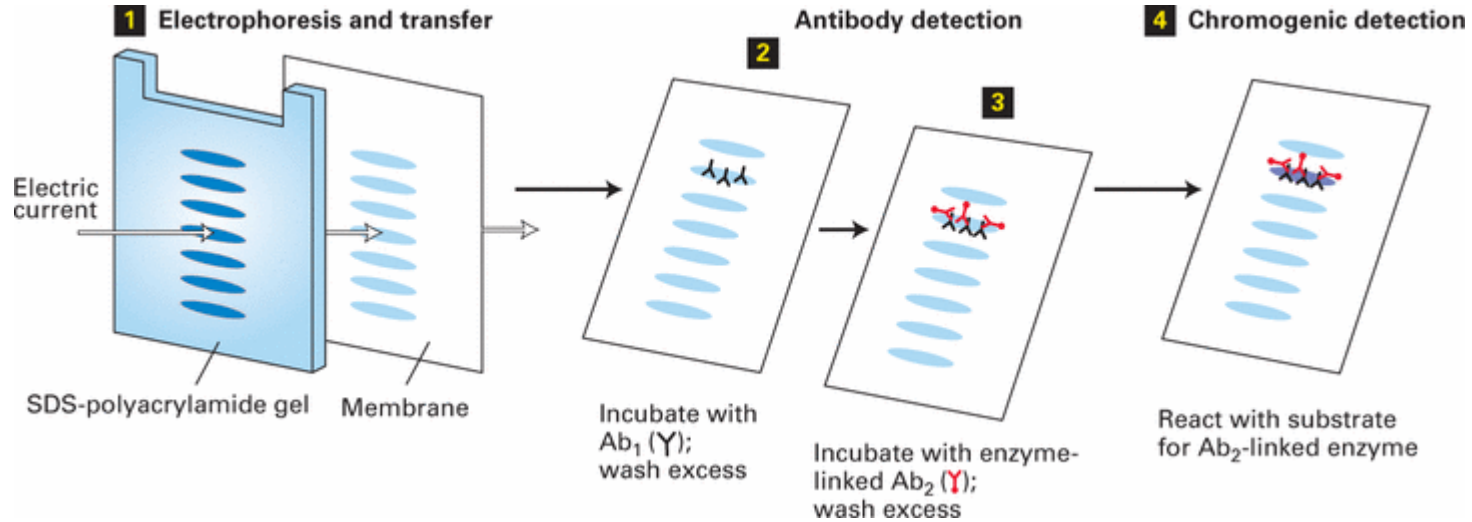
Without denaturing detergent - **native gel electrophoresis**, keeps protein complexes intact, migration in an electric field is dependent upon **size, shape and charge** (determined by amino acid composition)

With denaturing detergent (sodium dodecyl sulfate (SDS) - denatures proteins so that they are no longer part of a protein complex. The negatively-charged **SDS binds uniformly** to proteins (1.4g SDS/g protein). Therefore, **all proteins have a similar charge density** and migration in an electric field is **strictly dependent upon size**. Larger proteins migrate through the polyacrylamide gel more slowly while smaller proteins migrate more quickly. A reducing agent (**β -mercaptoethanol or dithiothreitol (DTT)**) are included to break disulfide bonds.



- 1 Proteins are transferred from the SDS-polyacrylamide gel onto a thin membrane (nitrocellulose, PVDF).

- 2 An antibody (**primary antibody**) to a specific protein is added to a solution bathing the membrane.



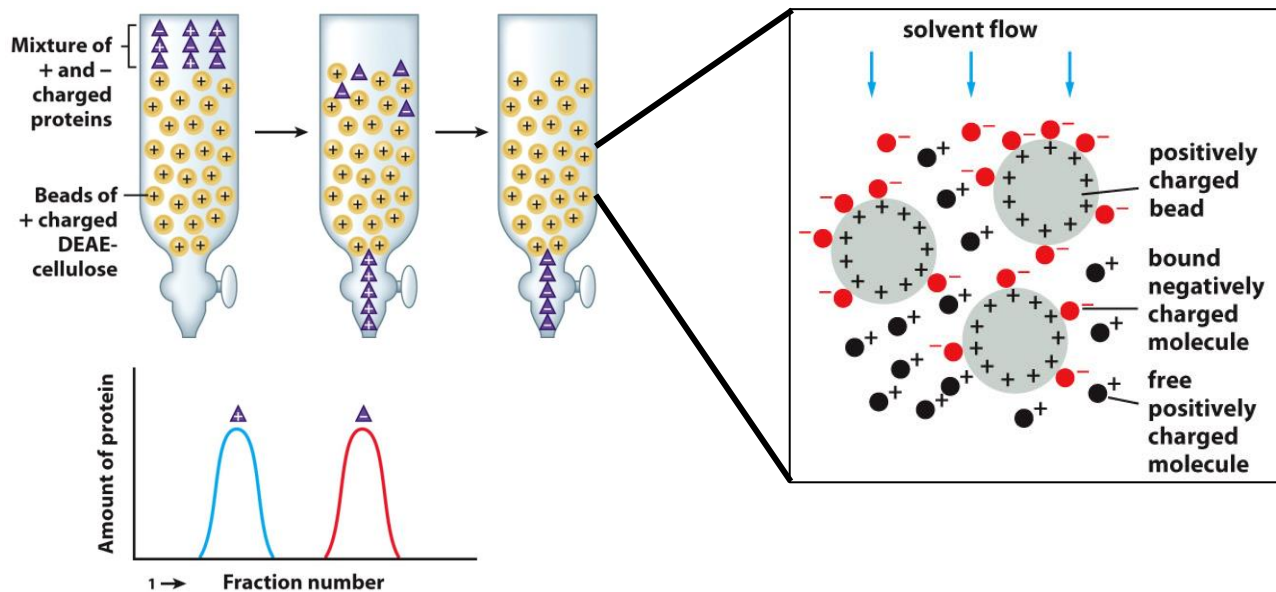
- 3 An antibody (**secondary antibody**) that binds to the primary antibody is added to the solution bathing the membrane. The secondary antibody is linked to an enzyme that produces colour or light. Like fluorescence microscopy, this **amplifies the signal**.

- 4 A solution containing a substrate for the enzyme linked to the **secondary antibody** is added to the solution bathing the membrane. If light is produced it can be detected using X-ray film.

Proteins can be separated from other cellular proteins using chromatography procedures such as **ion-exchange**, **size exclusion (gel filtration)** and **affinity chromatography**. All three chromatography procedures use **western analysis** to detect specific proteins of interest.

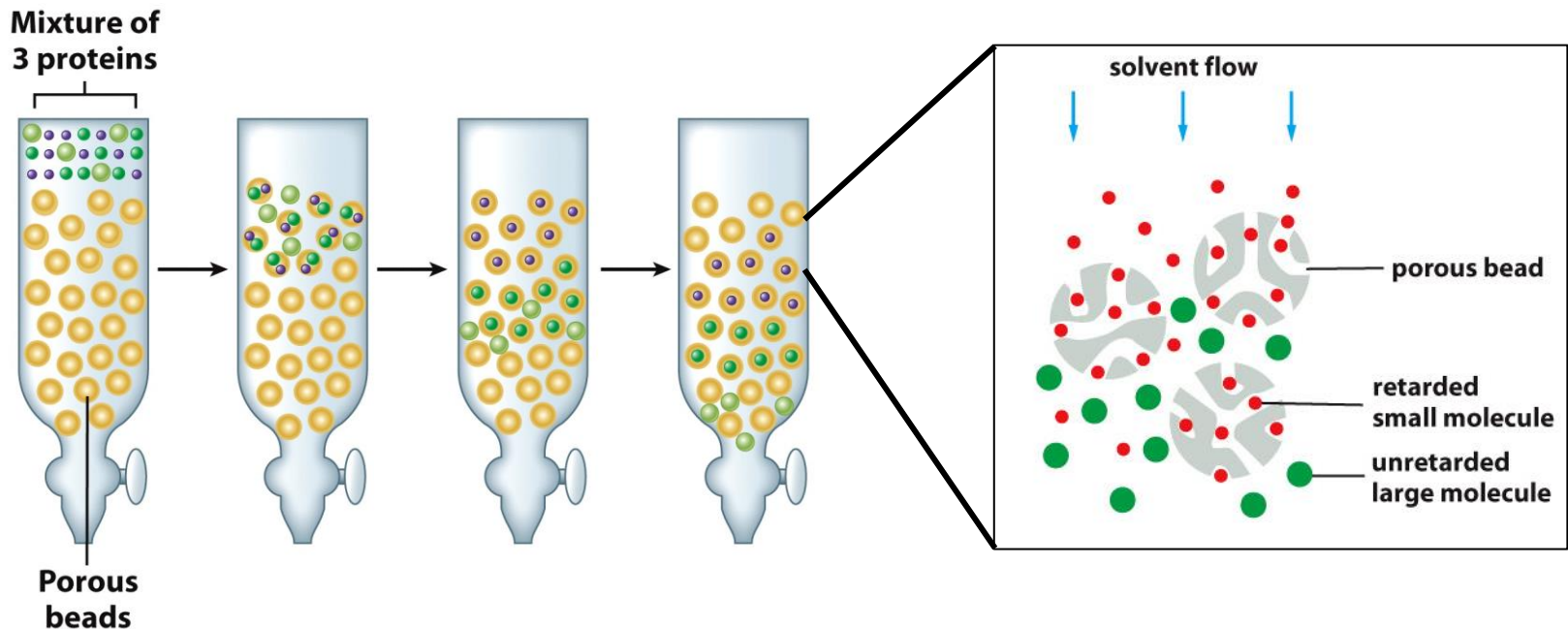
1. **Ion exchange chromatography** - separates proteins based on **charge**. The overall charge of a protein is the sum of all of the charges on the amino acids at a particular pH. When these charges are equal this is called the **isoelectric point**. At **pH > isoelectric point** proteins have a **net negative charge**, at **pH < isoelectric point** they have a **net positive charge**.

Proteins in the mixture interact with a charged bead and are eluted using a salt gradient of increasing concentration.



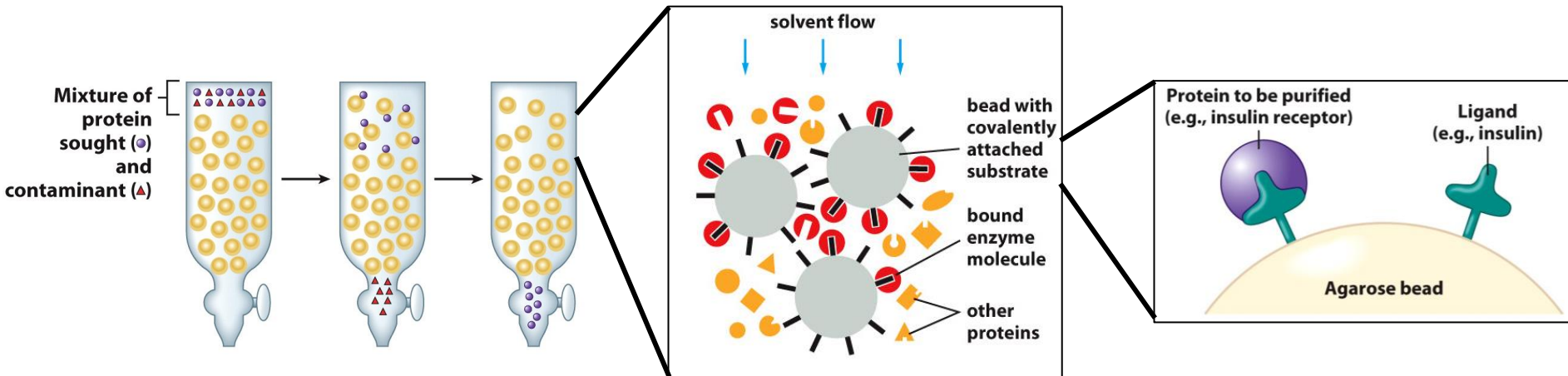
2. Size exclusion (gel filtration) chromatography - separates proteins based on **molecular size**. Can distinguish between monomeric and higher-ordered structures. If the protein (e.g. 80kDa) is part of a large complex, it will fractionate at a size greater than 80kDa (on a denaturing SDS-gel it will fractionate at 80kDa).

Proteins in the mixture pass through porous beads. Smaller proteins can access the pores more readily than larger proteins. Thus, large proteins pass through the entire column length faster and emerge first, the smaller proteins emerge last.



3. Affinity chromatography - separates proteins based on a **specific sequence within the protein**. The column has a ligand (e.g. substrate for a specific enzyme, antibody to a specific antigen, metal such as Ni^{2+} that binds to a stretch of histidine residues) bound to it that interacts with this sequence.

Proteins in the mixture pass through the column. Most flow right through. But the specific protein is attracted to the ligand and is retained. The specific protein is then eluted by adding an excess of soluble ligand or some other molecule to break the interaction. **Near total purification in a single step.**



Protein interactions can be studied by a number of methods including:

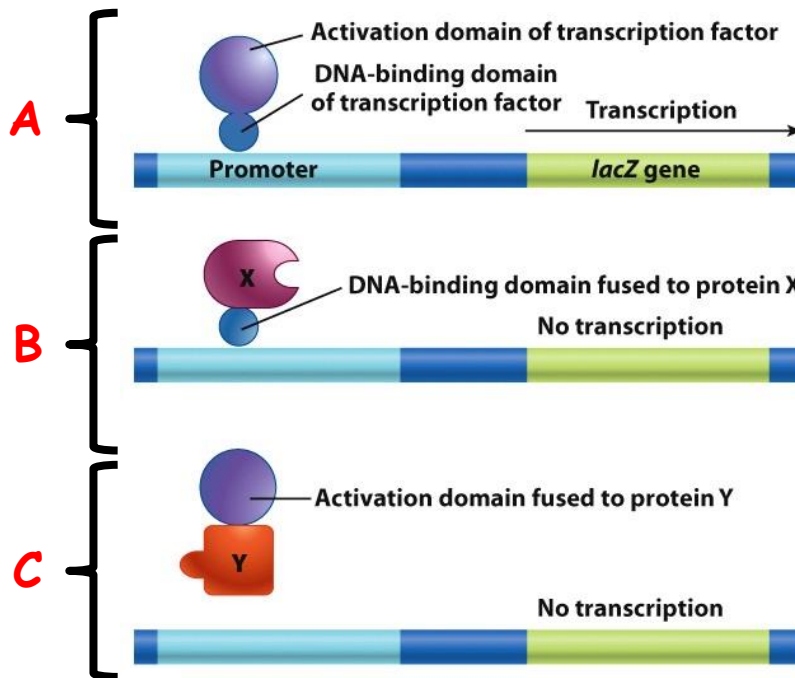
1. Immunoprecipitation
2. Yeast two-hybrid
3. FRET

1. **Immunoprecipitation** - variation of affinity chromatography using **antibodies**. Instead of packing a column, small amounts of beads with antibodies are incubated with a cellular extract under **non-denaturing conditions**.

The antibody binds to its antigen (protein X) and this immune complex is collected by centrifugation (the relatively large beads pellet, the supernatant contains the cellular extract). The only protein expected to pellet with the beads is protein X. If other proteins are detected, this indicates they interact with protein X. **Western analysis or mass spectrometry** can be used to detect co-immunoprecipitating proteins.

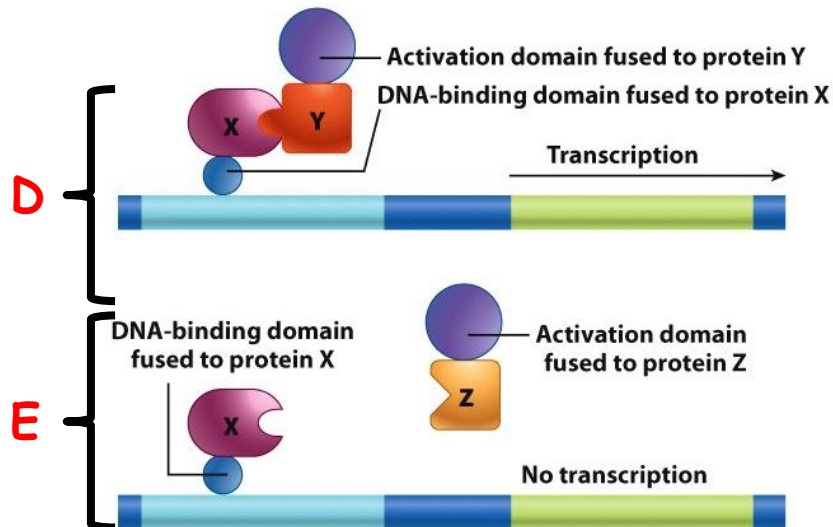
2. **Yeast two-hybrid - reconstitution of a transcription factor** by the interacting proteins leads to expression of a reporter gene (e.g. *lacZ* = β -galactosidase) whose activity can be easily measured.

(A) Transcription factor from yeast has two domains: **DNA binding domain** and **transcriptional activation domain**. Using molecular biological techniques, the DNA encoding each domain is linked to the DNA encoding two different proteins (**bait** and **fish**). The **bait** is linked to the **DNA binding domain (B)** and the **fish** to the **transcriptional activation domain (C)**.



2. **Yeast two-hybrid - reconstitution of a transcription factor** by the interacting proteins leads to expression of a reporter gene (e.g. lacZ = β -galactosidase) whose activity can be easily measured.

If the two hybrid proteins interact (**D**), the transcription factor activity will be reconstituted and transcription of lacZ will take place. The lacZ protein (β -galactosidase) will be produced and will function (in the presence of the appropriate substrate it will generate a **blue pigment**). If the two hybrid proteins do not interact, transcription of lacZ will not occur (**E**).



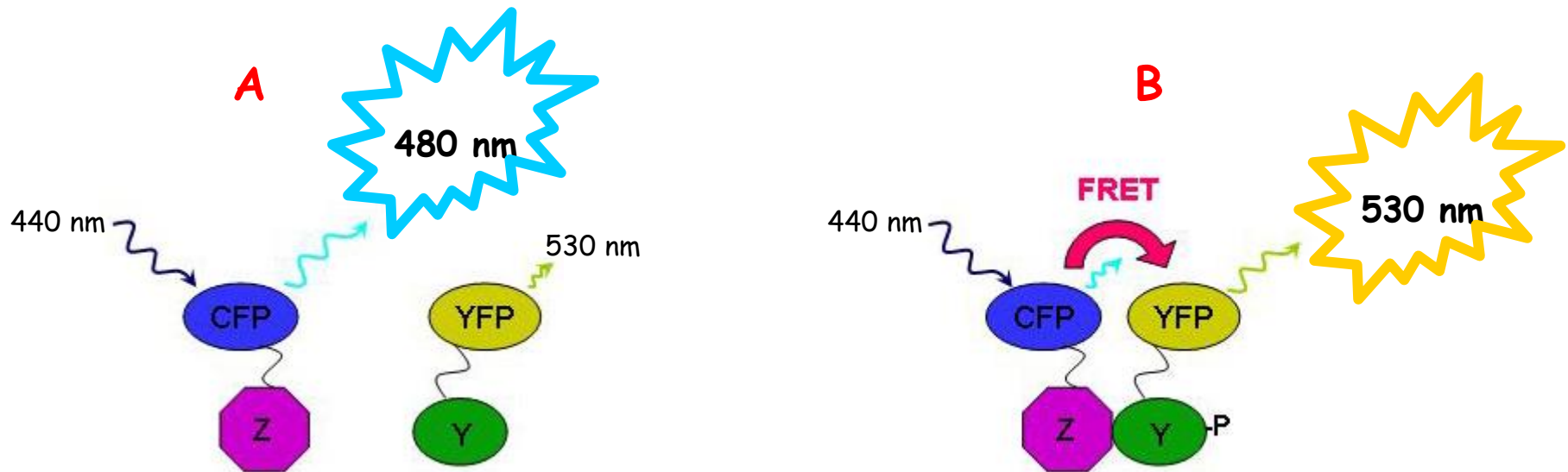
3. FRET (fluorescence resonance energy transfer) - can be used to examine protein-protein interactions within a cell.

Protein Z is fused to CFP and protein Y is fused to YFP.

λ_{ex} and λ_{em} of CFP: 440 nm and 480 nm

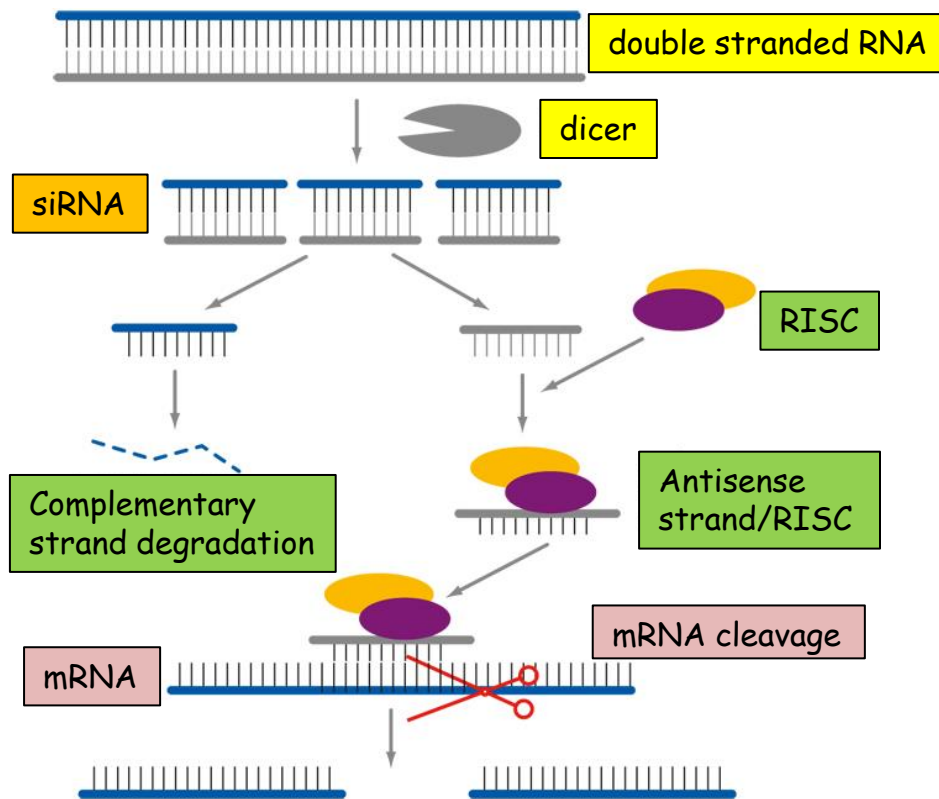
λ_{ex} and λ_{em} of YFP: 514 nm and 530 nm

(A) If the proteins do not interact, CFP absorbs the light at 440 nm and emits strongly 480 nm. Little emission at 530 nm is observed. (B) If the proteins do interact, CFP transfers some of the emitted energy to YFP which then emits strongly at 530 nm. Therefore, the cells are irradiated at 440 nm and the detector is set to detect light at 530 nm, indicating an interaction between the proteins. The two proteins must be within 5 nm for FRET to occur.



Gene silencing - RNA interference

Assessing the function of a protein is facilitated by examining the effects of removing that protein from the cell. One such method is called RNA interference (**RNAi**).

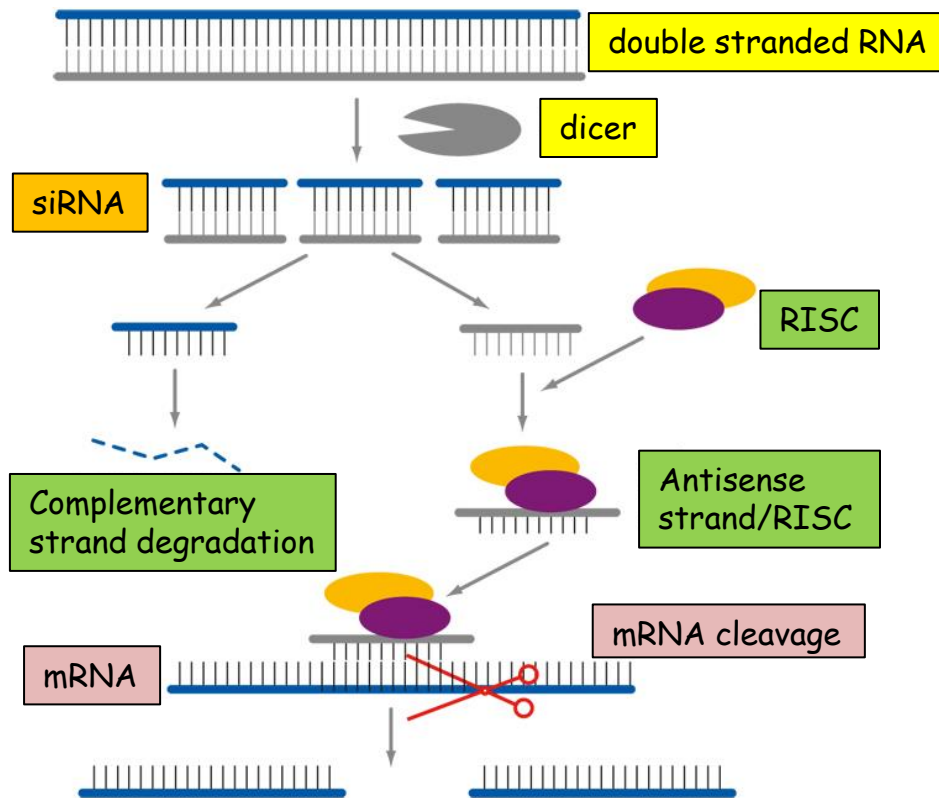


Double-stranded RNA related to a specific mRNA encoding the protein of interest is cleaved into small (~21bp) fragments by an enzyme called **dicer**. This RNA can be generated from a DNA plasmid after it is transfected into a cell. **Can be propagated in the laboratory.**

The resulting pieces of RNA are called small interfering RNA (**siRNA**). This type of RNA can be purchased from a company and transfected into a cell. **Cannot be propagated in the laboratory.**

Gene silencing - RNA interference

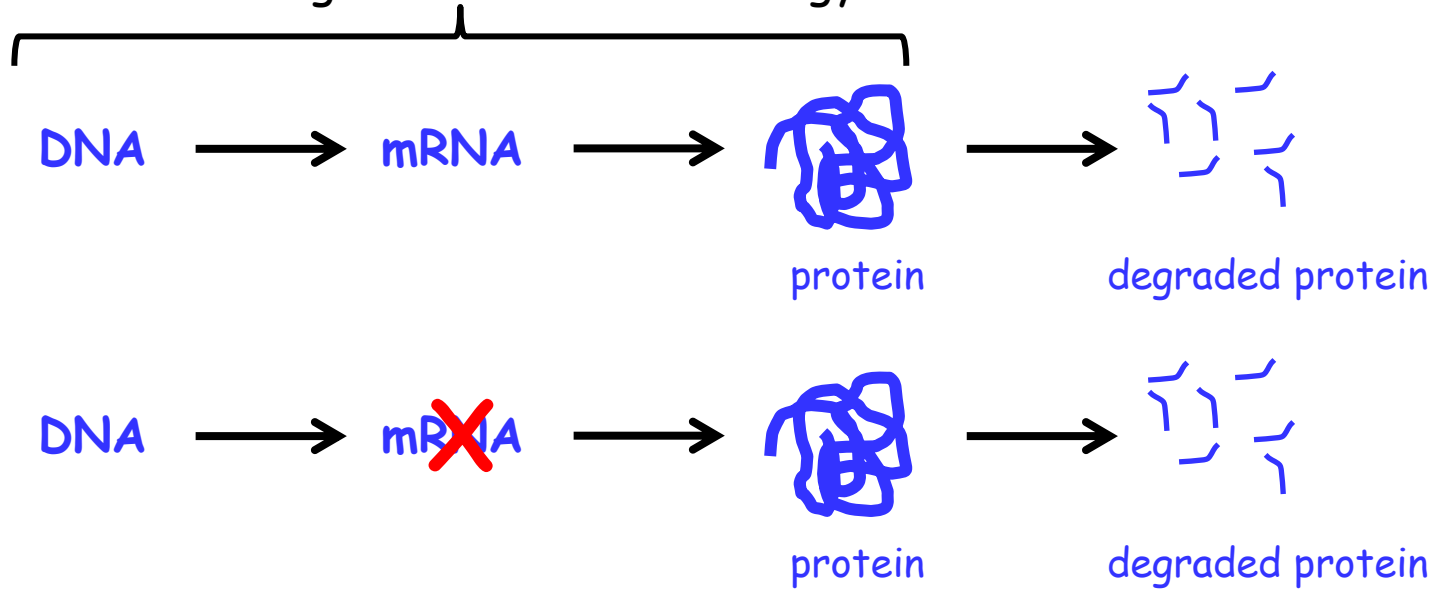
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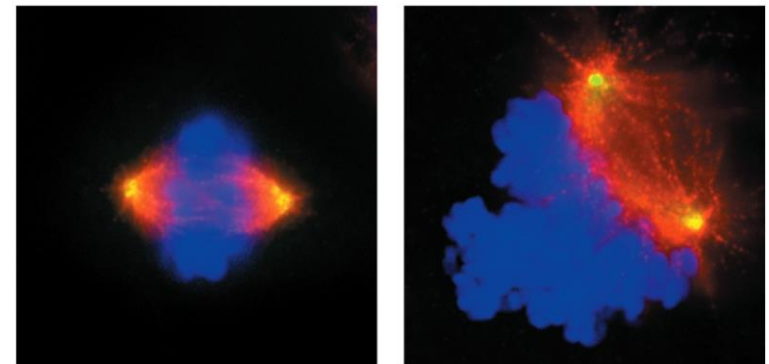
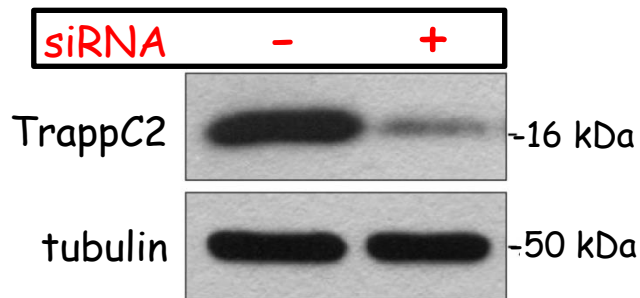
The antisense strand associates with a protein complex called **RISC**. The complementary strand is degraded.

The antisense strand/RISC complex then anneals with the mRNA encoding the protein of interest and cleavage of the mRNA ensues. **Argonaute**, a component of RISC, facilitates the annealing of the small RNA fragment to the mRNA.

central dogma of molecular biology



After hours or days (depending on protein stability), the protein is depleted and there is no mRNA present to replenish it. One can monitor the degree of depletion (**knockdown**) by western analysis.



Tubulin DNA

The effect of an Aurora B knockdown on mitotic cells