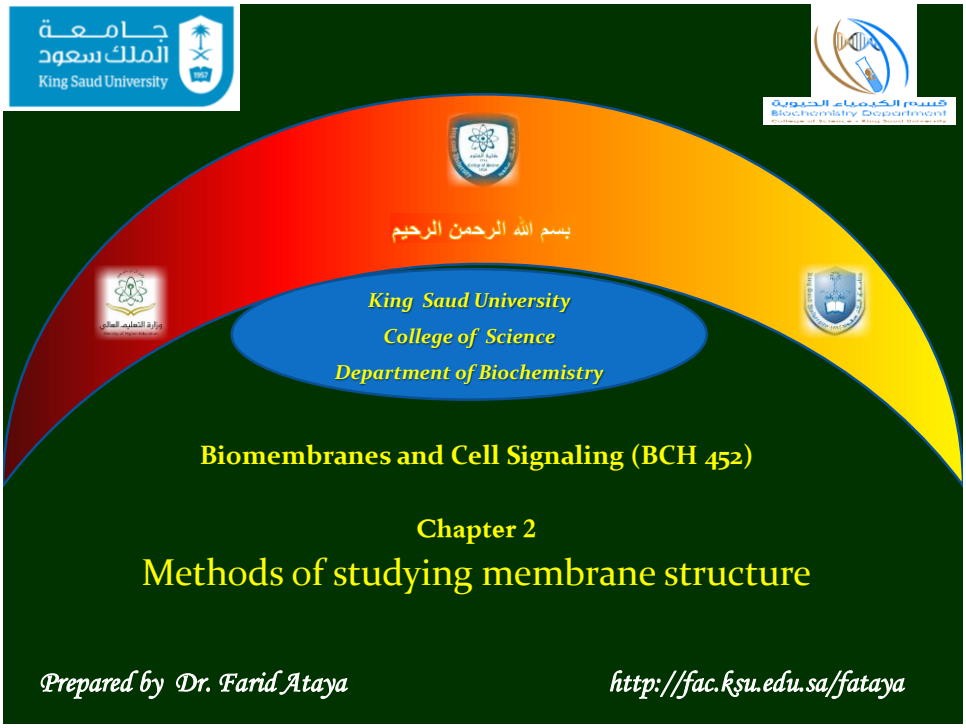


Topics to be covered	Lect No.
Membrane solubilisation with detergents. Visualization of membrane proteins with SDS-PAGE Visualization of membrane proteins with freeze fracture and freeze-etching electron microscopy. Factors affecting fluidity of membranes.	5-6

Techniques used to study biomembranes

- Study by Atomic Force Microscopy (AFM)
- Study the intact cell (whole cell) by:
  - Electron microscopy.
- Study the intact cell membrane by:
  - Simple fluorescent light microscopy.
- Study the disrupted membranes by freeze –etching E-microscopy.
- Study the membrane-bound matters:
  - Protein (integral and peripheral)
  - Carbohydrate

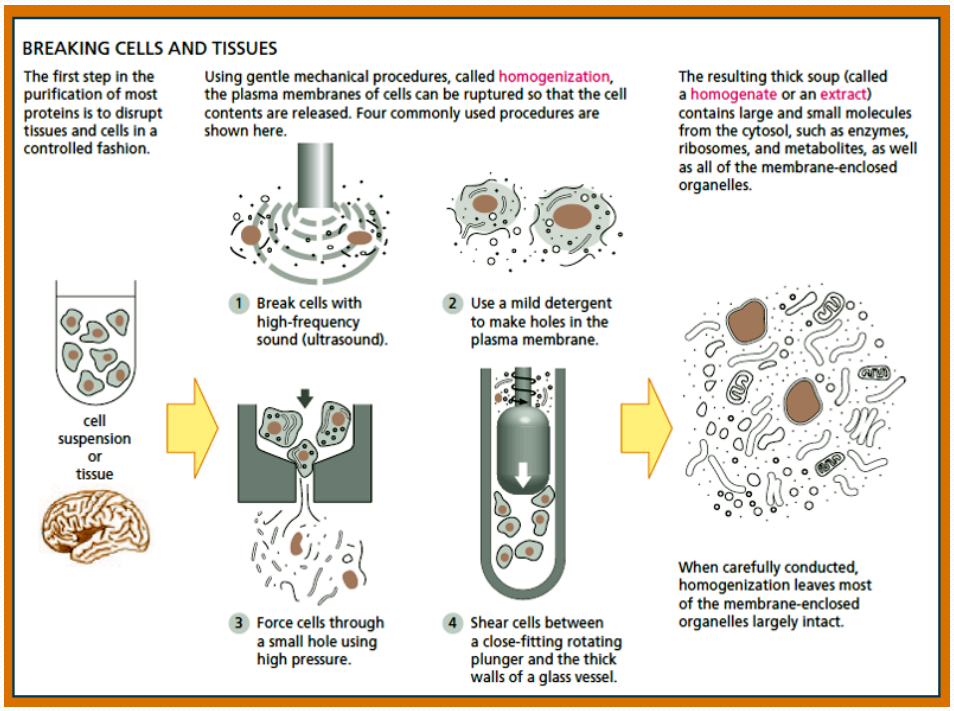
How can we isolate membrane?

1. Disruption of cell membrane by *Homogenisation*.
2. Separation of cell components by *Differential Centrifugation.*

Different methods for disrupting cell membrane

There are many methods for disrupting cell depending on the cell type

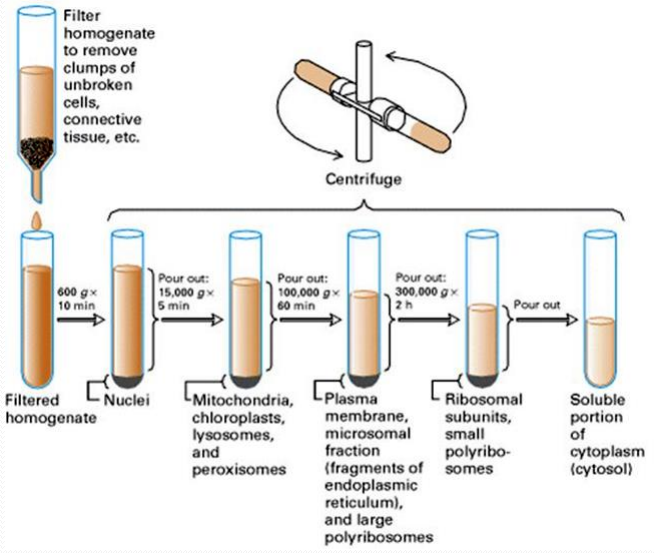
Technique	Principle	Advantages (+) / Disadvanatges (-)
Liquid shear pressure (e.g., French press)	Rapid pressure drop by transferring the sample from a chamber at high pressure through an orifice into a chamber at low pressure	+ Fast and efficient, also for large volumes - Causes heating of the sample (cooling is required)
Ultrasonication	Cells disrupted by high frequency sound	+ Simple - Causes heating of the sample, which can be difficult to control by cooling - Proteins may be destroyed by shearing - Noisy - Not for large volumes
Glass bead milling	Agitation of the cells with fine glass beads	+ Useful for cells that are more difficult to disrupt (e.g., yeast) - Somewhat slow and noisy
Osmotic shock	Change from high to low osmotic medium	+ Simple, inexpensive - Only useful for disruption of cells with less robust walls (e.g., animal cells)
Repeated freezing and thawing	Cells disrupted by repeated formation of ice crystals; usually combined with enzymatic lysis	+ Simple, inexpensive + Yields large membrane fragments - Slow - May damage sensitive proteins and dissociate membrane protein complexes - Low yield
Enzymatic lysis	Often used in combination with other techniques, e.g., freeze-thawing or osmotic shock; lysozyme is commonly used to break cell walls of bacteria	+ Gentle + Yields large membrane fragments - Slow - Low yield



Separation of cell components by differential centrifugation

- For studies of membrane composition, the first task is to isolate a selected membrane.
- When eukaryotic cells are subjected to mechanical shear, their plasma membranes are torn and fragmented, releasing cytoplasmic components and membrane-bounded organelles such as mitochondria, chloroplasts, lysosomes, and nuclei.
- Plasma membrane fragments and intact organelles can be isolated by centrifugal techniques.

Differential centrifugation



Why exploring membrane proteins is difficult?

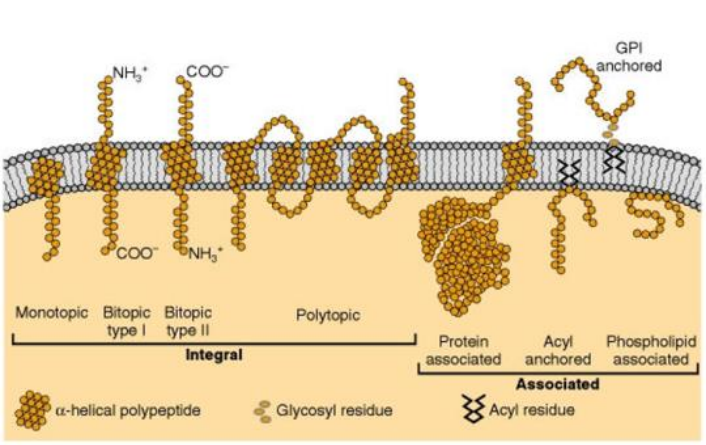
Membrane proteins are difficult to purify for the following reasons:

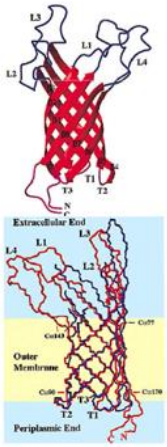
- It is associated with membranes (peripheral or integral).
- It has hydrophobic domains, and tend to form aggregates (why?).
- It is easily degraded by proteases following solubilization (why?).
- It is difficult to purify in high concentration in native form (why?).
- It is difficult to be expressed by recombinant DNA technology (why?).

Protein extraction depends on the type of protein

- To study the membrane proteins, it must be separated and purified.
- One have to consider the type of membrane protein of interest.
- **There are two main types of membrane proteins:**
  - ***Peripheral proteins,*** which bind noncovalently to the membrane surfaces by electrostatic and hydrogen bonds or covalently through lipids or PGI anchors.
  - ***Integral proteins.*** More strongly associated with the hydrophobic moiety of the phospholipid bilayer and contain one or more apolar domains (α-helix and β-sheet that span the lipid bilayer). It divides into :
    - Type I with the C-terminal protrude to the cytosol
    - Type II with the N-terminal protrude to the cytosol

Type of membrane proteins	
Integral	**Peripheral (associated)**
- Monotopic - Bitopic type I - Bitopic type II - Polytopic	- Protein associated - Acyl anchored - Phospholipid associated





Extraction of membrane proteins:

Peripheral proteins

- Peripheral proteins are dissociated by disrupting the electrostatic or hydrogen bonds between the protein in the surface of the phospholipid bilayer, without destroying the membrane.

Treatment	Example
Acid buffer	pH 3.0-5.0
Alkaline buffer	pH 8.0-12.0 (e.g. 100 mM Na_2CO_3, pH 11.3)
Chaotropic ions	I^-, ClO_4^-, SCN^-
Salt/ high ionic strength	1M NaCl or KCl

- *High pH* disrupts the sealed membrane structures without denaturing the lipid bilayer or the integral proteins.
- *Chaotropic ions* disrupts the hydrophobic bonds the membrane surface and promote the transfer of hydrophobic groups from nonpolar environment to the aqueous phase.
- *High salts* decreases the electrostatic interaction between protein and charged lipids.

Extraction of membrane proteins:

Integral proteins

- Extraction of integral proteins needs the use of strong agent that solubilize the phospholipid bilayer to liberate proteins.
- These agents can be classified into:
  - organic solvent,
  - detergent that solubilize the lipid bilayer
  - denaturing agents (8M Urea or 6 M Guanidine hydrochloride).

Extraction of membrane proteins:

Integral proteins (Cont.)

Detergent class	
Advantage	Disadvantage
Nonionic e.g. dodecyl maltoside	
Generally mild and non-denaturing Widely used	May give low solubilization yields
Ionic (anionic or cationic) e.g. SDS	
Can be extremely efficient in solubilization	Often denaturing Interfere with ion exchange separation
Zwitterionic , e.g. FOS-Choline 12	
Often used in membrane protein crystallization. Combines the advantages of ionic and non-ionic detergent	More denaturing than non-ionic detergent

Extraction of integral proteins:

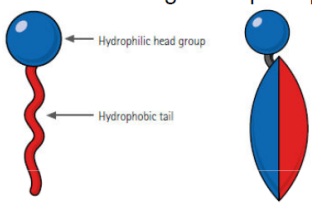
solubilisation with detergents

- The first step in this separation process involves solubilizing the membrane with agents that destroy the lipid bilayer by disrupting hydrophobic associations.
- The most widely used disruptive agents are detergents.
  - Detergent is characterized by its similarity to membrane phospholipids as it is amphipathic but they are small and have only a single hydrophobic tail, so it tends to aggregate into small *micelles* in water rather than forming a bilayer.
  - When detergents are mixed in great excess with membranes, its hydrophobic ends interact with hydrophobic regions of the transmembrane proteins that span the membrane, as well as with the hydrophobic tails of the phospholipid molecules. So, it disrupts the lipid bilayer and separates the proteins attached to the phospholipids.
  - The hydrophilic end of the detergent molecule interacts with water and brings the membrane proteins into solution and solubilizes the phospholipids. Hence it can then be separated further analysis.

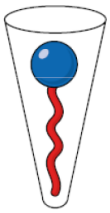
Essential Cell Biology, 4th ed. Albert et al., 2014 page 372

Detergents

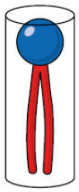
Detergents: amphipathic substances with a polar (hydrophilic) head group and a nonpolar, (hydrophobic) tail.
measurable aqueous solubility as both aggregates and as monomers
Classified according to the polar part: nonionic, anionic, cationic, or zwitterionic

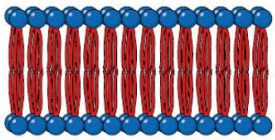


Some detergents contain both polar and nonpolar "faces";



Traditional detergent monomers are generally cone shaped; hydrophilic head groups occupying more molecular space than the linear alkyl chains





Lipids generally cylindrical; area occupied by the two alkyl chains is similar to the area occupied by the polar head group. Lipids have low solubility as monomers and tend to aggregate into planar bilayers that are water insoluble.

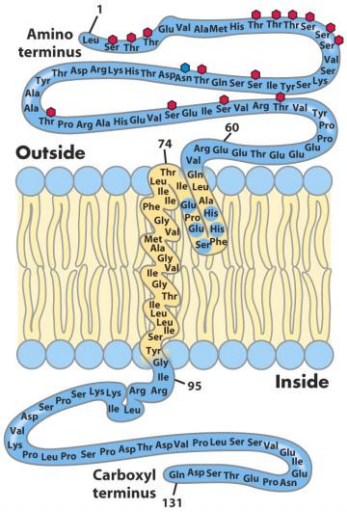
http://grad.md.chula.ac.th/english%20old/data/PEP_2011_8_membrane_protein_secure.pdf

Extraction of integral proteins:

Digestion by protease

A simple example of the of integral proteins is the erythrocyte glycoprotein **glycophorin.**

It is composed of hydrophilic sugar-containing carboxyterminal exposed to the cytoplasm; middle hydrophobic part (residues 75 to 93) impeded in the phospholipid bilayer of the membrane and other hydrophilic aminoterminal exposed to the extracellular **aqueous** medium**.**



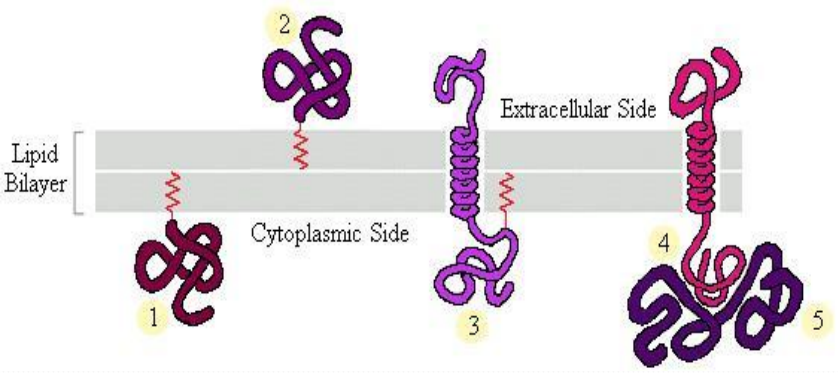
Essential Cell Biology, 4th ed. Albert et al., 2014

Extraction of integral proteins:

Digestion by protease (Cont.)

The structure of the extracellular domain can be determined with reagents that react with R groups of the amino acids.

- Trypsin cleaves the extracellular domains of proteins because it contains lysine residue targeted by the enzyme but does not affect domains buried within the bilayer or exposed on the inner surface.
- The segment in the center of the protein (residues 75 to 93, mainly hydrophobic amino acid residues and impeded part as it is in the phospholipid bilayer) and the protein segment in the inner face of the plasma membrane (carboxyl-terminal domains, hydrophilic) can not be cleaved because the membrane is impermeable to trypsin.



If you expose an intact cell to a protease enzyme, you can shave off the protein found on the outside of the cell.
If the cell is broken, you can remove proteins from the inside of the membrane as well, leaving only the parts of proteins within the cell wall intact.

Visualization of membrane proteins with SDS-PAGE

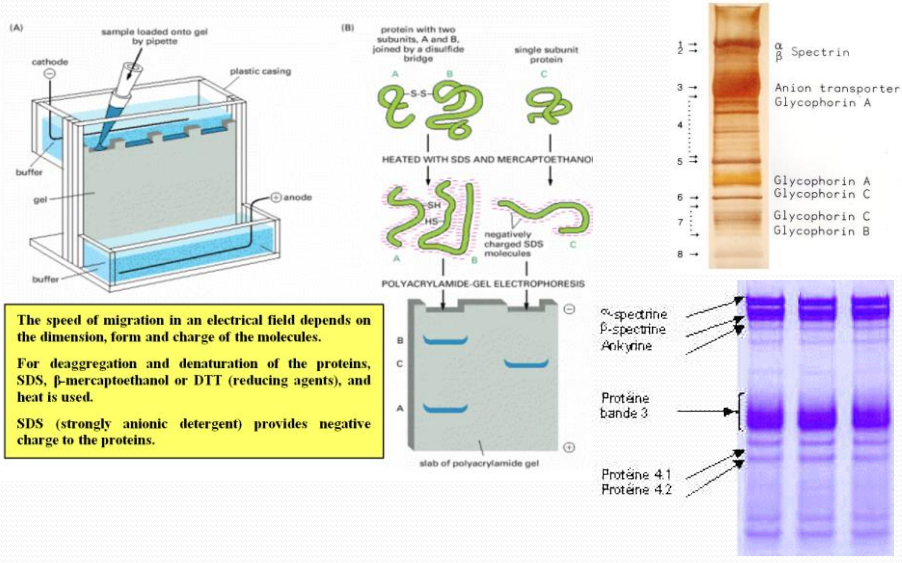
- The **number of different proteins** in a membrane varies from less than a dozen in the sarcoplasmic reticulum of muscle cells to over 100 in plasma membranes.
- Membrane proteins can be separated from one another using **sodium dodecyl sulfate polyacrylamide gel electrophoresis** (SDS-PAGE), a technique that separates proteins based on their molecular mass.
- The technique is based upon the principle that a negatively charged denatured proteins (due to SDS) migrate in an electric field towards the positively charged electrode (anode).
- SDS is a powerful detergent (Why?) In the absence of SDS, few membrane proteins remain soluble.
- After the visualization by a staining the protein Molecular Weight can be estimated using standard proteins of known molecular mass

Visualization of membrane proteins with SDS-PAGE (cont.)

SDS-PAGE for human erythrocyte cell membrane:

- There are more than 50 known membrane proteins in RBCs.
- These membrane proteins can perform a wide diversity of functions, such as transporting ions and molecules across the red cell membrane, adhesion and interaction with other cells such as endothelial cells, as signaling receptors, as well as other currently unknown functions.
- Approximately 25 of these membrane proteins carry the various blood group antigens,
- 15 major protein bands are detected, (Mwt from 15-250 kDa) when the human red blood cell plasma membrane proteins are separated by SDS polyacrylamide-gel electrophoresis.
- Three of these proteins *spectrin*, *glycophorin*, and *band 3* account for > 60% (by weight) of the total membrane protein.

Analyses of protein on SDS-PAGE



Visualization of membrane proteins with freeze fracture

- This technique provides a way of visualizing the interior of cell membranes.
- Cells are frozen and then the frozen block is cracked with a knife blade.
- The fracture plane often passes through the hydrophobic middle of lipid bilayers, thereby exposing the interior of cell membranes.
- The resulting fracture faces are shadowed with platinum, the organic material is dissolved away, and the replicas are floated off and viewed in the electron microscope.
- Such replicas are studded with small bumps, called *intramembrane particles,* which represent large transmembrane proteins.
- The technique provides a convenient and dramatic way to visualize the distribution of such proteins in the plane of a membrane.

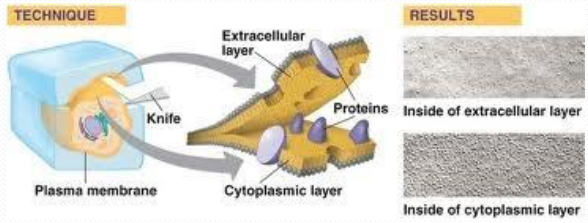
Molecular Biology of the Cell. 4th edition **https://www.youtube.com/watch?v=wxZxLMpxvTM**

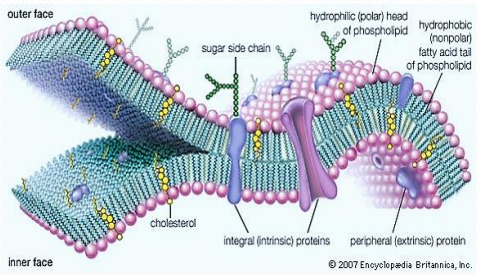
Visualization of membrane proteins with Freeze-etch electron microscopy

- **Freeze-etch electron microscopy**, is used to examine either the exterior or interior of cells.
- In this technique, the frozen block is cracked with a knife blade.
- The ice level is lowered around the cells (and to a lesser extent within the cells) by the sublimation of ice in a vacuum as the temperature is raised—a process called *freeze-drying*.
- The parts of the cell exposed by this *etching* process are then shadowed as before to make a platinum replica.
- This technique exposes structures in the interior of the cell and can reveal their three-dimensional organization with exceptional clarity

Molecular Biology of the Cell. 4th edition **https://www.youtube.com/watch?v=WitmkyrrUNI**

Visualization of membrane proteins with freeze-etching electron microscopy





Fluidity of biomembranes

- **The meaning of membrane fluidity:**
  - The fluidity of a cell membrane means the ease of its lipid molecules to move within the plane of the bilayer.

- **Importance of membrane fluidity.**
  - It enables many membrane proteins to diffuse rapidly in the plane of the bilayer and to interact with one another.
  - It permits membrane lipids and proteins to diffuse from sites of their synthesis into other regions of the cell.
  - It ensures that membrane molecules are distributed evenly between daughter cells when a cell divides.
  - It allows membranes to fuse with one another like in endocytosis, exocytosis and phagocytosis.
  - It enables the cell grow, change its shape and move and reproduce.

Essential Cell Biology, 4th ed. Albert et al., 2014 page 365

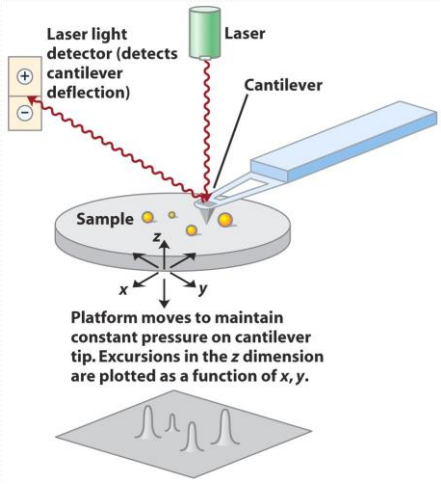
Factors affecting fluidity of membranes

- **The increase in unsaturated FA content increases membrane fluidity.**
  - The Phospholipids contain one saturated FA and one unsaturated FA. The unsaturated FA has one or more double bonds in the hydrocarbon tail. Each double bond creates a small kink in the tail which makes it more difficult to pack against one another. More than one double bond more kinks, less ordered packing and more fluid membrane.
- **The presence of sterols reduces the fluidity in the core of the bilayer, and increases the thickness of the lipid leaflet.**
  - Cholesterol represent about 20% of the membrane lipids by weight.
  - The rigid planar structure of cholesterol, inserted between fatty acyl side chains, reduces the movement of neighboring fatty acyl chains to move.
- **Shorter FA chains more fluid structure and Longer chain less fluidity.**

Essential Cell Biology, 4th ed. Albert et al., 2014 page 365

Visualization of Membrane Proteins by Atomic Force Microscopy (AFM)

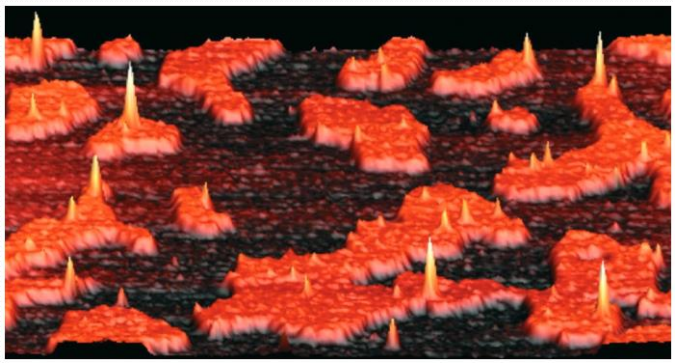
Write short essay about AFM
Use the following four slides in the essay.



28

essay

Raft regions visualized by Atomic force microscopy (AFM)

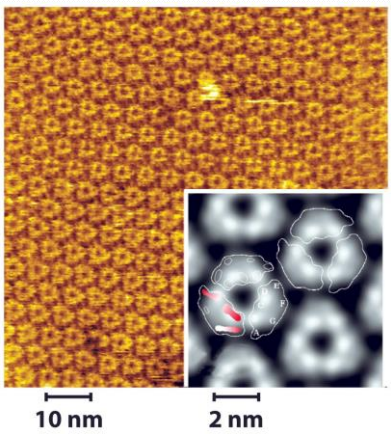


29

essay

AFM

Single molecules of bacteriorhodopsin in the purple membranes of the bacterium *Halobacterium salinarum*

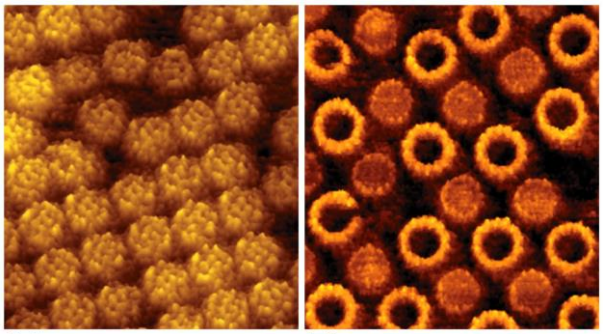


30

essay

AFM

Purified *E.coli* aquaporin F_0-chloroplast ATP synthase



(b) (c)

31

References and resources:

http://grad.md.chula.ac.th/english%20old/data/PEP_2011_8_membrane_protein_secure.pdf

Visualization of membrane proteins with freeze fracture and etching EM.
https://www.youtube.com/watch?v=wxZxLMpxvTM
https://www.youtube.com/watch?v=WitmkyrrUNI

Essential Cell Biology, Arbers etal., 4th Ed.
Lehninger, Biochemistry, 6th Ed.

- https://quizlet.com/123617448/chapter-11-biological-membranes-and-transport-flash-cards/
- https://quizlet.com/104726299/chapter-11-biological-membranes-and-transport-flash-cards/ (MCQ)
- https://quizlet.com/169548375/chapter-11-biological-membranes-and-transport-flash-cards/ (fill in the space)
- http://wenku.baidu.com/view/e4a071c65fbfc77da269b155.html###
- https://quizlet.com/85889411/chapter-11-biological-membranes-and-transport-flash-cards/ (MCQ)
- https://quizlet.com/109358583/chem-4551-chapter-11-biological-membranes-and-transport-flash-cards/ (written questions)