

BIOL 151 CELL STRUCTURE

EXAMINER:

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COVID-19 AWARENESS

- COVID-19: Caused by a virus known as Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2). Spreads very **easily** from person to person.
- **Signs and symptoms:** Fever or chills, cough, difficulty in breathing, cold, headache, diarrhea, loss of taste/smell, and several non-specific symptoms.
- **Transmission:** Respiratory droplets, airborne, contaminated surfaces.

COVID-19 AWARENESS CONT'D.

■ **Prevention:** Adhere to the COVID-19 safety protocols

- **Respiratory hygiene:** Wear a nose mask, cough etiquettes
- **Hand hygiene:** Frequent hand washing, hand sanitizing
- Maintain ‘safe’ physical distancing
- Avoid crowds and confined/poorly ventilated spaces

Virus is changing itself so it is important we all adhere to the safety protocols

ATTENDANCE AT LECTURES

- Attendance at lectures is an integral part of the requirements for the assessment for the course.
- 10 % of the marks will be awarded to students at the end of the semester for 100 % attendance at lectures.

ATTENDANCE AT LECTURES

CONT'D.

However, if any student absents herself/himself for a cumulative total of three (3) lecture periods before mid-semester, she/he will not be eligible to take part in both the mid-semester and end of semester examinations or will not be eligible to write the end of semester examination if this occurs after the mid-semester examination.

ATTENDANCE AT LECTURES CONT'D.

- Any student(s) affected by this regulation will however, be scored deferred (Df) and not zero per cent (0 %) for the course.
 - Any student who absents herself or himself from lectures with the **express permission of the lecturer** or for any reason acceptable to **the lecturer** will not be adversely affected.
-

MODE OF ASSESSMENT

- Pass mark for the course is 40 %. Mid-Semester is 30 % and End of Semester Examination is 70 %.
- The Mid-Semester Examinations shall be conducted from **Monday, March 01, 2021 to Friday, March 05, 2021**. Watch out for the timetable on your Notice Boards for exactly when BIOL 151 Cell Structure examination will be taken.
- The examination shall be fill in the blanks.

MODE OF ASSESSMENT CONT'D.

- The duration for the examination shall be one hour.
 - The examination questions shall be set from Monday/Tuesday/Thursday, January 18/19/21, 2021 to Monday/Tuesday/Thursday, February 22/23/25, 2021 for HB (Medicine), Biological Sciences and HB (Dental Surgery) and Physician Assistanship and Vet. Medicine students respectively.
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MODE OF ASSESSMENT CONT'D.

- The End of Semester Examination shall take place from Monday, April 12, 2021 to Friday, April 23, 2021.
- The examination questions shall be 100 or 120 multiple choice questions for a duration of two (2) hours.
- You are advised to consult the Notice Boards for both the Draft/Provisional and Final timetables.

COURSE ASSESSMENT

- The End of Semester Examinations questions will be made of the following:
 - *C1 – Knowledge,*
 - *C2 – Comprehension,*
 - *C3 – Application,*
 - *C4 – Analysis,*
 - *C5 – Synthesis,*
 - *C6 – Evaluation.*

COURSE ASSESSMENT CONT'D.

- Attendance and Participation in Lectures
→ 10%
- Assignment/presentation/ Term paper
→ 5%
- Practicals/Labs
→ 5%
- Mid-Semester Examinations
→ 20%
- Final Examinations
→ 70%

GRADING

<input type="checkbox"/>	70 – 100	→	A	→	Excellent
<input type="checkbox"/>	60 – 69	→	B	→	Very Good
<input type="checkbox"/>	50 – 59	→	C	→	Good
<input type="checkbox"/>	40 – 49	→	D	→	Pass
<input type="checkbox"/>	0 – 39	→	F	→	Fail
<input type="checkbox"/>			I	→	Incomplete

CLASSIFICATION OF DEGREE

- 70.00 – 100.00 → 1st Class
- 60.00 – 69.99 → 2nd Class (Upper Division)
- 50.00 – 59.99 → 2nd Class (Lower Division)
- 45.00 – 49.99 → Pass

What Industry looks for in potential employees: Employability Skills

- Some examples of employability skills are:
 - i. Communication and Interpersonal Skills
 - ii. Problem Solving skills
 - iii. Initiative
 - iv. Working under pressure
 - v. Organisational skills

What Industry looks for in potential employees: Employability Skills Cont'd

- vi. Team working
- vii. Adaptability
- viii. Numeracy
- ix. Valuing diversity and difference
- x. Critical thinking

READING OR REFERENCE MATERIAL

- Principles and techniques of practical Biochemistry. Fourth Edition and edited by Keith Wilson and John Walker.
- Techniques used in Bioproduct Analysis. Published by Butterworth-Heinemann Ltd.
- Structure and Function of cells, by Colin R. Hopkins.

READING OR REFERENCE MATERIAL CONT'D.

- Molecular and Cell Biochemistry: Cell Biology, 1st Edition by C. A. Smith and E. J. Wood.
 - Molecules of Life, Structure and Function at a glance, 2nd Edition, by J. P. Adjimani.
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READING OR REFERENCE MATERIAL CONT'D.

- Biology, Concepts and Connections, 2nd Edition, by Neil A. Campbell, Lawrence G. Mitchell and Jane B. Reece.
 - Microbiology: An Introduction (8th Edition) by Gerard J. Tortora, Berdell R. Funke and Christine L. Case.
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READING OR REFERENCE MATERIAL CONT'D.

- Cell Biology, Structure, Biochemistry and function (2nd edition), by Philip Sheeler and Donald E. Bianchi etc.
- Plant Cell Structure and Metabolism by J. L. Hall, T. J. Flowers and R. M. Roberts.

READING OR REFERENCE MATERIAL CONT'D.

- ❑ Cell and Molecular Biology, Concepts and Experiments, 3rd Edition, by Gerald Karp.

 - ❑ Biology, 8th Edition, Pearson International, by N. A. Campbell, J. B. Reece, L. A. Urry, M. L. Cain, S. A. Wasserman, P. V. Minorsky and R. B. Jackson.
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BROAD COURSE OBJECTIVES

- At the end of the course, you will be required to:
- Know the theory and uses of a variety of types of light microscopes.

BROAD COURSE OBJECTIVES CONT'D.

- Describe the advances made over the last three centuries in studying cell structure and function.
 - Know the theory and uses of the electron microscope in Biology.
 - Evaluate the advantages and disadvantages of electron microscopy in Biology.
-

BROAD COURSE OBJECTIVES CONT'D.

- Discriminate between organisms on the basis of their cellular structure.
 - Describe the structure and functions of organelles of eukaryotic cells.
 - Summarize the major methods of disrupting and fractionating cells and tissues.
-

SPECIFIC COURSE OBJECTIVES ON MICROSCOPY

- At the end of this chapter on microscopy, you should be able to:
- Know why there is the need to invent new microscopes that continue to be more powerful than the earlier ones.
- Know what a simple microscope is.
- Know the defects of simple microscopes.

SPECIFIC COURSE OBJECTIVES ON MICROSCOPY CONT'D.

- Know how to overcome the defects of the simple microscopes.
- Classify modern/conventional light microscopes.
- Know the uses of compound and dissecting microscopes.
- Define magnification.
- Distinguish between ‘useful’ and ‘empty’ magnification.

SPECIFIC COURSE OBJECTIVES ON MICROSCOPY CONT'D.

- Contrast:
- Identify a use for darkfield, phase-contrast, fluorescence, ultra-violet, ultra violet – fluorescence microscopy and compare each with brightfield microscopy.

SPECIFIC COURSE OBJECTIVES ON MICROSCOPY CONT'D.

- Know what parfocal is.
- Define resolution.
- Distinguish between limit of resolution and the resolving power of the microscope.
- Know the factors that determine the resolving power of a microscope.

UNITS OF MEASUREMENTS OF CELLS IN MICROSCOPY

- 1000 microns or micrometers (μm)
= 1mm
- 1000 $\text{m}\mu$ or nanometers (nm) = 1 μm
- 10 Angstrom (\AA) = 1 $\text{m}\mu$
- $10^6 \text{ m}\mu$ = 1mm

UNITS OF MEASUREMENT CON'T.

- ❑ Because microorganisms and their components are very small, they are measured in small units such as micrometers and nanometers. A micrometer (μm) is equal to 0.000001 m (10^{-6} m). A nanometer (nm) is equal to 0.000000001 m (10^{-9} m). Angstrom (\AA) was previously used for 10^{-10} m or 0.1 nm.

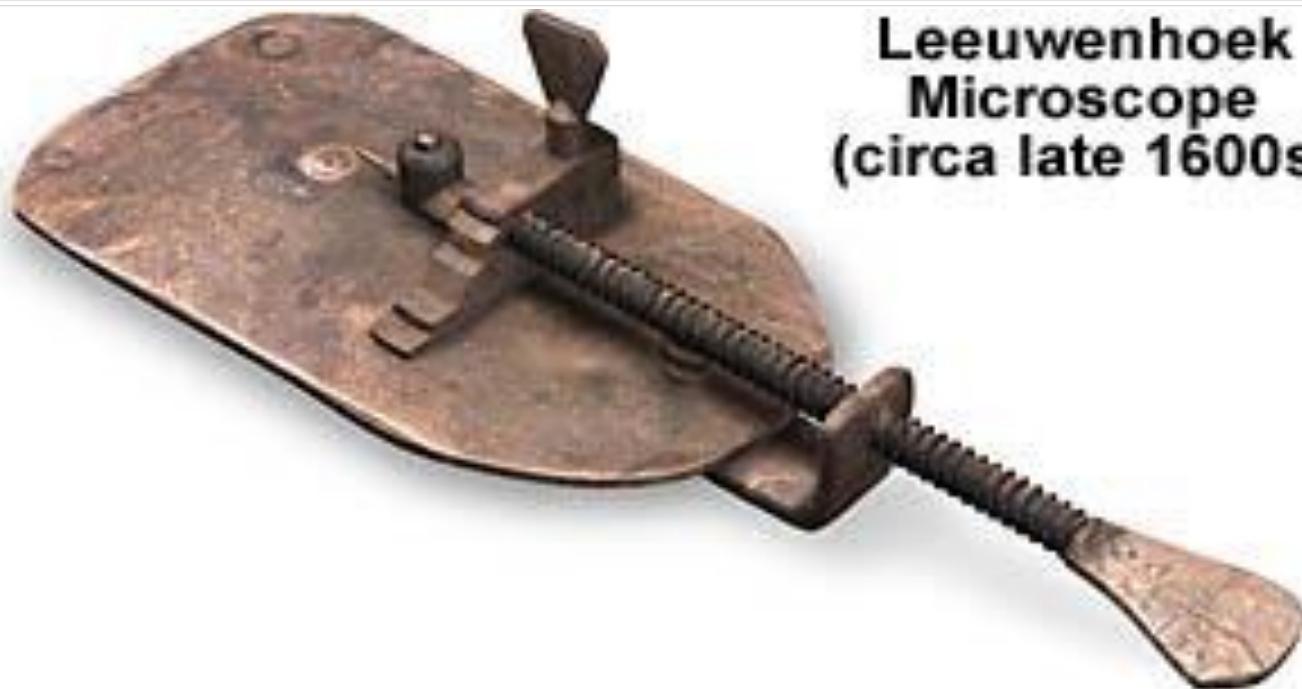
MICROSCOPY

- The unaided human eye can distinguish objects as small as 0.1 mm (100 μ m), but not smaller than this.
- To be able to view cell structure down to the molecular level while counteracting the transparency of the cell by increasing contrast requires the invention of microscopes with high resolving power.

TYPES OF LIGHT MICROSCOPES

- There are two types of light microscopes:
- Simple microscopes and
- Compound microscopes

Simple Microscope

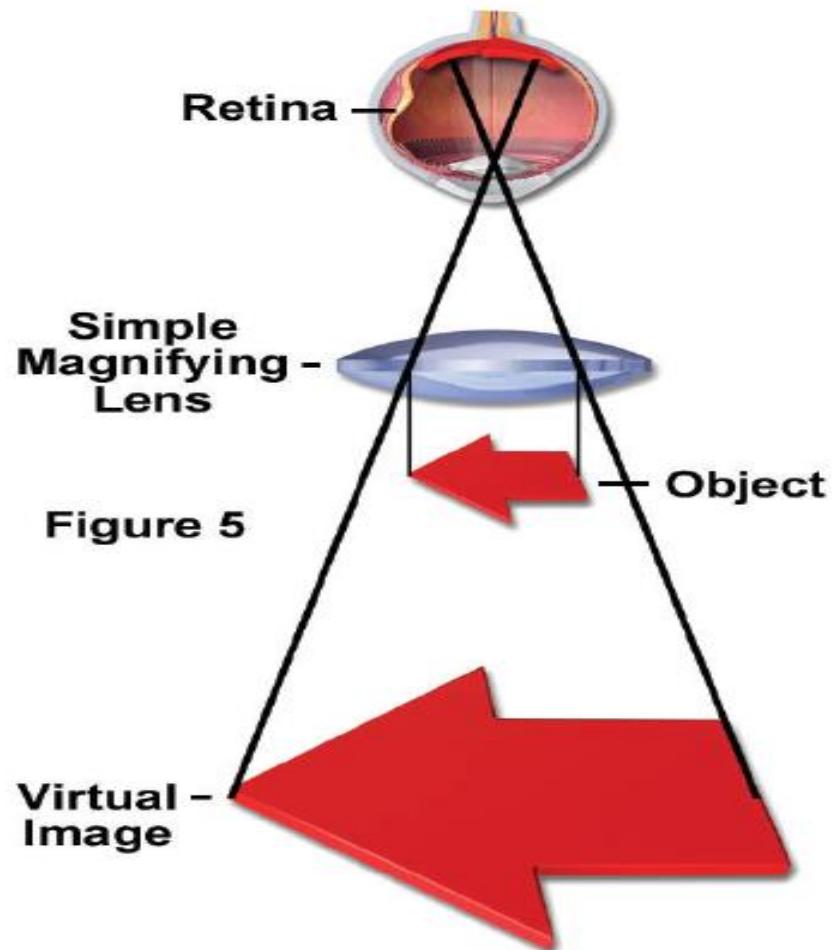
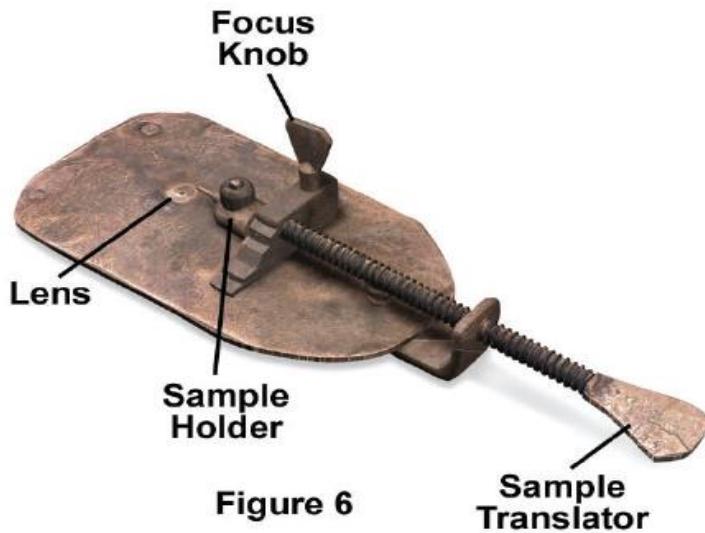


**Leeuwenhoek
Microscope
(circa late 1600s)**

Simple Microscope Cont'd



Simple magnifier – forming an image



SIMPLE MICROSCOPES

□ A simple microscope is a microscope which only has one lens, as opposed to the compound lenses used in more complex microscope designs. Magnifying glasses and loupes are two well-known examples of the simple microscope.

SIMPLE MICROSCOPES CONT'D.

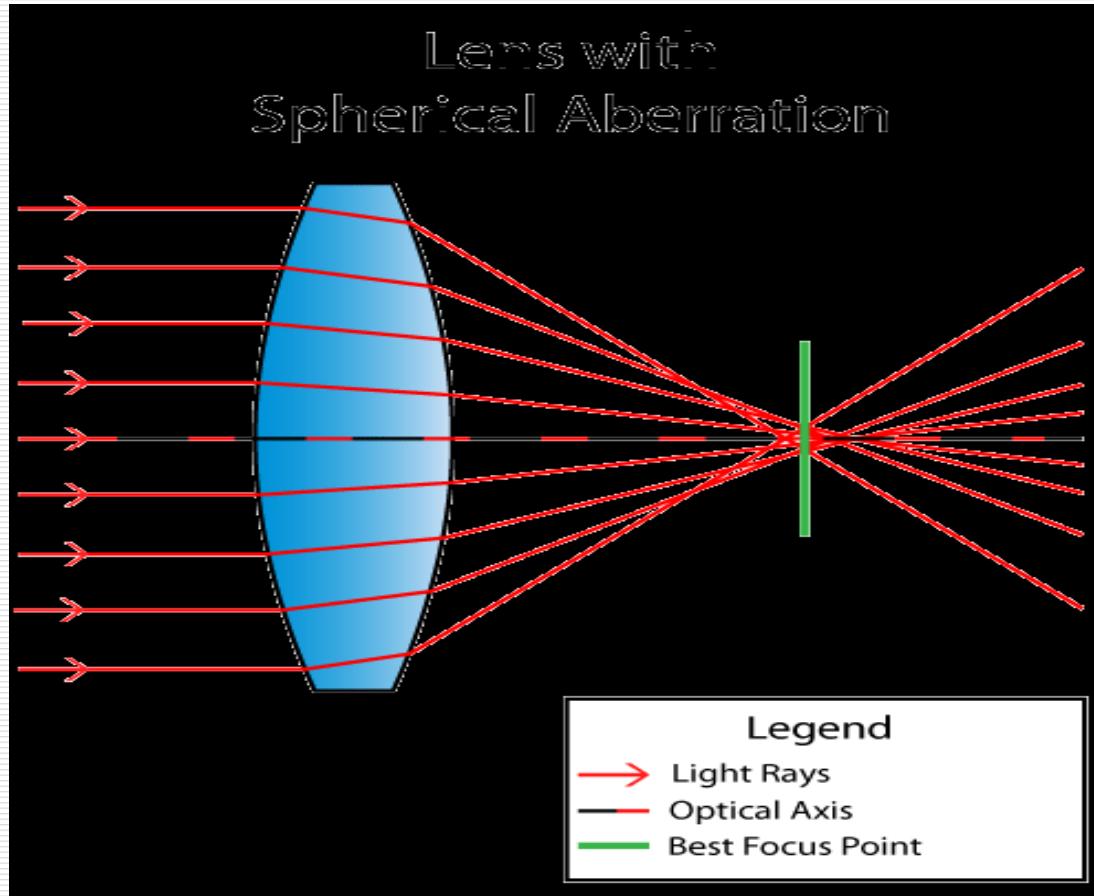
- Modern simple microscopes are usually hand held, designed for field work or quick viewing of objects which require magnification. Historical designs included mounts which resemble those used with modern microscopes, although instead of viewing the object through a series of lenses, the user had only one lens to use.

DEFECTS OF SIMPLE MICROSCOPES

- There are two inherent optical defects associated with simple microscopes:
 - a) Spherical aberration: Blurred images are formed because light rays from a specimen are not brought to simultaneous focus.

DEFECTS OF SIMPLE MICROSCOPES

CONT'D



DEFECTS OF SIMPLE MICROSCOPES

CONT'D

- Spherical Aberration is an optical problem that occurs when all incoming light rays end up focusing at different points after passing through a spherical surface. Light rays passing through a lens near its horizontal axis are refracted less than rays closer to the edge or “periphery” of the lens and as a result, end up in different spots across the optical axis.

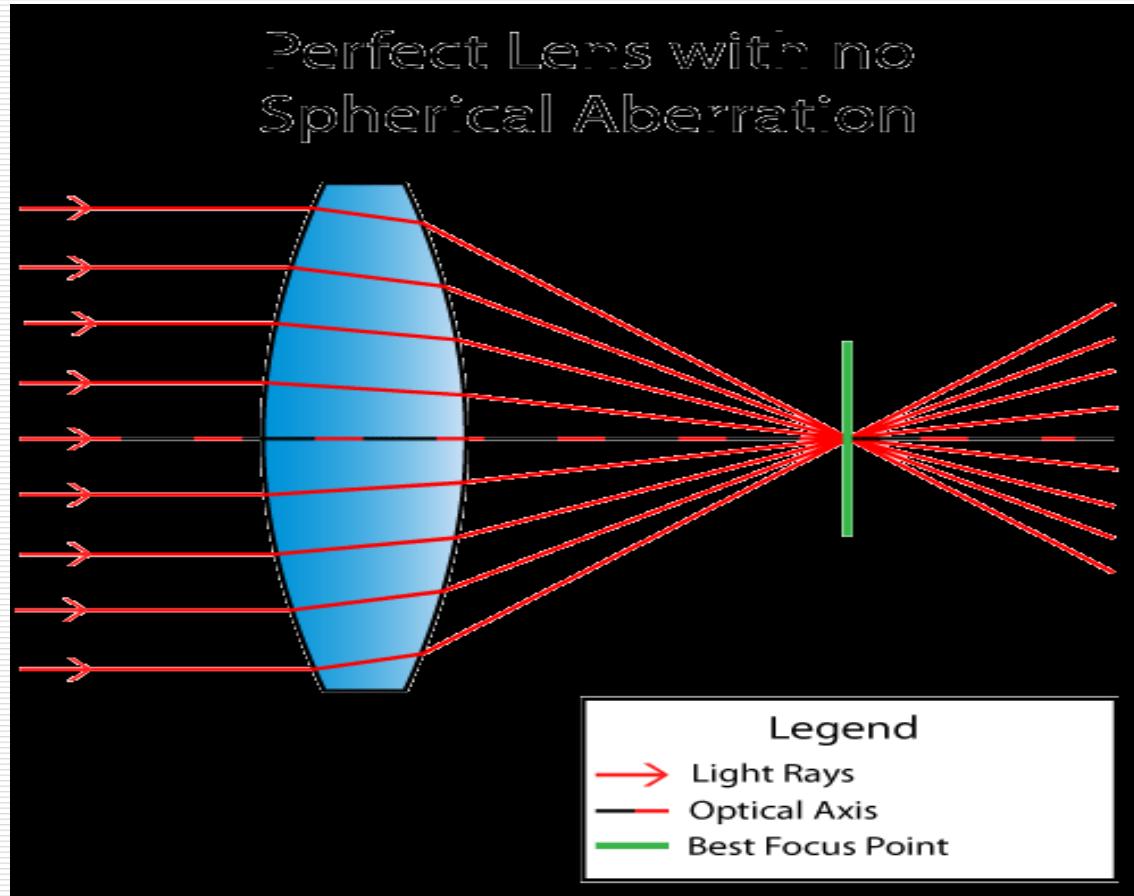
DEFECTS OF SIMPLE MICROSCOPES

CONT'D

- In other words, the parallel light rays of incoming light do not converge at the same point after passing through the lens. Because of this, Spherical Aberration can affect resolution and clarity, making it hard to obtain sharp images.
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DEFECTS OF SIMPLE MICROSCOPES

CONT'D



DEFECTS OF SIMPLE MICROSCOPES

CONT'D

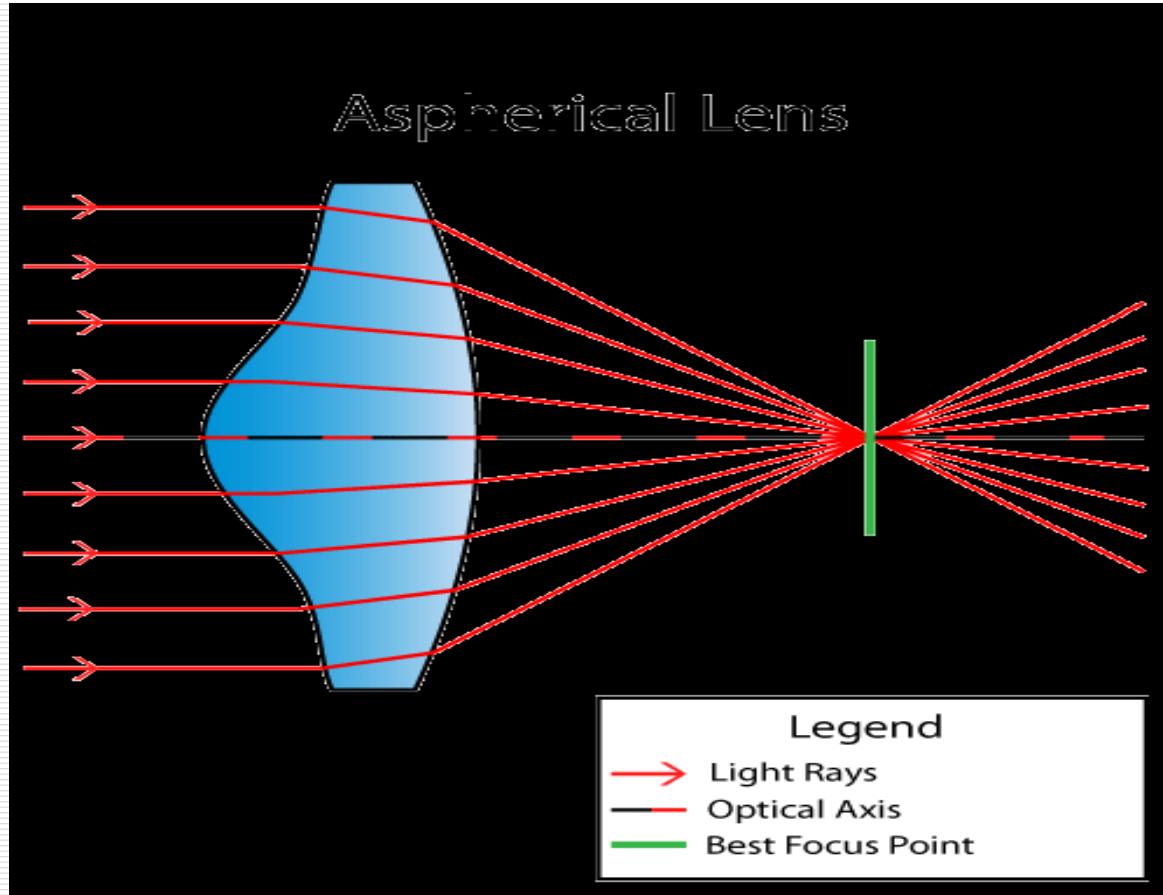
- Spherical aberration also occurs when the specimen and the objective lens are separated by several materials of different refractive indices e.g. air, glass etc. This results in the repeated bending of the light rays at different angles.

Ways to Reduce Spherical Aberration

- Modern lenses employ different techniques to dramatically reduce spherical aberration. One of the methods employs using a specialized **aspherical** (meaning non-spherical) lens surface, which is curved outwards on one side for the sole purpose of converging light rays into a single focal point.

Ways to Reduce Spherical Aberration

Cont'd



Ways to Reduce Spherical Aberration

1. This can be corrected by coating the periphery of the lens with opaque materials
2. Using spherical lenses such as plano-convex lenses
3. Using oil immersion lens since this will fill the entire space between the specimen and the lens with substances of the same refractive index to eliminate spherical aberration

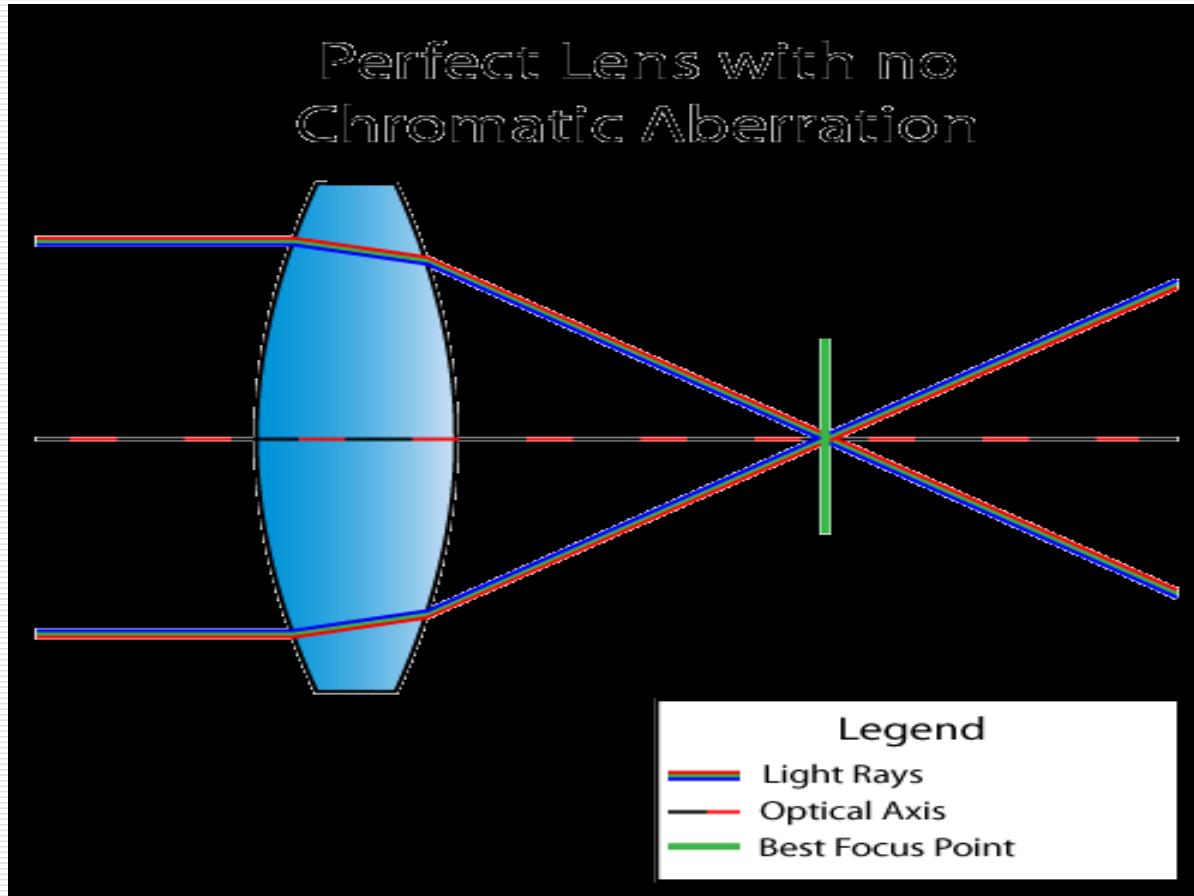
b) Chromatic Aberration

□ Chromatic Aberration, also known as “colour fringing” or “purple fringing”, is a common optical problem that occurs when a lens is either unable to bring all wavelengths of color to the same focal plane, and/or when

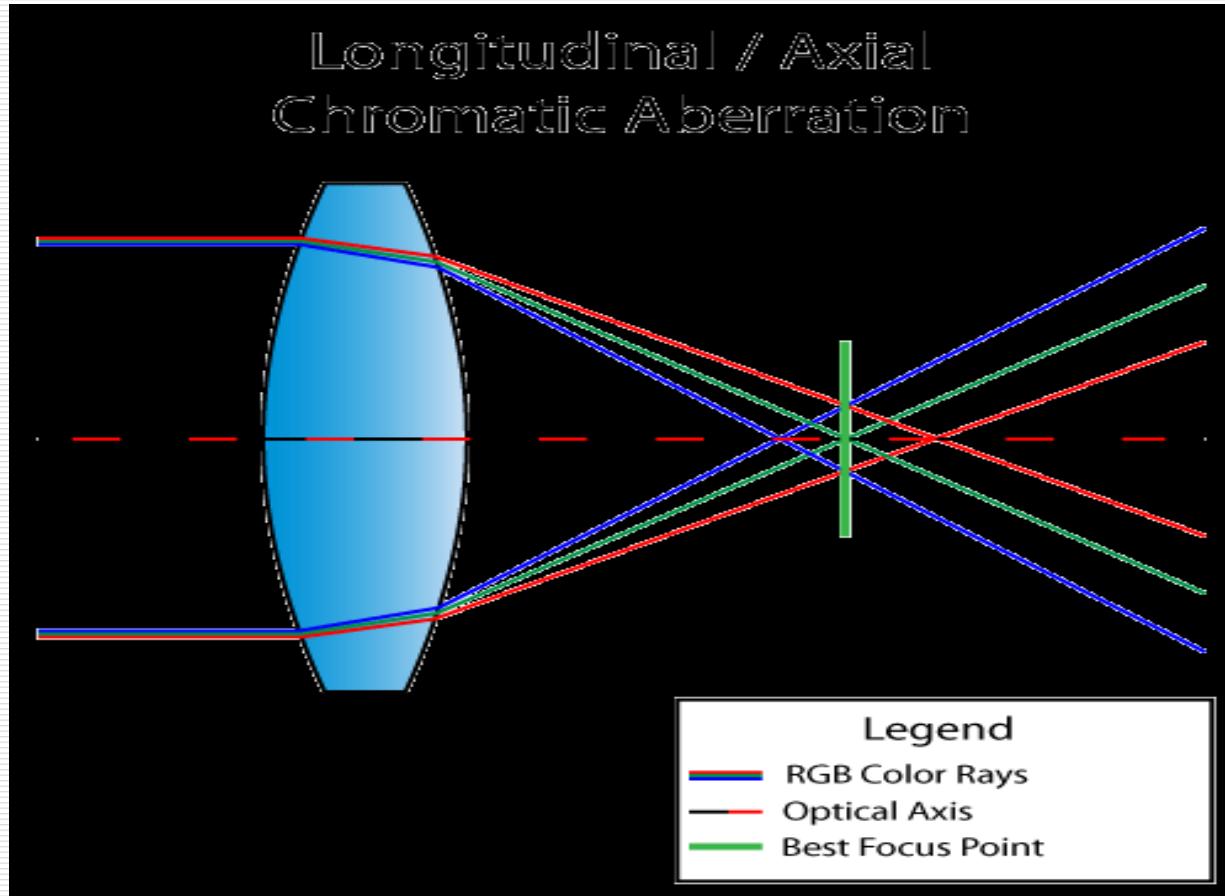
Chromatic Aberration Cont'd

- wavelengths of color are focused at different positions in the focal plane. Chromatic aberration is caused by **lens dispersion**, with different colors of light travelling at different speeds while passing through a lens. As a result, the image can look blurred or noticeable colored edges (red, green, blue, yellow, purple, magenta) can appear around objects, especially in high-contrast situations.

Chromatic Aberration Cont'd



Chromatic Aberration Cont'd



Ways of correcting chromatic aberration

1. Using flint glass and crown which have equal but opposite focal lengths. This combination of crown and flint is known as **Acromatic Doublet**.
2. Using more complex systems of lenses.

LIGHT MICROSCOPY.

- Light microscopy refers to the use of any kind of microscope that uses visible light to observe specimens. Here we examine several types of light microscopy.
- The curvatures of the lens materials and their composition are designed to minimize distortion of image shapes and colours.
- Light microscopes are of two different basic types:

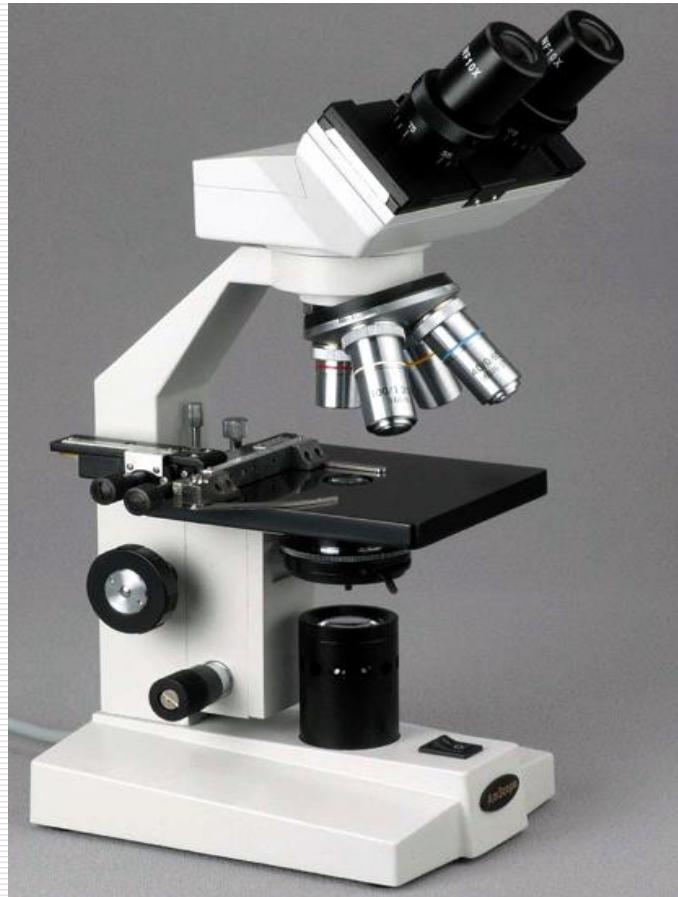
TYPES OF LIGHT MICROSCOPES

- 1. Compound Microscopes:** A modern compound light microscope has a series of lenses and uses visible light as its source of illumination. These microscopes require that the specimen to be examined, is cut/sliced thinly enough to allow light to pass through it.
- 2. Dissecting Microscopes:** These microscopes permit opaque specimens to be viewed.

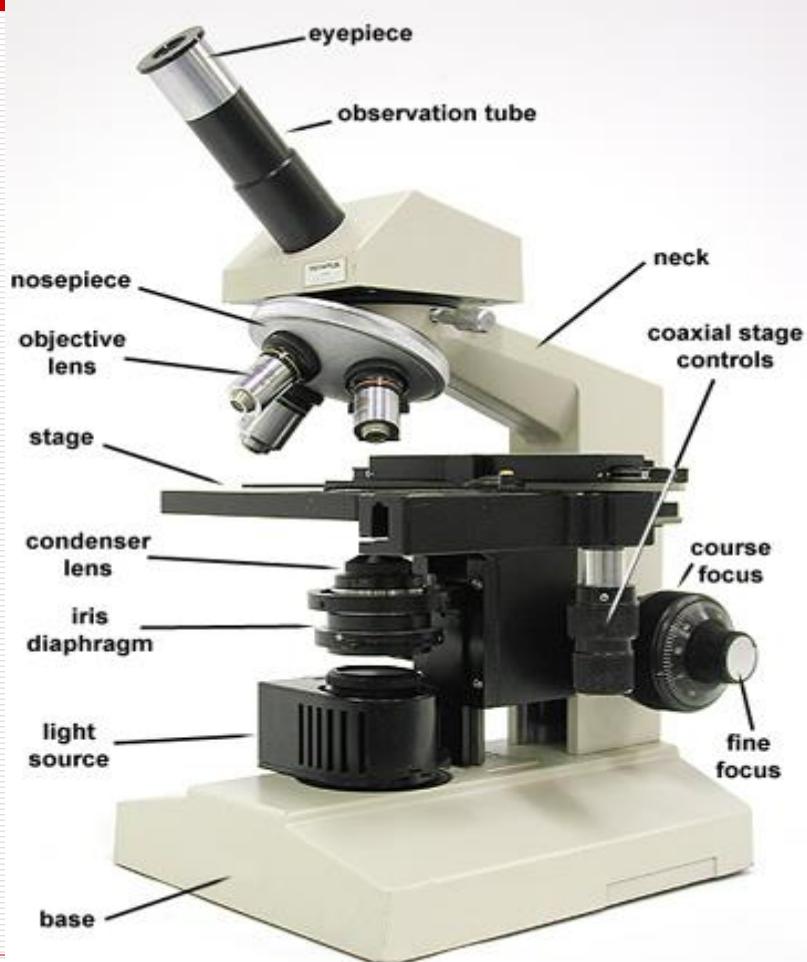
MONOCULAR COMPOUND MICROSCOPE



BINOCULAR COMPOUND MICROSCOPE



COMPOUND MICROSCOPE



FUNCTIONS OF MICROSCOPES

□ Microscopy serves two independent functions of enlargement (magnification) and improved resolution (the rendering of two objects as separate entities).

MAGNIFICATION OF LIGHT MICROSCOPES

- In their most basic essentials, all microscopes aim at:
 - a) Magnifying the specimen and
 - b) Displaying the specimen in greater detail.

These aims are interdependent and it is important to realise that to increase magnification without a commensurate improvement in the degree of discernible detail

Magnification of light microscopes Cont'd

is of little advantage.

- a) **Magnification:** The magnification of any optical system is dependent upon the focal length of the lenses in the system and their mutual arrangement.

Magnification of light microscopes Cont'd

It is usually expressed as the ratio of the length of the final image to that of the specimen, and for the ordinary class microscope it is between 25x and 1500x. Some compound microscopes can achieve a **useful** magnification of 2000x with the oil immersion lens.

Magnification of light microscopes Cont'd

Most dissecting microscopes magnify up to 30x.

Magnifications of more than 2000x for compound microscopes however, is considered “empty” because resolution does not improve with magnification beyond a certain point.

PARFOCAL

- Parfocal microscope objectives stay in focus when magnification is changed; i.e., if the microscope is switched from a lower power objective (e.g., $10\times$) to a higher power objective (e.g., $40\times$), the object stays in focus.

Objective working and Parfocal distance



Objective working and Parfocal distance

Cont'd.

□ *Working distance* is the distance between the objective lens and the specimen. At low magnification the working distance is relatively long. As you increase the magnification the working distance *decreases* considerably.

Objective working and Parfocal distance

Cont'd.

- Oil immersion lenses practically touch the specimen. Be aware of this change in working distance with increasing magnification so as to prevent damage to your specimens and the lens in particular.

Relationship between working distance and Magnification

- Generally speaking, the working distance of any given microscope is reduced as magnification increases. As in most compound microscopes, the lens is moved physically closer to the specimen to increase magnification; the working distance available between the lens and the specimen is reduced considerably as magnification increases.

Resolution of light microscopes

- b) Resolution: The resolving power of a lens indicates the fineness of detail that it allows to be seen. Thus, if one examines two small specimens with a microscope lens, provided the specimens are well separated, they will be resolved as separate entities. If, however, they are then gradually moved closer together, a situation will eventually arise in

Resolution of light microscopes Cont'd

which the two specimens, though still separate, can no longer be seen to be distinct from each other. In this situation, only by improving the resolution i.e. by using a lens with better resolving power, will it again be possible to render the two specimens as separate entities.

Factors that determine resolution

1. Numerical aperture: When light rays pass through a specimen containing fine detail they interfere with each other and they are variously diffracted; increasingly fine detail increases their angles of diffraction. Since the resolving power of a lens depends upon its ability to collect these diffracted rays, the

Factors that determine resolution Cont'd

wider the angle of rays collected, the better is the resolution.

The capacity of a lens to collect rays emerging from a specimen is defined by its numerical aperture (NA), and this depends upon both its angular aperture (U) and the refractive index (η) of the medium through which the rays pass.

Factors that determine resolution Cont'd

The relationship is expressed as:

$$NA = \eta \sin U$$

In any given lens, the NA and thus the resolution, is at its best when the cone of rays emerging from the specimen just fills the angular aperture. When setting up a microscope this optimum requirement is only obtained by careful focusing of the illumination system.

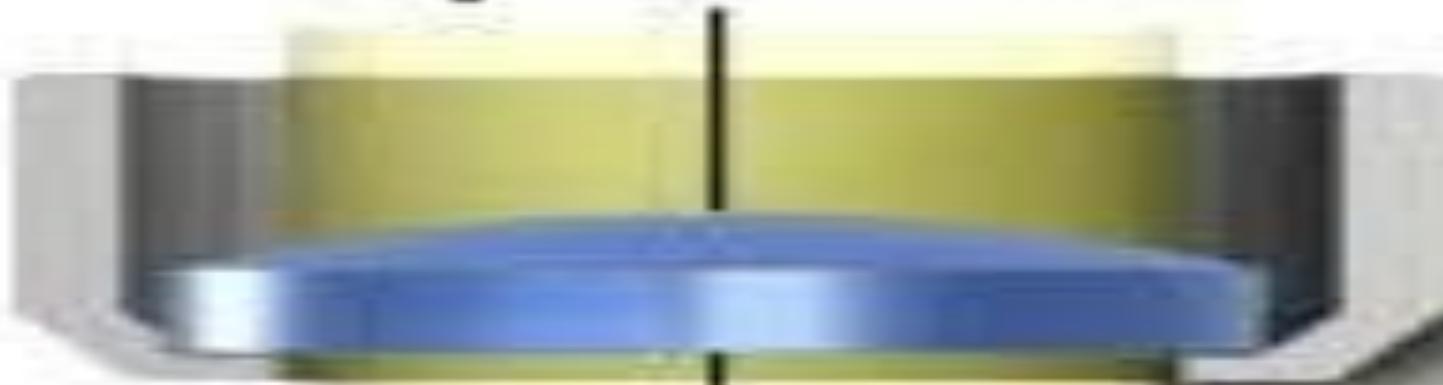
Factors that determine resolution Cont'd

In the conventional light microscope, the medium between the low power (less than x100) objective lenses and the specimen is air (i.e. the refractive index, $\eta = 1$). However, for lenses of higher power, where maximum resolution is required, the refractive index may be increased by filling this space with a special “immersion” oil. The refractive index of the immersion oils used with

Factors that determine resolution Cont'd

glass-covered microscope slides is optimally about 1.55. This arrangement increases the NA and results in fewer light rays being lost due to refraction. Resolution is thus improved.

Angular Aperture



A → A

Light Cone

H

Figure 1

Factors that determine resolution Cont'd

2. Wavelength:

Resolution also depends upon the wavelength of the transmitted wave form; the smaller the wavelength the better is the resolution.

Factors that determine resolution Cont'd

3. Limit of resolution (r):

Resolution is the ability of a lens to separate or distinguish between small objects that are close together. With reference to the numerical aperture (NA) and

Factors that determine resolution Cont'd

wavelength (λ), it is expressed as:

$$r = 0.61 \lambda / \text{NA}$$

In practice, the maximum NA available for light microscope objective lenses is about 1.4. The component of white light to which the human eye is most sensitive is green light ($\lambda = 560\text{nm}$);

Factors that determine resolution Cont'd

- Because $\sin(U)$ is always smaller than 1 and n cannot rise above 1.7, the maximal resolving power of a microscope is about

$$r = \lambda/2$$

and thus only depends on the wavelength of the light used, which normally will be about 600nm.

Factors that determine resolution

therefore, this equation indicates that the best resolution limit obtainable when using white light is about 240 nm ($0.24\mu\text{m}$). A red blood cell has a diameter of about $7\mu\text{m}$.

BRIGHT-FIELD MICROSCOPY

- A standard brightfield microscope relies upon light from the lamp source being gathered by the substage condenser and shaped into a cone whose apex is focused at the plane of the specimen. Specimens are seen because of their ability to change the speed and the path of the light passing through them. This ability is dependent upon the refractive index and the opacity of the specimen.

BRIGHT-FIELD MICROSCOPY CONT'D.

- To see a specimen in a brightfield microscope, the light rays passing through it must be changed sufficiently to be able to interfere with each other which produces contrast (differences in light intensities) and, thereby, build an image. If the specimen has a refractive index too similar to the surrounding medium between the microscope stage and the objective lens, it will not be seen.

BRIGHT-FIELD MICROSCOPY CONT'D.

- To visualize biological materials well, the materials must have this inherent contrast caused by the proper refractive indices or be artificially stained. These limitations require instructors to find naturally high contrast materials or to enhance contrast by staining them which often requires killing them. **Adequately visualizing transparent living materials or thin unstained specimens is not possible with a bright-field microscope.**

DARKFIELD MICROSCOPY

- The structure of many biological specimens are of low contrast that cannot be revealed by the brightfield compound microscopes.
 - Microscopes that improve the contrast of these specimens through special optics are prohibitively expensive. An inexpensive darkfield microscope is one that is modified from a brightfield microscope.
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DARKFIELD MICROSCOPY CONT'D.

□ Dark field microscopy is a very simple yet effective technique and well suited for uses involving live and **unstained** biological samples, such as a smear from a tissue culture or individual water-borne single-celled organisms. Considering the simplicity of the setup, the quality of images obtained from this technique is impressive.

DARKFIELD MICROSCOPY CONT'D.

- A darkfield microscope is used for examining live microorganisms that either are invisible in the ordinary light microscope, cannot be stained by standard methods, or are so distorted by staining that their characteristics then cannot be identified. Instead of the normal condenser, a darkfield microscope uses a

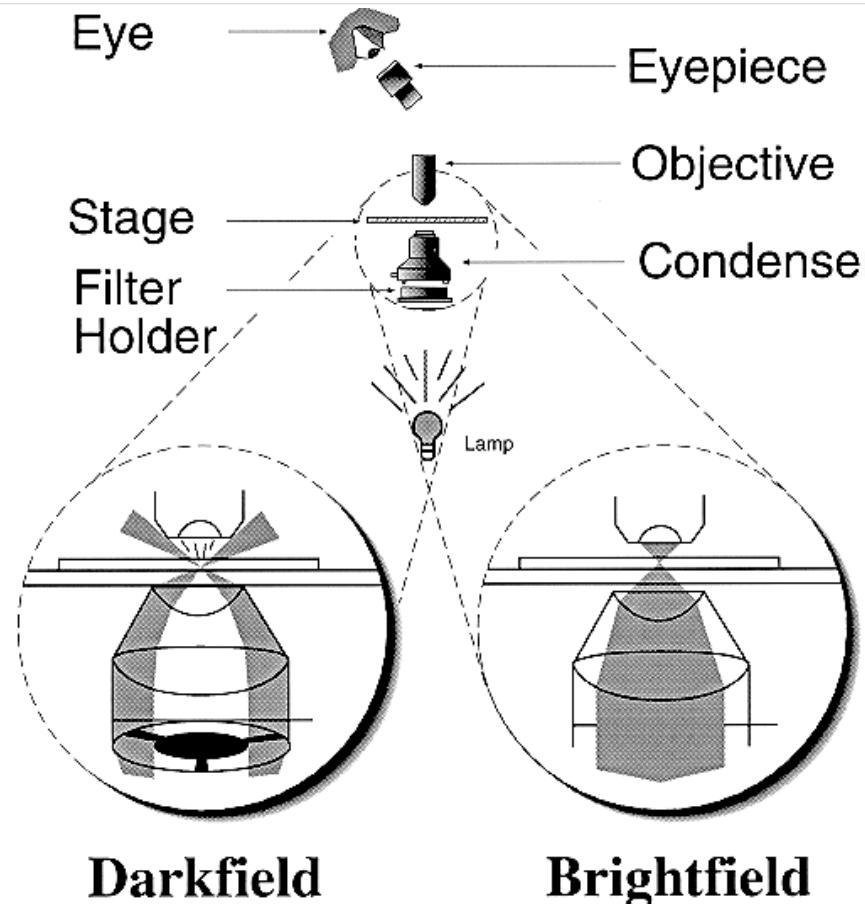
DARKFIELD MICROSCOPY CONT'D.

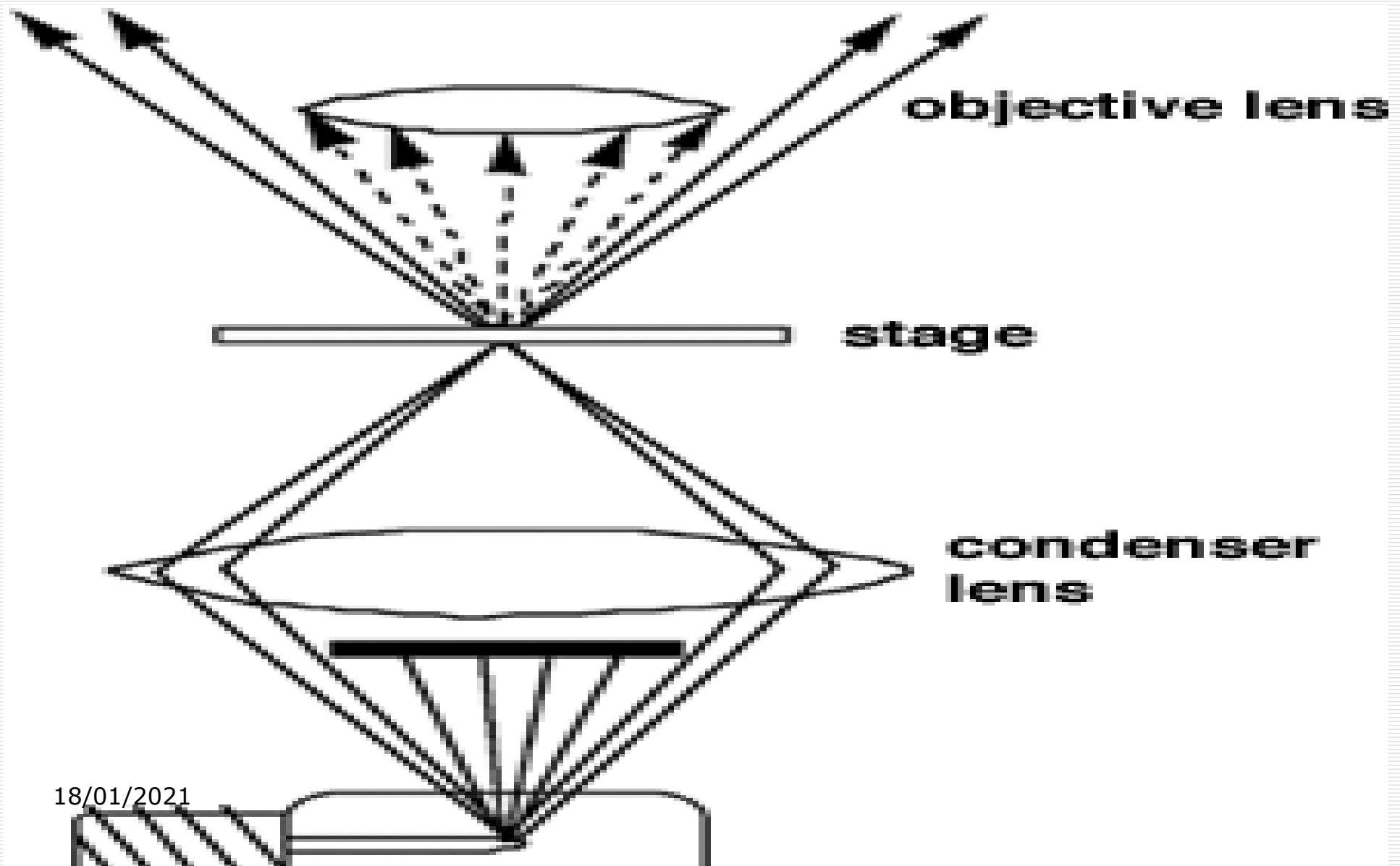
darkfield condenser that contains an opaque disc. The disc blocks light that would enter the objective lens directly. Only light that is reflected off the specimen enters the objective lens. Because there is no direct background light, the specimen appears light against a dark background. This technique is frequently used to examine unstained microorganisms

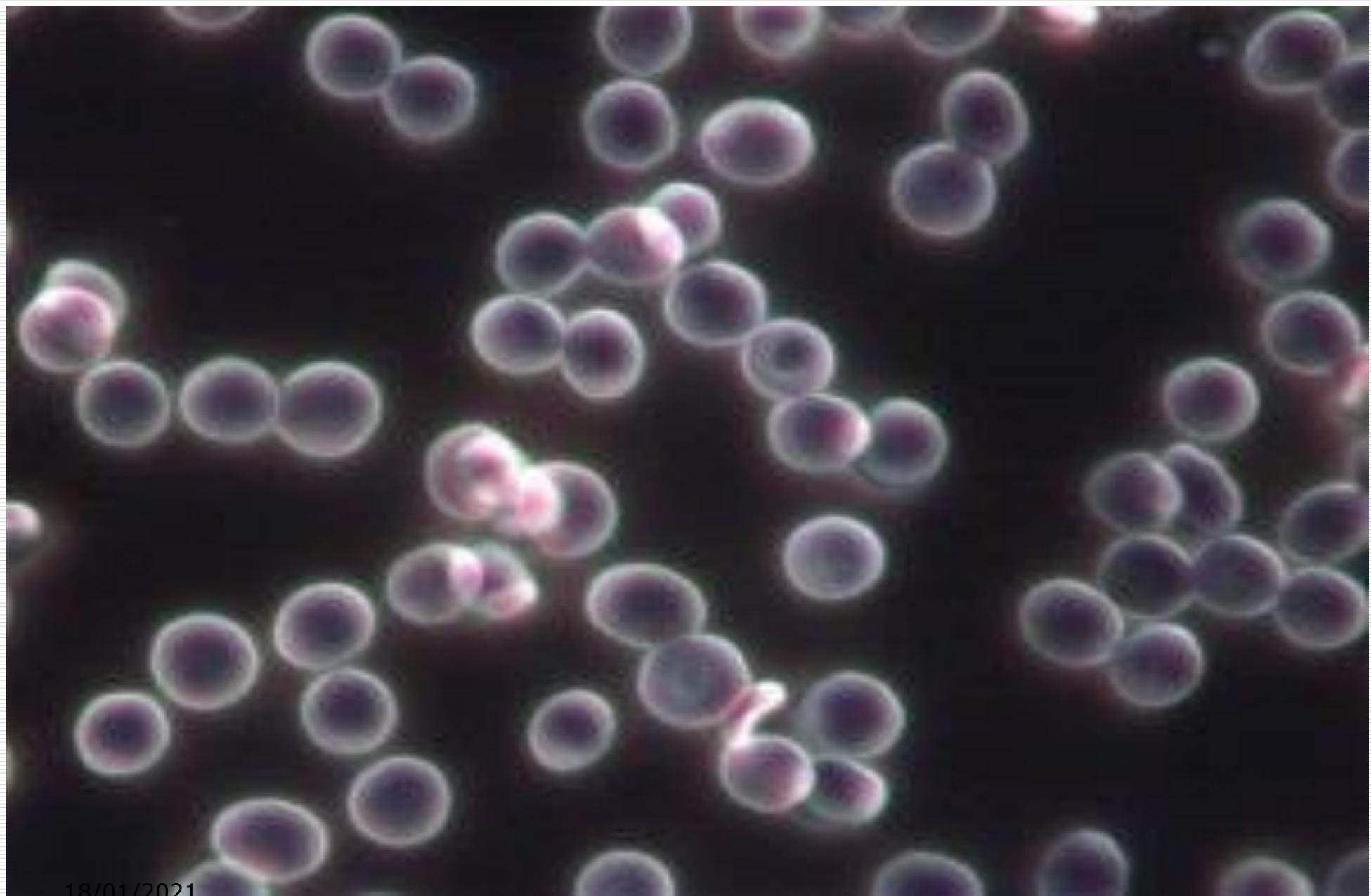
DARKFIELD MICROSCOPY CONT'D.

suspended in liquid such as yeast cells.

Bright-field and Dark-field compared







18/01/2021

89

89

PHASE-CONTRAST MICROSCOPY

- Differences in light absorption are often negligible between living cells and their surrounding nutrient medium, as well as between the various intracellular components and plasma membranes, rendering these entities barely visible when observed by brightfield illumination.

PHASE-CONTRAST MICROSCOPY

CONT'D.

When living cells are examined by normal light microscopy it is frequently difficult to see cell contents. This is because the human eye normally detects contrast by differences in colour or in intensity of illumination, and living cells are normally colourless and more or less transparent. One common method of overcoming this difficulty is to stain cells

PHASE-CONTRAST MICROSCOPY

CONT'D.

with various dyes but, as this usually results in death or disruption, it cannot be used to examine living cells. One technique which is now widely used to solve this problem is known as phase contrast microscopy.

The wavelength of light is a measure of colour whilst amplitude is a measure of brightness. When a specimen is examined

PHASE-CONTRAST MICROSCOPY

CONT'D.

with a light microscope, the light passing through the specimen will be affected in two ways in relation to the light passing through the surroundings, and will produce the contrast in the image. Firstly, the light will be diffracted or scattered, and may be lost to the image; the greater the diffraction, the darker the image. Secondly, the diffracted rays that

PHASE-CONTRAST MICROSCOPY

CONT'D.

do pass through the microscope will be retarded in relation to the light that did not pass through the specimen, the degree of retardation depending on the thickness of the specimen. Thus two sets of rays will arrive at the eye, the diffracted and undiffracted. The slight phase change produced in the diffracted rays will interfere with the other rays

PHASE-CONTRAST MICROSCOPY

CONT'D.

Resulting in a net reduction in amplitude, and so a lowering of brightness. The image seen by the eye is, in fact a complex interference pattern of diffracted and undiffracted rays. Normally the change in phase, and amplitude, is slight, and produces little contrast. The phase contrast microscope functions by further retarding the deffracted rays

PHASE-CONTRAST MICROSCOPY

CONT'D.

producing a greater change in amplitude on interference, and so a greater reduction in brightness. In the phase contrast microscope the light rays from the specimen background pass through the thinner portion of the positive phase plate and those that pass through the specimen will pass through the thicker portion of the positive phase plate

PHASE-CONTRAST MICROSCOPY

CONT'D.

and so suffer a greater diffraction (retardation), enhancing the phase difference produced by passage through the specimen. In addition, further light will be lost by the greater scatter produced by the thicker part of the positive phase plate, and so contrast will be enhanced by both greater amplitude changes and loss of light.

PHASE-CONTRAST MICROSCOPY

CONT'D.

Phase contrast microscopy is now widely used to study living cells and tissues. Considerable cytological detail of cell movement, changes in nuclei and other organelles, and cytoplasmic flow have been observed although resolution is still limited by the wavelength of visible light. Phase contrast microscopy has been particularly useful when used in conjunction with time-lapse motion photography of living cells.

PHASE-CONTRAST MICROSCOPY

CONT'D.

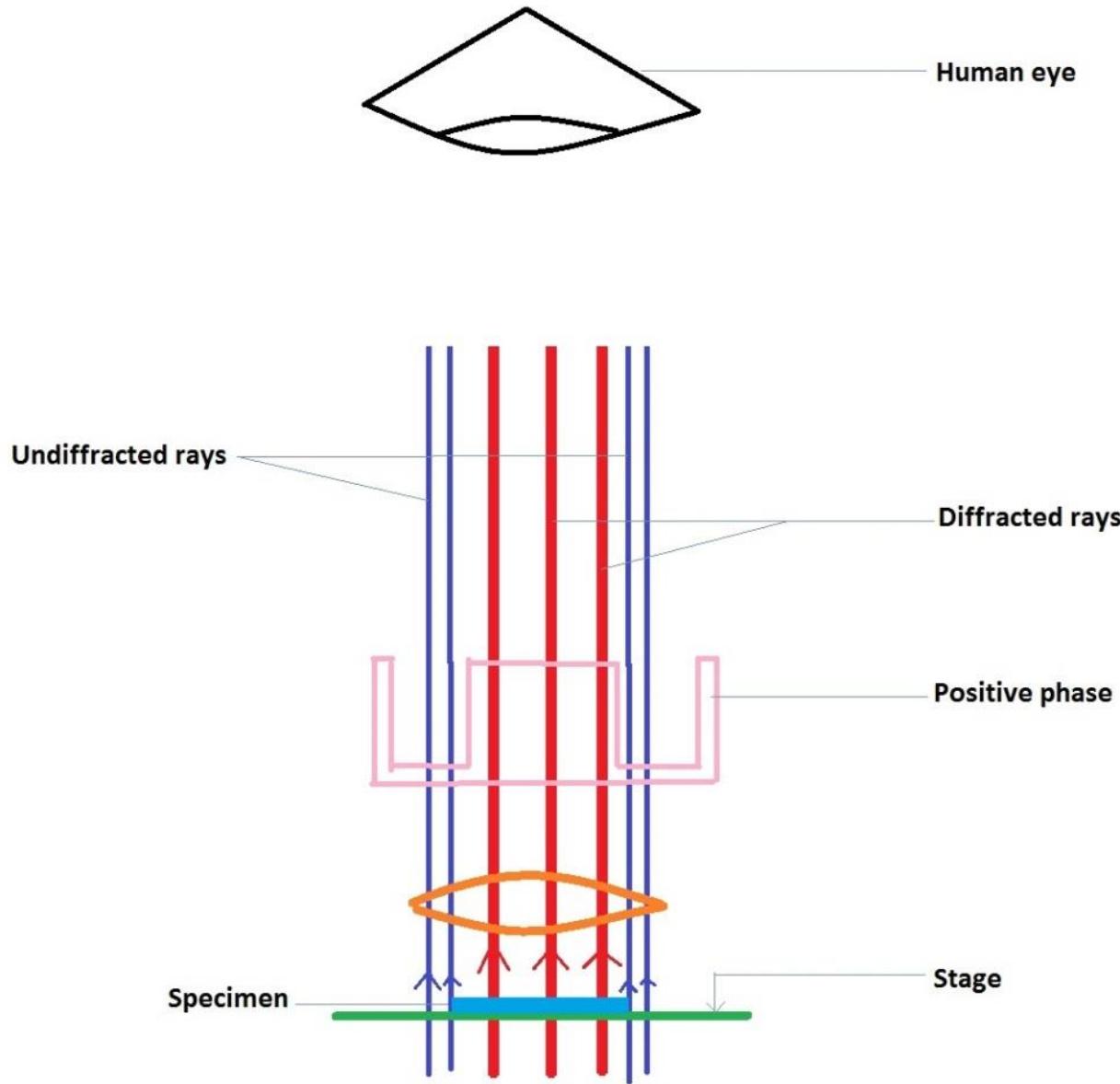
PRINCIPLE

- Light rays passing through a transparent specimen emerge as either direct rays or diffracted rays.
- In phase microscopy, this effect is amplified by using a microscope equipped with special annulus (below the stage) and phase plates (located in the objectives) which accentuate the phase changes produced by the specimen

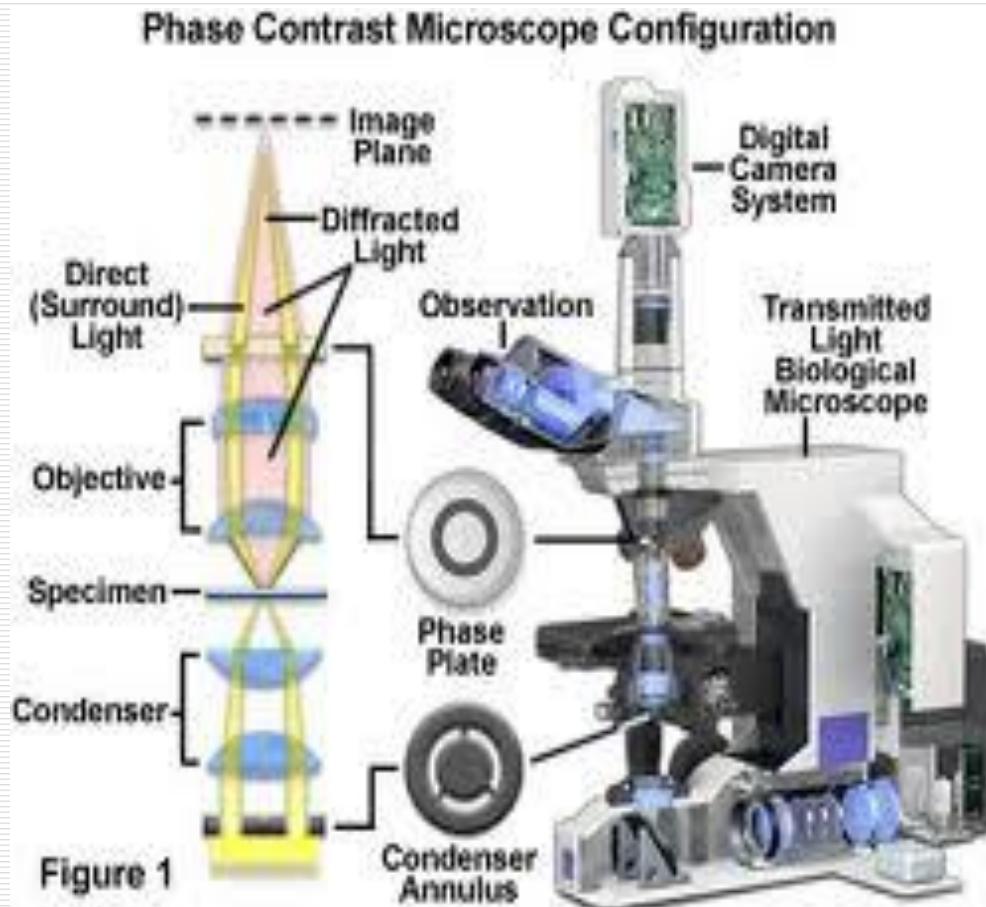
PHASE-CONTRAST MICROSCOPY

CONT'D.

- Direct rays, unimpeded by the phase plate (blue rays) are of higher intensity, making the background bright and the diffracted rays (red rays) impeded by the phase plate are of lower intensity, making parts of the specimen darker.



PHASE-CONTRAST MICROSCOPE



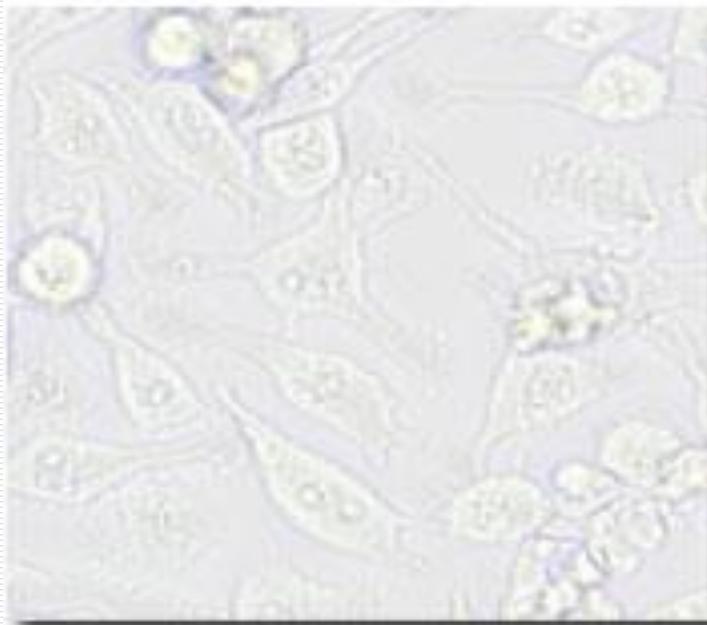
PHASE-CONTRAST MICROSCOPY

CONT'D.

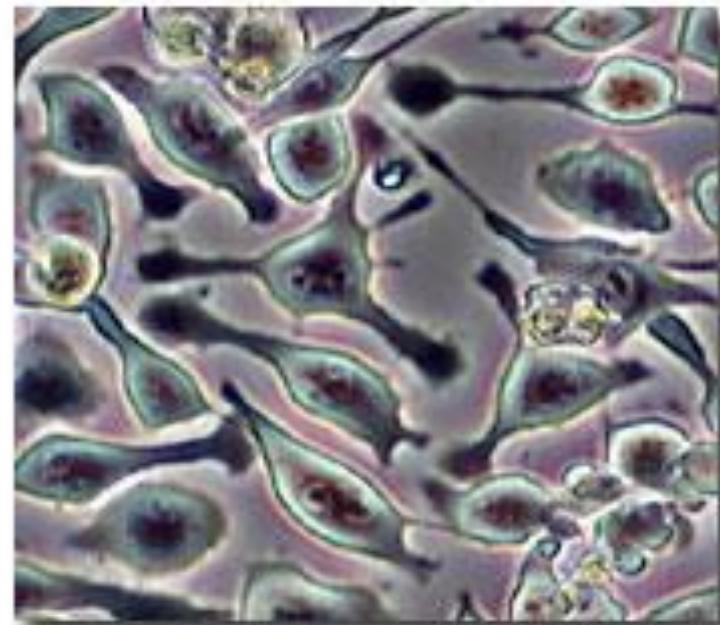
- Figure 2 in the slide below is a comparison of living cells in culture imaged in both brightfield and phase contrast illumination.
- In brightfield illumination (Figure 2(a)), the cells appear semi-transparent with only highly refractive regions, such as the membrane, nucleus, and unattached cells (rounded or spherical), being visible.

PHASE-CONTRAST MICROSCOPY CONT'D.

Living Cells in Brightfield and Phase Contrast



(a)



(b)

Figure 2

ULTRA VIOLET MICROSCOPY

This type of microscope uses U/V light that tends to increase resolution two-fold compared with a microscope that uses white light.

This type of microscope makes use of specimens that either have the natural ability to absorb U/V light and re-emit it as light of longer wavelength or stained with specific dyes to aid them absorb U/V light and re-emit it as light of longer wavelength visible to the human eye.

ULTRA VIOLET MICROSCOPY CONT'D

The main disadvantages of using this microscope are:

- U/V cannot pass through glass lenses and hence the lenses have to be replaced with quartz lenses which are expensive.
- The U/V light carrying the image of the specimen is not visible to the human eye.

ULTRA VIOLET MICROSCOPY CONT'D

U/V light has the tendency to damage the eye and requires that the microscope be fitted with a camera or the image of the specimen projected onto a fluorescent screen.

What is a Fluorescence Microscope?

- A fluorescence microscope is basically a conventional light microscope with added features and components that extend its capabilities.
- Basic Requirements of Fluorescence Microscope Optics

Nearly all fluorescence microscopes use the objective lens to perform two functions:

Basic Requirements of Fluorescence Microscope Optics Cont'd

1. Focus the illumination (excitation) light on the sample. In order to excite fluorescent species in a sample, the optics of a fluorescent microscope must focus the illumination (excitation) light on the sample to a greater extent than is achieved using the simple condenser lens system found in the illumination light path of a conventional microscope.

Basic Requirements of Fluorescence Microscope Optics Cont'd

2. Collect the emitted fluorescence. This type of excitation-emission configuration, in which both the excitation and emission light travel through the objective, is called epifluorescence. The key to the optics in an epifluorescence microscope is the separation of the illumination (excitation) light from the fluorescence emission emanating from the sample.

Basic Requirements of Fluorescence Microscope Optics Cont'd

In order to obtain either an image of the emission without excessive background illumination, or a measurement of the fluorescence emission without background "noise", the optical elements used to separate these two light components must be very efficient.

The Dichroic Mirror

In a fluorescence microscope, a **dichroic mirror** is used to separate the excitation and emission light paths. Within the objective, the excitation/emission share the same optics.

FLUORESCENCE MICROSCOPY

Certain chemical substances emit visible light when they are illuminated with ultra violet light. The effect is termed fluorescence and is put to use in the fluorescence microscopy in which ultraviolet light rays are focused on the specimen. Some cellular components possess a natural fluorescence and appear in various colours such as the chromosomes, nuclei etc.

FLUORESCENCE MICROSCOPY CONT'D

Other, nonfluorescing structures can be made to fluoresce by staining them with fluorescent dyes (fluorochromes). One of the most popular contemporary uses of fluorescence microscopy involves the preparation of antibodies that will bind to specific cellular proteins. The antibodies are first complexed with fluorescein (a fluorescent dye), and the fluorescein-labelled antibody is then applied to the cells.

FLUORESCENCE MICROSCOPY CONT'D

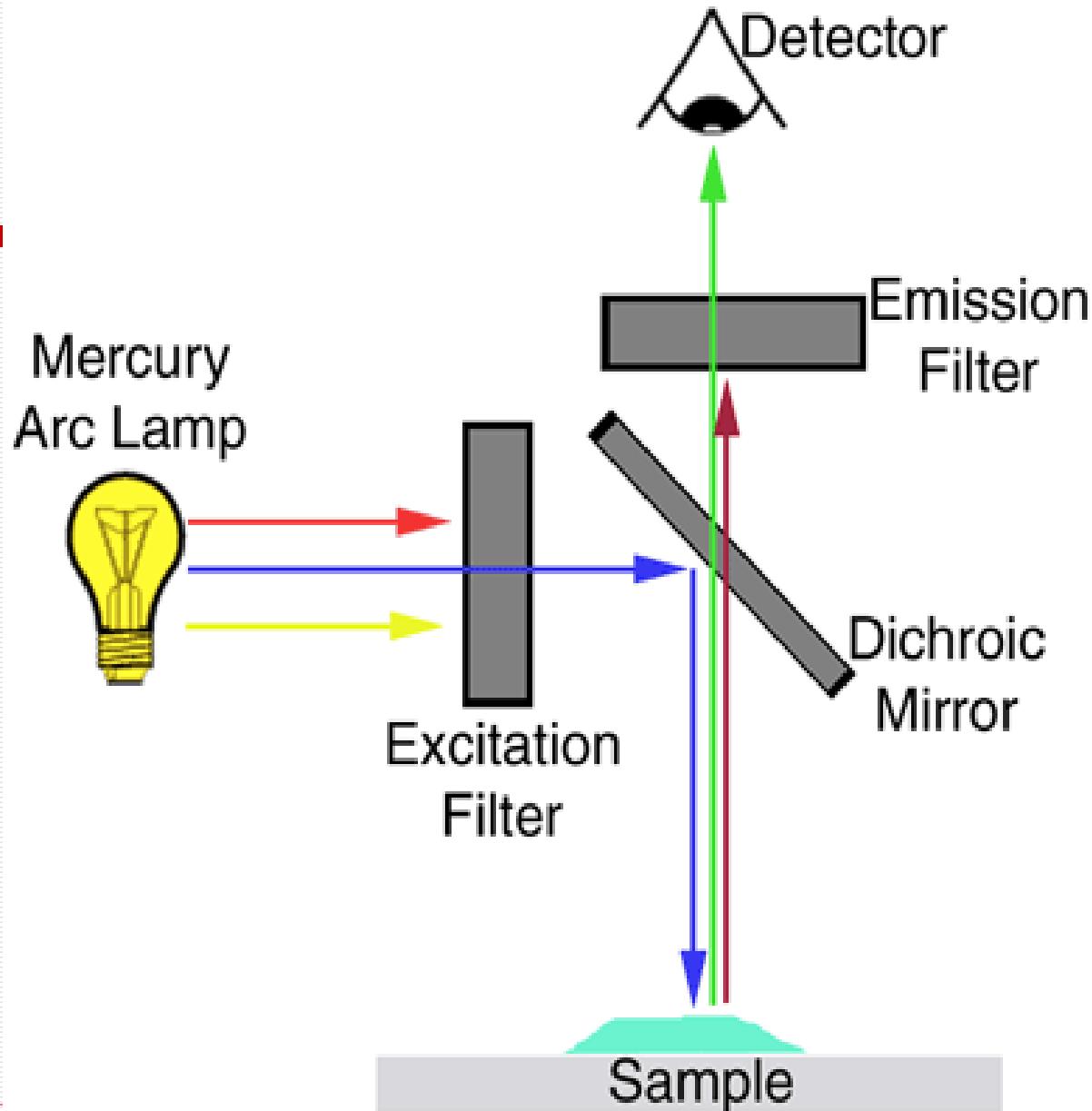
Cell structures containing the specific proteins capable of binding the fluorescein-labelled antibody are caused to fluoresce when examined with fluorescence microscope. This technique in microbiology is called immunofluorescence.

U/V – FLUORESCENCE MICROSCOPY

Fluorescence microscopy is used to detect structures, molecules or proteins within a cell. Fluorescent molecules absorb light at one wavelength and emit at another, longer wavelength. When fluorescent molecules absorb a specific absorption wavelength for an electron in a given orbital, the electron rises to a higher energy level. Electrons in this state are unstable and will return to the ground state, releasing energy in the form of

U/V – FLUORESCENCE MICROSCOPY CONT'D

light and heat. This emission of energy is fluorescence. In fluorescence microscopy, a cell is stained with a dye and the dye is illuminated with filtered light at the absorbing wavelength; the light emitted from the dye is viewed through a filter that allows only the emitted wavelength to be seen. The dye glows brightly against a dark background because only the emitted wavelength is allowed to reach the eye pieces or camera port of the microscope.



ELECTRON MICROSCOPY

All matter has a dual nature, both particulate and wave-like. Thus, electrons have wave-like properties and their wave-length depends upon the speed at which they are moving. The approximate λ of electrons, over a potential difference of V volts, can be calculated from:

ELECTRON MICROSCOPY CONT'D

$$\lambda = \sqrt{1.5/V} \text{ nm}$$

Thus, electrons at 60 KV have an apparent λ of 0.005 nm, which should give a huge increase in resolution over the light microscope. Thus, phenomenally high resolving power **theoretically** available with electron microscopy unfortunately is not realizable. The resolving power is limited by specimen contrast and the highly imperfect electromagnetic lenses.

ELECTRON MICROSCOPY CONT'D

There are two types of electron microscopes:
The Transmission electron microscope (TEM)
and the Scanning electron microscope (SEM).

Transmission electron microscope (TEM)

□ In transmission electron microscope, a finely focussed beam of electrons from an electron gun passes through a specially prepared, ultrathin section of the specimen. The ray of



ELECTRON MICROSCOPY CONT'D

electrons is produced by a pin-shaped cathode heated up by current. The electrons are vacuumed up by a high voltage at the anode. The acceleration voltage is between 50 and 150 kV. The higher it is, the shorter are the electron waves and the higher is the power of resolution. But this factor is hardly ever limiting. The power of resolution of electron microscopy is

ELECTRON MICROSCOPY CONT'D

usually restrained by the quality of the lens-systems and especially by the technique with which the preparation has been achieved. Modern gadgets have powers of resolution that range from 0.2 – 0.3 nm. The useful resolution is therefore around 300,000 x. The accelerated ray of electrons passes a drill-hole at the bottom of the anode.

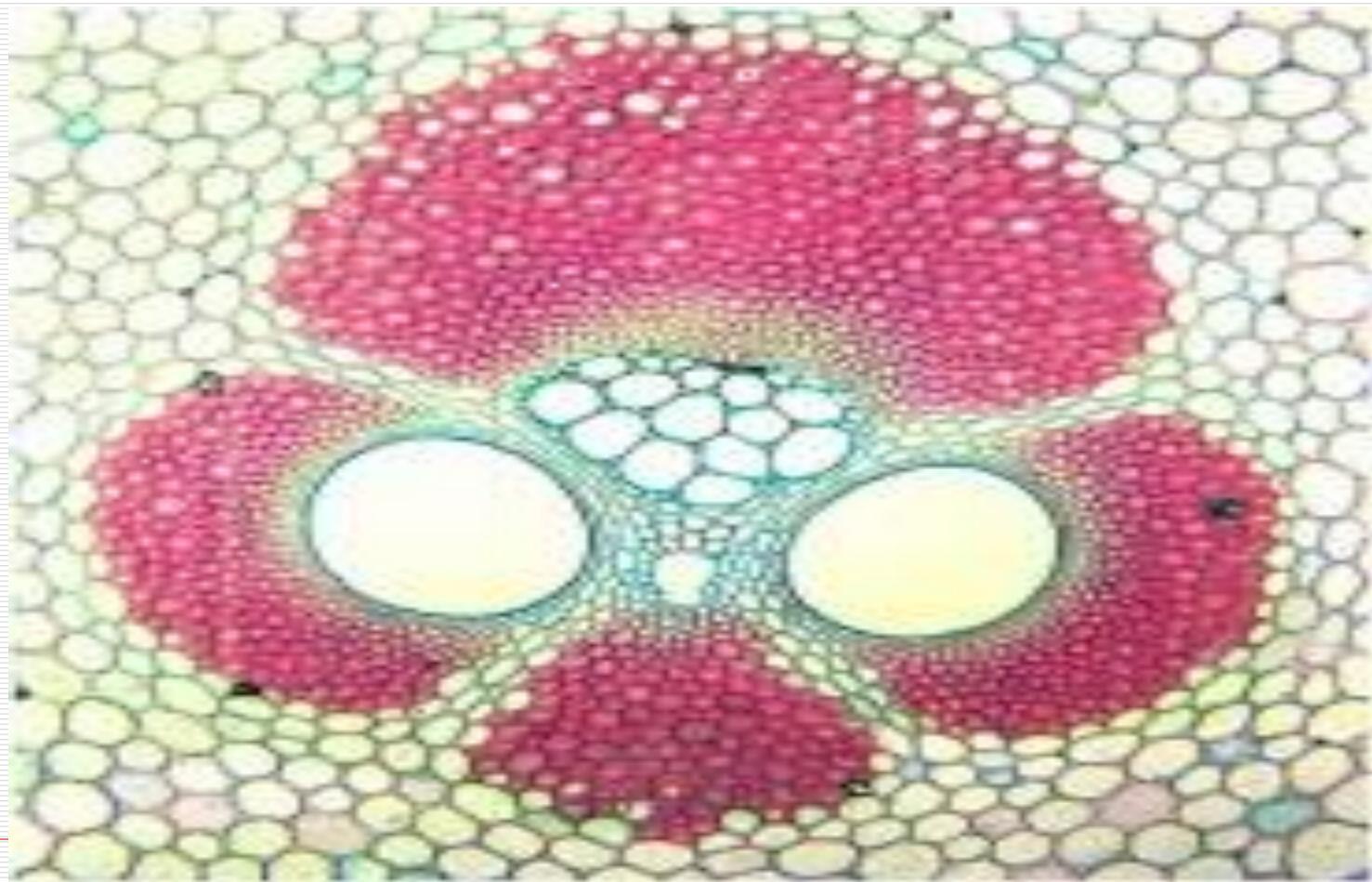
ELECTRON MICROSCOPY CONT'D

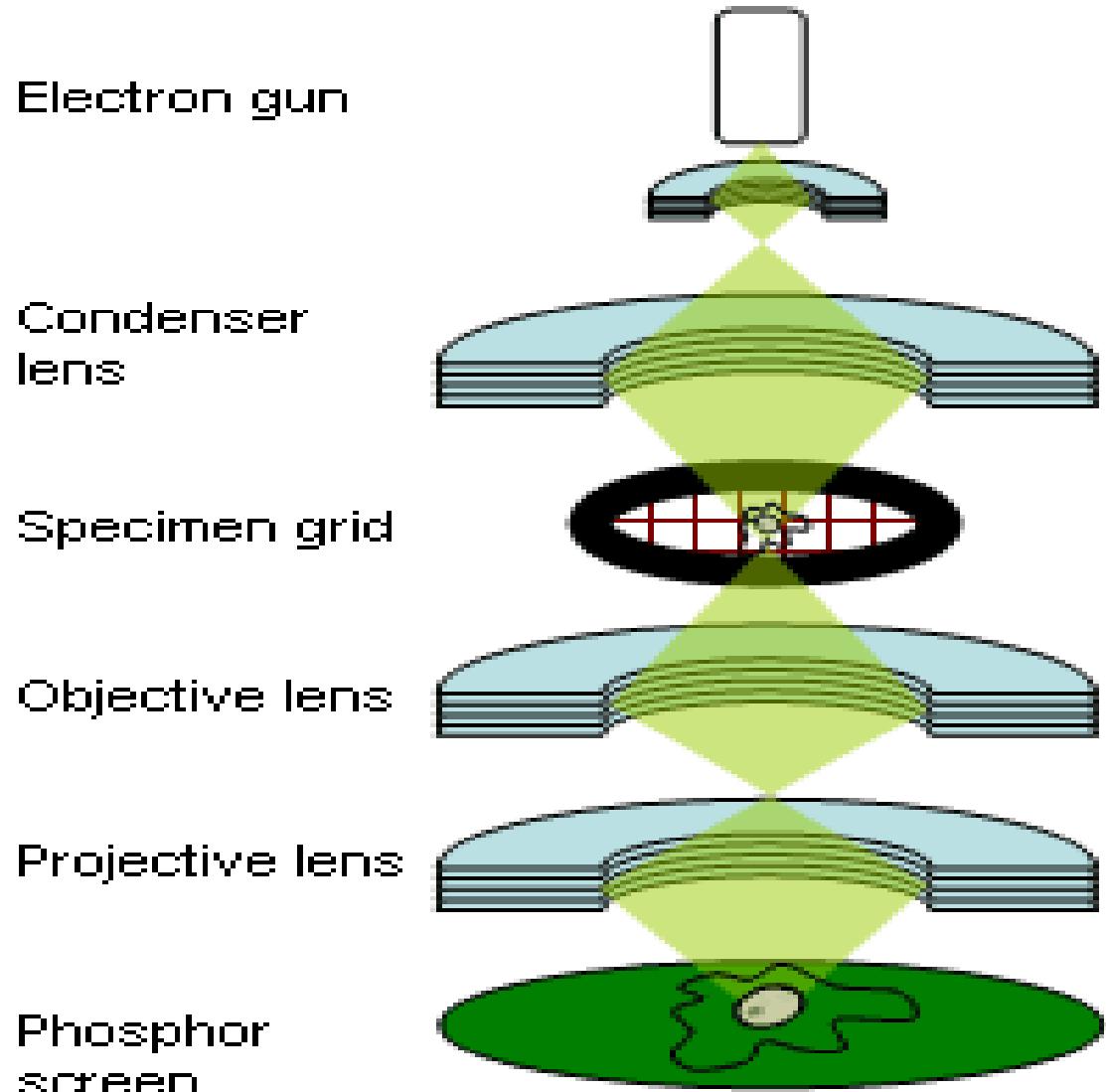
It follows a way analogous to that of a ray of light in a light microscope. The lens-systems consist of electronic coils generating an electromagnetic field. The ray is first focused by a condenser. It then passes through the object, where it is partially deflected. The degree of deflection depends on the electron density of the object. The greater the mass of the atoms, the greater is the degree of deflection. Biological objects have only weak contrasts since

ELECTRON MICROSCOPY CONT'D

they consist mainly of elements with low atomic numbers (C, H, N, O). After passing through the object the scattered electrons are collected by an objective. Thereby an image is formed, that is subsequently enlarged by an additional lens-system (called projective with electron microscopes). The thus formed image is made visible on a fluorescent screen or it is documented on photographic material.

TRANSMISSION ELECTRON MICROSCOPY





SCANNING ELECTRON MICROSCOPY

The Scanning Electron Microscope (SEM) is a microscope that uses electrons rather than light to form an image. There are many advantages to using the SEM instead of a light microscope. The SEM has a large depth of field, which allows a large amount of the sample to be in focus at one time.

SCANNING MICROSCOPY CONT'D

The SEM also produces images of high resolution, which means that closely spaced features can be examined at a high magnification. The combination of higher magnification, larger depth of focus, greater resolution, and ease of sample observation makes the SEM one of the most heavily used instruments in research areas today.

SCANNING MICROSCOPY CONT'D

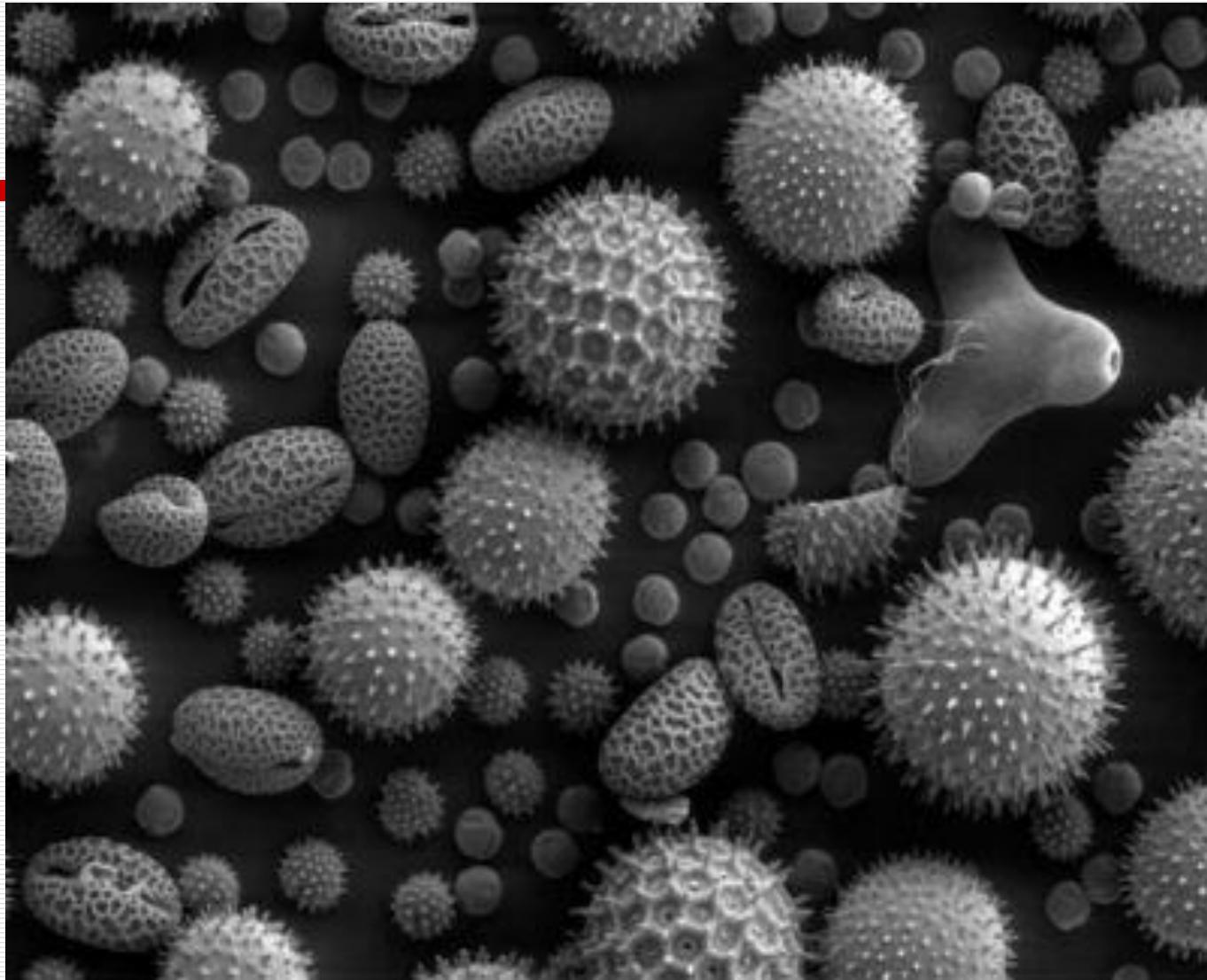
A scanning electron microscope (SEM) is a powerful microscope that uses electrons rather than light to form an image of objects such as fractured metal components, foreign particles and residues, polymers, electronic components, biological samples, and countless others.

SCANNING MICROSCOPY CONT'D

The shorter wavelength of electrons permits image magnifications of up to 100,000X, as compared to about 2,000X for conventional light microscopy. An SEM also provides a greater depth of field than a light microscope, allowing complex, three-dimensional objects to remain sharp and in focus. This capability reveals details that cannot be resolved by light microscopy.

SCANNING MICROSCOPY CONT'D

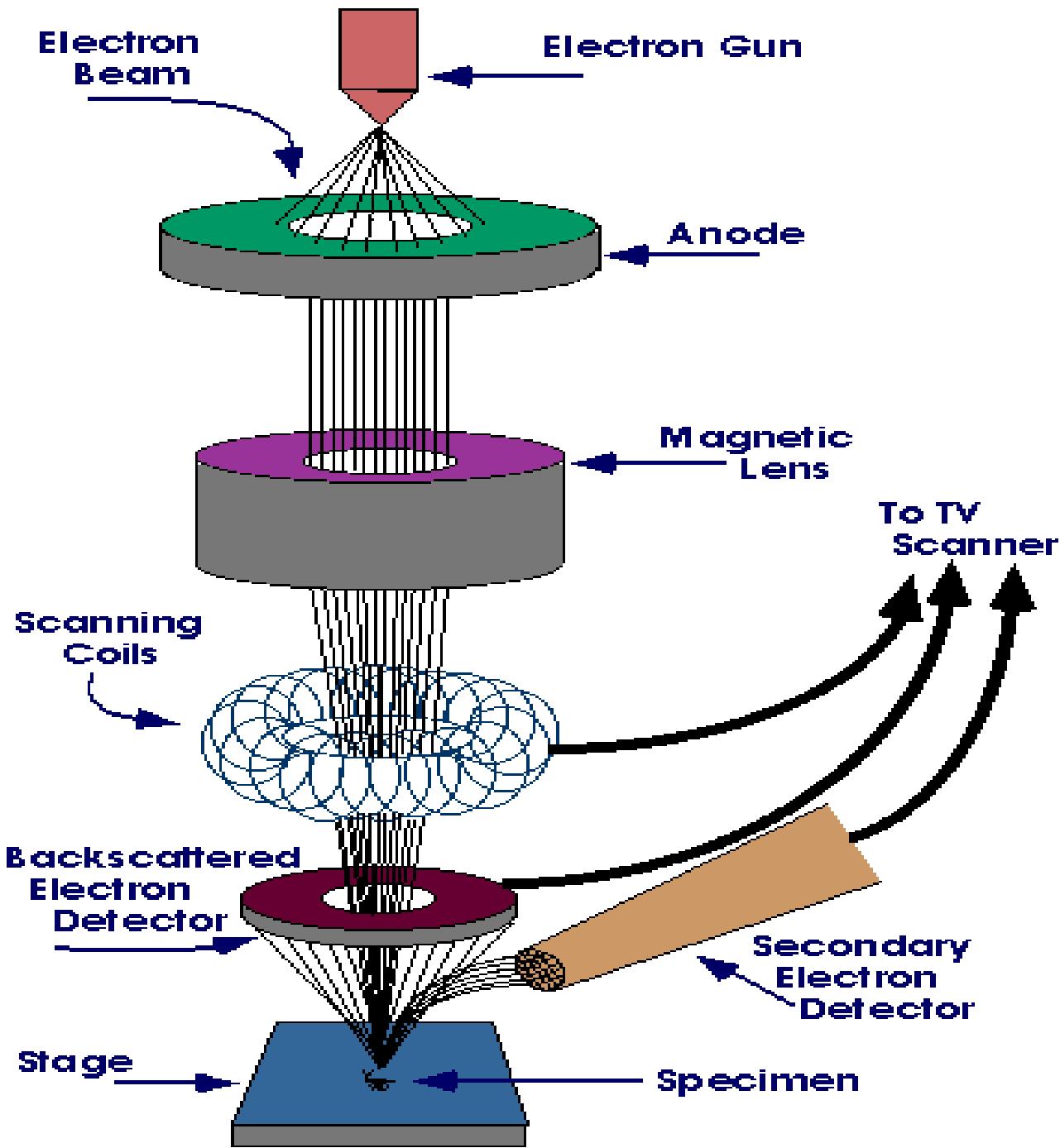
SEM is used to look *at* the surface of a solid specimen. The resolution is usually only 10 nm (unless FESEM is used), but with 20 000 × magnification. We get very attractive 3D-looking images because of the large depth of field.



Scanning electron microscope image of pollen.

18/01/2021

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PREPARATION OF SPECIMENS FOR LIGHT AND ELECTRON MICROSCOPY

There are problems in analysing cell structure and consequently complex preparative procedures must be used. These include the following:

1. FIXATION:

It is the rapid killing and preservation of a tissue. Correct fixation is the fundamental basis of all histological work.

Fixation Cont'd

The tissue must be fixed to prevent autolysis, bacterial or fungal attack and also to make the tissue resistant to any damage that might be caused by later procedures. Fixation may be by either physical and chemical means. Physical methods involve immersing the specimen in liquid nitrogen, thereby, freezing it so rapidly that ice crystals which would disrupt and distort the tissue, do not form.

Fixation Cont'd

This method of fixation is often essential if it is necessary to preserve the tissue structure and to prevent any damage occurring to the enzymatic components of cells. Frozen tissue is only fixed while frozen and if brought to room temp. would rapidly undergo autolysis. Thus, if it is necessary to keep permanent preparations of frozen sections, they must be chemically fixed.

Fixation Cont'd

Chemical methods of fixation involve chemicals that would stabilise proteins and lipids, which are the major structural components of cells. If the specimen is to be examined with the electron microscope, it is essential to use fixatives whose reactions lead to the formation of precipitates that do not obscure the structure of the cell organelles.

Fixation Cont'd

The most widely used fixatives for light microscopy include acetic acid, alcohol, acetone and mercuric chloride, chromic acid, formaldehyde, while formaldehyde and glutaraldehyde (which fix proteins) and osmium tetroxide and potassium permanganate, (which fix lipids) are used for electron microscopy.

PREPARATION OF SPECIMENS FOR LIGHT AND ELECTRON MICROSCOPY CONT'D

2. EMBEDDING:

An electron beam has a low penetrating power and so, the column of the microscope is kept under high vacuum. This means that water must be removed from the specimens otherwise the vacuum will be destroyed. Therefore, specimens are dehydrated after fixation, usually by immersion in a series of ethanol – water mixtures of increasing alcohol concentration up to 100% alcohol which

PREPARATION OF SPECIMENS FOR LIGHT AND ELECTRON MICROSCOPY CONT'D

replaces the water in the specimen. A wax solvent such as xylene is used to replace the alcohol in the specimen in another series of baths and finally replacing the xylene with molten paraffin wax. A similar dehydration procedure is used to embed material in epoxy resin, but instead of xylene, a resin solvent such as propylene oxide is used. The resin-impregnated specimen is then baked in an oven to polymerise it.

PREPARATION OF SPECIMENS FOR LIGHT AND ELECTRON MICROSCOPY CONT'D

Associated with the low penetration property of the electron beam is the question of specimen thickness. The formation of the image in an electron microscope depends upon the electrons passing through the specimen. If the specimen is too thick, all of the electrons will be scattered to some extent, resulting in an image with poor contrast and considerable loss of detail.

PREPARATION OF SPECIMENS FOR LIGHT AND ELECTRON MICROSCOPY CONT'D

Therefore, it is necessary to cut thin slices/sections of the material to be examined, and these must be of the order of 50 nm thick. To cut these ultra-thin sections, the tissue must first be embedded in a hard support medium since biological material has little mechanical strength. The supporting medium used depends on the thickness of the section required.

PREPARATION OF SPECIMENS FOR LIGHT AND ELECTRON MICROSCOPY CONT'D

For relatively thick sections of plant material, specimens may be supported by sandwiching them between easily cut material such as raw carrot or raw unripe pawpaw fruit. Thinner sections can be cut if the specimen is embedded in paraffin wax and for the ultra sections required for electron microscopy, a more rigid support such as the epoxy resin, araldite etc can be used.

PREPARATION OF SPECIMENS FOR LIGHT AND ELECTRON MICROSCOPY CONT'D

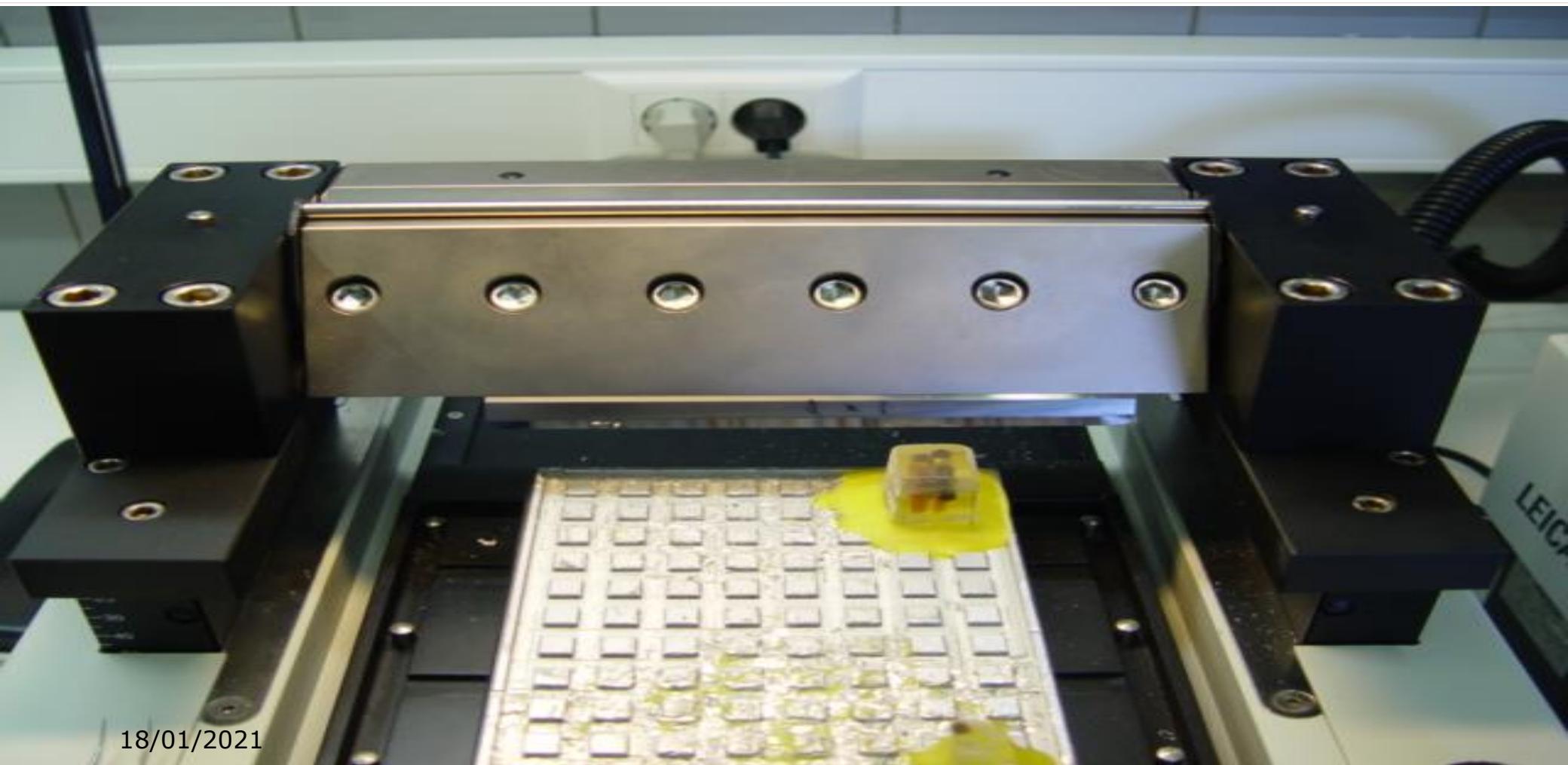
3. SECTIONING:

To produce thin sections (1000 – 20000 nm for light microscopy, but 50 – 100 nm for electron microscopy), an instrument called a microtome is used. All microtomes of whatever pattern consist of a specimen holder, a sharp cutting edge and a means of regulating the thickness of the section being cut. The sharp cutting edge

PREPARATION OF SPECIMENS FOR LIGHT AND ELECTRON MICROSCOPY CONT'D

may be that of a steel razor for wax - embedded specimens or of a glass or diamond knife for araldite – embedded specimens. Sections of frozen specimens are cut with a microtome mounted in a freezer maintained at – 20°C. When the sections have been cut, they are supported by being mounted either on glass microscope slides for examination with light microscopy or on a grid of fine copper strands for electron microscopy.

TYPES OF MICROTOMES







4. STAINING:

Thin sections of cells or tissues are usually transparent or nearly so and it is therefore, usually necessary to stain the sections before they are examined.

For light microscopy, most of the commonly used stains are organic aromatic dyes originally produced for use in the textile industry. Some, like toluidin blue colour all tissues; others colour only particular components of cells or parts of tissues.

STAINING contd

Of these more specific stains, there are essentially two groups; basic stains and acidic stains. The specificity of these stains depends on the difference in the charge of the different cell components.

A commonly used basic stain is haematoxylin – the colour imparting (chromogenic) group is cationic (+vely charged) and reacts primarily with negatively charged molecules, such as the nucleic acids in the nucleus, to produce colour.

STAINING contd



STAINING contd



STAINING contd

Haematoxylin is most frequently used in combination with a second dye, eosin. In an acidic solution, the chromogenic group of eosin is anionic (-vely charged) and it will react with basic groups in the cell, which are largely present in the cytoplasm, staining them red. Haematoxylin and eosin are used as routine stains for most animal tissues.

STAINING contd

In electron microscopy, different stains are used to emphasise different cell components as they are for light microscopy, but in this case, the stains must impart contrast to the sections instead of colour. This is because in electron microscopy, the formation of an image depends on a beam of electrons, which passes through the section and hits a fluorescent screen. The image is formed by

STAINING contd

the removal of electrons from this beam by the specimen, but because the electrons pass easily through the resin and the unstained tissue, the image is faint and with low contrast. By staining cell components with elements of high atomic number like Pb and Uranium, their contrast is increased because their electron density is increased.

STAINING contd

Uranium binds preferentially to nucleic acids and proteins, whereas Pb binds to lipids and membranes. Hence, cell components rich in lipids or nucleic acids appear dark or electron-dense and areas of cytoplasm or the resin surrounding the tissue appear light. Osmium will impart electron density to membranes as well as to lipids and nucleic acids.

STAINING contd

This is known as positive staining technique which is frequently used in the examination of fragments of isolated organelles and membranes and of small particles such as viruses and ribosomes. The double staining technique of Uranium and Pb is to electron microscopy as H and E is to light microscopy.

STAINING contd

Negative staining produces an electron –dense background against which the less dense specimens are observed. Negative stains consist of the salts of heavy metals and include potassium phosphotungstate, Uranyl acetate and ammonium molybdate. The electron-dense stain appears to dry more rapidly on the support film than on the specimen and forms a sharp boundary; the specimen appearing bright on a dark background.

Cellular level of organisation: Specific Objectives

- Appreciate the cell as the basic functional unit of life
- Basic differences between prokaryotes and eukaryotes
- Describe the structure of the plasma membrane and explain its functional significance.
- Describe the structure of the cell nucleus and explain its significance.
- Describe the structure & function of the cellular organelles in the cytoplasm.

Fundamentals of the cell

- Wide range of cell types but generally two kinds of cell types:
 - Cells without a nucleus = **Prokaryotes**
 - Cells with a nucleus = **eukaryotes**

What is the cell

- Like ourselves, the individual cells that form our bodies can
- grow, reproduce, process information, respond to stimuli and
- carry out an amazing array of chemical reactions. These
- abilities define life.

What is the cell Cont'd

- We and other multi-cellular organisms contain billions and
- trillions of cells organized into complex structures, but many
- organisms consist of a single cell.
- • Even simple unicellular organisms exhibit all the major
- properties of life,

What is the cell Cont'd

- This indicates that the cell is the fundamental unit of life.
- **Definition of Cell**
- A cell is the smallest unit of life that is capable of *independently* performing life functions.

Cellular Level of Organization

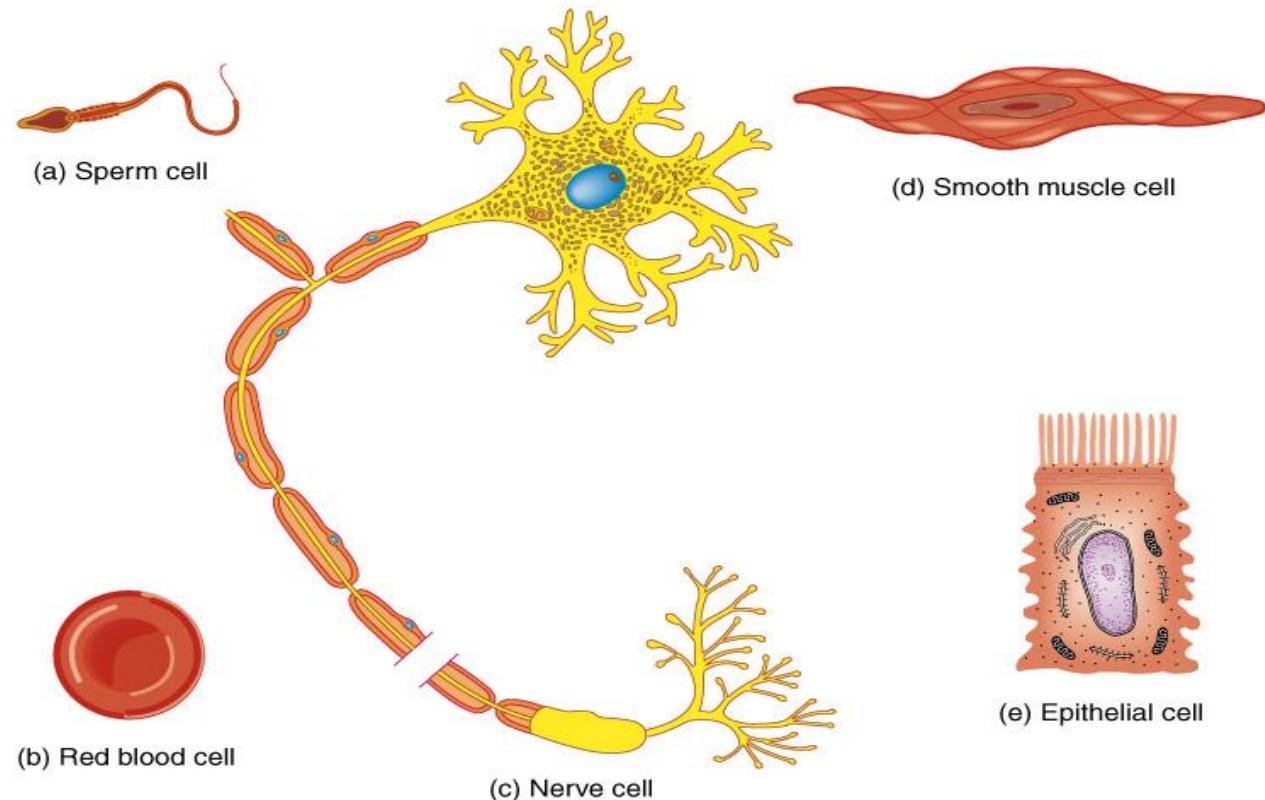
□ The cell:

- Basic, living, structural and functional unit of life, capable of independent reproduction
- Organismal activity depends on individual and collective activity of cells
- Biochemical activities dictated by subcellular structure.

General functions of the cell

- Compartmentalization of chemical reactions within specialized structures
- Regulate inflow & outflow of materials
- Use genetic material to direct cell activities
- Continuity of life originates from the cell

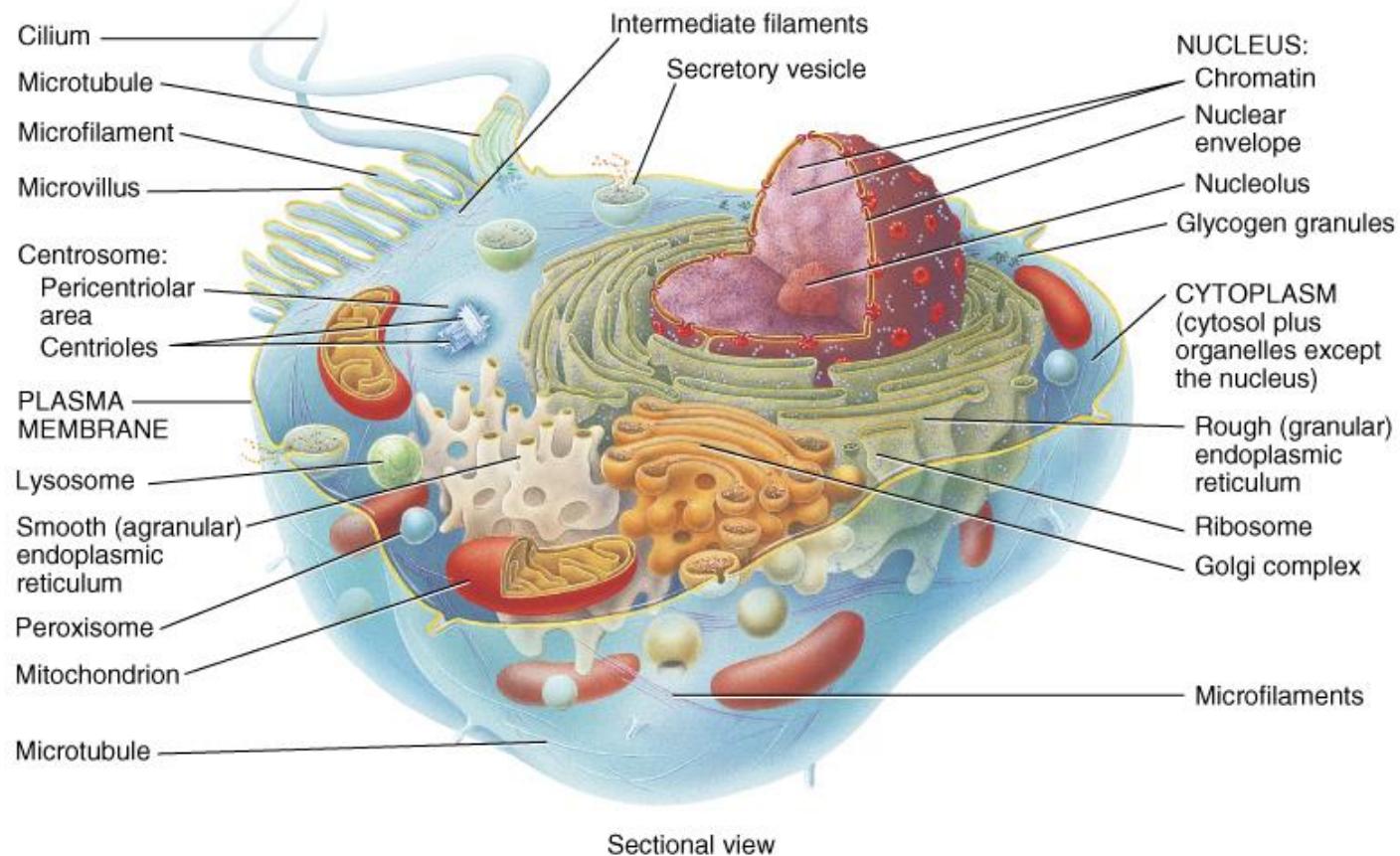
Cellular Diversity



Cellular Diversity Cont'd.

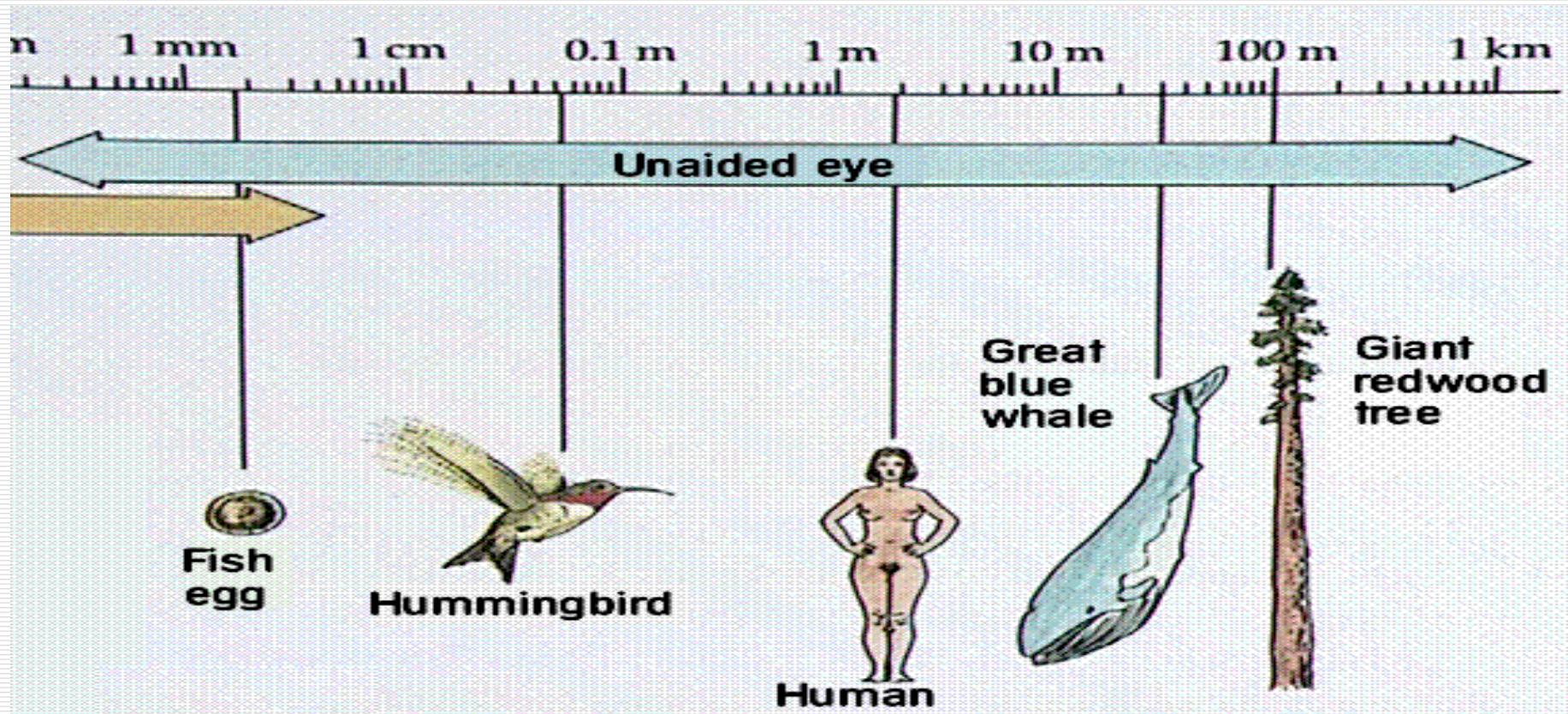
- 100 trillion cells in the body -- 200 different types
- Vary in size and shape related to their function

A typical cell



Cell Structure Cont'd

Cell Structure Cont'd



Factors that determine cell size and shape

The shape of the cell depends partly on the surface tension and viscosity of the cytoplasm, the mechanical action which the adjoining cells exert, the rigidity of the membrane and the functional adaptation.

Cell Structure Cont'd

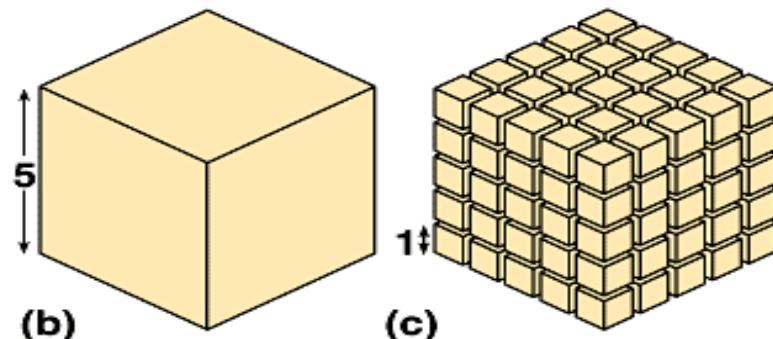
- ASSIGNMENTS:
- Cell Theory.
- Factors that determine cell size and cell shape.
- Differences between Prokaryotic and Eukaryotic cells.

Surface Area to Volume ratio

No.	Size	Area	Vol.	Surface area	Surface Area/Vol.
1	1 cm	1 cm ²	1 cm ³	6 cm ²	6/1=6 cm ⁻¹
2	2 cm	4 cm ²	8 cm ³	24 cm ²	24/8=3 cm ⁻¹
3	4 cm	16 cm ²	64 cm ³	96 cm ²	96/64=1.5 cm ⁻¹

Use of Geometric relationships to explain why most cells are microscopic

Surface area increases while total volume remains constant



Total surface area (height \times width \times number of sides \times number of boxes)	6	150	750
Total volume (height \times width \times length \times number of boxes)	1	125	125
Surface-to-volume ratio (area \div volume)	6	1.2	6

Detailed Structure of some of the organelles

MICROBODIES:

Microbodies are a heterogeneous group of small vesicle-like organelles, concerned largely with oxidation. They are usually oval or spherical and bounded by a single membrane. Microbodies are found in the liver and kidneys of vertebrates, in the leaves and seeds of plants as well as in protozoa, yeasts and other fungi. Microbodies consist of peroxisomes, glyoxysomes, hydrogenosomes and glycosomes.

PEROXISOMES

These contain the enzymes urate oxidase, D-amino acid oxidase and catalase.

Urate oxidase is a purine – catabolising enzyme, converting urate into allantoin, CO_2 and H_2O_2 . Thus, peroxisomes not only play a role in the break down of nitrogenous bases derived from nucleic acids but they are also involved in the degradation of L- α -hydroxy acids to oxoacids and H_2O_2 and of oxoacids to smaller products including acetyl CoA.

PEROXISOMES CONT'D

- D-Amino acid oxidase and catalase have protective functions. D-amino acids may be absorbed from the gut following their release by the break down of cell walls of gut bacteria. These “unnatural” amino acids are degraded to give oxoacids and H_2O_2 .
- H_2O_2 is a powerful oxidising agent and is potentially toxic. Catalase catalyses the rapid degradation of H_2O_2 to water and O_2 .

Peroxisomes Cont'd.

- **Membranous vesicles**
 - smaller than lysosomes
 - form by division of pre-existing peroxisomes
 - contain enzymes that oxidize organic material
- **Function**
 - part of normal metabolic breakdown of amino acids and fatty acids
 - oxidizes toxic substances such as alcohol and formaldehyde

MICROBODIES CONT'D

GLYOXYSOMES:

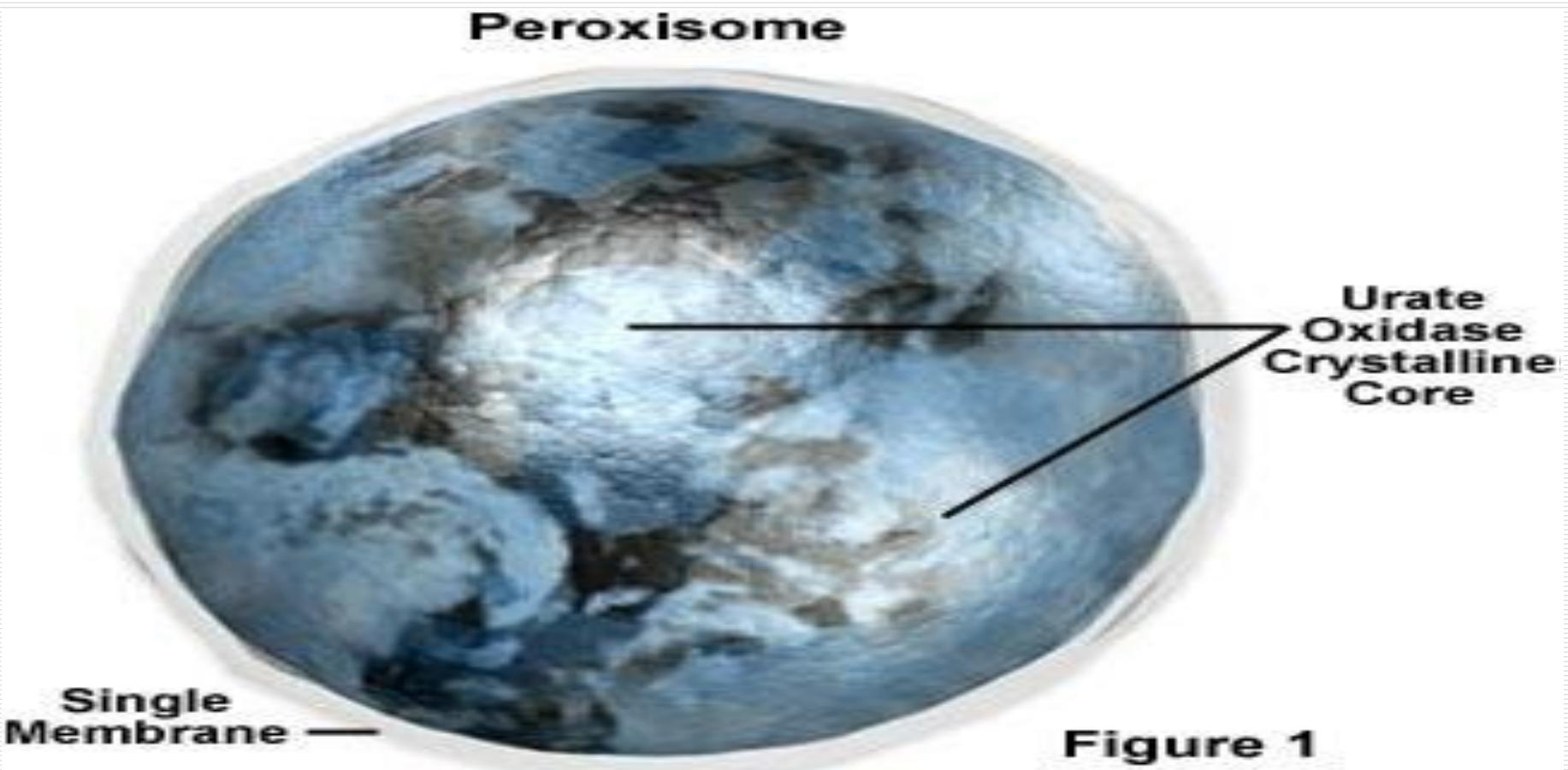
These are microbodies of plant cells in which the enzymes of the glyoxylate cycle are functionally more important than those involved in oxidative mechanisms. The glyoxylate cycle allows the relatively immobile fatty reserves of e.g. seeds to be converted to sugars and therefore, more easily transported to growing tissues.

The fats are degraded to acetyl CoA which feeds into the glyoxylate cycle to give succinate as a net product.

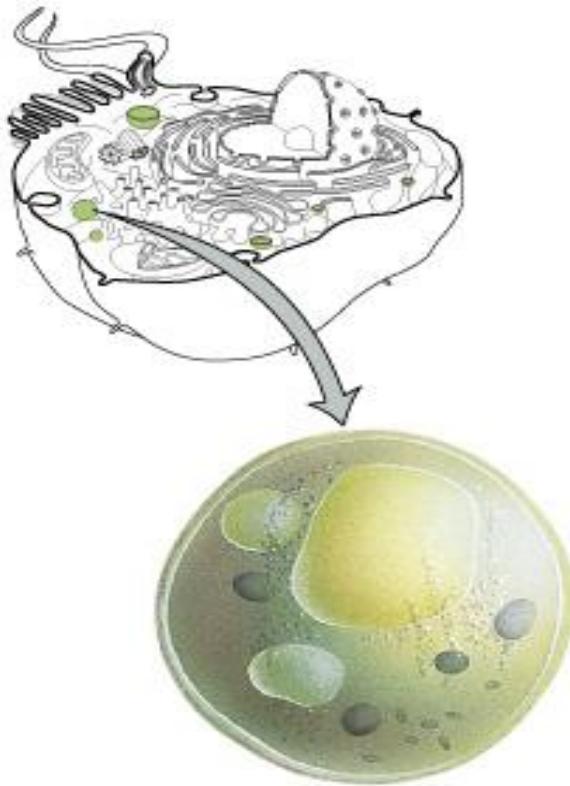
MICROBODIES CONT'D

Succinate is then transported to the mitochondria where it becomes a substrate for gluconeogenesis.

PEROXISOME



Lysosomes



- Membranous vesicles
 - _ formed in Golgi complex
 - _ filled with digestive enzymes
 - _ pumps in H⁺ ions until internal pH reaches 5.0
- Functions
 - _ digest foreign substances
 - recycles own organelles
 - _ autolysis
 - lysosomal damage after death

Lysosomes Cont'd.

Lysosomes are vesicular structures, limited by a single smooth membrane, and containing hydrolases active at acid pH values. Lysosomes arise initially as primary lysosomes, which appear to be derived from coated vesicles released from *trans* cisternae of the Golgi apparatus. Lysosomes contain about 60 hydrolytic enzymes whose concerted action will degrade most biological materials. Coated vesicles arising from receptor-mediated endocytosis deliver their contents to a

LYSOSOMES CONT'D:

vesicle called an endosome. Fusion of an endosome with a primary lysosome forms a secondary lysosome. The activity of a H⁺ - ATPase in the lysosome membrane pumps protons into the intralysosomal space producing a pH of about 5.0 which activates the lysosomal enzymes.

Phagocytic vesicles (phagosomes) also fuse with primary lysosomes to give secondary lysosomes.

LYSOSOMES CONT'D:

This allows digestion of the ingested material, and subsequently absorption of the products into the cytosol. Following digestive activity, a residual body containing non-degradable material may remain. Residual bodies are retained within the cell and may accumulate. The digestion of extracellular material is called heterophagy. However, lysosomes can also degrade material of intracellular origin, such as mitochondria and ribosomes. This process

Lysosomes Cont'd.

is called autophagy.

It is apparent that the lysosomal membrane is unusual. Not only is it resistant to digestion by the hydrolases of the lysosome, but also, under normal circumstances, it is impermeable to both the enzymes and their substrates. Despite this, the lysosomal membrane is freely permeable to the low molecular weight products of hydrolysis.

Lysosomes Cont'd.

Lysosomes are important in many clinical and medical aspects of biochemistry. For example, phagocytic cells in tissues of lungs and liver contain large lysosomes which are important in digesting foreign materials. Silicosis is a condition resulting from the inhalation of silica particles into the lungs which are taken up by phagocytes. Reactions between the silica and the lysosomal membranes lead to the rupture of the membrane, the release of the lysosomal enzyme and eventually

Lysosomes Cont'd.

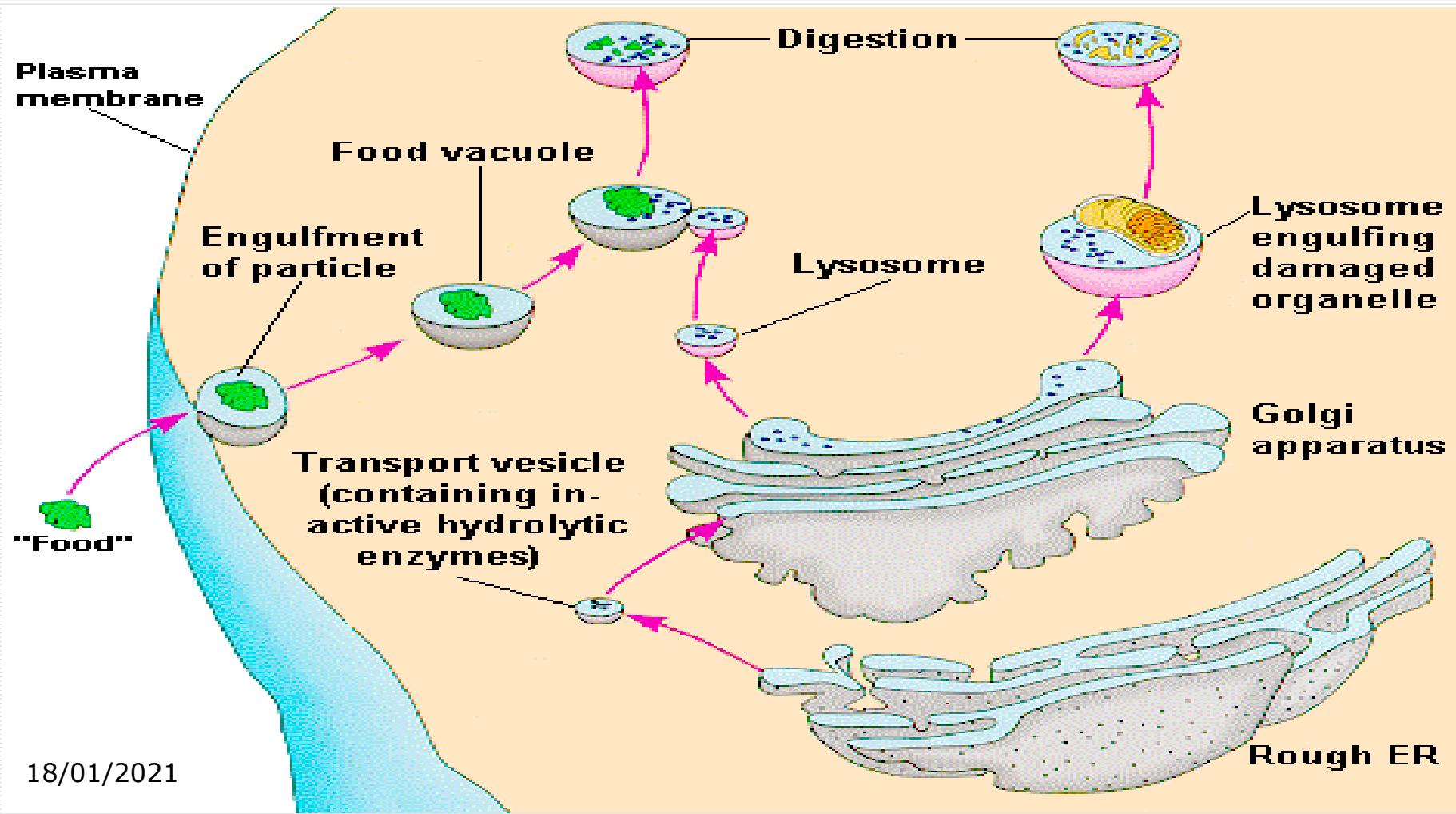
the death of the phagocyte. The death of large numbers of phagocytes stimulates fibroblasts to deposit collagen fibres which decrease lung elasticity, impair breathing and contribute to the pathology of disease. Silicosis is similar to asbestos and black lung disease, conditions caused by breathing in asbestos fibres and coal dust respectively.

The absence of specific hydrolase enzymes from lysosomes leads to the accumulation of substrate

Lysosome Cont'd.

for that enzyme within the lysosomes. This usually has severe medical consequences.

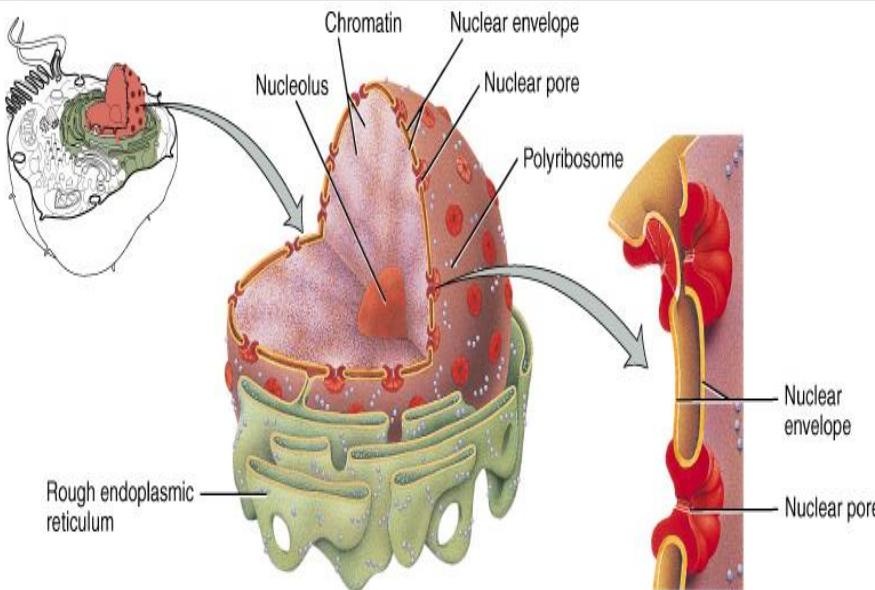
Lysosome Cont'd.



Tay-Sachs Disorder

- Affects children of Eastern European descent
 - seizures, muscle rigidity, blindness, demented and die before the age of 5
- Genetic disorder caused by absence of a single lysosomal enzyme
 - enzyme normally breaks down glycolipid commonly found in nerve cells
 - as glycolipid accumulates, nerve cells lose functionality

Nucleus



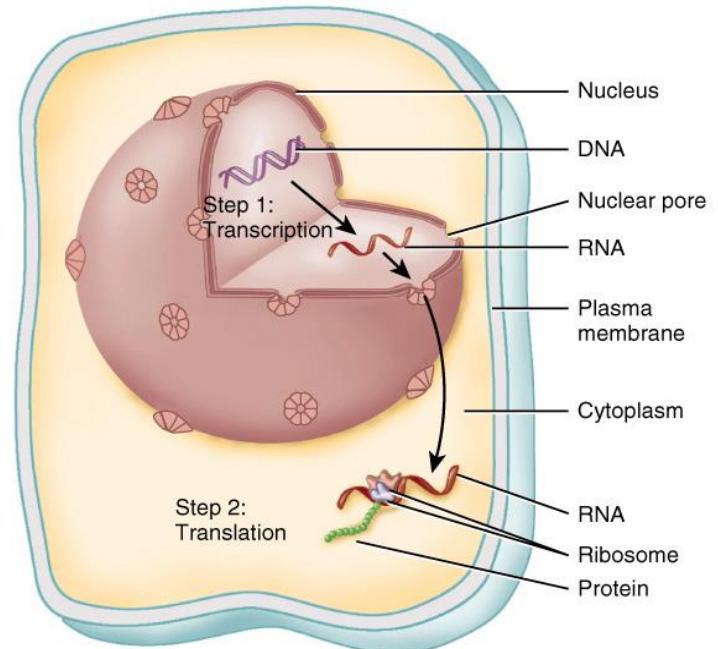
- Large organelle with double membrane nuclear envelope
 - outer membrane continuous with rough ER
 - perforated by water-filled nuclear pores
- Nucleolus
 - spherical, dark bodies within the nucleus (no membrane)
 - site of ribosome assembly

Function of Nucleus

- 46 human DNA chromosomes
 - genes found on chromosomes
 - gene directs synthesis for a specific protein
- Non-dividing cells contain nuclear chromatin
 - loosely packed DNA
- Dividing cells contain chromosomes
 - tightly packed DNA
 - it doublescopes itself before condensing

Protein synthesis

- Instructions for making specific proteins is found in the DNA (your genes)
 - transcribe that information onto a messenger RNA molecule
 - translate the “message” into a sequence of amino acids in order to build a protein molecule



THE NUCLEUS:

In eukaryotic organisms, the DNA is protected in the nucleus which has a surrounding envelope. The nuclear envelope consists of a double membrane separated by a perinuclear space.

The nuclear envelope is interrupted by pores, found at regions where the outer and inner membranes fuse. The pores are surrounded by highly organised annulus consisting of eight protein granules symmetrically arranged around the pore. The term

THE NUCLEUS CONT'D:

porosome has been applied to the nuclear pore and its associated annulus. There are molecules that are much bigger than the nuclear pores but are freely able to pass through yet there are molecules far smaller than the nuclear pores but are prevented from entering the nucleus. Nuclear proteins, like all proteins, are produced in the cytosol but must accumulate in the nucleus for functional reasons. Such proteins contain sequences of amino acid residues which act as signals, enabling them to be concentrated selectively in the nucleus.

THE NUCLEUS CONT'D:

Glycoproteins present in the porosome appear to regulate the entry of material into the nucleus.

Contained in the nucleus is the nucleolus and the nucleoplasm. Found embedded in the nucleoplasm are chromatin materials which are precursors of chromosomes. Eukaryotic chromosomes consist of about one-third DNA and two-thirds protein. The complex of chromosomal DNA and protein (a nucleoprotein) is called chromatin. The protein of chromatin consists of small, basic proteins known as histones and additional proteins generally called

THE NUCLEUS CONT'D:

chromatin is made up of repeating structural units called nucleosomes.

The nuclear envelope is perforated with holes called nuclear pores. These pores regulate the passage of molecules between the nucleus and cytoplasm, permitting some to pass through the membrane, but not others. Building blocks for building DNA and RNA are allowed into the nucleus as well as molecules that provide the energy for constructing genetic material.

THE NUCLEOLUS

The nucleolus is a membrane-less organelle within the nucleus that manufactures ribosomes, the cell's protein-producing structures. Through the microscope, the nucleolus looks like a large dark spot within the nucleus. A nucleus may contain up to four nucleoli, but within each species the number of nucleoli is fixed. After a cell divides, a nucleolus is formed when chromosomes are brought together into nucleolar organizing regions. During cell division, the nucleolus disappears.

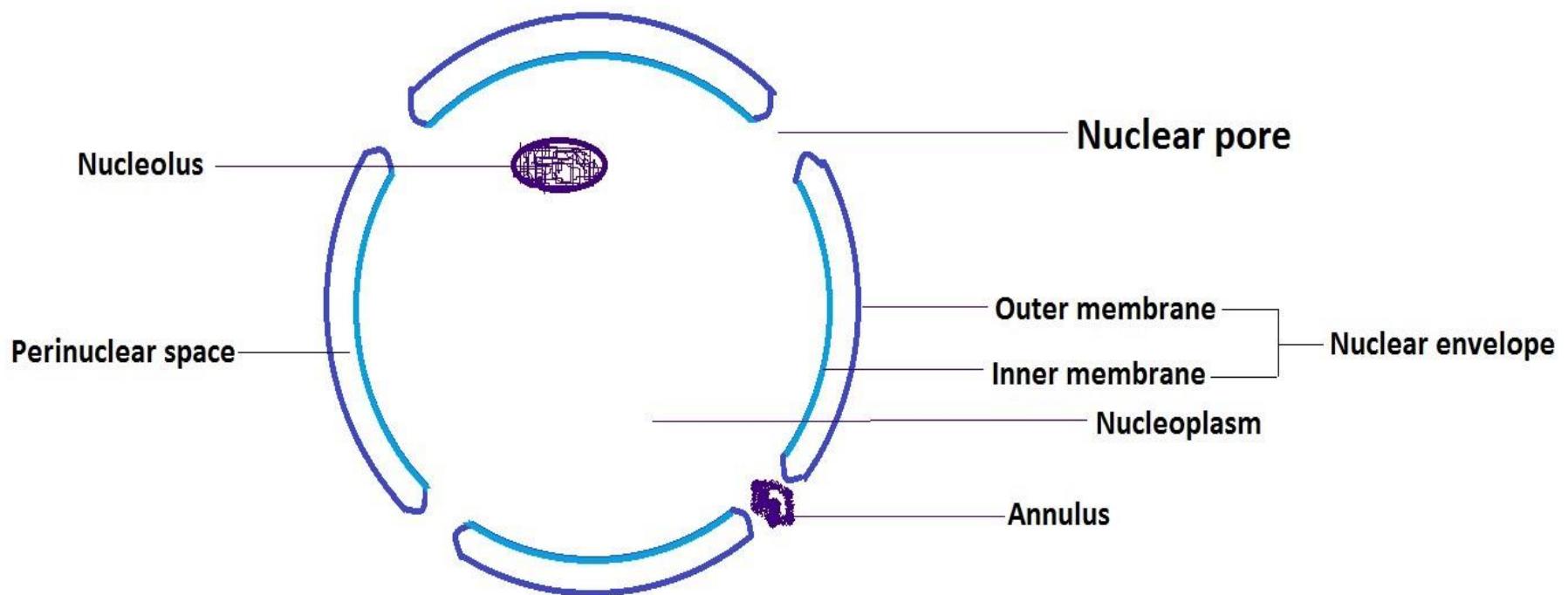
THE NUCLEOLUS CONT'D:

Nucleoli are prominently staining regions of the nucleoplasm. They are composed of groups of ribosomal genes surrounded by their rRNA transcripts, together with many proteins. Nucleoli are sites of synthesis of rRNA molecules and for the assembly of ribosomal subunits using rRNA molecules, and the ribosomal proteins produced in the cytoplasm.

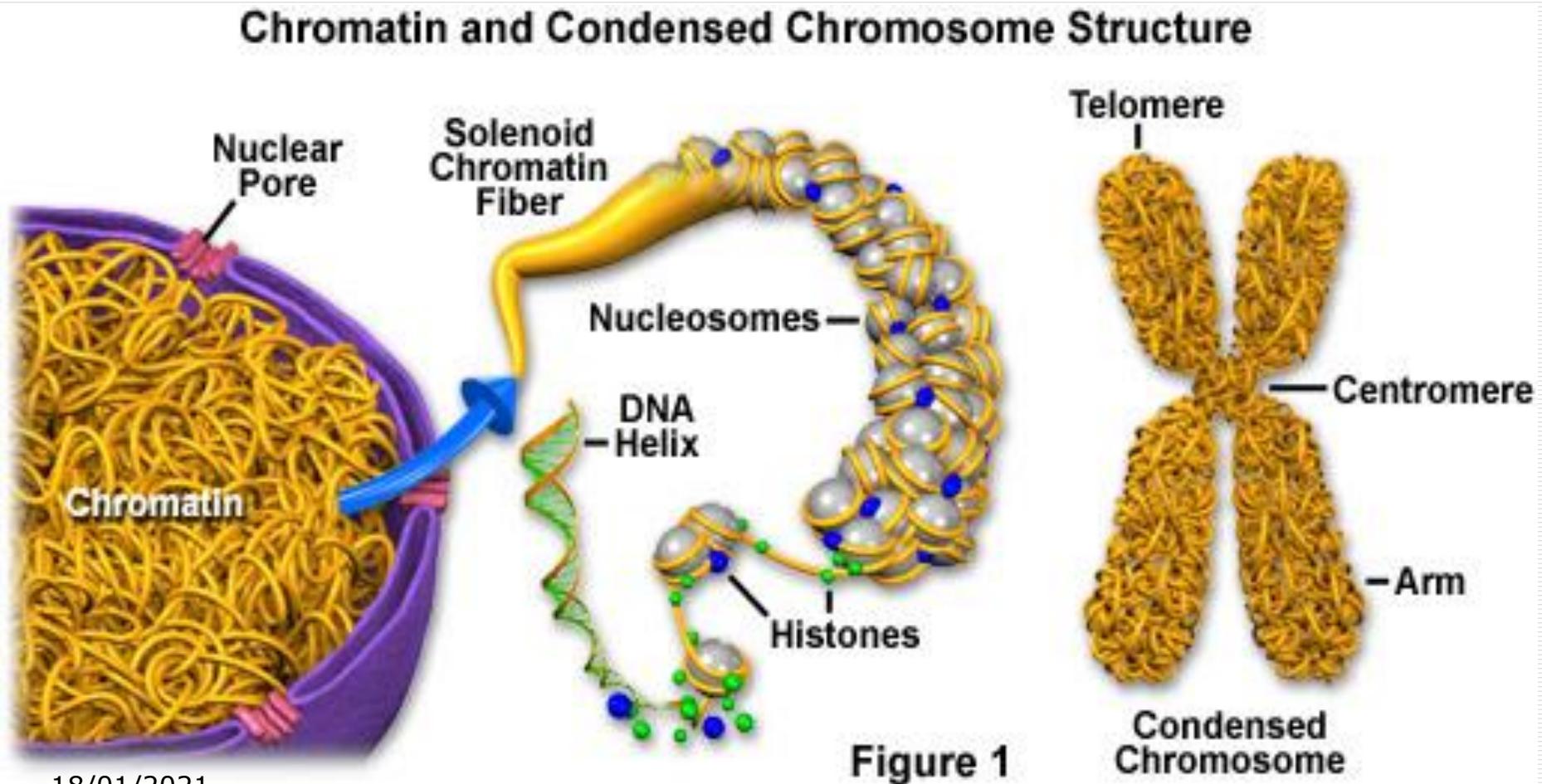
THE NUCLEOLUS CONT'D:

Some studies suggest that the nucleolus may be involved with cellular aging and, therefore, may affect the senescence of an organism.

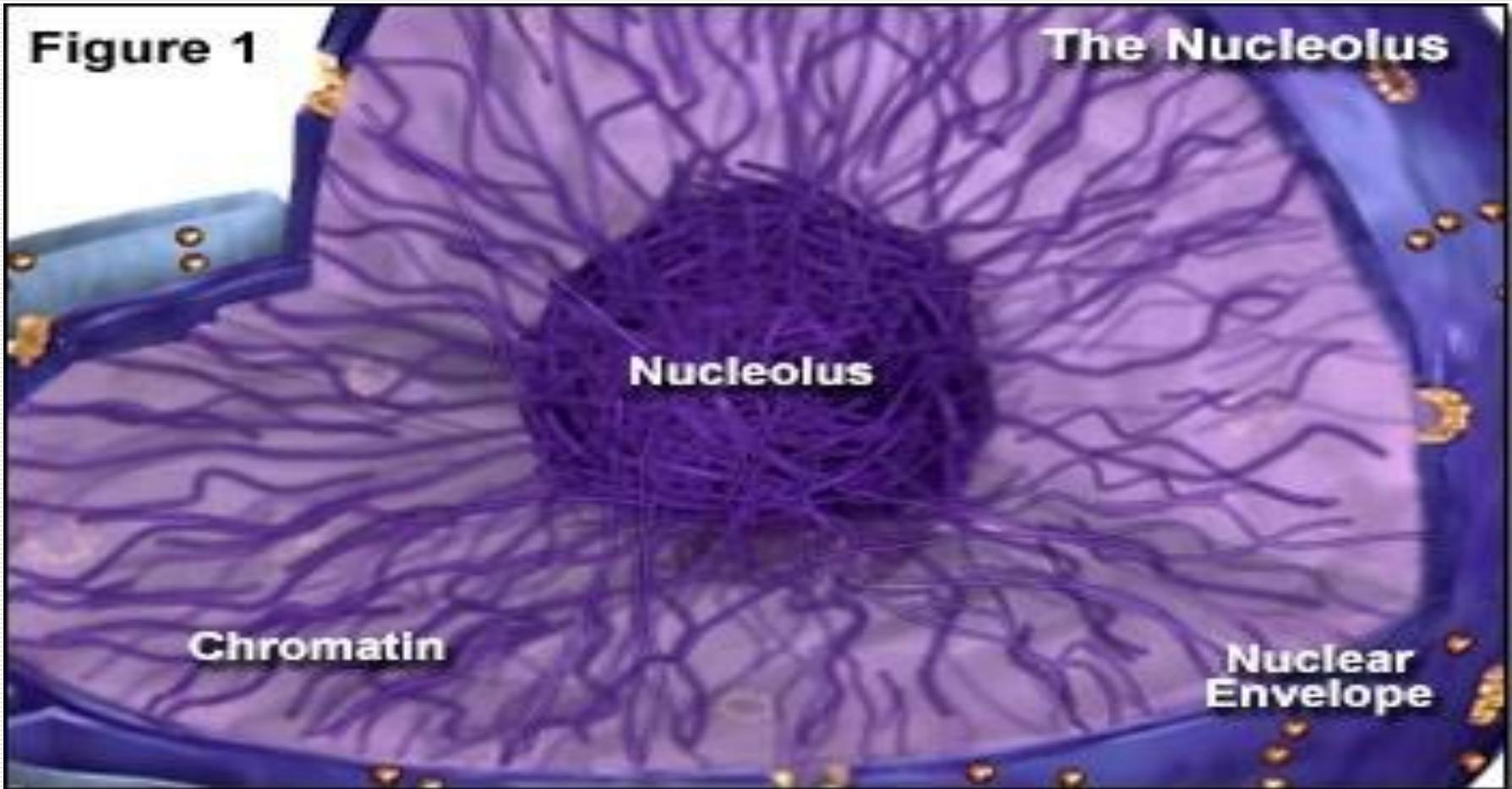
Structure of the nucleus



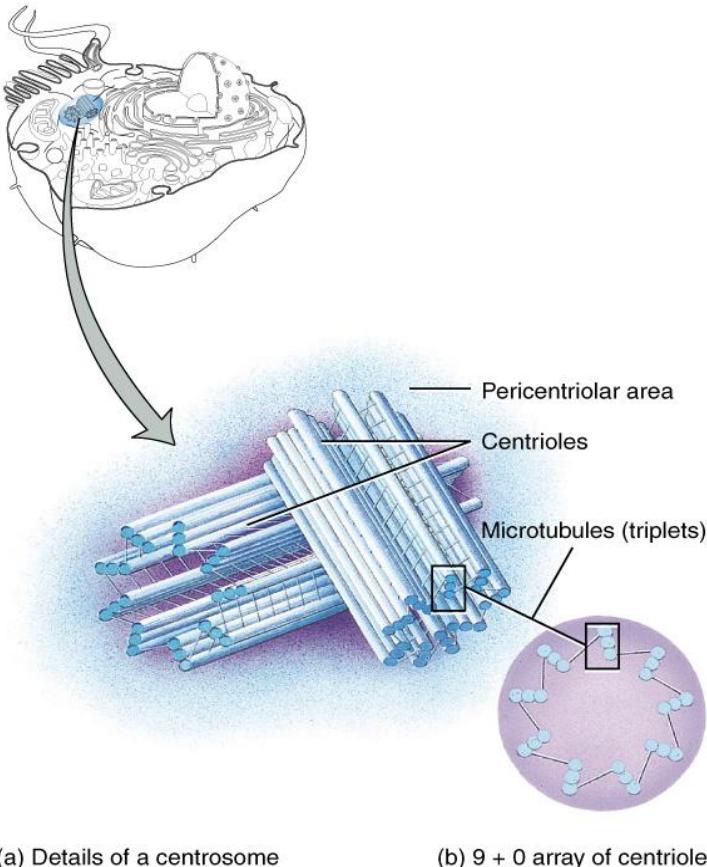
Chromatin and Chromosomes



Chromatin and Chromosomes Cont'd



Centrosome



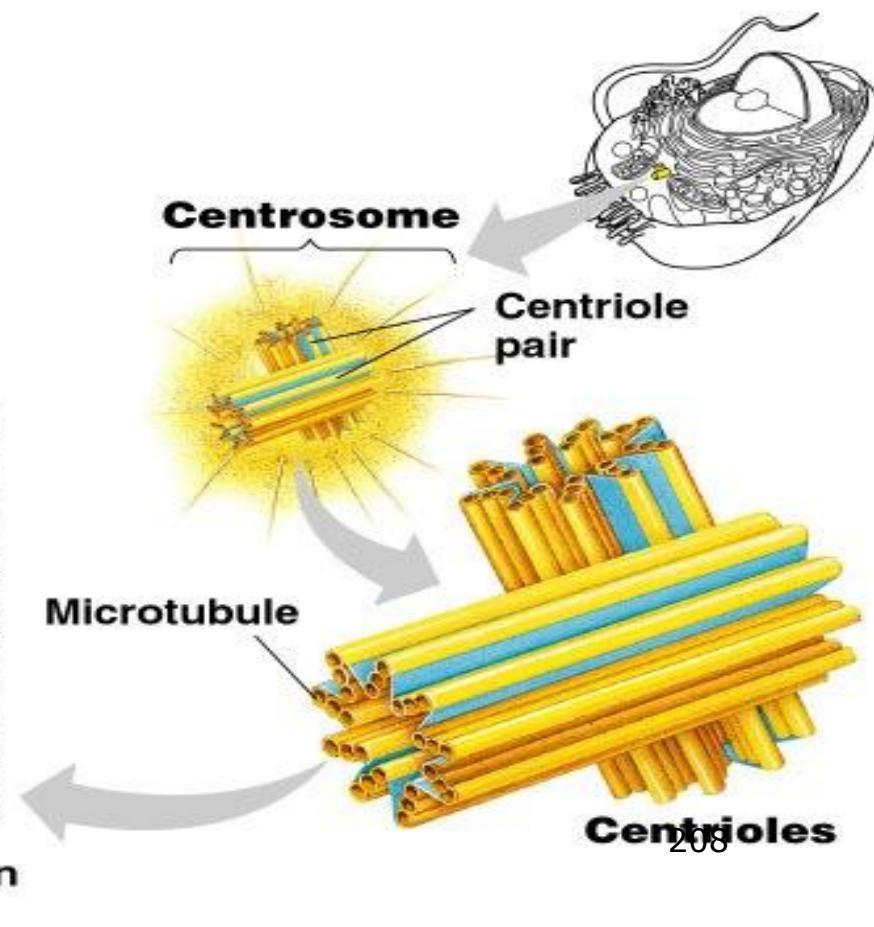
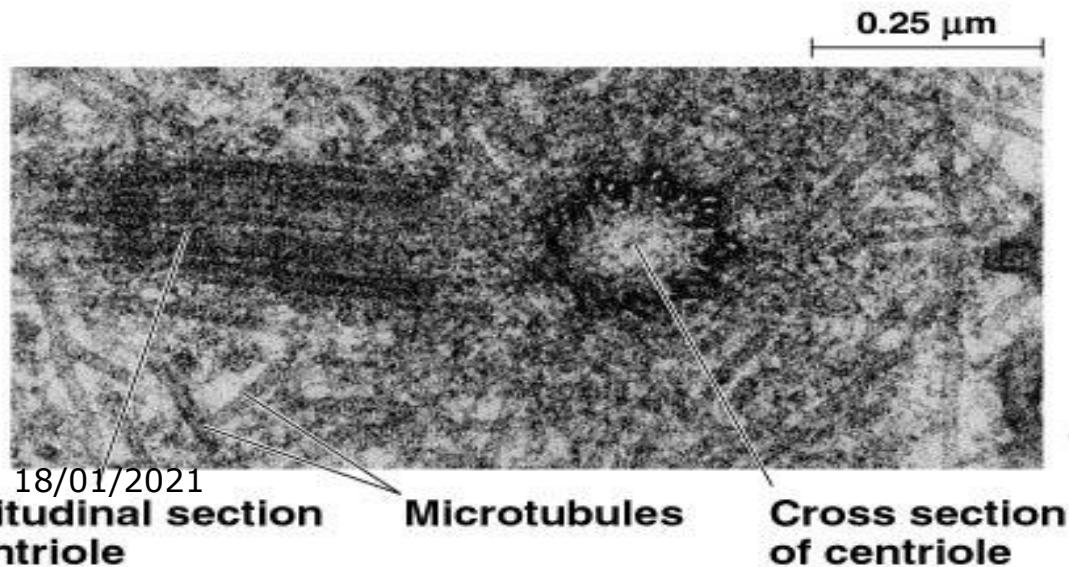
- Found near nucleus
- Pericentriolar area
 - formation site for mitotic spindle and microtubules
- Centrosome
 - 2 centrioles (90 degrees to each other)
 - 9 clusters of 3 microtubules
 - role in formation of cilia & flagella

Centrosome

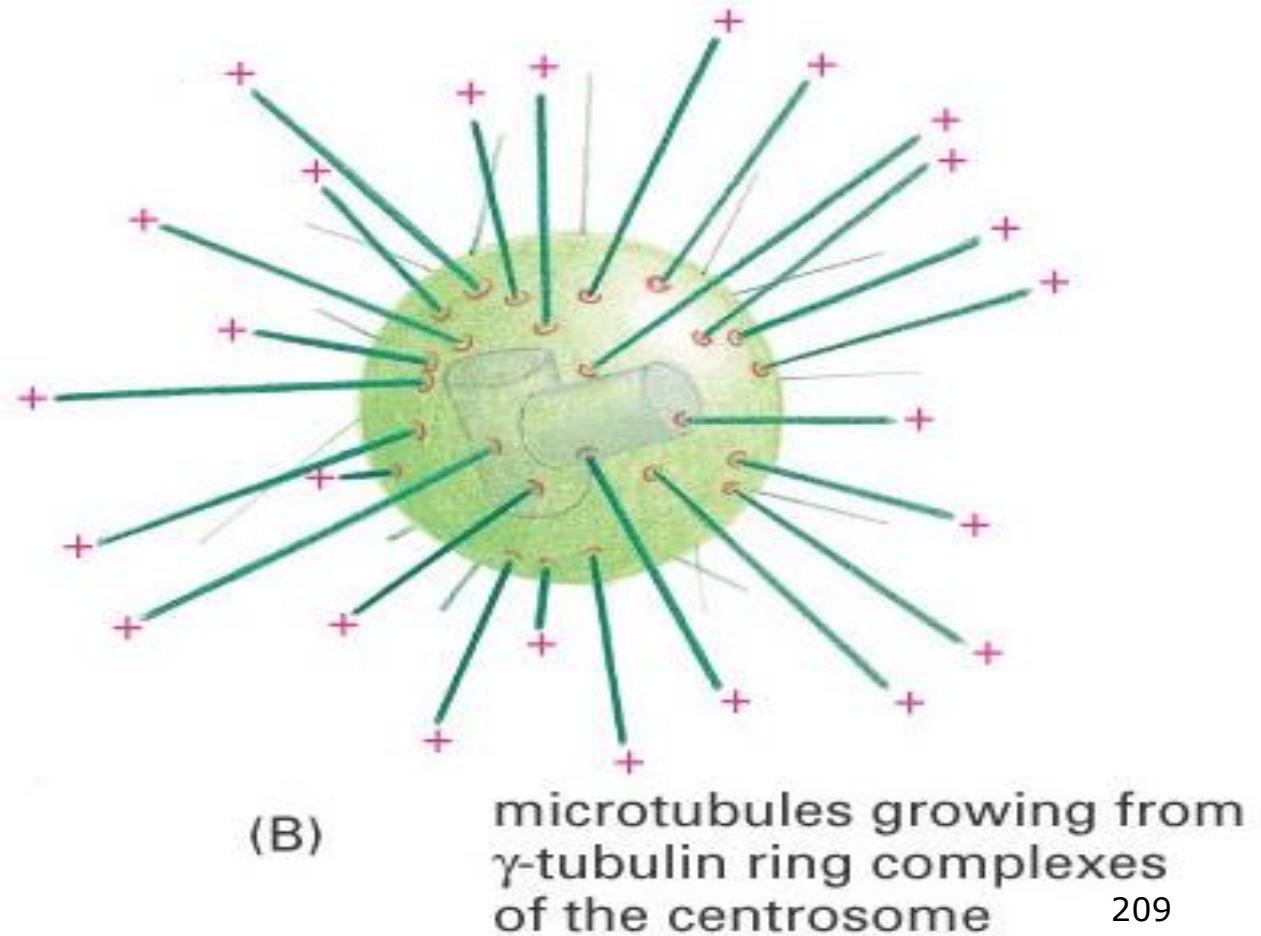
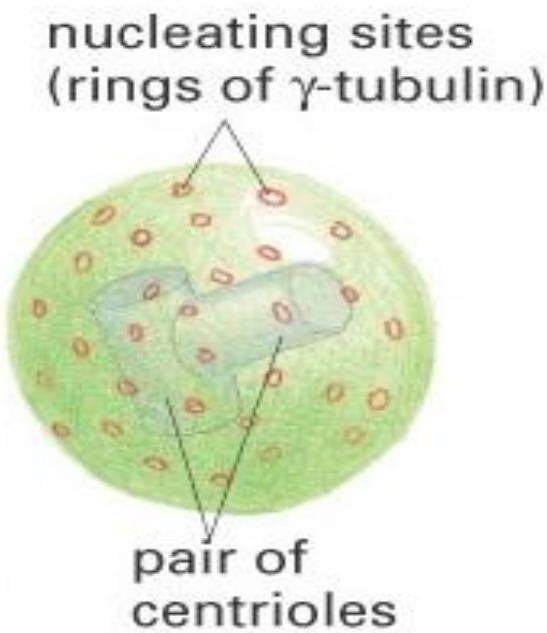
- Centrosomes often lie close to the cell nucleus and microtubules radiate from here in all directions towards the edge of the cell (plasma membrane).

- The 'plus' end of the microtubule is furthest away from the centrosome. This is where microtubules rapidly lengthen or shorten in response to signals.

- In animal cells, the centrosome has a pair of **centrioles**, each with nine triplets of microtubules arranged in a ring.
- During cell division the centrioles replicate.



Centrosome



Cytoskeleton

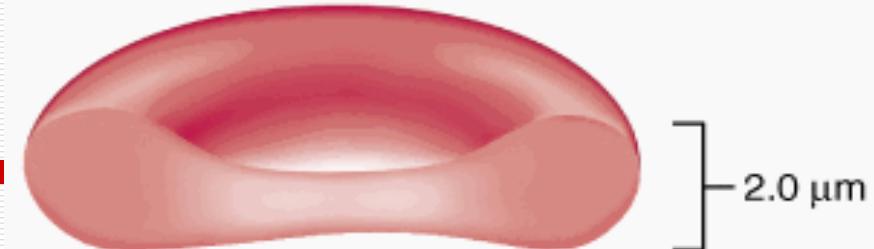
Eukaryotic cells have a wide variety of distinct shapes and internal organizations. Cells are capable of changing their shape, moving organelles, and in many cases, move from place to place. This requires a network of protein filaments placed in the cytoplasm known as the cytoskeleton.

The two most important protein filaments are called the actin filaments and the microtubules. The actin is responsible for contraction (like in muscles) and the microtubules are for structural strength.

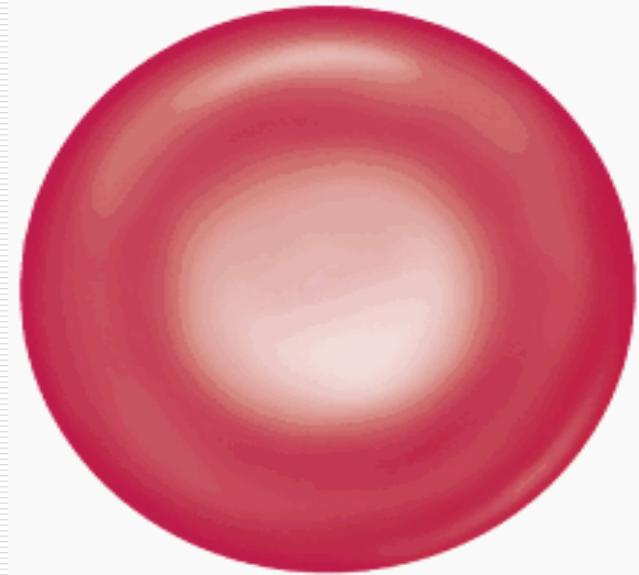
In this lecture, you will learn

1. What a cytoskeleton is?
2. Functions of the cytoskeleton
3. Composition and associated proteins
4. How cytoskeleton brings about movement
5. Dynamic instability of microtubules
6. Centrosome, Cilia and flagella
7. Microtubules and medicine/disease
8. Actin filaments and associated binding proteins
9. Actin filament and RBC cytoskeletal architecture
10. Actin filaments and muscle contraction

Red Blood Cells



Side view

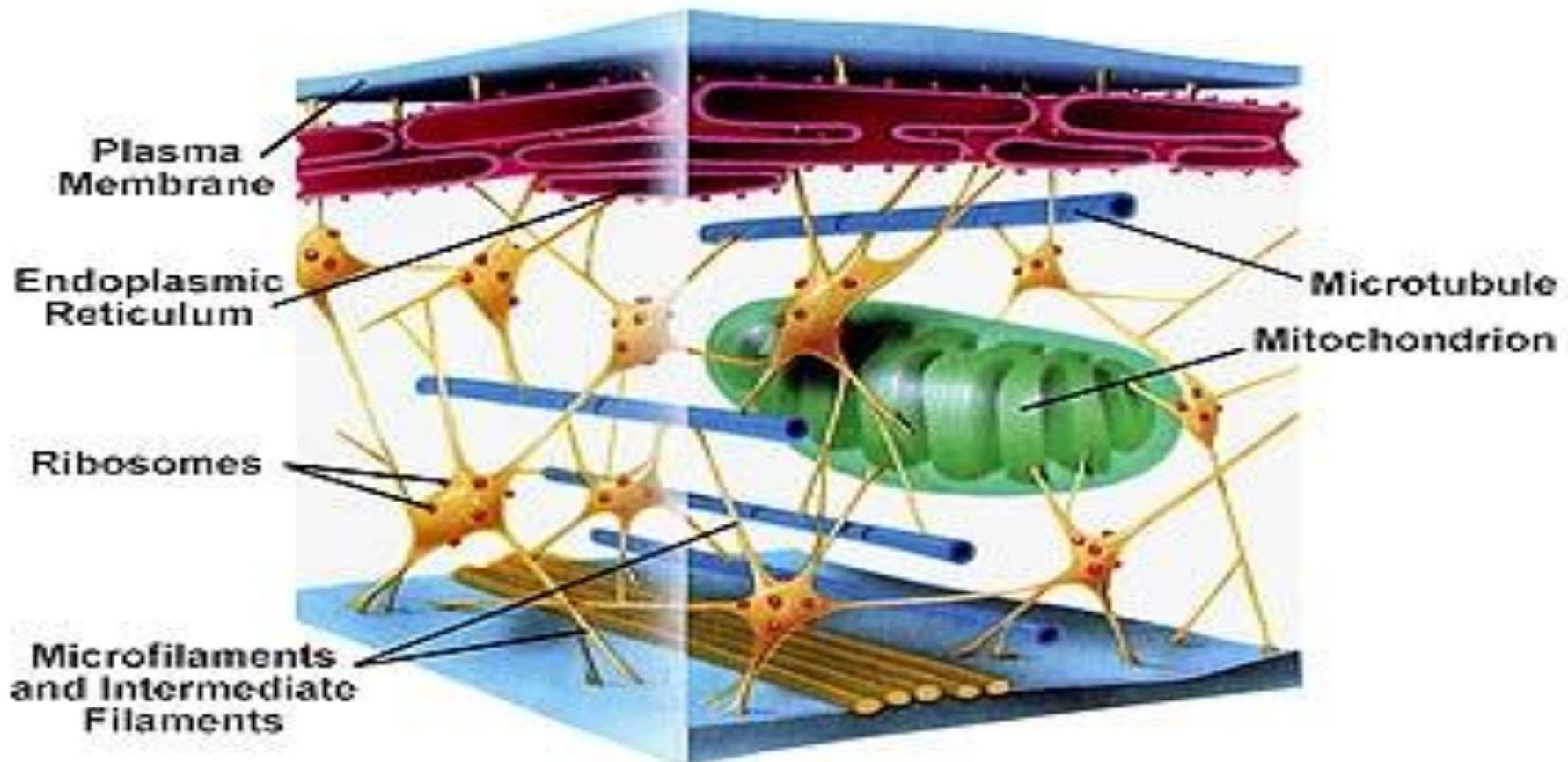


Top view

Why aren't they spherical?

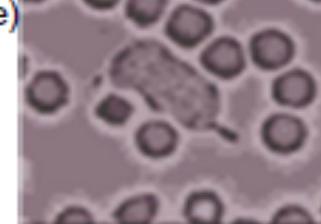
They have an internal cytoskeleton

Cytoskeleton Cont'd.



Why cells need to be motile?

Neutrophil (macrophage)
chasing bacterium



- **Development:** cells migrate inside the embryo to their defined locations
- **Host defense:** motile cells constantly **search for pathogens** inside the adult animal
- **Wound healing:** injured tissues are immediately invaded by highly motile cells to secret extracellular matrix (ECM) proteins

Why do people need to study the basics of cell movement?

Uncontrolled cell migration contributes to several pathologies:

- **Vascular diseases**
- **Chronic inflammatory diseases**
- **Cancer:** tumor formation and metastasis

What is the basis of cell migration?

A **cytoskeleton** composed of fibers which dynamically **reorient, shrink and grow**.

Based on this mechanism:

- axons of neurons can grow and connect to other neurons
- muscle **cells can contract** and produce force
- cells can send out small filopodia to sense their environment
- cells can divide during **mitosis**

What is the Cytoskeleton

- The cytoskeleton acts much like our own skeletons in supporting the general shape of a cell.
 - Unlike our skeletons though, the cytoskeleton is highly dynamic and internally motile, shifting and rearranging in response to the needs of the cell.
- It also has a variety of purposes beyond simply providing the shape of the cell.
- Generally, these can be categorized as
 - Structural
 - Transport.
- All 3 major components performs each of these functions, but not equally, because their biophysical characteristics are quite different

The Cytoskeleton

- With respect to ***structure***, at some point in the life of every cell, it must change shape,
 - whether simply increasing or decreasing in size,
 - May be a drastic alteration like the super-elongated form of neurons with axons,
 - the cytoskeleton must be able to respond by dynamically increasing and decreasing the size of the internal structures as needed.

Structure and Function

■ Structure

- also applies to the relative position of internal cellular elements, such as organelles or proteins, to one another.
- In many highly specialized cells, the segregation of particular structures within certain parts of the cell is crucial for it to function.

■ Transport

- Refers to the movement of molecules and organelles within the cell
- Movement of the cell as a whole.

Structural Support

□ **Mechanical support**

- Maintains shape

□ Fibers act like a geodesic dome to stabilize and balance opposing forces

□ Provides anchorage for organelles

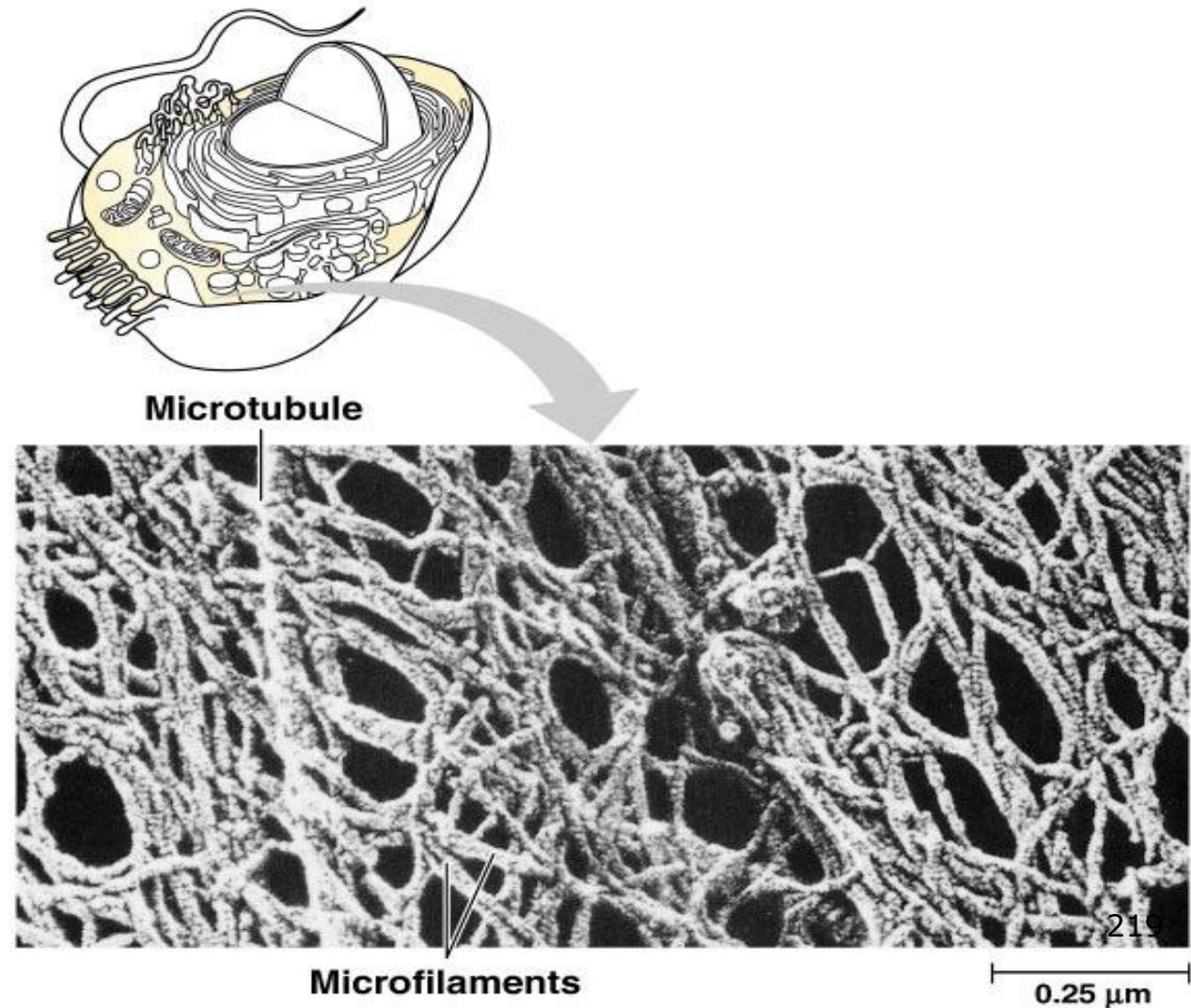
□ **Dynamic**

- Dismantles in one spot and reassembles in another to change cell shape

□ Introduction

- The **cytoskeleton** is a network of fibers extending throughout the cytoplasm.

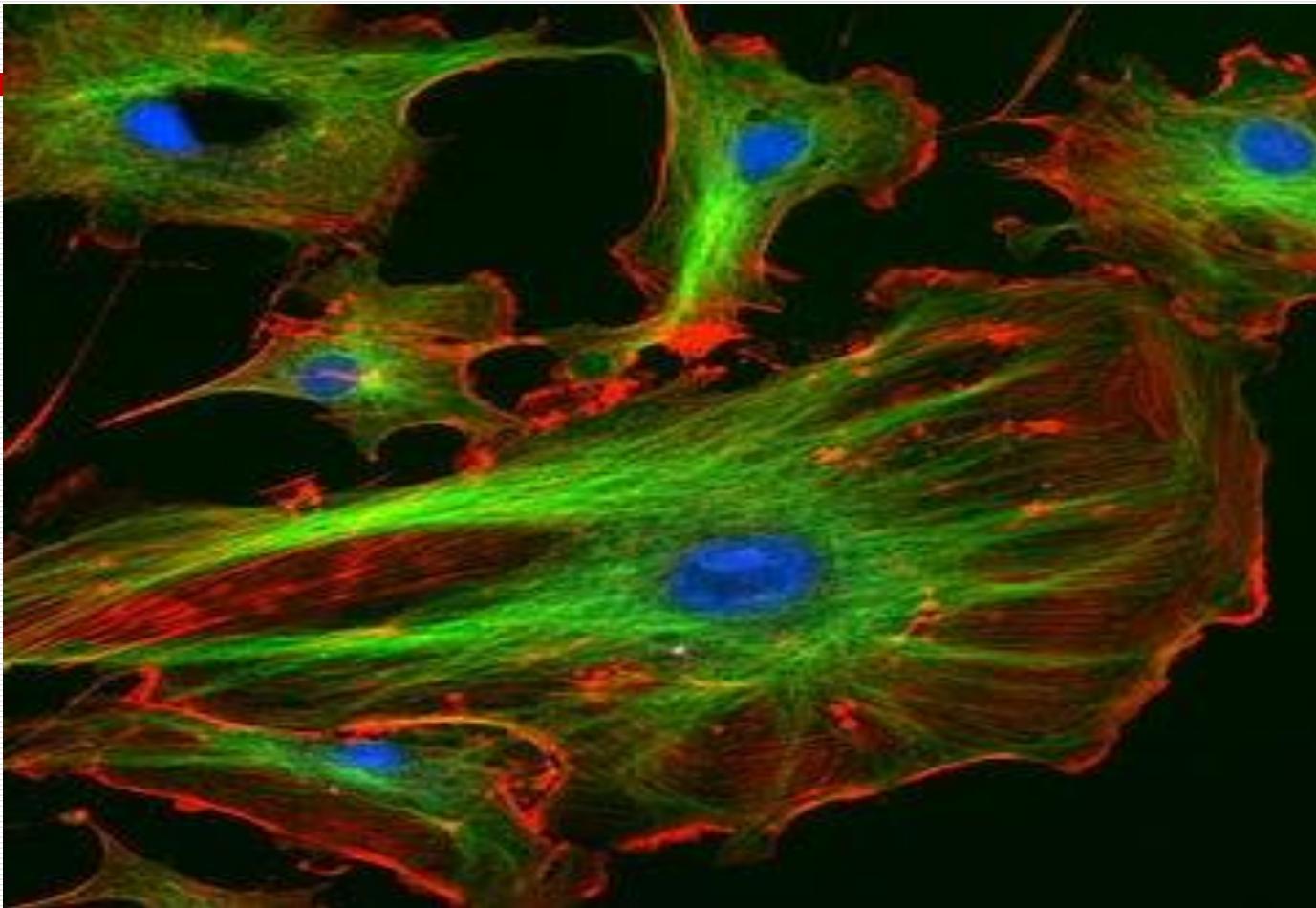
- The cytoskeleton organizes the structures and activities of the cell.



Cytoskeleton

- *This is the overall name given to **protein filaments** and **motor proteins** in the cell.*
- **These protein filaments form an enormous three dimensional (3D) meshwork.**
- *Filaments can be cross linked to other similar filaments, and to membranes, by means of **accessory proteins**.*
- *This inter-linking greatly increases rigidity and provides mechanical strength*
- *Some filaments are used as **trackways** for motor proteins to transport cargoes*

The eukaryotic cytoskeleton



The eukaryotic cytoskeleton. Actin filaments are shown in red, microtubules are in green, and the nuclei are in blue.

Functions of the cytoskeleton

- They help the cell remain rigid but also help it move and change its shape when instructed to do so.
- Components of the cytoskeleton also enable cilia, flagella and sperm to swim, cell organelles to be moved and positioned, and ***muscles contraction***.
- During cell division these components also assist by pulling the daughter chromosomes to opposite 'poles' in the dividing process.
- Throughout the life of the cell various molecules and cargo containing vesicles are transported around the cell by motor proteins.
- These move along the protein filaments using them as trackways rather like a railway locomotive runs on rail tracks.
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Cytoskeleton forms the architecture and transport system of the cell

- During the evolution of eukaryotic cells, the compartmentalization of cell organelles into membrane bounded structures, was accompanied by the evolution of a system that positioned and anchored them.
- This system therefore contributes to the architecture of the cell, its rigidity and in some cases to its ability to move.
- It also contributes by providing a physical transport system that enables cargo filled vesicles, some individual molecules, and even some cell organelles to be moved within the cell.

Cytoskeleton: Movers and shapers

- Three main groups of shapers, the protein filaments: ***microtubules, intermediate filaments and actin filaments.***
- There are three groups of movers, the motor proteins: ***kinesin, dynein and myosin,***

Shapers (protein filaments) come in three sizes

- The variable shape and rigidity of the cell and its ability to move is largely dependent on three groups of cytoskeletal protein filaments:
- ***Microtubules*** - size: about 25nm external diameter
- ***Intermediate filaments*** – size: about 10nm external diameter
- ***Actin filaments*** – size: about 8nm external diameter
- All three groups of protein filaments are polymers made up of protein sub-units.

There are three kinds of intracellular fibers

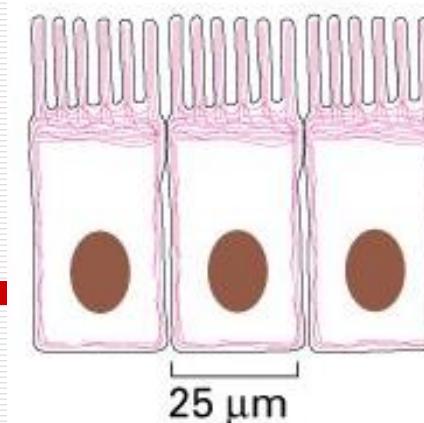
- **Microtubules** - long tubes made from the protein, tubulin
 - **Microfilaments** - thinner than microtubules; they are made from actin
 - **Intermediate filaments** - intermediate in diameter; they are made from a family of proteins that are all similar to keratin, the protein of hair
-

The Cell Cytoskeleton

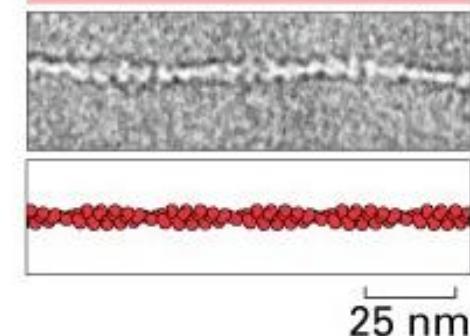
What are the 3 primary types of cytoskeletal proteins?

Actin filaments

- membrane contraction
- muscle cells
- cytokinesis



ACTIN FILAMENTS

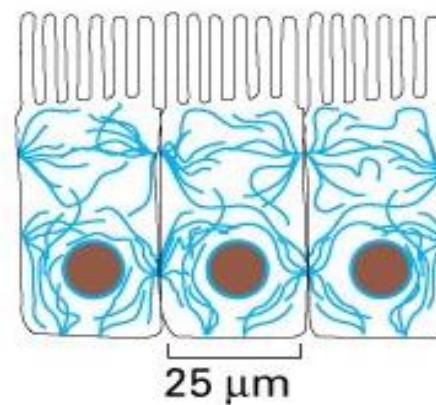


Intermediate filaments

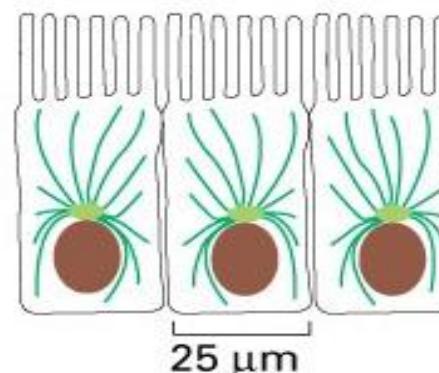
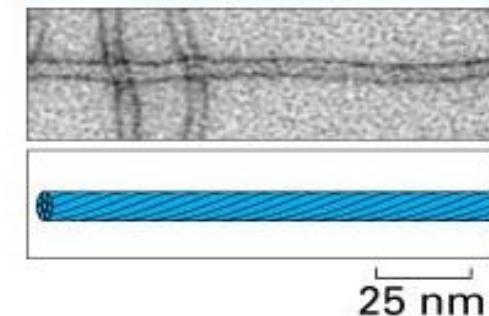
- resist mechanical stress

Microtubules

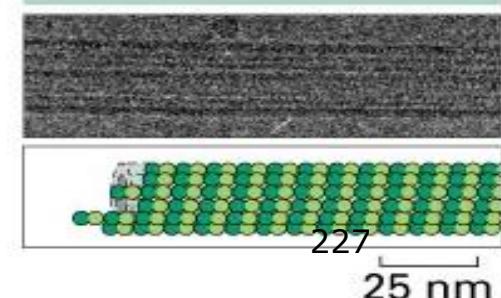
- cytoplasmic transport
- axoneme movement
- chromosome movement



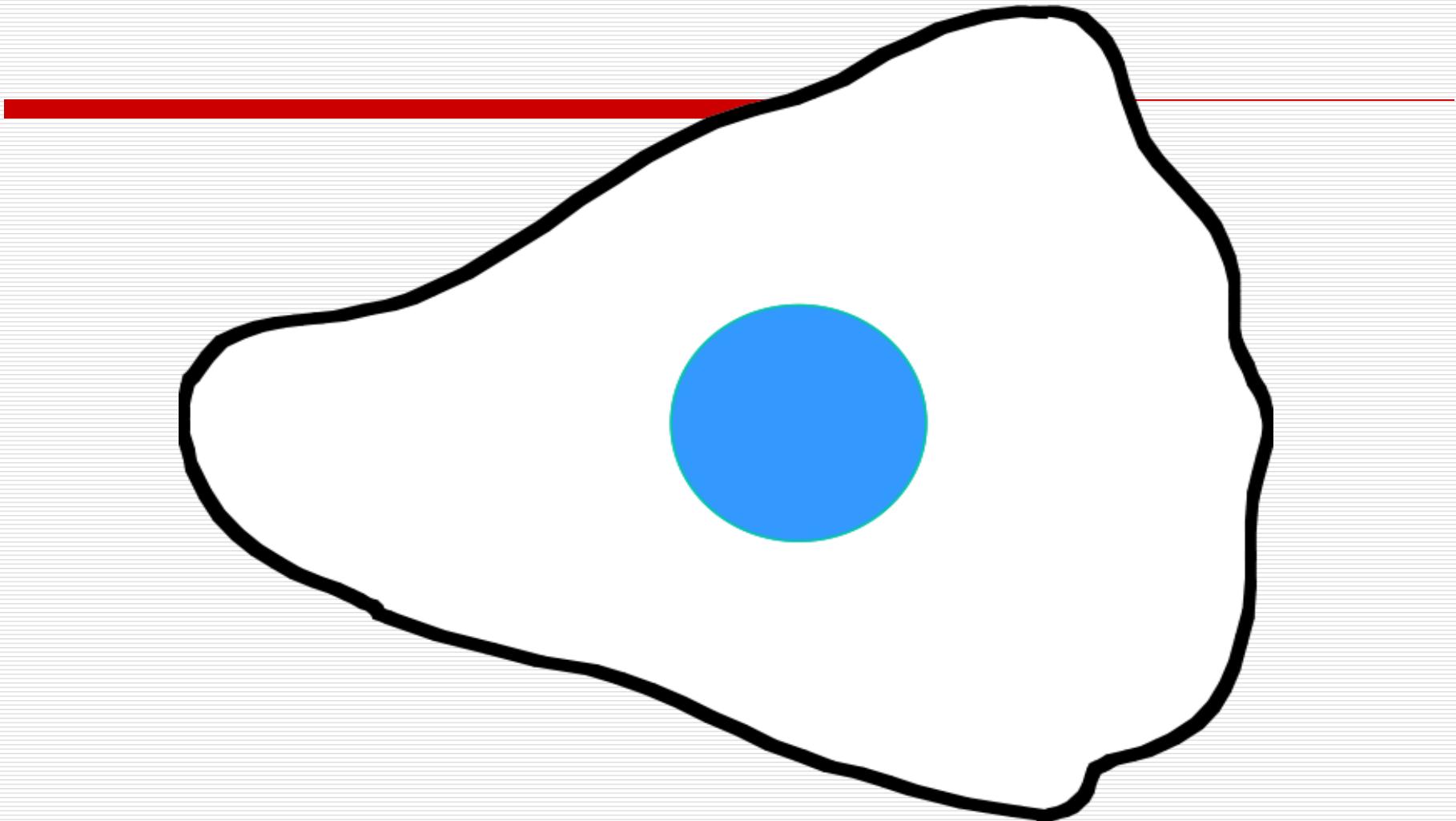
INTERMEDIATE FILAMENTS



MICROTUBULES

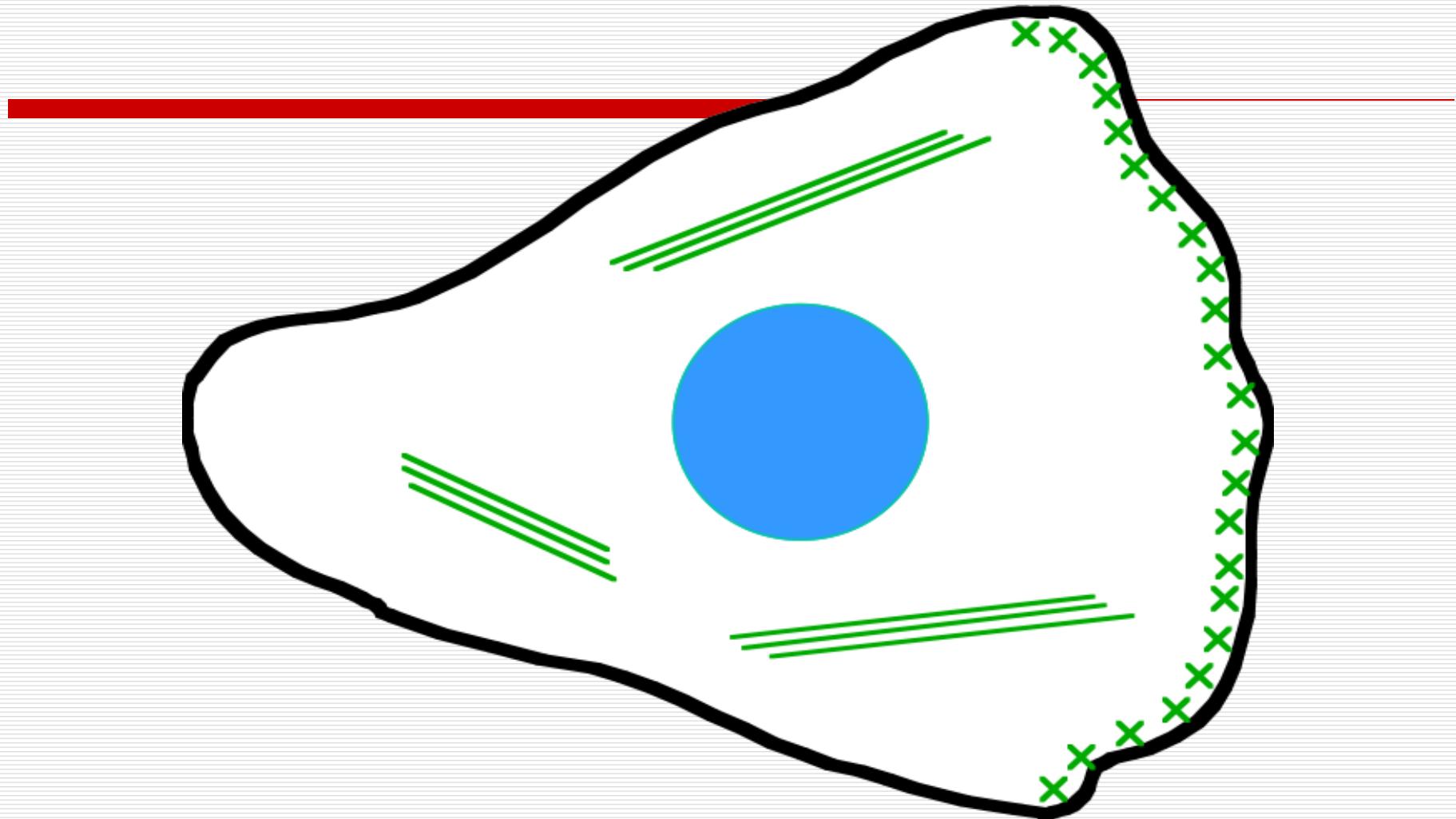


Cytoskeleton – mammalian cell



Cytoskeleton – mammalian cell

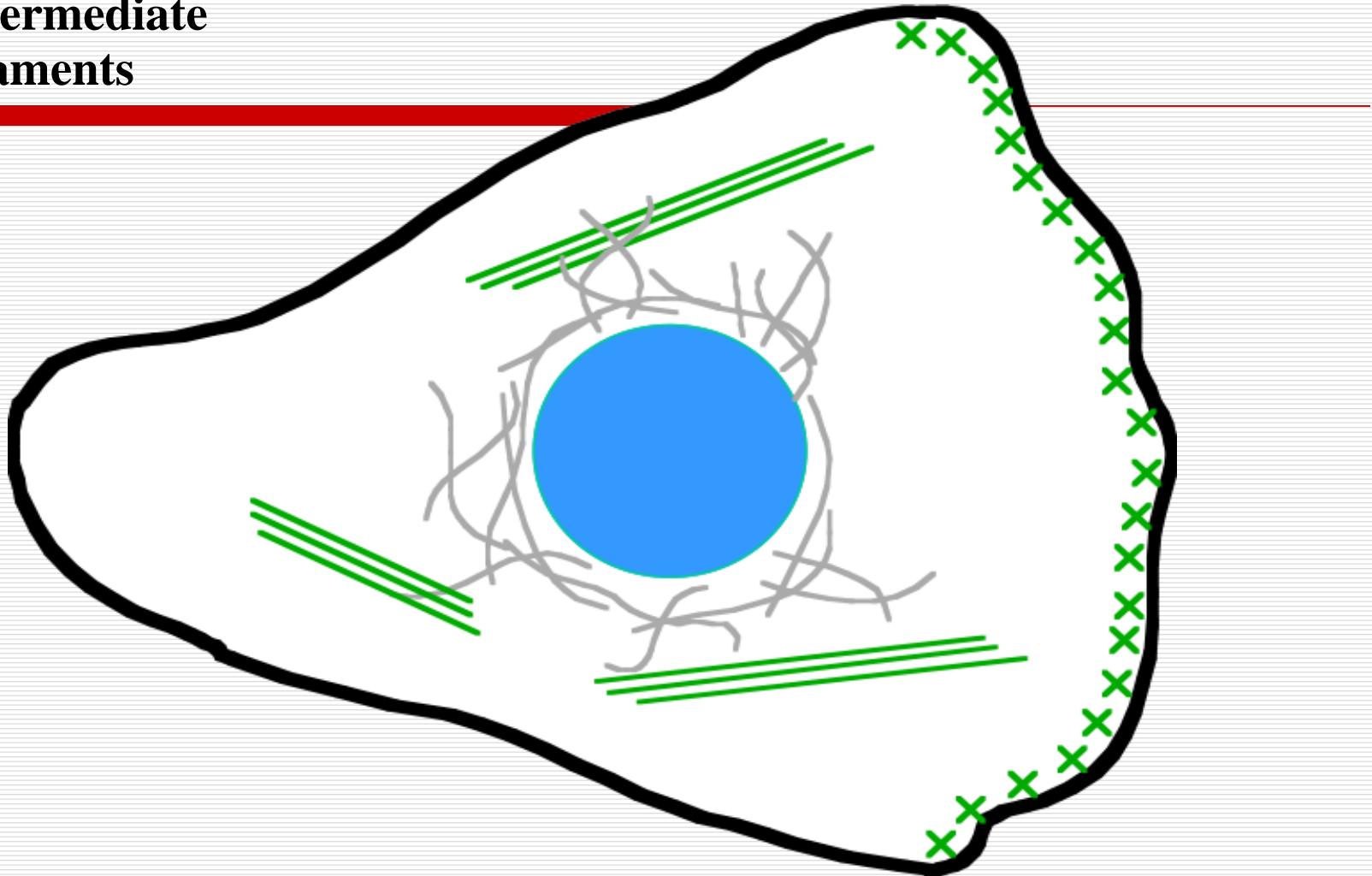
actin



Cytoskeleton – mammalian cell

actin

intermediate
filaments

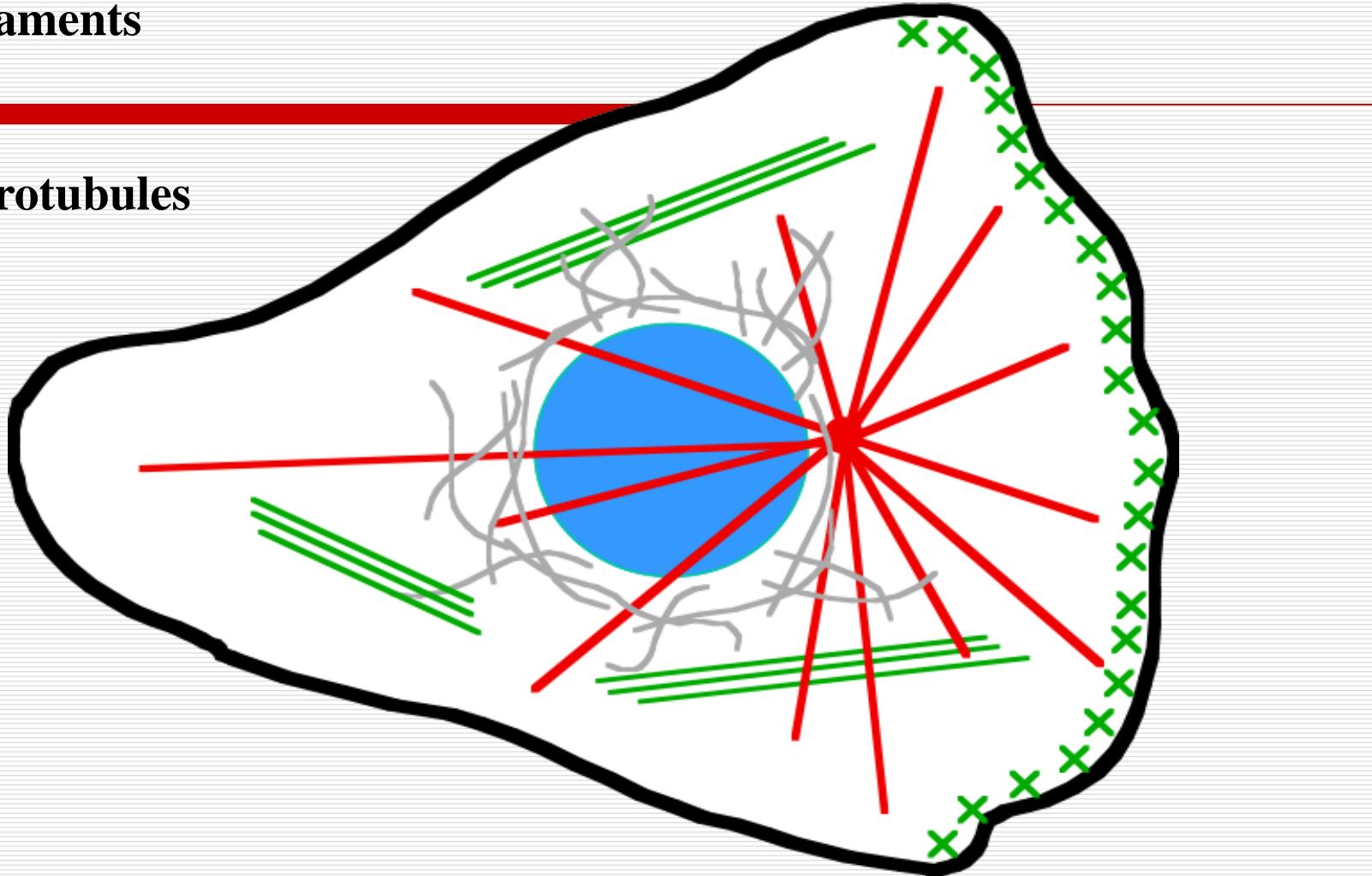


Cytoskeleton – mammalian cell

actin

intermediate
filaments

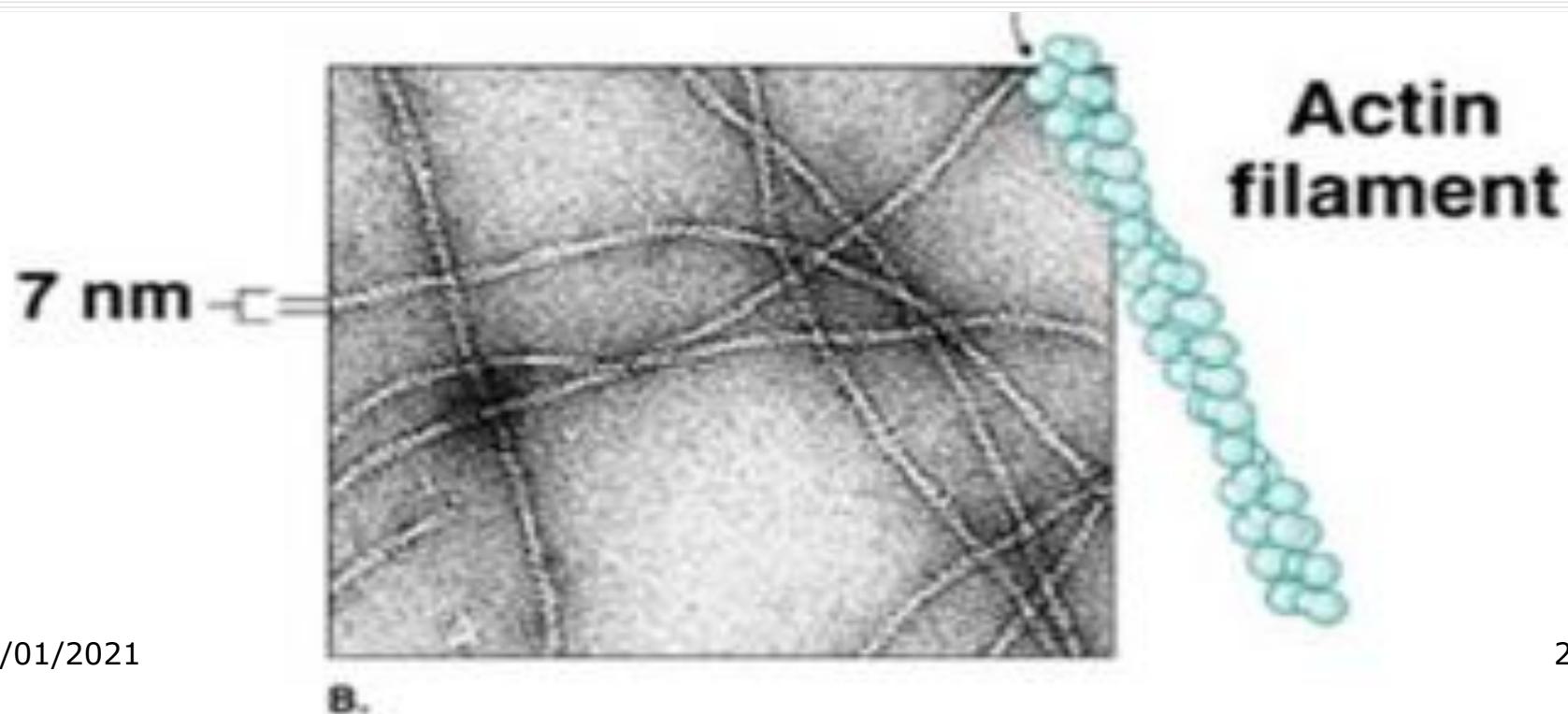
microtubules



Actin

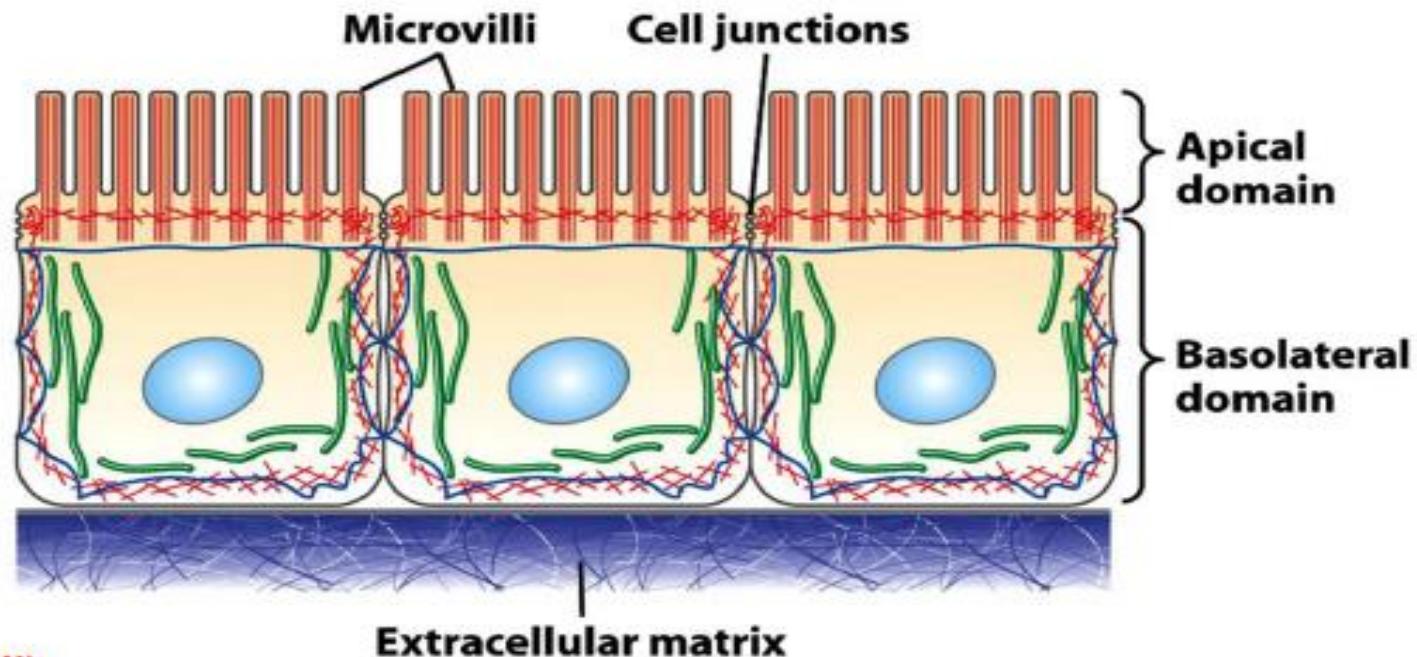
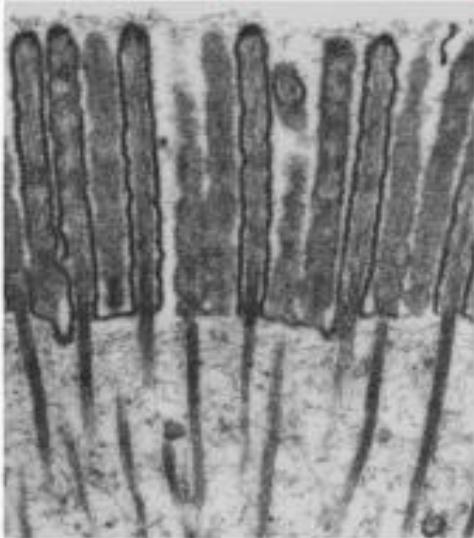
Actin: twisted, two-stranded (pearl-string like) structure

⇒ cell cortex, microvilli, stereocilia, adherens belt, filopodia

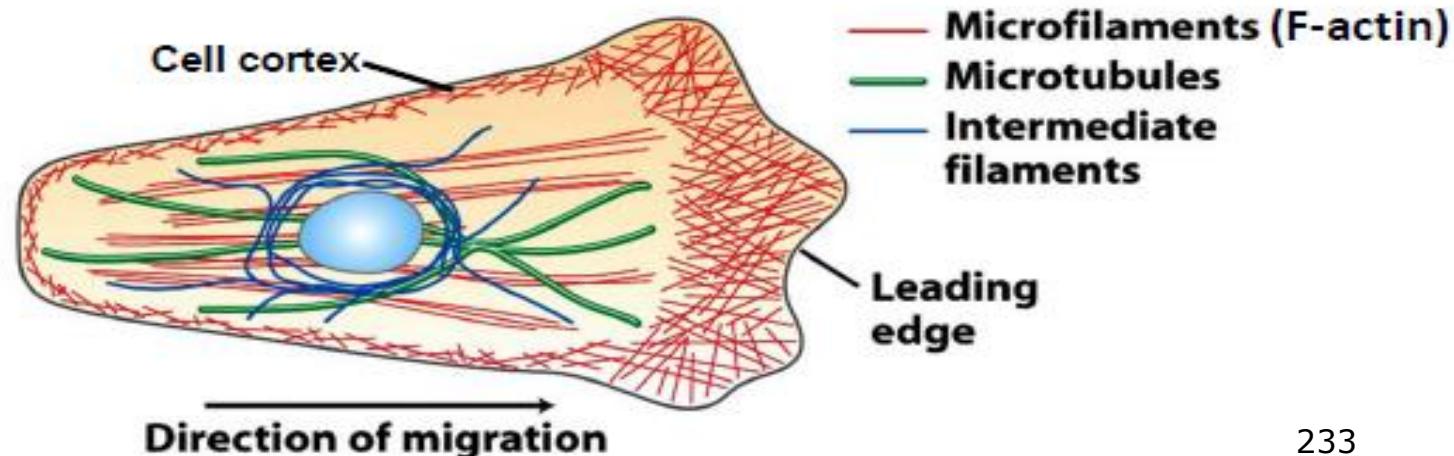
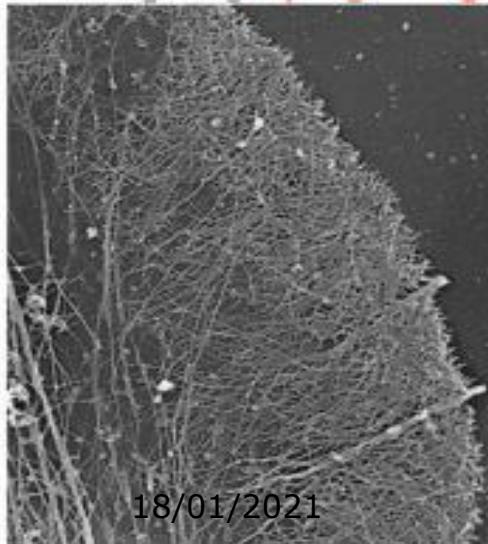


Cytoskeletal organization differs between cell types

Microvilli (epithelial cell)

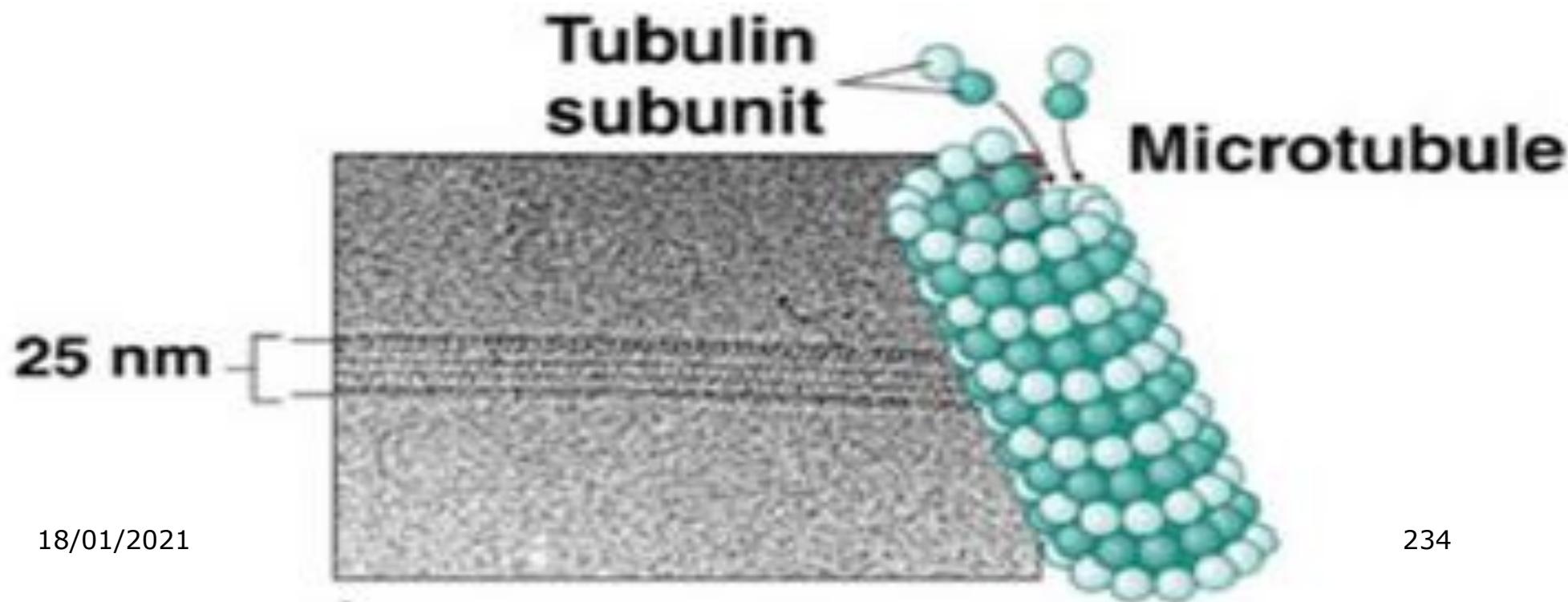


Leading edge (migrating cell)



Microtubules

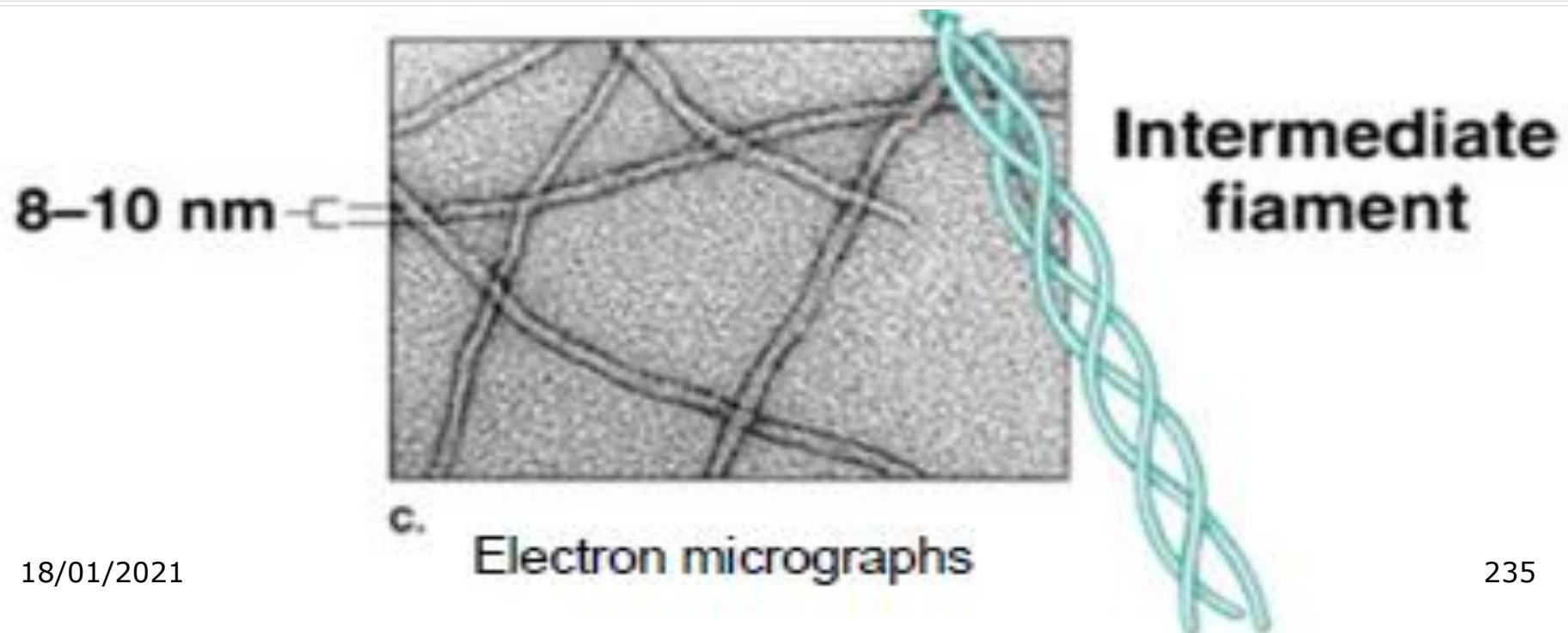
MT: hollow cylinder formed by proto-filaments made of tubulin-subunits
⇒ positioning of Golgi, ER, vesicles etc.;
cilia/flagella; chromosome separation



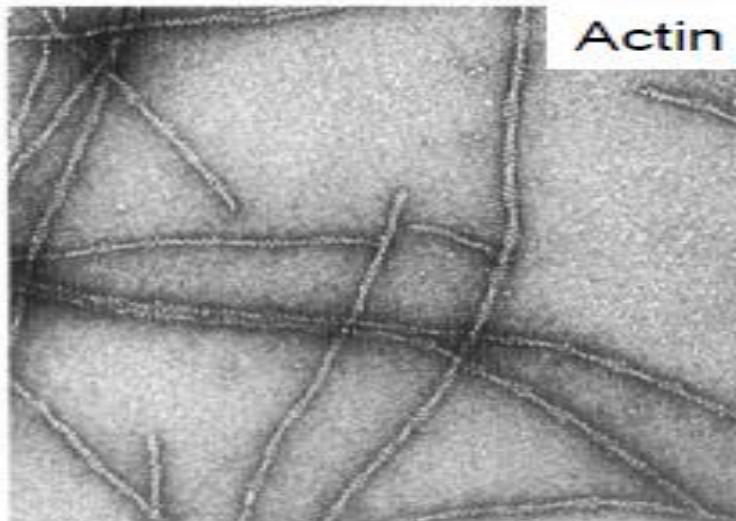
Intermediate filaments

IFs: rope-like structure

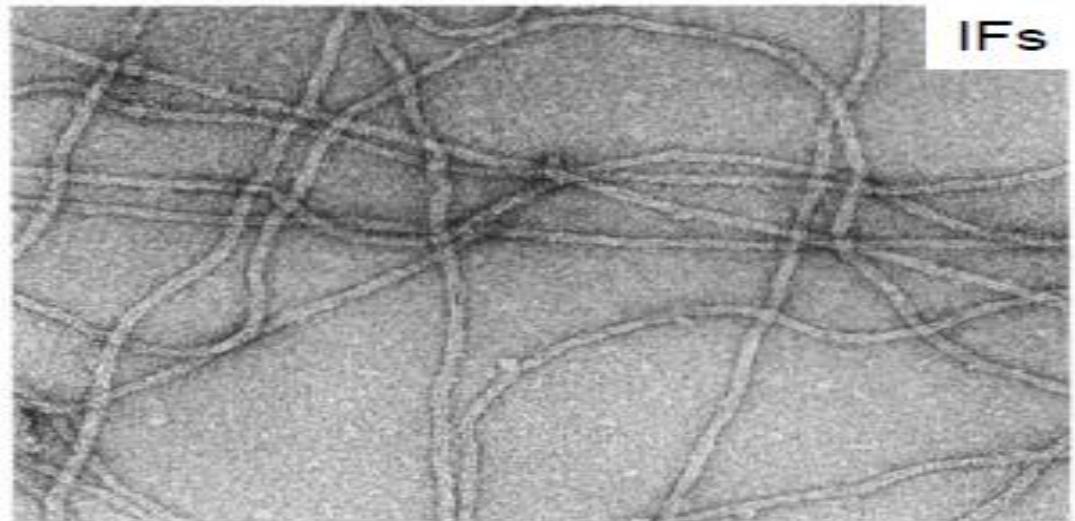
⇒ **lamins** (nucleus support), **keratin** (in stiff epithelial cells), **vimentin** (in soft mesenchymal cells), **desmin** (bundles myofibers in muscle cells)



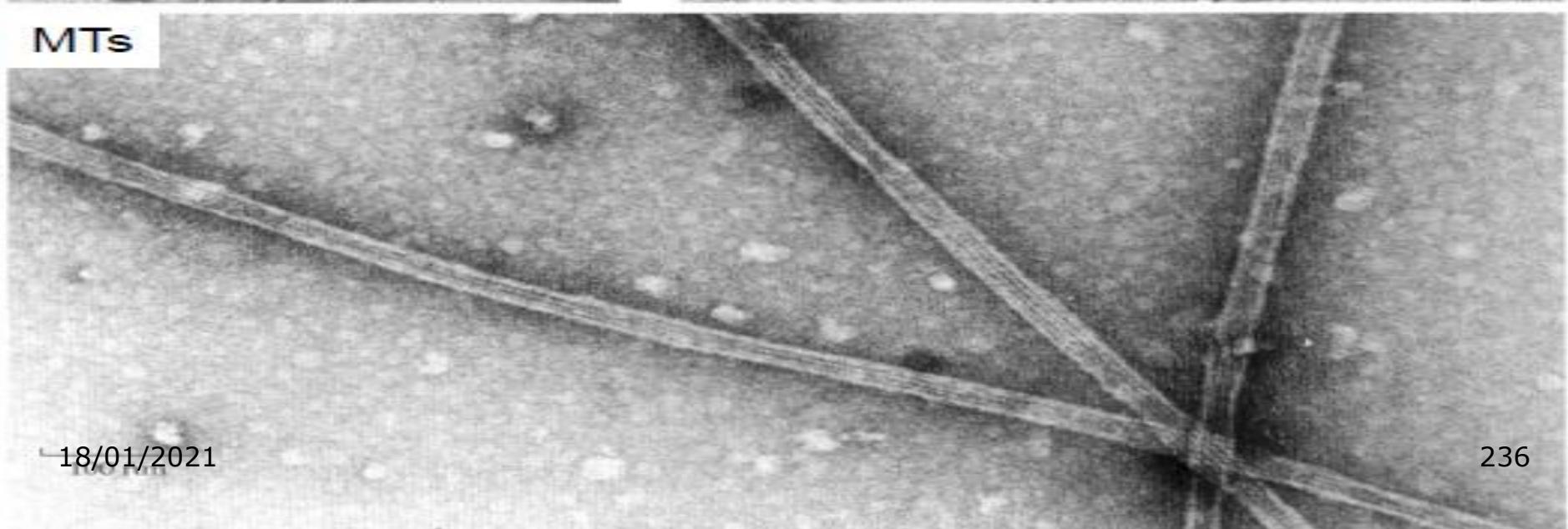
Compared!



Actin



IFs



MTs

Microtubules

- Microtubules are hollow tubes of variable length and about 25nm diameter.
- They are stiff but flexible.
- Microtubules carry cargoes along the length of nerve axons; in humans axons these can be more than a meter in length.
- Microtubules are assembled linearly from building blocks of tubulin molecules grouped into pairs called a **dimer**.
- **Dimers** are joined end-to-end by the process of polymerization to form a linear polymer called a **protofilament**.

Microtubules

- The **microtubule consist of thirteen (13)** protofilaments lying in parallel are formed into a circular tube with the duct running down the middle.
- For microtubule assembly to take place the concentration of tubulin molecules in solution must exceed a critical level.
- Each tubulin building block molecule is said to be 'polar' (*has polarity*) since it has a different molecular configuration at each end.
- One end is called the 'plus' end; the other the 'minus' end. The 'plus' end of one molecule can only link to the 'minus' end of another to form a linear polymer.

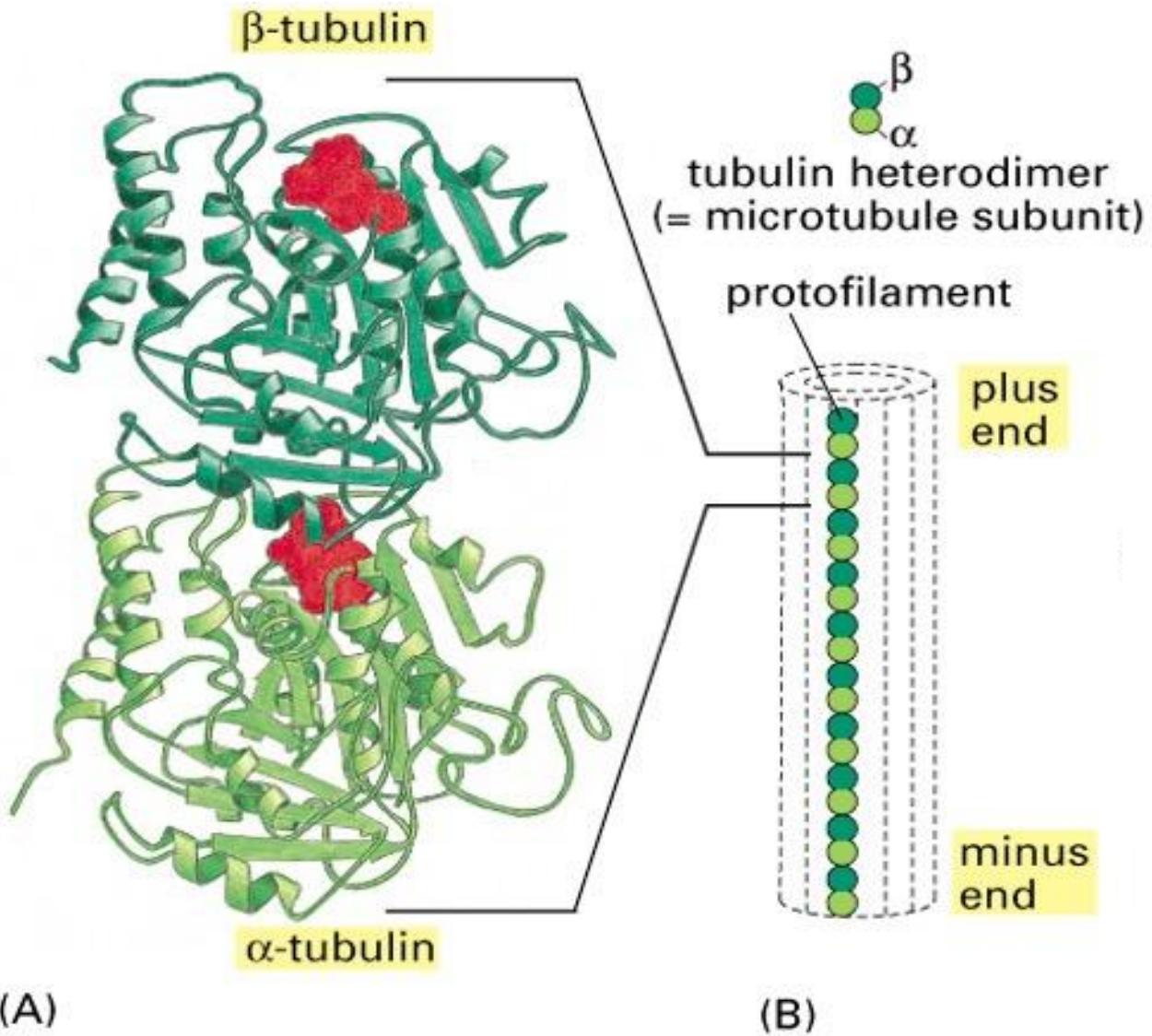


Figure 16–6. Molecular Biology of the Cell, 4th Edition.

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What is the fundamental structure of microtubules?

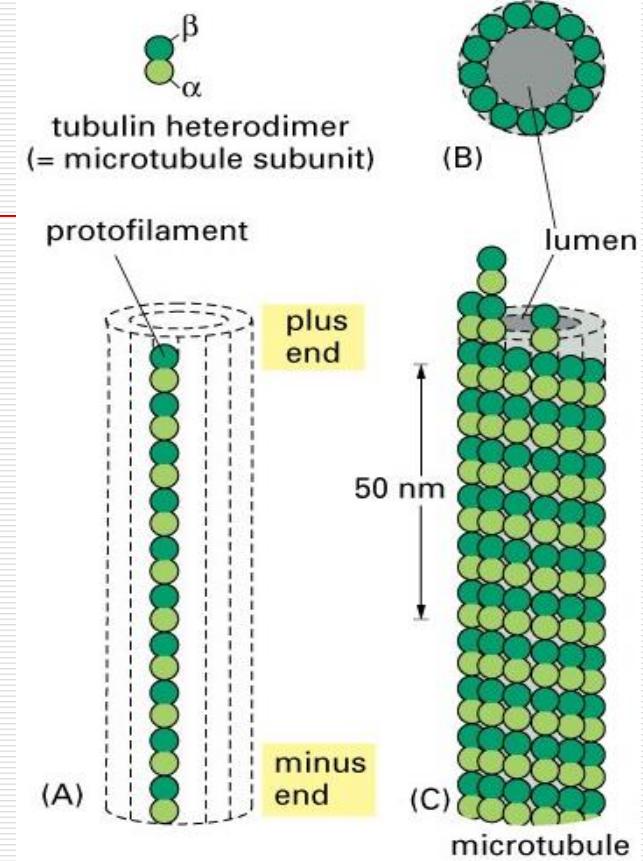
Alpha and beta tubulin subunits

13 member ring

Why do MTs have polarity?

•MT Organizing Center

Why are MTs said to display ‘Dynamic Instability’?



Cytosol

- 55% of cell volume
- 75-90% water with other components
 - large organic molecules (proteins, carbohydrates & lipids) suspended by electrical charges
 - small organic molecules (simple sugars) & ions dissolved

Cytosol Cont'd.

- inclusions (large aggregates of one material)
 - lipid droplets
 - glycogen granules
- Site of many important chemical reactions
 - production of ATP, synthesis of building blocks (amino Acids)

Plasma Membrane

- The plasma membrane is the membrane surrounding a cell that separates the cell from its external environment, consisting of a **phospholipid bilayer** and associated membrane lipids and proteins.
- Each organelle is also surrounded by a **Biomembrane** and each type of organelle contains a unique complement of proteins – some embedded in its membranes other in its **aqueous interior space** or **lumen**
- These proteins enable each organelle to carry out its unique cellular functions.

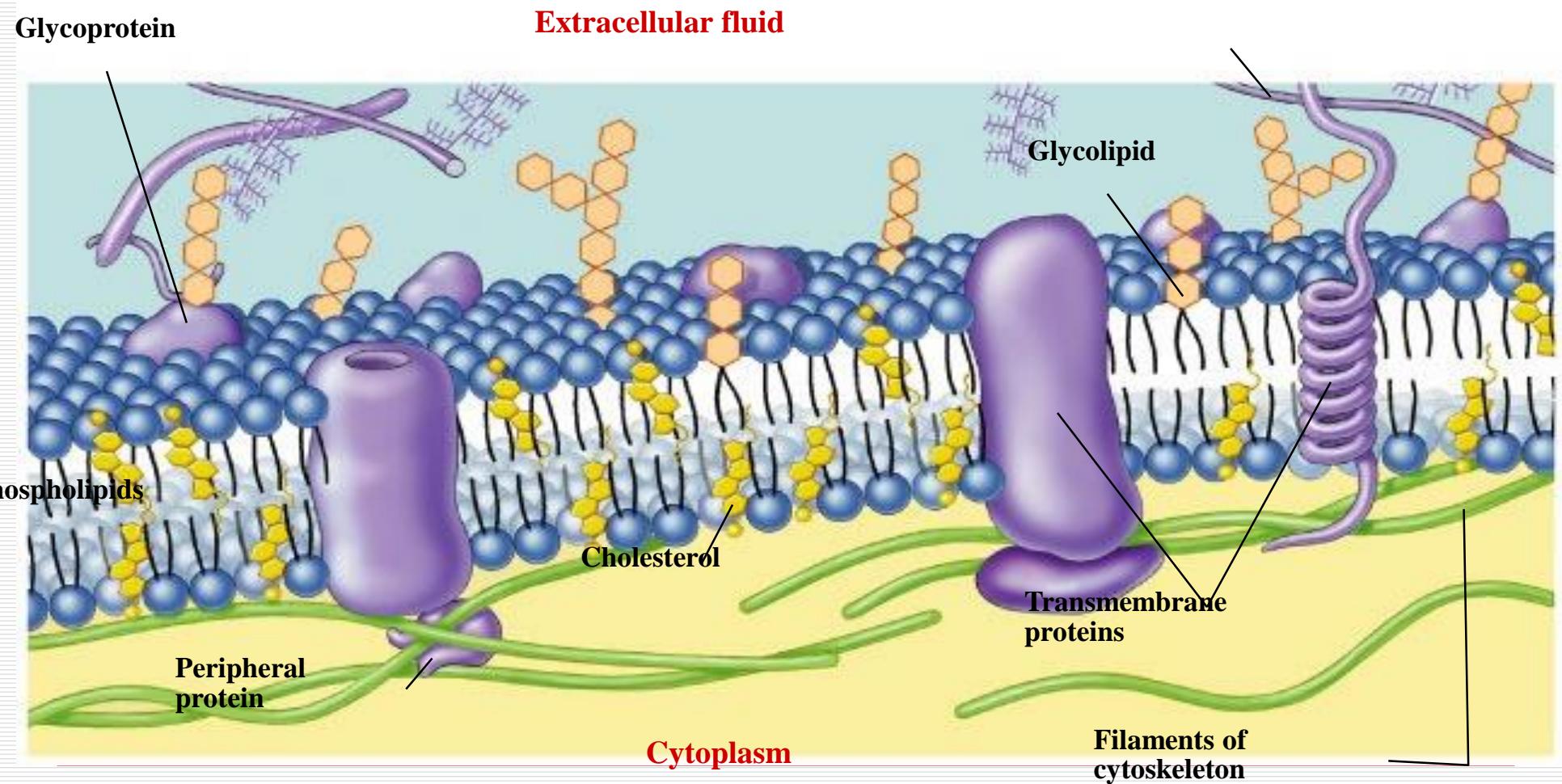
Biomembranes

- **The cytoplasm** is the part of the cell outside the largest organelle, the nucleus.
- **The Cytosol** is the aqueous part of the cytoplasm outside all of the organelles; it also contains its own distinctive proteins.
- All Biomembranes form closed structures, **separating** the lumen on the inside from the outside and are based on a similar bilayer structure
- They control the **movement of molecules** between the inside and outside of all cells and into and out of the organelles of eukaryotic cells.

Cellular membranes are fluid mosaics of lipids and proteins

- **Phospholipids** are the most abundant lipid in the plasma membrane
- Phospholipids are **amphipathic molecules**, containing **hydrophobic and hydrophilic regions**
- The **fluid mosaic** model states that a membrane is a fluid structure with a “mosaic” of various proteins embedded in it

Membrane is a collage of proteins & other molecules embedded in the fluid matrix of the lipid bilayer

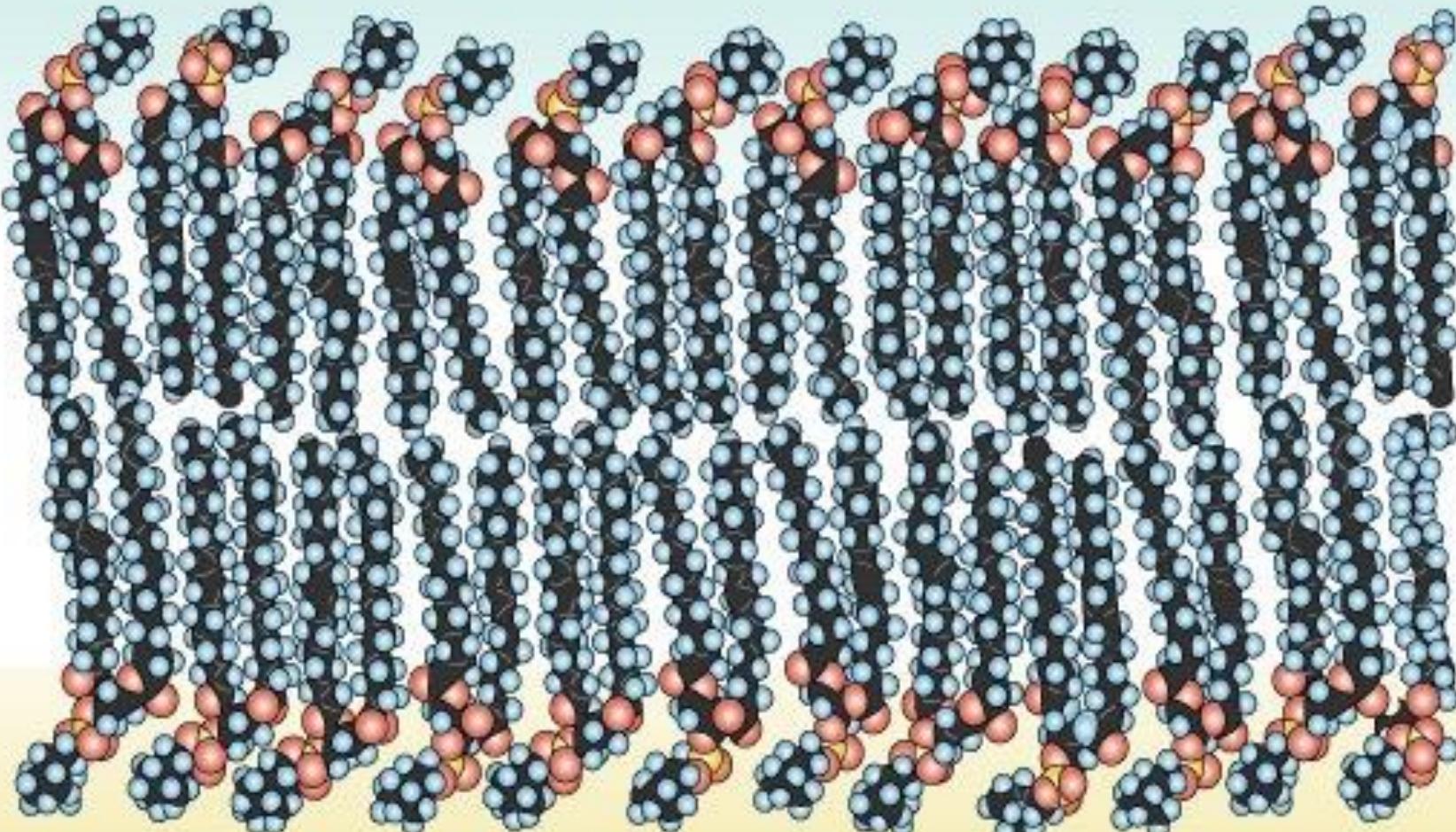


Phospholipid bilayer

polar
hydrophilic
heads

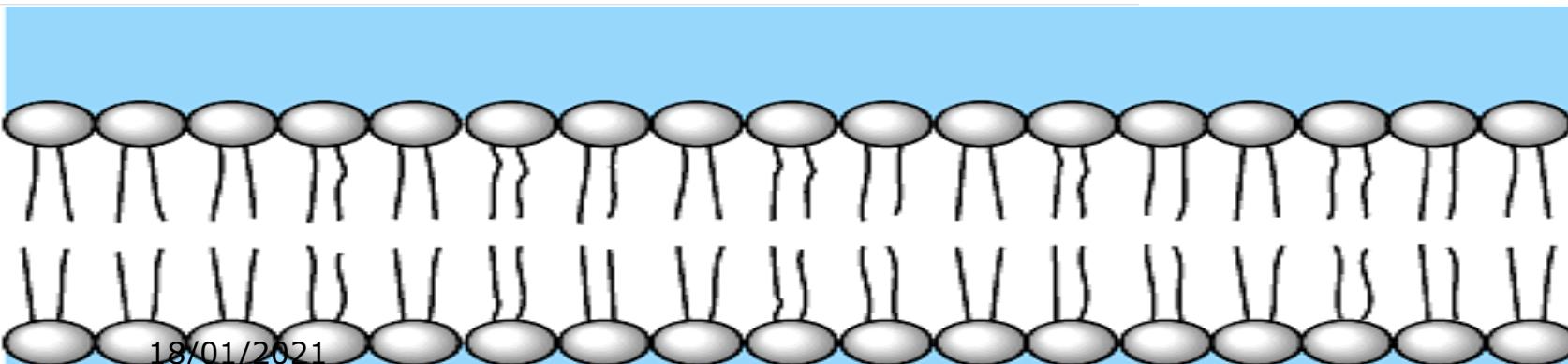
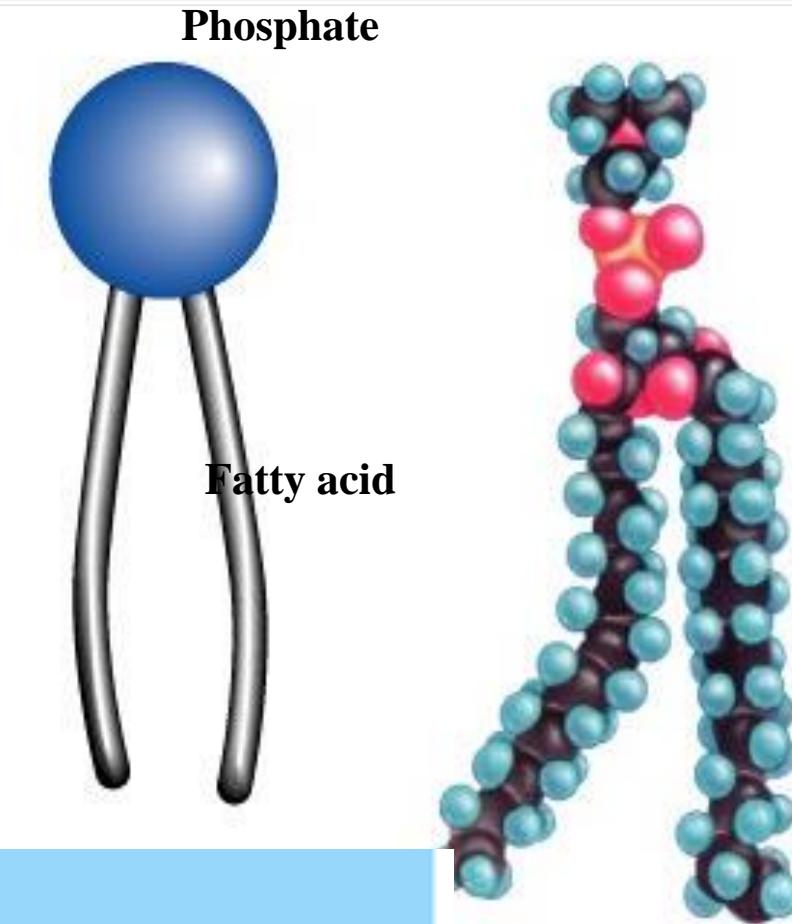
nonpolar
hydrophobic
tails

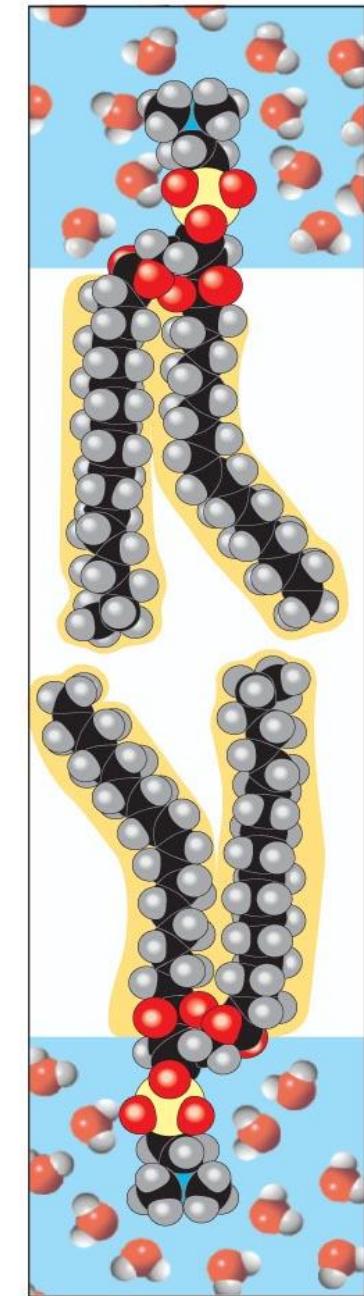
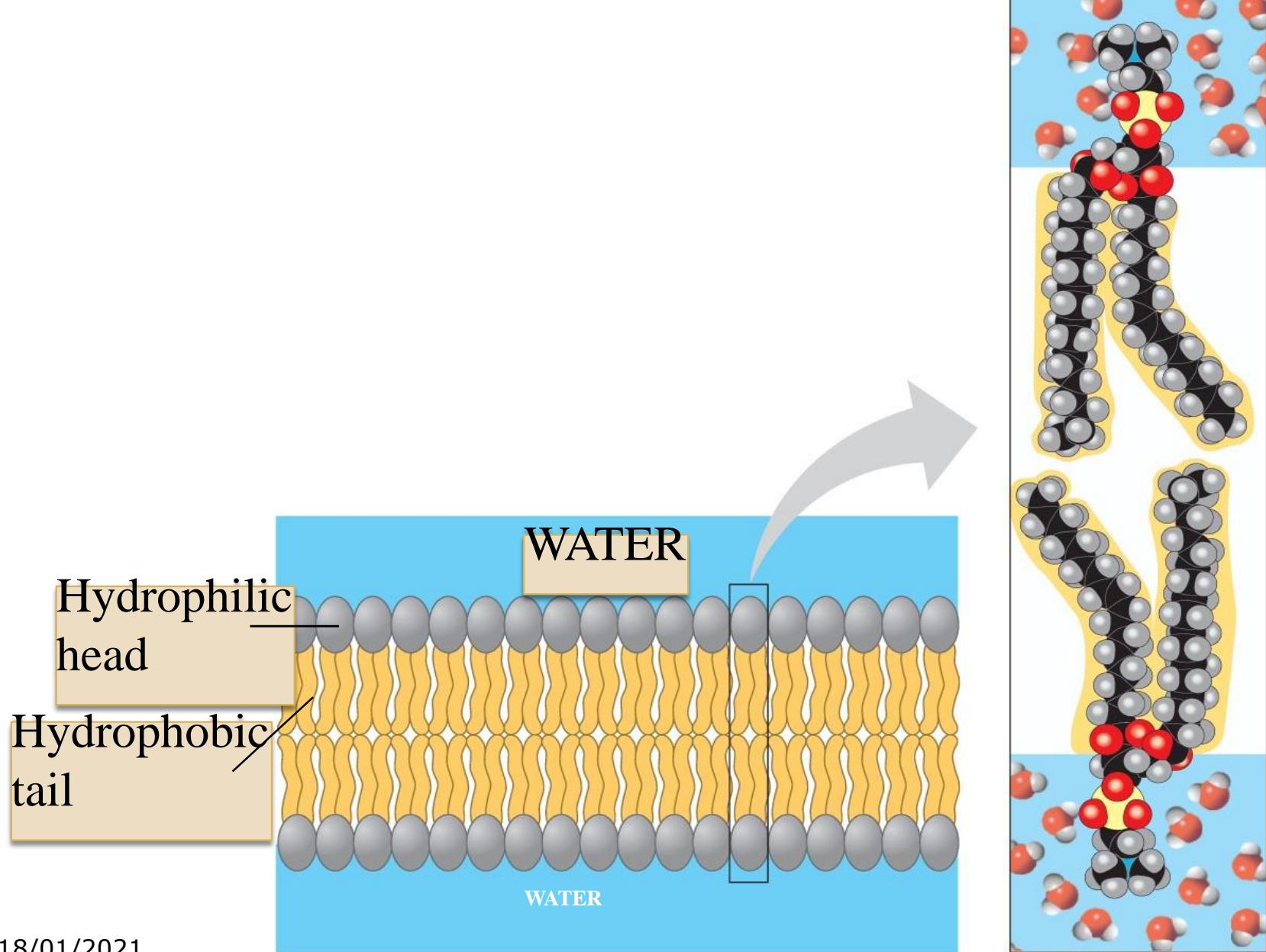
polar
hydrophilic
heads



Phospholipids

- Fatty acid tails
 - hydrophobic
- Phosphate group head
 - hydrophilic
- Arranged as a bilayer

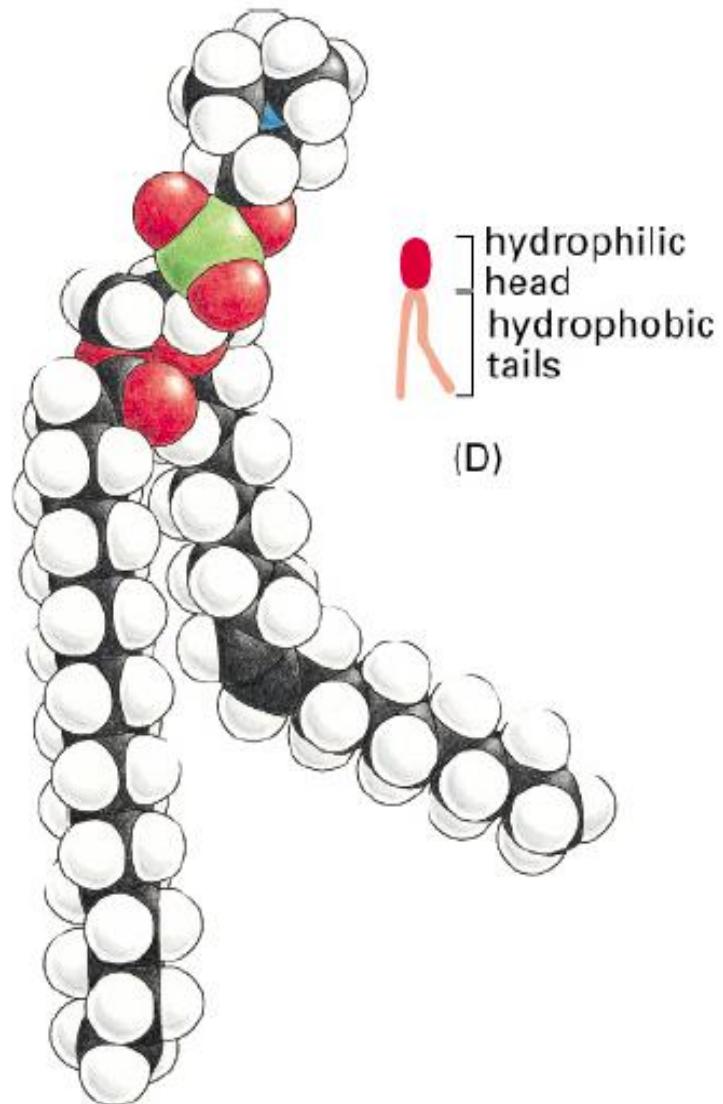
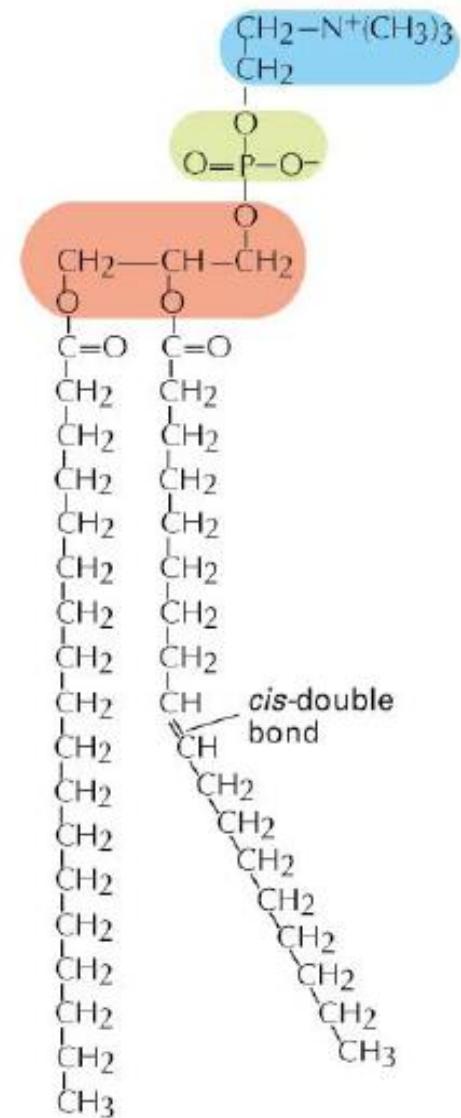
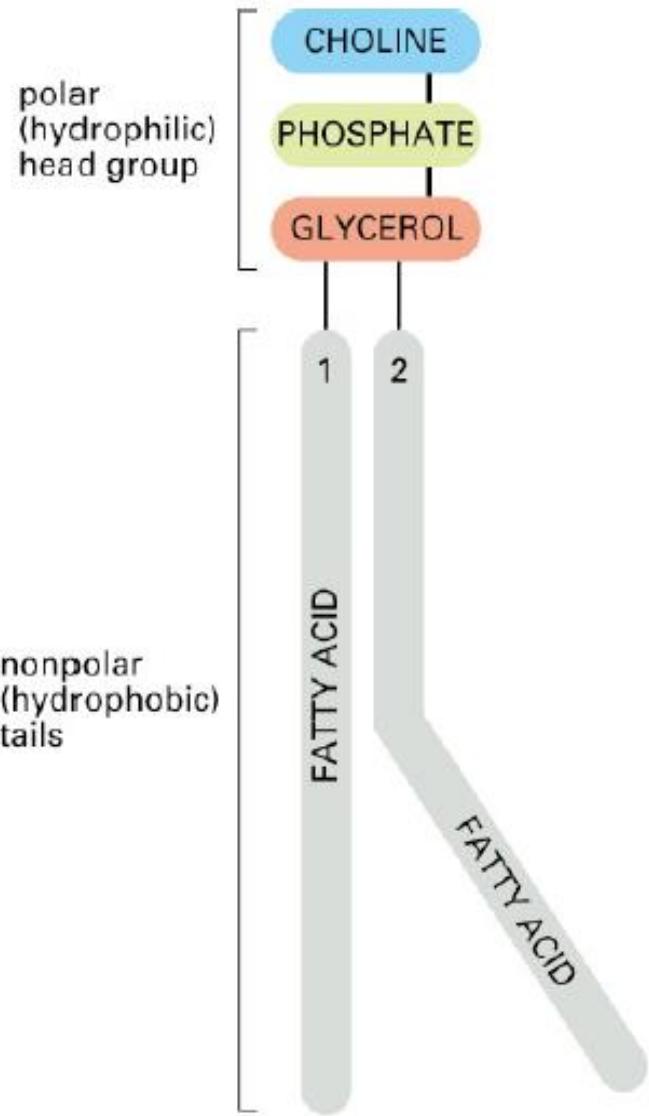


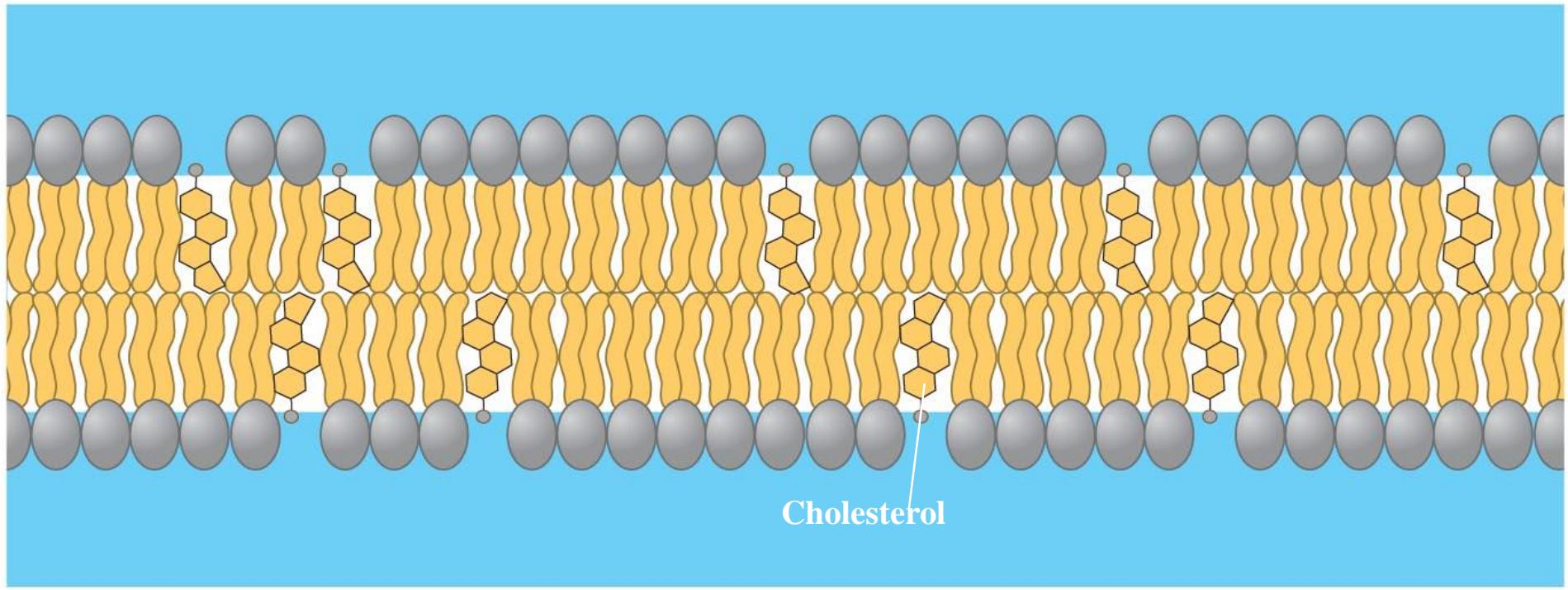


Phospholipids Are the Main Lipid Constituents of Most Biomembranes

- The most abundant **lipid** components in most membranes are **phospholipids**, which are **amphipathic** molecules (i.e., they have a **hydrophilic** and a **hydrophobic** part).
- In ***phosphoglycerides***, a principal class of **phospholipids**, **fatty acyl** side chains are ***esterified*** to two of the three hydroxyl groups in **glycerol**, and the third hydroxyl group is **esterified to phosphate**.

Phospholipid molecule





(c)

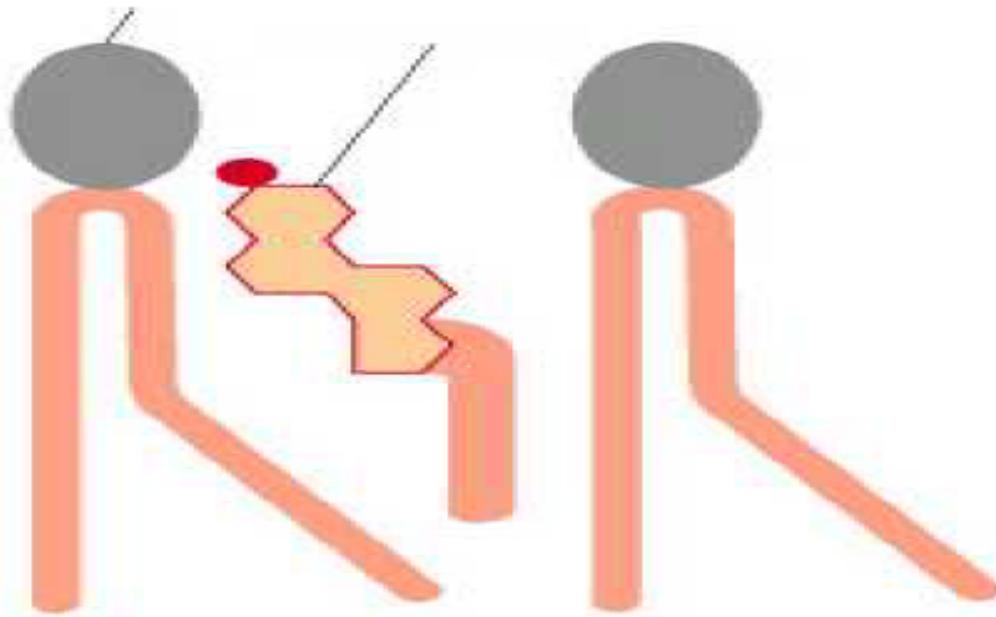
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Cholesterol → Membrane fluidity ↓

In animal cells, cholesterol used to modulate membrane fluidity - fills gaps between kinks of unsaturated tails

Used particularly in plasma membrane ⇒ closer packing ⇒ less fluidity/permeability

phospholipid cholesterol



18/01/2021

Adapted from ECB Fig 11-16

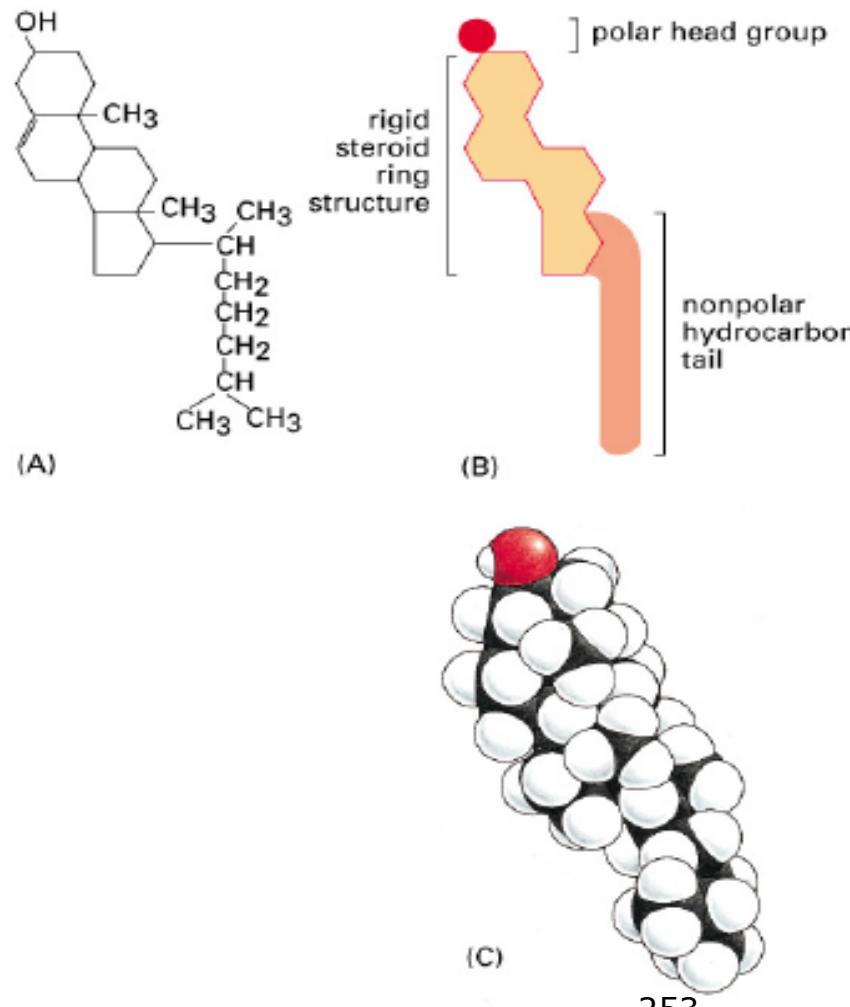
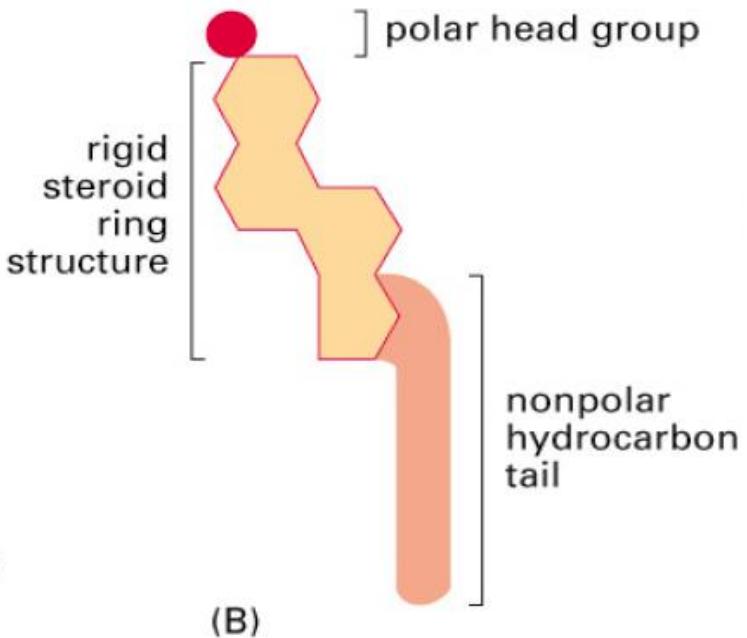
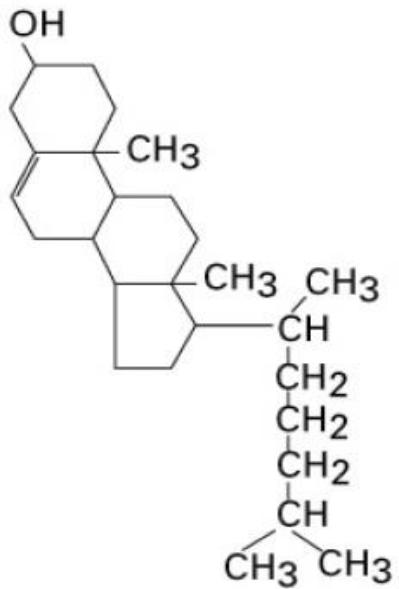
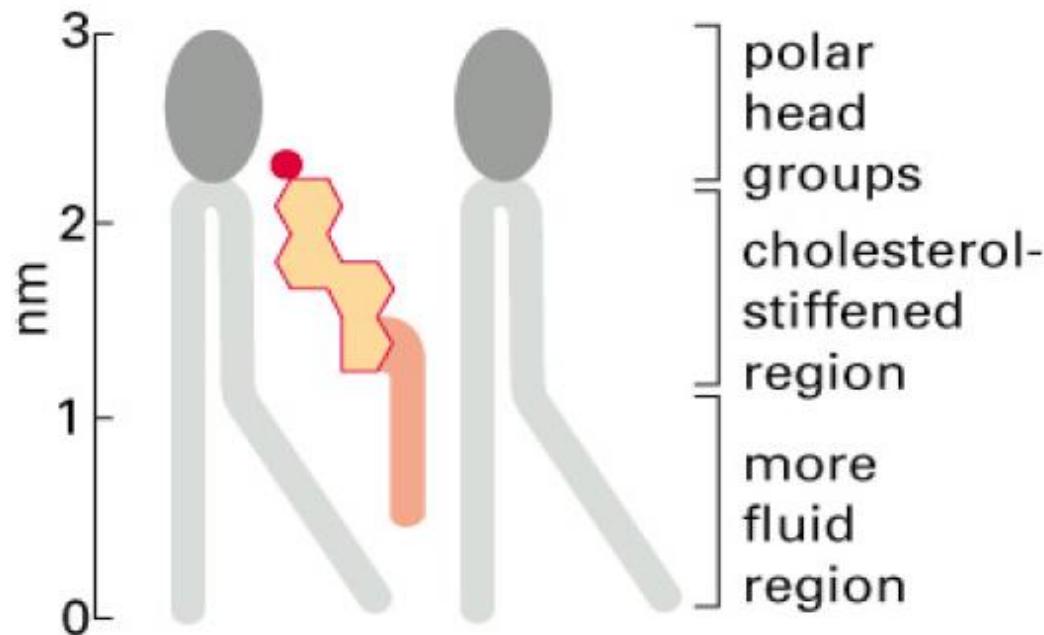


Figure 10–10. Molecular Biology of the Cell, 4th Edition.

Cholesterol

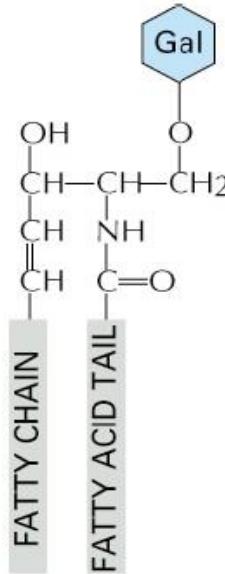


Cholesterol

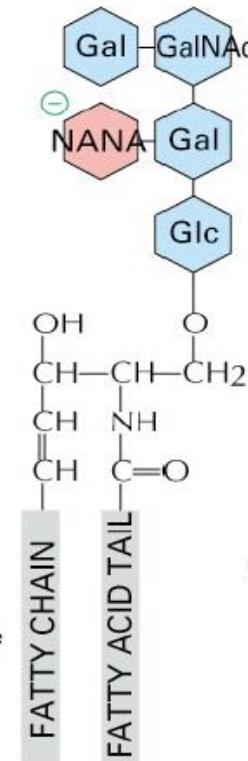


Cholesterol in the membrane decreases the permeability of the bilayer to small water-soluble molecules and at high concentration inhibits possible phase transition to crystallizing.

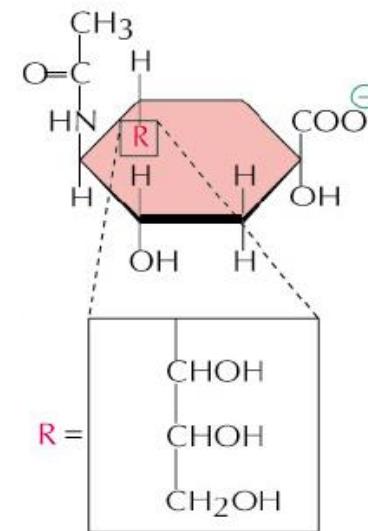
Glycolipids are found on the surface of all plasma membranes



(A) galactocerebroside



(B) GM1 ganglioside



(C) sialic acid (NANA)

Why does membrane need to be **fluid**?

Enables **rapid diffusion** of membrane proteins within plane of bilayer and permits interaction (important for cell signalling)

Facilitates **distribution** of membrane lipids and proteins from insertion site (following synthesis) to other regions of cell

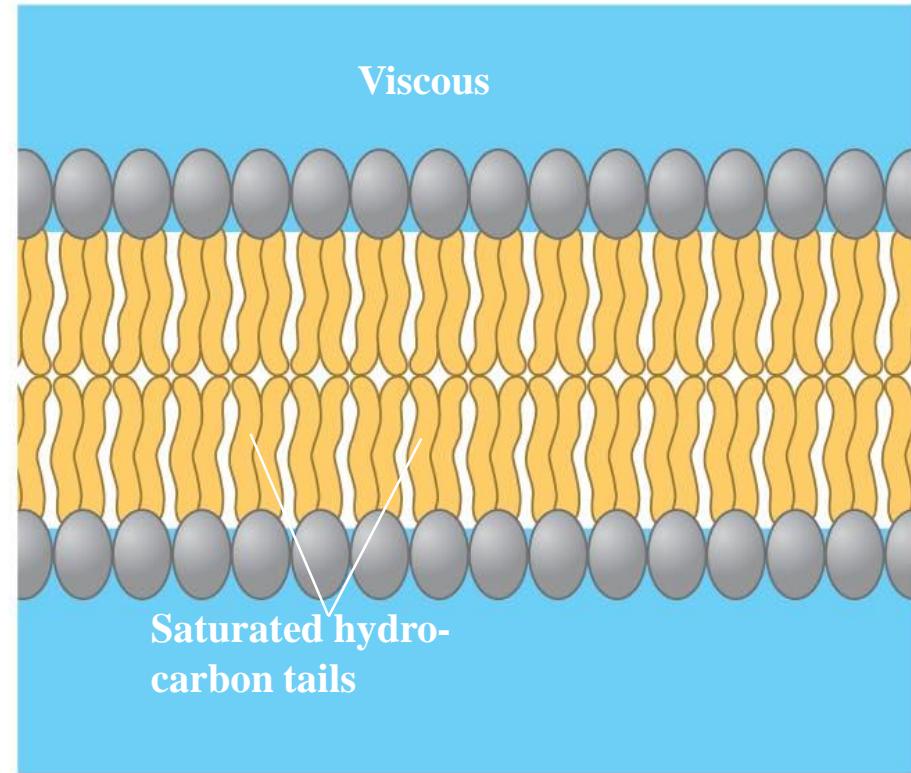
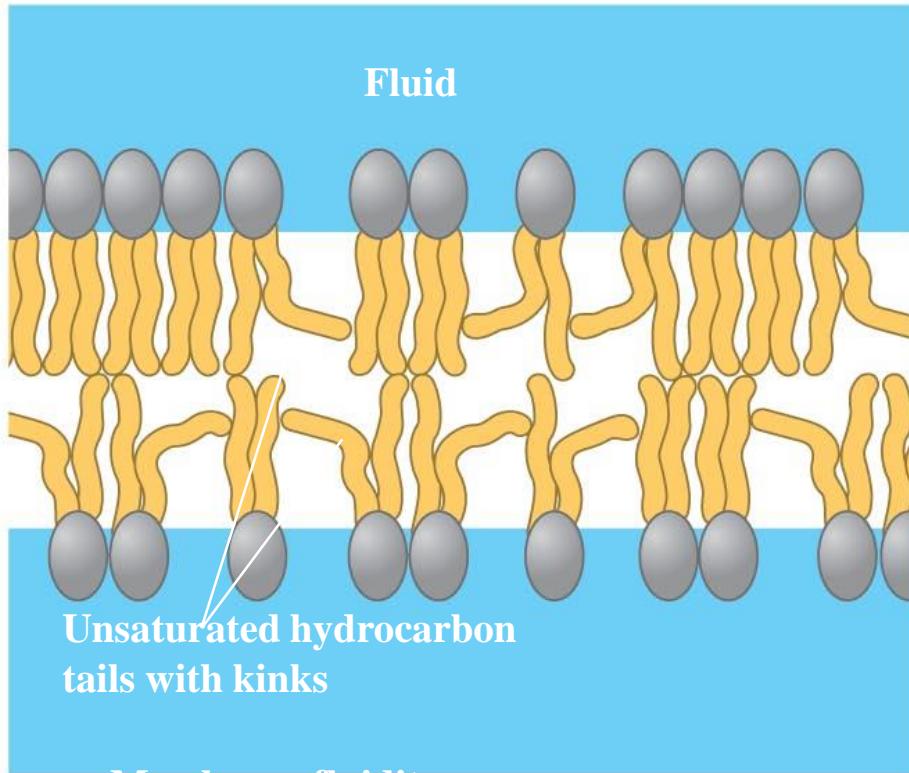
Allows membranes to **fuse and mix** molecules

Ensures even distribution of membrane molecules between daughter cells following **division**

The Fluidity of Membranes

- Phospholipids in the plasma membrane **can move** within the bilayer
- Most of the lipids, and some proteins, **drift laterally**
- Rarely does a molecule flip-flop transversely across the membrane

The Fluidity of Phospholipid



(b)

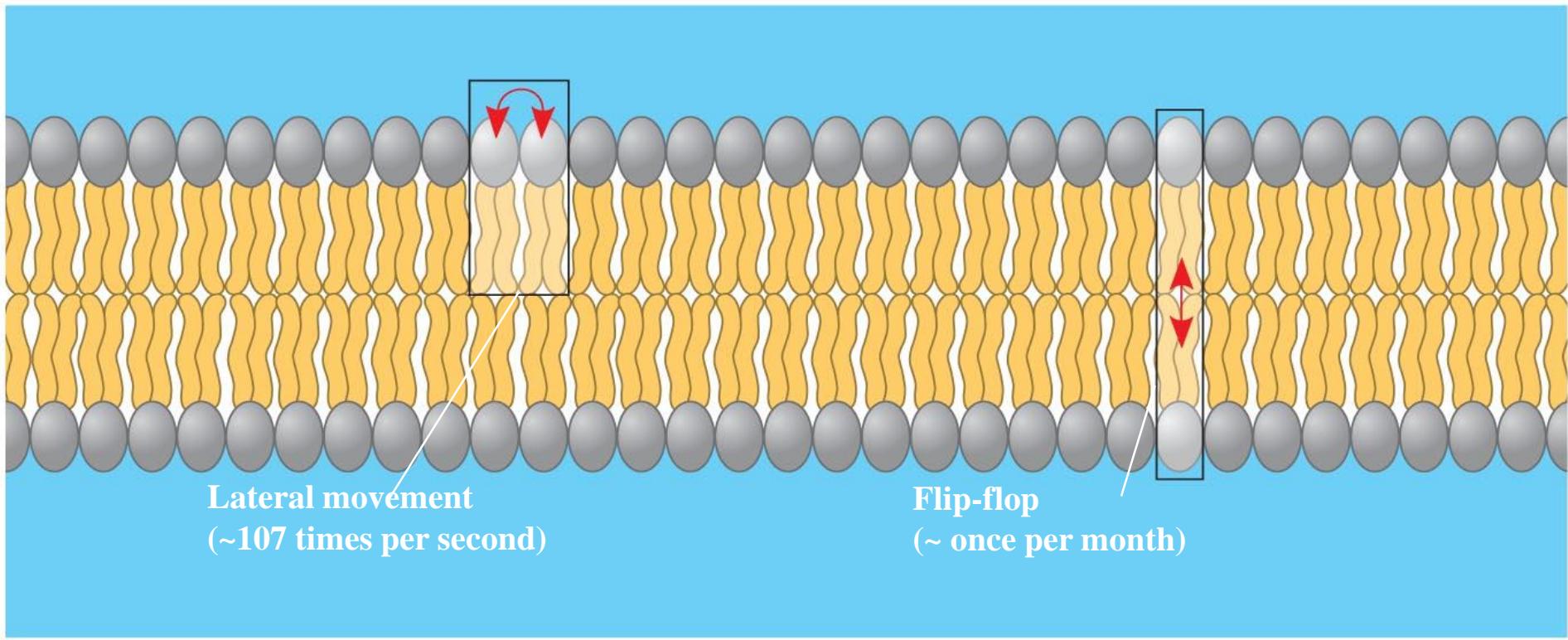
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The Fluidity of Membranes

- As temperatures cool, membranes switch from a fluid state to a solid state
- The temperature at which a membrane solidifies depends on the types of lipids
- Membranes rich in unsaturated fatty acids are more fluid than those rich in saturated fatty acids
- Membranes must be fluid to work properly; they are usually about as fluid as salad oil

The Fluidity of Membranes

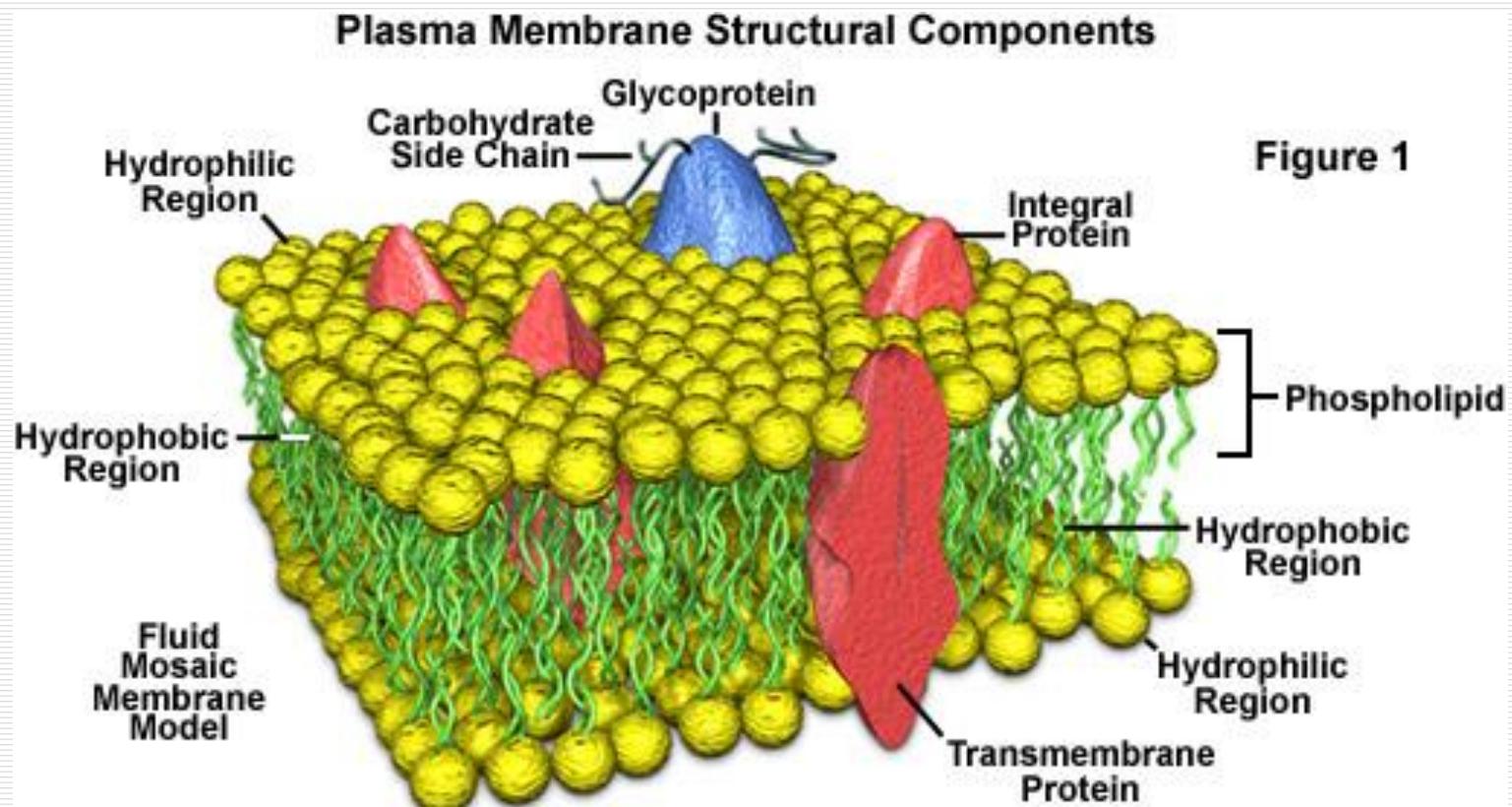
- The **steroid cholesterol** has different effects on **membrane fluidity** at different temperatures
- At warm temperatures (such as 37°C), cholesterol restrains movement of phospholipids
- At cool temperatures, it maintains fluidity by preventing tight packing



(a)

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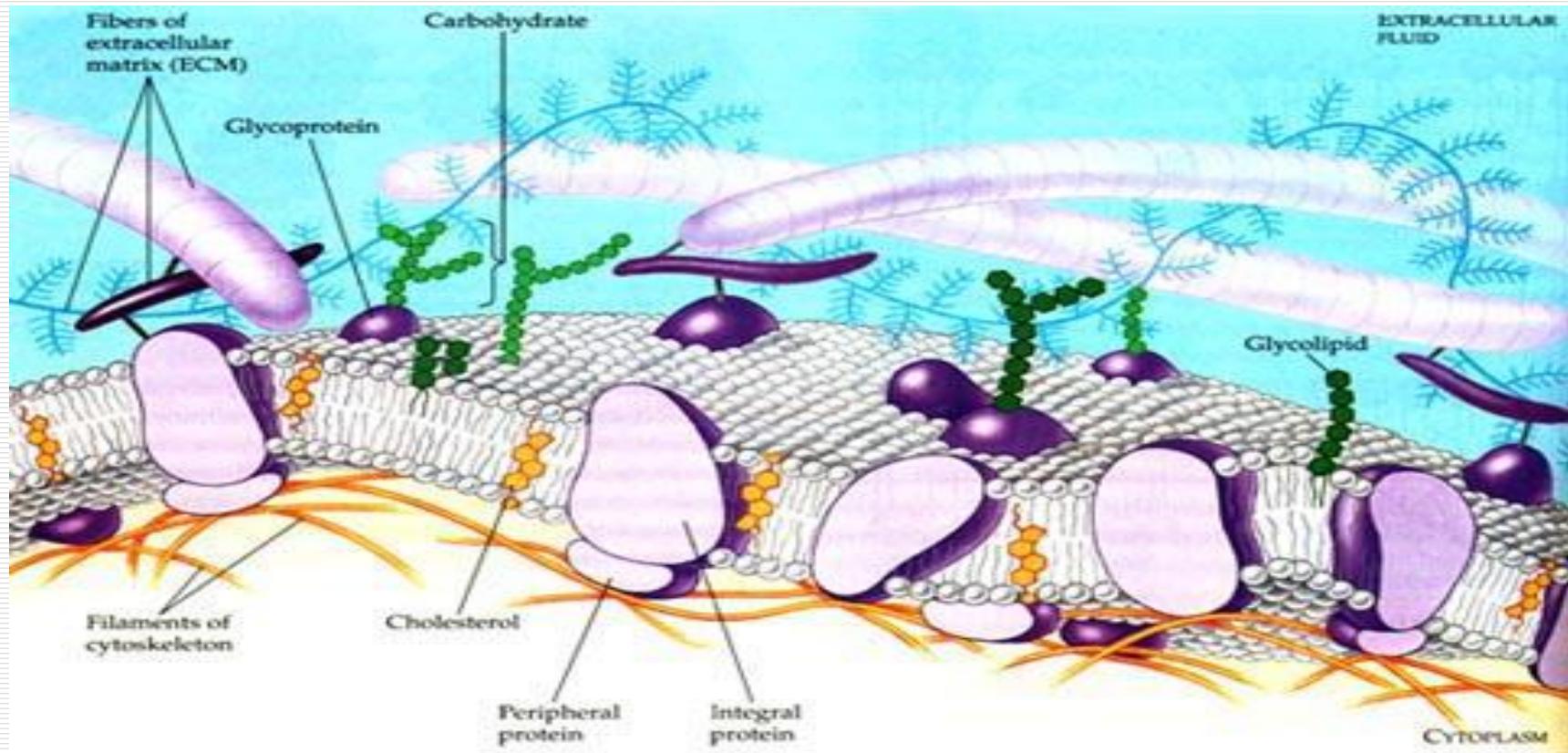
Plasma Membrane Structural Components



Plasma Membrane Components Cont'd.

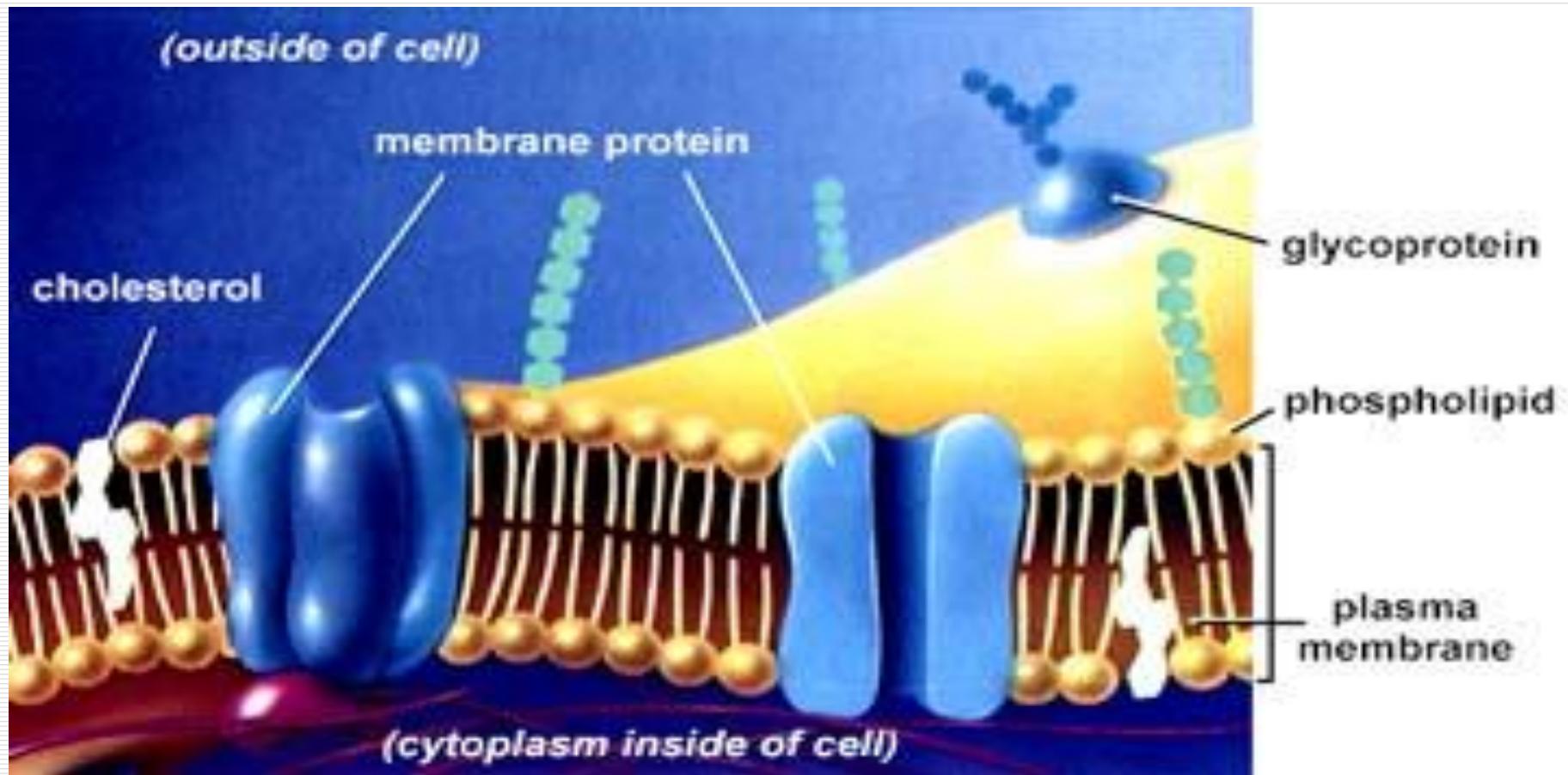
- Phospholipids
- Comprises 75% of lipids
- Phospholipid bilayer = 2 parallel layers of molecules
- Each molecule is amphipathic (has both a polar & non-polar region)
 - polar parts (heads) are hydrophilic and face on both surfaces of a watery environment
 - nonpolar parts (tails) are hydrophobic and line up next to each other in the interior

Membrane Structure Components Cont'd.

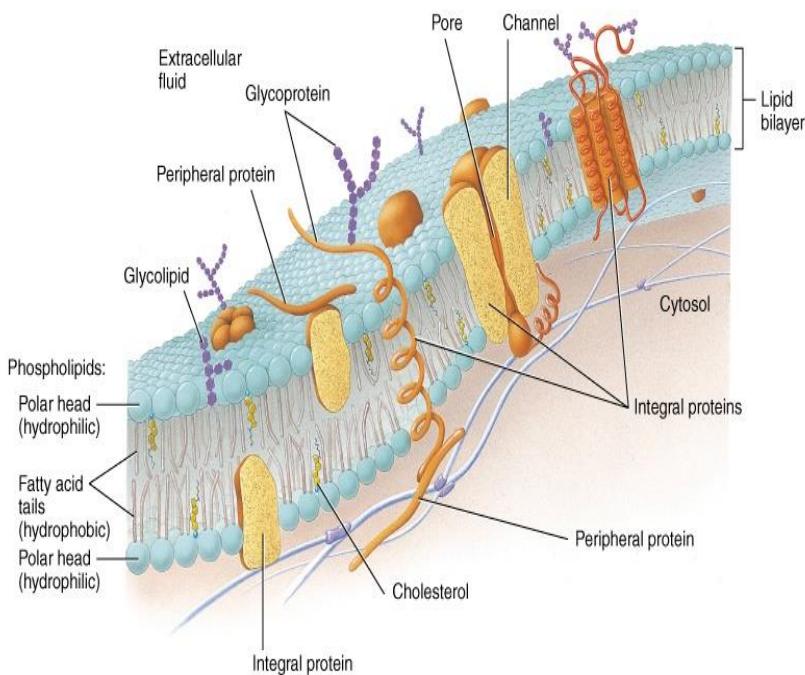


The detailed structure of an animal cell's plasma membrane, in cross section.

Membrane Structure Components Cont'd.



Glycolipids within the Cell Membrane



- Comprises 5% of the lipids of the cell membrane
- Carbohydrate groups form a polar head only on the side of the membrane facing the extracellular fluid

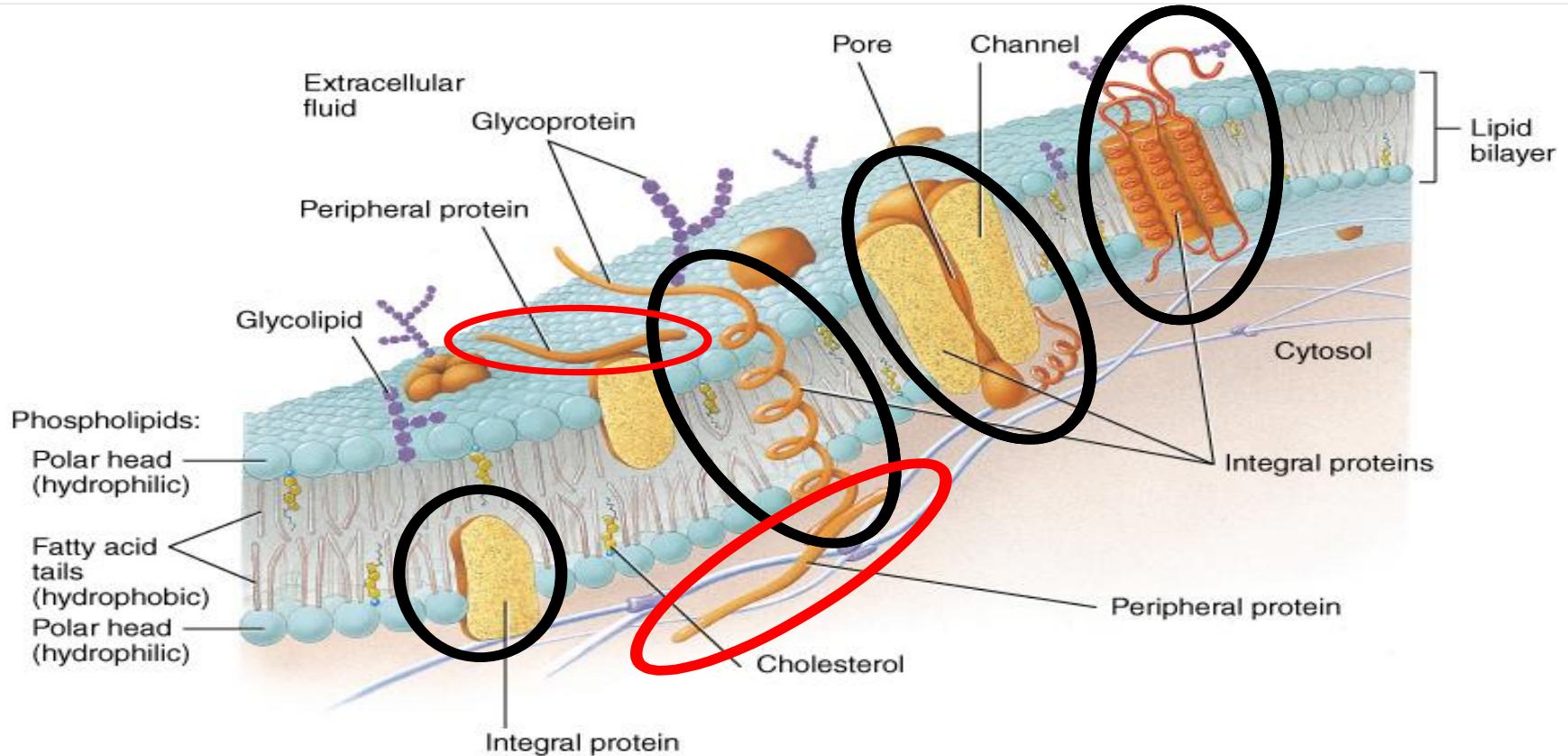
Types of Membrane Proteins

- Integral proteins
- Extend into or completely across cell membrane
 - -if it extends completely across = transmembrane protein
- All are amphipathic with hydrophobic portions
 - hiding among the phospholipid tails

Types of Membrane Proteins Cont'd.

- Glycoproteins have the sugar portion facing the extracellular fluid to form a glycocalyx
 - Gives cell “uniqueness”; creates a stickiness to hold it to other cells and also it can hold a fluid layer creating a slippery surface
- Peripheral proteins
 - Attached to either inner or outer surface of cell membrane and are easily removed from it

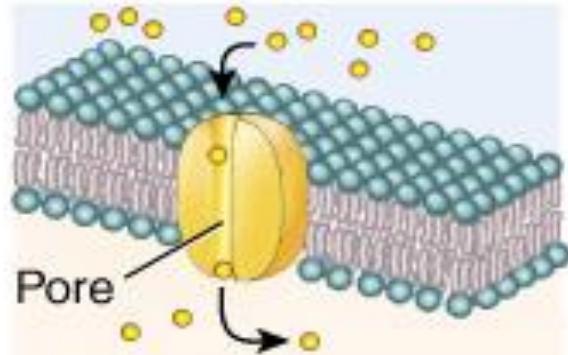
Membrane Proteins



Integral versus Peripheral Proteins

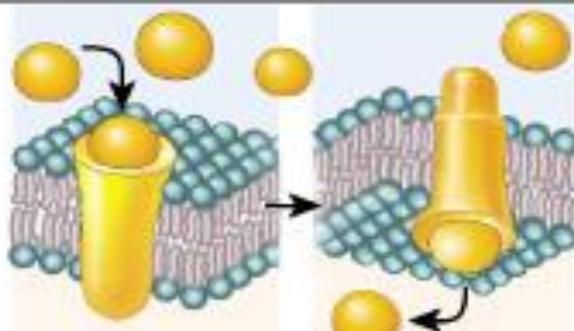
Functions of Membrane Proteins (1)

Extracellular fluid Plasma membrane Cytosol



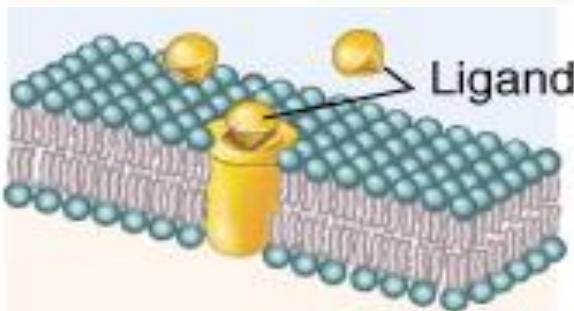
Channel

Allows specific substance (●) to move through water-filled pore. Most plasma membranes include specific channels for several common ions.



Transporter

Transports specific substances (●) across membrane by changing shape. For example, amino acids, needed to synthesize new proteins, enter body cells via transporters.



Receptor

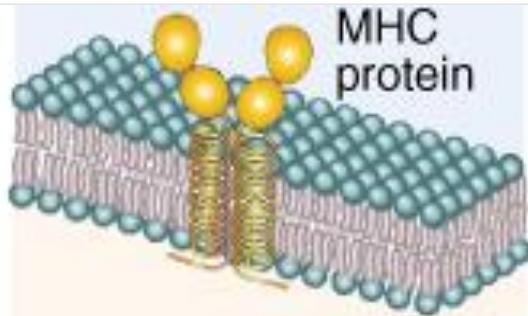
Recognizes specific ligand (●) and alters cell's function in some way. For example, antidiuretic hormone binds to receptors in the kidneys and changes the water permeability of certain plasma membranes.

Summary of functions of membrane proteins

(I)

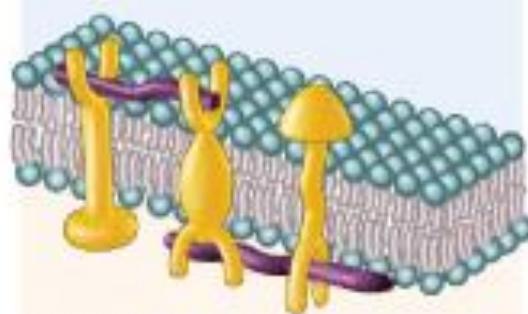
- **Formation of Channel**
 - -passageway to allow specific substance to pass through
- **Transporter Proteins**
 - -bind a specific substance, change their shape & move it across membrane
- **Receptor Proteins**
 - -cellular recognition site -- bind to substance

Functions of Membrane Proteins (2)



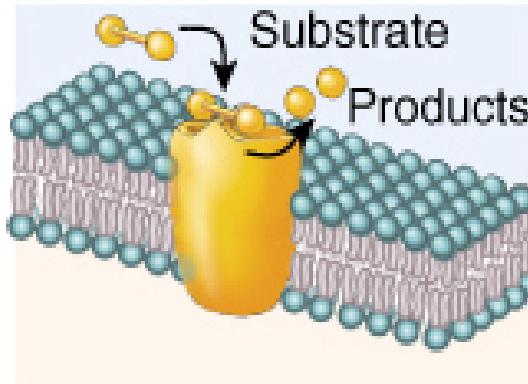
Cell Identity Marker

Distinguishes your cells from anyone else's (unless you are an identical twin). An important class of such markers are the major histocompatibility (MHC) proteins.



Linker

Anchors filaments inside and outside to the plasma membrane, providing structural stability and shape for the cell. May also participate in movement of the cell or link two cells together.



Enzyme

Catalyzes reaction inside or outside cell (depending on which direction the active site faces). For example, lactase protruding from epithelial cells lining your small intestine splits the disaccharide lactose in the milk you drink.

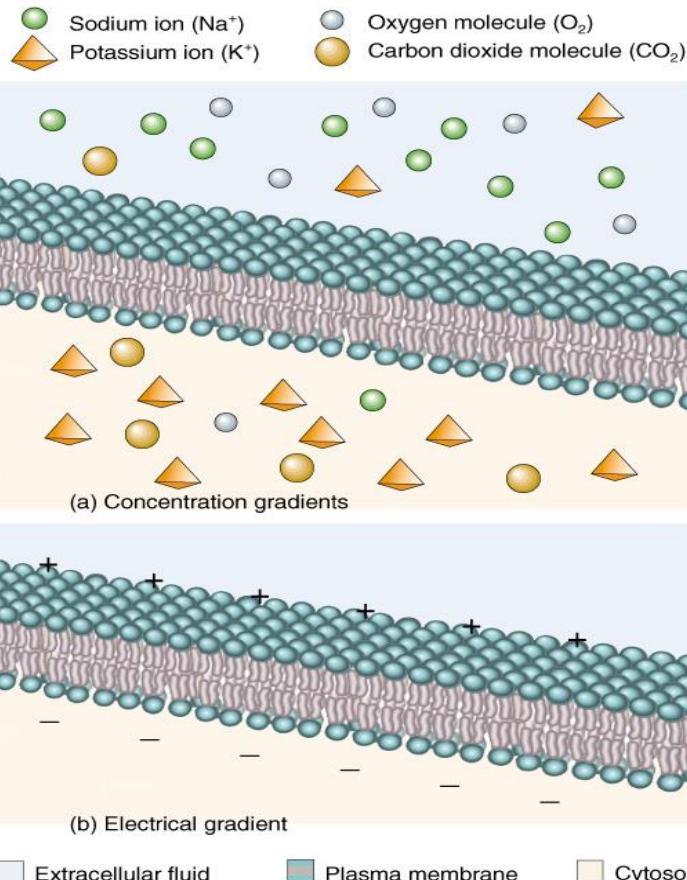
Selective Permeability of Membrane

- **Lipid bilayer**
 - permeable to non-polar, uncharged molecules -- oxygen, CO₂, steroids
 - permeable to water which flows through gaps that form in hydrophobic core of membrane as phospholipids move about
 - **Transmembrane proteins** act as specific channels
 - for small and medium polar & charged particles
 - **Macromolecules unable to pass through the membrane**
-

Gradients across the Plasma Membrane

- Membrane can maintain difference in concentration of a substance inside and outside of the membrane (**concentration gradient**)
 - more O₂ & Na⁺ outside of cell membrane
 - more CO₂ and K⁺ inside of cell membrane
- Membrane can maintain a difference in charged ions between inside & outside of membrane (**electrical gradient or membrane potential**)
- Thus, substances move down their concentration gradient and towards the oppositely charged area
 - E.g. ions have electrochemical gradients

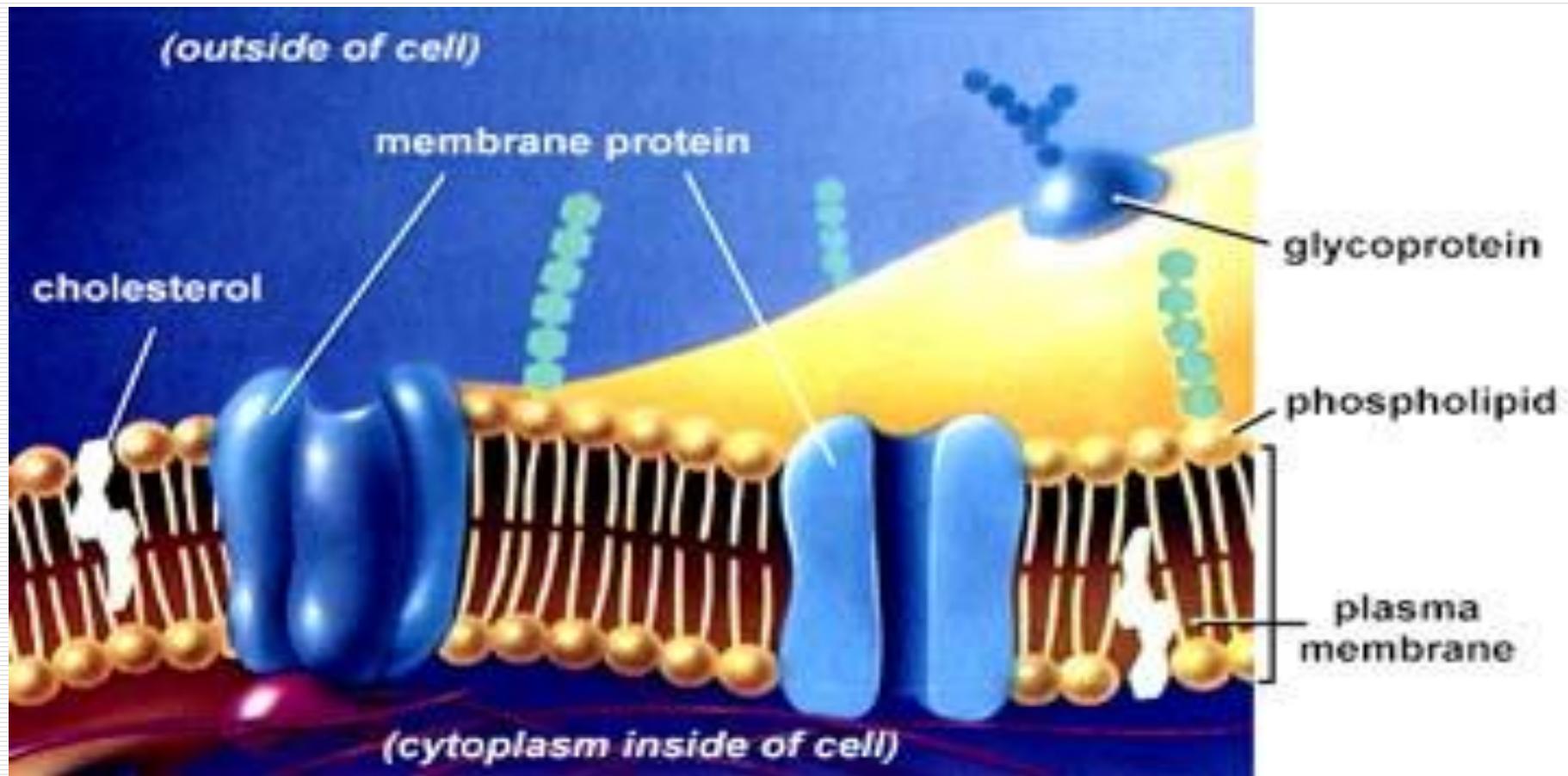
Gradients across membrane



- **Concentration gradient**

- **Electrical gradient**

MEMBRANE STRUCTURE CONT'D



Cell Fractionation

The functions of different parts of the cell may be investigated by disrupting the cell and separating it into its individual compartments. The process is called cell fractionation and consists of:

Disruption or homogenisation of cells, usually in a supportive iso-osmotic medium, to give a homogenate

Fractionation or separation of the various cell fragments and organelles in the homogenate for individual study and

Analysis of chemical activity of the separated fragments.

CELL HOMOGENISATION

Cell lysis is the first step in cell fractionation and protein purification and as such opens the door to a myriad of biological studies. Many techniques are available for the disruption of cells, including physical and detergent-based methods.

Cell Lysis Using Traditional (Non-detergent) Methods:

Several methods are commonly used to physically lyse cells, including mechanical disruption, liquid homogenization, high frequency sound waves, freeze/thaw cycles and manual grinding.

Mechanical Disruption

These methods have been reviewed extensively. Mechanical methods rely on the use of rotating blades to grind and disperse large amounts of complex tissue, such as liver or muscle. The Waring blender and the Polytron are commonly used for this purpose. Unlike the Waring blender, which is similar to a standard household blender, the Polytron draws tissue into a long shaft containing rotating blades. The shafts vary in size to accommodate a wide range of volumes, and can be used with samples as small as 1 ml.

Liquid Homogenization

Liquid-based homogenization is the most widely used cell disruption technique for small volumes and cultured cells. Cells are lysed by forcing the cell or tissue suspension through a narrow space, thereby shearing the cell membranes. Three different types of homogenizers are in common use. A Dounce homogenizer consists of a round glass pestle that is manually driven into a glass tube. A Potter-Elvehjem homogenizer consists of a manually or mechanically driven Teflon pestle shaped to fit a rounded or conical vessel.

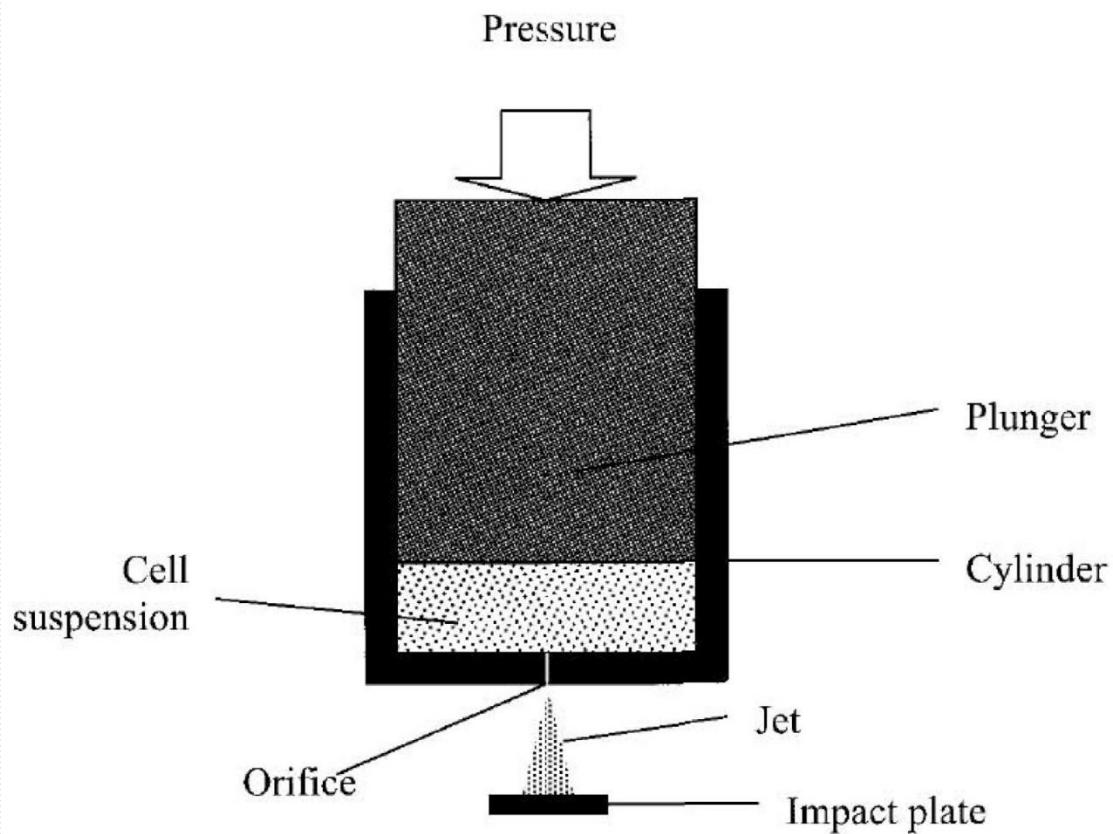
Potter Homogeniser



Liquid Homogenization Cont'd

The number of strokes and the speed at which the strokes are administered influences the effectiveness of Dounce and Potter homogenization methods. Both homogenizers can be obtained in a variety of sizes to accommodate a range of volumes. A French press consists of a piston that is used to apply high pressure to a sample volume of 40 to 250 ml, forcing it through a tiny hole in the press. Only two passes are required for efficient lysis due to the high pressures used with this process. The equipment is expensive, but the French press is often the method of choice for breaking bacterial cells mechanically.²⁸³

French Press



Sonication

Sonication is the third class of physical disruption commonly used to break open cells. The method uses pulsed, high frequency sound waves to agitate and lyse cells, bacteria, spores and finely diced tissue. The sound waves are delivered using an apparatus with a vibrating probe that is immersed in the liquid cell suspension. Mechanical energy from the probe initiates the formation of microscopic vapour bubbles that form momentarily and implode, causing shock waves to

Sonication Cont'd

radiate through a sample. To prevent excessive heating, ultrasonic treatment is applied in multiple short bursts to a sample immersed in an ice bath. Sonication is best suited for volumes <100 ml.

Sonicator



Freeze and thaw

The freeze and thaw method is commonly used to lyse bacterial and mammalian cells. The technique involves freezing a cell suspension in a dry ice/ethanol bath or freezer and then thawing the material at room temperature or 37°C. This method of lysis causes cells to swell and ultimately break as ice crystals form during the freezing process and then contract during thawing. Multiple cycles are necessary for efficient lysis, and the process can be

Freeze and thaw Cont'd

quite lengthy. However, freeze and thaw has been shown to effectively release recombinant proteins located in the cytoplasm of bacteria and is recommended for the lysis of mammalian cells in some protocols.

Mortar and Pestle

Manual grinding is the most common method used to disrupt plant cells. Tissue is frozen in liquid nitrogen and then crushed using a mortar and pestle. Because of the tensile strength of the cellulose and other polysaccharides comprising the cell wall, this method is the fastest and most efficient way to access plant proteins and DNA.

Additives/Facilitators

Cells can be treated with various agents to aid the disruption process. Lysis can be promoted by suspending cells in a hypotonic buffer, which cause them to swell and burst more readily under physical shearing. 200 µg/ml can be used to digest the polysaccharide component of yeast and bacterial cell walls. Alternatively, processing can be expedited by treating cells with glass beads in order to facilitate the crushing of cell walls. This treatment is commonly used with yeast cells.

Additives/Facilitators Cont'd

Viscosity of a sample typically increases during lysis due to the release of nucleic acid material. DNase can be added to samples (25-50 µg/ml) along with RNase (50 µg/ml) to reduce this problem.

Nuclease treatment is not required for sonicated material since sonication shears chromosomes. Finally, proteolysis can be a problem whenever cells are manipulated; therefore, protease inhibitors should be added to all samples undergoing lysis.

Method of cell lysis	disadvantages
freeze-thaw	very slow
chemical lysis	can cause changes in protein structure, difficulties in purification, expensive detergents
enzymatic lysis	often not reproducible, enzyme stability, long incubation time, necessity of removing the lysis enzymes, expensive scale-up, often combination with other method necessary
cell bomb	only applicable to specific cell types
high pressure homogenizer (e.g. french press)	expensive equipment, high maintenance due prone of orifices' clogging
centrifugation	only for very weak cell walls
ball mill / bead mill	uneven processing = incomplete lysis, protein denaturation, low efficiency whilst relatively high energy consumption, complex separation of milling medium and product, time-consuming cleaning

Fractionation or Centrifugation

Centrifugation is the process of isolating components of a cell. There are two common centrifugation techniques for separating bacteria components. The two methods are differential centrifugation and buoyant density centrifugation.

Fractionation or Centrifugation Cont'd

Once the homogenate has been prepared it is then necessary to fractionate or separate the various intracellular components from each other. The most widely used method for doing this is centrifugation. When a centrifugal force is applied to a suspension of cellular particles the rate of sedimentation of any given particle will depend on its size, density and shape.

Differential Centrifugation

Differential centrifugation is the process where a homogenate undergoes repeated centrifugations at increasing centrifugal force. Centrifugation is the use of increased gravity to quicken the sedimentation of substances to the bottom. The tool used here is the centrifuge that spins at various speeds.

Differential Centrifugation Cont'd

The centrifuge separates the cell's parts into pellets and supernatant. The pellets are the large cell structures that are settled at the test tube's bottom. The supernatant are smaller parts of the cell suspending in liquid, the supernatant is decanted and undergoes another centrifugation. The process is repeated at increased speed with each trial to collect successively smaller parts of a cell in pellets.

Differential Centrifugation Cont'd

Separation is achieved primarily based on the size of the particles in differential centrifugation. This type of separation is commonly used in simple pelleting and in obtaining partially-pure preparation of subcellular organelles and macromolecules.

Differential centrifugation Cont'd.

For the study of subcellular organelles, tissues or cells are first disrupted to release their internal contents. This crude disrupted cell mixture is referred to as a homogenate. During centrifugation of a cell homogenate, larger particles sediment faster than smaller ones and this provides the basis for obtaining crude organelle fractions by differential centrifugation.

Differential centrifugation Cont'd.

A cell homogenate can be centrifuged at a series of progressively higher g-forces and times to generate pellets of partially-purified organelles.

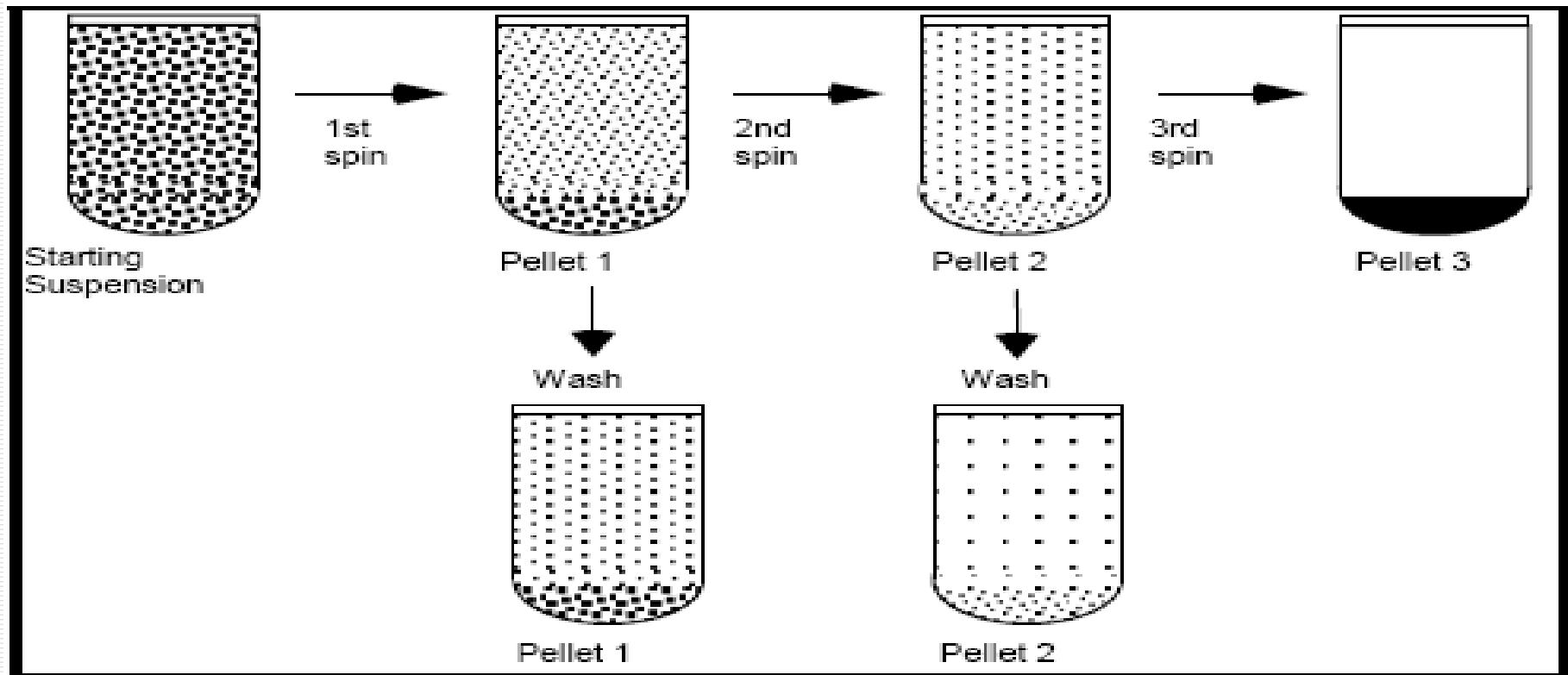
When a cell homogenate is centrifuged at $1000 \times g$ for 10 minutes, unbroken cells and heavy nuclei pellets move to the bottom of the tube. The supernatant can be further centrifuged at $10,000 \times g$ for 20 minutes to pellet subcellular organelles of intermediate velocities such as mitochondria, lysosomes, and microbodies.

Differential centrifugation Cont'd.

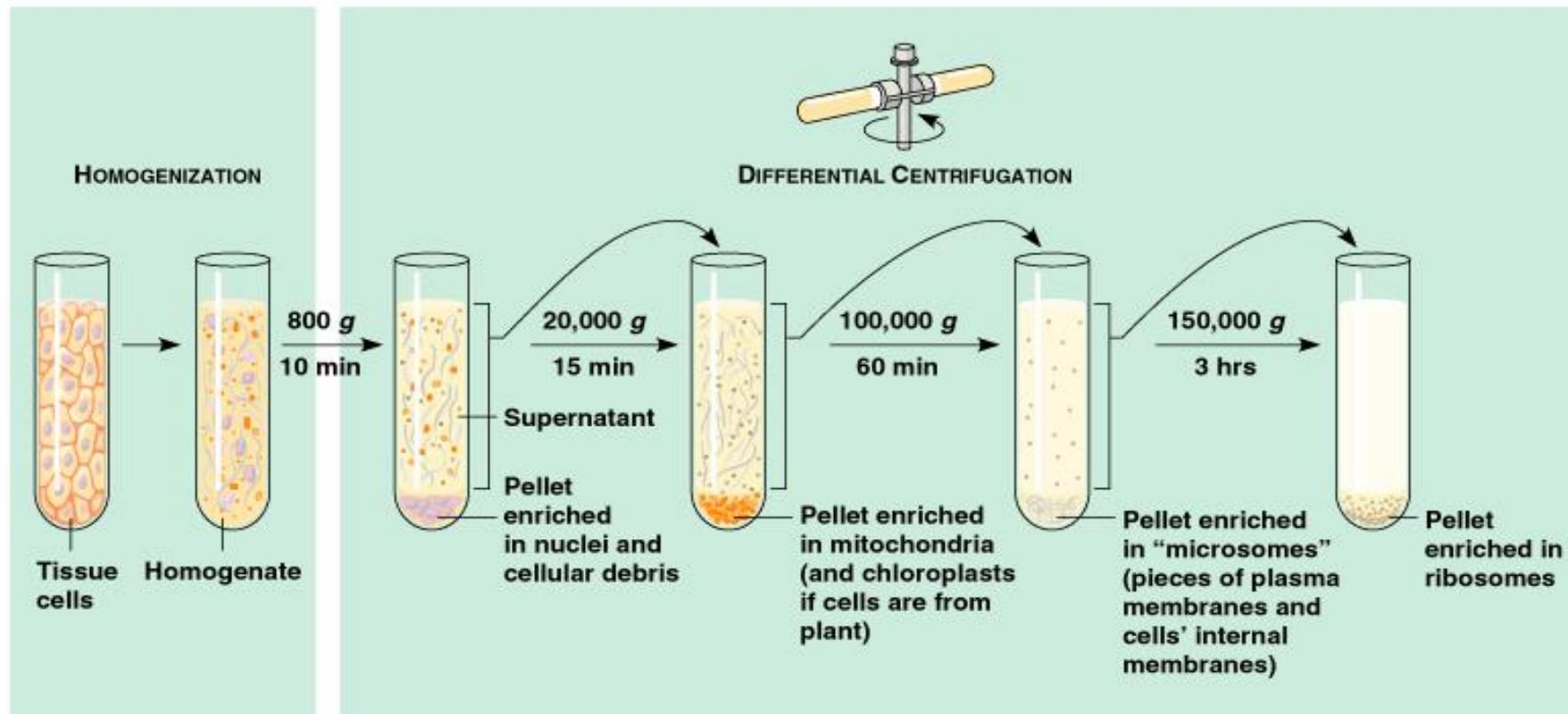
Some of these sedimenting organelles can be obtained in partial purity and are typically contaminated with other particles. Repeated washing of the pellets by re-suspending in isotonic solvents and re-pelleting may result in removal of contaminants that are smaller in size (Figure 1 on slide # 203). Obtaining partially-purified organelles by differential centrifugation serves as the preliminary step for further purification using other types of centrifugal separation (density gradient separation).

Differential centrifugation Cont'd.

Figure 1. Differential Centrifugation



Differential Centrifugation



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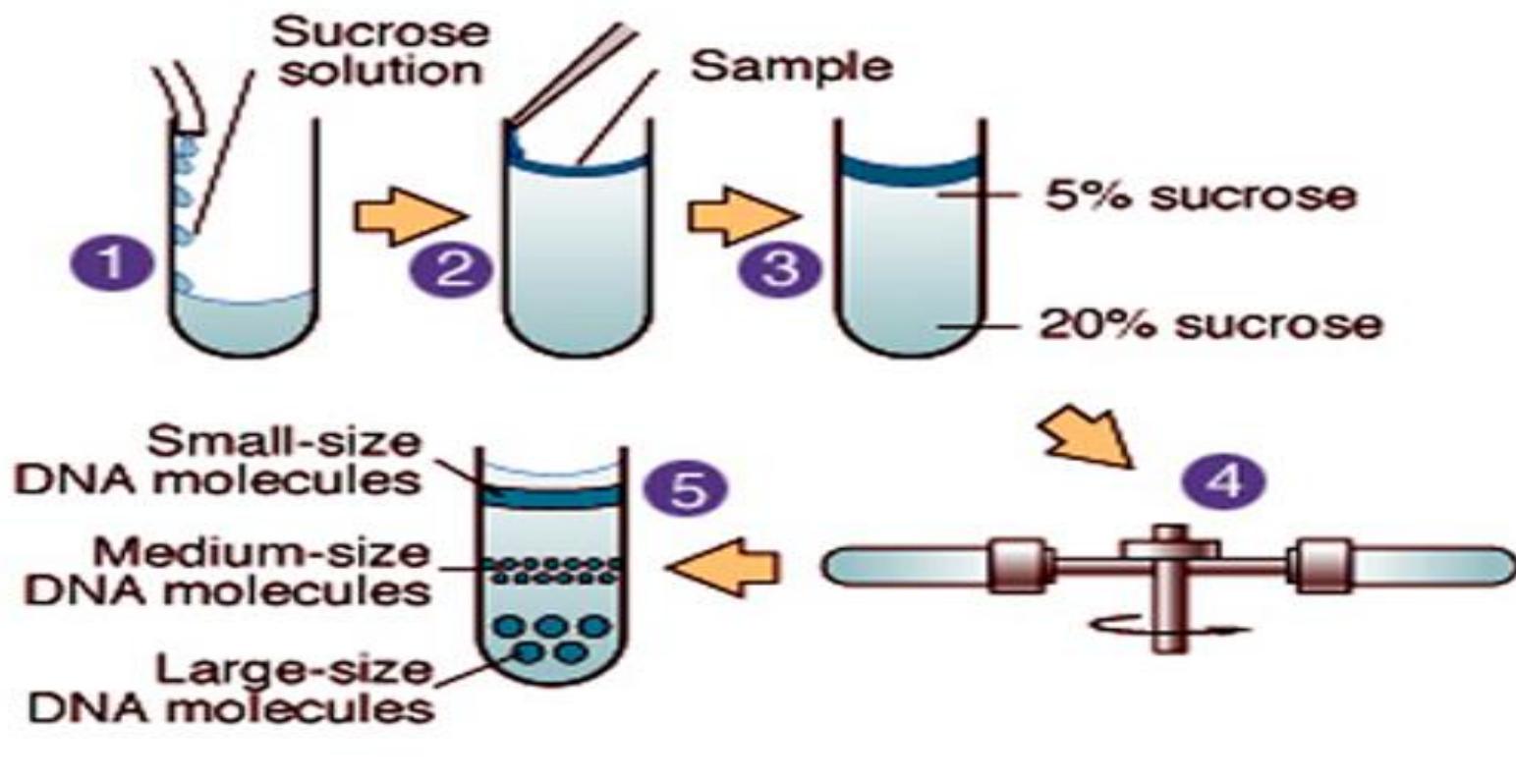
Density gradient centrifugation or Buoyant density centrifugation

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 (Table 2 on slide # 214) such as sucrose in a tube with the heaviest layer at the bottom and the lightest at the top in either a discontinuous or continuous mode.

Density gradient centrifugation or Buoyant density centrifugation Cont'd

The buoyant density centrifugation involves viruses with densities of 1.1-1.2 g/cm and a sucrose gradient. The cell suspension is added to the top of the sucrose gradient. In this centrifugation the densest components move fastest down the tube and stops at the sucrose density equal to its own. The sucrose gradient bands at the bottom contain cell components with high buoyant densities and the components at the top have low buoyant densities.

Buoyant density centrifugation



Density gradient centrifugation Cont'd.

The cell fraction to be separated is placed on top of the layer and centrifuged. Density gradient centrifugation can be classified into two categories. (a) Rate-zonal (size) separation. (b) Isopycnic (density) separation.

(a) Rate zonal (size) separation

Rate-zonal separation takes advantage of particle size and mass instead of particle density for sedimentation.

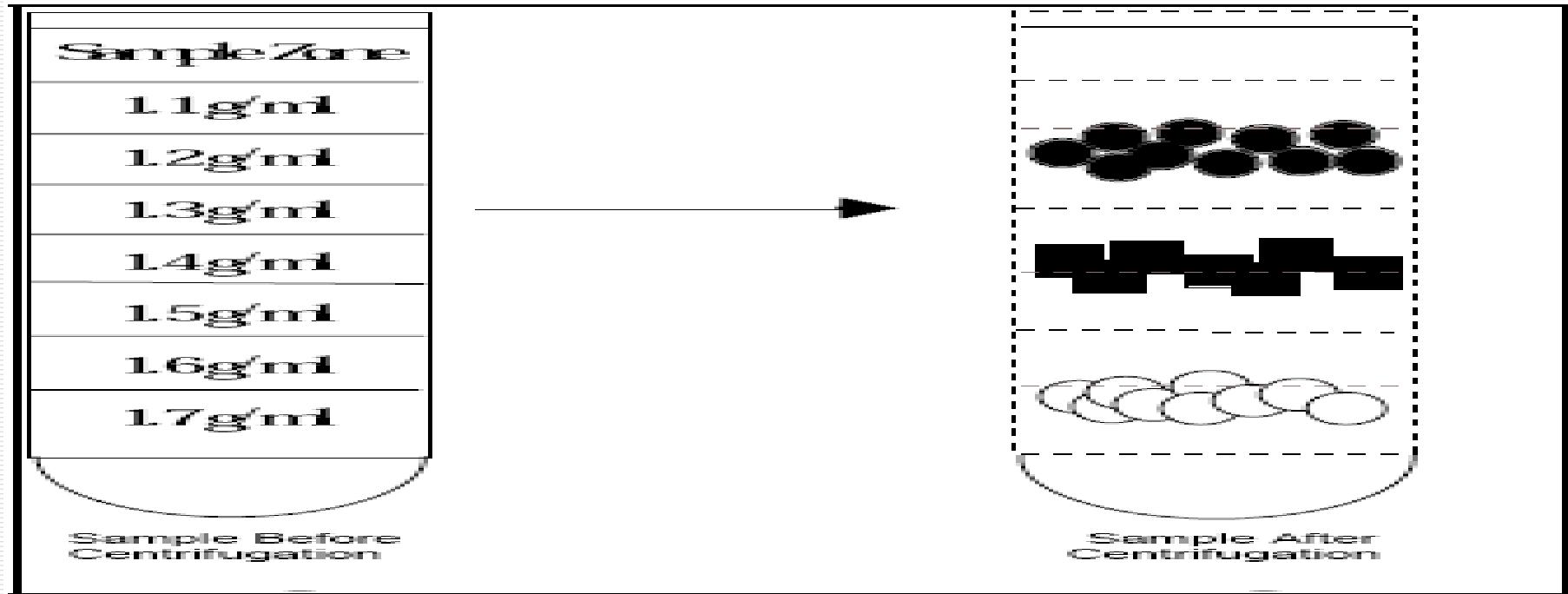
Rate zonal (size) separation Cont'd.

Examples of common applications include separation of cellular organelles such as endosomes or separation of proteins, such as antibodies. For instance, antibody classes all have very similar densities, but different masses. Thus, separation based on mass will separate the different classes, whereas separation based on density will not be able to resolve these antibody classes (Figure 2, slide # 210).

Rate zonal (size) separation

Cont'd.

Figure 2. RATE-ZONAL (SIZE) SEPARATION



(b) Isopycnic separation

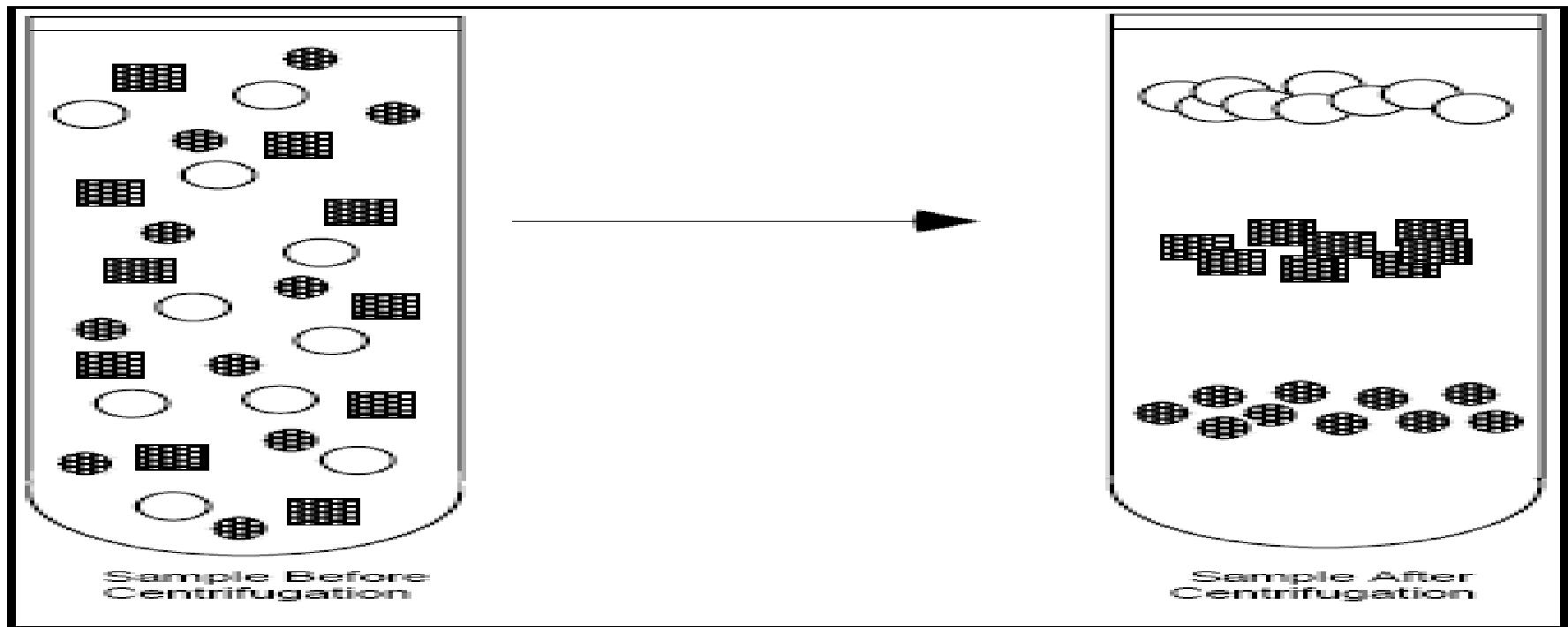
In this type of separation, a particle of a particular density will sink during centrifugation until a position is reached where the density of the surrounding solution is exactly the same as the density of the particle. Once this quasi-equilibrium is reached, the length of centrifugation does

Isopycnic separation Cont'd

not have any influence on the migration of the particle. A common example for this method is separation of nucleic acids in a CsCl gradient (Figure 3, slide # 213).

Isopycnic separation Cont'd.

Figure 3. ISOPYCNIC (DENSITY) SEPARATION



Criteria for successful isopycnic separation:

Table 2. Applications of density gradient media for isopycnic separations.

Gradient media	Cells	Viruses	Organelles	Nucleoproteins	Macro-molecules
Sugars (e.g sucrose)	+	+++	+++	+	-
Polysaccharides (e.g Ficoll)	++	++	++	-	-
Colloidal silica (e.g Percoll)	+++	+	+++	-	-
Iodinated media (e.g Nycodenz)	++++	++	++++	+++	+
Alkali metal salts (e.g. CsCl)	-	++	-	++	++++

++++ excellent, ++ good, + good for some applications, + limited use, - unsatisfactory

Source: D. Rickwood, T.C. Ford, J. Steensgard (1994) *Centrifugation essential data*, John Wiley & Sons Ltd. U.K.

Analytical Ultra-Centrifuge

Analytical ultracentrifugation is a classical method of biochemistry and molecular biology. Because analytical ultracentrifugation relies on the principal property of mass and the fundamental laws of gravitation, it has broad applications. It is a primary technique requiring no standards for comparison.

Analytical Ultra-Centrifuge Cont'd.

Sedimentation can be used to analyze the solution behavior of nearly any molecule over a wide range of solute concentrations and in a wide variety of solvents.

It is a further refinement of the preparative centrifuges. It can be used to separate and characterize very small molecules and macromolecules such as ribosomes and proteins.

Analytical Ultra-Centrifuge Cont'd.

The specimen is mixed with a sucrose or caesium chloride medium in a quartz centrifuge cell and centrifuged at very high speeds to generate forces of up to 500,000g. Such extreme forces causes small particles and macromolecules to sediment from the solution leaving pure solvent at the top of the cell and forming a sharp boundary between the pure solvent and solvent containing specimen.

Centriflex Centrifuge



Gilson GmCLab Mini Centrifuge



Analysis

Once cell fractions have been isolated, they can then be examined by standard biochemical procedures such as spectrophotometry, autoradiography, chromatography etc. in an attempt to characterize their biochemical activities.

Spectrophotometry

□ Principles of Spectrophotometry:

A spectrophotometer consists of two instruments, namely a *spectrometer* for producing light of any selected colour (wavelength), and a *photometer* for measuring the intensity of light. The instruments are so arranged that liquid in a cuvette can be placed between the spectrometer beam and the photometer.

Spectrophotometry Cont'd

The amount of light passing through the tube is measured by the photometer. The photometer delivers a voltage signal to a display device, normally a galvanometer. The signal changes as the amount of light absorbed by the liquid changes.

If development of colour is linked to the concentration of a substance in solution

Spectrophotometry Cont'd

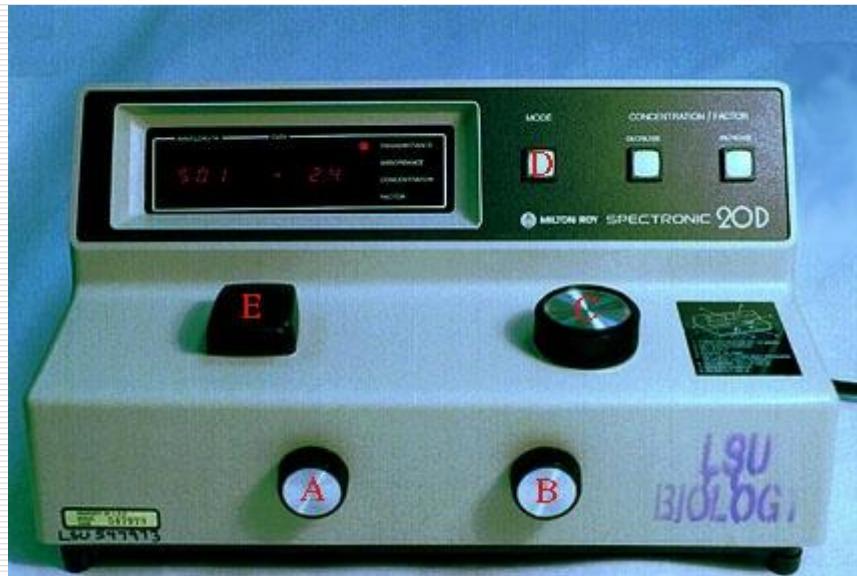
then the concentration can be measured by determining the extent of absorption of light at the appropriate wavelength.

For example haemoglobin appears red because the hemoglobin absorbs blue and green light rays much more effectively than red. The degree of absorbance of blue or green light is proportional to the concentration of haemoglobin in solution.

Spectrophotometer



Spectrophotometer



Spectrophotometry Cont'd

Water quality photometers and colorimeters are ion-specific computer-interfaced probes designed to determine the concentration of a solution from its colour intensity.

Autoradiography

This technique, which is now widely used in cell biology, involves the visual localization of radioactive substances in cells and tissues. Tissues are incubated in solutions containing radioactive isotopes which become incorporated into the cells. The tissue is sectioned and the sites of incorporation are traced by placing a photographic emulsion over the section.

Autoradiography Cont'd.

The radioactive particles emitted from the isotope sensitizer react with silver halide crystals in the emulsion and these are converted to metallic silver by normal photographic development. The unsensitized crystals are washed away during the subsequent fixation and the site

Autoradiography Cont'd.

of radioactive incorporation is then seen as a deposit of silver grains on the sections.

- The most widely used isotopes in biological research are tritium (^3H), ^{14}C , ^{32}P , ^{45}Ca , ^{35}S etc.
- These can be used to label a wide range of molecules which, when incubated with the

Autoradiography Cont'd.

appropriate tissues, are incorporated into various cellular constituents.

- Sites of protein synthesis in tissues can be detected by immersing the tissue in a radioactive precursor to proteins i.e. amino acids.
- In medicine, barium meal is used to test for ulcer in the GIT, which is an isotope of barium.

Chromatography

Chromatography is a technique used to separate components of mixtures. It is based on the partitioning of compounds between a stationary phase and a moving phase. In partition chromatography, the stationary phase is a liquid whilst in adsorption chromatography the stationary phase is a solid.

Chromatography Cont'd

Partition chromatography depends primarily on the relative solubility of substances in two or more immiscible solvents. When a solute is added to a mixture of equal volumes of two immiscible solvents, it may dissolve entirely in one or other of the solvents. If the solute dissolves in both solvents, it may not dissolve to the same extent in each solvent.

Chromatography Cont'd

- The ratio:

$$\frac{\text{Concentration of solute in solvent 1}}{\text{Concentration of solute in solvent 2}}$$

at equilibrium is called the partition coefficient. In partition chromatography, use is mainly made of differences in the

Chromatography Cont'd

partition coefficient to separate components of mixtures.

- In contrast, adsorption chromatography relies mainly on electrostatic interaction between solutes and the stationary phase. Some adsorption also occurs in partition chromatography.

Paper chromatography

Paper chromatography is an analytical technique for separating and identifying mixtures that are coloured or can be made coloured, especially pigments. This method has been largely replaced by thin layer chromatography, however it is still a powerful research tool.

Paper chromatography Cont'd.

□ Technique:

A small concentrated spot of solution that contains the sample is applied to a strip of chromatography paper about 2 cm away from the base of the plate, usually using a capillary tube for maximum precision. This sample is absorbed onto the paper and may form interactions with it.

Paper chromatography Cont'd.

Any substance that reacts or bonds with the paper cannot be measured using this technique. The paper is then dipped in to a suitable solvent, such as **ethanol** or **water**, or even **acetone** taking care that the spot is above the surface of the solvent, and placed in a sealed container.

Paper chromatography Cont'd.

The solvent moves up the paper by capillary action, which occurs as a result of the attraction of the solvent molecules to the paper and to one another. As the solvent rises through the paper it meets and dissolves the sample mixture, which will then travel up the paper with the solvent. Different compounds in the sample mixture travel at different rates due to differences in

Paper chromatography Cont'd.

solubility in the solvent, and due to differences in their attraction to the fibre or cellulose in the paper. Paper chromatography takes anywhere from several minutes to several hours to complete. In some cases, paper chromatography does not separate pigments completely; this occurs

Paper chromatography Cont'd.

when two substances appear to have the same values in a particular solvent. In these cases, two-way chromatography is used to separate the multiple-pigment spots. The chromatogram is turned by ninety degrees 90° , and placed in a different solvent in the same way as before; some spots separate in the presence of more than one pigment.

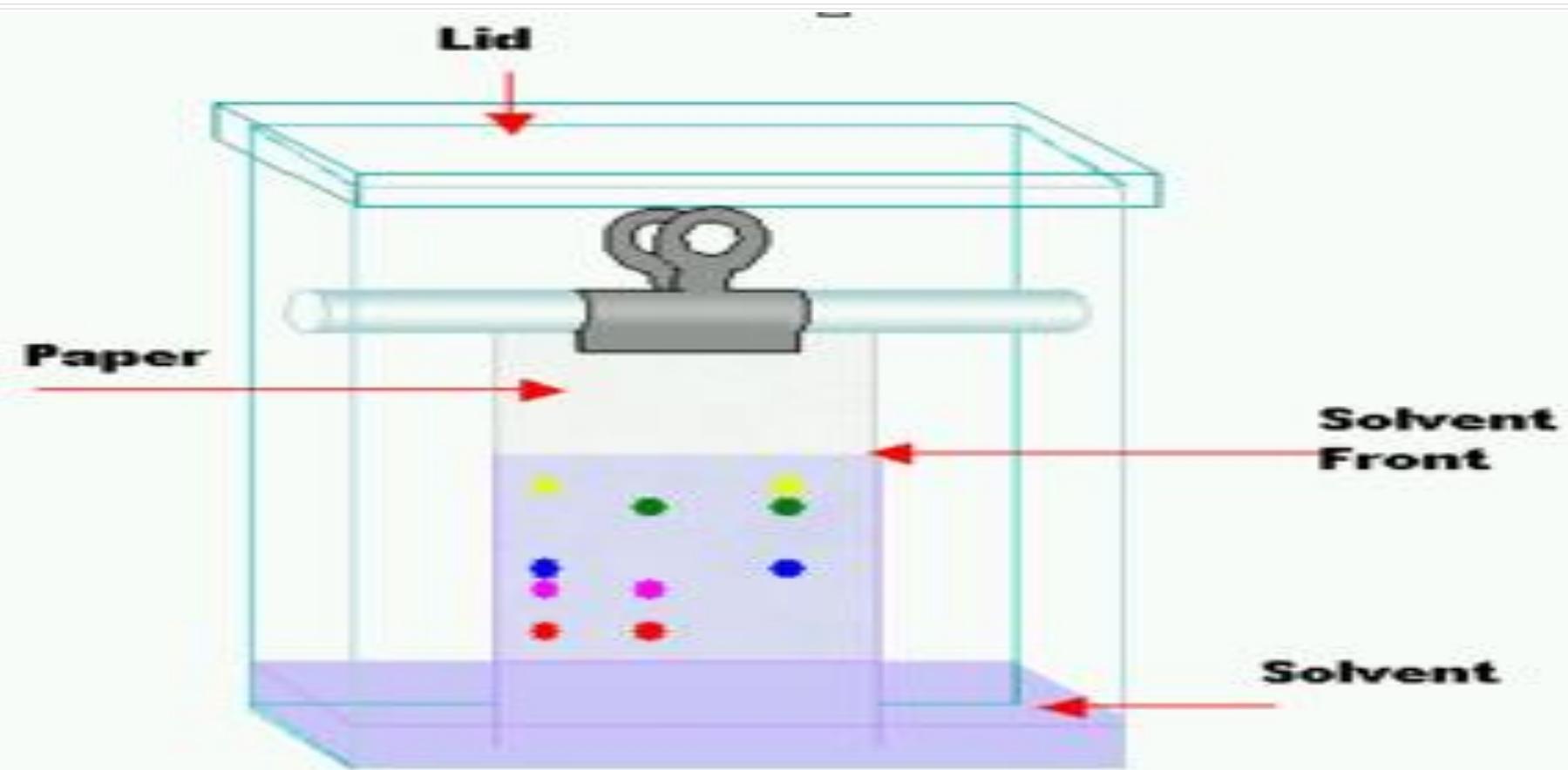
Paper chromatography Cont'd.

As before, the value is calculated, and the two pigments are identified. The R_f value (retention factor) is the distance travelled by a particular component from the origin (where the sample was originally spotted) as a ratio to the distance travelled by the solvent front from the origin. R_f values for each substance will be unique, and can be

Paper chromatography Cont'd.

used to identify components. A particular component will have the same R_f value if it is separated under identical conditions.

Paper chromatography

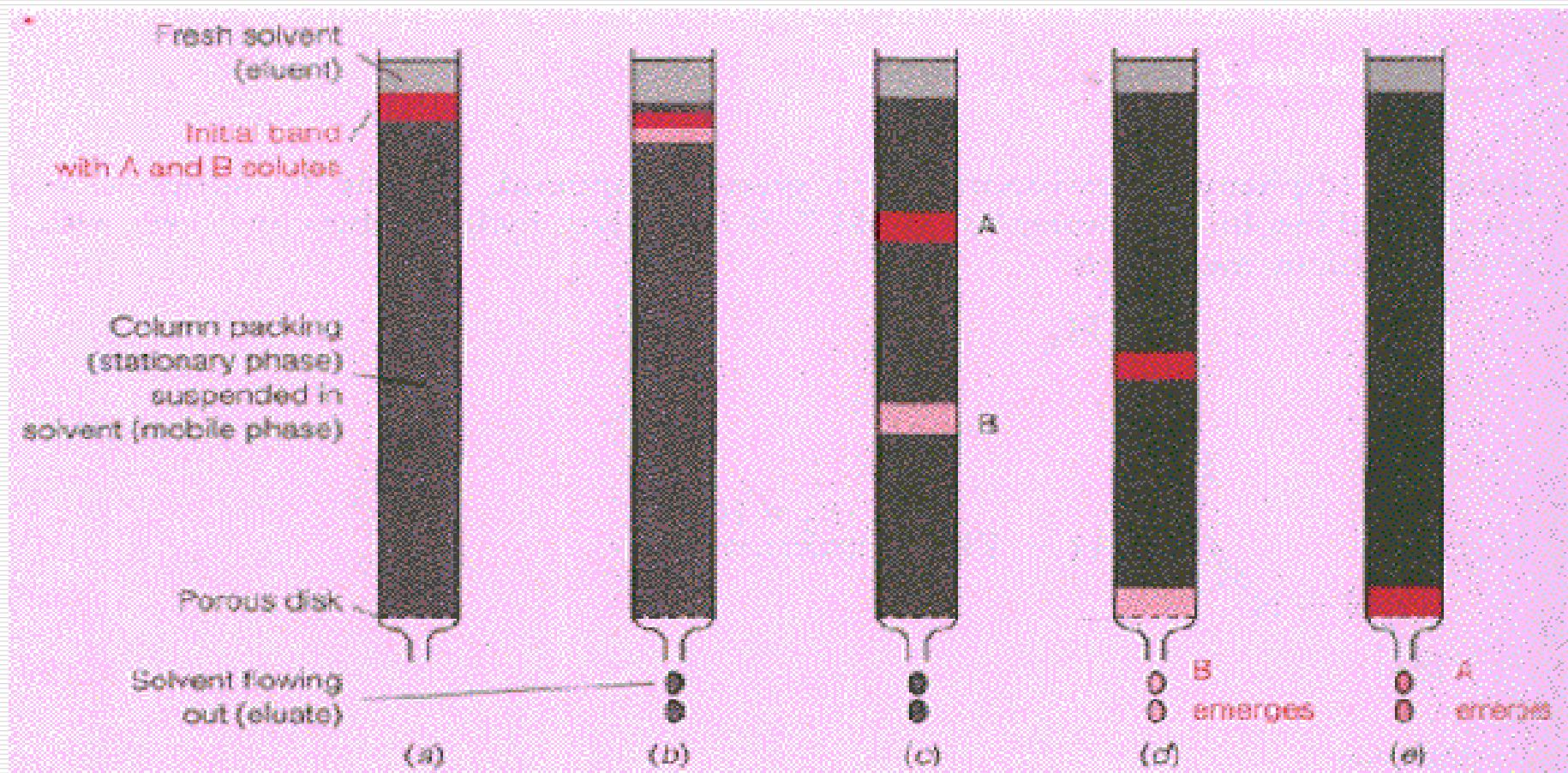


CHROMATOGRAM

The figure on the next slide shows a simple separation by chromatography. A continuous flow of solvent carries a solution of solutes A and B down a column.

(a) As the solvent carries the two solutes down the column, we begin to see some separation of the solution. (b) At some later point in time, it can be seen that solute B is moving at a much faster rate than A. (c) In (d), solute B emerges first, while solute A finally emerges in (e). Thus, solute A has a greater affinity for the stationary phase than solute B. By varying the pH of the solvent or temperature of the column, the output of the column can be significantly altered, such as the timing of when individual species emerge.

CHROMATOGRAM



Interpreting the Data

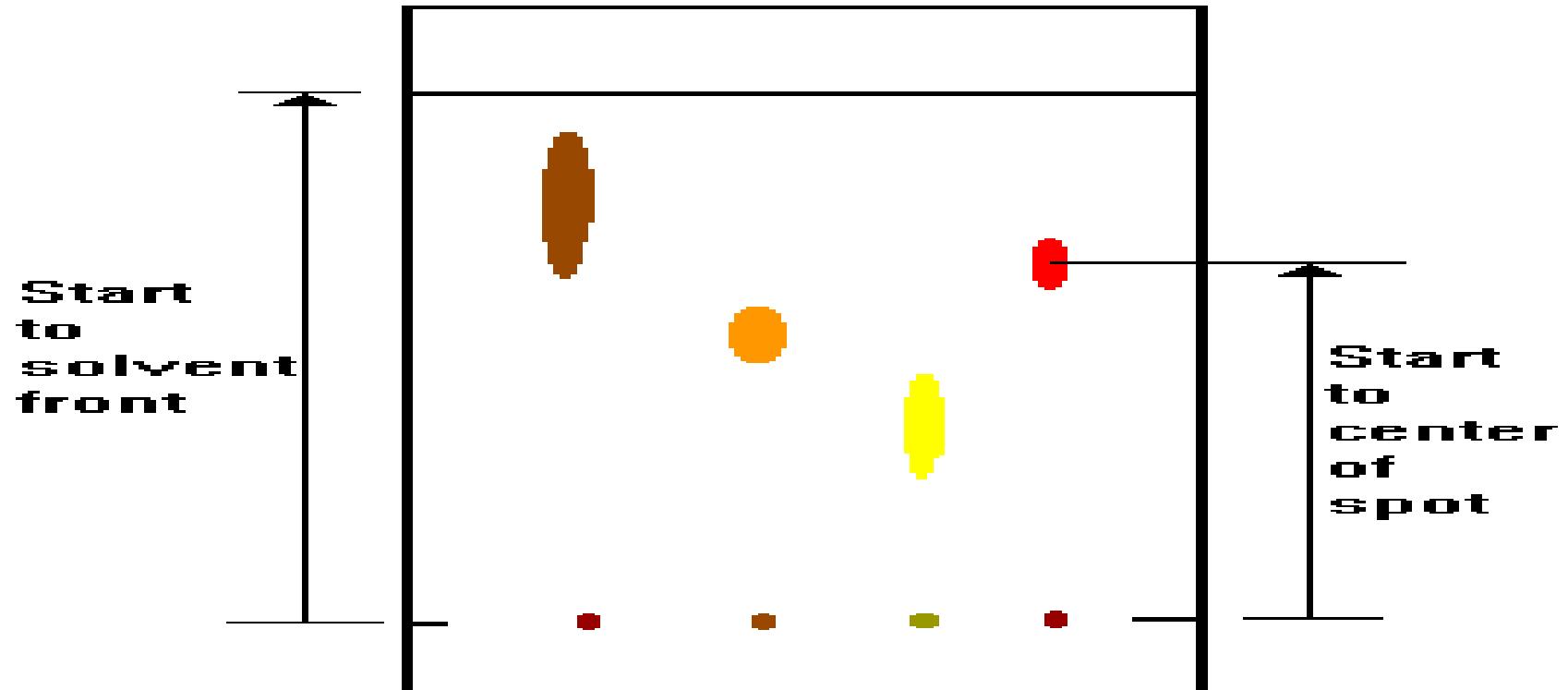
The R_f value for each spot should be calculated. R_f stands for "ratio of fronts" and is characteristic for any given compound on the same stationary phase using the same mobile phase for development of the plates.

Interpreting the Data Cont'd

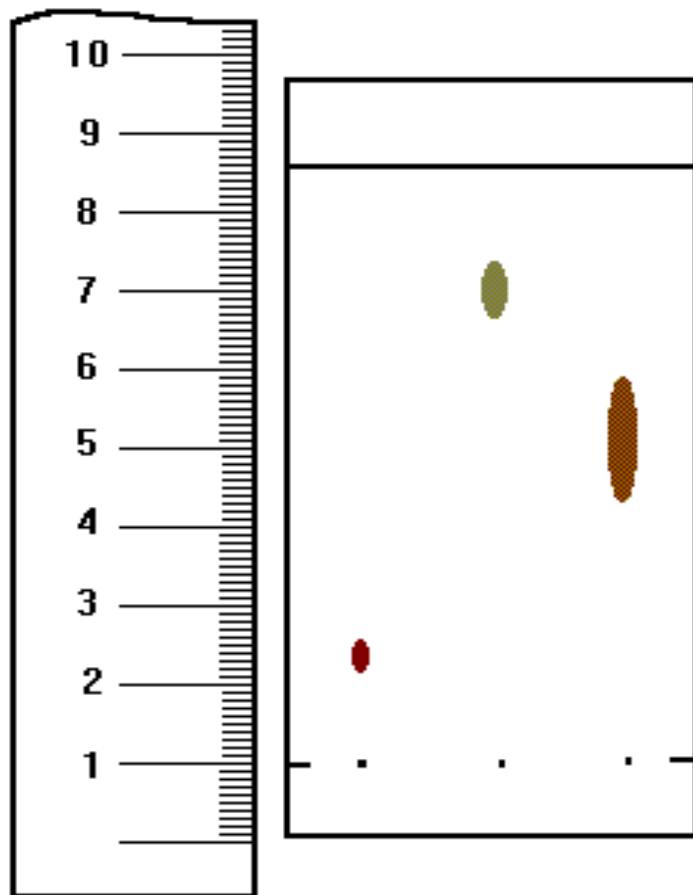
Hence, known R_f values can be compared to those of unknown substances to aid in their identifications.

$$R_f = \frac{\text{Distance from start to centre of substance spot}}{\text{Distance from start to solvent front}}$$

Interpreting the Data Cont'd.



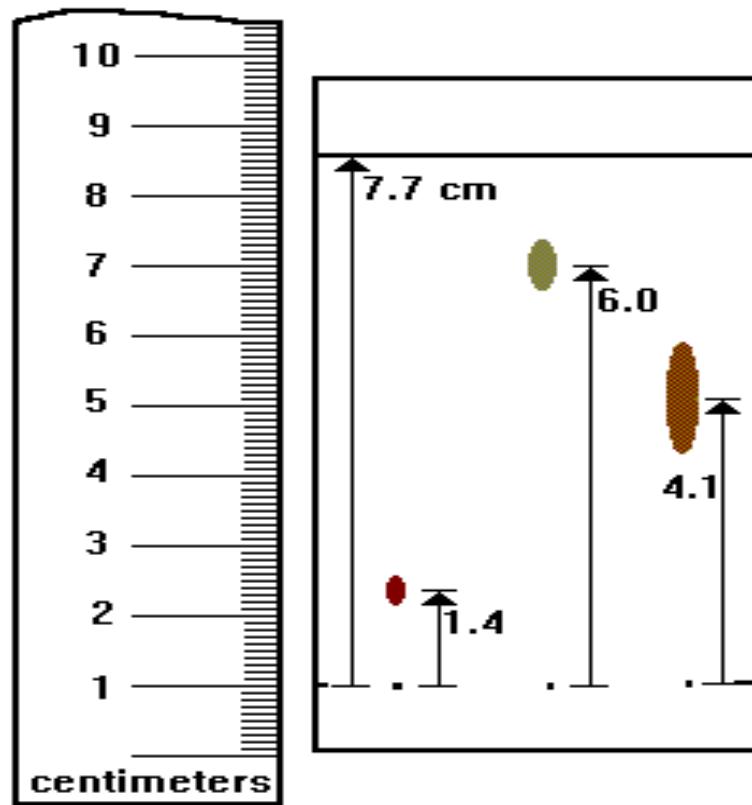
Calculate the R_f values for the spots on the TLC slide below.



Solution:

- Use the equation:
- Measure the distances from the starting point to the solvent front and from the starting point to the center of each spot, and calculate the R_f values:

Solution Cont'd:



Solution Cont'd:

Spot 1: $R_f = \frac{1.4 \text{ cm}}{7.7 \text{ cm}} = 0.18$

Spot 2: $R_f = \frac{6.0 \text{ cm}}{7.7 \text{ cm}} = 0.78$

Spot 3: $R_f = \frac{4.1 \text{ cm}}{7.7 \text{ cm}} = 0.53$