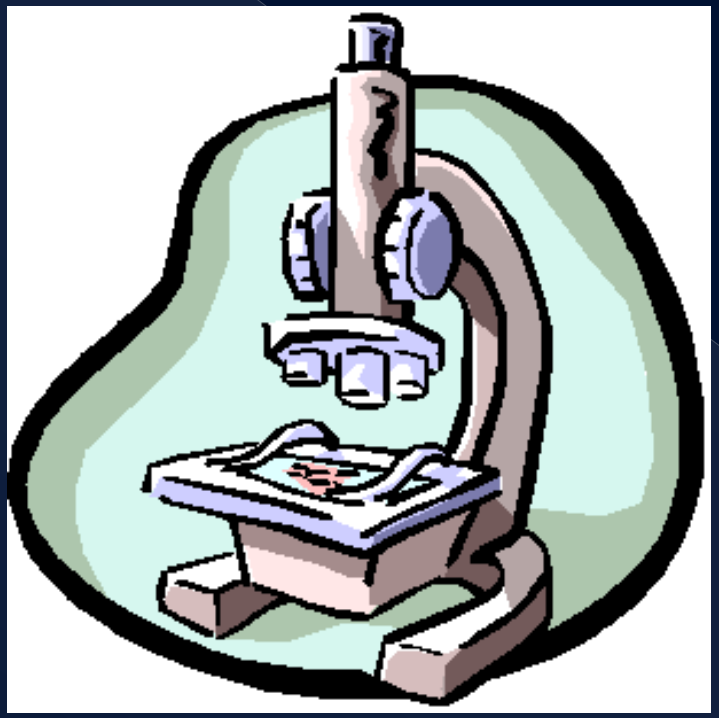
METHODS OF CELL CULTURE AND TECHNIQUES USED IN MEDICAL RESEARCH

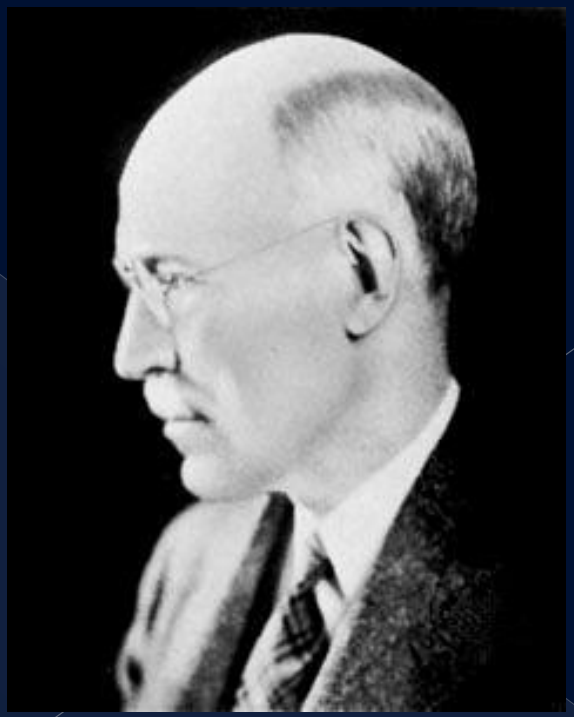


HISTORY

- 1885 - chicken neural plate cultured in a warm saline solution (Wilhelm Roux)
- 1907 - frog neural tube cultured in frog lymph clot. The formation of nerve processes (Ross Harrison).
- optimization of culture conditions through the use of different culture media (plasma, serum, extract from chicken embryos) and determination the needs of the cells: osmotic pressure, pH, salts (Burrows, Carrel, Ebeling, Lewis).

Professor Wilhelm Roux (1850 –1924) German zoologist

Professor Ross Granville Harrison (1870 –1959) American biologist and anatomist

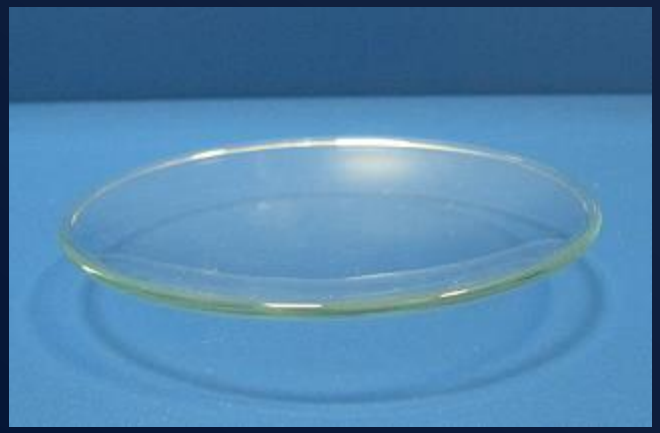


HISTORY

-20s of the 20th century - embryonic tissue cultures. The ability of cells to differentiate in culture. Culture in clot on the slide watch.

-40s of 20th century - the first artificial medium comprising mineral salts, amino acids, vitamins.

-1952 – the use of trypsin to isolate single cells from tissue and to separate them from the glass (A. Moscona, H. Moscona)

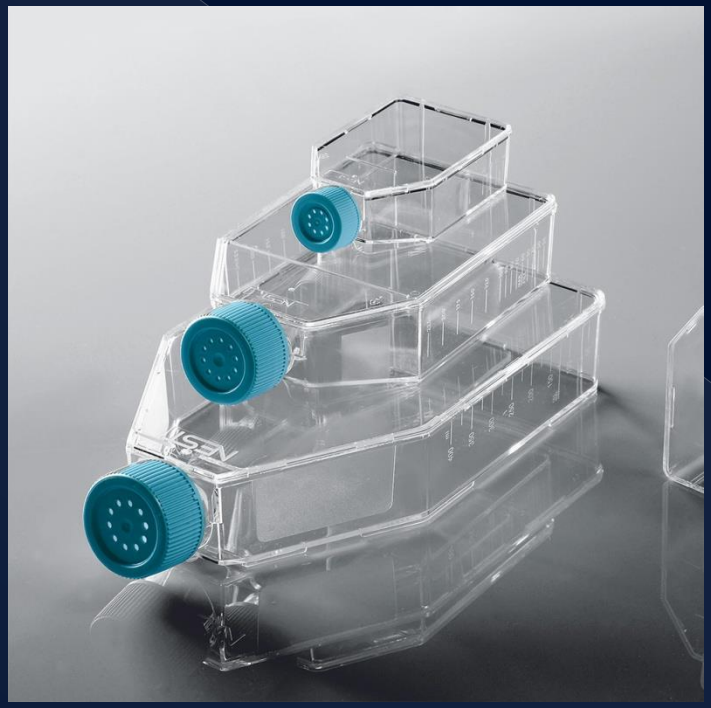


MODERN CELL/TISSUE CULTURE

-special devices of laboratory allow to save sterility of culture

Biosafety cabinet - enclosed, ventilated laboratory workspace for safely working with materials contaminated with (or potentially contaminated with) pathogens. All exhaust air is filtered (vertical air flow)

MODERN CELL/TISSUE CULTURE



plastic disposable dishes, with properly prepared surface

the use of appropriate growth factors for proliferation and growth of different types of cells

established cell lines

APPLICATIONS OF CELL/TISSUE CULTURE

1. Basic research *in vitro*
2. Diagnostics
3. Production of vaccines (polio, measles, mumps, rubella)
4. Production of enzymes, hormones, cytokines
5. Production of monoclonal antibodies
6. Getting the cells for transplantation

BASIC DEFINITIONS

Tissue culture is the growth of tissues or cells *in vitro* more than 24h (less then 24h – incubation)

Primary cell culture - cells derived directly from the parent tissue

Cell line - the cells growing in the first or later subculture derived from a primary culture (after first passage).

Cell strain - cells adapted to culture, but with finite division potential

Established cell line - cells that proliferate indefinitely if they have appropriate fresh medium and space (immortal line)

CELL LINES

DIPLOID CELL LINE

line, wherein at least 75% of the cells have the same karyotype as normal somatic cells of the species from which derived cells

HETEROPLOID CELL LINE

cell line, in which less than 75% of the cells has a diploid karyotype

Definitions cont.

POPULATION DOUBLING TIME
period of time, wherein the number of cells doubles

TRANSFORMATION OF CELLS
change of normal cells to tumor cells,
due to the introduction of new genetic material
or mutation.

CELL CULTURE GROWTH
increase in cell mass (volume) (formerly term related to increase of the number)

PROLIFERATION
increase of the number of cells

CELL CULTURE MEDIA

1. Osmotic pressure of 340 ± 5 mOsm /kg H_2O and the pH in the range 7.2 to 7.4.

COMPOSITION OF THE MEDIUM:

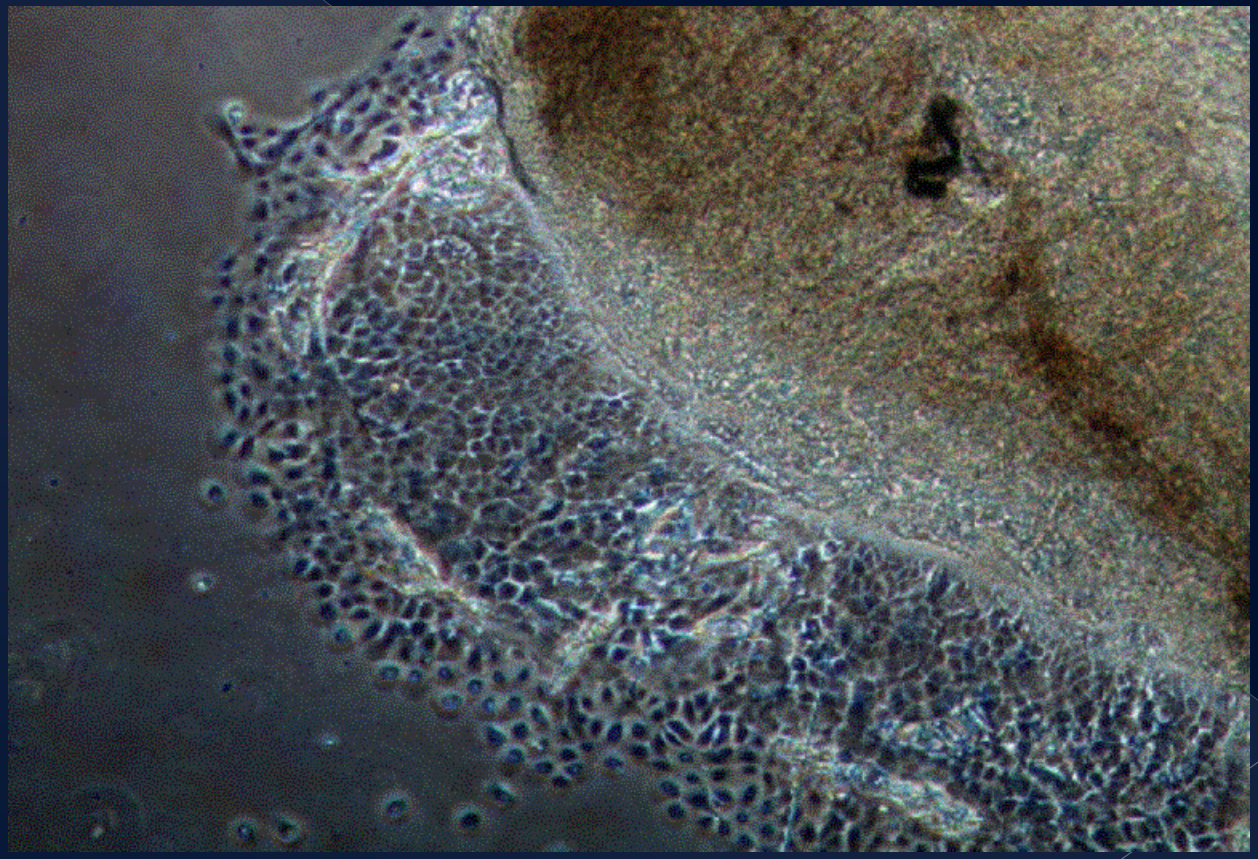
- balanced salt solution
- amino acids
- glucose
- fatty acids
- vitamins
- purine and pyrimidine bases
- phenol red
- fetal calf serum – a source of growth factors and hormones

CO_2 incubator for cell culture

maintains optimal temperature, humidity, the carbon dioxide and oxygen content of the atmosphere inside

ISOLATION OF SINGLE CELLS OR THEIR GROUPS

Culture of the explant (isolated portion of the tissue or organ) for a time sufficient to get out the cells.



ISOLATION OF SINGLE CELLS OR THEIR GROUPS

Separation of single cells from explant by enzymatic methods:

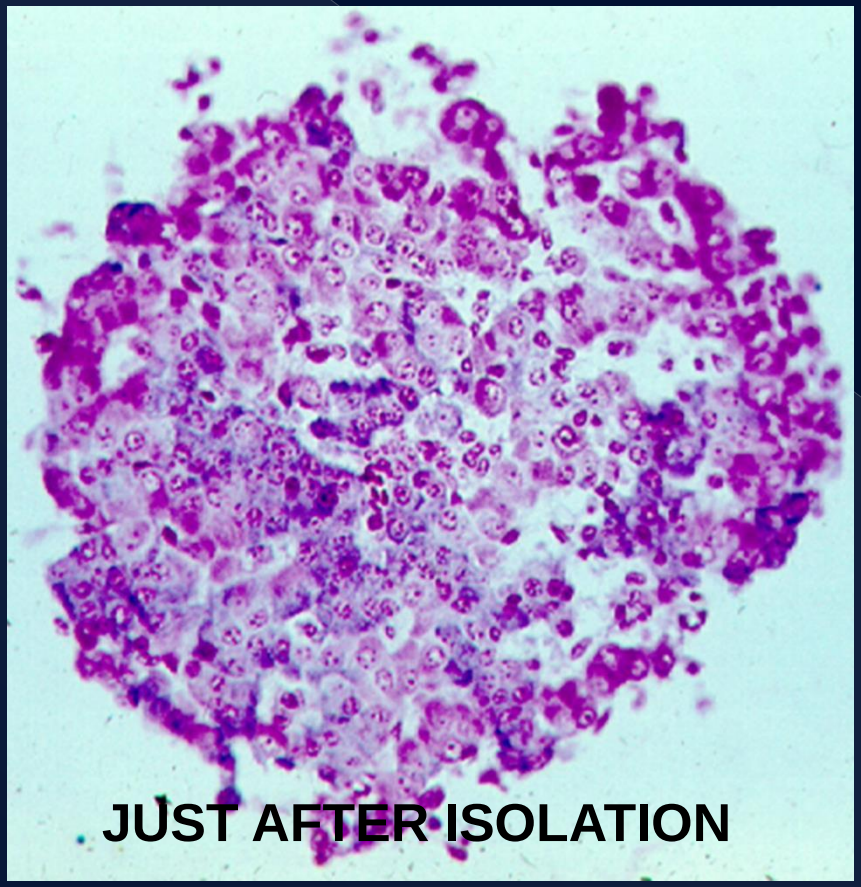
a) When small amount extracellular matrix eg. fetal tissues – by trypsin

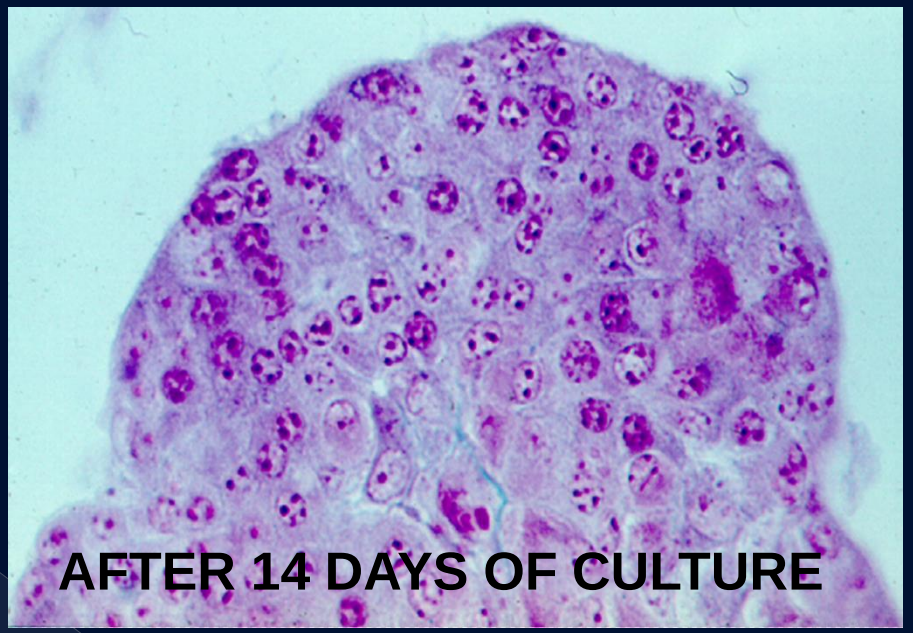
b) when large amount of extracellular matrix eg. cartilage – by collagenase with deoxyribonuclease

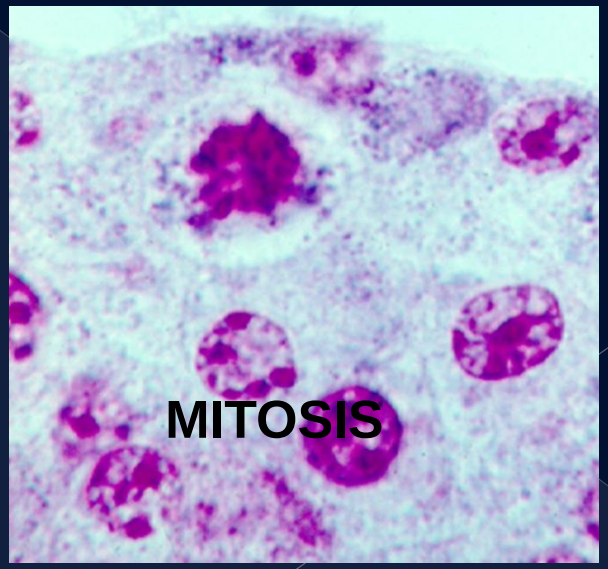
Separation the larger fragments eg. thyroid follicles or pancreatic islets – by collagenase

Isolation and culture of the islets of Langerhans of the guinea pig.
Stanislaw Moskalewski Department of Histology and Embryology, Medical University of Warsaw
General and Comparative Endocrinology 1965; 44(3):342-53. DOI: 10.1016/0016-6480(65)90059-6

LANGERHANS ISLET



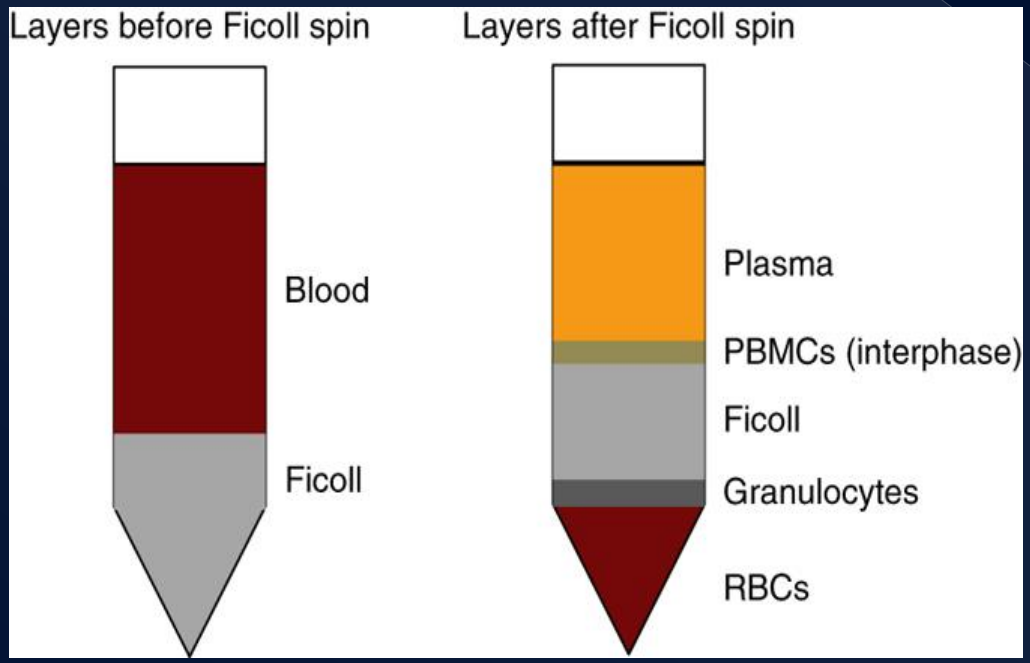




ISOLATION OF SINGLE CELLS OR THEIR GROUPS

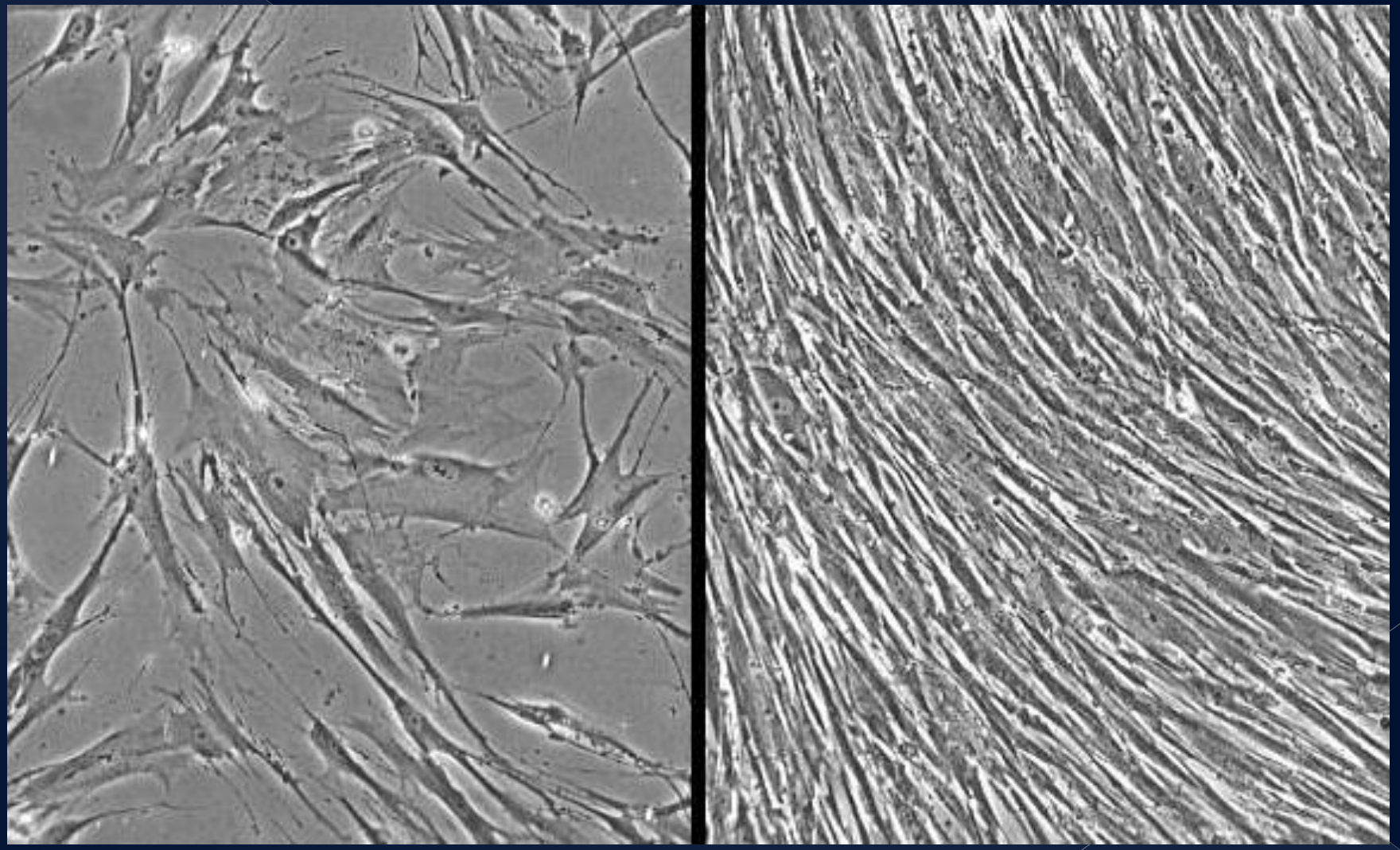
Separation of a heterogeneous population of cells, eg. blood cells into more homogenous populations by density gradient centrifugation. Cells are sorted according to their sedimentation constant.

Blood with anticoagulant, diluted 1 : 1 is layered on neutral, highly branched, high-mass, hydrophilic polysaccharide in aqueous solution (1.075g/ml density) and centrifuged.



Differential migration of cells results in the formation of layers containing particular cell types.

CULTURED FIBROBLASTS



CONTACT INHIBITION

mechanism which functions to keep cells growing into a layer one cell thick (a monolayer). If a cells have plenty of free space, they divide rapidly. This process continues until there is no any free surface. At this point, normal cells will stop dividing.

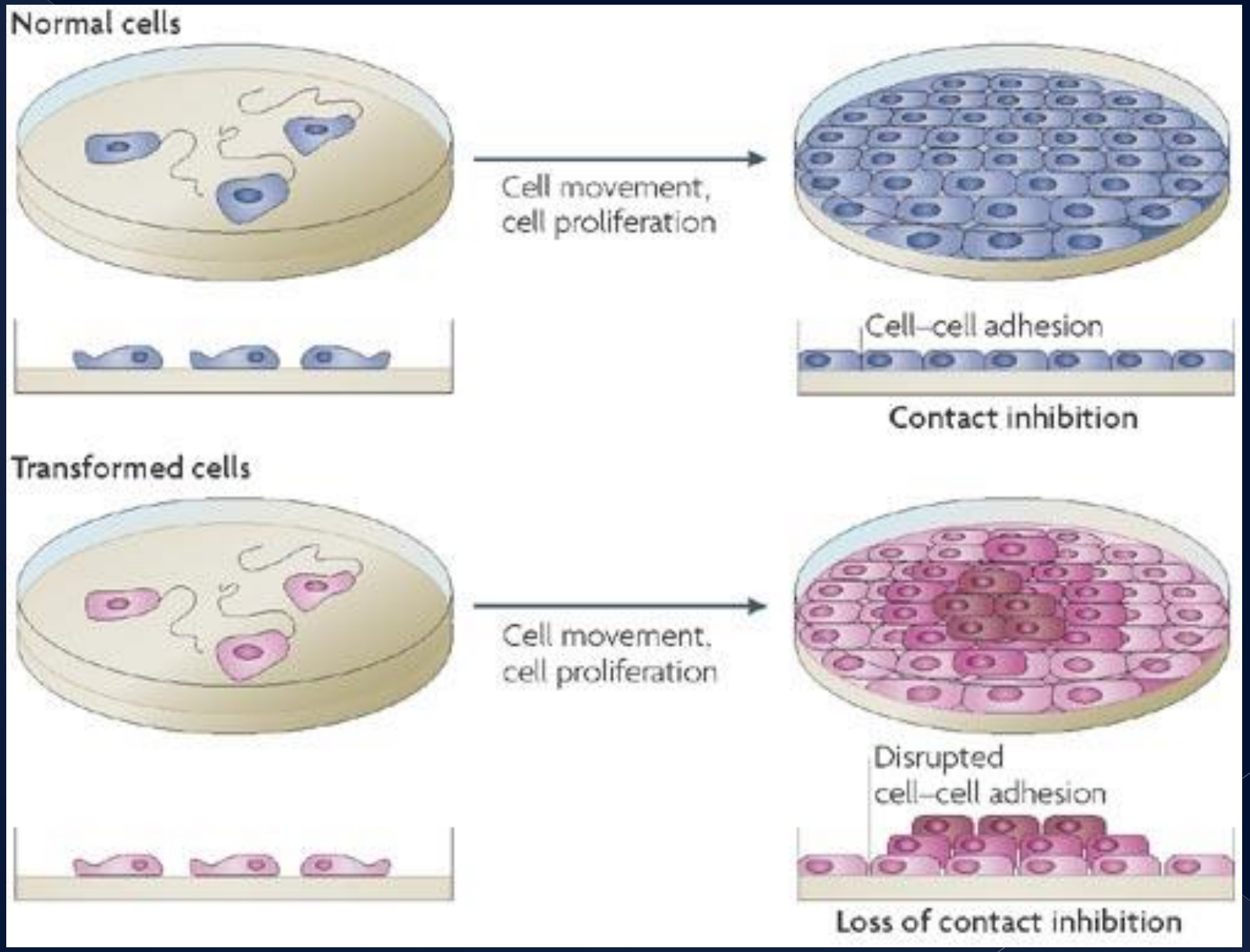
CONTACTINHIBIN – membrane glycoprotein of human fibroblasts, causes a reversible inhibition of proliferation in confluent culture. This effect is seen also with other cell types, with the exception of transformed cells, which do not show the phenomenon of contact inhibition.

CiR – plasma membrane protein - a receptor for contactinhibin mediating the contact-dependent inhibition of growth of cultured cells

The ligand-receptor binding causes changes in the cyclin-dependent kinases (inactive Cdk4), which leads to inhibition of cell division.

Transformed fibroblasts – lack of CiR

CONTACT INHIBITION – NORMAL AND TRANSFORMED CELLS



NUMBER OF POPULATION DOUBLINGS

Normal human fibroblasts derived from young person can divide – several dozen times (50-60) = several dozen population doublings - Hayflick limit.

Professor Leonard Hayflick
American anatomist

TELOMERES

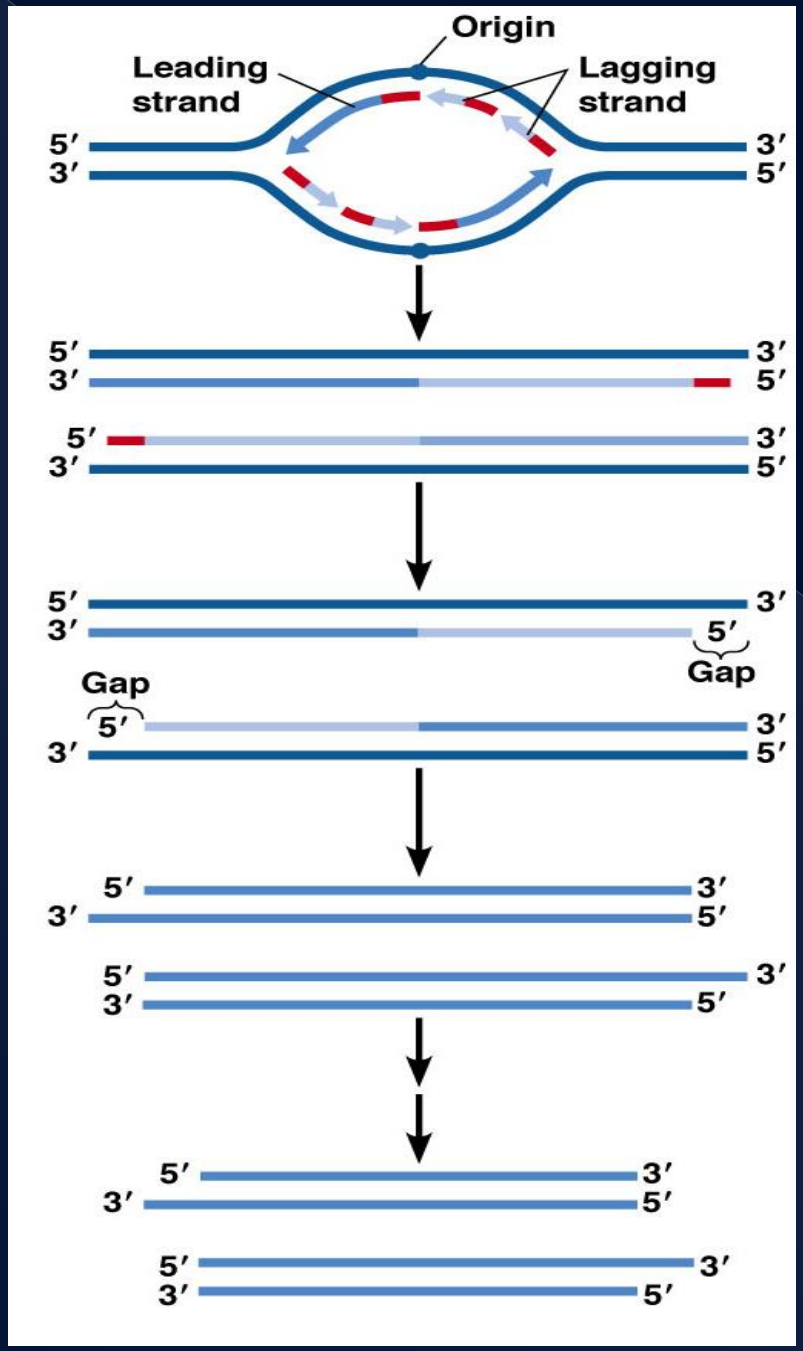
Linear chromosomes are less stable than circular, but determine the genetic diversity of living organisms (recombinations).

However, the 3 'and 5' ends are sensitive to DNA-degrading enzymes, as well as linear chromosomes may undergo fusion.

Telomeres - repetitive non-coding DNA sequences (TTAGGG) with proteins, located at the ends of chromosomes, prevent the damage of chromosomes.

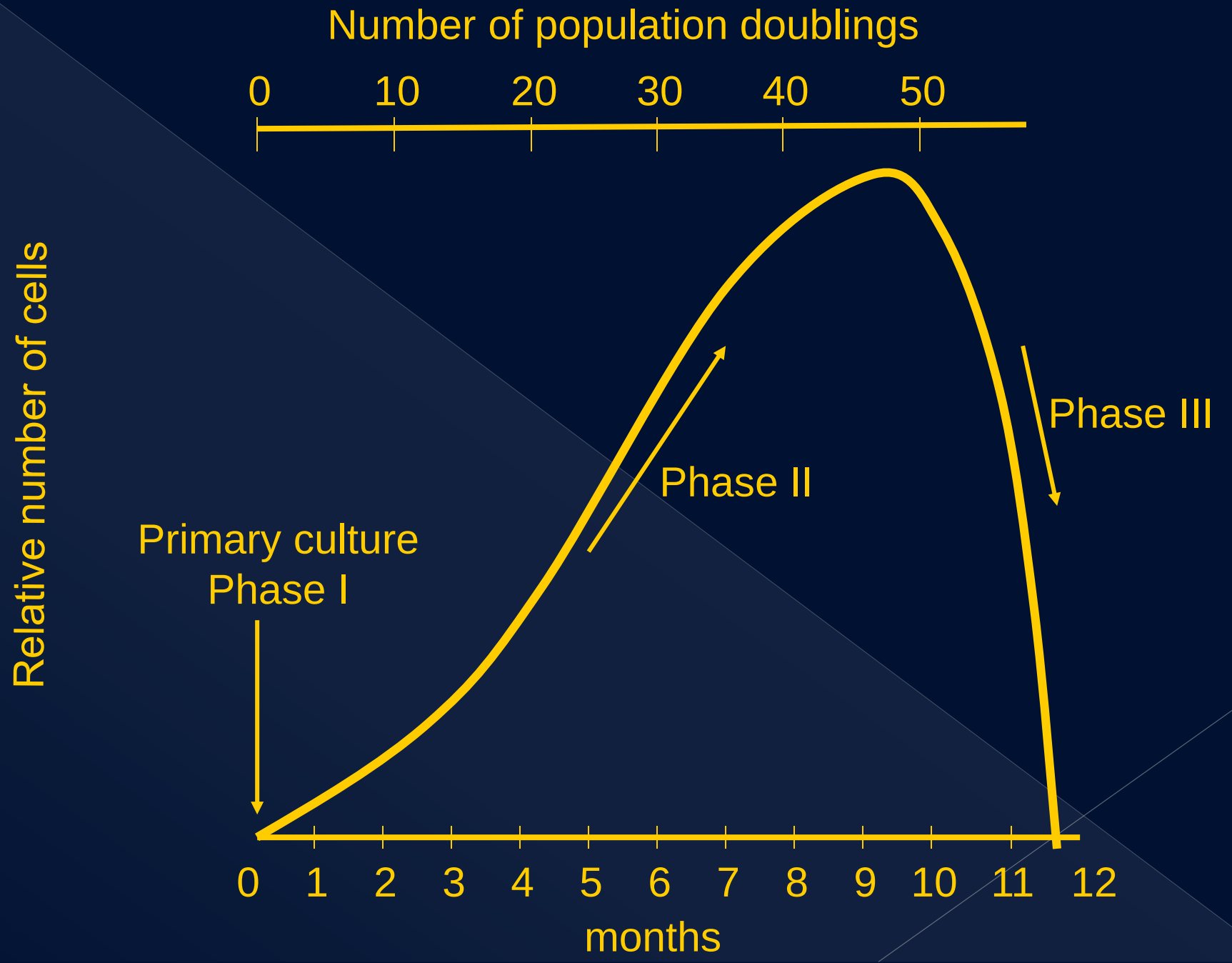
During each replication cell loses 50-200 bp telomeric DNA.

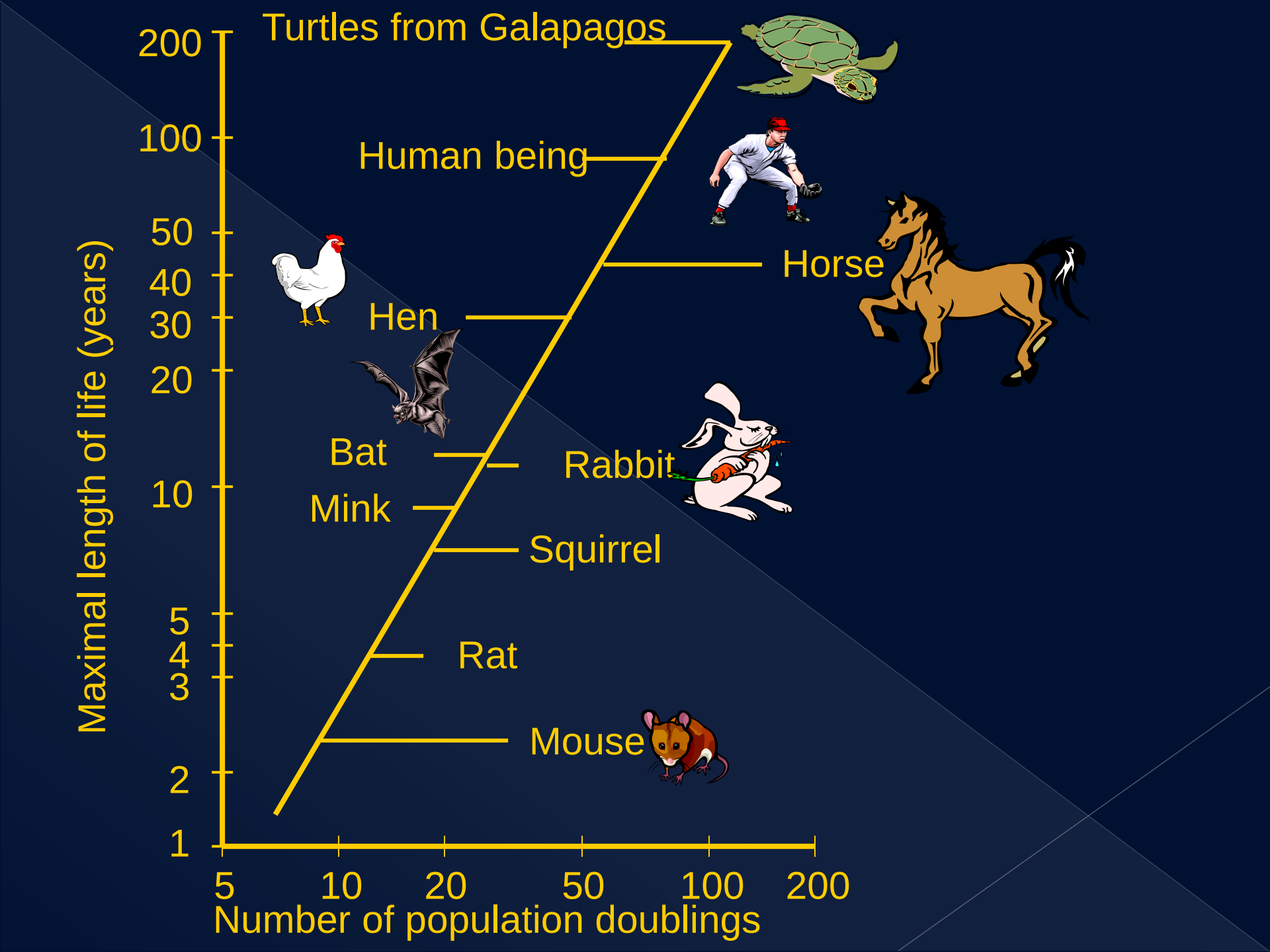
When the telomeres reach a critical length - STAGE OF CELLULAR SENESCENCE (p53-dependent inhibition of cell cycle)



End replication problem - the shortening of telomeres during replication

DNA polymerase can read and synthesize DNA only in one direction, starting from primer.

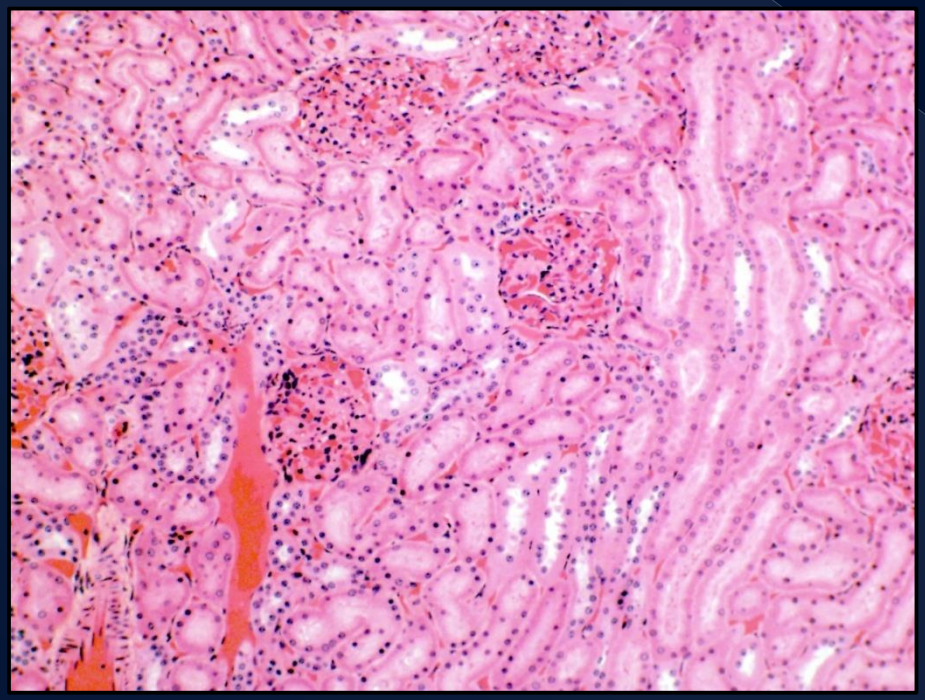

Number of population doublings
0
10
20
30
40
50
Relative number of cells
Primary culture
Phase I
Phase II
Phase III
0
1
2
3
4
5
6
7
8
9
10
11
12
months


Turtles from Galapagos
Human being
Horse
Hen
Bat
Rabbit
Mink
Squirrel
Rat
Mouse
Maximal length of life (years)
200
100
50
40
30
20
10
5
4
3
2
1
5
10
20
50
100
200
Number of population doublings

How Cells Are Studied?

What we can study?

Cell (tissue) structure

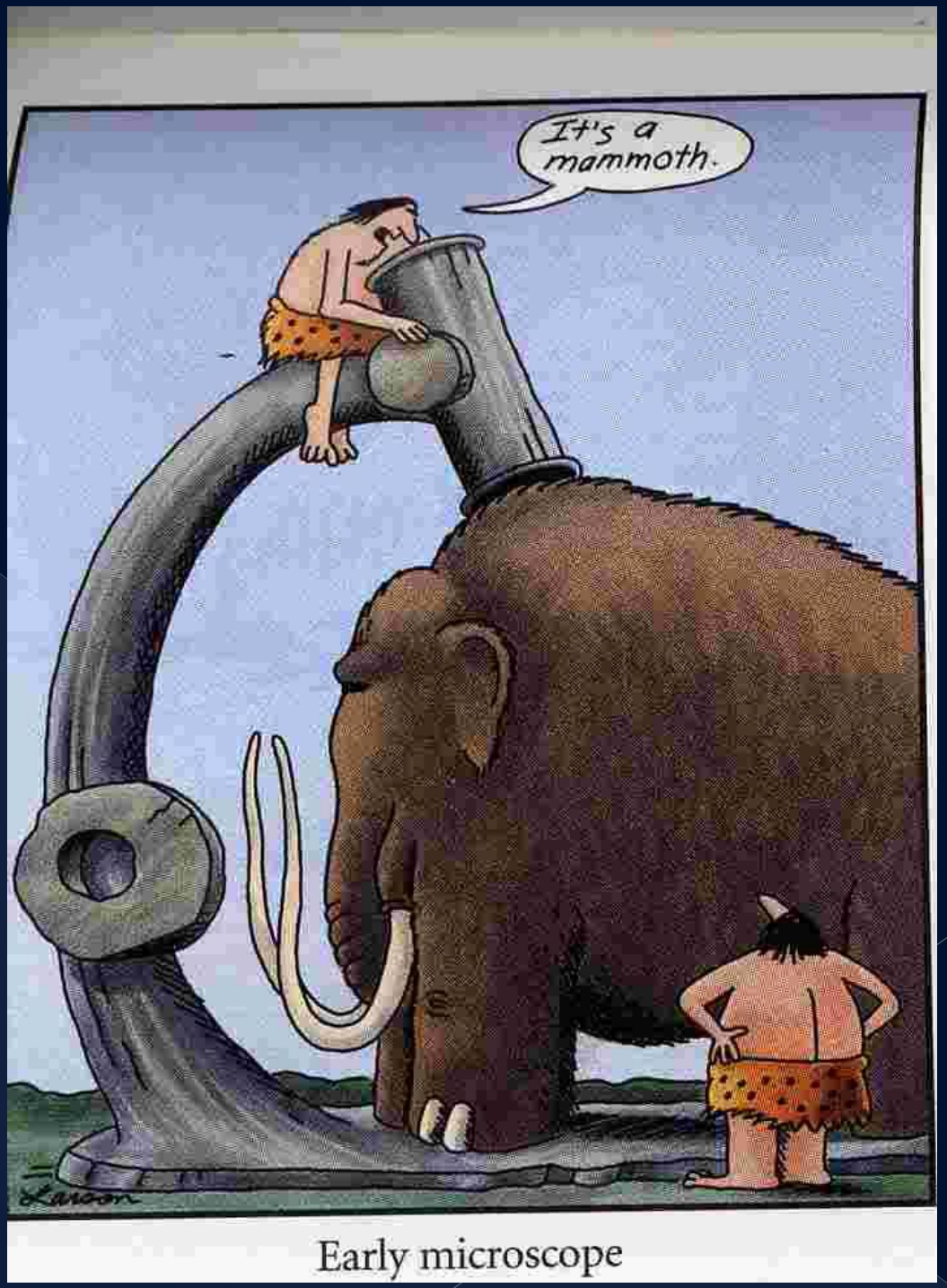


Light/electron microscopy

HISTORY

MICROSCOPY: ONE OF THE EARLIEST TOOLS OF THE CELL BIOLOGISTS

The first compound microscope appearing in 17th century in Netherlands (inventor Cornelis Drebbel, Hans Lippershey, Zacharias Janssen). Antonie van Leeuwenhoek discovered red blood cells and spermatozoa and helped popularize microscopy as a technique. In 1676, Van Leeuwenhoek reported the discovery of microorganisms



Early microscope

CYTO- AND HISTOCHEMISTRY

(detection of chemical compounds present in the cells, tissues, and in the matrix)

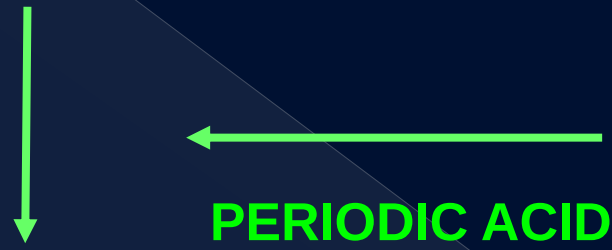
Histochemical color reaction

Autoradiography

Immunohistochemistry

REACTION P.A.S PERIODIC ACID- SCHIFF STAIN-
DETECTION OF POLYSACCHARIDES

POLYSACCHARIDES



PERIODIC ACID

(oxidation of the glycol groups – vicinal diols)

ALDEHYDE GROUPS

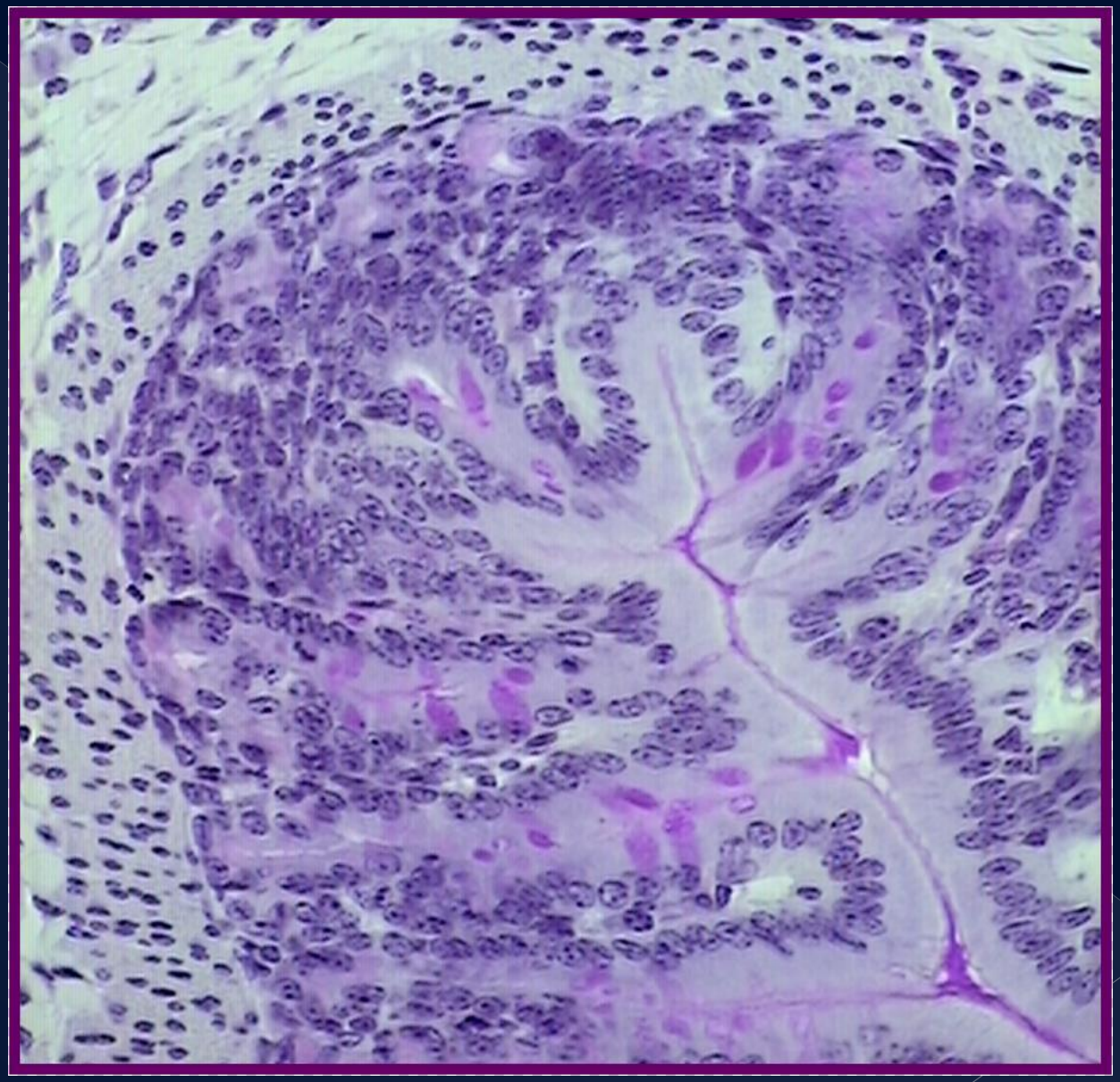
SCHIFF REAGENT

(colorless basic fuchsin)

RED COLOR OF SCHIFF REAGENT

(GLYCOGEN STORAGE DISEASES, CANCERS)

PERIODIC ACID- SCHIFF STAIN - goblet cells in intestine



HISTOENZYMOLOGY

ENZYME

(eg. alkaline phosphatase,

succinic acid dehydrogenase)

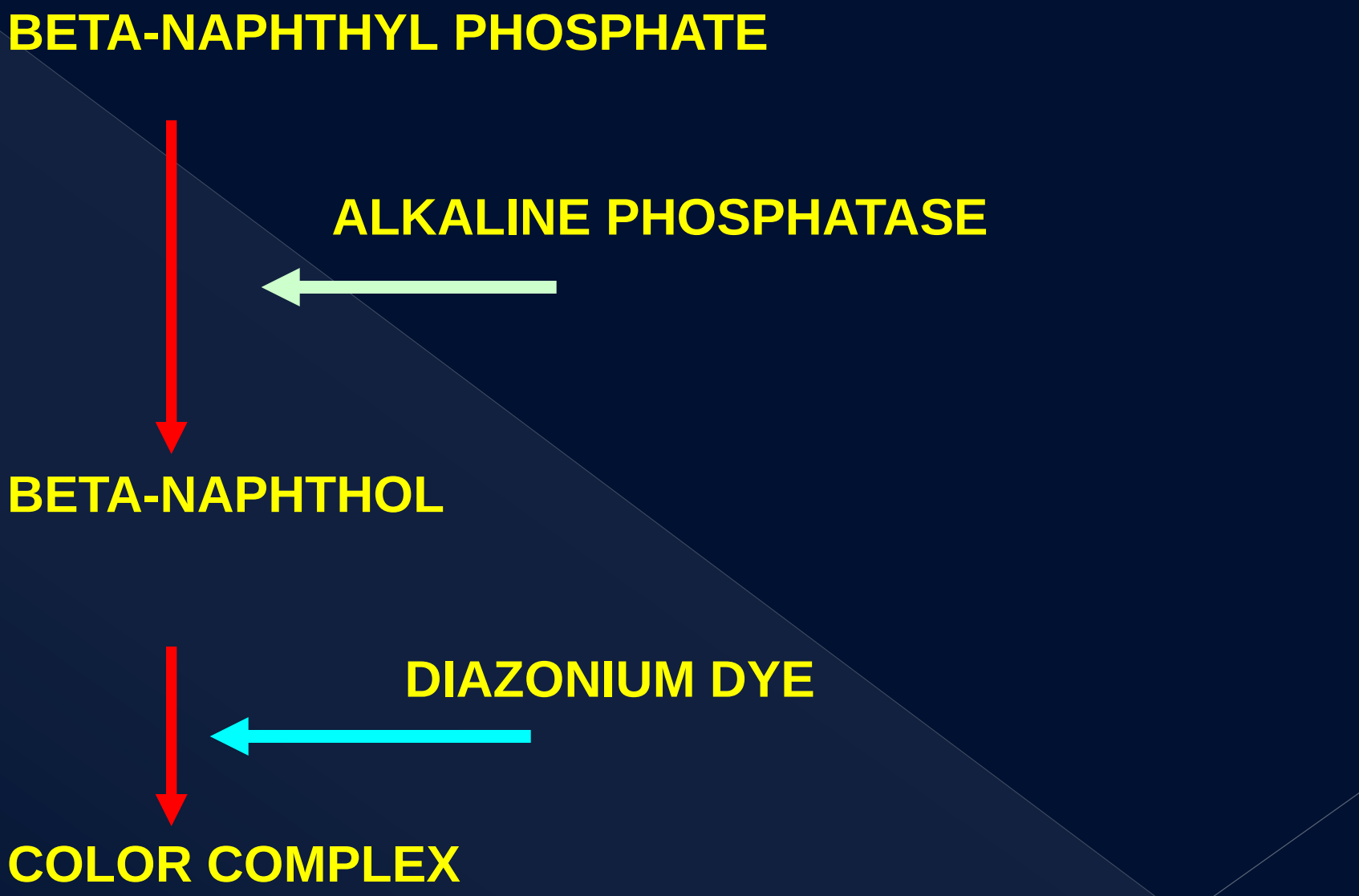
SUBSTRATE

PRODUCT

INTERMEDIATE COMPOUND

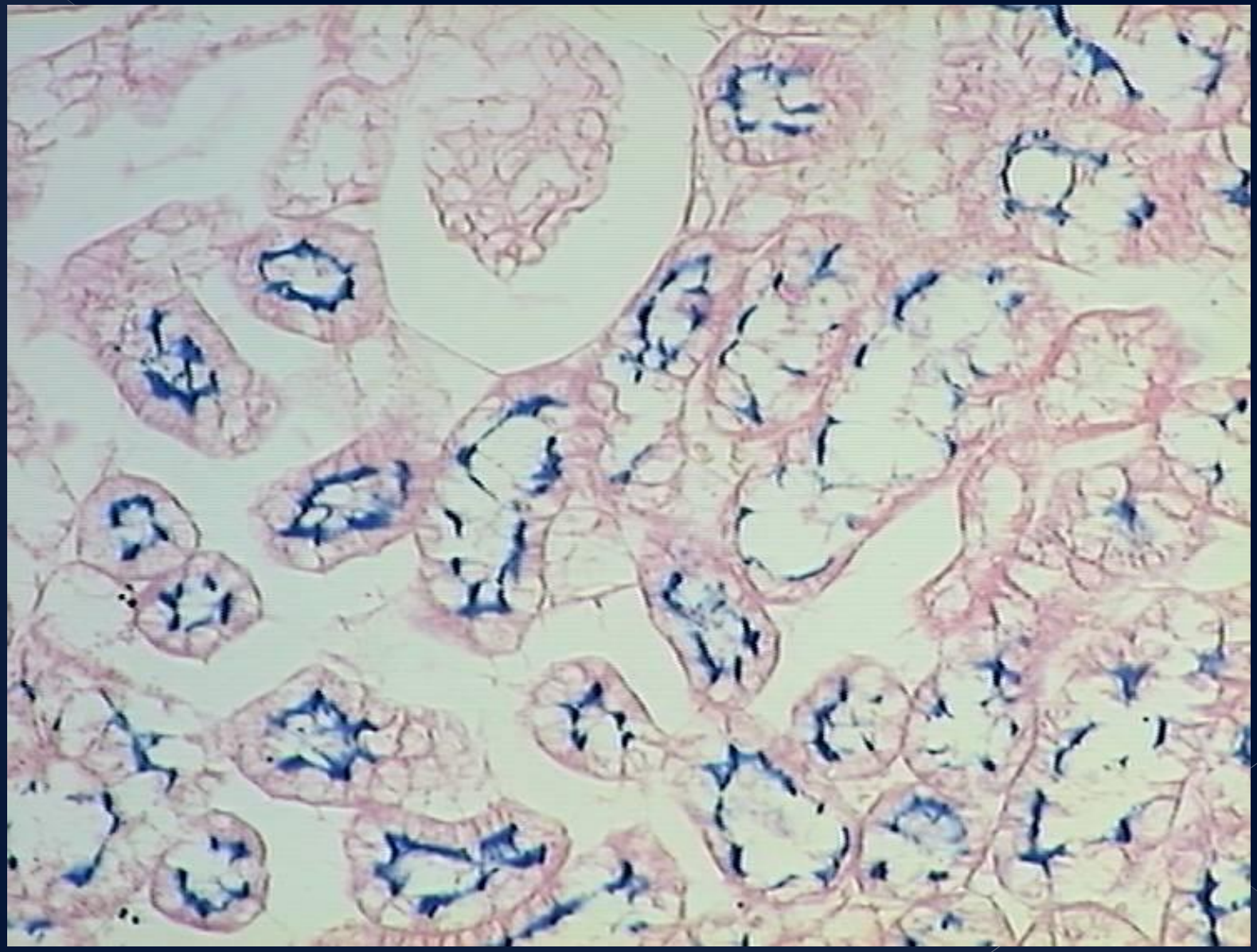
COLORFUL PRECIPITATE

(insoluble)



(TUMORS DERIVED FROM THE TROPHOBLAST, METASTASIS OF PROSTATE CANCER)

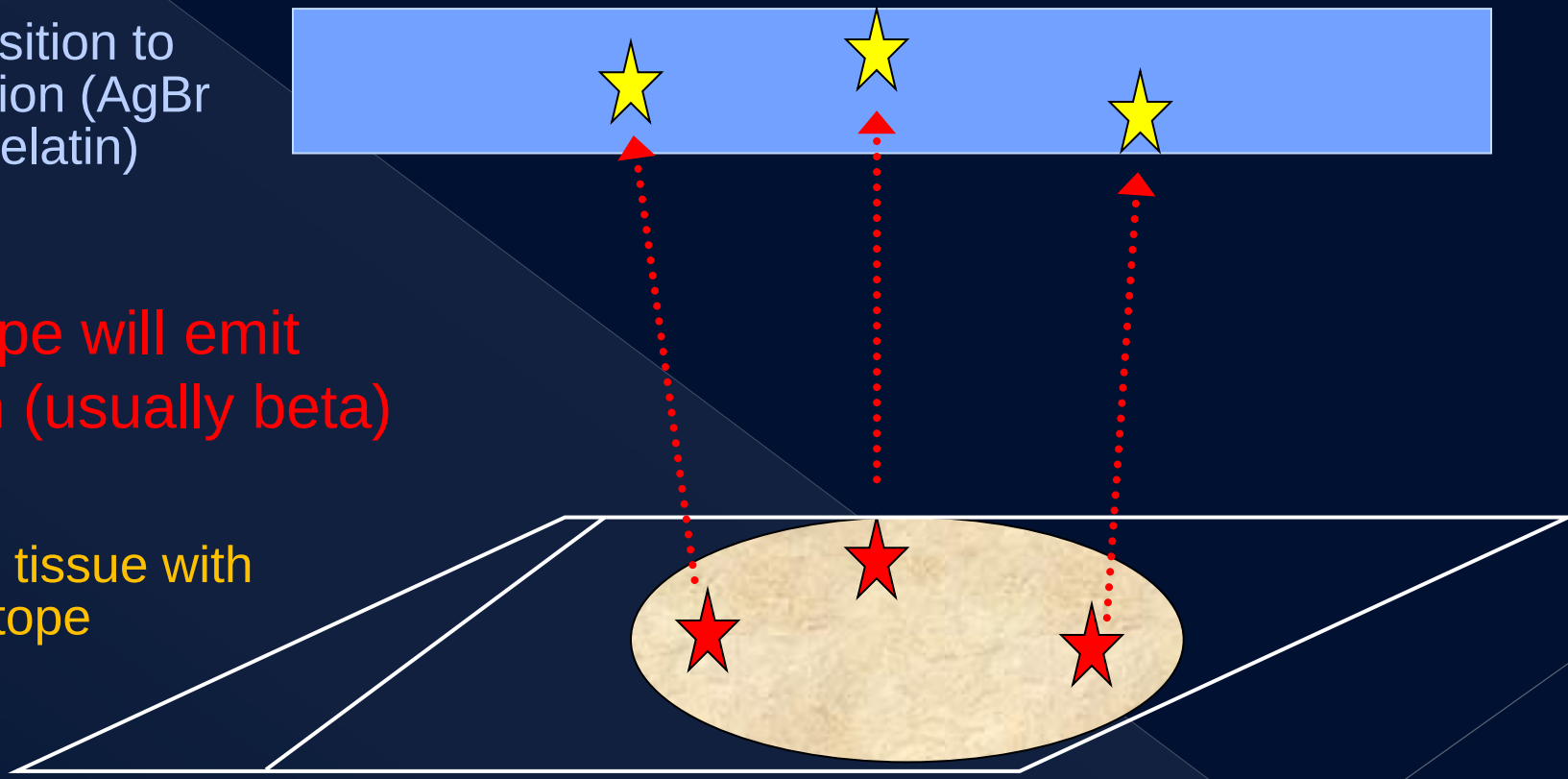
Staining for alkaline phosphatase, a marker for proximal tubule in normal kidney.



AUTORADIOGRAPHY - technique used to visualize the distribution of radioactive labeled substance with isotope in a biological sample

Radiation will hit silver grains in emulsion and expose them.
Formation of latent image; metallic silver

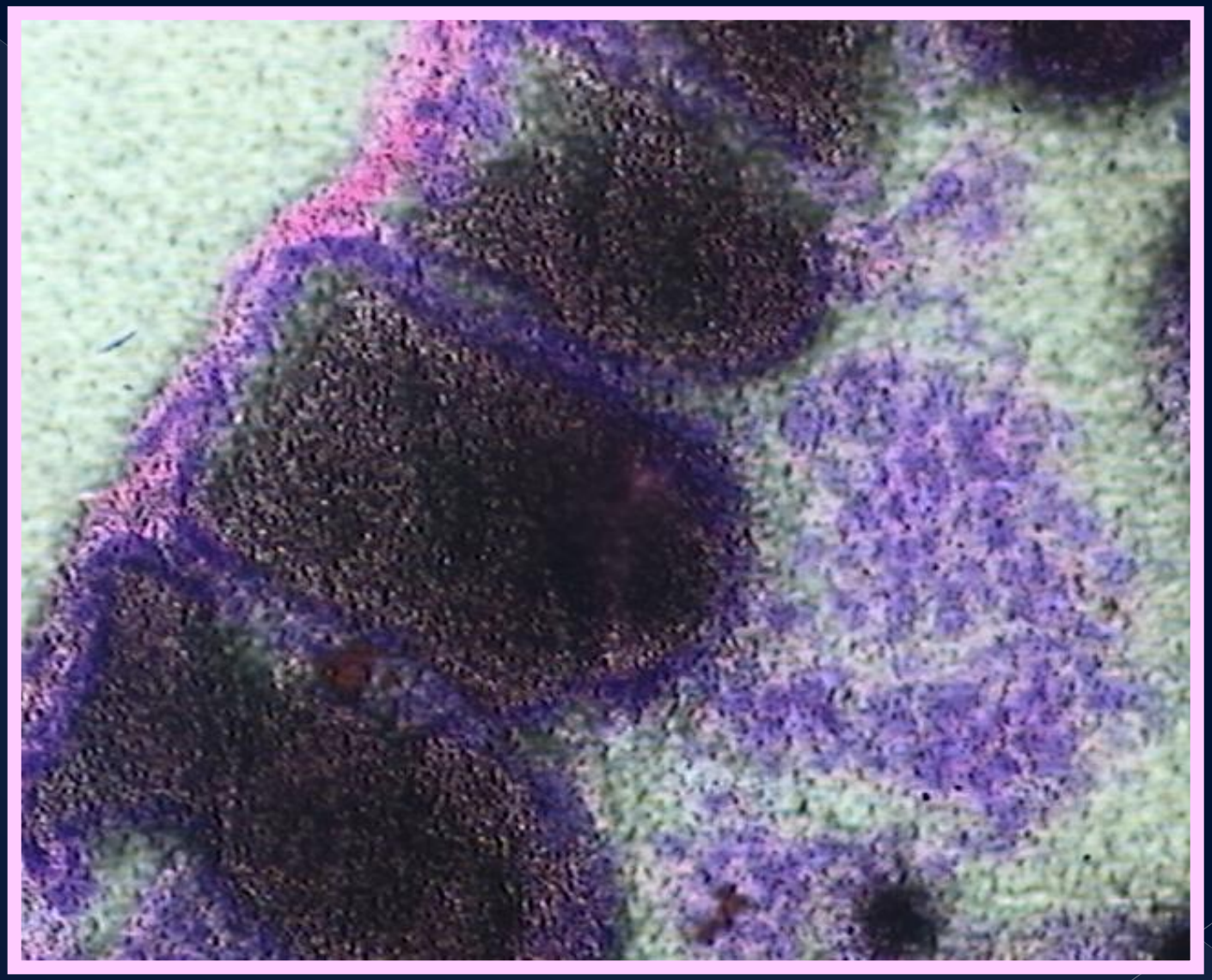
Exposition to emulsion (AgBr in gelatin)



Isotope will emit radiation (usually beta)

Incubation tissue with isotope

Photographic processing transforms the latent image into a visible image and makes this permanent. Over the place where was isotope incorporated are visible black grains of silver.



Thyroid gland- iodine^{131}

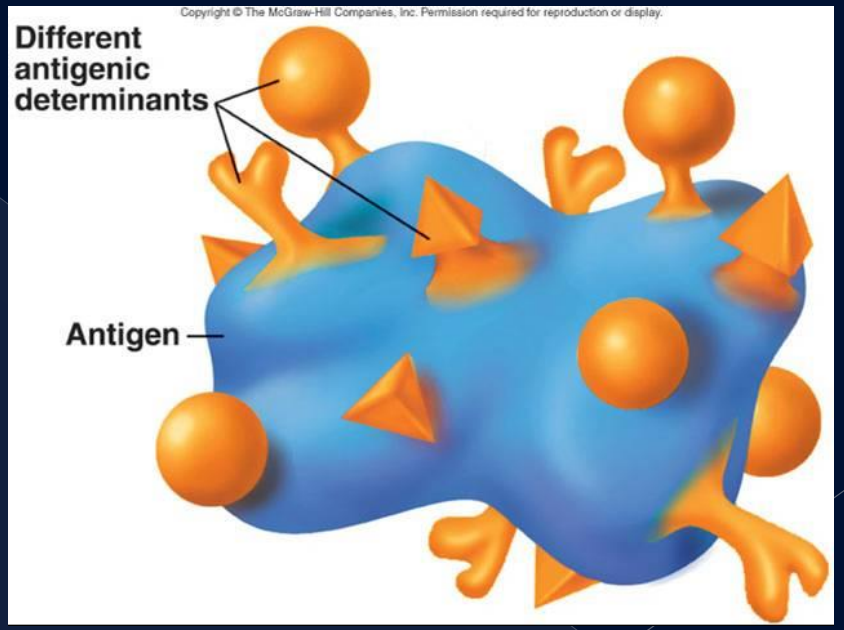
IMMUNOHISTOCHEMISTRY

Detection of the antigens present in cells and tissues using antibodies:

Monoclonal or polyclonal

$F(ab)_2$

Fc



METHODS FOR LABELING ANTIBODIES

- FLUOROCHROMES (eg. fluorescein derivative)

- METALS (eg. ferritin, colloidal gold)

- ISOTOPES (eg. ^{3}H, ^{125}I, ^{35}P, ^{14}C)

- ENZYMES (eg. alkaline phosphatase, peroxidase)

IMMUNOHISTOCHEMISTRY

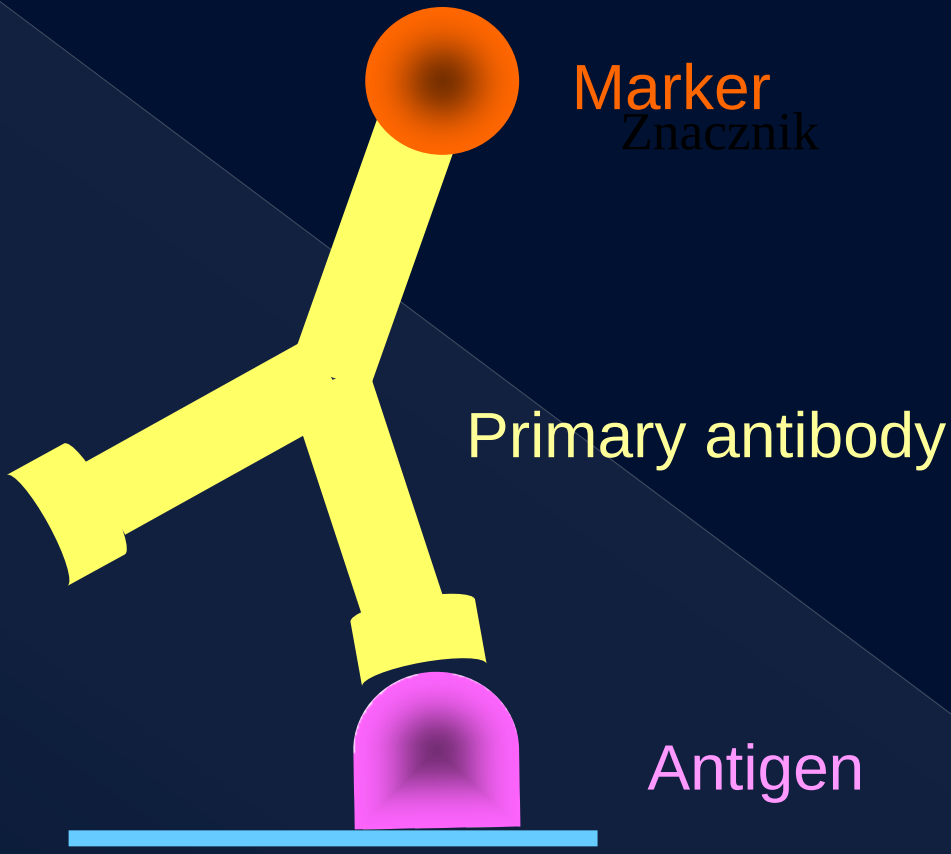
- DIRECT METHOD

-with using primary antibodies

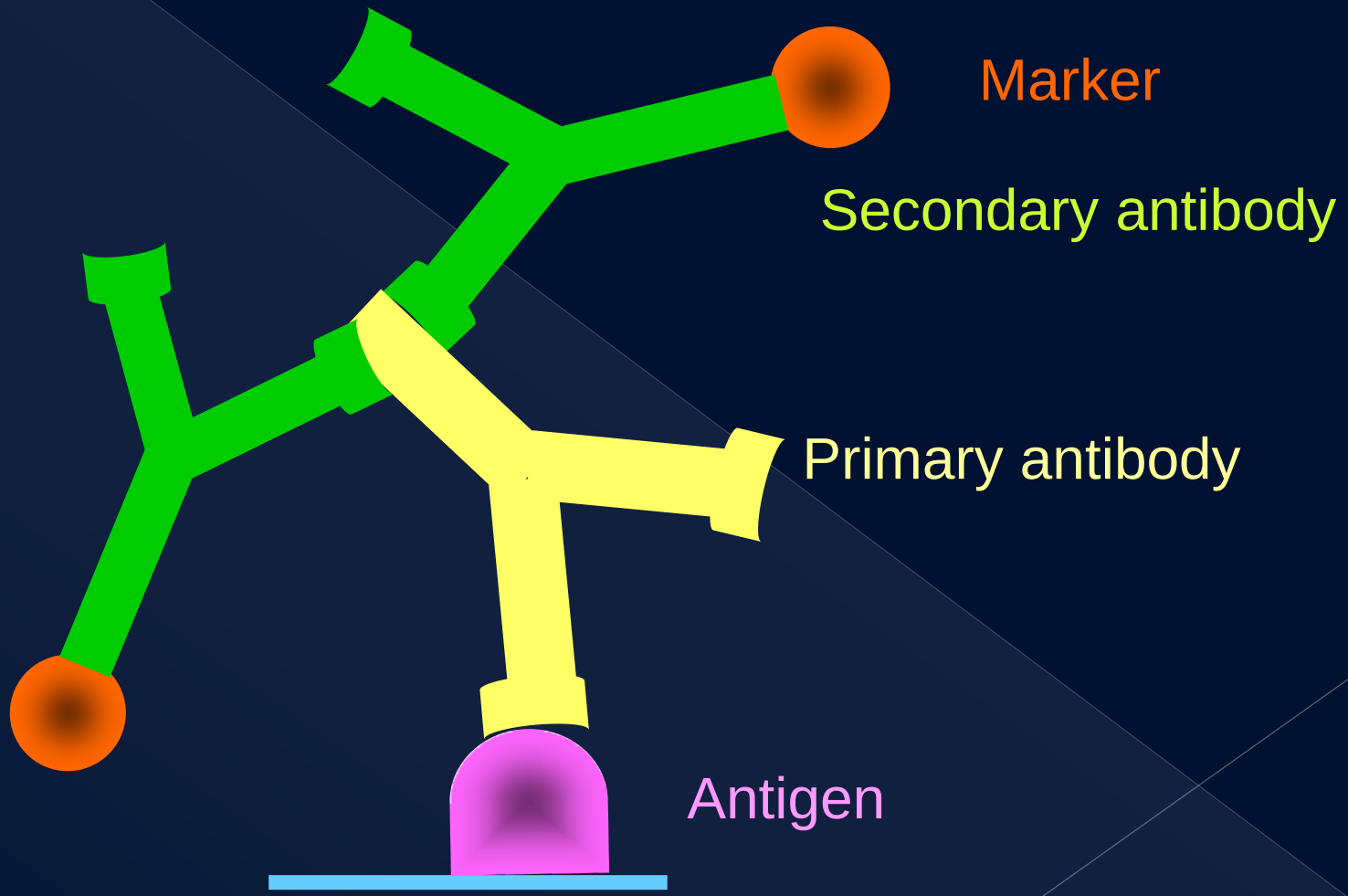
- INDIRECT METHOD

with using primary and secondary antibodies

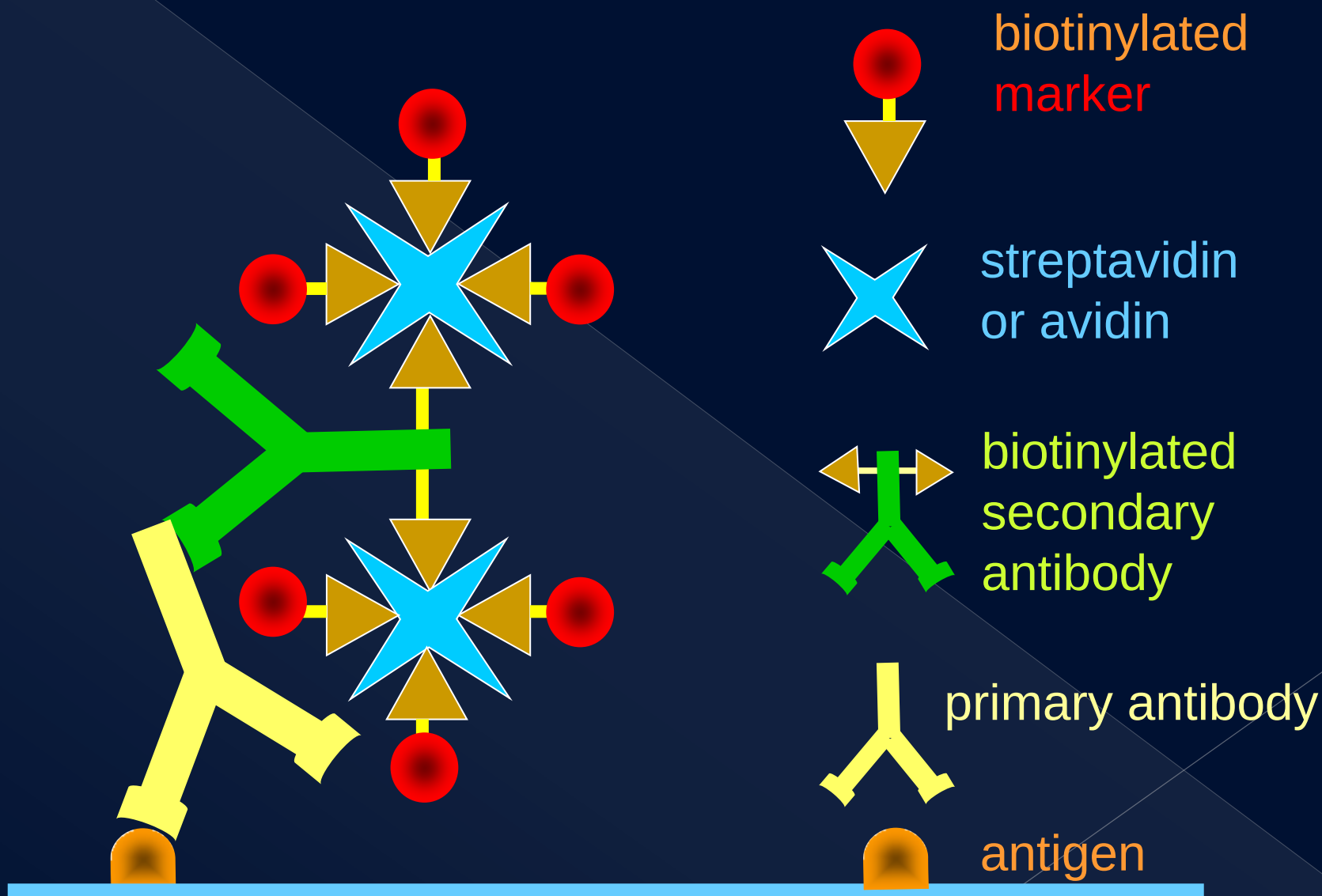
DIRECT IMMUNODETECTION



INDIRECT IMMUNODETECTION



INDIRECT IMMUNODETECTION



PEROXIDASE

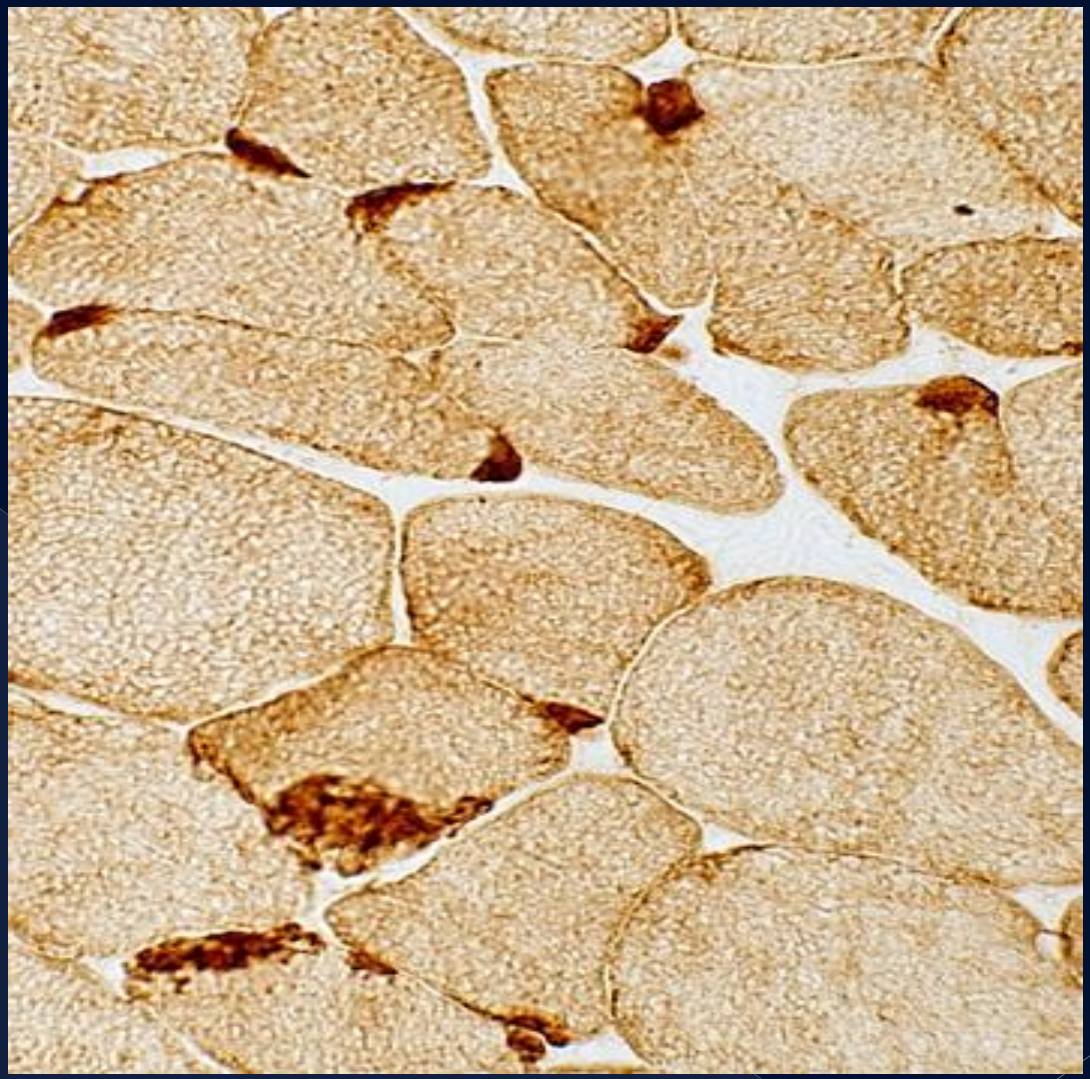
SUBSTRATE H_2O_2

Diaminobenzidine (DAB)

(chromogen)

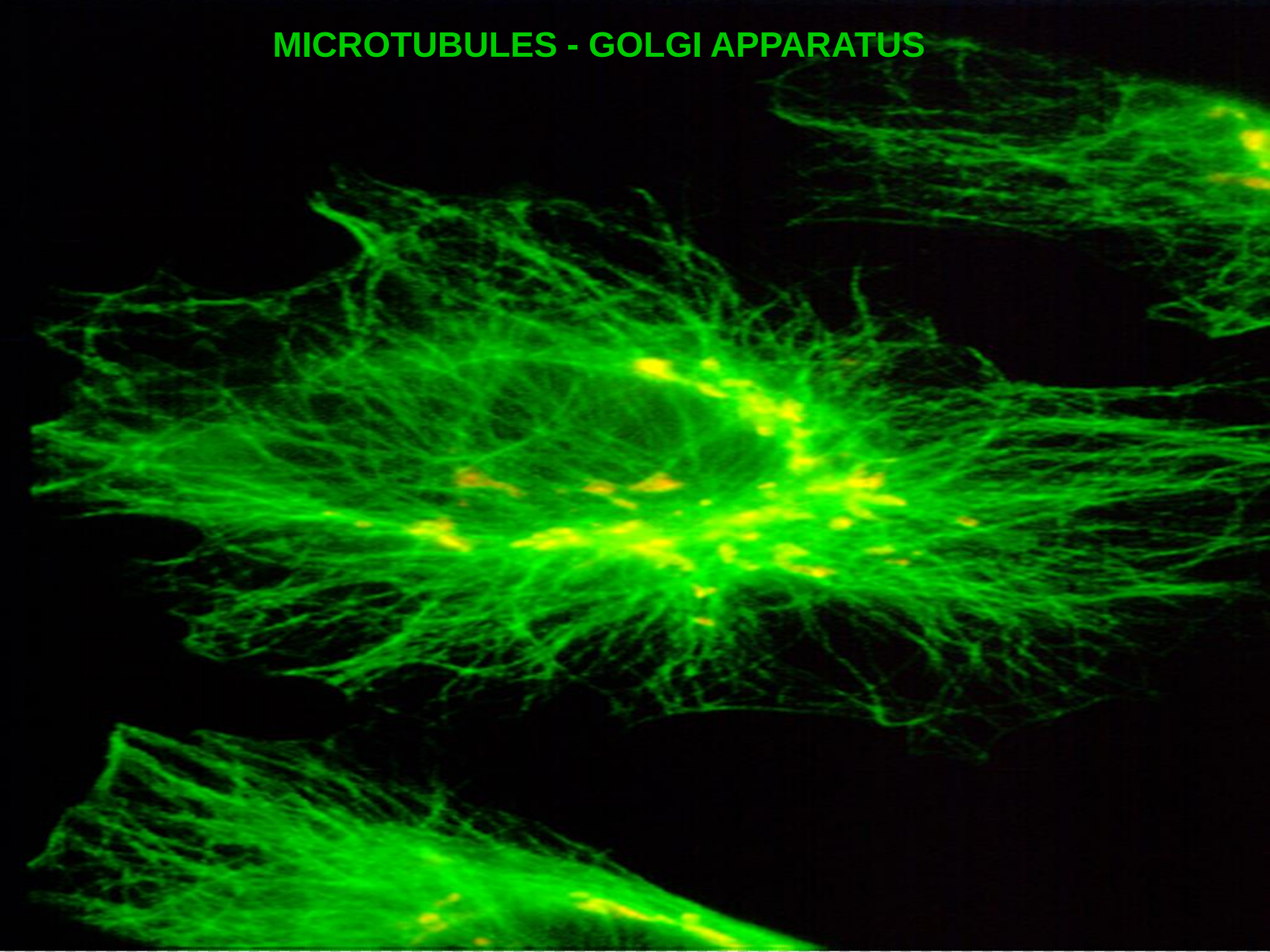
Oxidation

BROWN PRECIPITATE



Detection of desmin in skeletal muscle cells

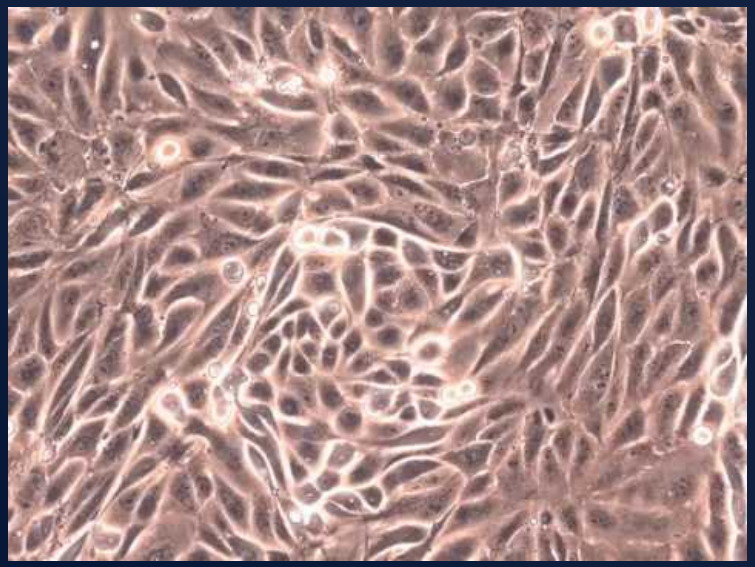
MICROTUBULES - GOLGI APPARATUS



How Cells Are Studied?

What else we can study?

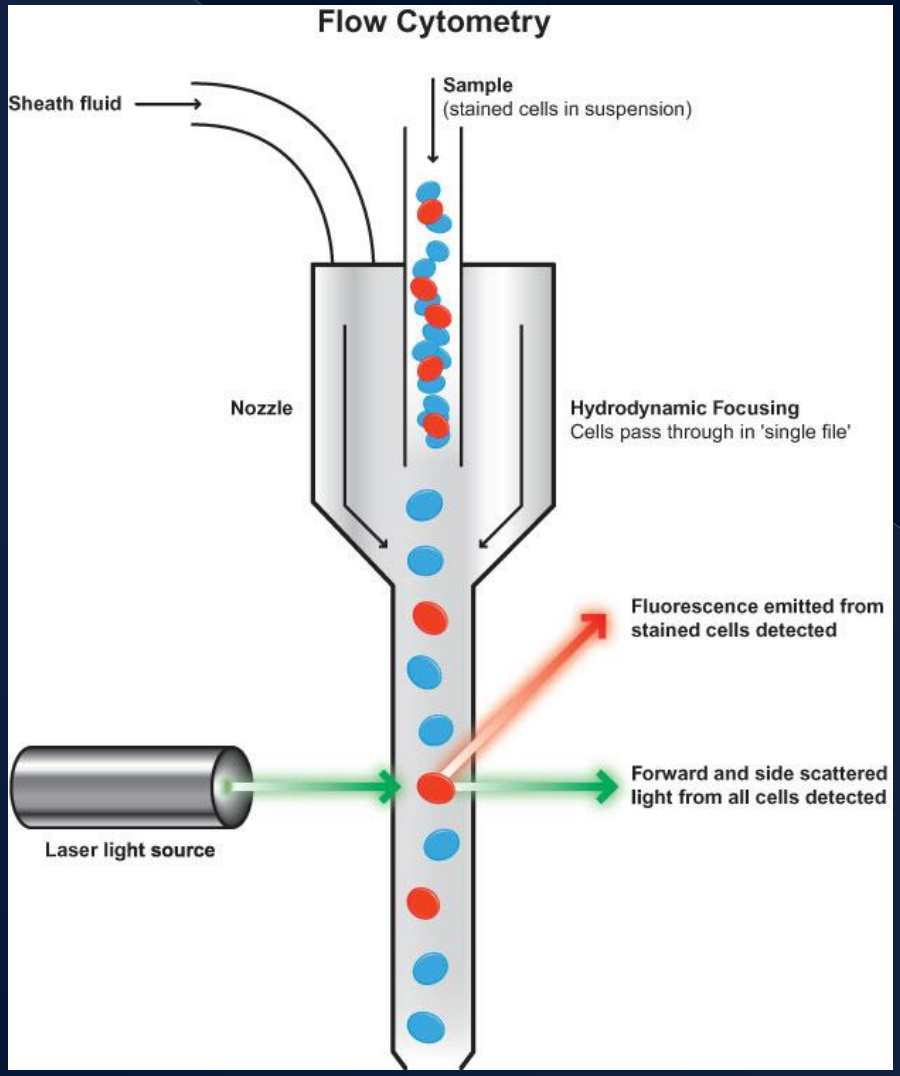
Cell/tissue culture



human kidney epithelial cell culture

Inverted microscope

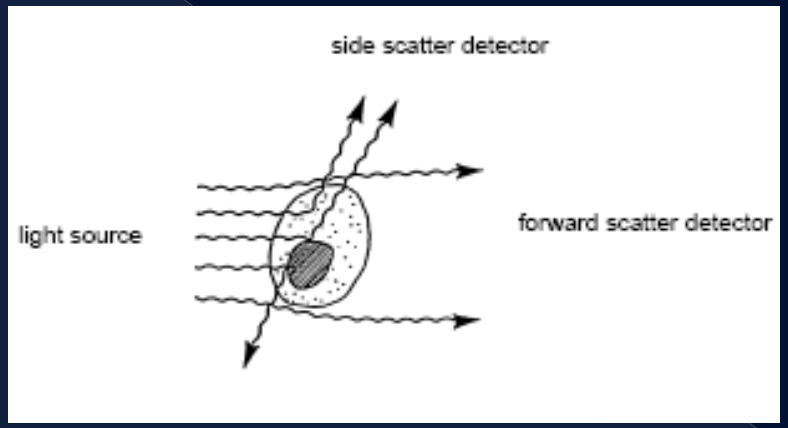
Flow Cytometry



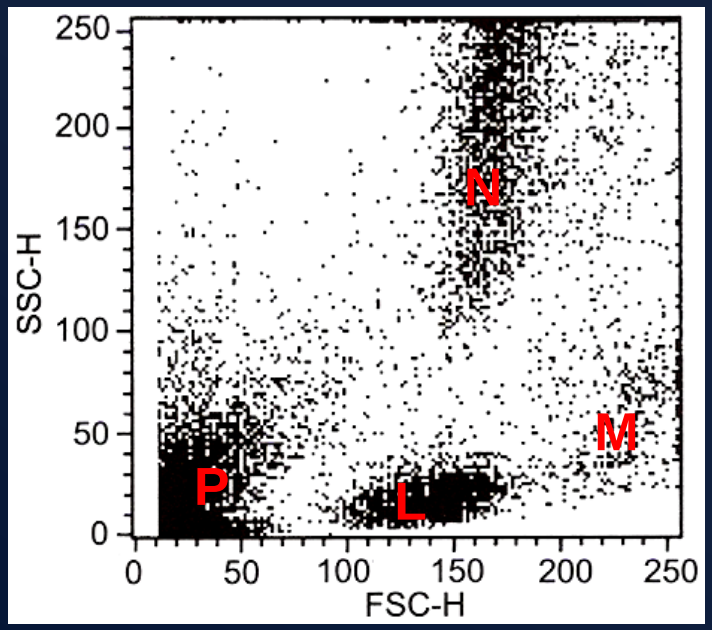
A flow cytometer has an optical system – lamps, lasers, a detector, measuring system and Analogue-to-Digital Conversion (ADC) system - which converts forward-scattered light (FSC), side-scattered light (SSC) and fluorescence signals into electrical signals that are processed by a computer.

The single cell passes through the light beam and causes its deflection (FSC) and dispersion (SSC)

Flow Cytometry



FSC – Forward-scattered light is proportional to cell-surface area or size - the greater cell the greater deflection or FSC.



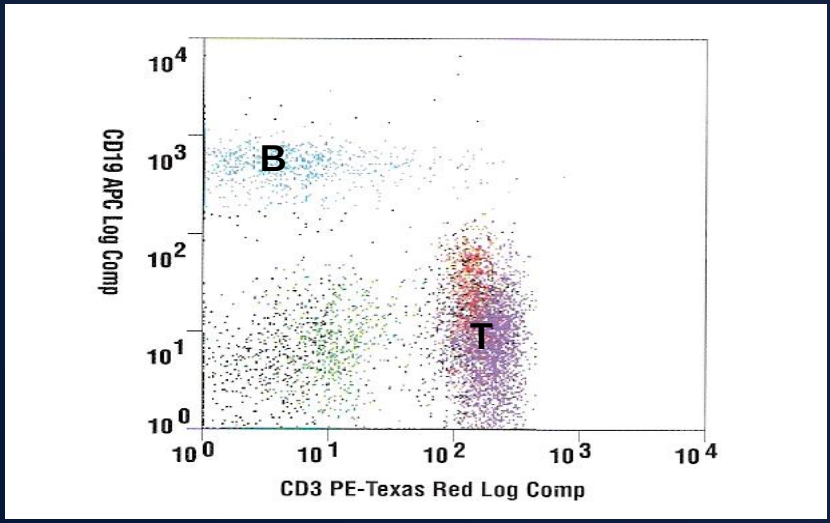
SSC - Side-scattered light is proportional to cell granularity or internal complexity. The more complicated cell the greater dispersion of light or SSC.

Human blood leukocytes FSC/SSC.

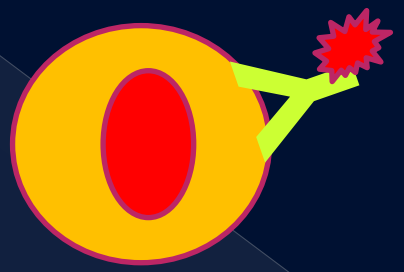
Flow Cytometry

When a fluorescent dye is conjugated to a monoclonal antibody, it can be used to identify a particular cell type based on the surface markers of the cell.

Different fluorochromes can be used to distinguish subpopulations. The staining pattern of each subpopulation, combined with FSC and SSC data, can be used to identify cells.



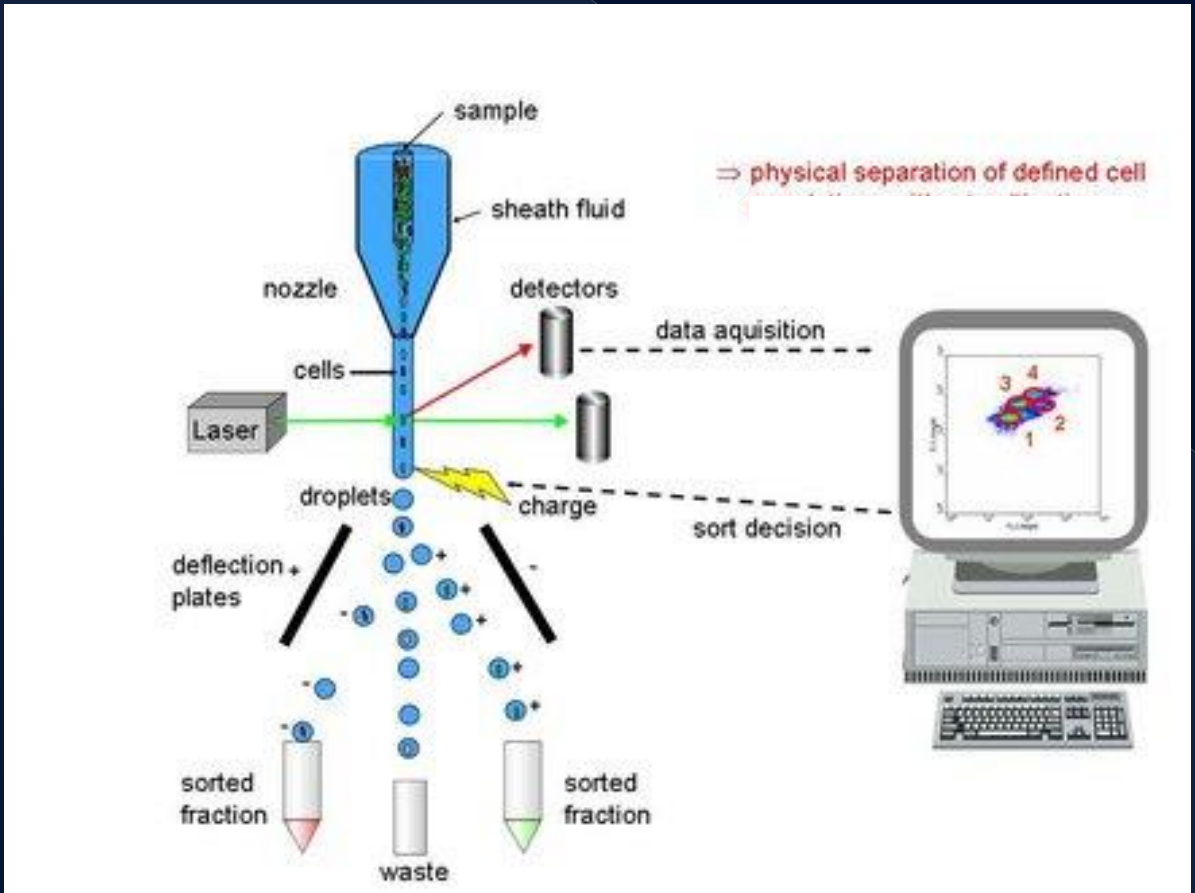
Anti-CD3 and anti-CD19.



Antibody with fluorochrome

SORTING

Sorting allows us to capture and collect cells of interest for further analysis. Once collected, the cells can be analyzed microscopically, biochemically, or functionally.



Cytometer first needs to identify the cells of interest, then separates out the individual cells.

FLOW CYTOMETRY APPLICATIONS

DIAGNOSIS:
proliferative diseases of the hematopoietic and lymphatic system
congenital and acquired immunodeficiency
autoimmune diseases
analysis of cells for bone marrow transplantation

MONITORING:
- the treatment of leukemias
- the immune system of HIV patients
- immunosuppressive treatment of transplanted patients

How Cells Are Studied?

What we can study?

Organelles

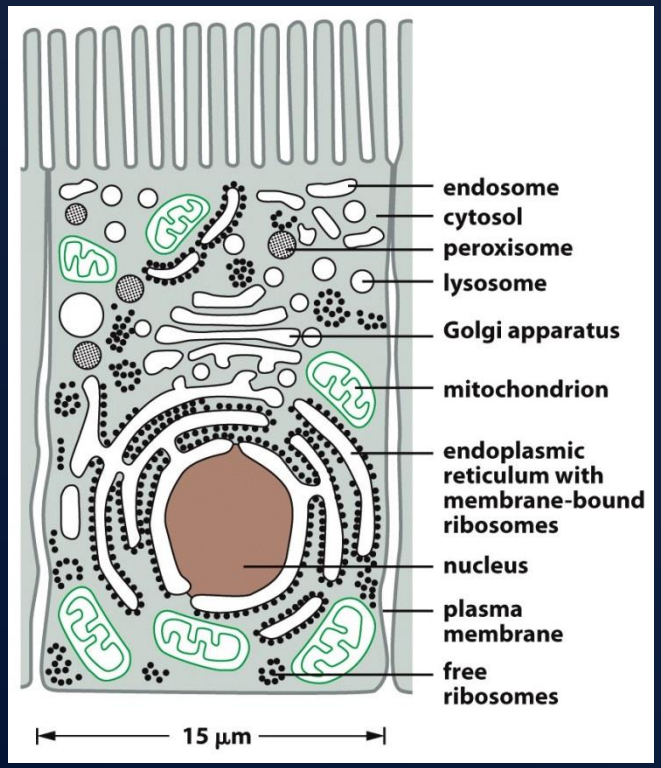
Proteins

Saccharides

Nucleic Acids

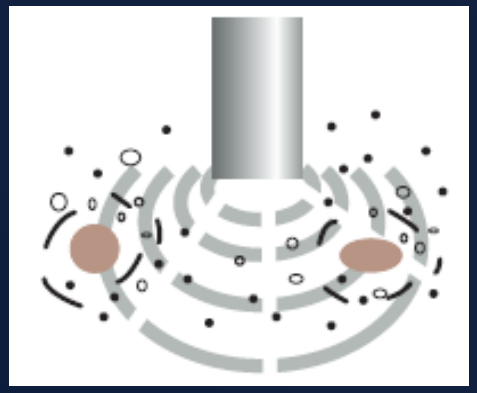
Lipids

Cell Ingredients:

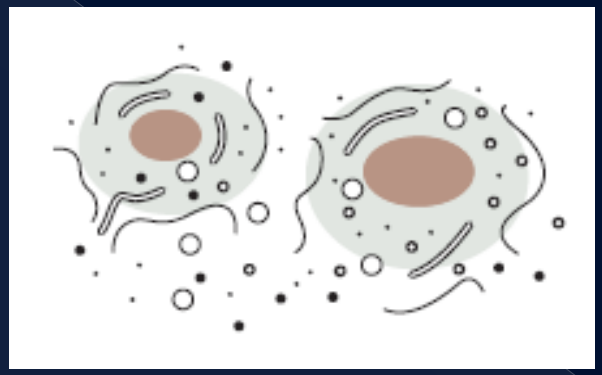


How Cells Are Studied?

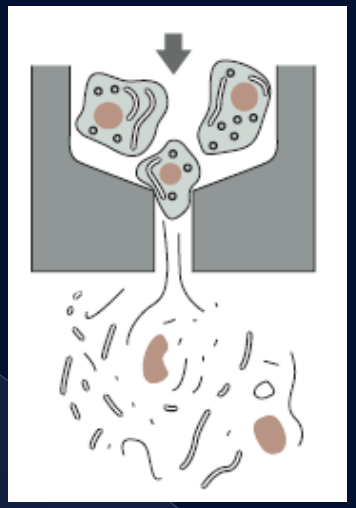
Material Isolation

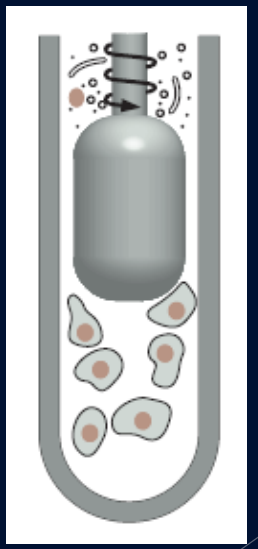


Ultrasonic cell disruption



The breakdown of cell membranes by detergents

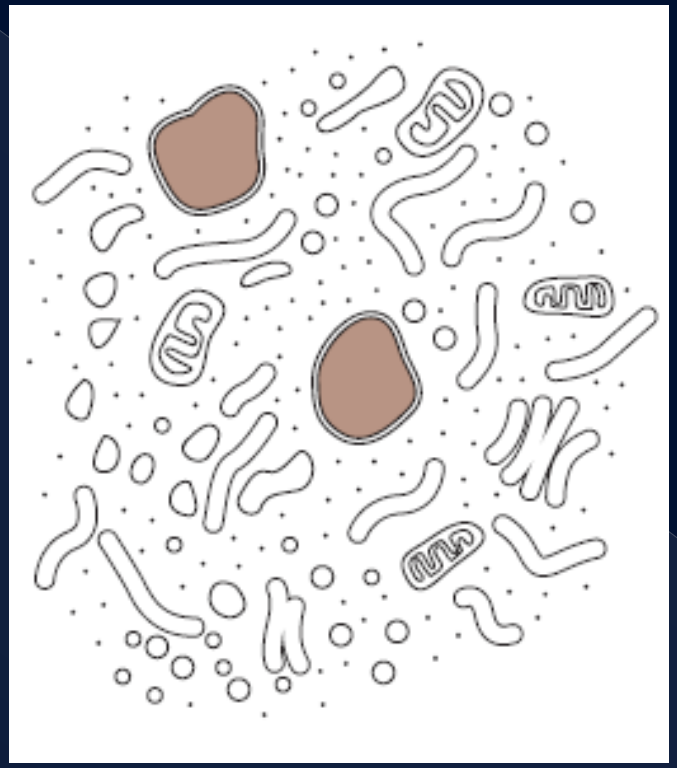




Mechanical disruption

How Cells Are Studied?

Material Isolation

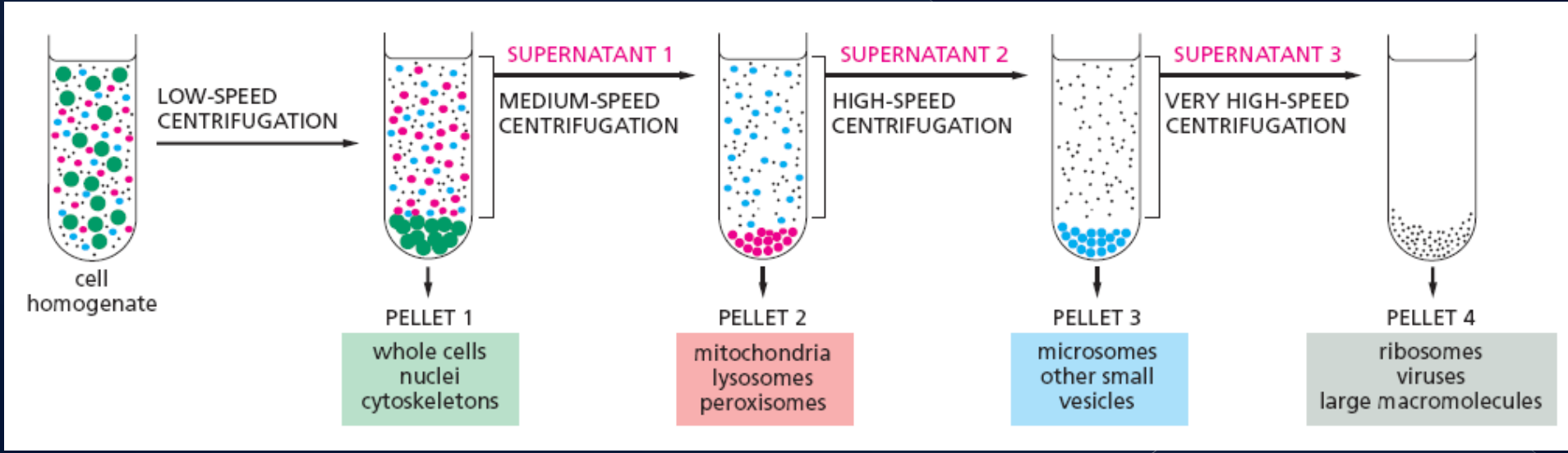


Cell homogenate

How Cells Are Studied?

Fractionation

Differential Centrifugation
is based on differences in size and density, with larger and denser particles pelleting at lower centrifugal forces.



Protein analysis

WESTERN BLOT

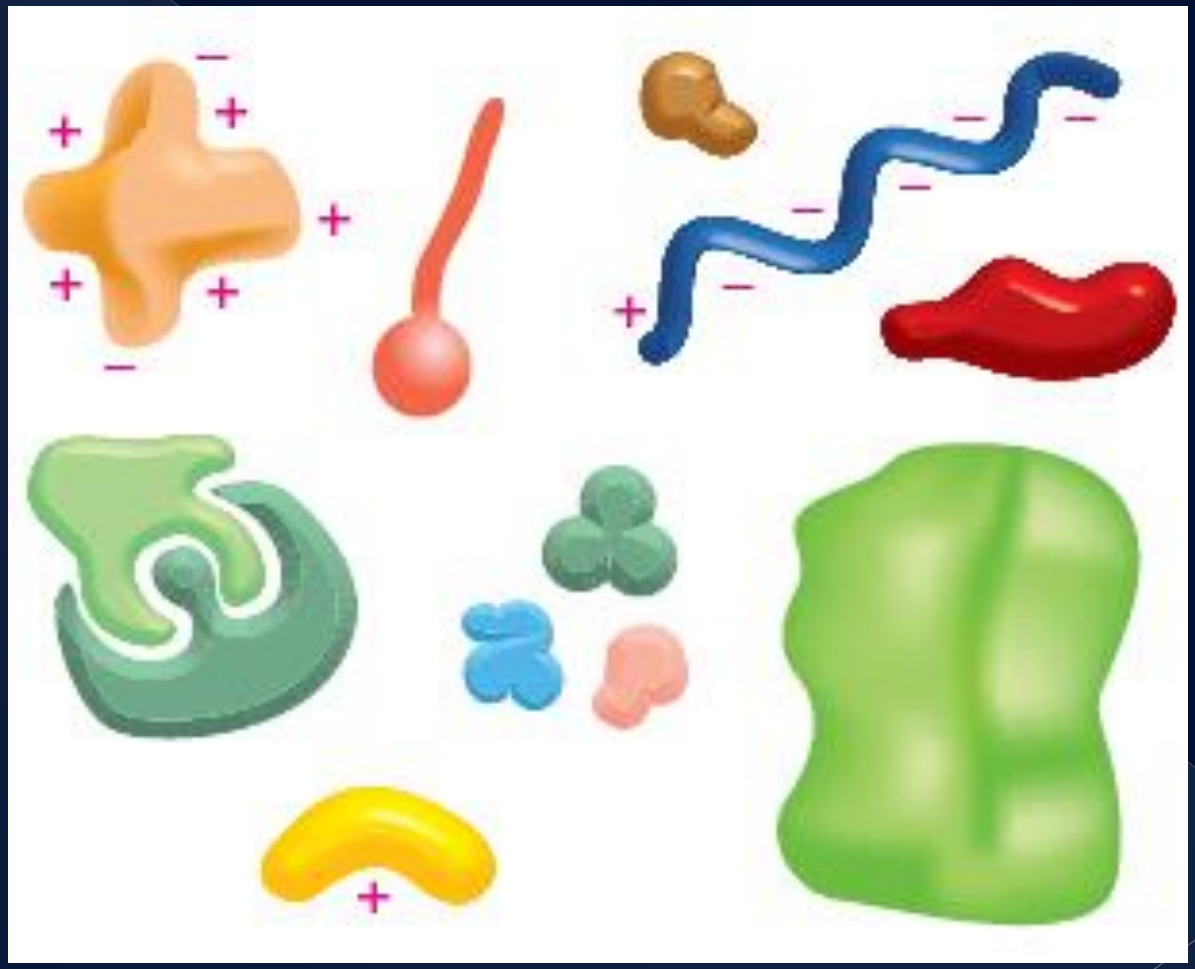
Method used to detect specific proteins in a sample of tissue homogenate, cellular extract or in body fluid.

W. Neal Burnette - American biochemist published a paper that described a technique called Western blotting (1981)

“I am happy to have done it and made a contribution to science that everybody uses. I could have never imagined that I would have my 15 minutes of fame last this long.”

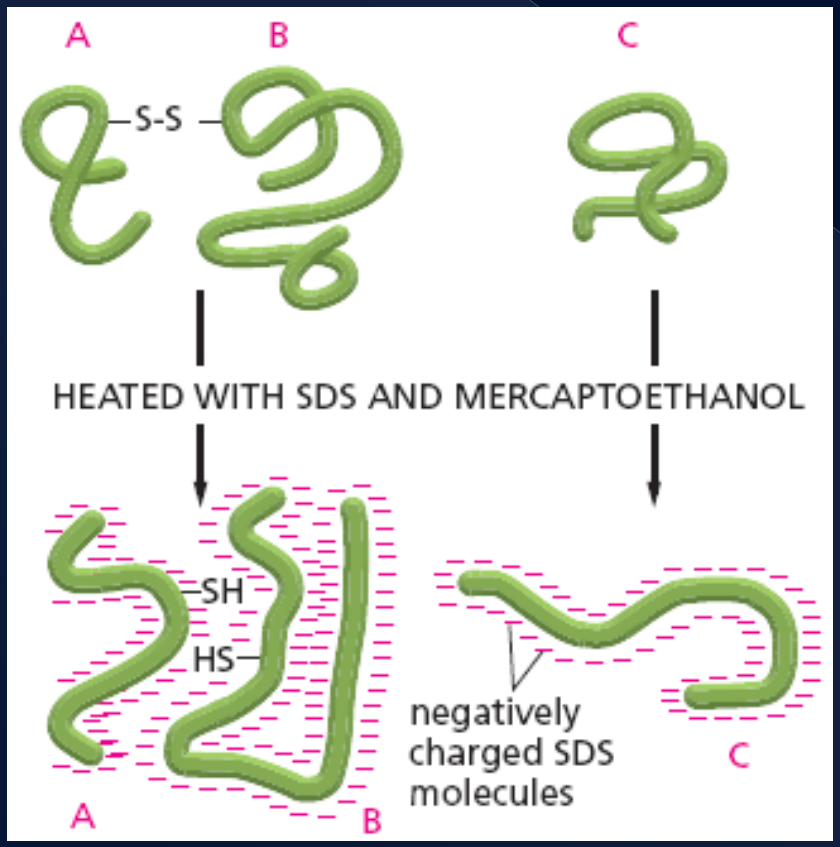
Protein analysis

Separation and detection



Protein analysis

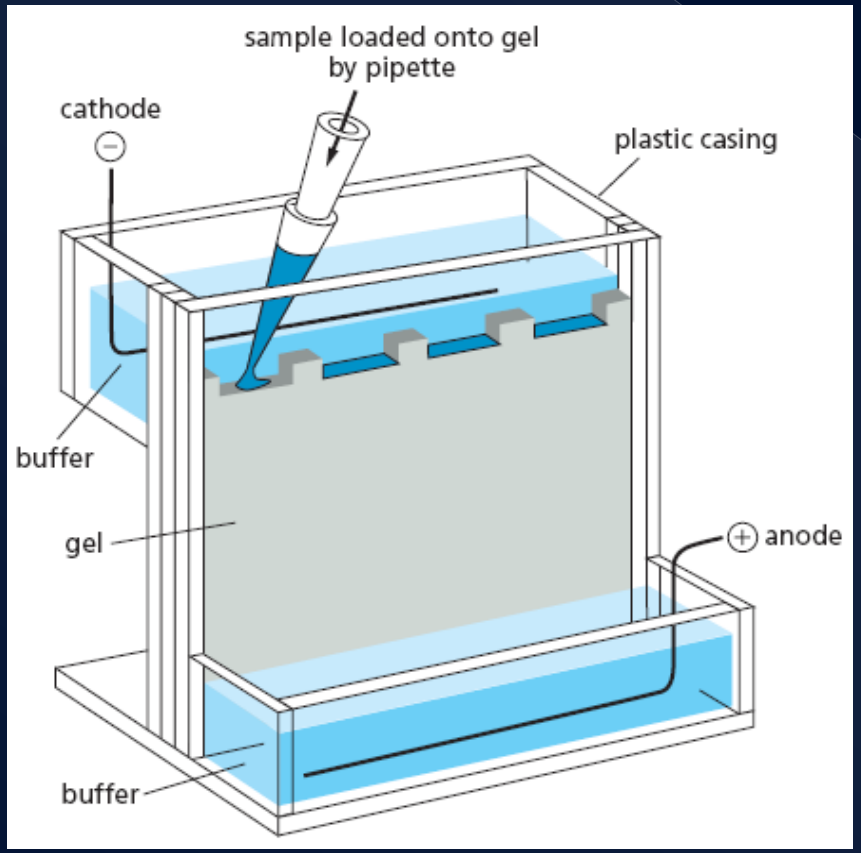
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis



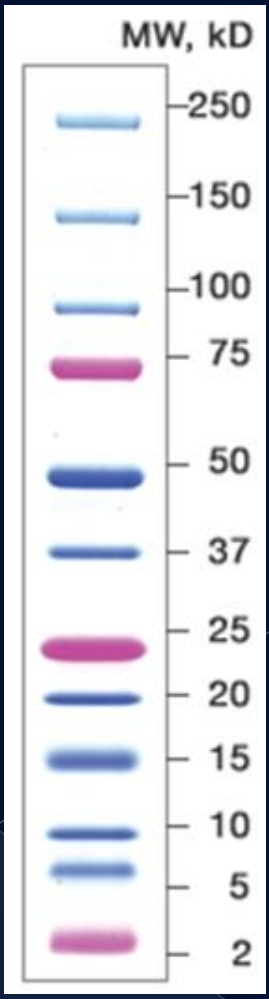
Proteins in the sample are dissolved and heated in buffer with SDS, 2-ME and bromophenol blue. The negatively charged detergent SDS (anionic surfactant) unfolds proteins and coats them with a uniform negative charge density; disulfide bonds (S-S) are reduced with mercaptoethanol. Bromophenol blue stains the sample.

Protein analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis

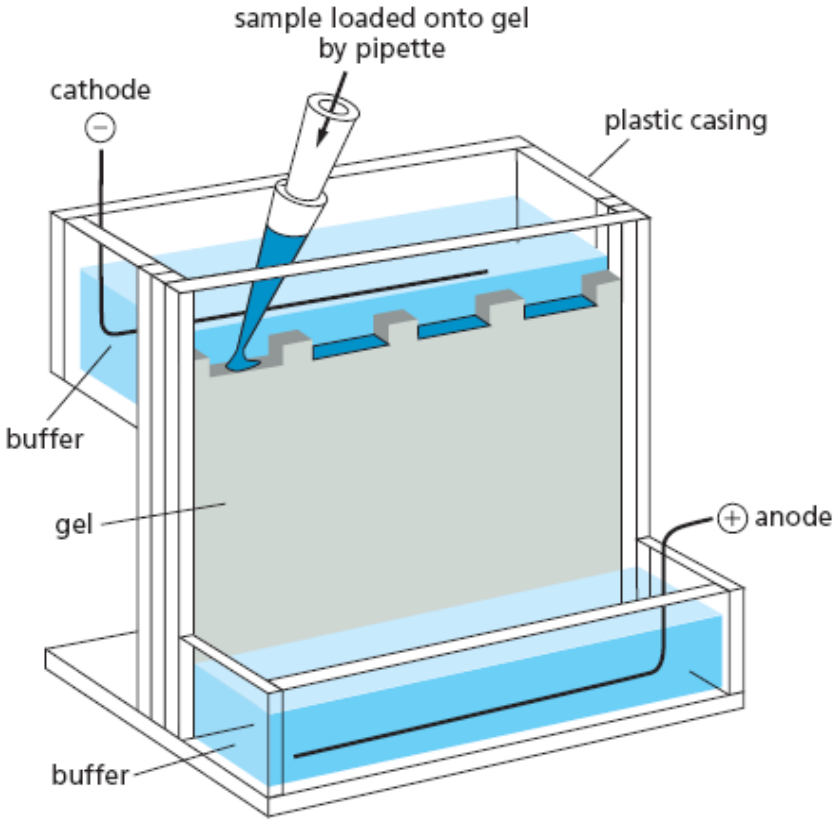


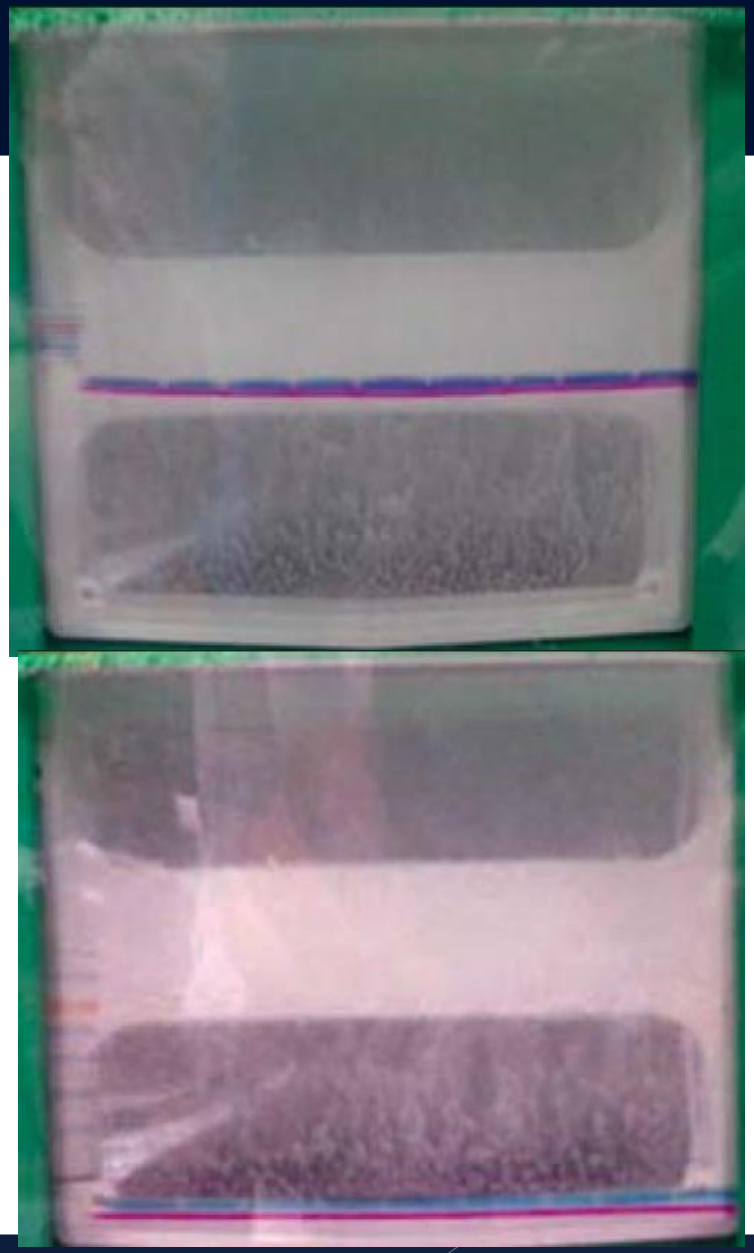
The sample is loaded to the well of a gel, and a voltage is applied. The negatively charged detergent-protein complexes migrate to the bottom of the gel, toward the positively charged anode. Small proteins move faster, larger proteins move slower, so proteins are separated by size, smaller toward the bottom and larger toward the top.



Protein analysis

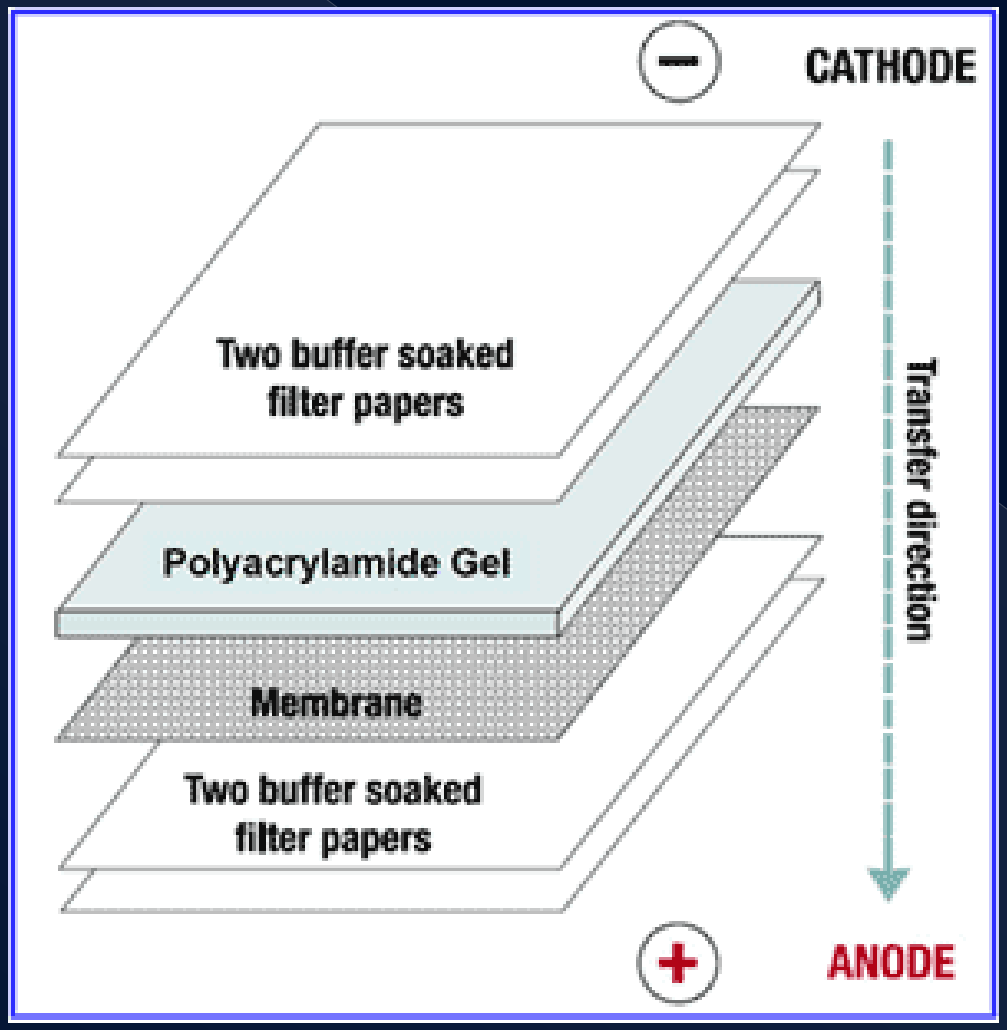
Electrophoresis





Protein analysis

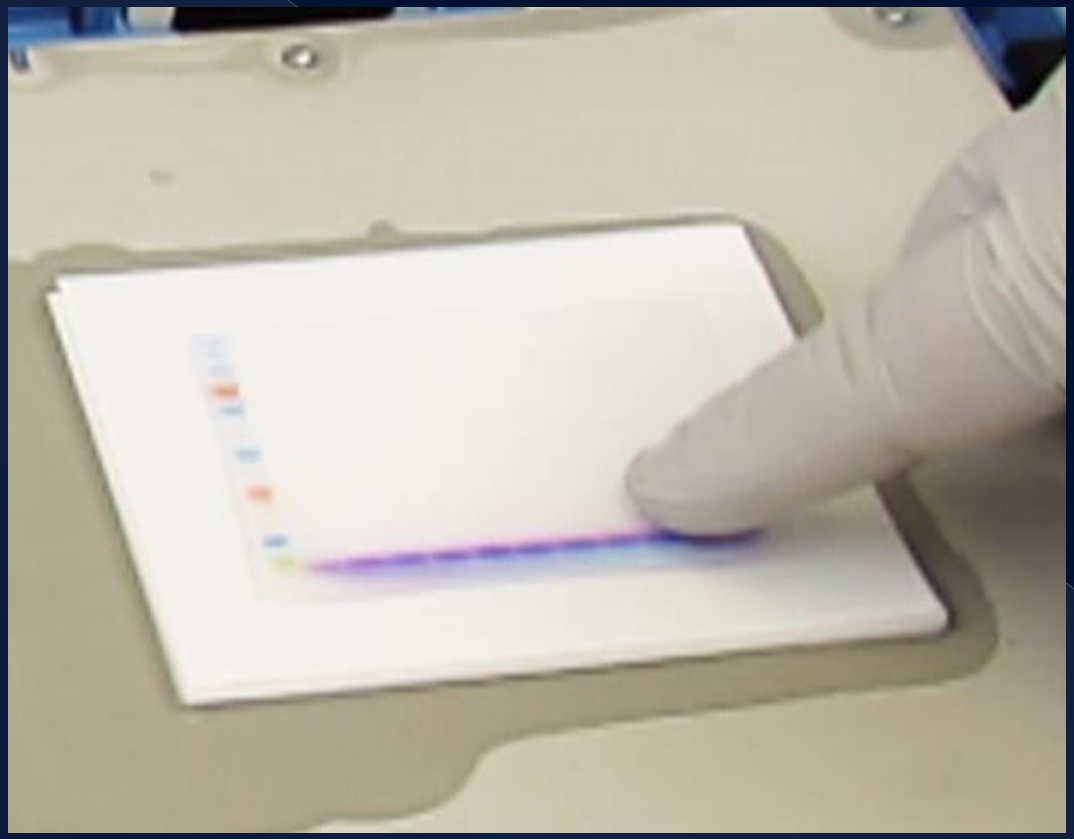
TRANSFER PROTEINS FROM GEL TO MEMBRANE



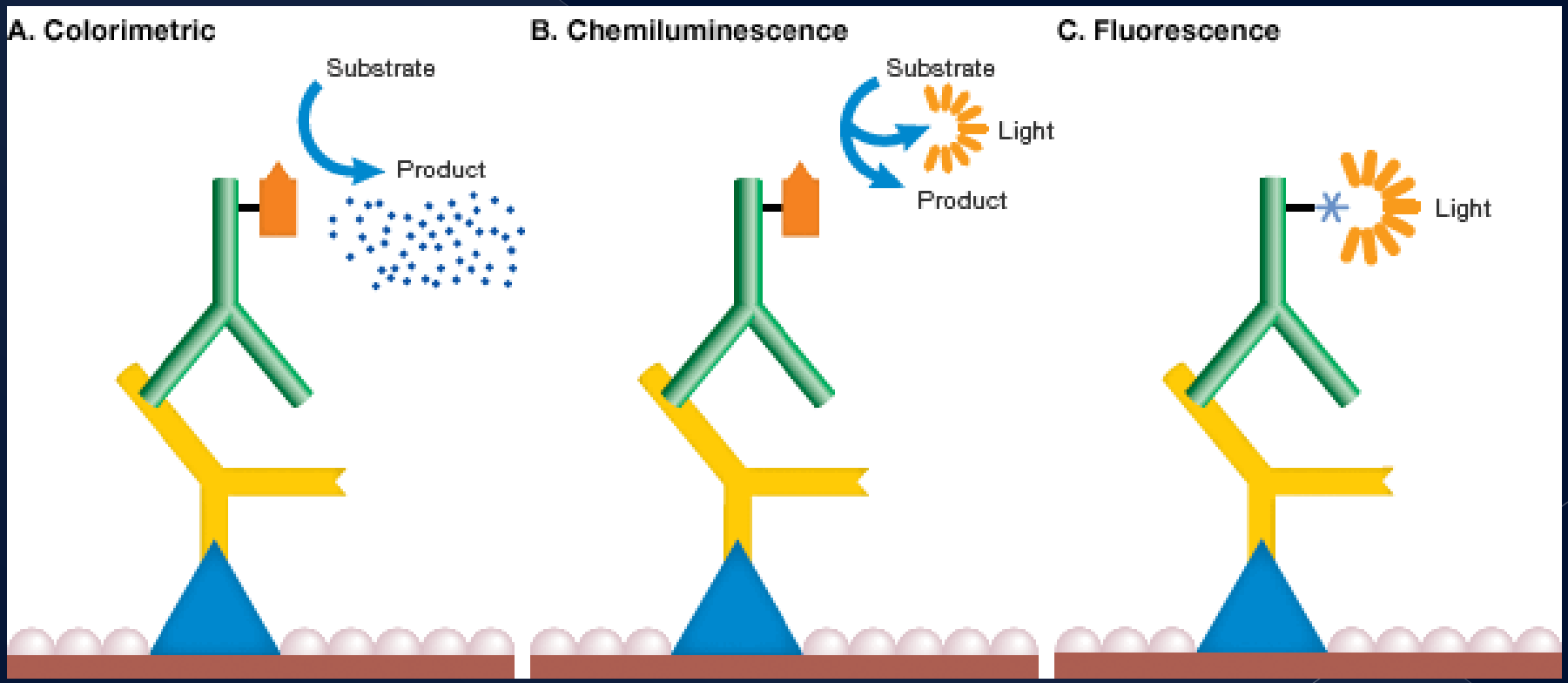
The gel with the resolved set of proteins is then placed in an apparatus that permits electrophoretic transfer of the proteins from the gel onto the surface of a special paper (e.g., nitrocellulose paper, polyvinylidene fluoride (PVDF) membrane) to which proteins strongly adsorb.

Protein analysis

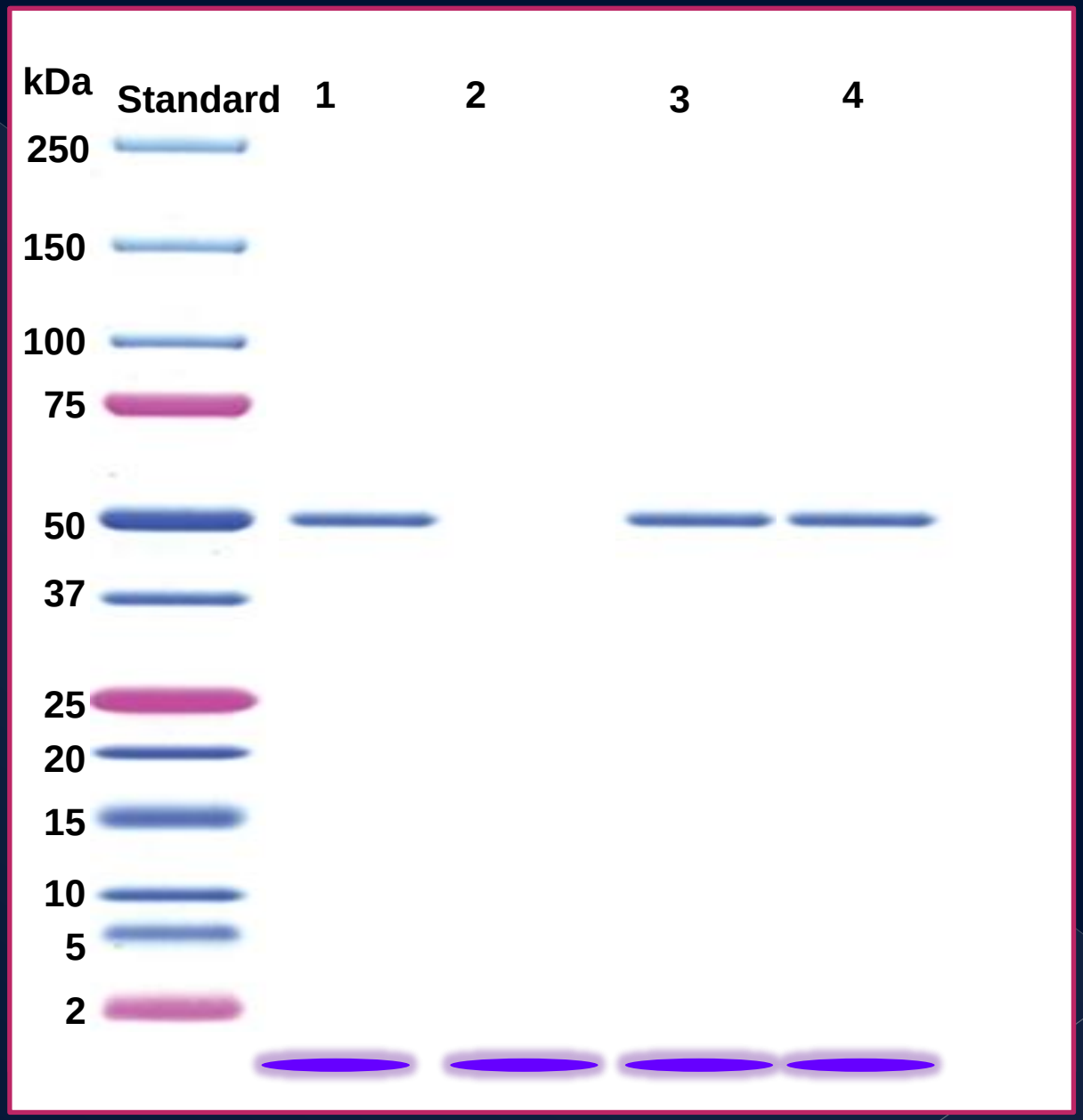
WESTERN BLOT – PVDF MEMBRANE AFTER TRANSFER



WESTERN BLOT - METHODS OF DETECTION



WESTERN BLOT - THE RESULT



Protein analysis

ELISA – detection and quantification of specific protein using antibodies conjugated with enzymes

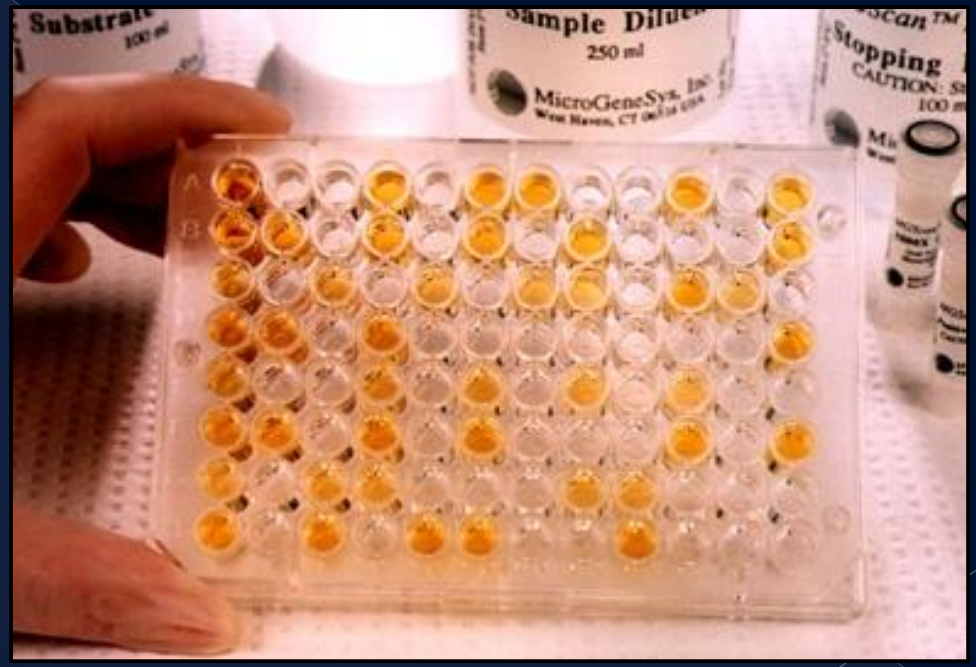
1966 - fixation of antibody or antigen to the surface of a container - Wide and Jerker Porath (Uppsala University)
1971 - Peter Perlmann and Eva Engvall at Stockholm University invented ELISA

Enzyme

Linked

Immunosorbent

Assay

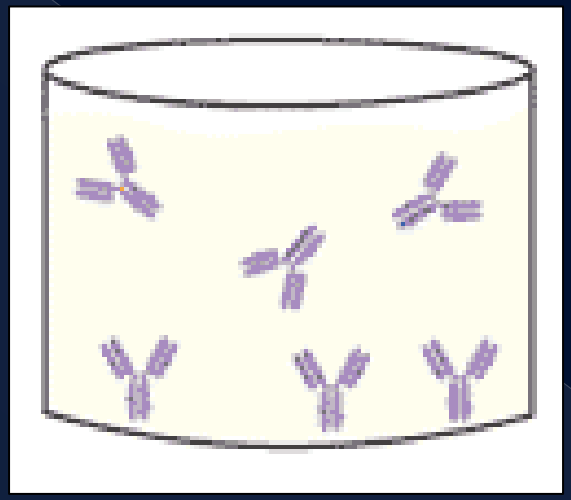


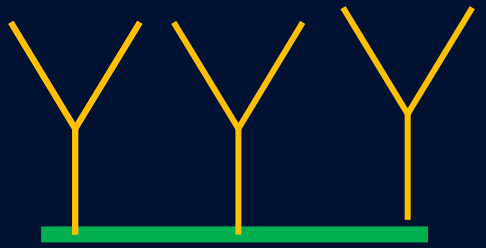
Protein detection and quantification

Protein analysis

ELISA

96-well plate

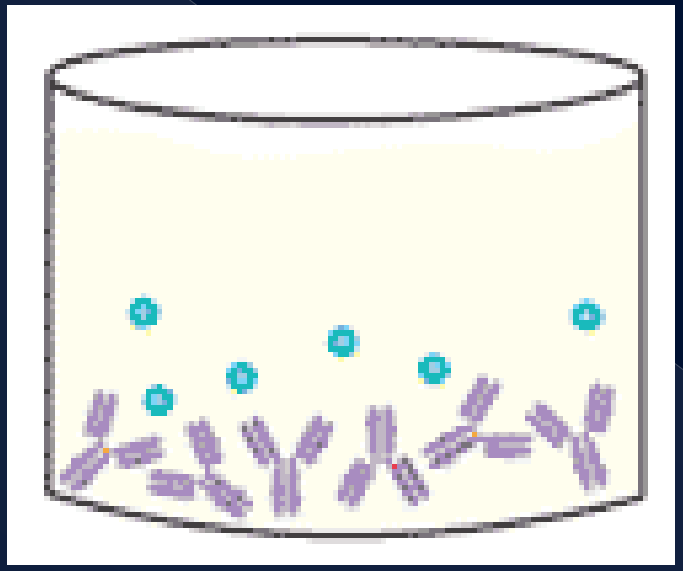


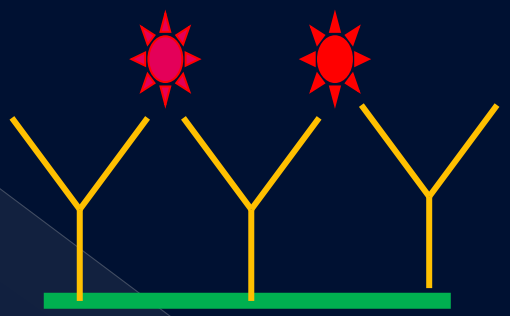


Specific capture antibody coating

Protein analysis

ELISA

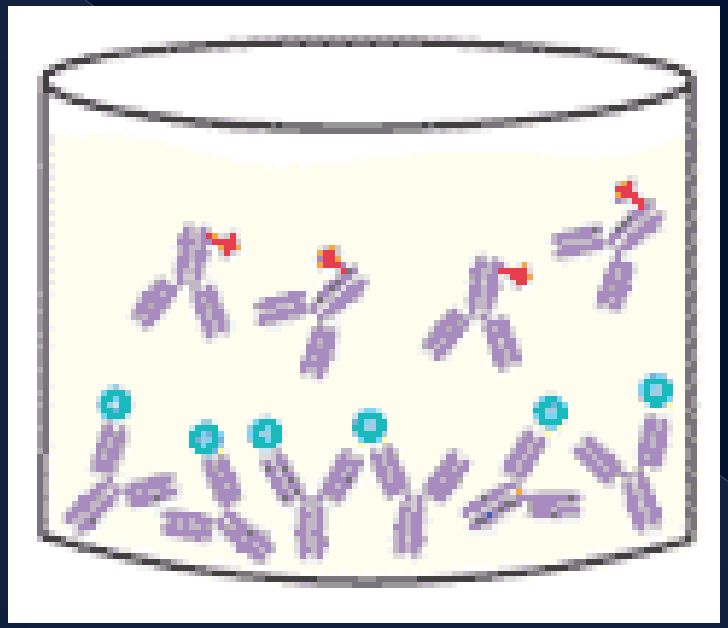


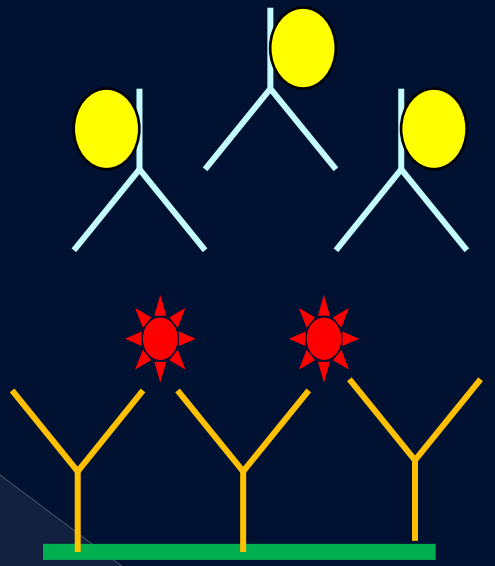


Sample with target protein

Protein analysis

ELISA

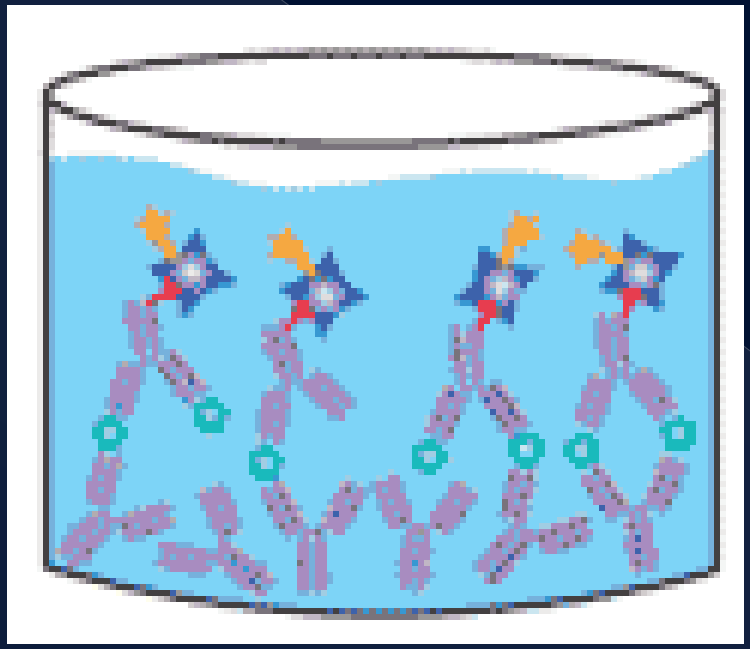


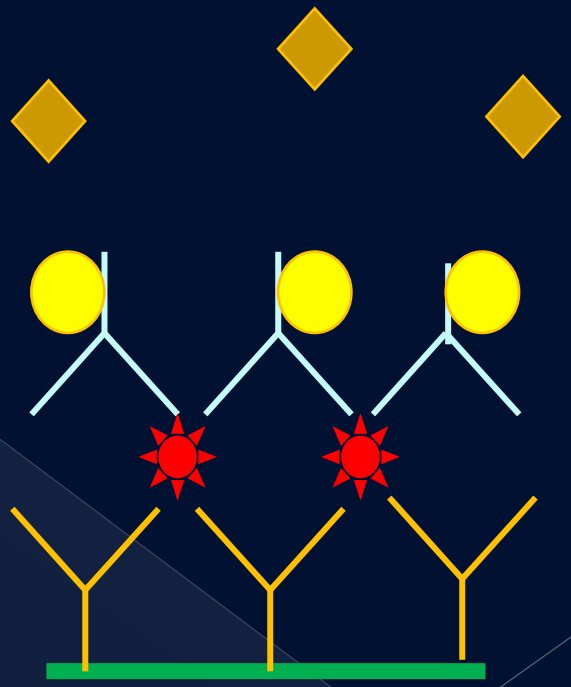


Antibodies conjugated with enzyme

Protein analysis

ELISA

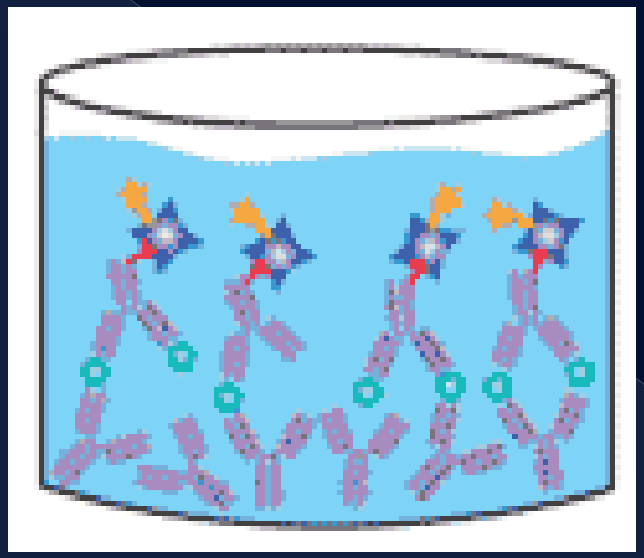


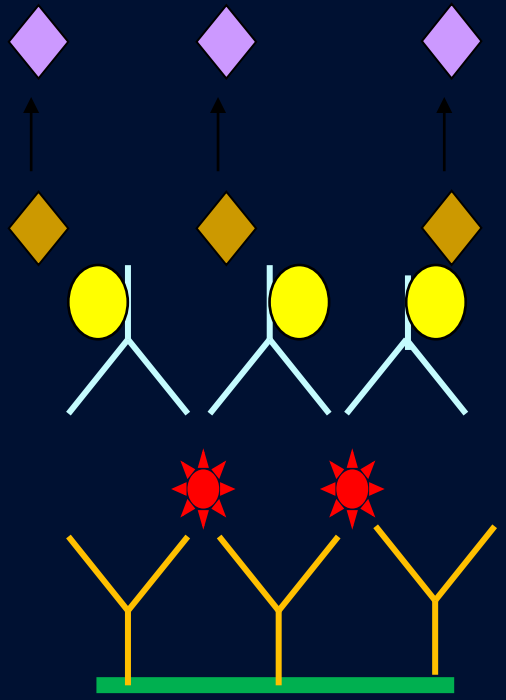


Substrate addition

Protein analysis

ELISA

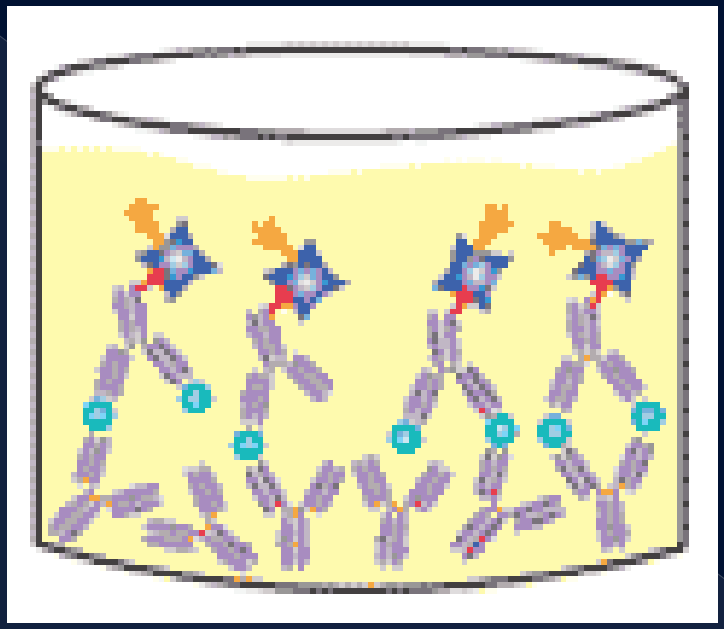




Conversion of colorless substrate into a colored product.
The intensity of color depends on amount of target protein.

Protein analysis

ELISA



Stop solutions

Reading absorbance

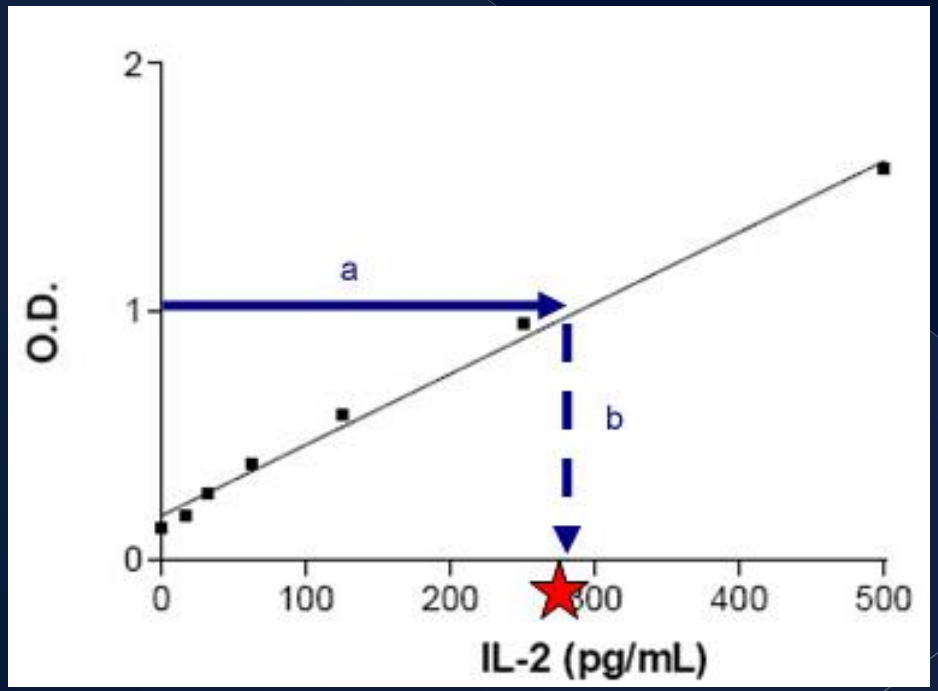
Protein analysis

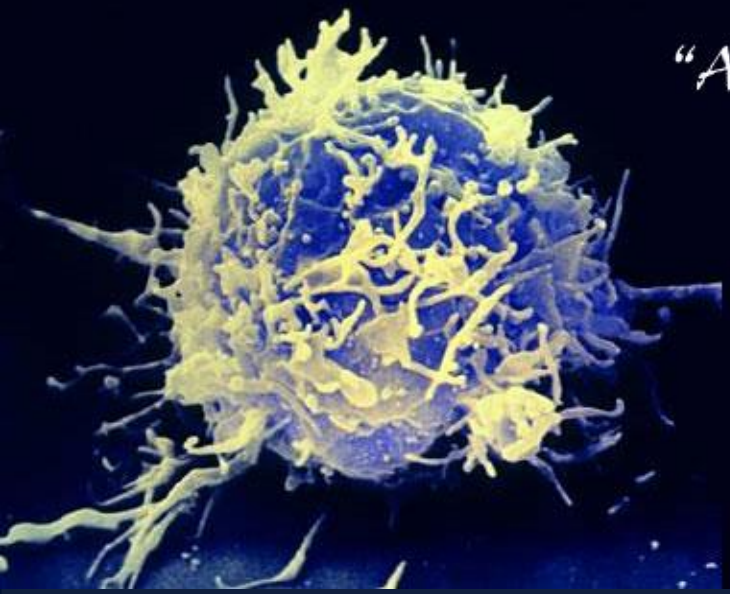
ELISA

Standard

Unknown Sample

1:2 1:4 1:8 1:16 1:32 1:64 1:128 0



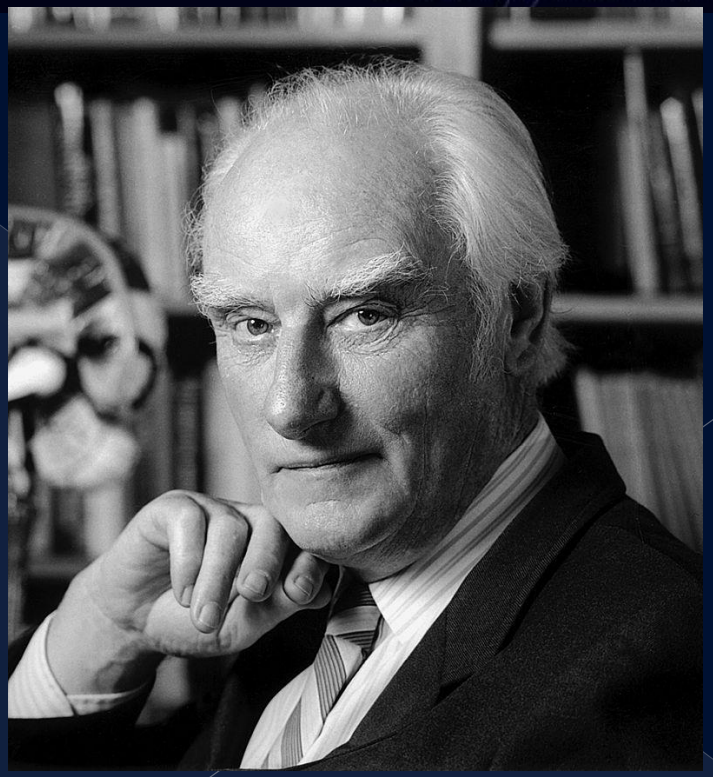


"Almost all aspects of life are engineered at the molecular level, and without understanding molecules we can only have a very sketchy understanding of life itself."

Francis Crick

University of Texas MMBSS

Francis Crick (1916 – 2004) British molecular biologist, biophysicist, and neuroscientist, co-discoverer of the structure of the DNA in 1953 with James Watson. They were awarded the 1962 Nobel Prize in Physiology or Medicine "for their discoveries concerning the molecular structure of nucleic acids and its significance for information transfer in living material".



CONTACT INHIBITION

100% confluence means the surface is completely covered by the cells, and no more room is left for the new cells in a monolayer.

Confluent cell culture is cell culture in which all the cells are in contact and the entire surface of the culture dish is covered by cells.

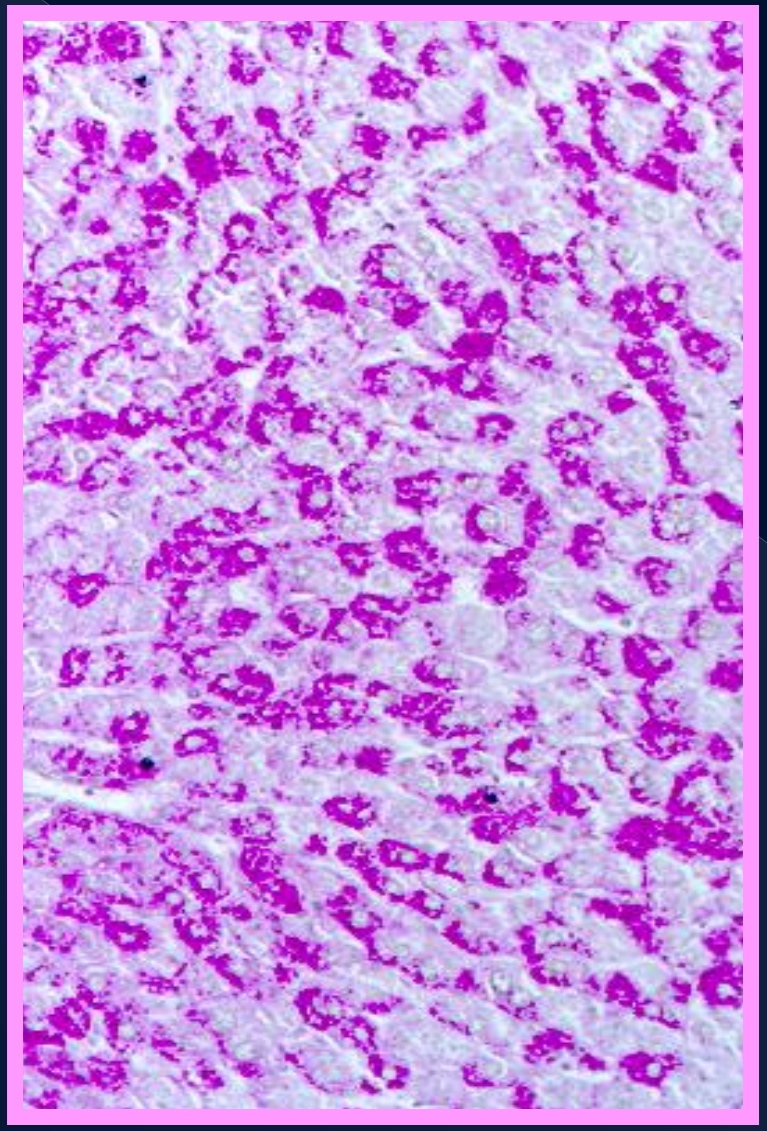
Sparsely seeded cells

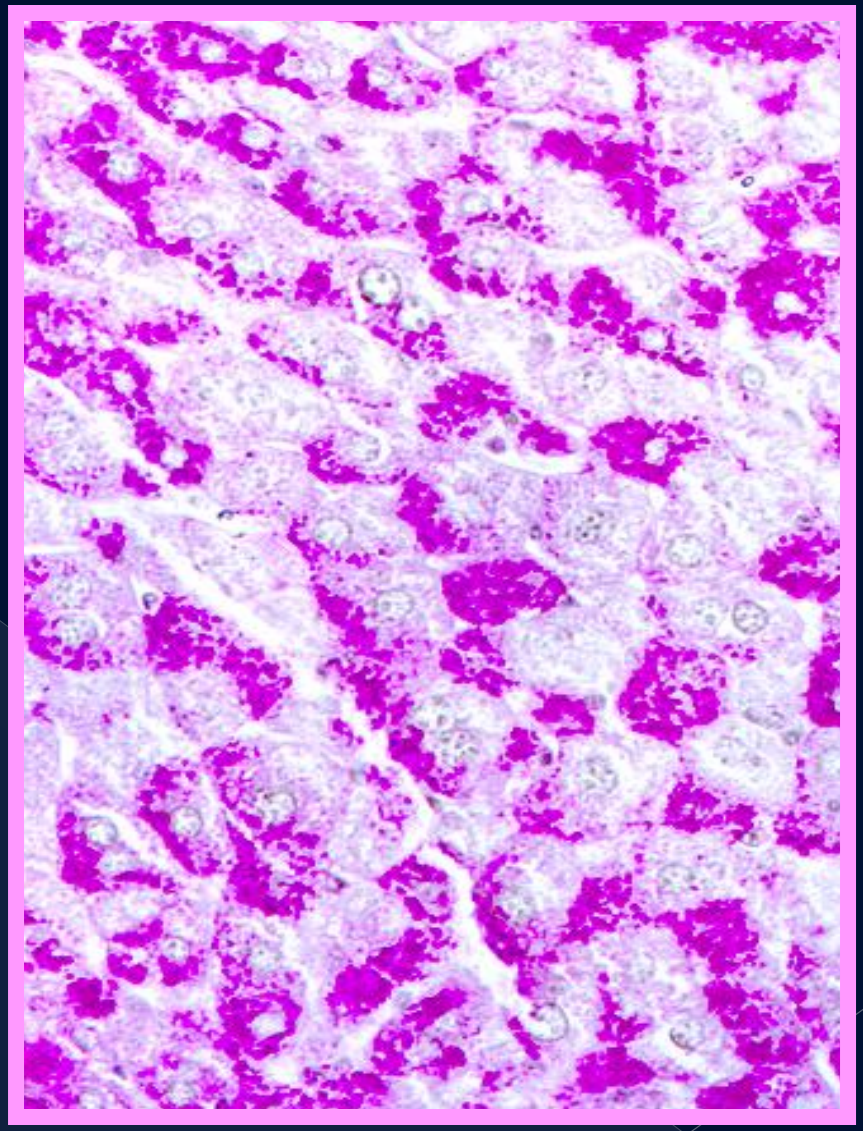
Confluent culture (monolayer). Cells do not divide

Cells removed by scraping

Cells start dividing

up to fill the free space





PERIODIC ACID- SCHIFF STAIN - glycogen granules in hepatocytes

Cell suspended in agar

Cell placed on a adhesive clump

small

large

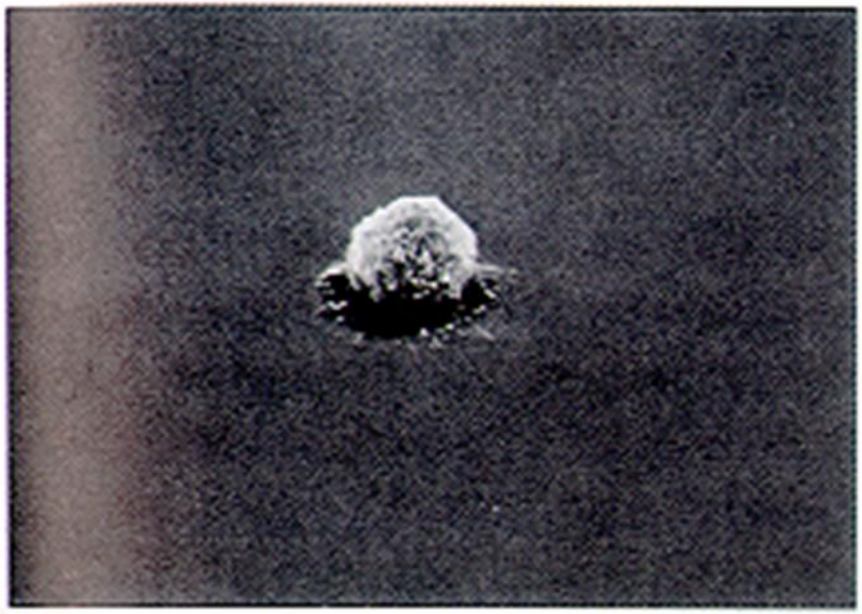
8%

30%

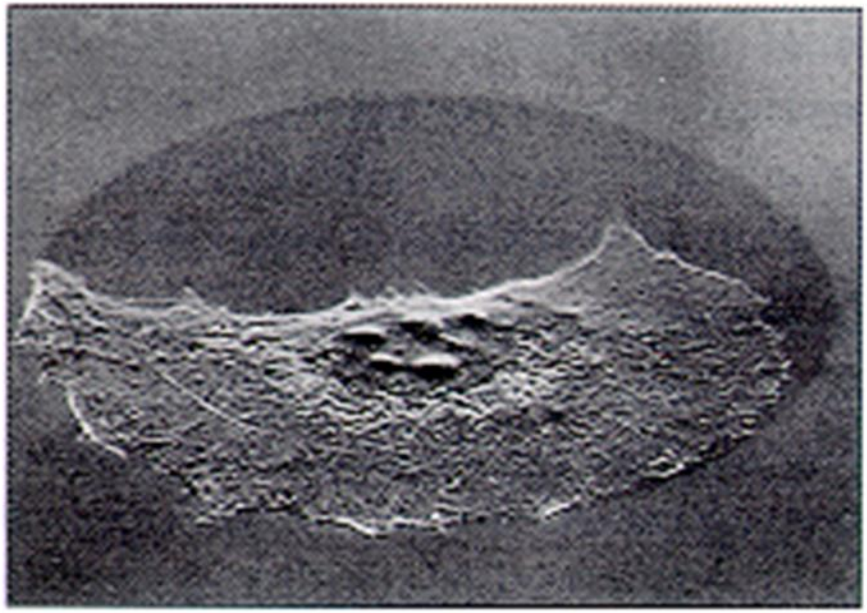
90%

probability of entry the cell into phase S

(A)



(B)



(C)

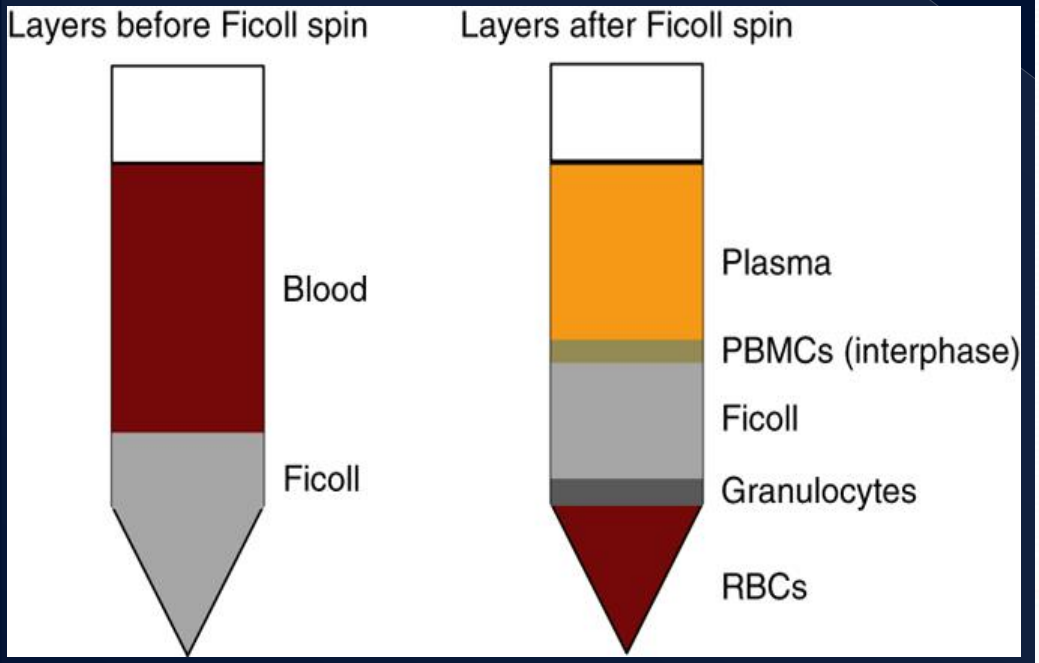
50 μm

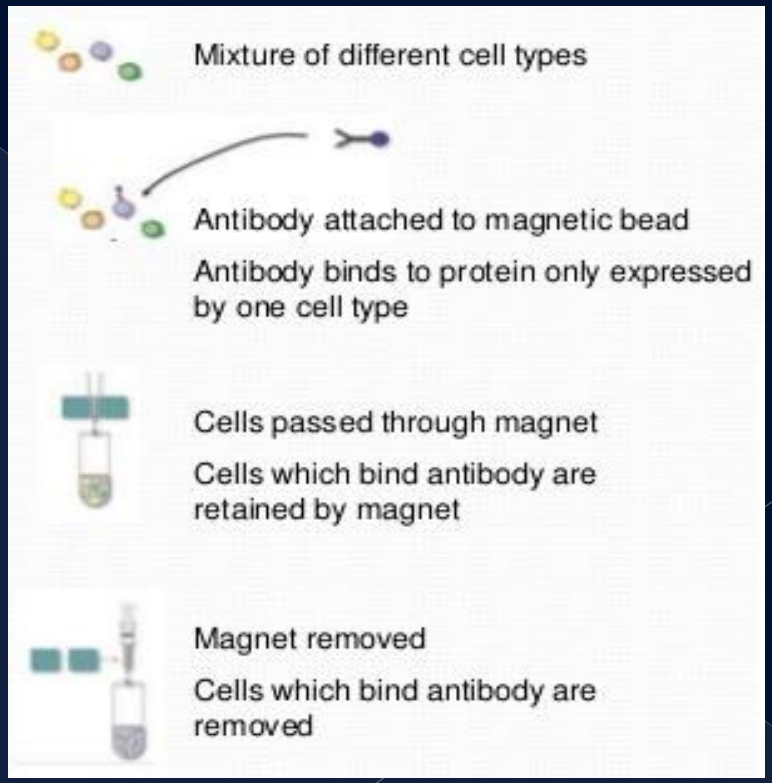
ISOLATION OF SINGLE CELLS OR THEIR GROUPS

Separation of a heterogeneous population of cells, eg. blood cells into more homogenous populations

a) by density gradient centrifugation. Cells are sorted according to their sedimentation constant.

b) by passing through a magnetic column with beads conjugated with antibodies. Cells are sorted depending on their surface antigens (*magnetic assisted cell sorting)*





NUMBER OF POPULATION DOUBLINGS

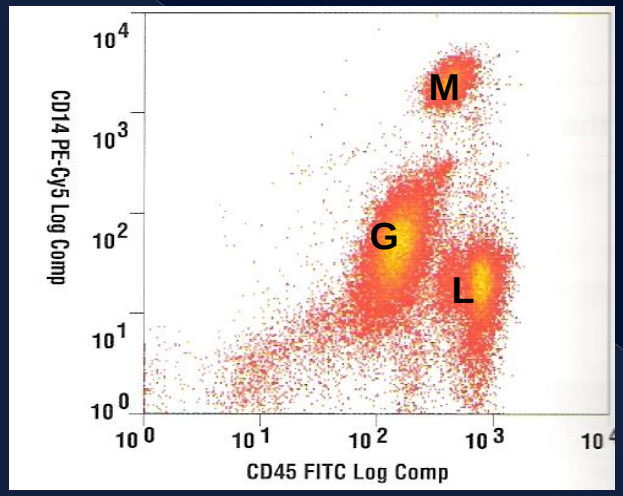
Normal human fibroblasts derived from young person can divide – several dozen times (40-60) = several dozen population doublings - Hayflick limit.

Professor Leonard Hayflick
American anatomist

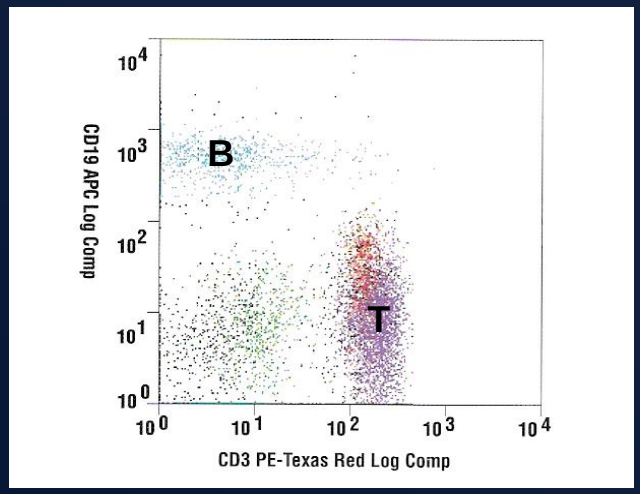
Transformed cell lines (by viruses, oncogenic substances, mutations) are immortal and divide continuously, show a lack of contact inhibition (no CiR), are less dependent on the presence of growth factors and interactions with extracellular matrix, neighboring cells or surface of culture vessels.

Flow Cytometry

Peripheral blood leukocytes



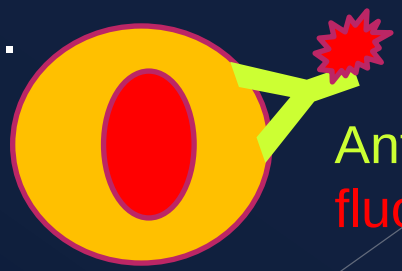
Anti-CD45 and anti-CD14.



Anti-CD3 and anti-CD19.

When a fluorescent dye is conjugated to a monoclonal antibody, it can be used to identify a particular cell type based on the surface markers of the cell.

Different fluorochromes can be used to distinguish subpopulations. The staining pattern of each subpopulation, combined with FSC and SSC data, can be used to identify cells.



METHODS OF CELL CULTURE AND TECHNIQUES USED IN MEDICAL RESEARCH

1. **TISSUE AND CELL CULTURE – HISTORY AND PRESENT**
   - 1885 - chicken neural plate cultured in a warm saline solution (Prof. Wilhelm Roux)
   - 1907 - frog neural tube cultured in frog lymph clot. The formation of nerve processes (Prof. Ross Harrison).
   - optimization of culture conditions through the use of different culture media (plasma, serum, extract from chicken embryos) and determination the needs of the cells: osmotic pressure, pH, salts (Burrows, Carrel, Ebeling, Lewis). 20 years of the 20th century - embryonic tissue cultures. The ability of cells to differentiate in culture. Culture in clot on the slide watch.
   - 40 years of 20th century - the first artificial medium comprising mineral salts, amino acids, vitamins.
   -1952 – the use of trypsin to isolate single cells from tissue and to separate them from the glass (A. Moscona, H. Moscona).
   - modern cell/tissue culture - special devices of laboratory allow to save sterility of culture. Biosafety cabinet - enclosed, ventilated laboratory workspace for safely working with materials contaminated with (or potentially contaminated with) pathogens. All exhaust air is filtered (vertical air flow). CO2 incubator for cell culture maintains optimal temperature, humidity, the carbon dioxide and oxygen content of the atmosphere inside. Plastic disposable dishes, with properly prepared surface, the use of appropriate growth factors for proliferation and growth of differents types of cells, established cell lines.

2. **APPLICATIONS OF CELL/TISSUE CULTURE**.
   Basic research in vitro, diagnostics, production of vaccines (polio, measles, mumps, rubella), production of recombinanat proteins (enzymes, hormones, cytokines), monoclonal antibodies, getting the cells for transplantation – for example autologous keratinocytes, isolated from a small piece of skin, which proliferate in cell cultures and are used as wound dressing in poorly healing wounds. Or we can use keratinocyte dressing for burns treatment. Also damage in articular cartilage caused by trauma can be treat by transplantation of autologous chondrocytes (after proliferation in cell culture) into defects in cartilage.

3. **BASIC DEFINITIONS.**
   Tissue culture is the growth of tissues or cells in vitro more than 24h (below 24h – incubation).
   Primary cell culture - cell line derived directly from the parent tissue
   Cell line - the cells growing in the first or later subculture from a primary culture (after first passage)
   Cell strain - cells adapted to culture, but with finite division potential
   Established cell line - permanently established cell culture that will proliferate indefinitely given appropriate fresh medium and space (immortal line)
   Diploid cell line - line, wherein at least 75% of the cells have the same karyotype as normal somatic cells of the species from which derived cells

Heteroploid cell line - cell line, in which less than 75% of the cells has a diploid karyotype.

Population doubling time - period of time, wherein the number of cells doubles

Transformation of cells - change of normal cells to tumor cells, due to the introduction of new genetic material or mutation.

Cell culture growth - increase in cell mass (volume) (formerly term related to increase of the number).

Proliferation - increase of the number of cells

4. **CELL CULTURE MEDIA.**

Osmotic pressure of 340 ± 5 mOsm /kg H_2O and the pH in the range 7.2 to 7.4. COMPOSITION OF THE MEDIUM: balanced salt solution, amino acids, glucose, fatty acids, vitamins, purine and pyrimidine bases, phenol red (phenolsulfonphthalein or PSP - a pH indicator, which helps in monitoring of the pH changes in the cell culture. It will change from red to yellow when the pH value decreases and the medium should be changed), fetal calf serum – a source of growth factors and hormones. FCS cannot be used in a laboratory where grown anything that is designed to provide or transplantation to the patient, due to the possibility of zoonotic infections.

5. **ISOLATION OF SINGLE CELLS OR THEIR GROUPS**.

Culture of the explant (isolated portion of the tissue or organ) for a time sufficient to get out the cells. Separation of explant into single cells by enzymatic method:

a) when small amount extracellular matrix eg. fetal tissues – by trypsin,

b) when large amount of extracellular matrix eg. cartilage – by collagenase with deoxyribonuclease. DNase is added to digest the DNA from destroyed cells.

c) separation the larger fragments eg. thyroid follicles or pancreatic islets – by collagenase.

d) separation of a heterogeneous population of cells, eg. blood cells into more homogenous populations a) by density gradient centrifugation. Cells are sorted according to their sedimentation constant. Blood with anticoagulant, diluted 1 to 1 is layered on Ficoll (neutral, highly branched, high-mass, hydrophilic polysaccharide which dissolves readily in aqueous solutions density from 1.075 to 85g/ml). Differential migration of cells during centrifugation results in the formation of layers containing different cell types: The bottom layer contains erythrocytes, The layer immediately above the erythrocyte layer contains mostly granulocytes, at the interface between the plasma and the Ficoll layer, mononuclear cells - monocytes and lymphocytes (buffy coat) are found together with other slowly sedimenting particles (e.g., platelets) with low density.

6. **CONTACT INHIBITION**.

when cells occupy entire surface of culture dish and are in contact with each other - cell culture is called confluent culture. In confluent culture normal cells stop dividing (monolayer cell culture). Mechanism which functions to keep cells growing into a layer one cell thick is called contact inhibition. If a cell has plenty of free space, it divides rapidly. This process continues until there is no any free space in the layer for

cells. At this point, normal cells will stop dividing. **Contactinhibin** – membrane glycoprotein from human diploid fibroblasts, causes a reversible inhibition of proliferation in confluent culture. **CiR** – plasma membrane protein - a receptor for contactinhibin mediating the contact-dependent inhibition of growth of cultured cells. The ligand-receptor binding causes changes in the cyclin-dependent kinases (inactive Cdk4), which leads to inhibition of cell division. This effect is seen also with other cell types, with the exception of transformed cells, which do not show the phenomenon of contact inhibition. Transformed fibroblasts – lack of CiR.
Sometimes normal cells can also form multilayered culture for example chondrocytes. But they produce a lot of ECM, and particular layers of cells are separated by layers of matrix – the cells of different layers are not in direct contact.

7. **HAYFLICK LIMIT**
   - normal human fibroblasts derived from young person can divide – several dozen x (40 to 60) = several dozen population doublings (shortening of telomeres, replicative senescence).
   - phases of normal cell culture and population doublings. Primary cultures – phase I, after first passage – phase II – phase of proliferation, and phase III – cell death phase.
   - linear chromosomes are less stable than circular, but determine the genetic diversity of living organisms (recombinations). However, the 3 'and 5' ends are sensitive to DNA-degrading enzymes, as well as linear chromosomes may undergo fusion. Telomeres - repetitive non-coding DNA sequences (TTAGGG) with proteins, located at the ends of chromosomes, prevent the damage of chromosomes. During each replication cell loses 50-200 bp telomeric DNA. When the telomeres reach a critical length - STAGE OF CELLULAR SENESCENCE (p53-dependent inhibition of cell cycle). **End replication problem** - the shortening of telomeres during replication. DNA polymerase can read and synthesize DNA only in one direction, starting from primer.
   - the longer the lifetime of the representative of a given species, the more population doublings undergo the cells of this species in culture. The life span of representative of given species is directly proportional to the cell population doublings.

8. **MICROSCOPY**
   one of the earliest tools of the cell biologists. The first compound microscope appearing in 17th century in Netherlands (inventor Cornelis Drebbel, Hans Lippershey, Zacharias Janssen). Antonie van Leeuwenhoek discovered red blood cells and spermatozoa and helped popularize microscopy as a technique. On 9 October 1676, Van Leeuwenhoek reported the discovery of microorganisms.

9. **CYTO- AND HISTOCHEMISTRY** –
   detection of chemical compounds present in the cells, tissues, and in the matrix. Histochemical color reaction, autoradiography, immunohistochemistry.

10. **HISTOCHEMICAL COLOR REACTION. REACTION P.A.S.** –

Periodic Acid- Schiff stain- detection of polysaccharides. Polysaccharides, periodic acid, (oxidation of the glycol groups – vicinal diols), aldehyde groups, Schiff reagent (colorless basic fuchsin), red color of Schiff reagent. Periodic acid will cleave vicinal diols into two aldehyde fragments. A diol is a chemical compound containing two hydroxyl groups (−OH groups). In a vicinal diol, the two hydroxyl groups occupy vicinal positions, that is, they are attached to adjacent atoms. These compounds are called glycols. An aldehyde or alkanal is an organic compound containing a formyl group. The formyl group is a functional group, with the structure R-CHO. Under influence of aldehyde groups colorless basic fuchsin (fjuksyn) gives a purple-magenta

11. **HISTOENZYMOLOGY**.

Enzyme, (eg. alkaline phosphatase, succinic acid dehydrogenase), substrate, product, intermediate compound, colorful precipitate (insoluble). Example: detection of alkaline phosphatase: substrat - beta-naphthyl phosphate (beta-naftyl, solution of sodium /3-naphthyl phosphate buffered to an appropriate pH (4.8 for acid phosphatase and 9.1 for alkaline phosphatase) at 37.5" for a standard period of time (2 hours and 1 hour respectively), 2 molecules of p-naphthol released in the course of the reaction are coupled with tetrazotized diorthoanisidine to yield an insoluble, purple diazonium dye dye.Alkaline phosphatase dephosphorylates substrat and beta-naphthol is formed. Diazonium dye is converted into color complex. (tumors derived from the trophoblast, metastasis of prostate cancer)

12. **AUTORADIOGRAPHY**.

Incubation tissue with isotope. Isotope will emit radiation (usually beta). Exposition to film or emulsion (AgBr in gelatin). Radiation will hit silver grains in emulsion and expose them. Formation of latent image; metallic silver. Photographic processing transforms the latent image into a visible image and makes this permanent. Over the place where was isotope incorporated are visible black grains of silver.

13. **IMMUNOHISTOCHEMISTRY.**

Very important and common technique used to detect proteins present in cells and tissues is method called immunohistochemistry. This method exploits the principle of antibodies that specifically bind to antigens. Due to the use of antibodies is called immunohistochemistry and target protein – is called antigen. The antibody is composed of two heavy chains and two light chains connected by disulfide bonds. We can distinguish 2 fragments of antibody: divalent (dajvejlent) $F(ab)_2$ - antigen binding fragment – two sites - decides about specificity of antibody. The epitope (antigenic determinant) is the fragment of target protein. The antibody can bind to the antigen epitope if the shape of epitope and shape of antigen-binding site correspond to each other. Second fragment - Fc – crystallized fragment serves for binding with cell receptors, activation of complement. In molecular biology this fragment is usually labeled. Monoclonal antibodies are identical antibodies (the same specificity, they binds the same epitope of target protein) that are made by identical B cells in contrast to polyclonal antibodies which are made by several different B cells and can bind to different epitopes of the same antigen.. Polyclonal antibodies are cheaper, but

monoclonal antibodies are considered more specific than polyclonal antibodies, and, typically, result in less background staining.

Methods for labeling antibodies: fluorochromes (eg. fluorescein derivative), metals (eg. ferritin, colloidal gold), isotopes (eg. ^{3}H, ^{125}I, ^{35}P, ^{14}C), enzymes (eg. alkaline phosphatase, peroxidase).

Direct method - the antibody against target protein is labeled. Such antibody is called primary antibody. It is one-step, simple, rapid and cheap method, but its sensitivity is relatively low.

Indirect method - involves an unlabeled primary antibody (first layer) that binds to the target protein and a labeled secondary antibody (second layer) that reacts with the Fc fragment of primary antibody. This method is more sensitive because of signal amplification due to the binding of several secondary antibodies to each primary.

Method with streptavidin or avidin.

Most often used in immunohistochemistry. Biotin (vitamin H, coenzyme R, is a water-soluble vitamin B7) is a coenzyme for enzymes, involved in the synthesis of fatty acids, amino acids, gluconeogenesis. Streptavidin is a tetrameric protein from the bacterium Streptomyces avidinii (in soil). Avidin is a tetrameric biotin-binding protein produced in the oviducts of birds, reptiles and amphibians and deposited in the whites of their eggs. Streptavidin and avidin homo-tetramers have an extraordinarily high affinity for biotin. The binding of biotin to these 2 proteins is one of the strongest non-covalent interactions known in nature. In this method we use biotinylated secondary antibody and biotinylated marker. Avidin or streptavidin is used as a bridge between the biotinylated secondary antibody and the biotinylated marker. Very sensitive method, strong signsl amplification.

14. OBSERVATION OF LIVING CELLS UNDER MICROSCOPE.

An inverted microscope is a microscope with its light source and condenser on the top, above the stage, while the objectives and turret are below the stage. Inverted microscopes are useful for observing living cells or organisms at the bottom of a large container (e.g., a tissue culture flask) under more natural conditions than on a glass slide. Phase contrast microscopy utilizes the difference between light rays propagating directly from the light source and light rays refracted by the specimen when light passes through it to add bright dark contrast to images of transparent specimens. Therefore the borders of images are surrounded by a characteristic bright "halo." The microscope is fitted with a phase-contrast objective and a condenser for observations.

15. FLOW CYTOMETRY.

A flow cytometer has a measuring system, optical systems – lamps, lasers resulting in light signals, a detector and Analogue-to-Digital Conversion (ADC) system - which generates forward-scattered light (FSC) and side-scattered light (SSC) as well as fluorescence signals from light into electrical signals that can be processed by a computer. The key point in this technique that the single cell passes through the light beam and causes its deflection FSC and dispersion SSC. FSC – Forward-scattered light is proportional to cell-surface area or size -the greater cell the greater deflection

or FSC. SSC - Side-scattered light is proportional to cell granularity or internal complexity. The more complicated cell the greater dispersion of light or SSC. When a fluorescent dye is conjugated to a monoclonal antibody, it can be used to identify a particular cell type based on the surface markers of the cell. Different fluorochromes can be used to distinguish subpopulations. The staining pattern of each subpopulation, combined with FSC and SSC data, can be used to identify cells. Cytometer first needs to identify the cells of interest, then separate out the individual cells. Sorting allows us to capture and collect cells of interest for further analysis. Once collected, the cells can be analyzed microscopically, biochemically, or functionally.

Flow cytometry applications:

DIAGNOSIS: proliferative diseases of the hematopoietic and lymphatic system, congenital and acquired immunodeficiency, autoimmune diseases, analysis of cells for bone marrow transplantation,

MONITORING: the treatment of leukemia, the immune system of HIV patients, immunosuppressive treatment of transplanted patients.

Disadvantage of flow cytometry – we can only examine cells in suspension, for example blood cells. If the cells are connected with the extracellular matrix epithelial cells, connective tissue proper cells, bone and cartilage cells, muscle cells we must separate them using enzymes before examination. For this reason we lose the information about the connection of these cells with the extracellular matrix and with other cells. But now there is newer technique – we can use laser scanning cytometer LSC that offers a analytical capabilities, not provided by flow cytometry (FCM). We can examined slides with specimens. The tissue should be stained with at least 2 fluorochromes. The slides are scanned and the fluorescence, the dispersion of light, the area and boundaries of cells and cell organelles are measured and analyzed. LSC can be used for assessment of chromatin condensation to identify mitotic, apoptotic or senescent cells; detection of nuclear or mitochondrial translocation of critical factors such as NF-κB, p53, or Bax; analysis of fluorescence in situ hybridization (FISH), enumeration and morphometry of nucleoli and other cell organelles.

16. **ISOLATION OF CELL ORGANELLES FOR DETECTION OF PROTEINS, NUCLEIC ACIDS, LIPIDS AND CARBOHYDRATES**.

Material isolation: cell disruption is a method of releasing biological molecules from a cell. We can use several different techniques for cell disruption. We can use ultrasounds. This process is called ultrasonication. We can destroy cell membranes using detergents - surfactants – lower the surface tension. Detergents are amphiphilic: partly hydrophilic (polar) and partly hydrophobic (non-polar). Solubilization of cell membrane bilayers requires a detergent that can enter the inner membrane monolayer. Or we can homogenise the cells. A homogeniser is a piece of laboratory equipment used for the homogenization of various types of material, such as tissue. Many different models have been developed. The 'mortarand pestle, already used for thousands of years, is a standard tool even in modern laboratories. More modern solutions are based on blender type of instruments (also known in the kitchen). We obtain cell homogenate.

Fractionation: differential centrifugation based on differences in size and density, with larger and denser particles pelleting at lower centrifugal forces. Cell fractionation

is the process in which cell homogenates are separated into fractions by spinning. The homogenate is then subjected to repeated centrifugations, each time removing the pellet and increasing the centrifugal force. We have to increase the speed of centrifugation .

17. **WESTERN BLOT**.

Method used to detect specific proteins in a sample of tissue homogenate, cellular extract or in body fluid.

1. **Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE**) electrophoresis. Proteins in the sample are heated with the negatively charged detergent SDS (anionic surfactant), which unfolds them and coats them with a uniform negative charge density; disulfide bonds (S-S) are reduced with mercaptoethanol. The bromophenol blue is added to dye the sample – to observe the front of electrophoresis. The sample is applied to the well of polyacrylamide gel, and a voltage is applied to the gel. The negatively charged detergent-protein complexes migrate to the bottom of the gel, toward the positively charged anode. Small proteins move faster, larger proteins move slower, so proteins are separated by size, smaller toward the bottom and larger toward the top. Western protein standard. Prestained standards permit to visual control of electrophoresis and transfer. Western Protein Standards are designed for protein molecular weight estimation directly on western blots. They contain proteins of known weight and we can compare target protein with standard and evaluate the weight of our protein.

2. **Transfer proteins from gel to membrane**. In Western blot proteins are detected by antibodies. It is regular immunohistochemical method. But antibody molecules are too large to penetrate the gel, and we must to transfer the proteins onto another carrier. The gel with the resolved set of proteins is then placed in an apparatus that permits electrophoretic transfer of the proteins from the gel onto the surface of a special paper (e.g., nitrocellulose paper, polyvinylidene fluoride (PVDF) membrane) to which proteins strongly adsorb.

3. **Method of detection of target protein - immunohistochemistry**.

Colorimetric, chemiluminescence, fluorescence.

The colorimetric detection method depends on incubation of the western blot with a substrate that reacts with the reporter enzyme that is bound to the antibody. This converts the soluble dye into an insoluble form of a different color that precipitates and thereby stains the membrane.

Chemiluminescent detection methods depend on incubation of the western blot with a substrate that will luminesces when exposed to the reporter on the antibody. The light is then detected by CCD cameras which capture a digital image of the western blot.

The fluorescently labeled probe is excited by light and the emission of the excitation is then detected by a photosensor such as a CCD camera equipped with appropriate emission filters which captures a digital image of the western blot.

18. **ELISA ASSAY**.

Detection and quantification of specific protein using antibodies conjugated with enzymes. 96-well plate with specific capture antibody coating. The target protein-containing samples and serial dilutions of standard with known concentration are applied to the plate, and molecules of target protein are captured by antibodies. Enzyme-linked antibodies are applied as detection antibodies that also bind specifically to target protein. Substrate addition. Conversion of colorless substrate into a colored product. The intensity of color depends on amount of target protein. The absorbance of the plate wells is measured to determine the presence and quantity of target protein. The absorbance (optical density) of each well is read with a plate reader (kind of spectrophotometer). Standard curve is prepared from the data produced from the serial dilutions of standard with concentration on the x axis (log scale) vs. absorbance on the Y axis (linear). The concentration of the sample is interpolated from this standard curve.

19. **PROPERTIES OF DNA WHICH HELP TO STUDY THEM**

Denaturation - or DNA melting is the separation of a double strand into two single strands, which occurs when the hydrogen bonds between the strands are broken. (thermal denaturation – by heat, chemical denaturation – by bases, acids). DNA strands caused by slow cooling. Hydrogen bonds are formed.

Hybridization - a phenomenon in which single-stranded DNA or RNA molecules spontaneously anneal to complementary DNA or RNA.

20. **SOUTHERN BLOT**

method used in molecular biology for detection of a specific DNA sequence in DNA samples. The method is named after its inventor, the British biologist Edwin Southern.

1.**Restriction endonucleases** are used to cut DNA into small fragments.

2**.The electrophoresis of DNA** fragments on an agarose gel to separate them by size.

3. **Alkaline DNA denaturation**.

4. **Transfer DNA from gel onto nitrocellulose or nylon membrane**.

5. **The membrane is exposed to a hybridization probe** - a DNA fragment with a specific sequence whose presence in the target DNA is to be determined. The probe DNA is labeled so that it can be detected.

Southwestern blot is used for identifying and characterizing DNA-binding proteins. The proteins are separated by electrophoresis, transferred to membrane and detected by DNA probes.

21. **NORTHERN BLOT**

- technique used to study gene expression by detection of RNA in a sample. It was used for determining the particular gene expression. Extraction of total RNA from cells. RNA samples are separated by gel electrophoresis and transferred to a nylon membrane. Complementary labeled probe is hybridized to the RNA on the membrane.

Analysis of gene expression can be done by several different methods for example RT-PCR. The problem in northern blotting is often sample degradation by RNases (both endogenous to the sample and through environmental contamination), which can be avoided by proper sterilization of glassware and the use of RNase inhibitors .The

chemicals used in most northern blots can be a risk to the researcher. Compared to RT-PCR, northern blotting has a low sensitivity, but it also has a high specificity, which is important to reduce false positive results.

22. **HYBRIDIZATION *IN SITU*.**

Receptor tyrosine-protein kinase erbB-2, (CD340), proto-oncogene Neu, Erbb2 (ERBB2) is encoded by the ERBB2 gene, called HER2 (human epidermal growth factor receptor 2) or HER2/neu. Amplification or overexpression of this oncogene plays an important role in the development and progression of certain aggressive types of breast cancer. This protein is also an important biomarker and target of therapy for approximately 30% of breast cancer patients. The use of eFISH HER2/CEN17 probe in interphase and normal chromosome 17 produces two HER2 signals and two chromosome 17 signals. These signals appear green with hybridized HER2 probe and orange when hybridized to alpha centromeric region of chromosome 17. In cases of increased gene amplification, an increased number of gene signals (green) are visible. Trastuzumab (Herceptin) is a monoclonal antibody that interferes with the HER2 receptor. It is used to treat certain breast cancers. The original studies of trastuzumab showed that it improved overall survival in late-stage (metastatic) HER2-positive breast cancer. However, it increases serious heart problems.

23. **POLYMERASE CHAIN REACTION.**

Thermus aquaticus is a species of bacterium that can tolerate high temperatures. It is the source of the heat-resistant enzyme Taq DNA polymerase, most important enzyme in molecular biology because of its use in PCR reaction - DNA amplification technique. It was first discovered in the Lower Geyser Basin of Yellowstone National Park.

To carry out the PCR reaction we have to have: DNA template that contains the DNA region to amplify, two primers forward and reverse, that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target fragment, Taq polymerase, DNA nucleotides, a chemical environment and thermocycler.

Denaturation step: DNA is heated to separate the strands (95 °C). It causes DNA melting of the DNA template by disrupting the hydrogen bonds and single-stranded DNA molecules are formed.

Annealing (anyling) step: The reaction temperature is lowered to 50–65 °C allowing annealing (hybridization) of the primers to the single-stranded DNA. The polymerase binds to the primer-template hybrid and begins DNA formation.

Extension/elongation step: DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand. At each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification (geometric progression) of the specific DNA fragment. In first cycle of amplification from 1 template we obtain 2 copies.In second cycle of amplification from 2 templates we obtain 4 copies. In third cycle of amplification from 4 templates we obtain 8 copies. Usually in PCR we carry out 30 - 40 cycles and we obtain from 10 to the power of twelfth to the 10 to the power of fifteenth copies.

24. REVERSE TRANSCRIPTASE-PCR REACTION

used to detect gene expression by detection of specific mRNA.

1. Isolation of the total RNA from tissue.
2. Ttranscription of mRNA into complementary DNA – cDNA using enzyme called reverse transcriptase.
3. Degradation of the mRNA template by RNase H. Ribonuclease H (RNase H) - endonuclease that catalyzes the cleavage of RNA. Because RNase H specifically degrades only the RNA in RNA:DNA hybrids, it is commonly used in molecular biology to destroy the RNA template after first-strand complementary DNA (cDNA) synthesis by reverse transcription.
4. Synthesis of the complementary DNA strand by DNA polymerase (double-stranded cDNA copy of original mRNA is formed).
5. Amplification of ds cDNA in polymerase chain reaction
6. The electrophoresis of PCR product and DNA electrophoresis size standard in agarose gel. DNA electrophoresis size standard contains DNA fragments of known length (known number of base pairs). DNA size standard is used as positive control for electrophoresis and for determining the sizes of target DNA fragments.
7. Ethidium bromide is commonly used to detect nucleic acids in molecular biology laboratories. When exposed to ultraviolet light, it will fluoresce, intensifying almost 20-fold after binding to DNA. Therefore we can see amplified product of PCR and DNA standard in agarose gel placed on or under a UV lamp.

25. REAL TIME PCR

-permits us not only to detect gene expression, but also to quantification of gene expression. In this method signals emitted from labeled with fluorescent dyes primers or probes are measured after each cycle and mathematical analysis of this signal is carried out. Using of probe increases the specificity of reaction The probe is designed so that bridges 2 adjacent exons and eliminates the false reaction with genomic DNA, which sometimes can be present in the sample. In genomic DNA the introns are present, they are removed in process called alternative splicing during mRNA maturation. The probe ccontains reporter dye and quencher. Quencher quenches the emission of reporter dye when they are connected by probe.

The reaction of DNA amplification in Real-time PCR is the same.