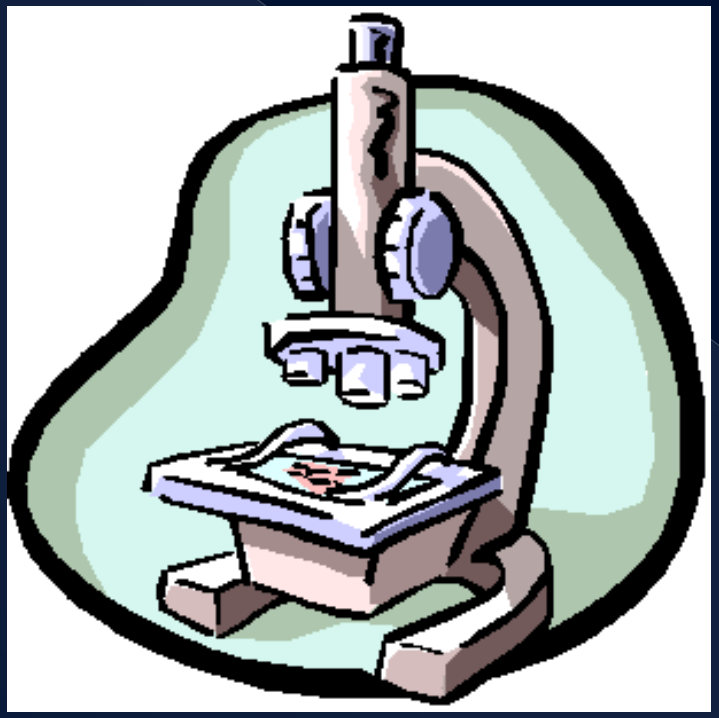
METHODS OF CELL CULTURE AND TECHNIQUES USED IN MEDICAL RESEARCH



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, 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:

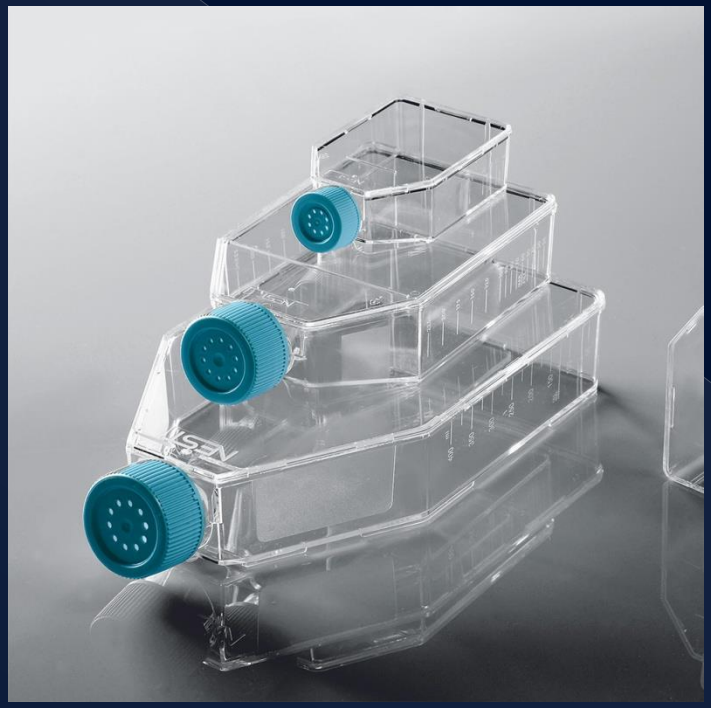
balanced salt solution
amino acids
glucose
fatty acids
vitamins
purine and pyrimidine bases
phenol red
fetal calf serum – a source of growth factors and hormones

CELL/TISSUE CULTURE

-special laboratory equipment 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

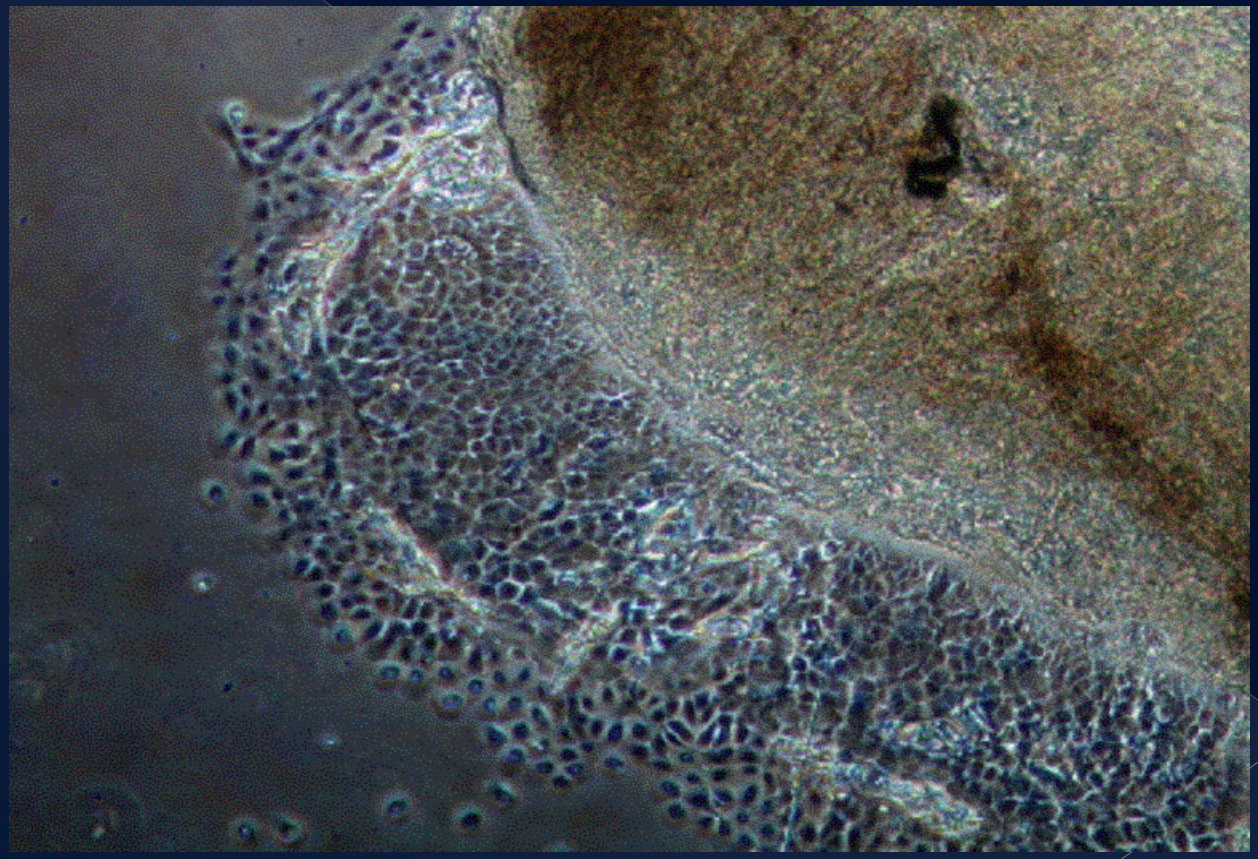
established cell lines

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:

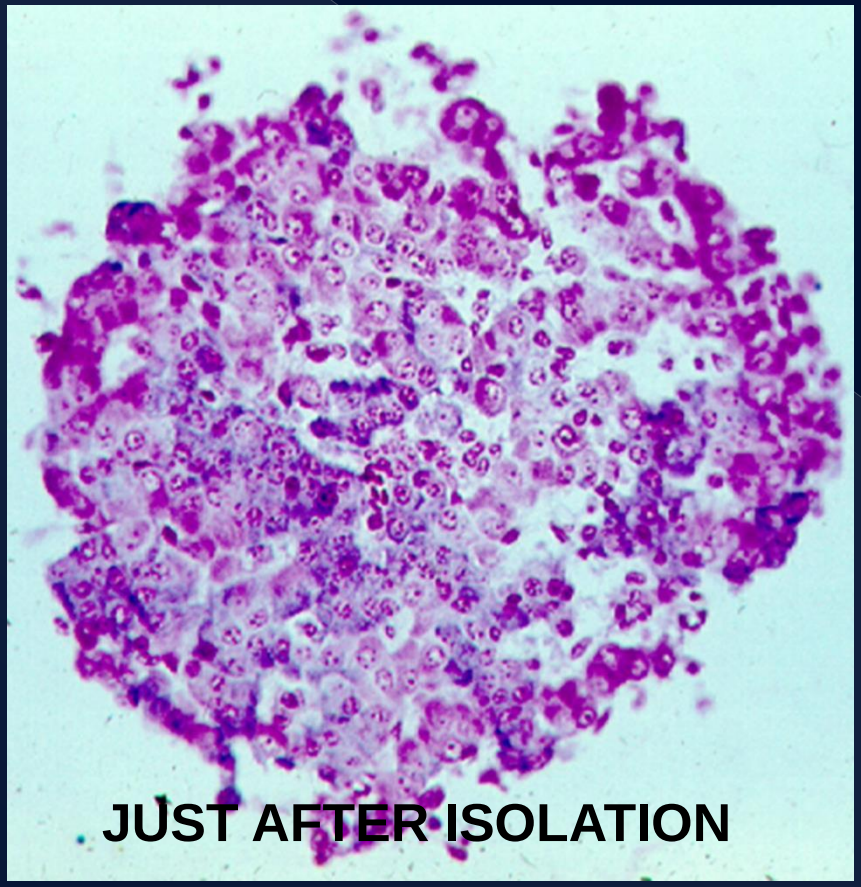
a) When small amount extracellular matrix eg. fetal tissues – by trypsin

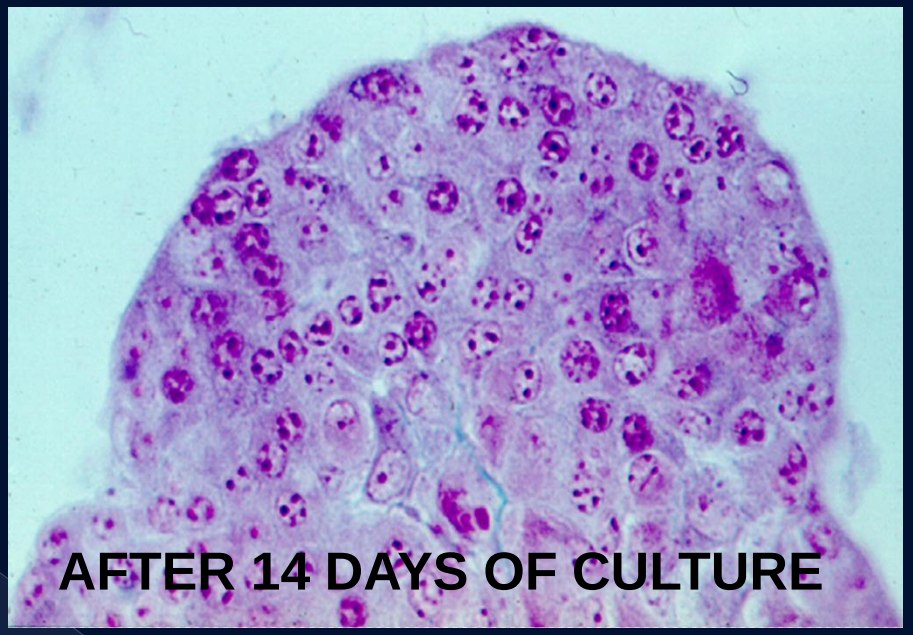
b) when large amount of extracellular matrix eg. cartilage – by collagenase with deoxyribonuclease

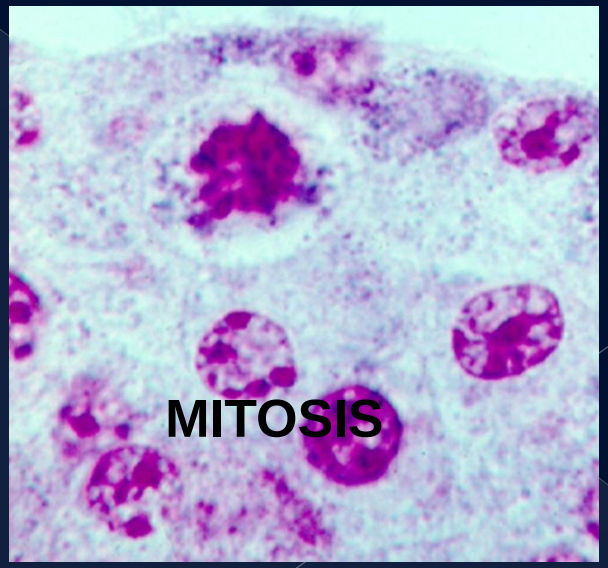
Separation the larger fragments eg. thyroid follicles or pancreatic islets – by collagenase

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

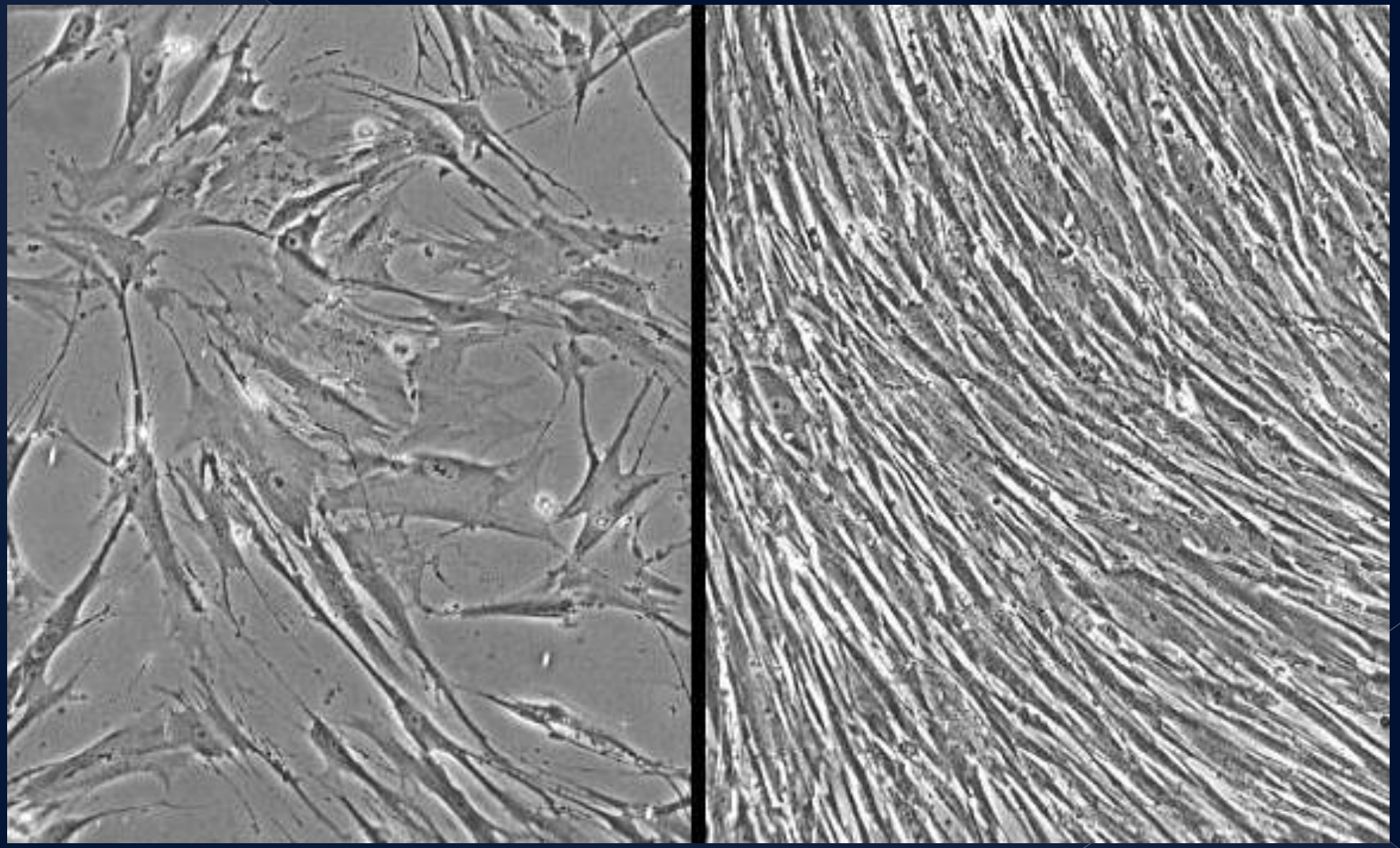
LANGERHANS ISLET







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

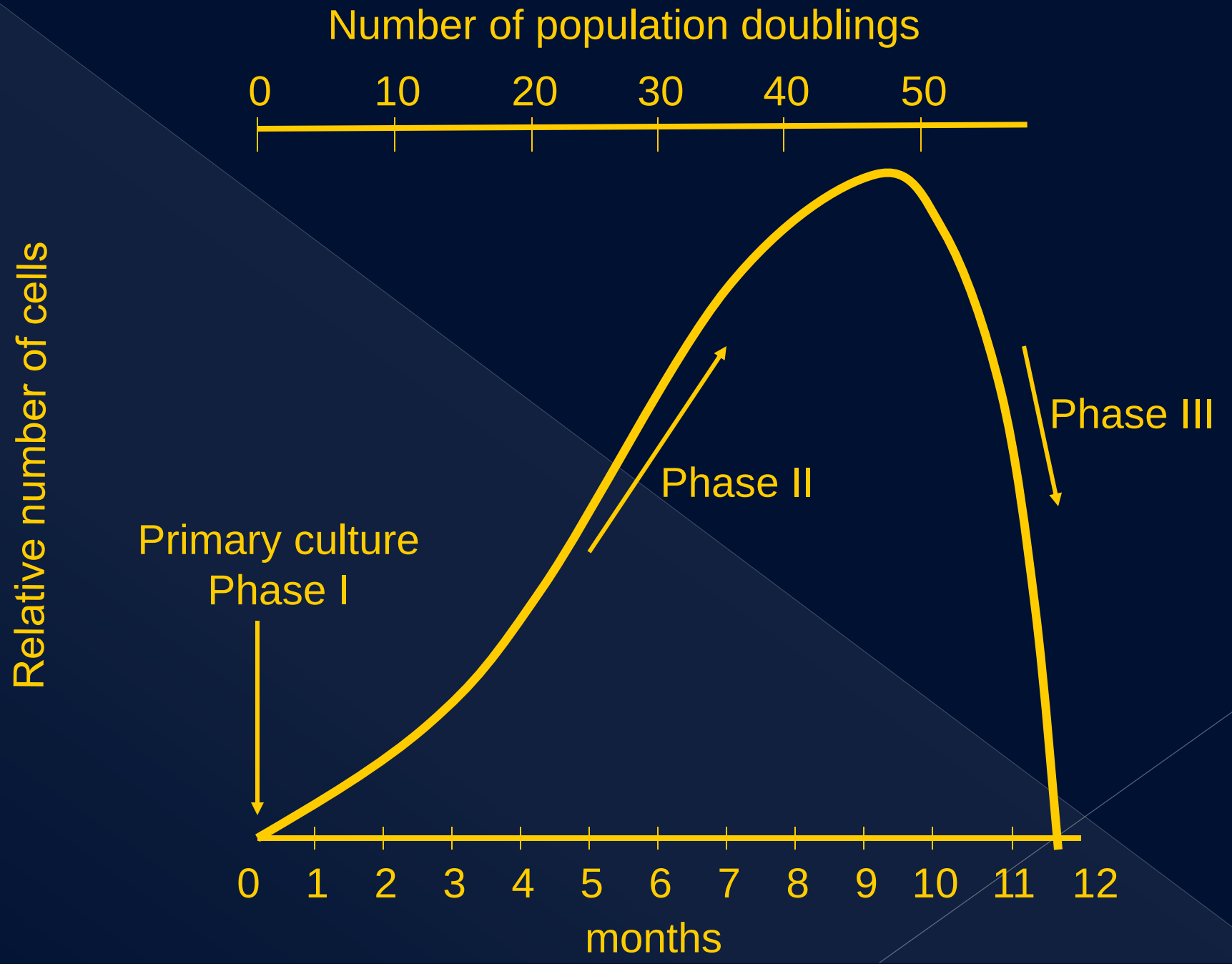
The ligand-receptor binding causes changes in the cyclin-dependent kinases (inactive Cdk4), which leads to inhibition of cell division.

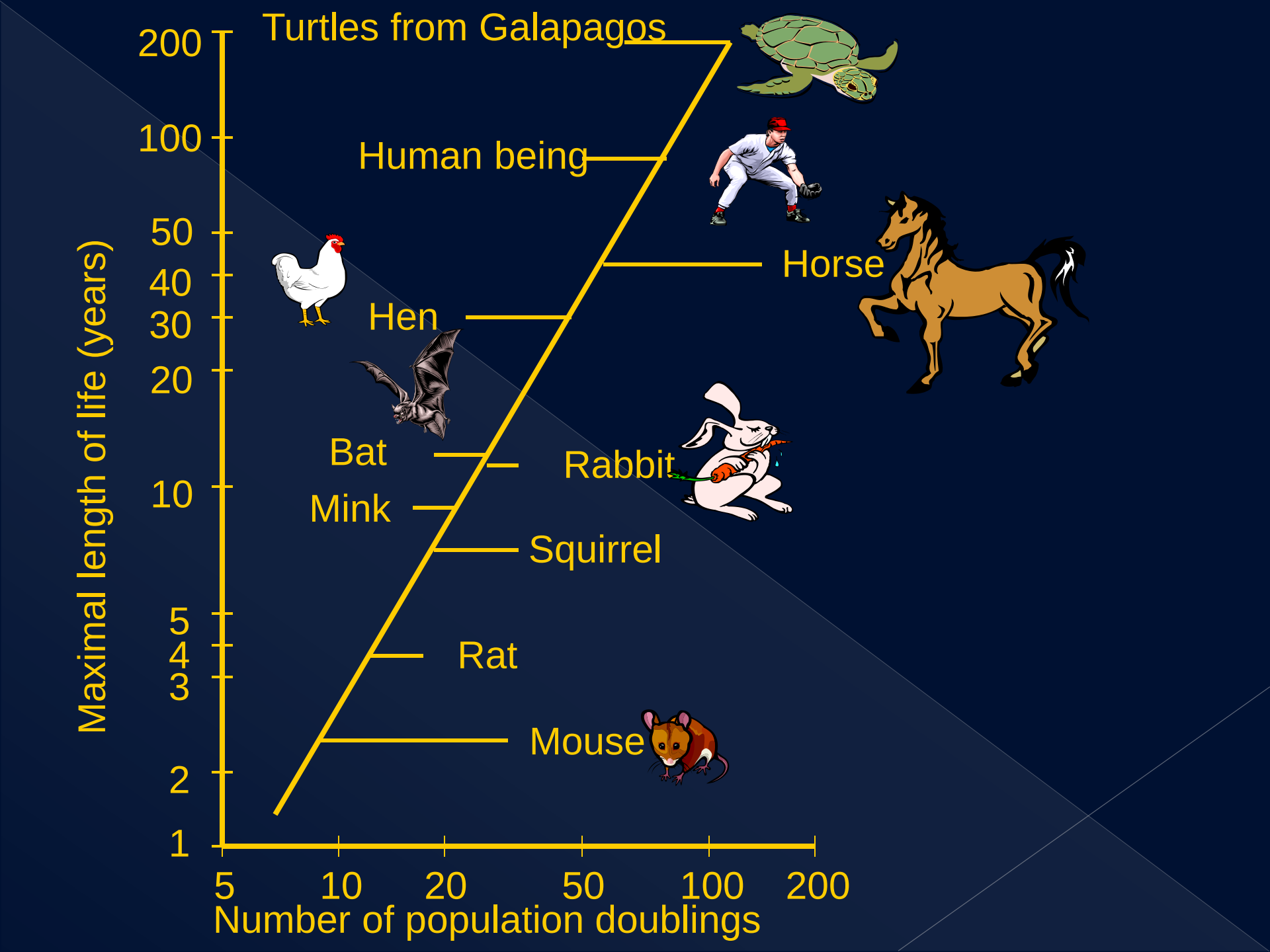
Transformed fibroblasts – lack of CiR

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

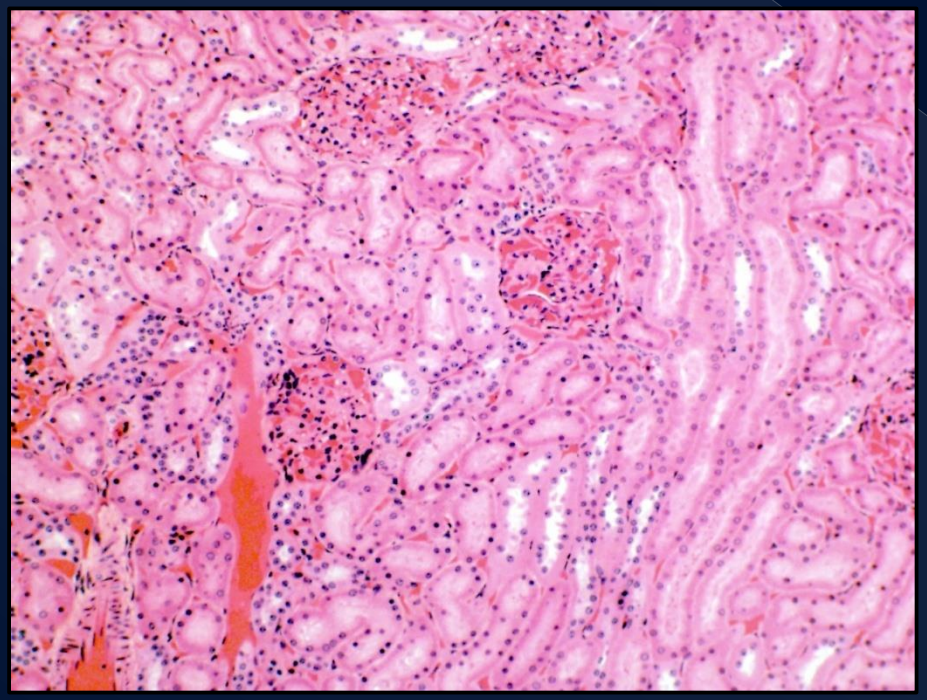

Number of population doublings
0
10
20
30
40
50
Relative number of cells
Primary culture
Phase I
Phase II
Phase III
0
1
2
3
4
5
6
7
8
9
10
11
12
months


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

How Cells Are Studied?

What can we study?

Cell (tissue) structure

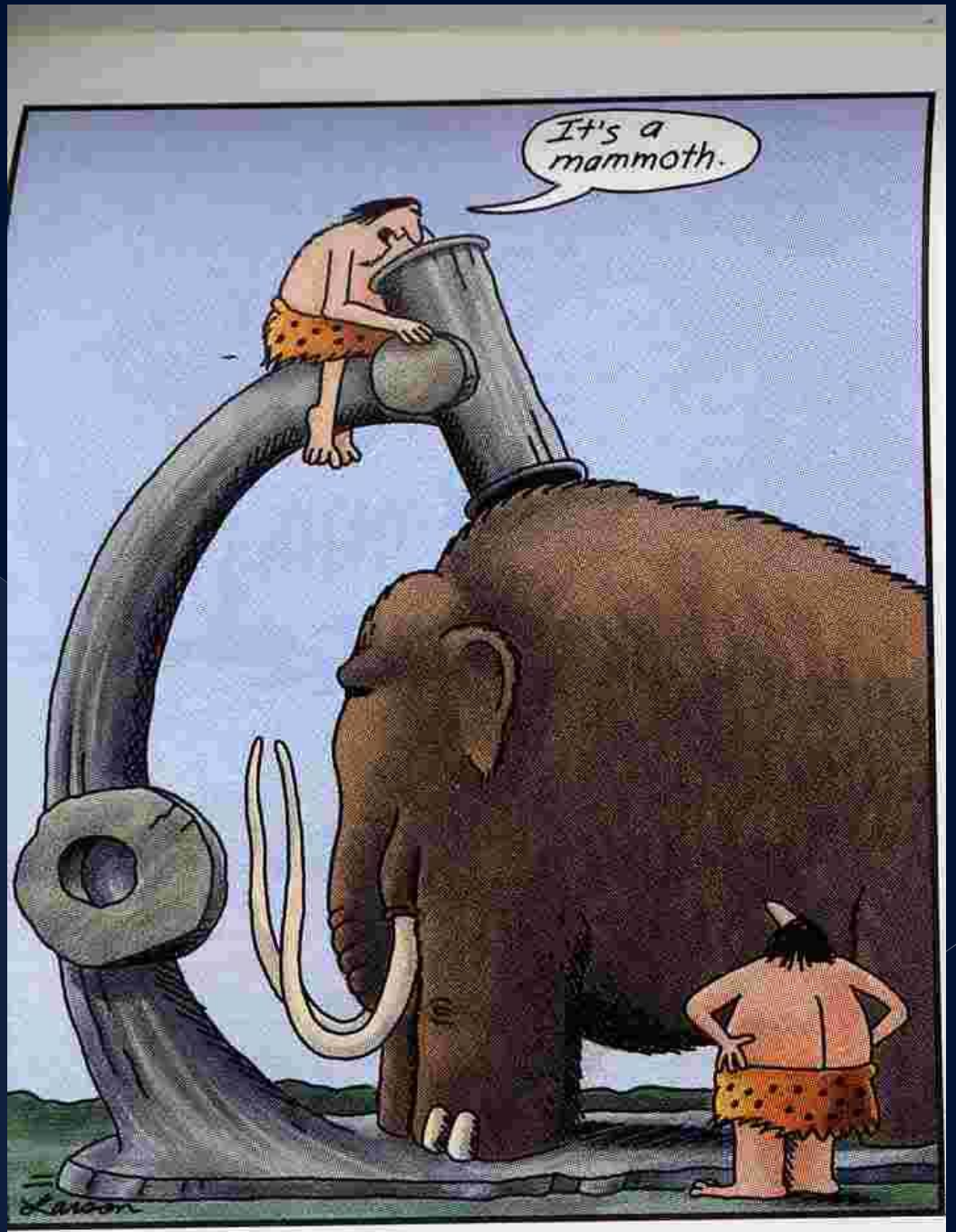


Light/electron microscopy

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)

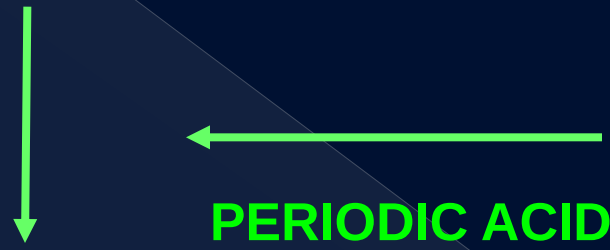
Histochemical color reaction

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

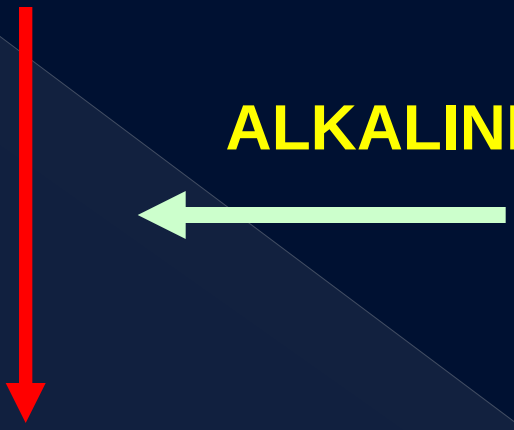
PRODUCT

INTERMEDIATE COMPOUND

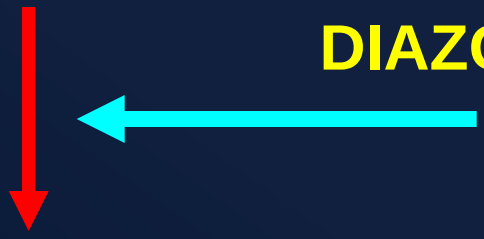
COLORFUL PRECIPITATE

(insoluble)

BETA-NAPHTHYL PHOSPHATE



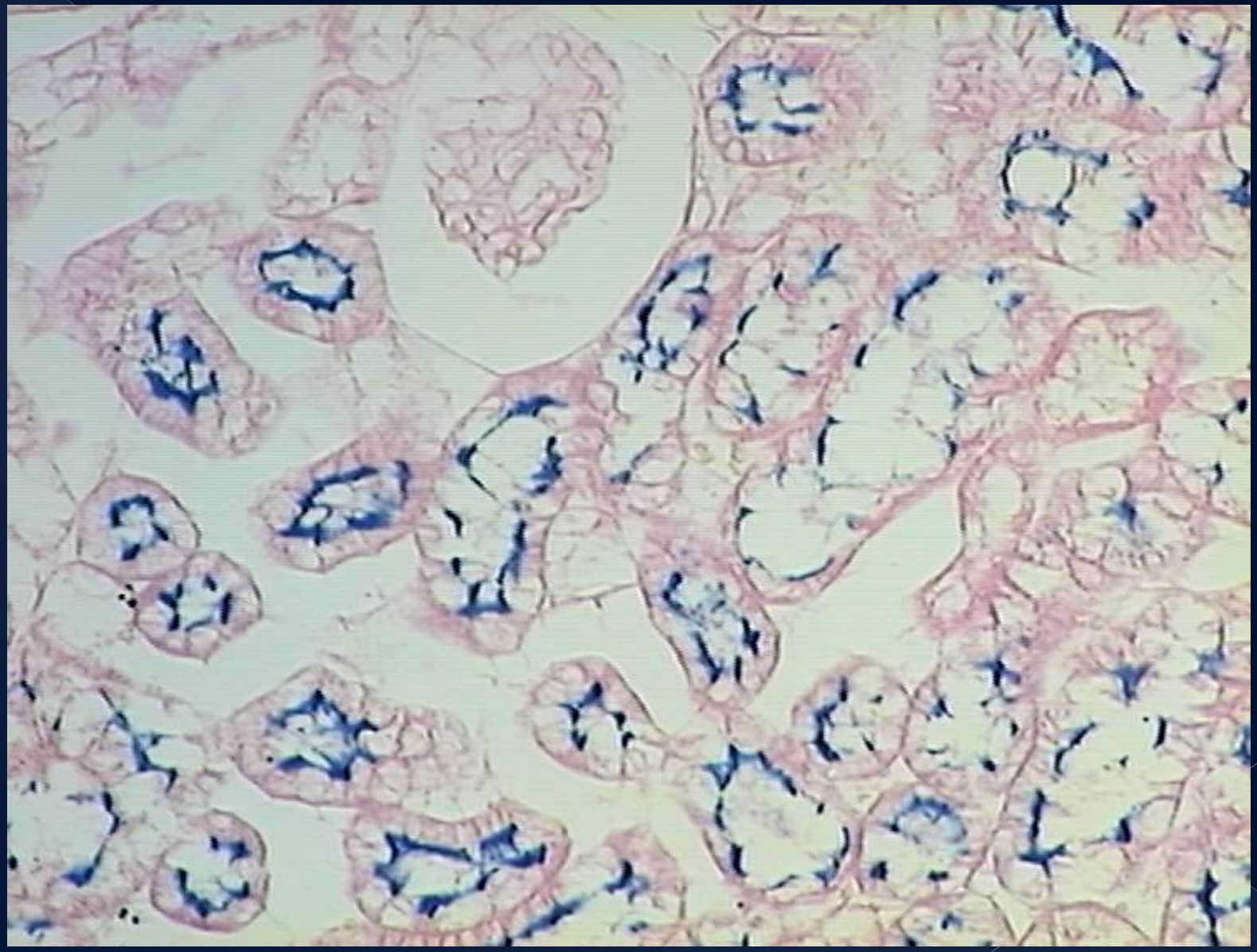
BETA-NAPHTHOL



COLOR COMPLEX

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

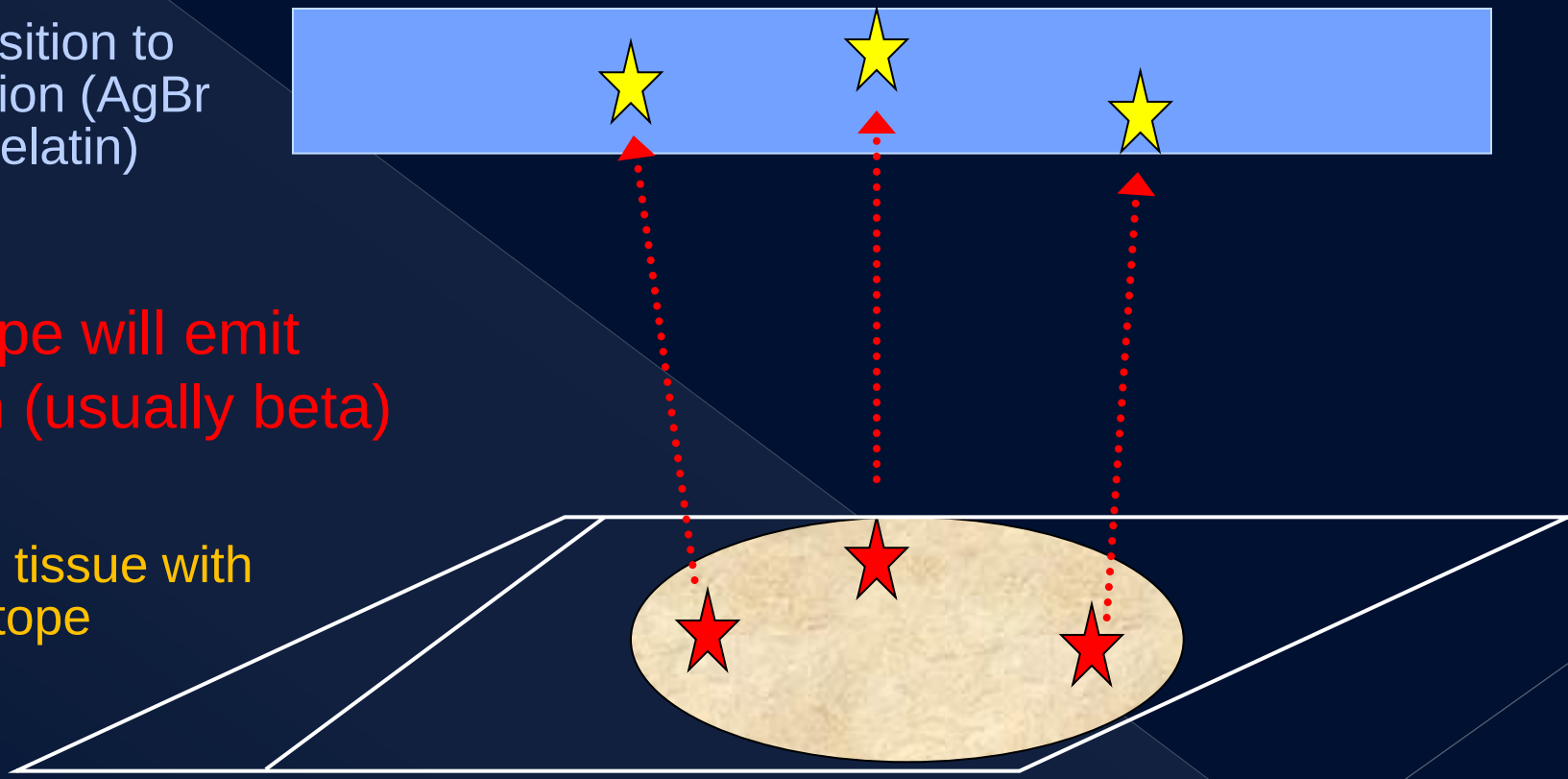
Staining for alkaline phosphatase, a marker for proximal tubule in normal kidney.



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

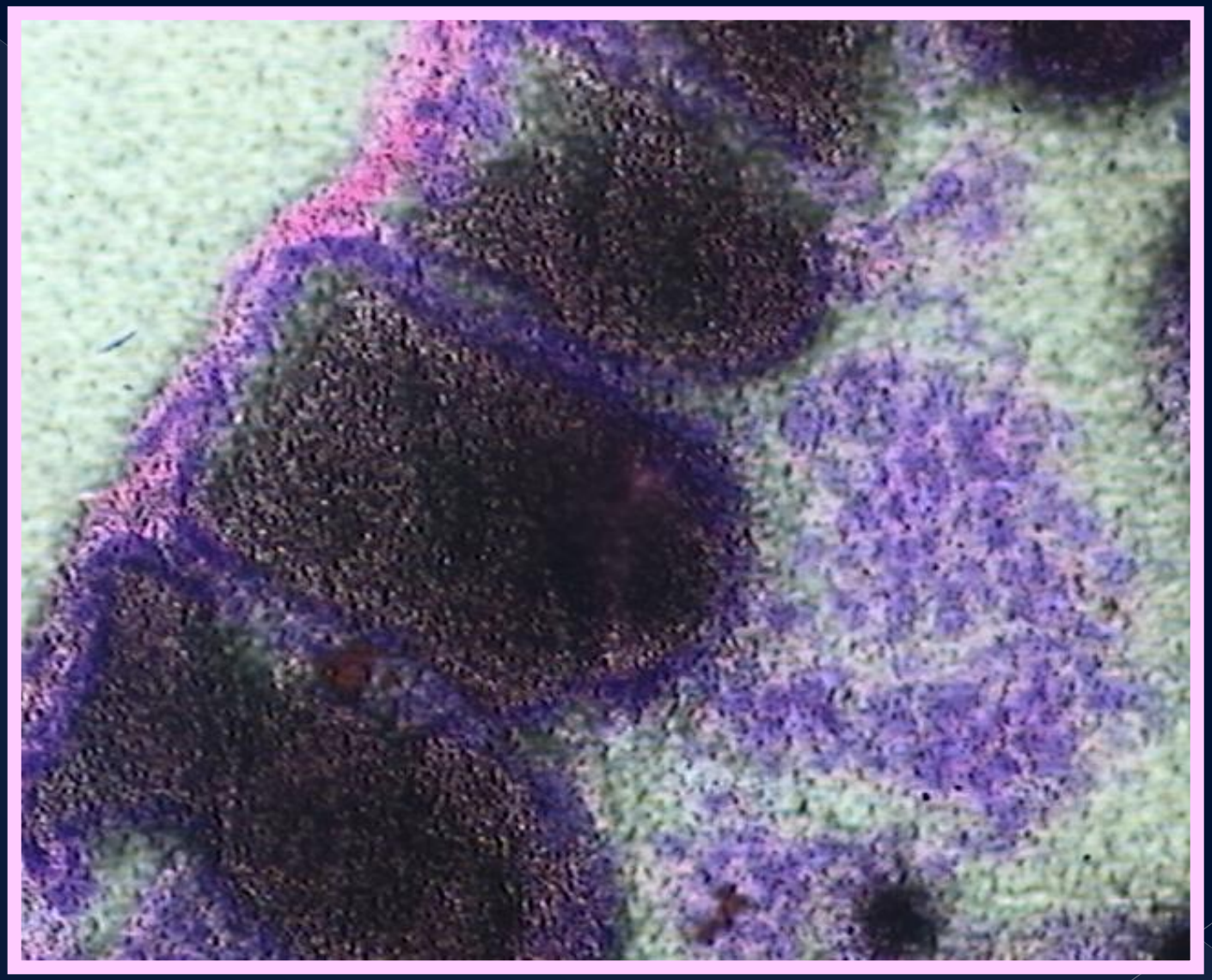
Exposition to emulsion (AgBr in gelatin)



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}

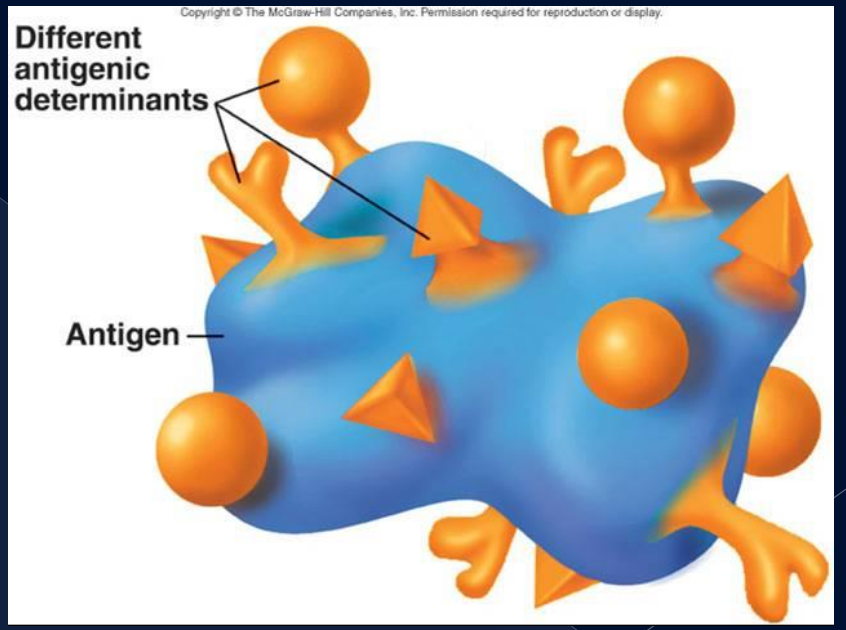
IMMUNOHISTOCHEMISTRY

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

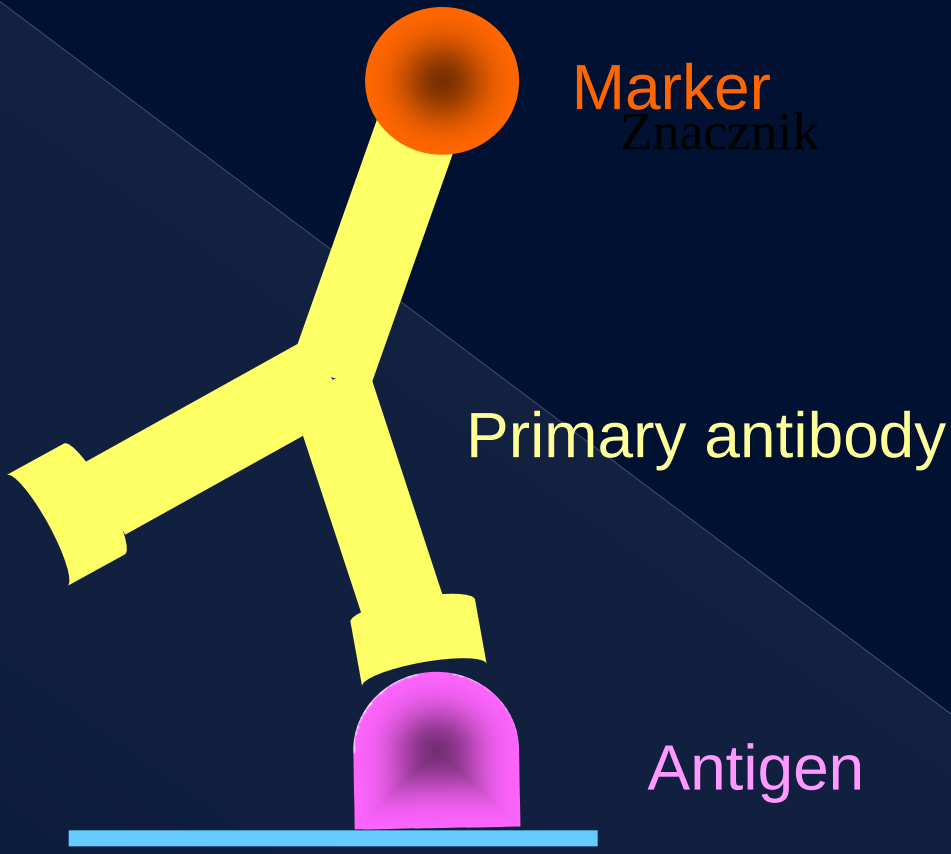
- DIRECT METHOD

-with using primary antibodies

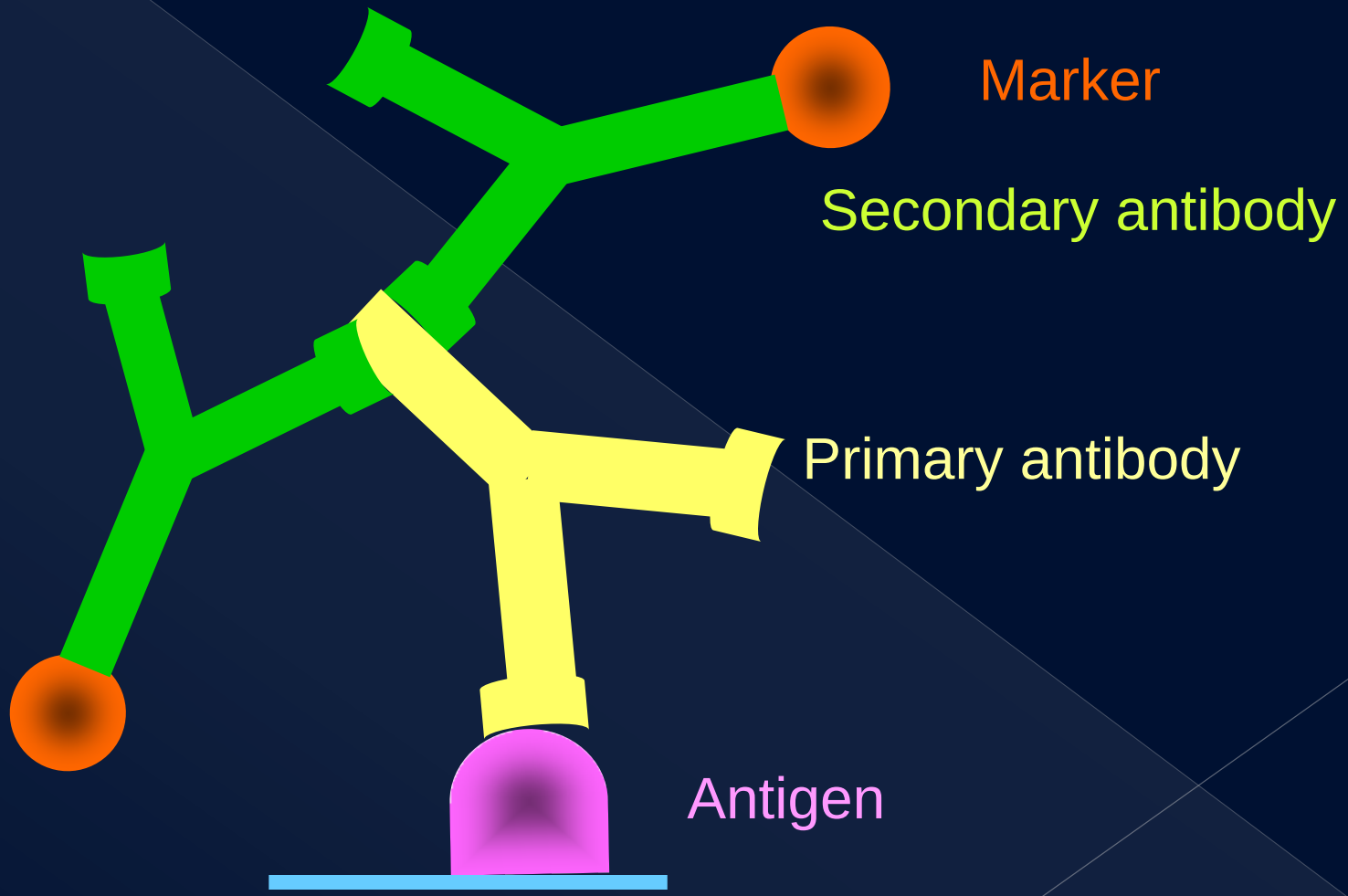
- INDIRECT METHOD

with using primary and secondary antibodies

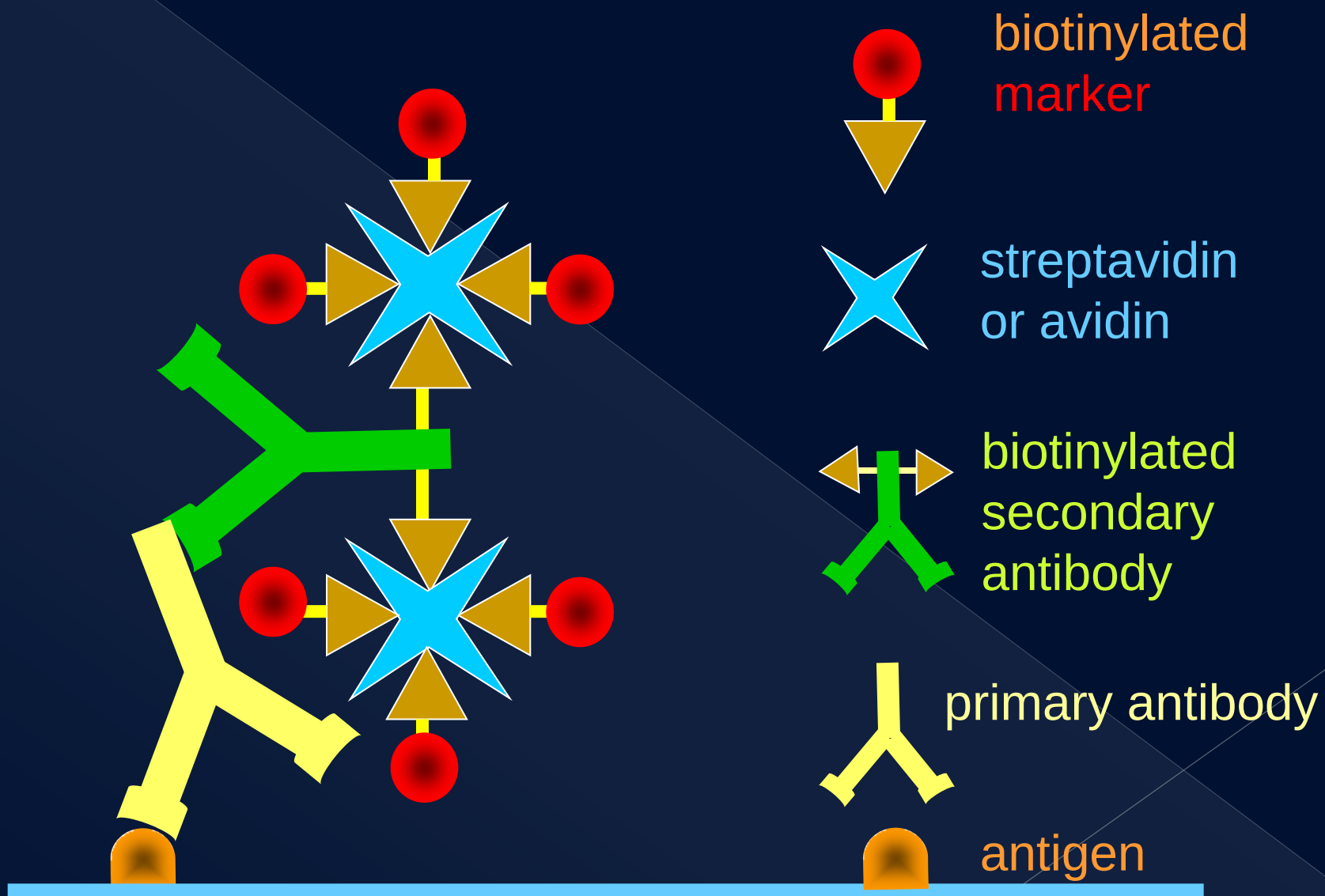
DIRECT IMMUNODETECTION



INDIRECT IMMUNODETECTION



INDIRECT IMMUNODETECTION



PEROXIDASE

SUBSTRATE H_2O_2

Diaminobenzidine (DAB)

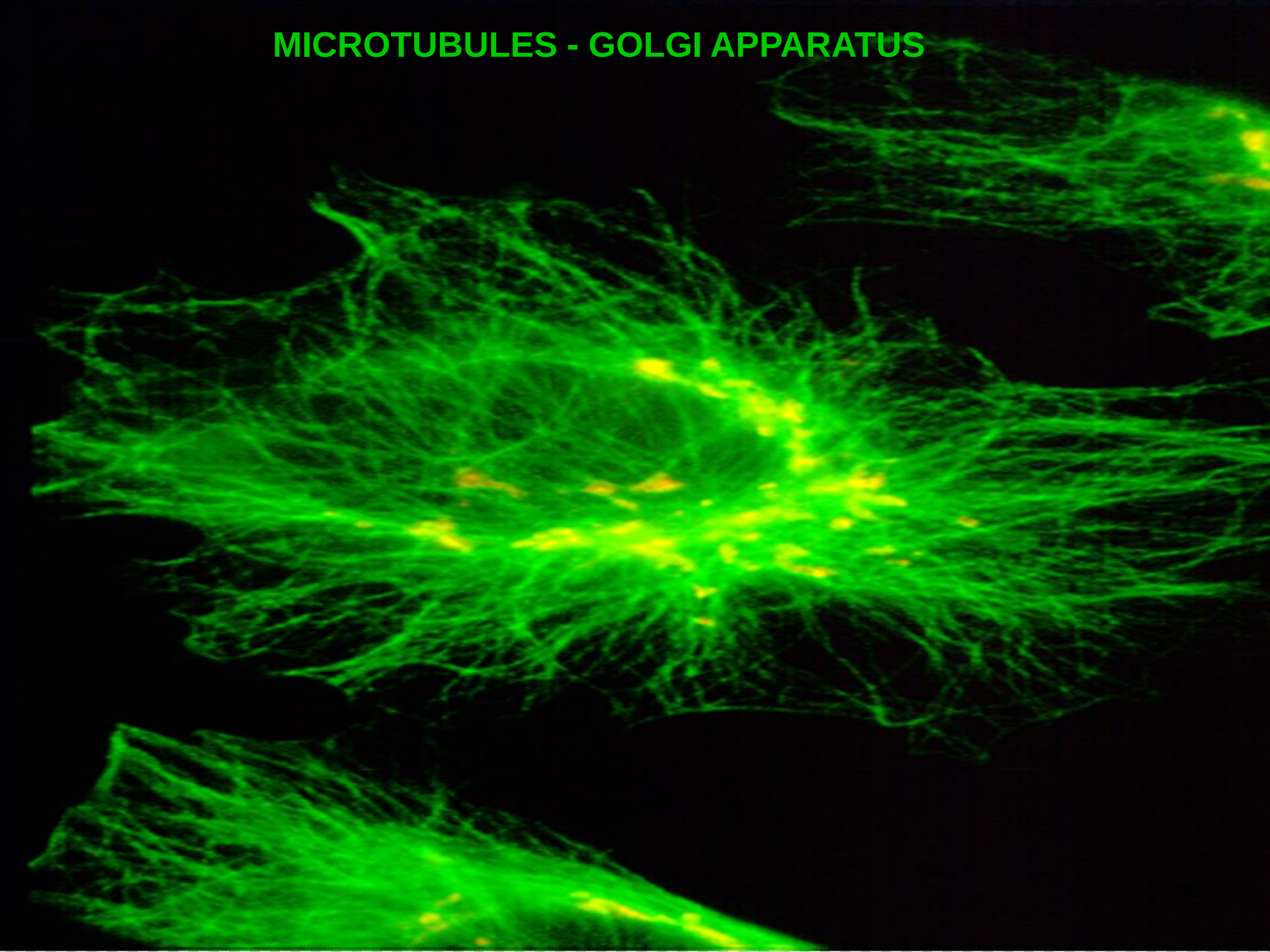
(chromogen)

Oxidation

BROWN PRECIPITATE

Detection of desmin in skeletal muscle cells

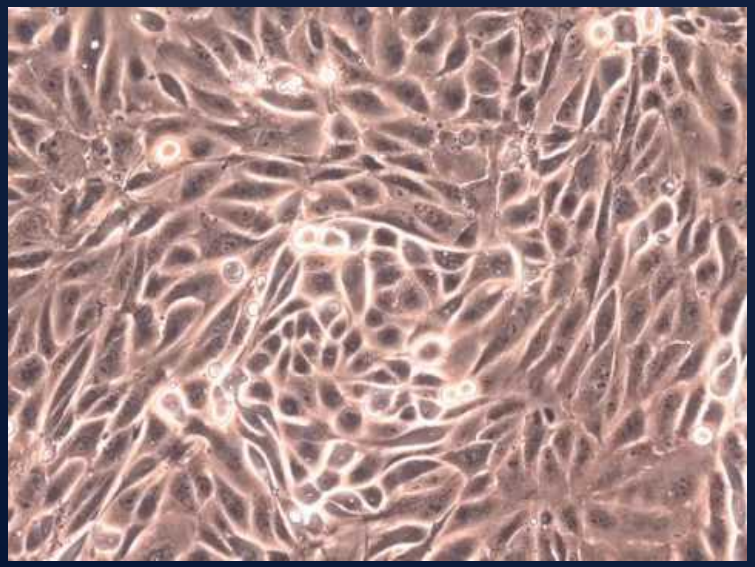
MICROTUBULES - GOLGI APPARATUS



How Cells Are Studied?

What else we can study?

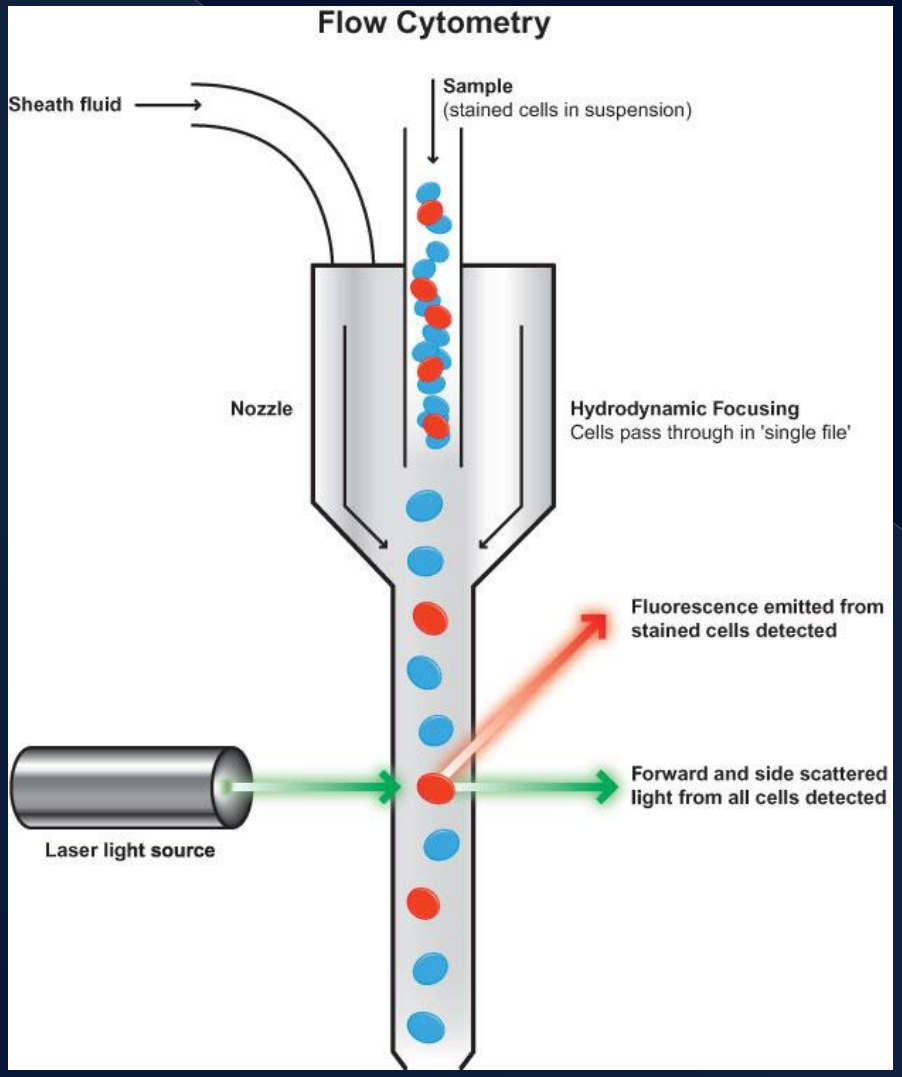
Cell/tissue culture



human kidney epithelial cell culture

Inverted microscope

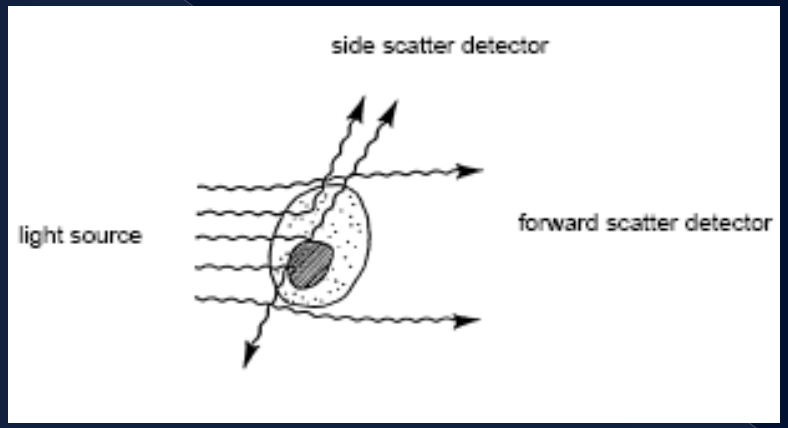
Flow Cytometry



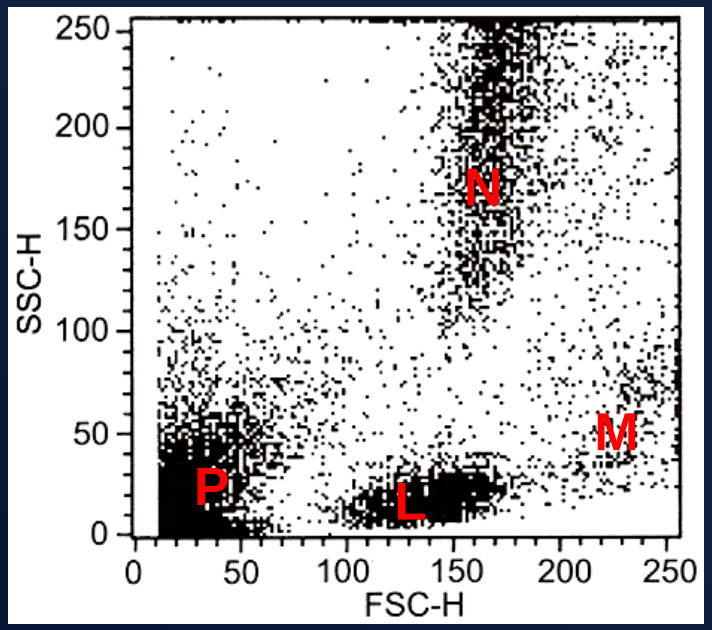
A flow cytometer has an optical system – lamps, lasers, a detector, measuring system and Analogue-to-Digital Conversion (ADC) system - which converts forward-scattered light (FSC), side-scattered light (SSC) and fluorescence signals into electrical signals that are processed by a computer.

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.



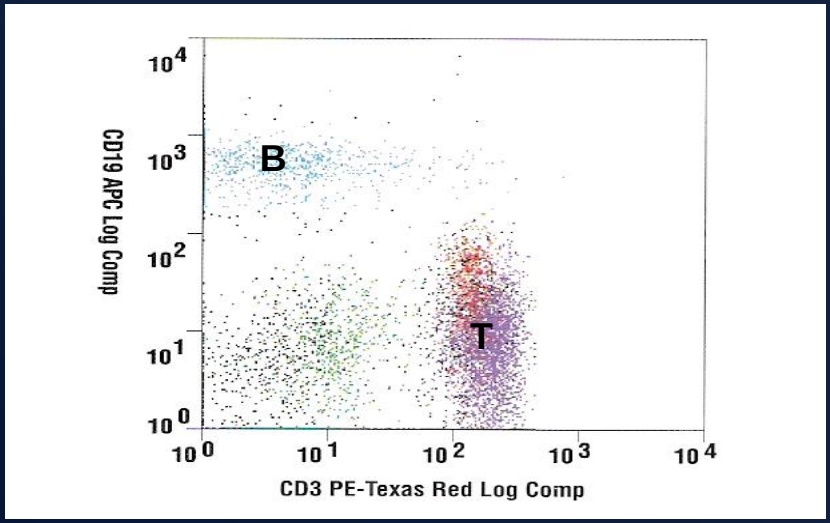
SSC - Side-scattered light is proportional to cell granularity or internal complexity. The more complicated cell the greater dispersion of light or SSC.

Human blood leukocytes FSC/SSC.

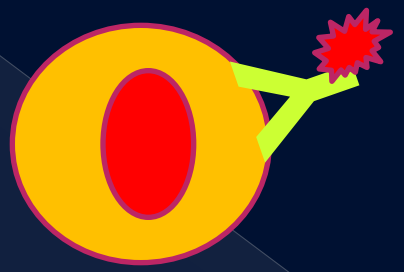
Flow Cytometry

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.



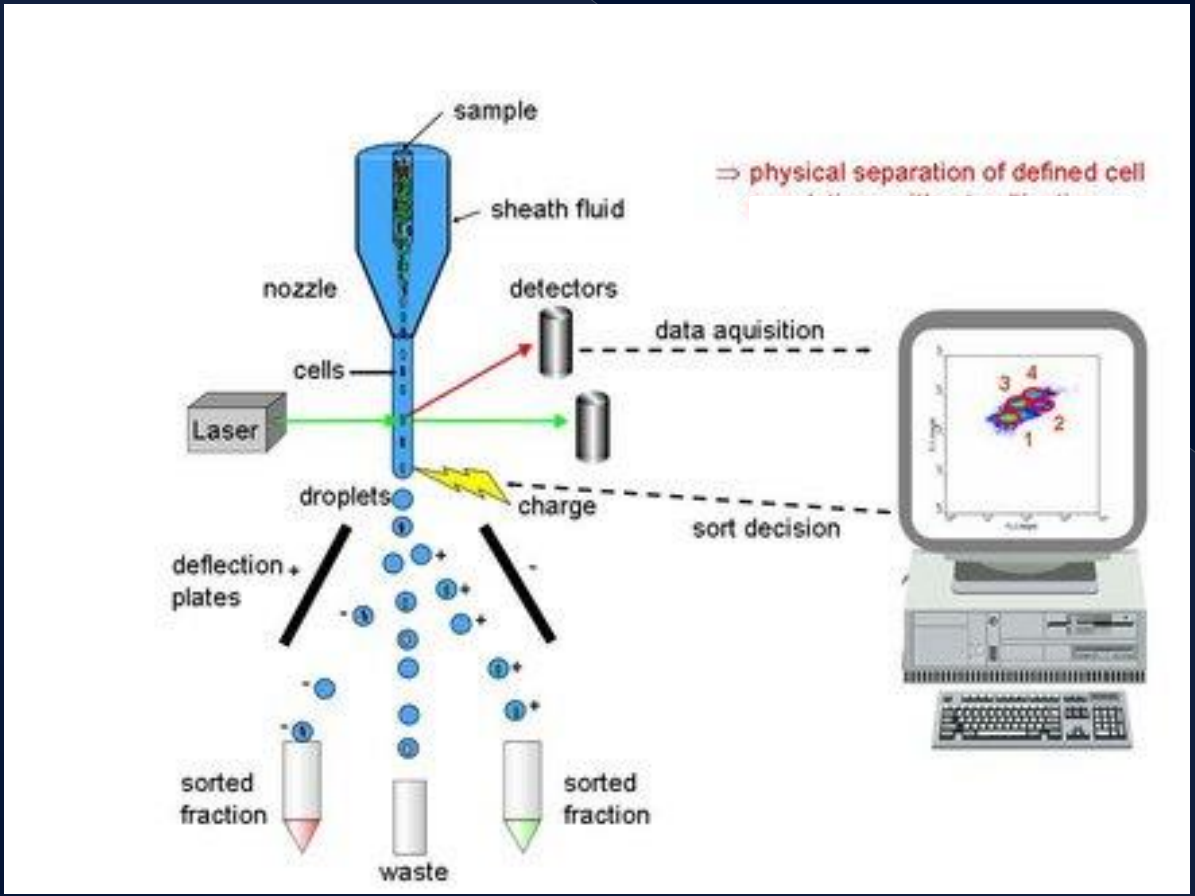
Anti-CD3 and anti-CD19.



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

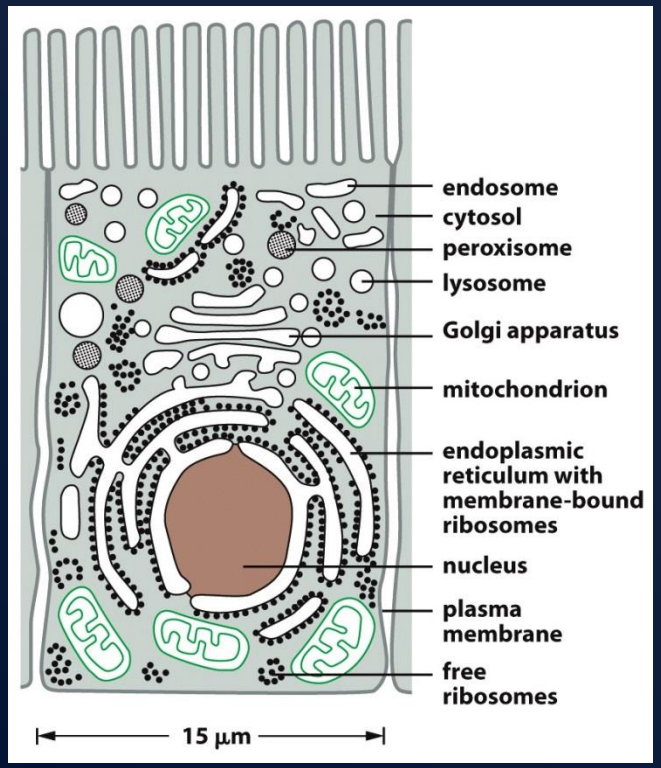
Proteins

Saccharides

Nucleic Acids

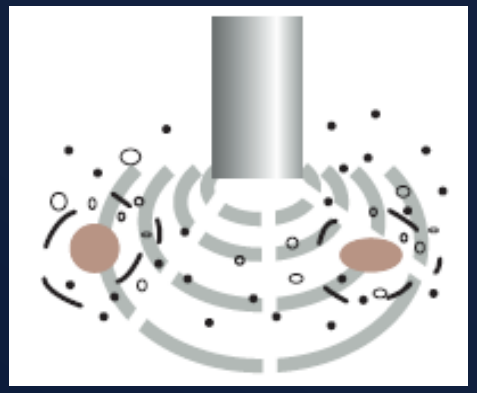
Lipids

Cell Ingredients:

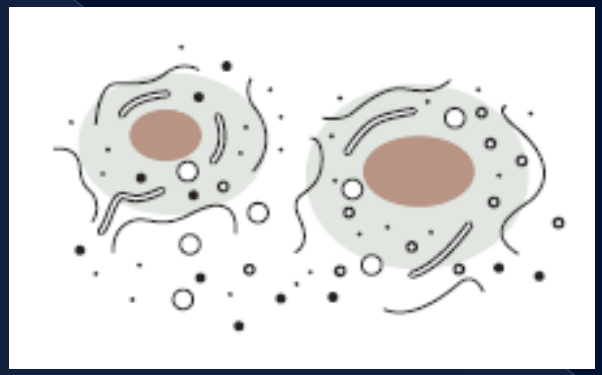


How Cells Are Studied?

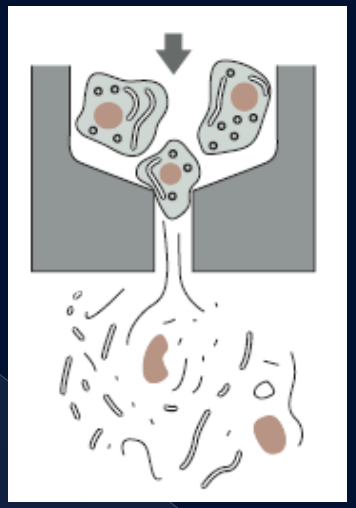
Material Isolation

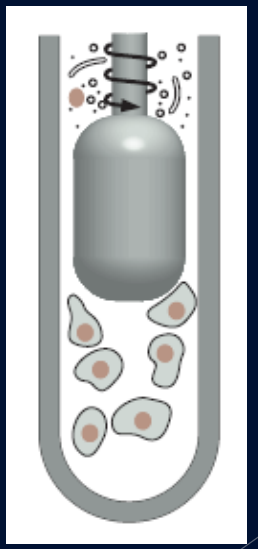


Ultrasonic cell disruption



The breakdown of cell membranes by detergents

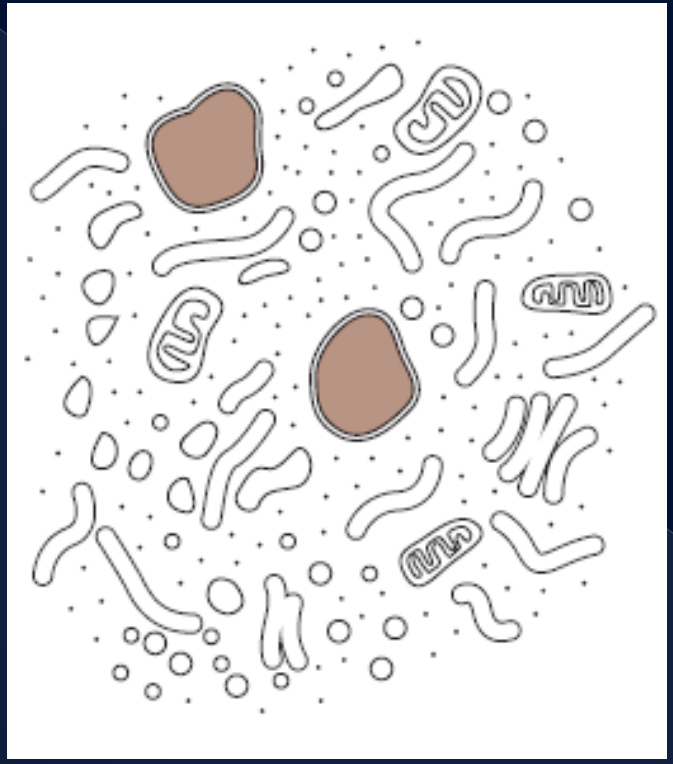




Mechanical disruption

How Cells Are Studied?

Material Isolation

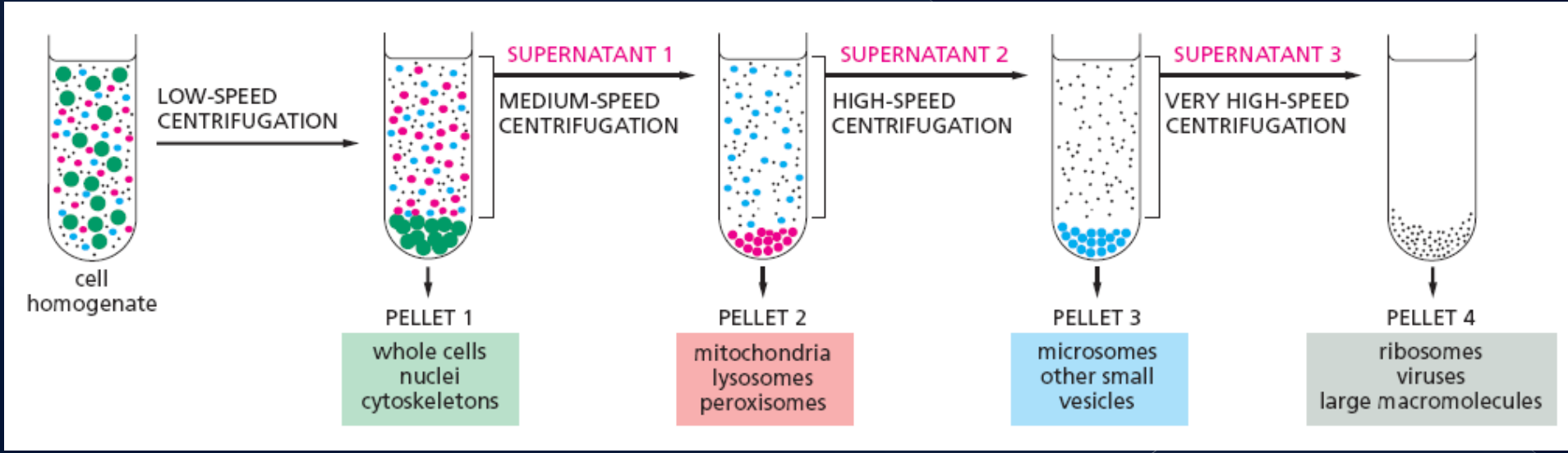


Cell homogenate

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

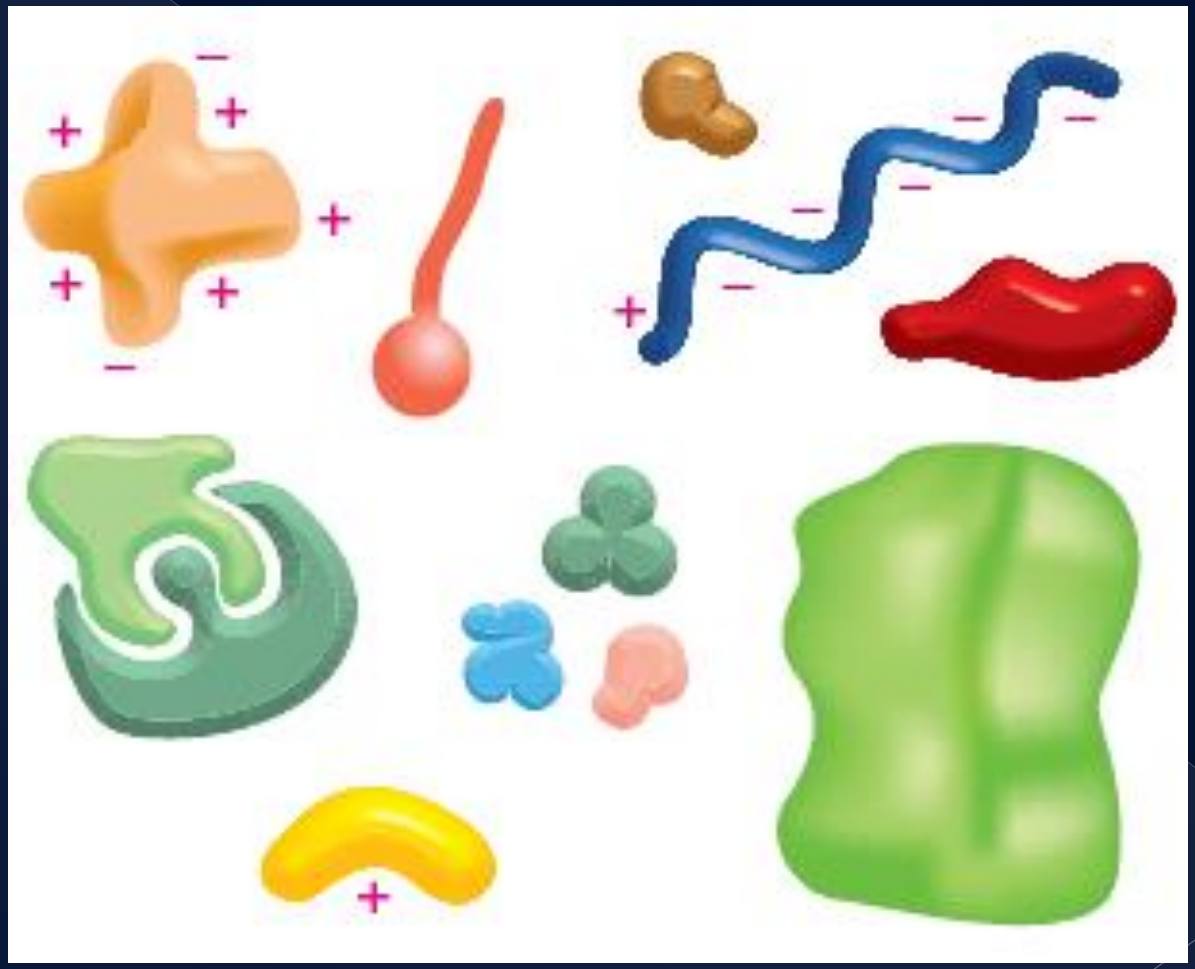
Method used to detect specific proteins in a sample of tissue homogenate, cellular extract or in body fluid.

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.”

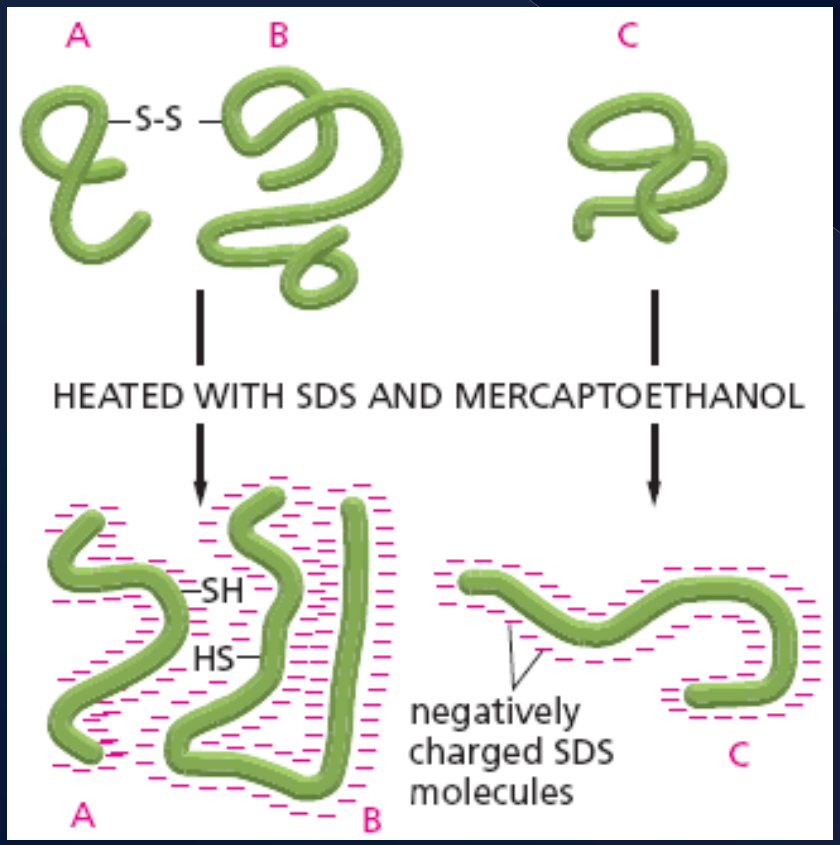
Protein analysis

Separation and detection



Protein analysis

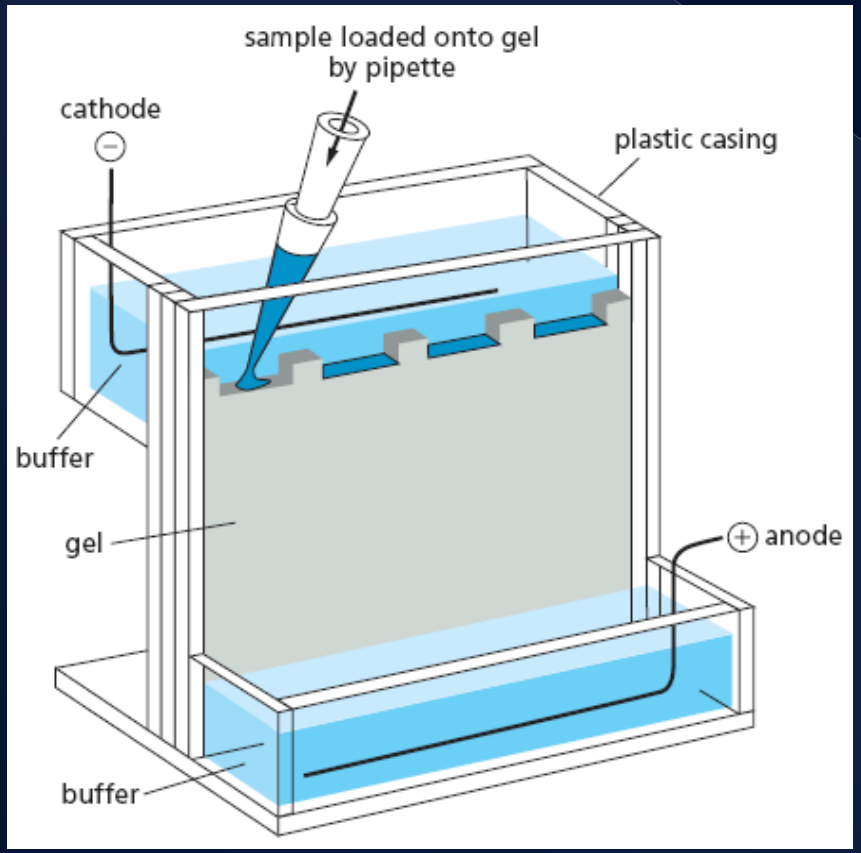
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis



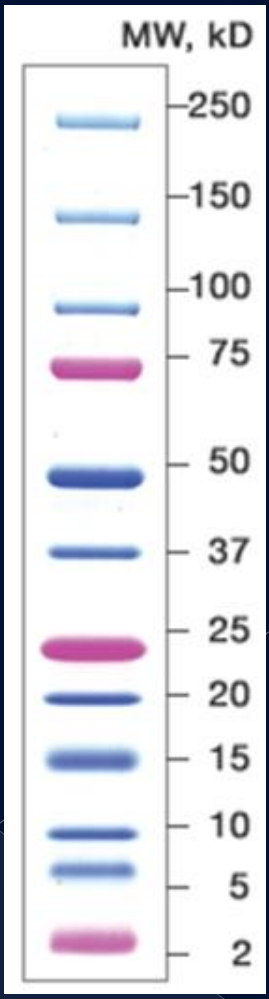
Proteins in the sample are dissolved and heated in buffer with SDS, 2-ME and bromophenol blue. The negatively charged detergent SDS (anionic surfactant) unfolds proteins and coats them with a uniform negative charge density; disulfide bonds (S-S) are reduced with mercaptoethanol. Bromophenol blue stains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

Protein analysis

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

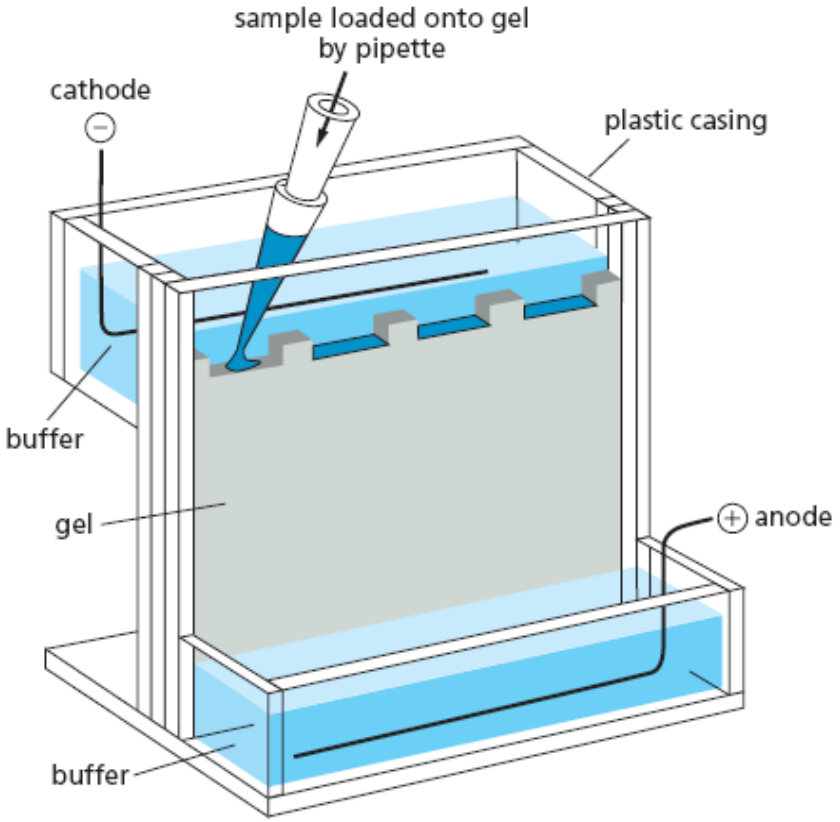


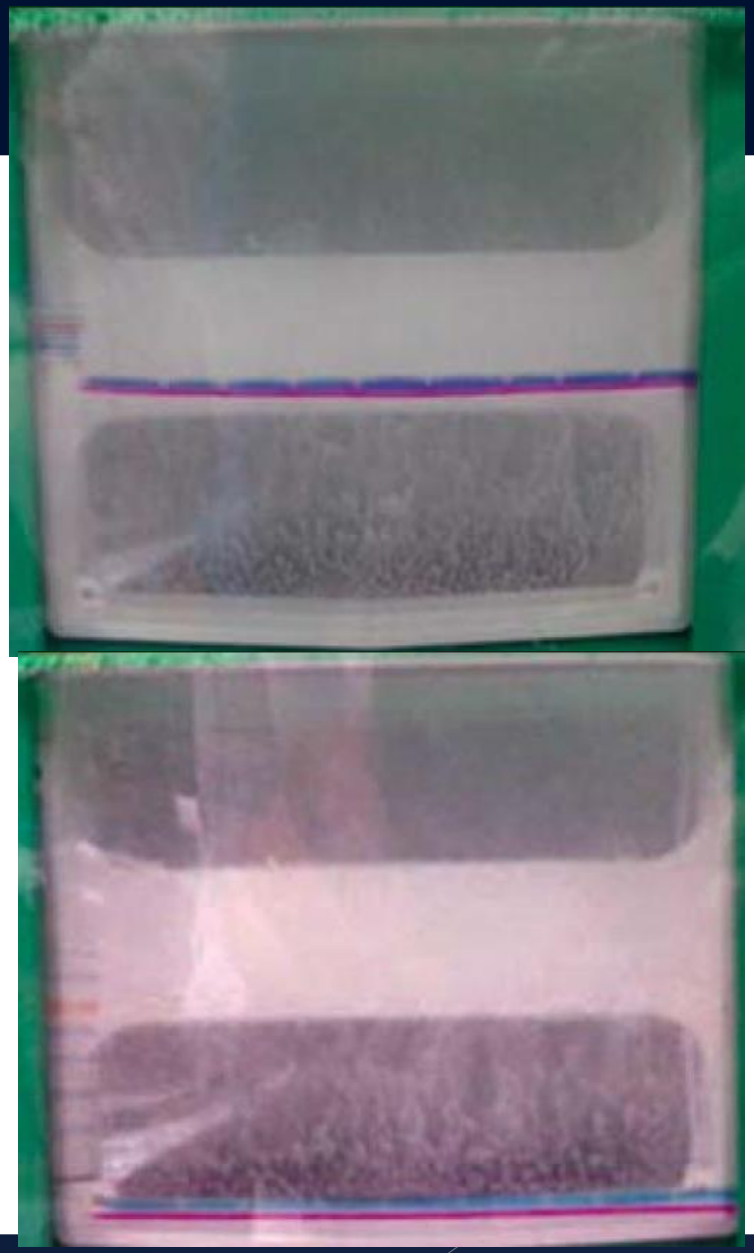
The sample is loaded to the well of a gel, and a voltage is applied. The negatively charged detergent-protein complexes migrate to the bottom of the gel, toward the positively charged anode. Small proteins move faster, larger proteins move slower, so proteins are separated by size, smaller toward the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



Protein analysis

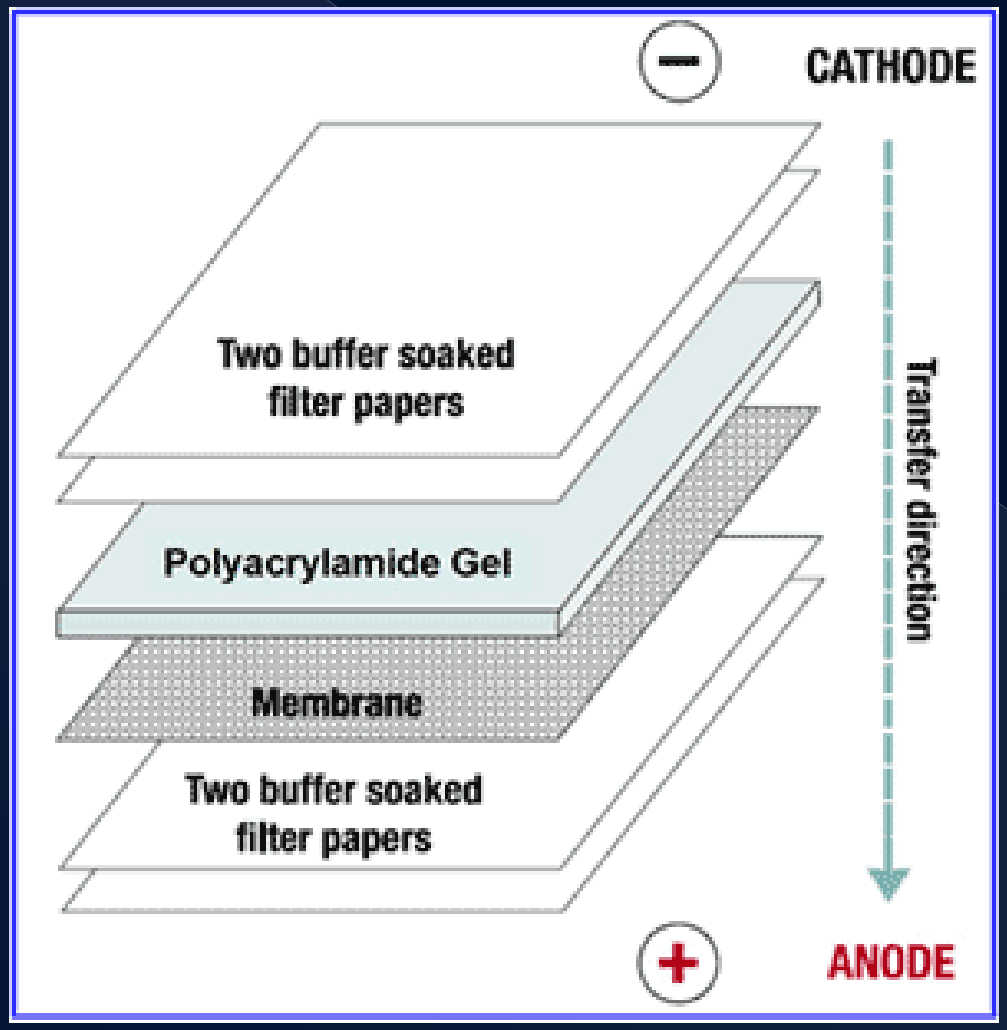
Electrophoresis





Protein analysis

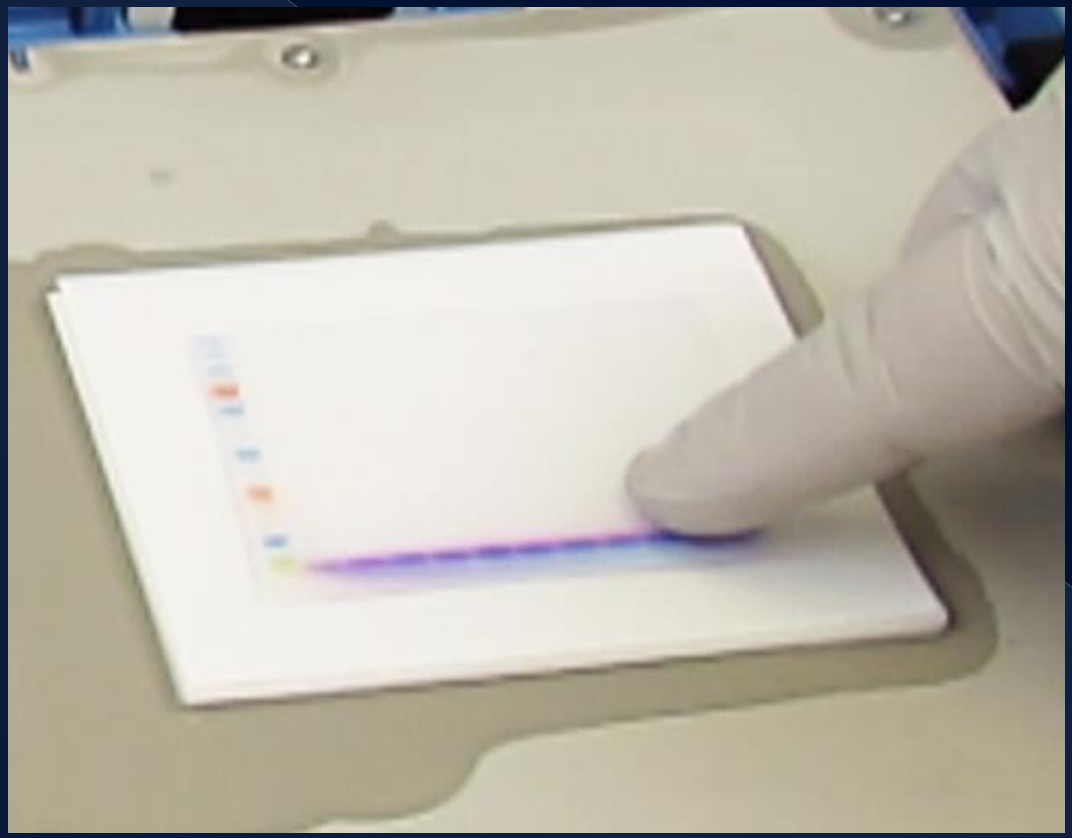
TRANSFER PROTEINS FROM GEL TO MEMBRANE



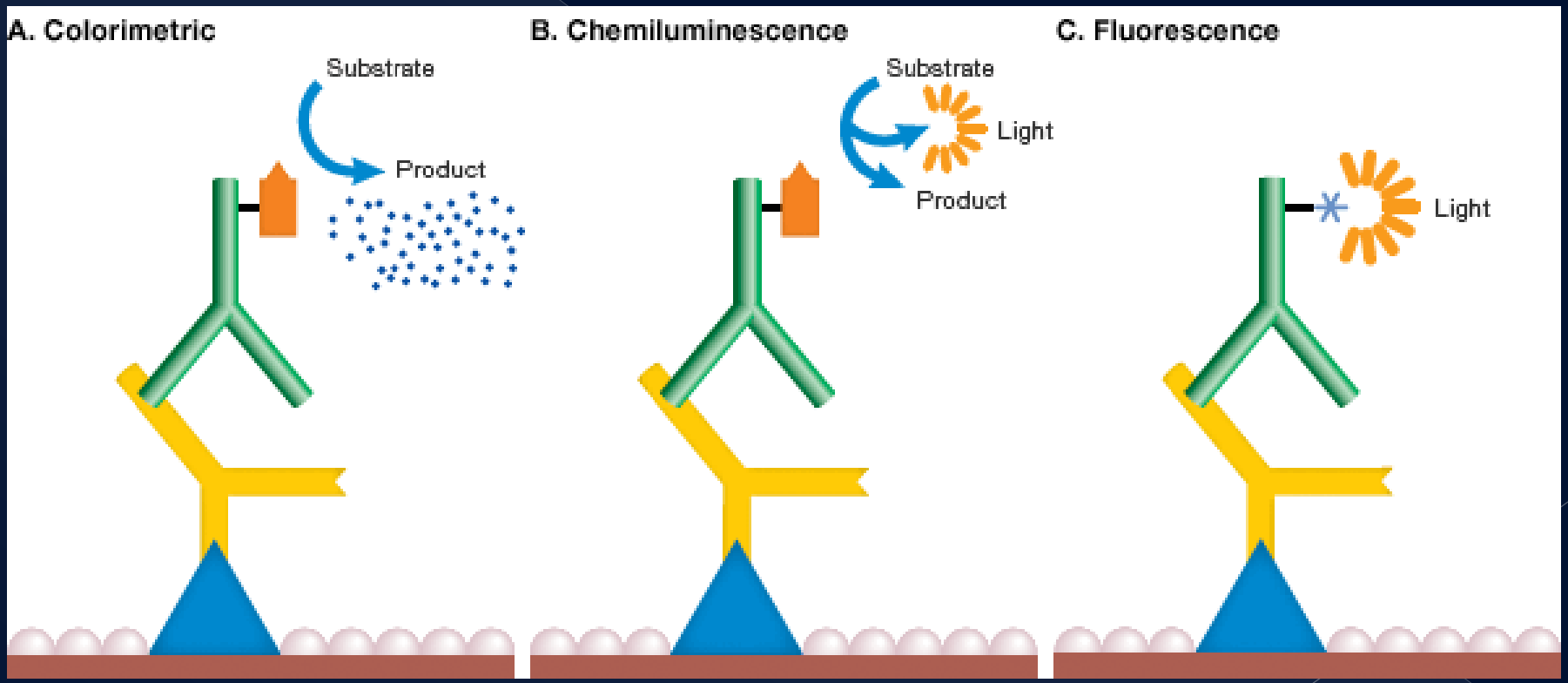
The gel with the resolved set of proteins is then placed in an apparatus that permits electrophoretic transfer of the proteins from the gel onto the surface of a special paper (e.g., nitrocellulose paper, polyvinylidene fluoride (PVDF) membrane) to which proteins strongly adsorb.

Protein analysis

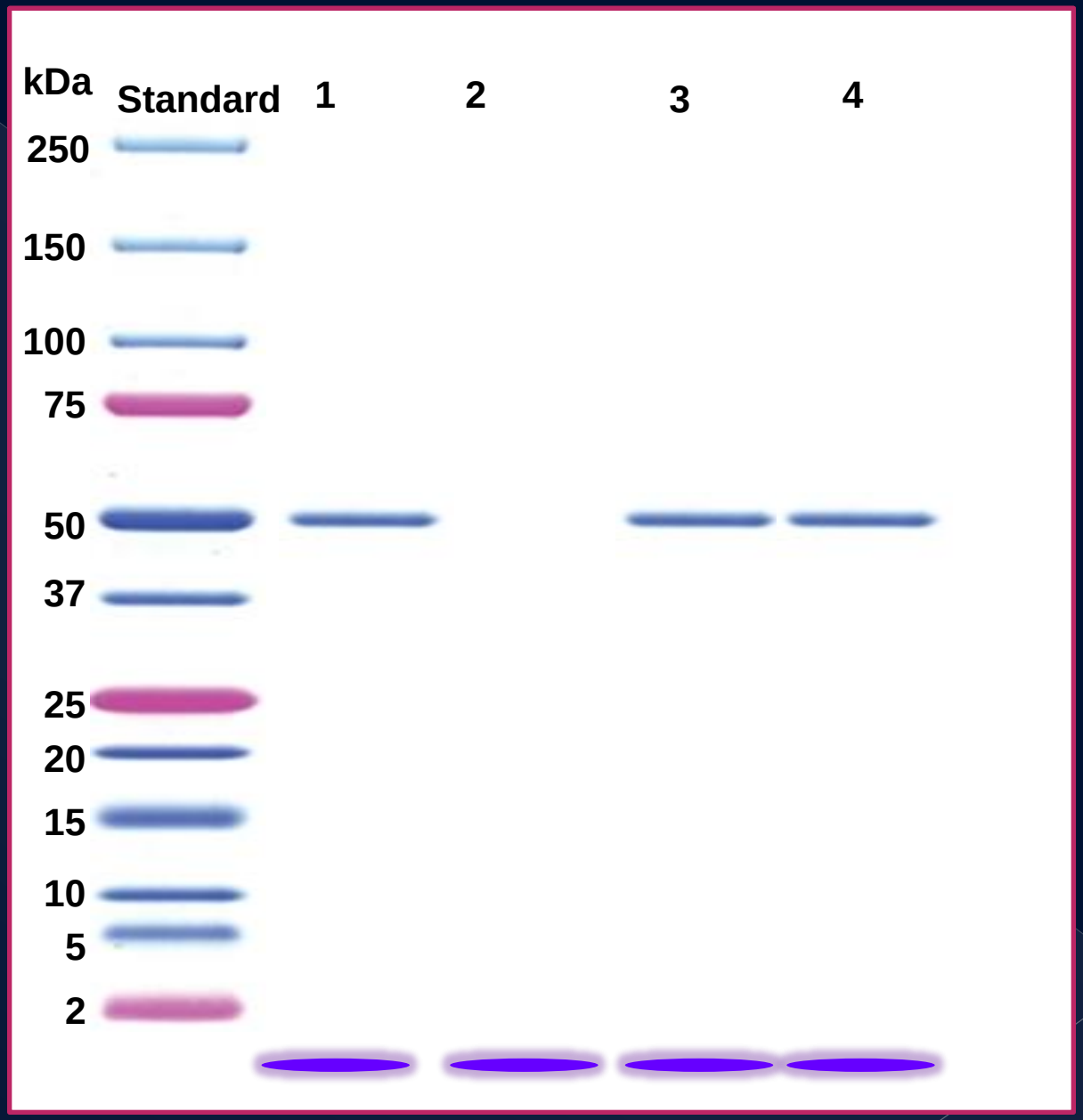
WESTERN BLOT – PVDF MEMBRANE AFTER TRANSFER



WESTERN BLOT - METHODS OF DETECTION



WESTERN BLOT - THE RESULT



Protein analysis

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

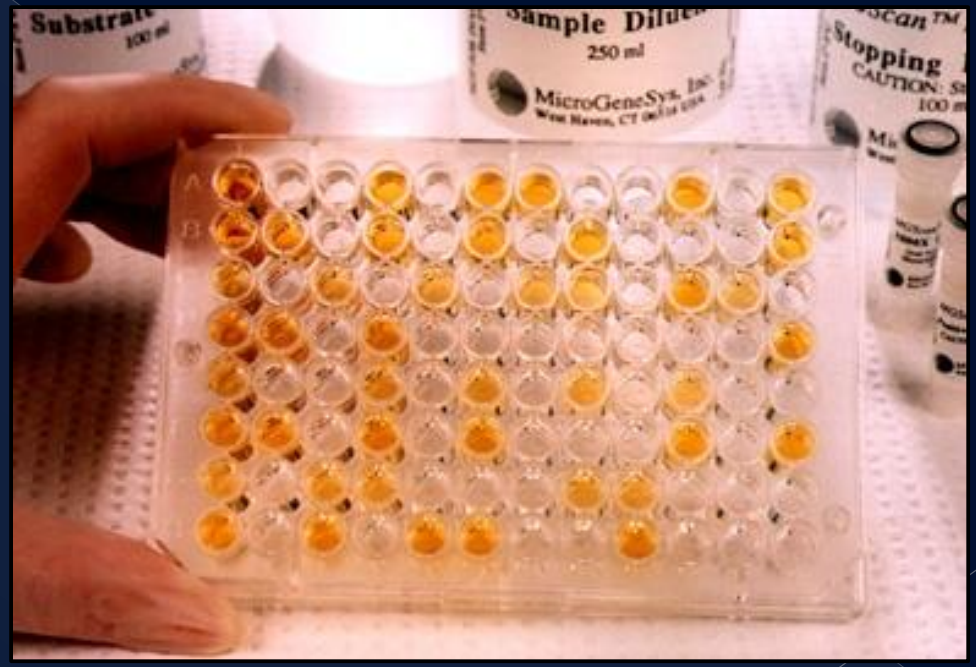
1966 - fixation of antibody or antigen to the surface of a container - Wide and Jerker Porath (Uppsala University)
1971 - Peter Perlmann and Eva Engvall at Stockholm University invented ELISA

Enzyme

Linked

Immunosorbent

Assay

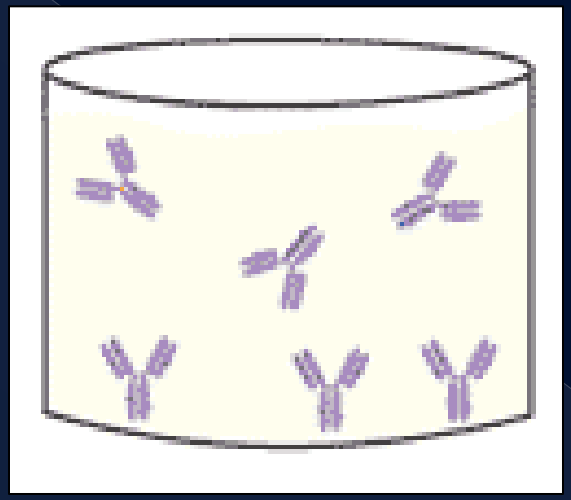


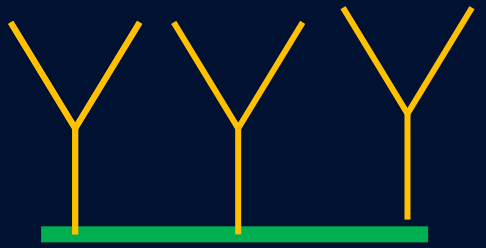
Protein detection and quantification

Protein analysis

ELISA

96-well plate

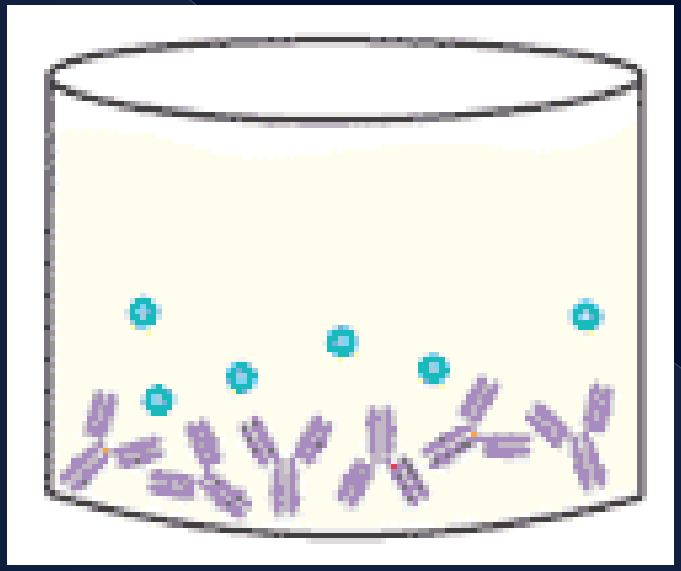


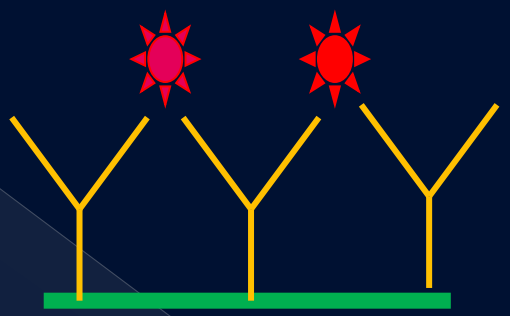


Specific capture antibody coating

Protein analysis

ELISA

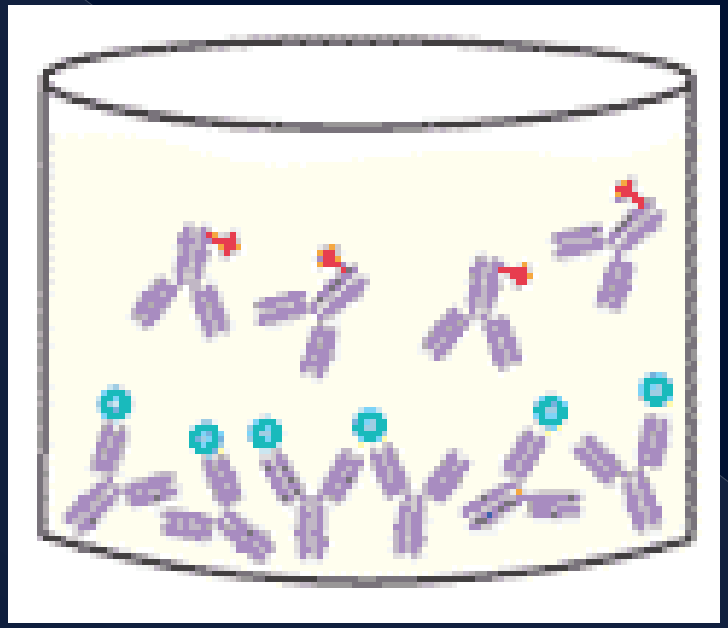


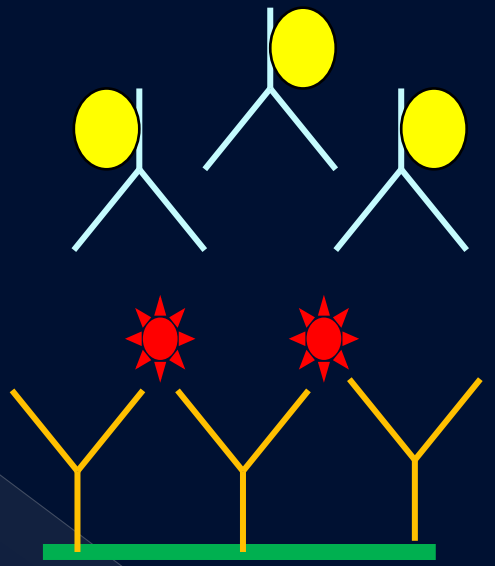


Sample with target protein

Protein analysis

ELISA

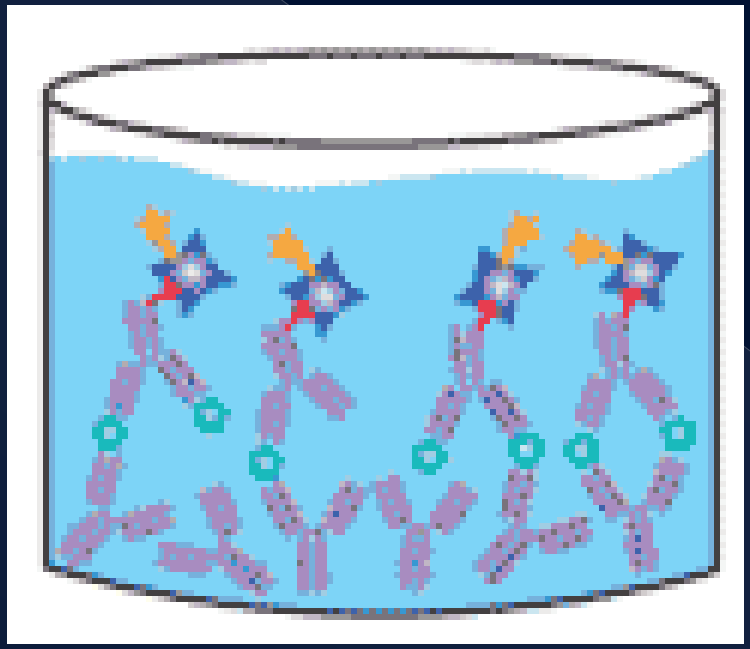


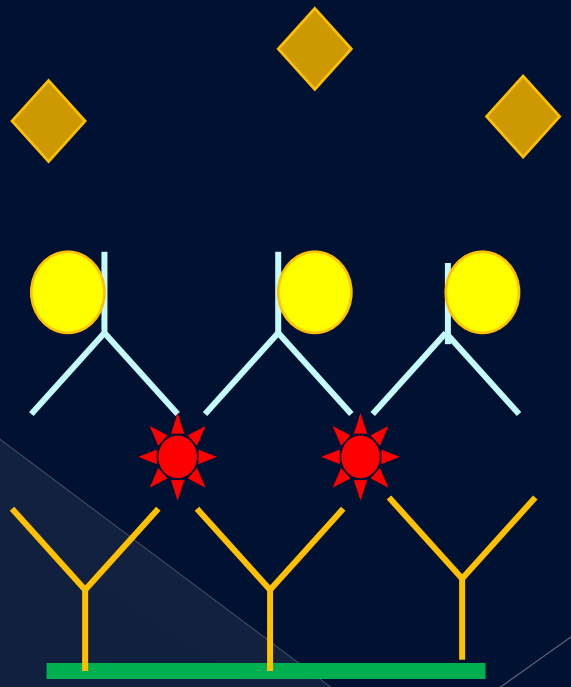


Antibodies conjugated with enzyme

Protein analysis

ELISA

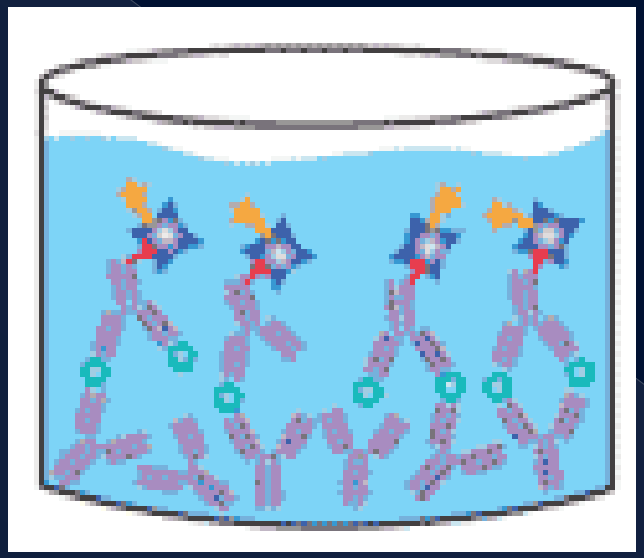


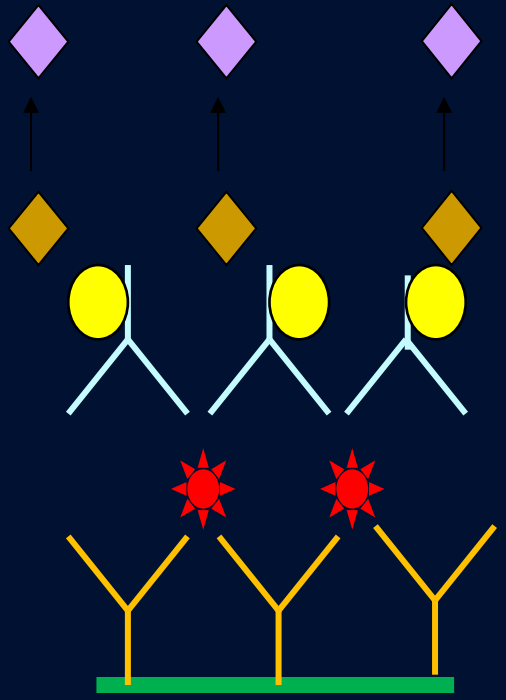


Substrate addition

Protein analysis

ELISA

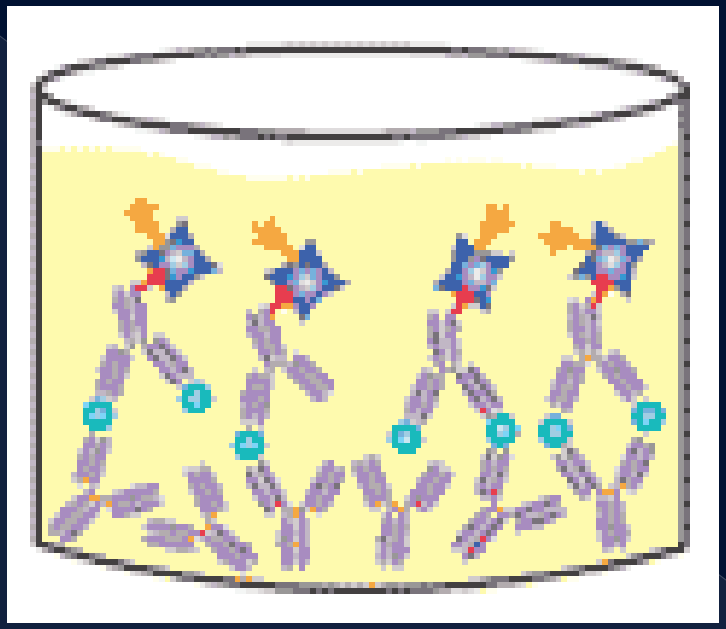




Conversion of colorless substrate into a colored product.
The intensity of color depends on amount of target protein.

Protein analysis

ELISA



Stop solutions

Reading absorbance

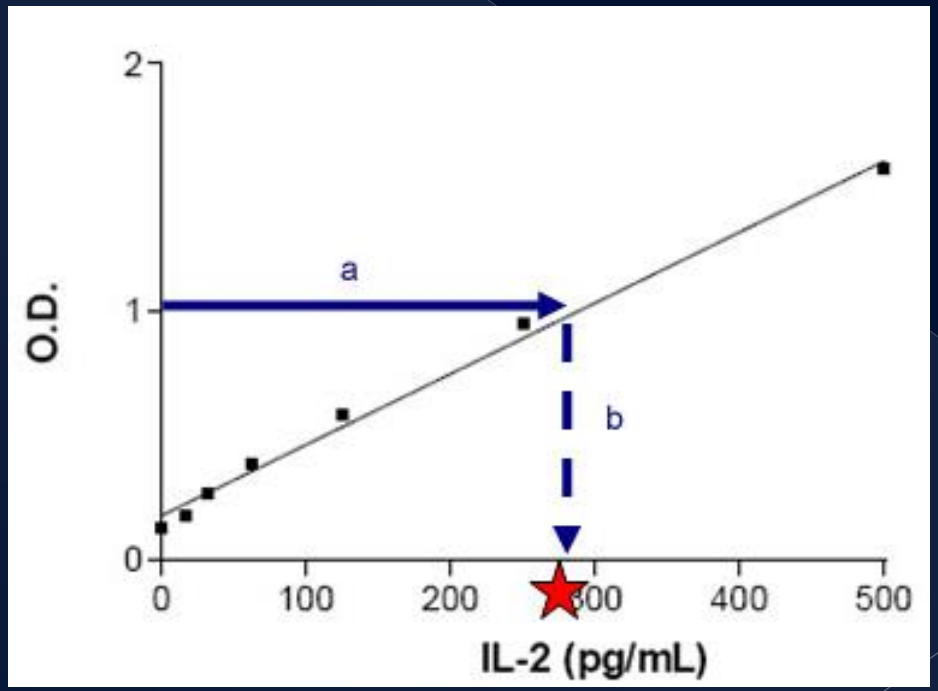
Protein analysis

ELISA

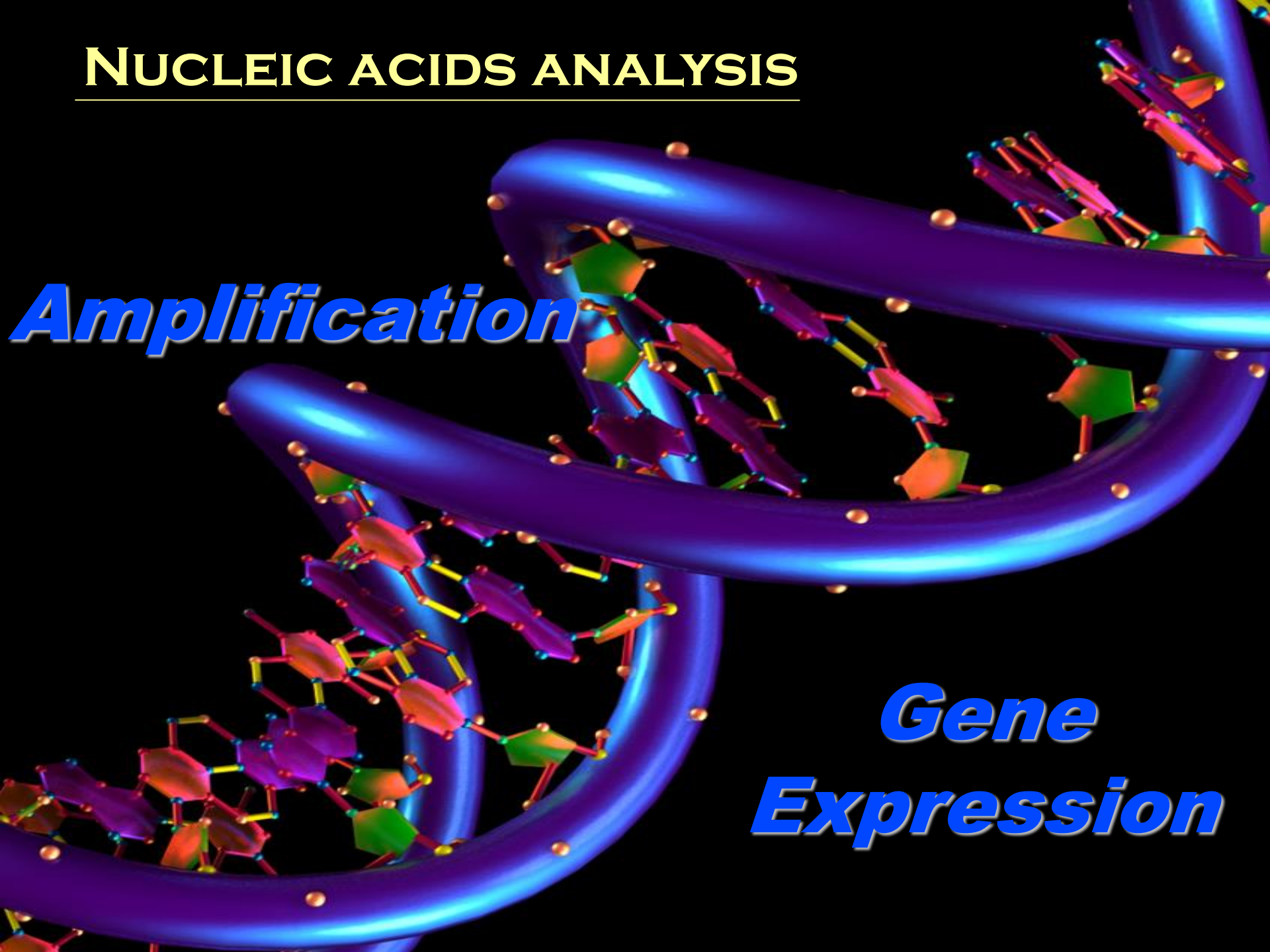
Standard

Unknown Sample

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

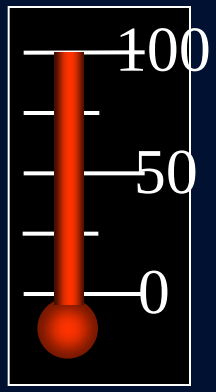


Nucleic acids analysis

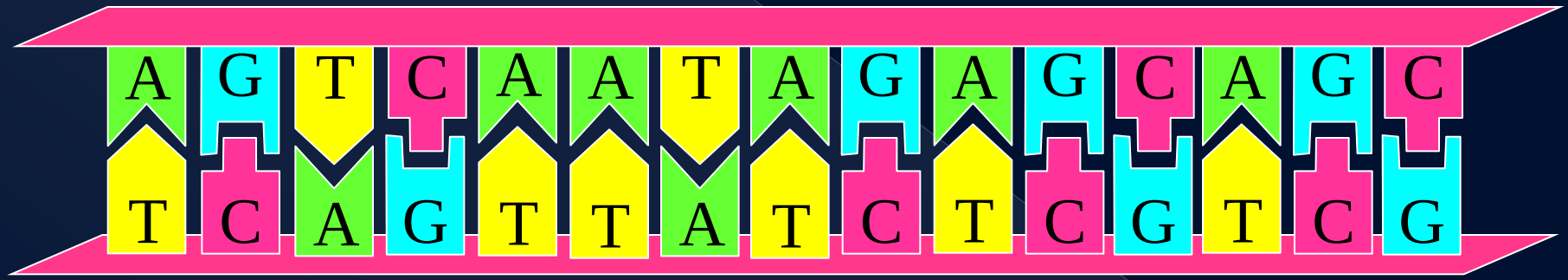


Denaturation

Renaturation

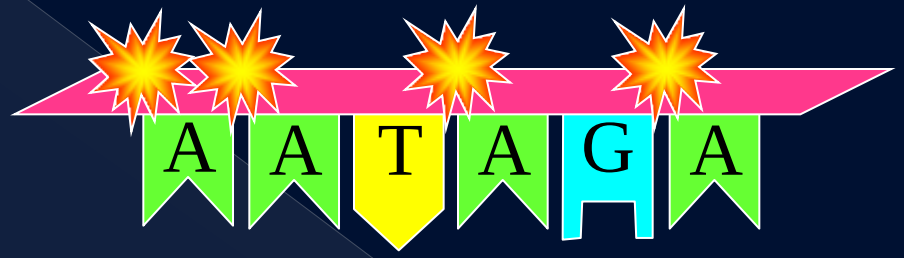


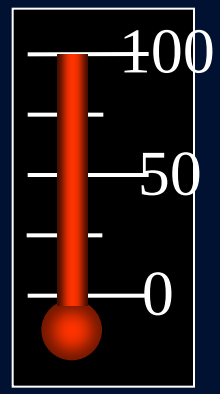
or DNA melting is the separation of a double strand into two single strands, which occurs when the hydrogen bonds between the strands are broken. (thermal denaturation – by heat, chemical denaturation – by bases, acids)

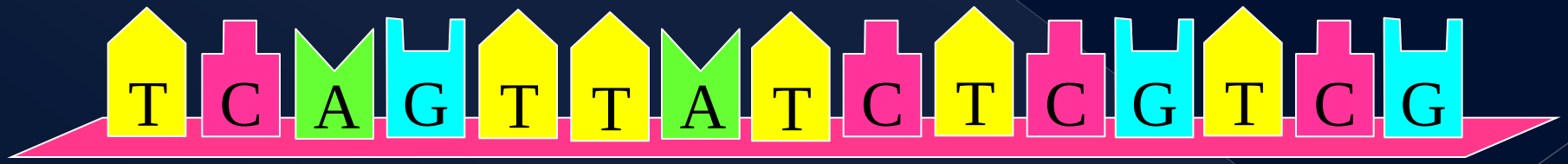


Formation of double stranded DNA from denatured complementary DNA strands caused by slow cooling. Hydrogen bonds are formed.

Hybridization







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

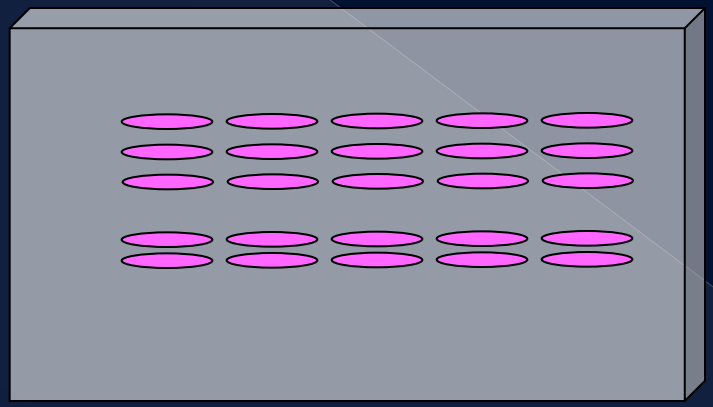
SOUTHERN BLOT

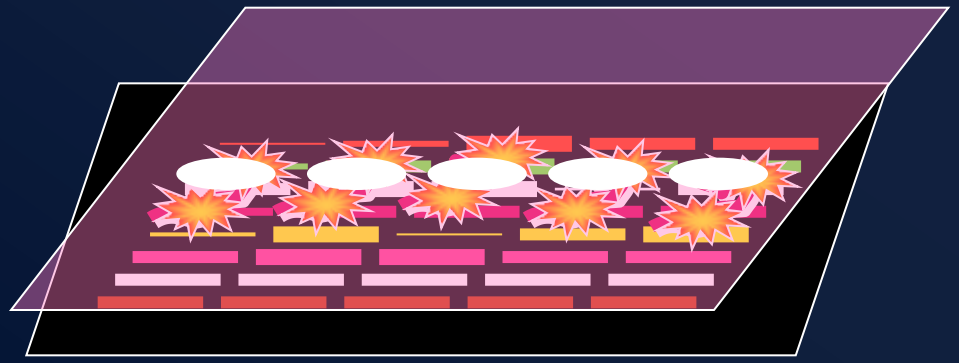
Sir Edwin Mellor Southern, the British molecular biologist.

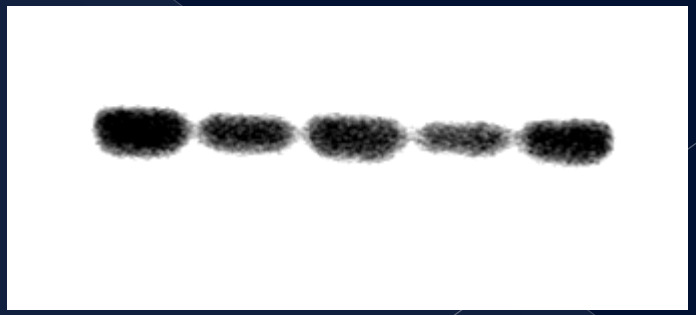
Southern blot is a method used in molecular biology for detection of a specific DNA sequence in DNA samples. The method is named after its inventor, the British biologist Edwin Southern.

SOUTHERN BLOT

1.Cutting of DNA into small fragments by restriction endonucleases
2.Separation of DNA fragments (by size) by the electrophoresis on an agarose gel.
3. Alkaline DNA denaturation.
4.Transfer DNA from gel onto nitrocellulose or nylon membrane.
5. Expositon of the membrane to a hybridization probe - a DNA fragment which can bind to a specific sequence whose presence in the target DNA is to be determined. The probe DNA is labeled so that it can be detected.



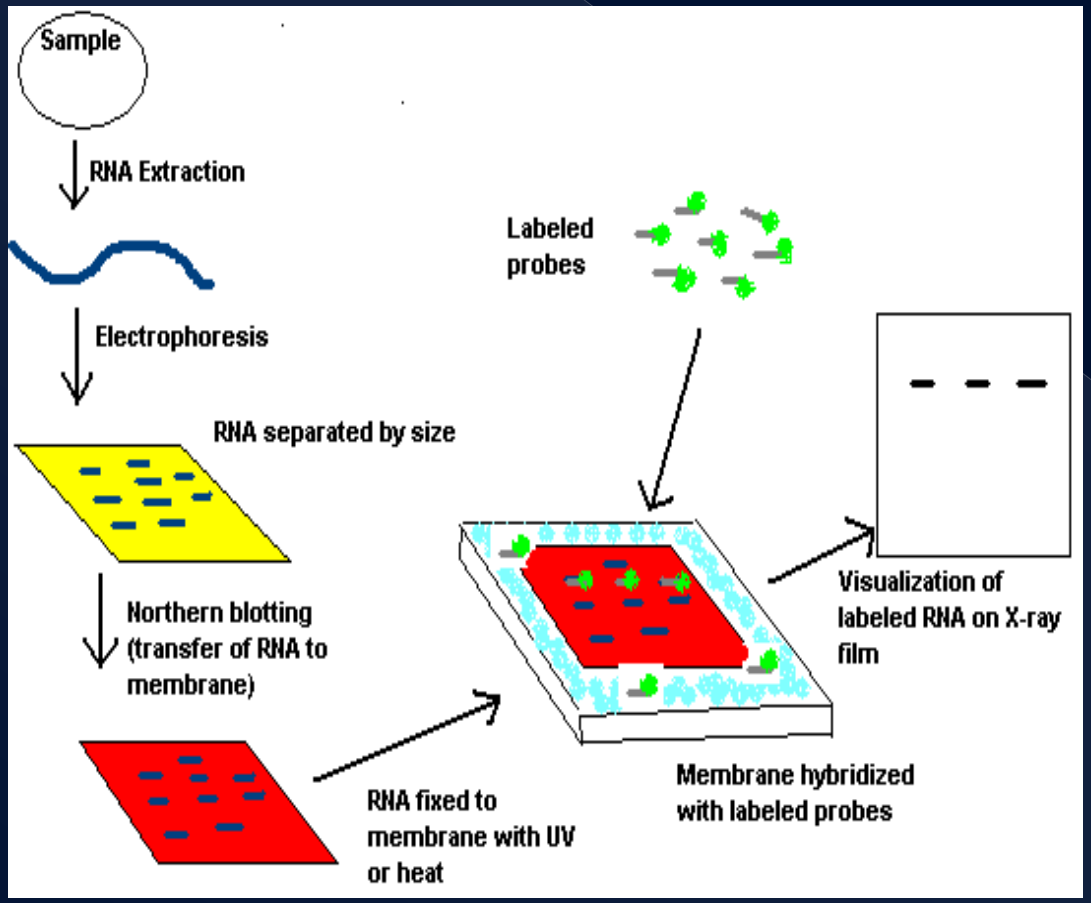




Southwestern blot is used for identifying and characterizing DNA-binding proteins. The proteins are separated by electrophoresis, transferred to membrane and detected by DNA probes.

NORTHERN BLOT

Technique used to study gene expression by detection of isolated mRNA in a sample. (James Alwine, David Kemp, George Stark – American biochemists)

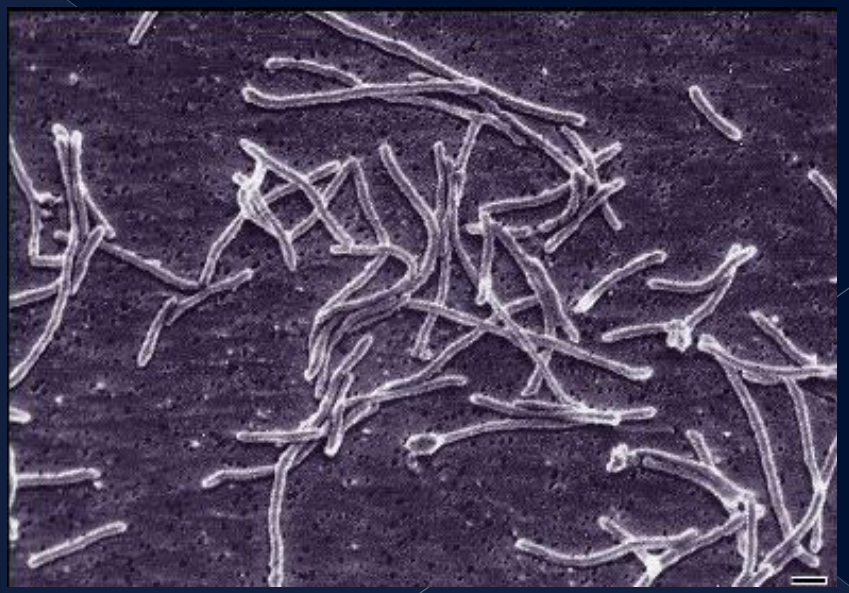


Extraction of total RNA from cells.
RNA samples are separated by gel electrophoresis and transferred to a nylon membrane.
Complementary labeled probes hybridize to the RNA on the membrane.

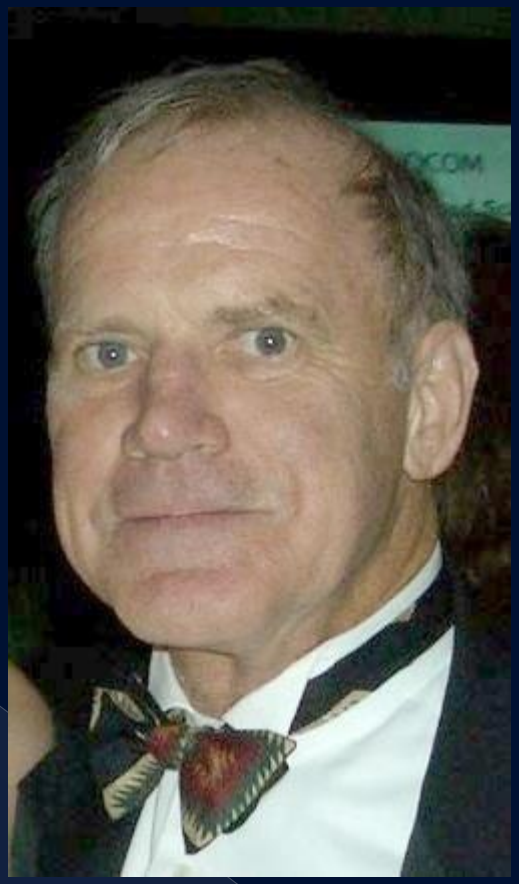
Nucleic acids analysis

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.

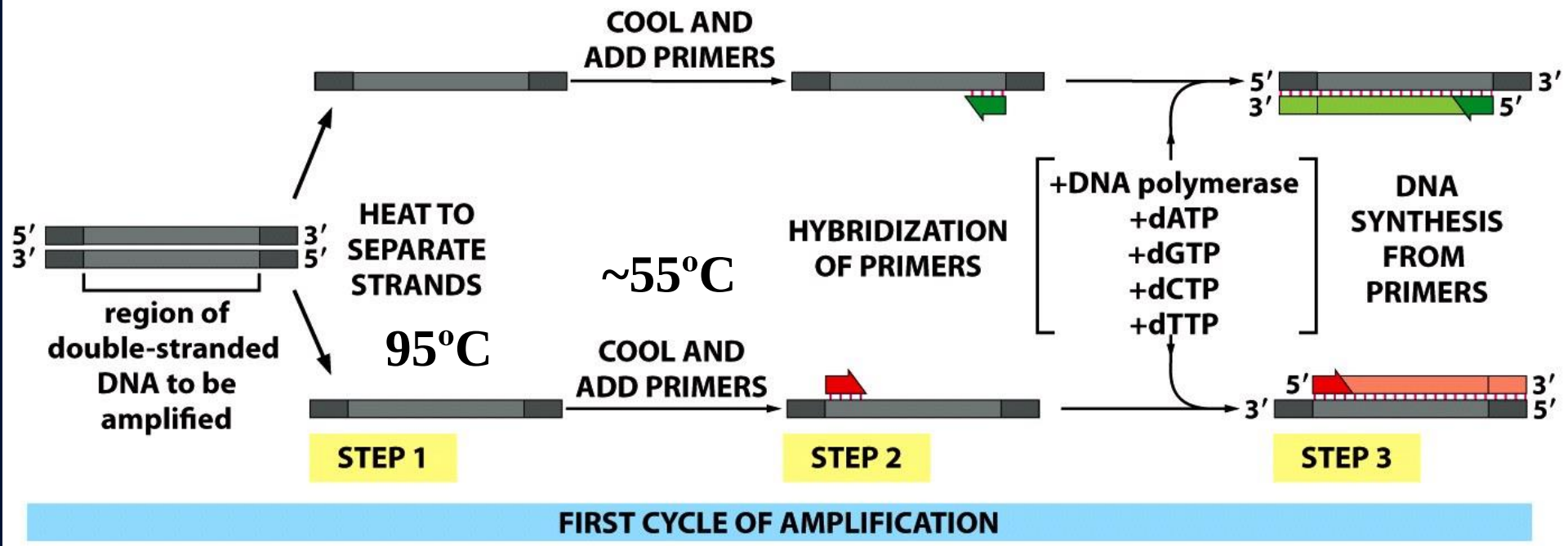


The process of polymerase chain reaction (PCR) described by Kjell Kleppe (Norwegian biochemist) Har Gobind Khorana (Indian-american biochemist) allows the amplification of specific DNA sequences. But Kary Banks Mullis American biochemist improved this technique and this improvements allowed PCR to become a central technique in biochemistry and molecular biology. Kary Banks Mullis was awarded the 1993 Nobel Prize in Chemistry.



Nucleic acids analysis

Polymerase Chain Reaction

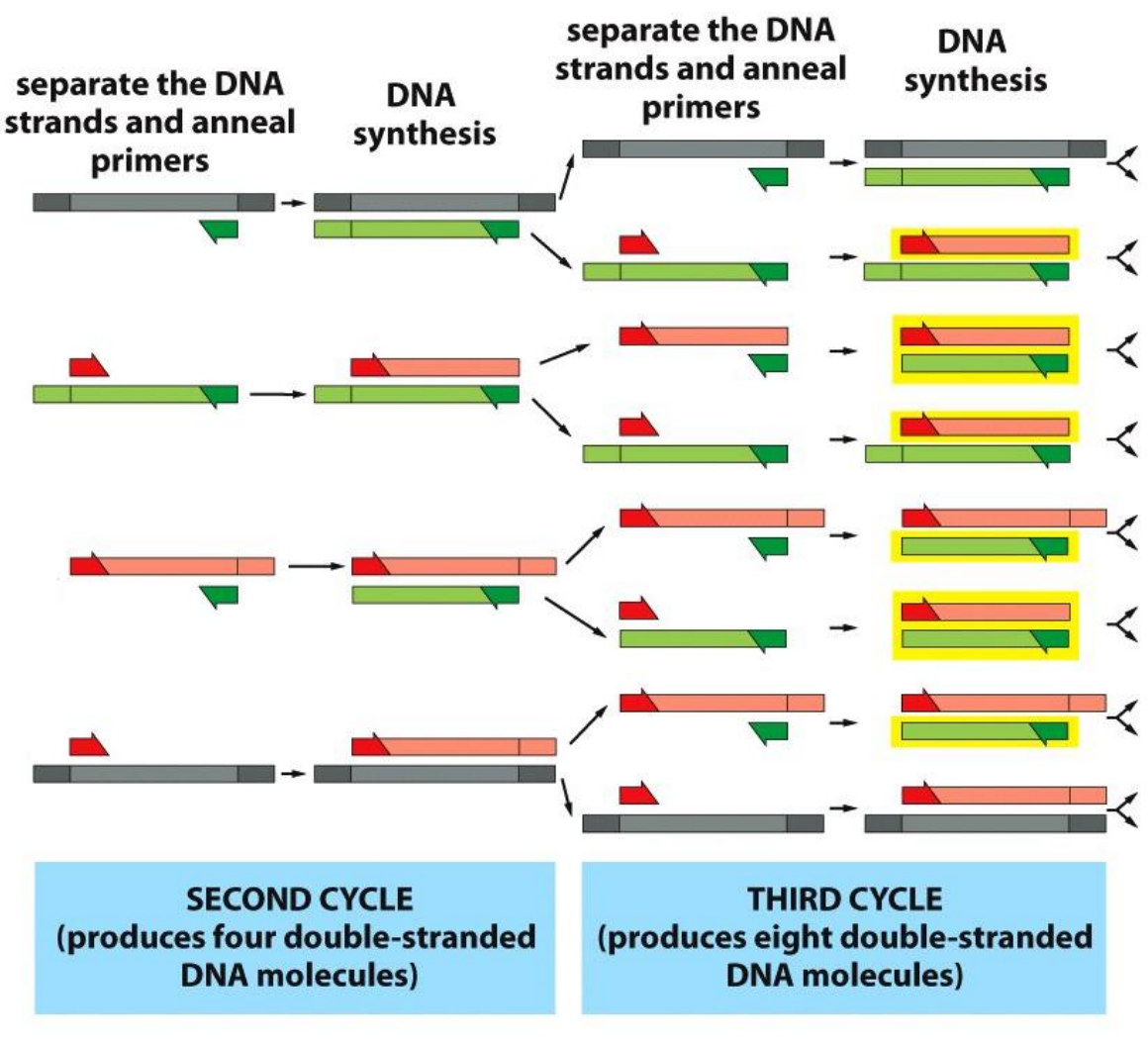


Heat-stable DNA polymerase - Taq polymerase enzymatically assembles a new DNA strand from the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides - primers, which are required for initiation of DNA synthesis.

72°C

Nucleic acids analysis

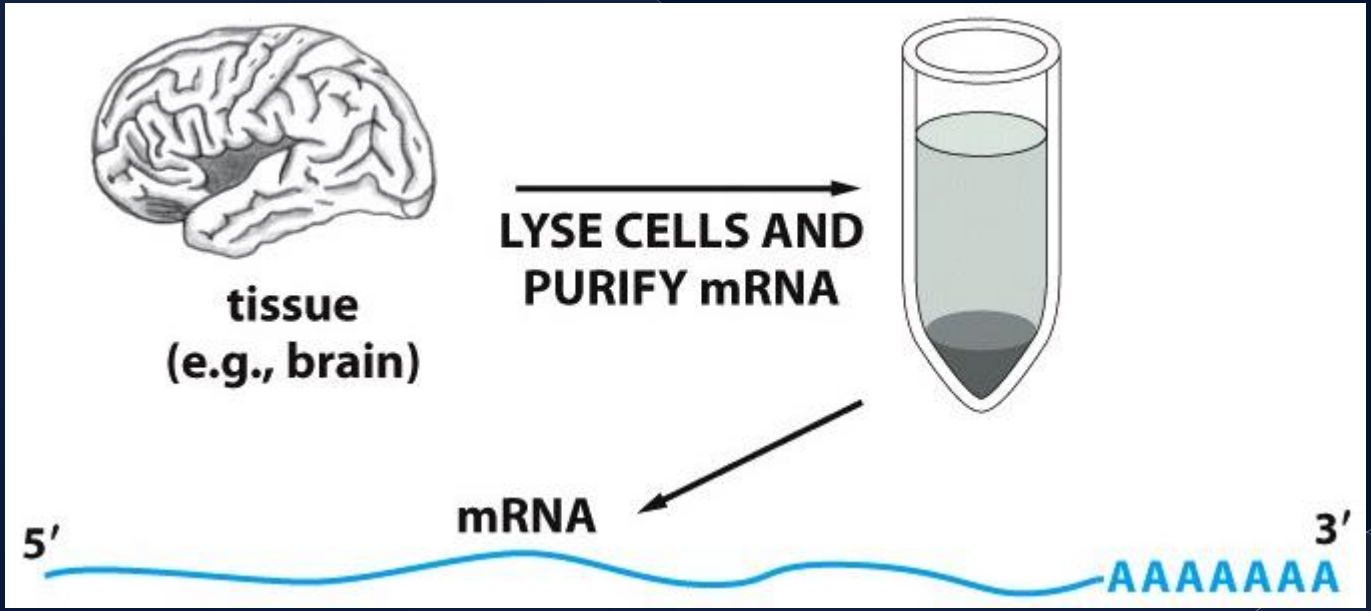
Polymerase Chain Reaction



Nucleic acids analysis

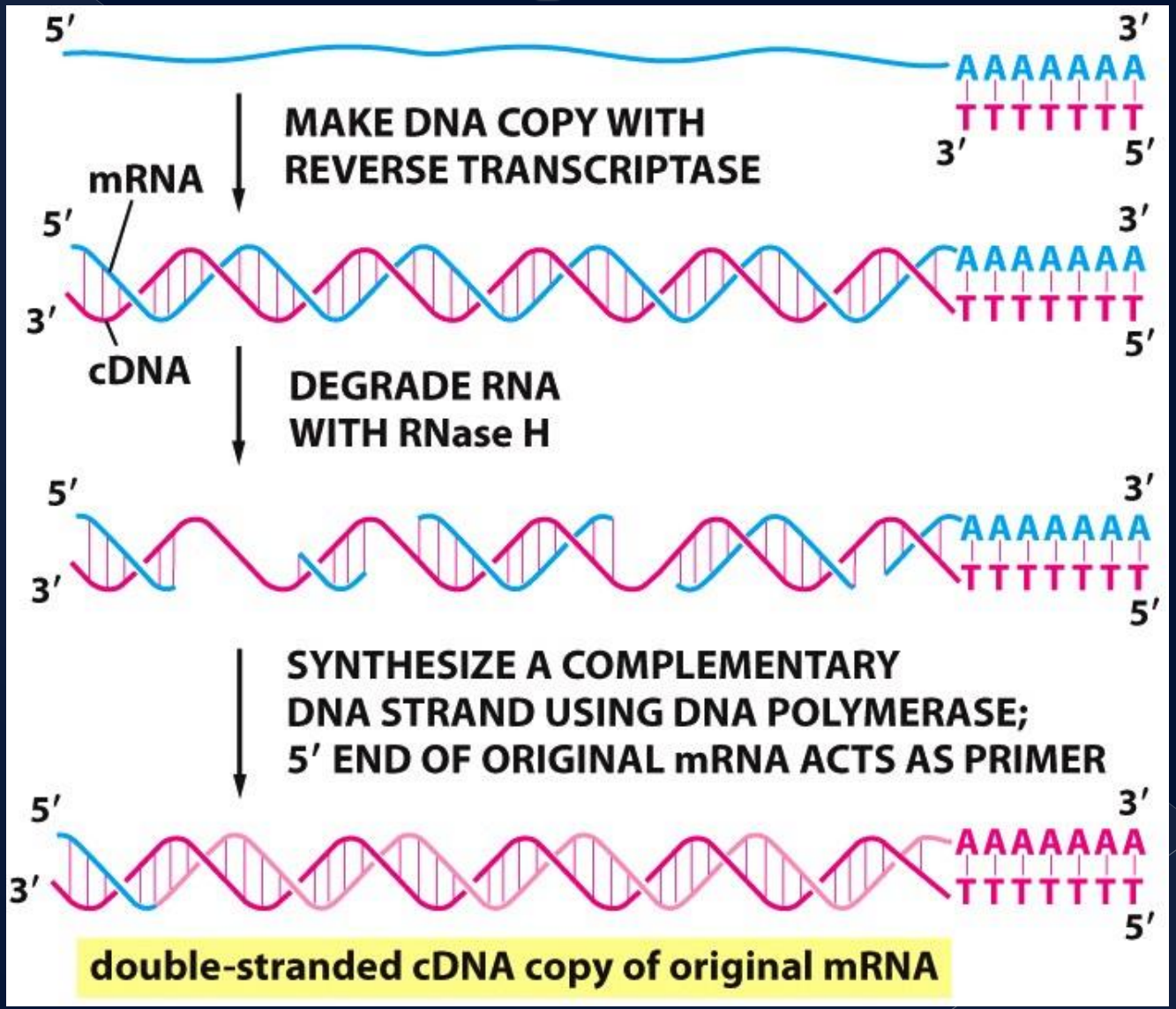
Gene Expression

Detection of specific mRNA by reverse transcription PCR



Nucleic acids analysis

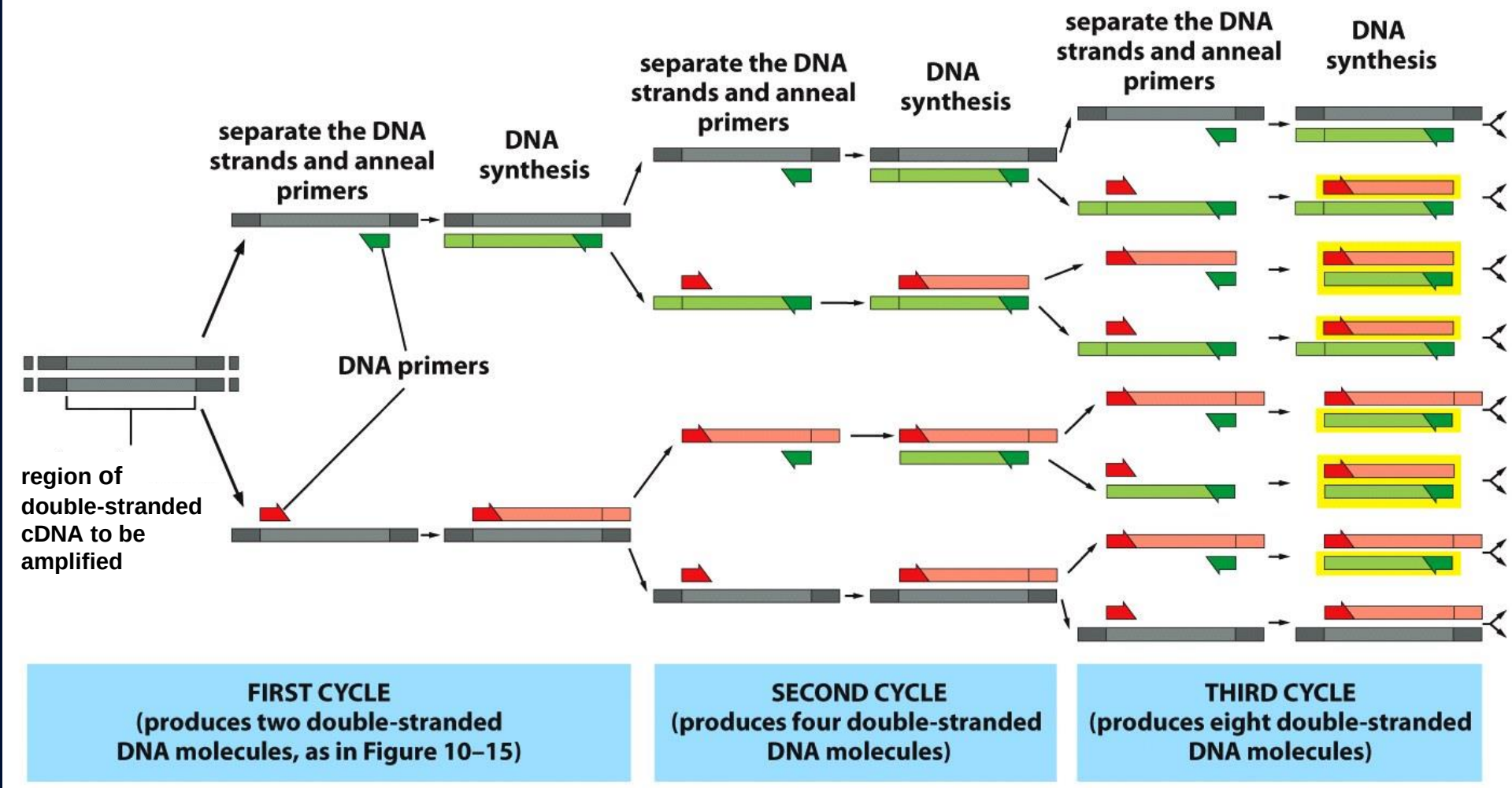
Detection of specific mRNA



Nucleic acids analysis

Detection of specific mRNA

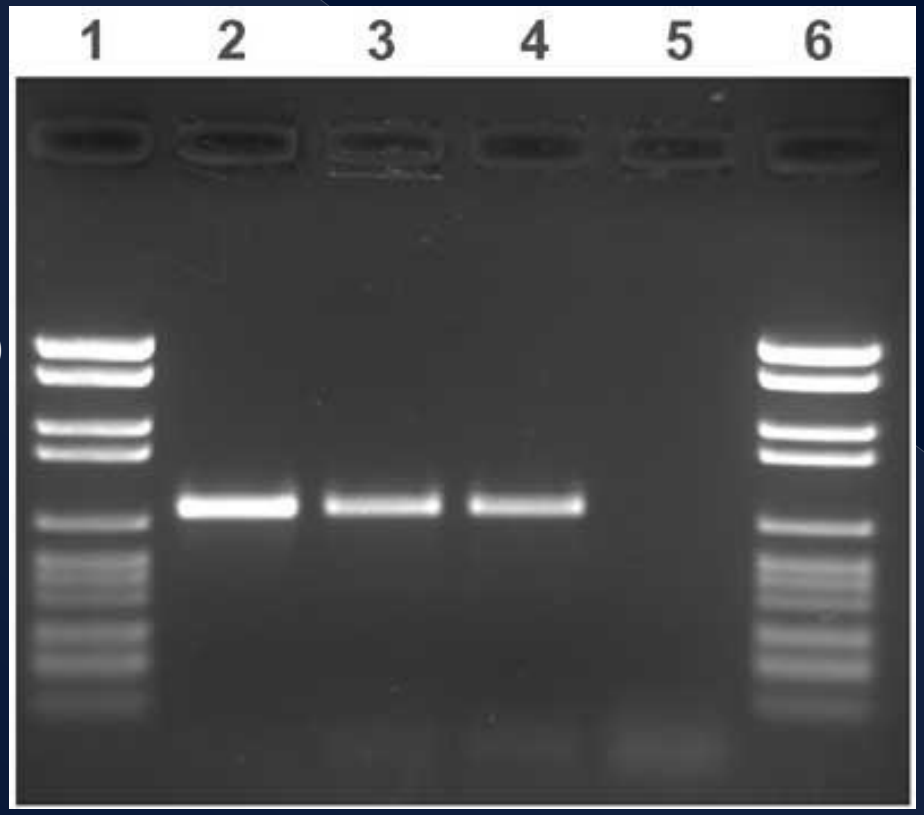
Performing a PCR with cDNA as a template

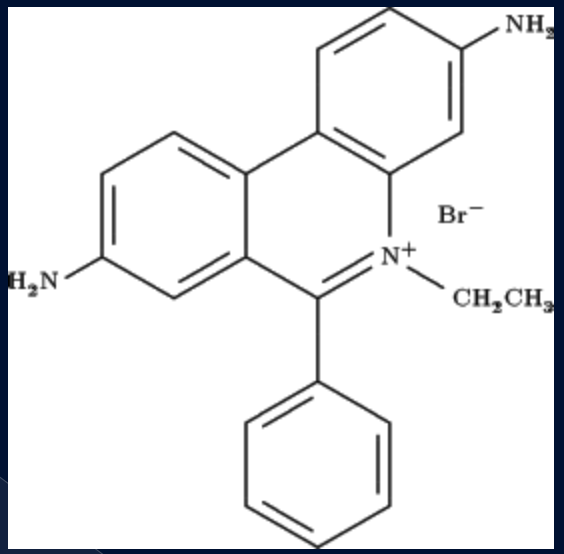


Nucleic acids analysis

Detection of specific mRNA

PCR product electrophoresis in agarose gel





DNA staining with ethidium bromide

Lanes 1 and 6 - DNA standard. PCR product is in lanes 2-5 with bands in lanes 2-4. In sample 5 target DNA fragment is absent .

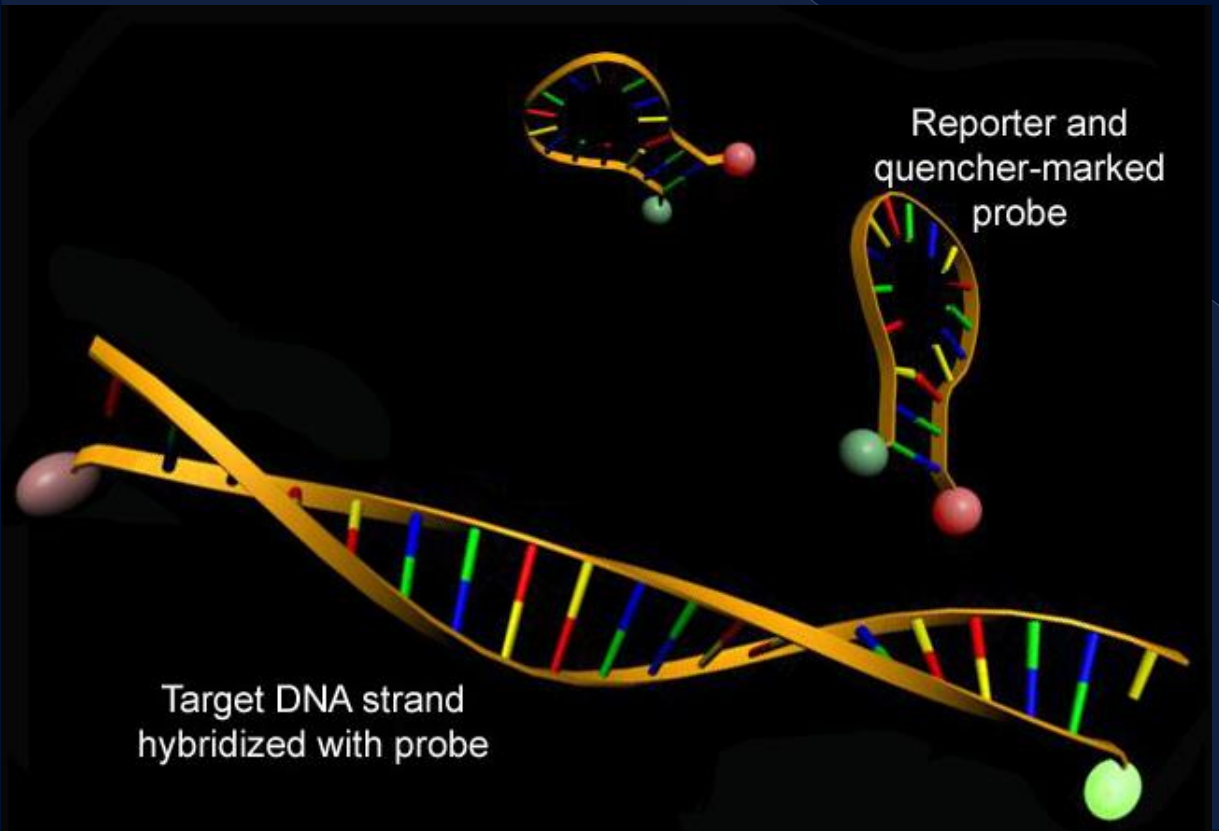
Nucleic acids analysis

Quantification of gene expression

Real-Time PCR

Measurement after each cycle

Mathematical quantification

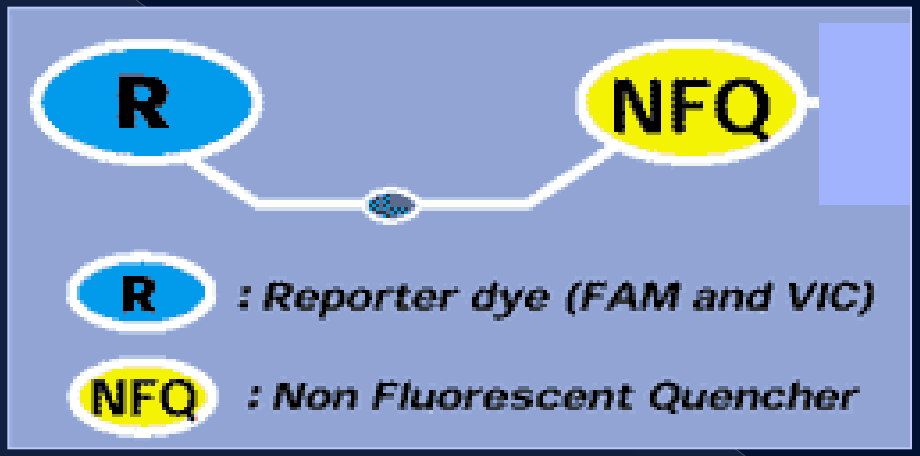


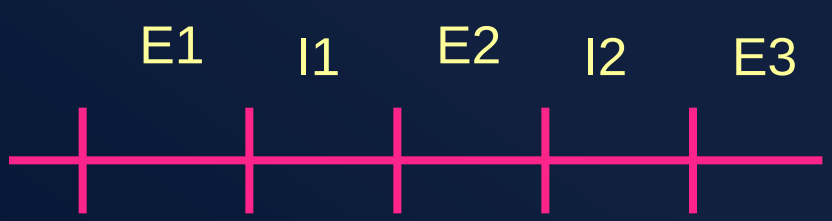
Labeled probes - appropriate DNA sequence

Nucleic acids analysis

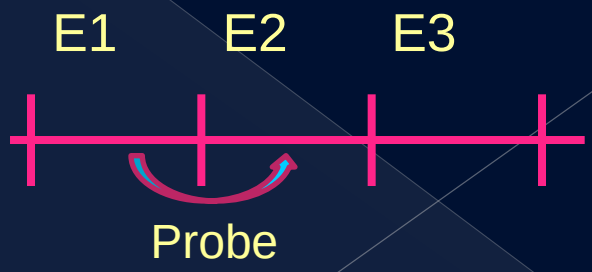
Quantification of gene expression

Real-Time PCR Labeled probe





genomic DNA



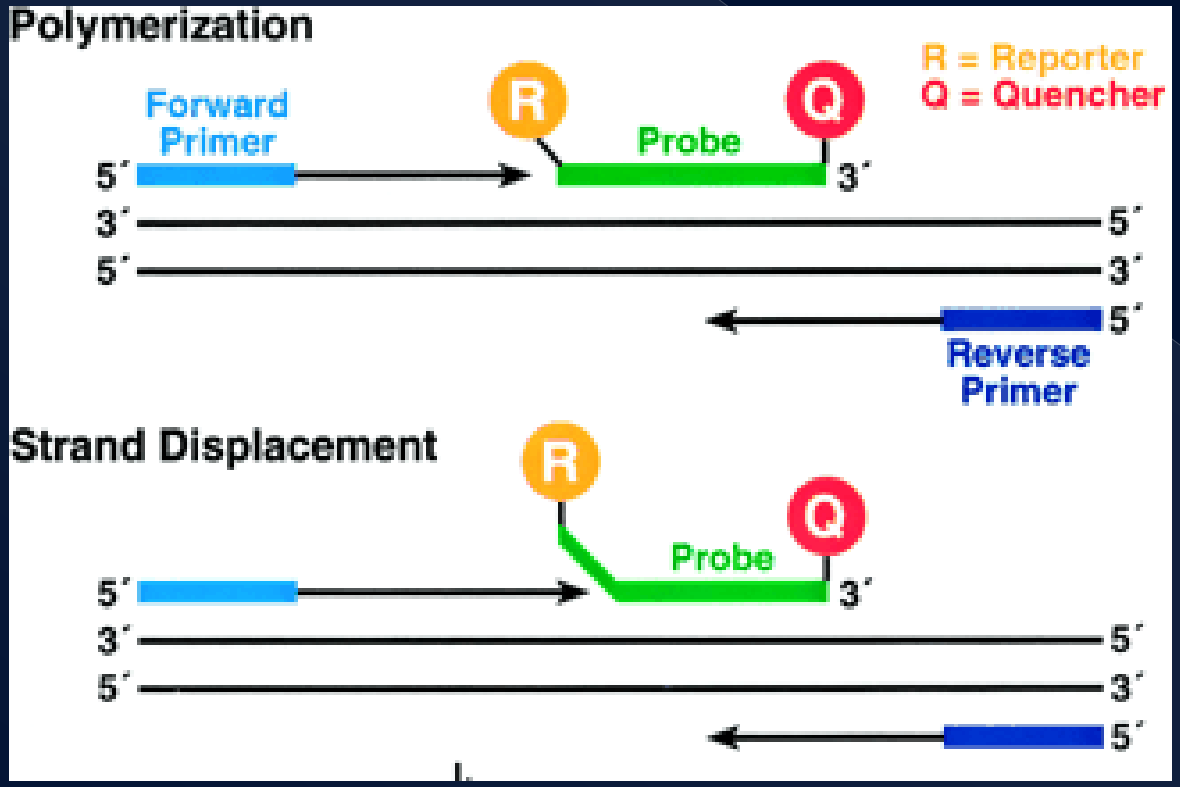
mRNA and cDNA

Nucleic acids analysis

Quantification of gene expression

Real-Time PCR

Fluorescent report dye (R), and quencher (Q) are attached to the 5' and 3" ends of probe.



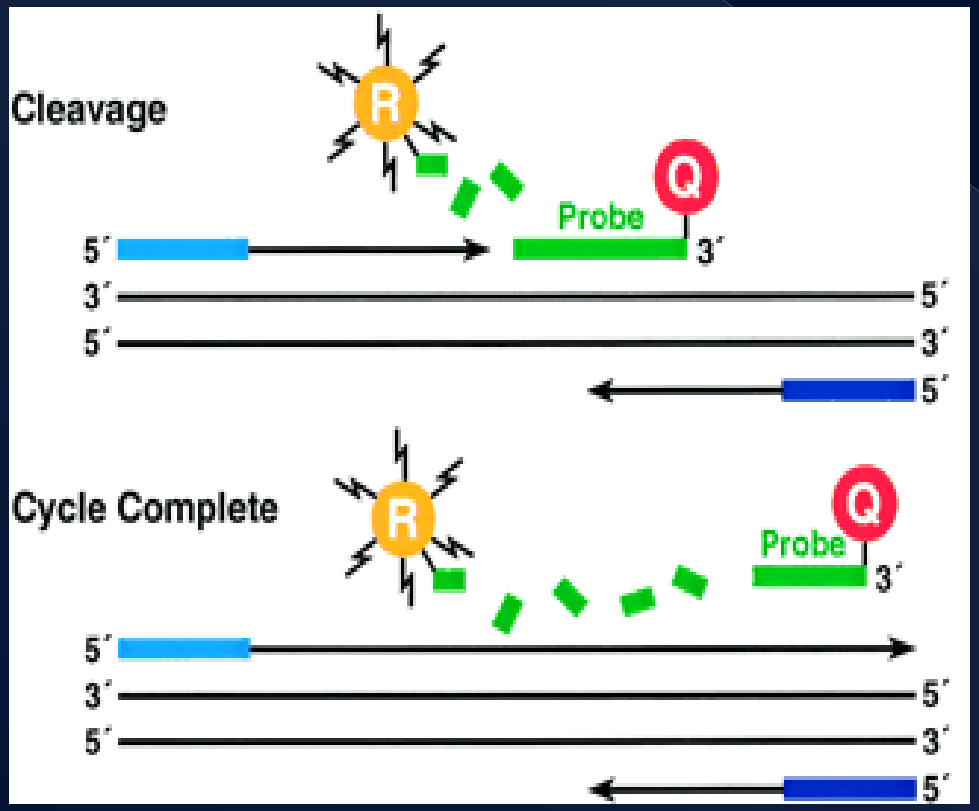
When the probe is intact the R emission is quenched.

Nucleic acids analysis

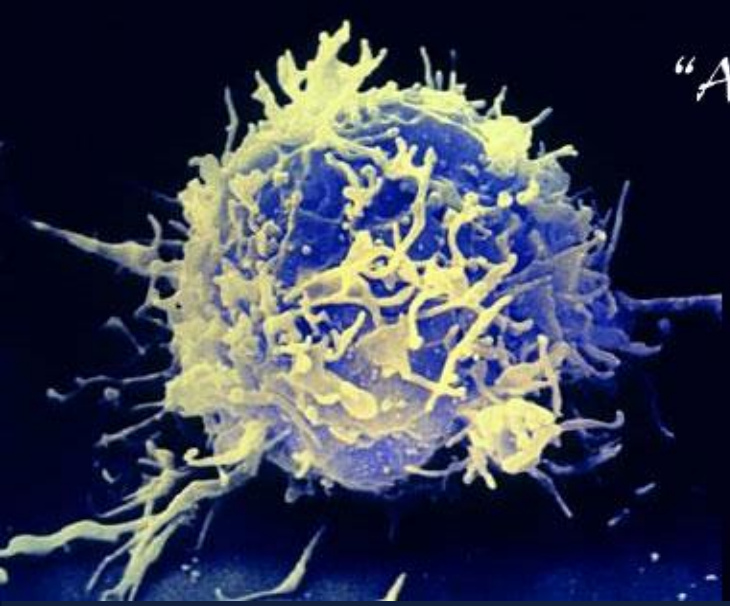
Quantification of gene expression

Real-Time PCR

During each cycle the DNA polymerase cleaves the report dye from the probe.



Once separated from the quencher, the report dye emits characteristic fluorescence.
This fluorescence is measured after each cycle and analyzed by special software for analyzing the data.

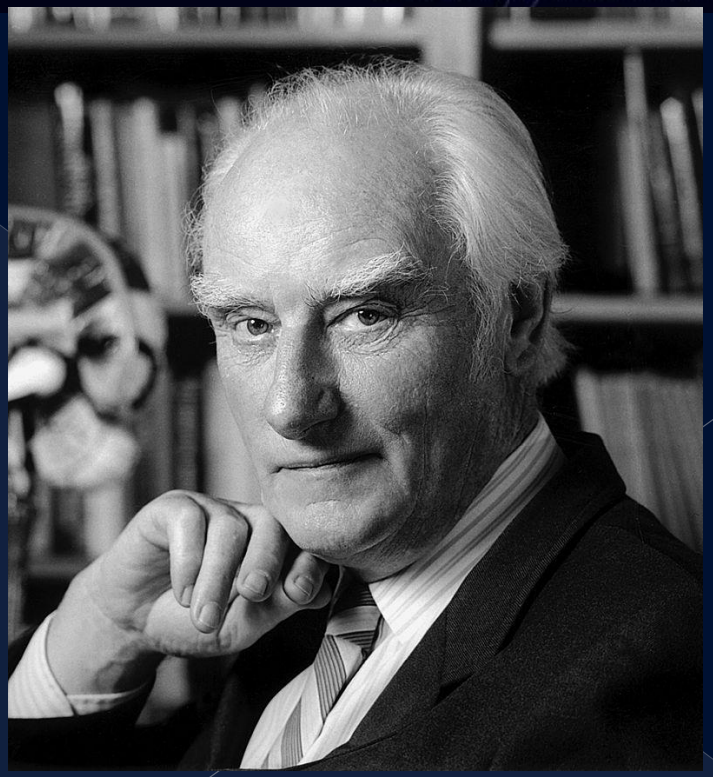


"Almost all aspects of life are engineered at the molecular level, and without understanding molecules we can only have a very sketchy understanding of life itself."

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".

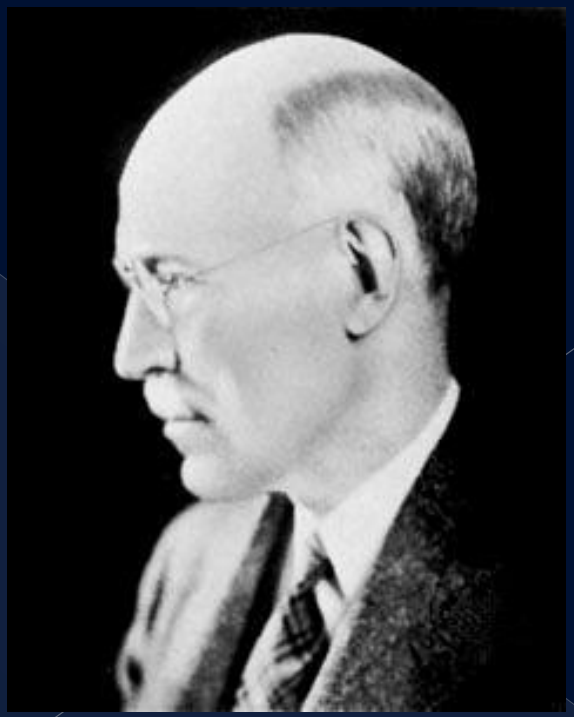


HISTORY

- 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

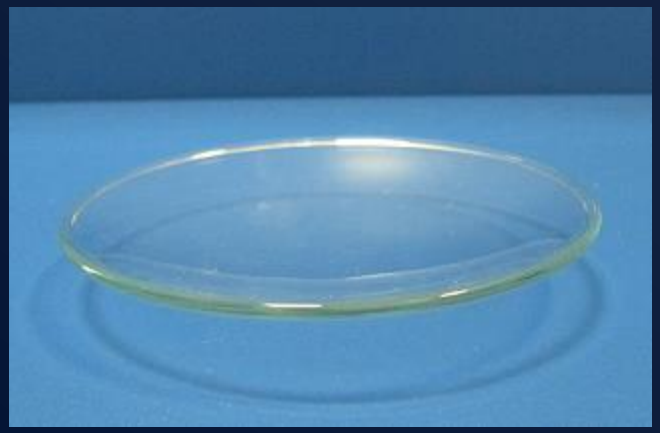


HISTORY

-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)



TELOMERES

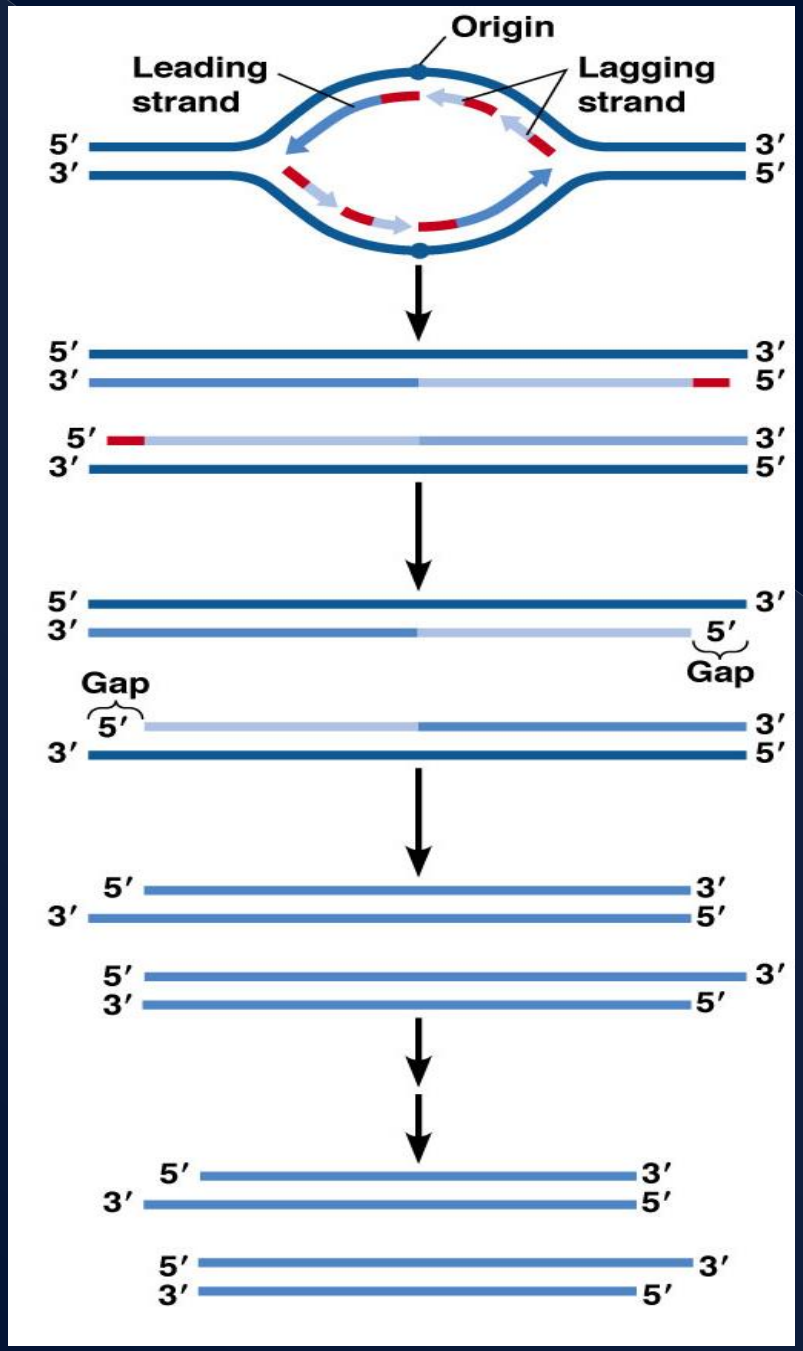
Linear chromosomes are less stable than circular, but determine the genetic diversity of living organisms (recombinations).

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.

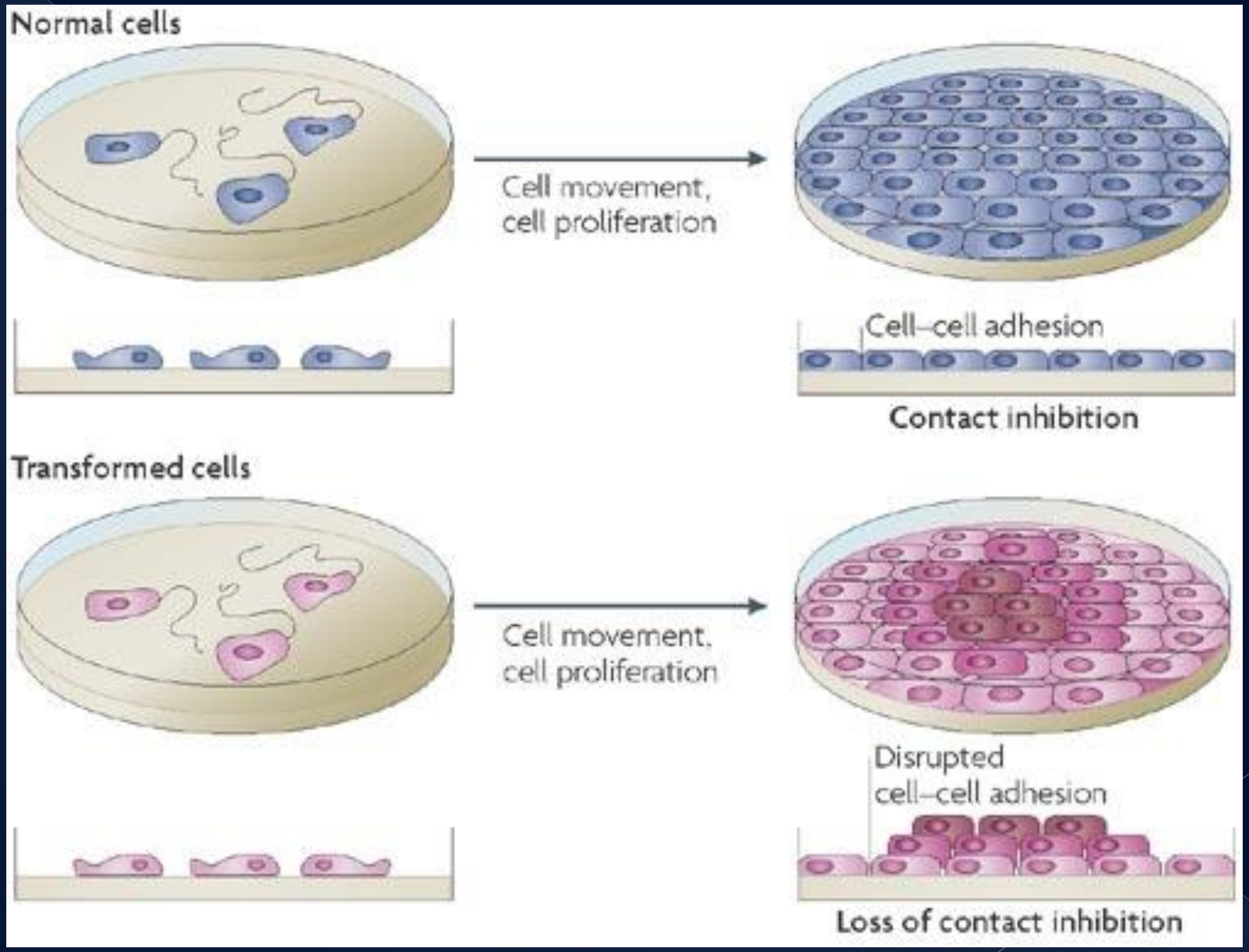
When the telomeres reach a critical length - STAGE OF CELLULAR SENESCENCE (p53-dependent inhibition of cell cycle)



End replication problem - the shortening of telomeres during replication

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

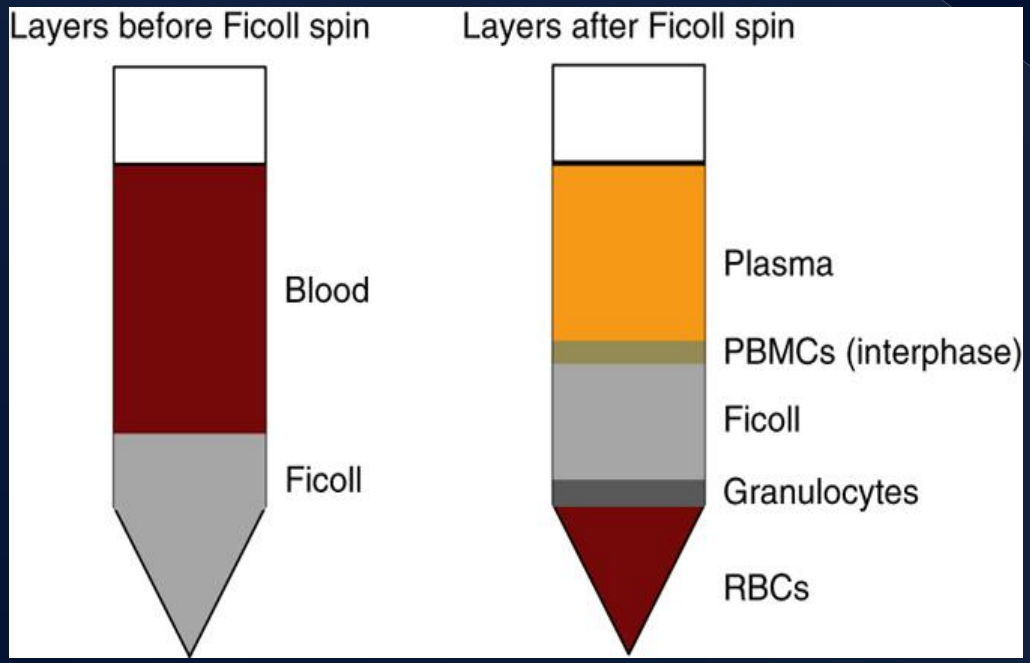
CONTACT INHIBITION – NORMAL AND TRANSFORMED CELLS



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.