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Unit-V
Immunotechniques

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Precipitation and Agglutination Reactions

- The combination of an antigen with a specific antibody plays an important role in the laboratory in diagnosing many different diseases.
- Immunoassays have been developed to detect either antigens or antibodies and vary from easily performed manual tests to highly complex automated assays.
- The first such assays were based on the principles of precipitation or agglutination.
- **Precipitation** involves combining soluble antigens with soluble antibodies to produce insoluble complexes that are visible.

Precipitation Reaction

- Precipitation reactions are based on the interaction of antibodies and antigens. In this reaction, two soluble reactants that come together to make one insoluble product, the precipitate.
- Any antibody which reacts with an antigen to form a precipitate is called **Precipitin.**
- Precipitation assays are performed in semi-solid media such as Agar or Agarose where antibodies and antigens can diffuse toward one another and form a visible line of precipitation.

- **Precipitation reactions** are serological assays for the detection of immunoglobulin levels from the serum of a patient with infection.
- Precipitation reactions are widely used in Medicine for analysis of Hormones, Enzymes, Toxins and Immune system products.

Some of the examples of Precipitation reactions are

1) Capillary Tube Precipitation (Ring Test),
2) Ouchterlony Double Immunodiffusion (Immunodiffusion or Agar gel Immunodiffusion or Passive Double Immunodiffusion),
3) Single Radial Immunodiffusion (SRID) (Mancini Method),
4) Immunoelectrophoresis (IEP),
5) Rocket electrophoresis.

Agglutination

- **Agglutination**, similar to precipitation, is a two-step process that results in the formation of a stable lattice network.
- The first step, sensitization, involves antigen–antibody combination through single antigenic determinants on the particle.
- The second step, lattice formation, involves the development of crosslinks that form visible aggregates. Lattice formation represents the stabilization of antigen-antibody complexes with the binding together of multiple antigenic determinant

Types of Agglutination reaction

Direct Reaction

- If an agglutination reaction involves RBCs, then it is called hemagglutination.
- The best example of this occurs in ABO blood group typing of human RBCs, one of the world's most frequently used immunoassays.
- Patient RBCs mixed with antisera of the IgM type can be used to determine the presence or absence of the A and B antigens; this reaction is usually performed at room temperature.

Group A RBCs will agglutinate with anti-A antibody, and

Group B RBCs will agglutinate with anti-B antibody.

- This type of agglutination reaction is simple to perform, relatively sensitive, and easy to read.

Indirect Reaction [Passive]

- Employs particles that are coated with antigens not normally found on their surfaces.
- A variety of particles, including latex and gelatin, are used for passive agglutination (indirect).
- Using synthetic beads or particles provides the advantages of consistency and uniformity.
- Reactions are easy to read visually and give rapid results.
- It is used to detect many antibodies, including rheumatoid factor (an antiIgG found in some autoimmune disorders), antibodies to Group A Streptococcus antigens, and antibodies to viruses such as cytomegalovirus and rubella.

Immunoelectrophoresis

- Immunoelectrophoresis refers to precipitation in agar under an electric field.
- It is a process of a combination of immuno-diffusion and electrophoresis.
- An antigen mixture is first separated into its component parts by **electrophoresis** and then tested by **double immuno-diffusion**.
- Antigens are placed into wells cut in a gel (without antibody) and electrophoresed. A trough is then cut in the gel into which antibodies are placed.

- The antibodies diffuse laterally to meet diffusing antigens, and lattice formation and precipitation occur permitting determination of the nature of the antigens.
- The term “immunoelectrophoresis” was first coined by **Grabar** and **Williams** in **1953.**

Principle

- When an electric current is applied to a slide layered with gel, the antigen mixture placed in wells is separated into individual antigen components according to their charge and size.
- Following electrophoresis, the separated antigens are reacted with specific antisera placed in troughs parallel to the electrophoretic migration and diffusion is allowed to occur.
- Antiserum present in the trough moves toward the antigen components resulting in the formation of separate precipitin lines in 18-24 hrs, each indicating reaction between individual proteins with its antibody.

Procedure of Immunoelectrophoresis

- Agarose gel is prepared on a glass slide put in a horizontal position.
- Using the sample template, wells are borne on the application zone carefully.
- The sample is diluted 2:3 with protein diluent solution (20µl antigen solution +10 µl diluent).
- Using a 5 µl pipette, 5 µl of control and sample is applied across each corresponding slit (Control slit and Sample slit).
- The gel is placed into the electrophoresis chamber with the samples on the cathodic side, and electrophoresis runs for 20 mins/ 100 volts.

- After electrophoresis completes, 20 µl of the corresponding antiserum is added to troughs in a moist chamber and incubated for 18- 20 hours at room temperature in a horizontal position.
- The agarose gel is placed on a horizontal position and dried with blotter sheets.
- The gel in saline solution is soaked for 10 minutes and the drying and washing repeated twice again.
- The gel is dried at a temperature less than 70°C and may be stained with protein staining solution for about 3 minutes followed by decolorizing the gel for 5 minutes in distaining solution baths.
- The gel is dried and results evaluated.

Results

1. The presence of elliptical precipitin arcs represents antigen-antibody interaction.
2. The absence of the formation of precipitate suggests no reaction.
3. Different antigens (proteins) can be identified based on the intensity, shape, and position of the precipitation lines.

Applications

- The test helps in the identification and approximate quantization of various proteins present in the serum. Immunoelectrophoresis created a breakthrough in protein identification and in immunology.
- Immunoelectrophoresis is used in patients with suspected monoclonal and polyclonal gammopathies.
- The method is used to detect normal as well as abnormal proteins, such as myeloma proteins in human serum.
- Used to analyze complex protein mixtures containing different antigens.

- The medical diagnostic use is of value where certain proteins are suspected of being absent (e.g., hypogammaglobulinemia) or overproduced (e.g., multiple myeloma).
- This method is useful to monitor antigen and antigen-antibody purity and to identify a single antigen in a mixture of antigens.
- Immunoelectrophoresis aids in the diagnosis and evaluation of the therapeutic response in many disease states affecting the immune system.

Advantages

- Immunoelectrophoresis is a powerful analytical technique with high resolving power as it combines the separation of antigens by electrophoresis with immunodiffusion against an antiserum.
- The main advantage of immunoelectrophoresis is that a number of antigens can be identified in serum.

Limitations

- Immunoelectrophoresis is slower, less sensitive, and more difficult to interpret than Immunofixation electrophoresis.
- The use of immunoelectrophoresis in food analysis is limited by the availability of specific antibodies.

RIA

- Radioimmunoassay is one of the sensitive immunoassay techniques which helps in the determination of antigens or antibodies in a sample with the use of radioisotopes.
- When radioisotopes instead of enzymes are used as labels to be conjugated with antigens or antibodies, the technique of detection of the antigen-antibody complex is called **radioimmunoassay (RIA).**
- Radioimmunoassay (RIA) is an *in vitro* assay that measures the presence of an antigen with very high sensitivity.
- Alternative names used for RIA include *saturation analysis*, *displacement analysis* and *competitive radioassay*.

- In this method, an unlabeled antigen competes with a radiolabeled antigen for binding to an antibody with the appropriate specificity. Thus, when mixtures of radiolabeled and unlabeled antigen are incubated with the corresponding antibody, the amount of free (not bound to antibody) radiolabeled antigen is directly proportional to the quantity of unlabeled antigen in the mixture.

Principle

- Antigens and antibodies bind specifically to form the Ag-Ab complex. The antigen can be labeled or conjugated with radioisotopes. The unlabeled antigens from the sample compete with radiolabeled antigens to bind on paratopes of specific antibodies. The unlabeled antigens replace labeled antigens that are already linked with the antibodies. The unlabeled antigens when bind with antibodies, increases the amount of free radiolabeled antigens in the solution. Hence the concentration of free labeled antigens is directly proportional to the bound unlabeled antigens.

It involves a combination of three principles.

- An immune reaction i.e. antigen, antibody binding.
- A competitive binding or competitive displacement reaction. (It gives specificity)
- Measurement of radio emission. (It gives sensitivity)

Applications

- It was first used for the detection of peptide hormones.
- Detection of different viral antigens
- Detection of many hormones and drugs
- Detection of Hepatitis B surface antigens
- Detection of mycotoxins
- Detection of the early stage of cancer

Advantages

- High specificity
- High sensitivity
- Can detect a very small amount (nanograms) of antigen or antibodies.

Limitations

- Working with radioactive substances makes it a bit risky.
- Disposal of radioactive substances can be problematic.
- Equipment and reagents are expensive.
- Radiolabeled substances used have a short shelf-life.

Procedure of RIA

1. Specific antibodies of known concentration are fixed in the microtitre well.
2. A known amount of hot antigens is then added to the well
3. Washed carefully to remove any unbound antigens
4. At this point, the radioactivity of the well will be maximum.
5. Unlabeled antigens are then added to the well
6. The unlabeled antigens will bind to the antibodies and there will be free labeled antigens in the well.
7. Again washed carefully to remove the free labeled antigens.
8. Radioactivity of well is then measured by gamma-counter

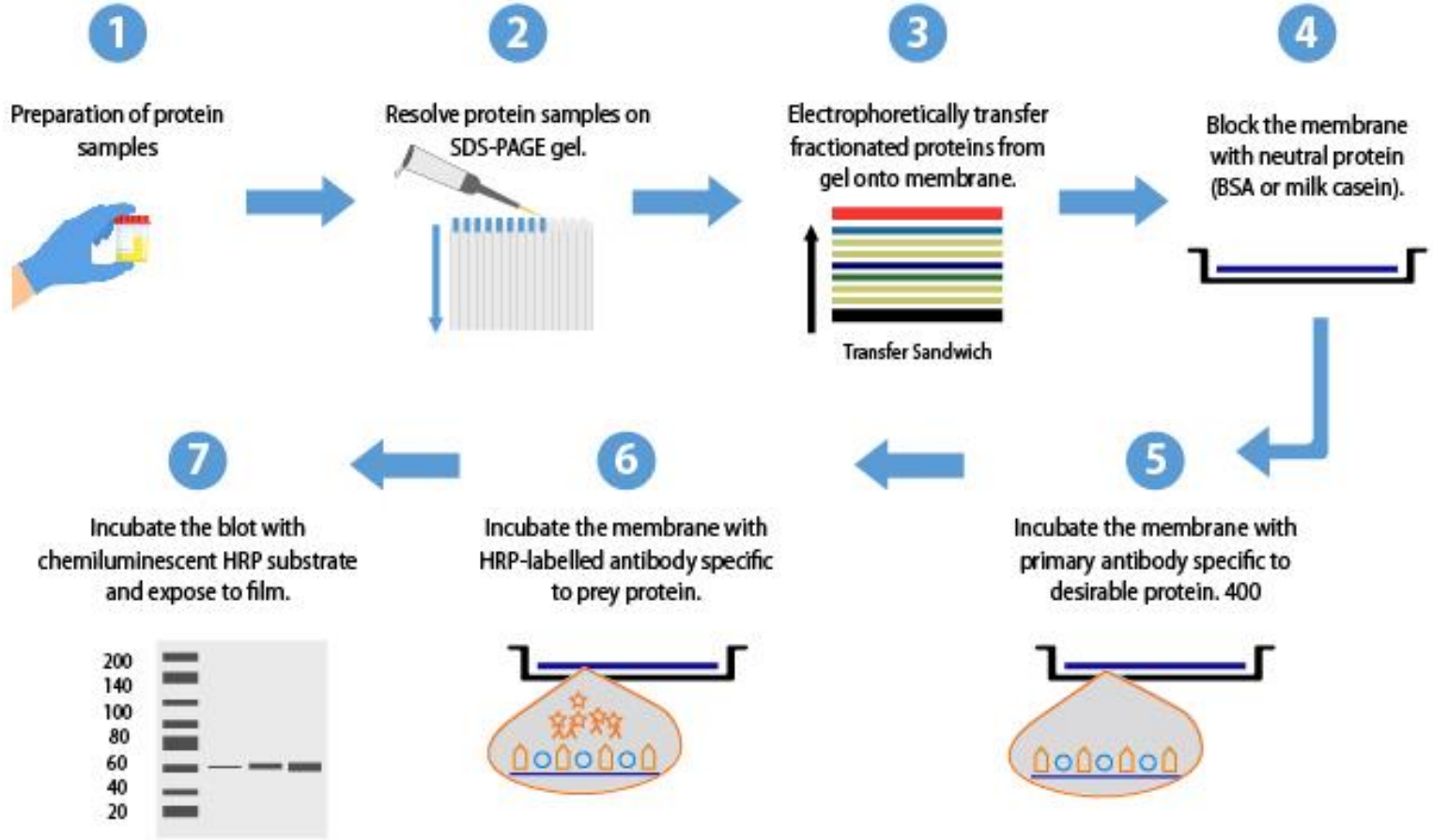
Radioimmunoassay (RIA) Result Interpretation

- At first, the labeled antigens will bind to the antibodies hence radioactivity will be maximum.
- If the sample contains specific antigens of interest, it will bind to the antibodies releasing labeled antigens and hence the radioactivity of the solution will decrease.
- So by observation of decreasing radioactivity, it can be confirmed that the antigen of interest is present in the sample. And if the radioactivity remains the same, it can be called a negative test.

- With the increasing concentration of unlabeled antigens, the radioactivity decreases. By plotting a graph of radioactivity(in percentage) vs concentration of unlabeled antigens, a standard curve is obtained.
- The sample to be assayed is run parallel following a similar procedure and the radioactivity measured is calibrated with the standard curve to determine the concentration of the antigen.

Immunoblotting

- **Western Blotting**, also known as Immunoblotting or Protein blotting, is a core technique in cell and molecular biology.
- The term “blotting” refers to the transfer of biological samples from a Gel to a Membrane and their subsequent detection on the surface of the membrane.
- It was first described by **Towbin et al.** (1979).
- It is used to detect the presence of a specific protein in a complex mixture extracted from cells or tissue.


1
Preparation of protein samples
2
Resolve protein samples on SDS-PAGE gel.
3
Electrophoretically transfer fractionated proteins from gel onto membrane.
Transfer Sandwich
4
Block the membrane with neutral protein (BSA or milk casein).
5
Incubate the membrane with primary antibody specific to desirable protein. 400
6
Incubate the membrane with HRP-labelled antibody specific to prey protein.
7
Incubate the blot with chemiluminescent HRP substrate and expose to film.
200
140
100
80
60
40
20

Steps in Western Blotting

Step – 1: Extraction of Protein

- Protein is extracted from cell by mechanical or chemical lysis of cell. This step is also known as Tissue preparation.
- To prevent denaturing of protein Protease inhibitor is used.
- The concentration of protein is determined by Spectroscopy.
- When sufficient amount of protein sample is obtained, it is diluted in loading buffer containing glycerol which helps to sink the sample in well.
- Tracking dye (Bromothymol blue) is also added in sample to monitor the movement of proteins.

Step – 2: Gel Electrophoresis

- The sample is loaded in well of SDS-PAGE.
- The proteins are separated on the basis of electric charge, isoelectric point, molecular weight, or combination of these all.
- The small size protein moves faster than large size protein.
- Protein are negatively charged, so they move toward positive (Anode) pole as electric current is applied.

Step – 3: Blotting

- The Nitrocellulose membrane is placed on the gel. The separated protein from gel get transferred to nitrocellulose paper by capillary action. This type of blotting is time consuming and may take 1-2 days.
- For fast and more efficient transfer of desired protein from the gel to nitrocellulose paper electro-blotting can be used.
- In electro-blotting nitrocellulose membrane is sandwich between gel and cassette of Filter paper and then electric current is passed through the gel causing transfer of protein to the membrane.

Step – 4: **Blocking**

- Blocking is very important step in Western blotting.
- Antibodies are also protein so they are likely to bind the nitrocellulose paper. So before adding the Primary antibody the membrane is non - specifically saturated or masked by using **casein or Bovine serum albumin (BSA).**

Step – 5: Treatment with Primary Antibody

- The Primary antibody (1° Ab) is specific to desired protein so it form Ag-Ab complex.

Step – 6: Treatment with Secondary Antibody

- The Secondary antibody is enzyme labelled. For eg. Alkaline phosphatase or Horseradish peroxidase is labelled with Secondary antibody.
- Secondary antibody (2° Ab) is antibody against primary antibody (anti-antibody) so it can bind with Ag-Ab complex.

Step – 7: Treatment with suitable Substrate

- To visualize the enzyme action, the reaction mixture is incubated with specific substrate.
- The enzyme convert the substrate to give visible colored product, so band of color can be visualized in the membrane.

Applications of Western Blotting

- To determine the size and amount of protein in given sample.
- Disease diagnosis - Detects antibody against virus or bacteria in serum.
- Western blotting technique is the Confirmatory test for HIV. It detects Anti - HIV antibody in patient's serum.
- Useful to detect Defective proteins. For eg Prions disease.
- Definitive test for Creutzfeldt-Jacob disease (Brain disorder), Lyme disease (Borreliosis caused by Borrelia sp.), Hepatitis B virus and Herpes virus.

AVIDIN-BIOTIN MEDIATED IMMUNOASSAY
IMMUNOHISTOCHEMISTRY
IMMUNOFLUORESENCE
IMMUNOFERRTIN TECHNIQUE

Avidin- Biotin mediated Immunoassay

- Avidin-biotin-mediated immunoassays (ABIAs) are a type of immunological test that uses the high affinity interaction between avidin and biotin to amplify the signal generated by the binding of an antibody to its target antigen.
- This technique leverages the strong binding affinity between avidin and biotin, which is one of the strongest non-covalent interactions in nature.

ABIAs are typically performed in a two-step process.

1. Antigen capture: The sample to be tested is incubated with an antibody that is specific for the target antigen. The antibody is typically labeled with biotin, a small molecule that binds avidly to avidin.
2. Detection: The sample is incubated with a solution of avidin that is conjugated to an enzyme. When the avidin binds to the biotin on the antibody, it brings the enzyme into close proximity to the target antigen. The enzyme then catalyzes a reaction that produces a detectable signal, such as a change in color or fluorescence.

Advantages:

- ABIAs are highly sensitive and specific, meaning that they can detect very small amounts of the target antigen.
- ABIAs are versatile and can be used to detect a wide range of different antigens, including proteins, peptides, carbohydrates, and nucleic acids.

Disadvantages:

- ABIAs can be time-consuming to perform, especially if multiple steps are involved.
- ABIAs can be affected by a variety of factors, such as the quality of the reagents used and the temperature of the reaction.
- ABIAs can be sensitive to non-specific binding, which can lead to false positive results.

Applications:

- Detection of infectious agents
- Detection of hormones and other proteins
- Food safety testing
- Immunohistochemistry

Immunohistochemistry (IHC)

- Immunohistochemistry (IHC) is a technique used to detect and localize specific antigens in cells and tissue sections. IHC is based on the principle of antibody-antigen binding.
- Antibodies are proteins that bind specifically to their target antigens. When an antibody binds to its target antigen in a tissue section, the antibody can be visualized using a variety of different methods, such as chromogenic staining, fluorescent staining, or electron microscopy.

- This is a technique for identifying cellular or tissue constituents (antigens) by means of antigen antibody interactions, the site of antibody binding being identified either by direct labeling of the antibody, or by use of a secondary labeling method.
- Immunohistochemistry- using tissue sections.
- Immunocytochemistry – cytological preparations

The basic Immunohistochemistry technique steps

- **Sample preparation:** The tissue sample is fixed to preserve its structure and then embedded in a paraffin wax block. Thin sections of the paraffin block are then cut and mounted on glass slides.
- **Deparaffinization and rehydration:** The paraffin wax is removed from the tissue sections using a series of organic solvents. The tissue sections are then rehydrated using a series of graded alcohols.
- **Antigen retrieval**: The formalin fixation process can mask some antigens, making them difficult for antibodies to bind to. Antigen retrieval is a process used to reverse the effects of formalin fixation and expose the antigens of interest.

- **Blocking**: The tissue sections are blocked with a solution containing proteins, such as bovine serum albumin (BSA), to prevent non-specific antibody binding.
- **Primary antibody application:** The primary antibody is applied to the tissue sections and allowed to incubate for a period of time. The primary antibody is specific to the antigen of interest.
- **Secondary antibody application:** The secondary antibody is applied to the tissue sections and allowed to incubate for a period of time. The secondary antibody is labeled with a reporter molecule, such as an enzyme or a fluorescent dye.
- **Detection**: The reporter molecule on the secondary antibody is visualized using a variety of different methods, such as chromogenic staining, fluorescent staining, or electron microscopy.

Immunofluorescence

- Fluorescent dyes such as fluorescrin and rhodamine can be coupled with antibody without destroying specificity.
- Fluorescein absorbs blue light at 490 nm and emits an intense yellowish green fluorescence at 517nm.
- Rhodamine absorbs light the yellow green range at 515nm and emits a deep red fluorescence at 546 nm.
- The emitted light is viewed through a fluorescent microscope ,which is fitted with UV light source and excitation filter.

- Differential staining of cells with two fluorescent substance can achieved by tagging one antibody with fluorescein an the other antibody with rhodamine and simultaneously two different antigen of the cell preparation can be identified.
- Staining of cell membrane molecules or tissue sections by fluorescent antibody molecules can be carried out in two ways. Direct and indirect.

❖ Direct method

❖ Indirect method

Direct immunofluorescence

Direct detection of Pathogens or their Ag's in tissues or in pathological samples Also used for localization of IgG in immune complexes along the dermal- epidermal junction of skin biopsies from patients suffering from systemic lupus erythematosus The aim is to identify the presence and location of an antigen by the use of a fluorescent labeled specific antibody

Indirect immunofluorescence

- The aim is to identify the presence of antigen specific antibodies in serum. The method is also be used to compare concentration of the antibodies in sera.
- Indirect test is a double-layer technique, uses two antibodies i.e the primary antibody and secondary antibody, carries the fluorochrome.The most widely used method of IF in pathology.

USES

- For the diagnosis of bacterial, viral and protozoan diseases including Borrelia burgdorferi.
- Bovine immunodeficiency like virus and Toxoplasma gonadii

Immunoferritin technique

- Immunoferritin technique is a type of immunocytochemistry used to localize antigens at the ultrastructural level using transmission electron microscopy (TEM). It was first developed in the 1960s and has been used to study a wide range of biological processes, including protein trafficking, cell-cell interactions, and viral infection.
- When the antibody-ferritin conjugate binds to the antigen of interest, the ferritin molecules produce a dark electron-dense signal under TEM(Transmission electron microscopy). This allows the precise localization of the antigen within the cell or tissue.

- Steps involved in the immunoferritin technique:

1. Fixation:

The tissue is fixed with a fixative that preserves the ultrastructure of the cells, such as glutaraldehyde.

2.Cryosectioning:

The tissue is frozen and then sectioned into ultrathin sections using a cryomicrotome.

3.Immunostaining:

The sections are incubated with the antibody-ferritin conjugate.

4.Washing:

The sections are washed to remove any unbound antibody-ferritin conjugate.

5.Negative staining:

The sections are stained with a heavy metal salt, such as osmium tetroxide or uranyl acetate, to increase the contrast of the electron micrograph.

6.Embedding:

The sections are embedded in a resin, such as Epon or Araldite.

7.Ultramicrotomy:

The embedded sections are cut into ultrathin sections using an ultramicrotome.

8.TEM:

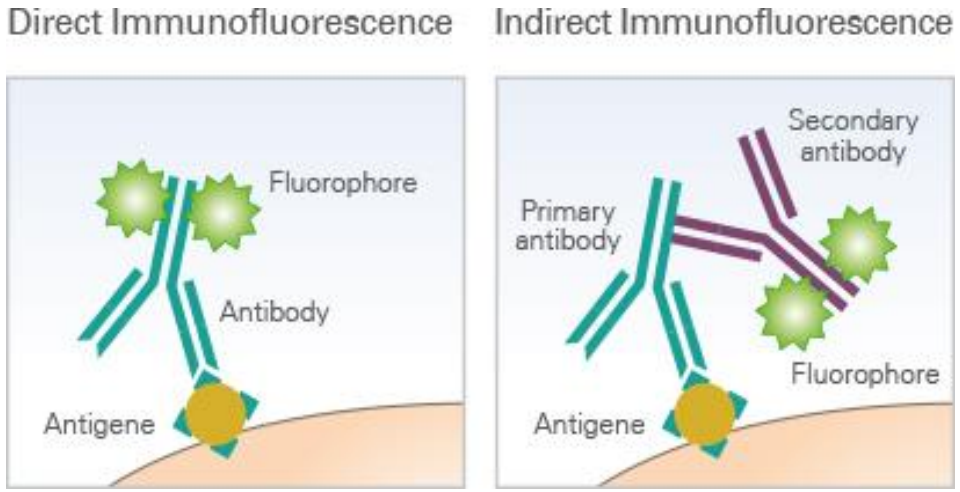
The sections are examined under TEM

FLUORESCENCE IMMUNOASSAY

- Immunofluorescence (IF) is a powerful approach for getting insight into cellular structures and processes using microscopy. Specific proteins can be assessed for their expression and location, making immunofluorescence indispensable for scientists to solve many cell biological questions.
- PRINCIPLE
- An Fluorescence immunoassay experiment is based on the following principal steps:
- Specific antibodies bind to the protein of interest.
- Fluorescent dyes are coupled to these immune complexes in order to visualize the protein of interest using microscopy.

TYPES

- It is distinguished between direct and indirect immunofluorescence. In direct immunofluorescence, the primary antibody is directly coupled to a fluorophore (also called fluorochrome), allowing for easy handling and quick visualization.
- In indirect immunofluorescence, a secondary fluorophore-coupled antibody, which specifically binds to the primary antibody, is used to visualize the structure of interest



- **Complement method**: use fluorescein to label anti-complement antibodies to identify unknown antigens or antibodies. This method only needs one labeled anti-complement antibody.
- Since complement binds to the antigen-antibody complex without species specificity.
- **Double labeling method**: use fluorescent yellow isothiocyanate (FITC) and tetraethyl rhodamine (RB200) to label different antibodies to detect the same matrix sample, if there are two corresponding antigens, then Displays different colors of fluorescence.

EXPERIMENT

Sample Preparation

- The optimal cell density has to be determined. In general, a confluence of 70%–80% is recommended for immunocytochemistry.

Sample Fixation

- The first step of an immunofluorescence staining protocol is to fixate the sample. This is usually done by incubating the sample for 10 minutes at room temperature in a 4% formalin solution (in PBS, pH 7.4), which crosslinks the proteins. The sample can also be fixated in 100% chilled methanol or acetone.

Cell Permeabilization

In order to stain intracellular proteins, the cell needs to be permeabilized. Without this step, it is not possible for the antibodies to enter the cell through the lipid membrane.

The permeabilization requires incubation in a detergent, for example Triton X-100 or Tween-20 (for a less harsh permeabilization) in a PBS solution.

Blocking

- In order to minimize intra- or extracellular background signals, non-specific antigens should be blocked by incubating the sample in (1) the serum of the host, in which the secondary antibody was made, (2) bovine serum albumin (BSA), or (3) milk. The first option is the most recommended because of its highest specificity.
- Typical blocking times are from 30 minutes up to one hour.

Primary Antibody Incubation

- A suitable primary antibody must have a high specificity for the antigen of interest. Furthermore, whether the antibody is mono- or polyclonal influences its specificity.
- Another highly relevant property of the primary antibody is its originating host, as it determines the secondary antibody.

Secondary Antibody Incubation

- In this assays, the secondary antibody is conjugated to a fluorophore(fluorescein), which emits light when excited at a defined wavelength.

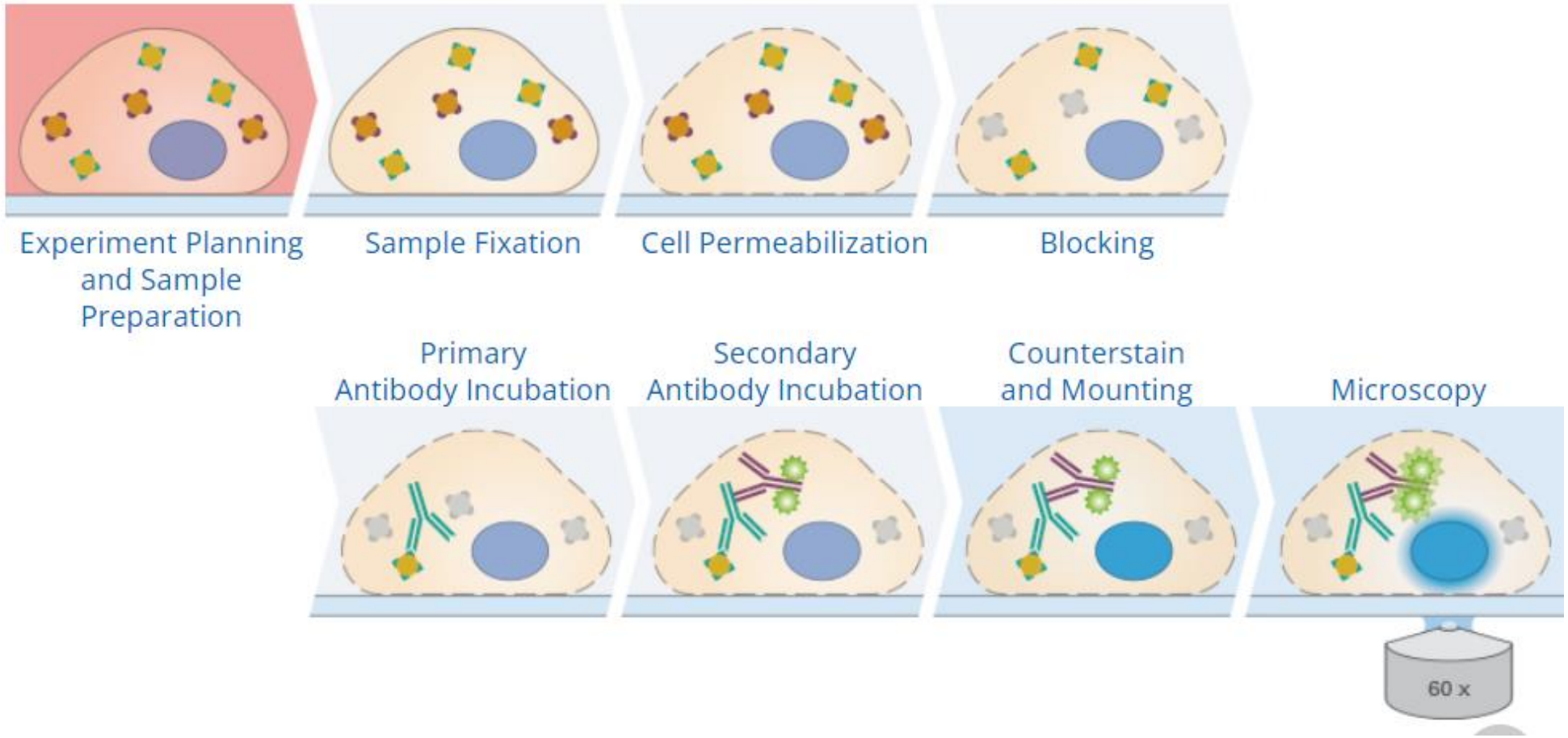
  The secondary antibody specifically binds to the first antibody. Therefore, it is essential that the secondary antibody is specific to the host in which the primary antibody was produced.
- To be suitable for fluorescence microscopy, stable fluorophores must be used, because the sample is generally exposed to a high number of photons.

Counterstain and Mounting

- The final step before microscopy is the counterstaining of the nuclei and the mounting. In order to avoid drying out of the samples, and to guarantee a stable refractive index of the cellular environment (a prerequisite for successful microscopy), the sample needs to be mounted.
- DAPI, (4',6-diamidino-2-phenylindole) the standard for nuclear counterstaining, is either included in the mounting medium

Microscopy

- To get optimal results, the microscopic analysis of the immunofluorescence staining should be done directly after the mounting.
- For standard immunofluorescence stainings, epifluorescence and confocal microscopy are widely-used methods


Experiment Planning and Sample Preparation
Sample Fixation
Cell Permeabilization
Blocking
Primary Antibody Incubation
Secondary Antibody Incubation
Counterstain and Mounting
Microscopy
60 x

FLUORESCENCE ACTIVATED CELL SORTING(FACS)

- Fluorescence-activated cell sorting (FACS) is a specialized form of flow cytometry that provides a method for sorting a heterogeneous mixture of cells into two or more containers based upon the fluorescence and/or light scattering properties of each cell.

Principles of Flow Cytometry

- Flow cytometry runs on the principles of light scattering, excitation and emission. Fluorescently tagged cell components get excited when they pass through a laser beam, producing lights of different wavelengths. The fluorescence is used to analyze cellular properties.
- FACS is usually capable of separating multiple cell populations simultaneously, which improves the efficiency and diversity of experiments.

- Different cell types usually express unique molecules, or a unique combination of several molecules, on the plasma membrane that can distinguish one cell population from another.
- Upon binding of these cell surface molecules by specific fluorescence-conjugated antibodies, a detecting machine called flow cytometer/sorter is able to excite and detect the light signals of different fluorescent dyes that represent different molecule markers on the cells at the single cell level.
- After passing through the detector, cells with the same phenotype of interest are diverted towards a designated collecting tube based on electrical charge

The FACS Process

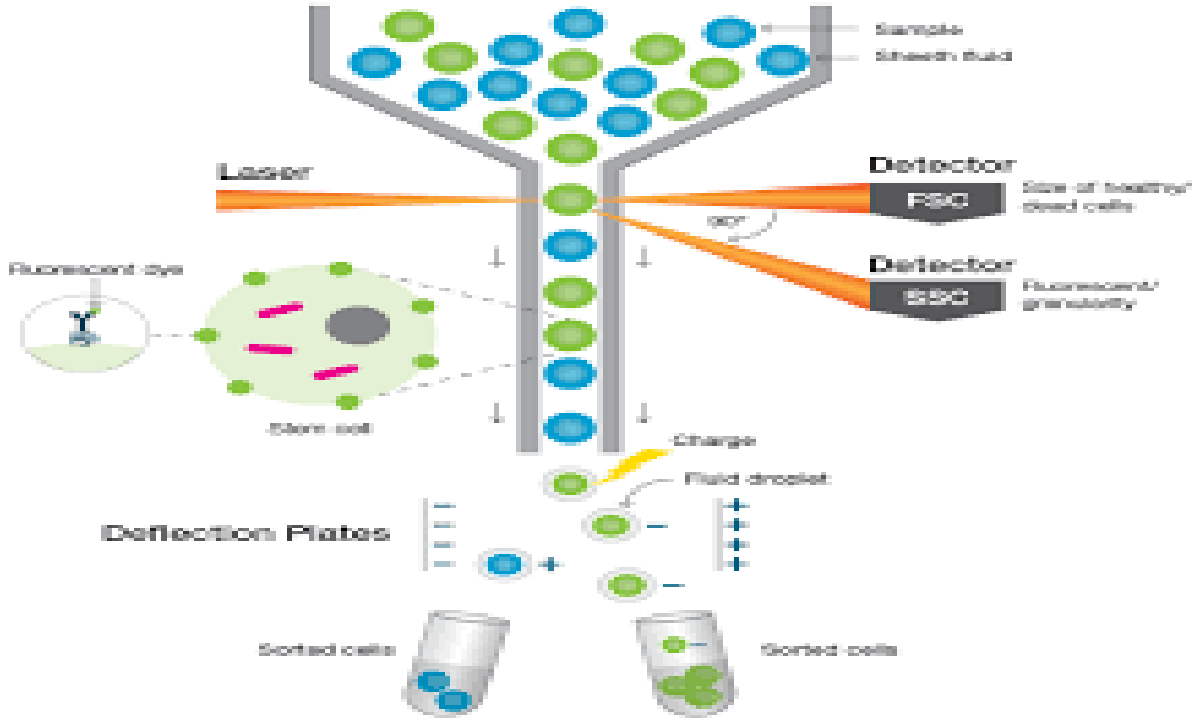
FACS protocols consist of four general phases:

- **Sample preparation and labeling**: cell sample is prepared. Fluorescent labels—dyes attached to antibodies—are introduced to the sample, adhering to specific surface features unique to each cell type.

  Laser excitation and cell interrogation: Once labeled, cells are funneled through the flow cytometer one at a time. They encounter a laser beam that excites the fluorescent tags, causing each cell to emit light at varying wavelengths depending on their labels.

  Signal detection and system analysis: Sophisticated detectors within the FACS system capture the emitted light and the scattered light from cells. Forward scatter reveals information about cell size, while side light scatter provides insights into the granularity or internal complexity of the cells. The emitted fluorescence is indicative of the bound fluorescent tag, thus revealing the presence and quantity of specific cell markers.

- **Cell sorting and collection**: Post-detection, cells are electrically charged and pass through an electromagnetic field within the sorter. This field diverts cells into different containers based on their charge, effectively sorting them according to the predefined fluorescence profiles.



ELISA

- Enzyme linked immunoassays (ELISAs) use the catalytic properties of enzymes to detect and quantify immunologic reactions.

  Two different research teams simultaneously invented the direct ELISA: scientists Engvall and Perlman and scientists Van Weemen and Schuurs in 1970.

  In ELISA technique, an aliquot of sample containing the antigen (Ag) to be quantified is added to and allowed to bind with a solid-phase antibody (Ab).

  After washing, an enzyme-labeled antibody is added and forms a "sandwhich complex" of solid-phase Ab-Ag-Ab enzyme. Unbound antibody is then washed away, and enzyme substrate is added.

- The amount of product generated is proportional to the quantity of antigen in the sample.

Procedure

- There are four main general steps to completing an ELISA immunoassay. These steps are:
- Coating (with either antigen or antibody)
- Blocking (typically with the addition of bovine serum albumin [BSA])
- Detection
- Final read
- Between each of the above four steps is a “wash” of the plate using a buffer, such as phosphate-buffered saline (PBS) and a non-ionic detergent, to remove unbound material.

- There are four major types of ELISA:

Direct ELISA

- The first binding step involves adding antigens to the plates, which are incubated for one hour at 37 °C or can be incubated at 4 °C overnight.
- The next step is to wash the plates of any potential unbound antigens and block any unbound sites on the ELISA plate using agents like BSA, ovalbumin, aprotinin, or other animal proteinsresults.
- After adding the buffer, the plate is rewashed, and a selected enzyme-conjugated primary detection antibody is added. The plate is further incubated for one hour.

- The primary detection antibody binds directly to the protein of interest.
- Next, the plate is rewashed to remove any unbound antibodies. An enzyme, such as alkaline phosphatase (AP) or horseradish peroxidase (HRP), is added to the plate.
- Then the substrate is added to the well. The colour change is noted. This method helps to determine the presence of supposed antigen in samples.

Indirect ELISA

- Indirect ELISA requires two antibodies: a primary detection antibody that sticks to the protein of interest and a secondary enzyme-linked antibody complementary to the primary antibody.

PROCEDURE

- The different steps of indirect ELISA used to detect the presence of antiserum antibodies against HIV are given below:
- Microtiter well is coated with HIV antigen.
- Blood is taken from a patient suffering from HIV infection and centrifuged to remove blood cells and large proteins. The remaining fluid is the antiserum.
- The antiserum is added to the antigen coated microtiter well. The microtiter plate is incubated at 4°C for 12 hrs or at 37°C for 30 minutes.
- The microtiter well is thoroughly washed with a wash buffer to remove unbound antibodies in the antiserum.

- HIV is injected into a goat. After 4 hrs, blood is taken and centrifuged to get HIV antiserum.
- This antiserum contains secondary antibodies. The enzyme horse radish peroxidase is linked to the secondary antibodies by mixing.
- This enzyme-linked secondary antibody is added to the microtiter well. It is incubated at 37°C for 30 minutes to facilitate antigen-antibody binding.
- The well is washed with a wash buffer.
- The substrate ortho-phenylene diamine dihydrochloride is added to the well and incubated for 30 minutes.
- Horse radish peroxidase reduces the substrate into a coloured compound. Colour change in the well indicates presence of anti-HIV antibodies.in the serum.
- Negative and positive control are used for this diagnosis For measuring the concentration of the anti-HIV antibodies, the colour change is read using a microassay plate reader at 492 nm.

Sandwhich ELISA

- The sandwich ELISA begins with a capture antibody coated onto the wells of the plate.[8] It is termed a “sandwich” because the antigens are sandwiched between two layers of antibodies (capture and detection antibodies).
- PROCEDURE
- The microtiter well is coated with a known quantity of antibody.
- The sample antigen is applied on the titer well.
- The well is washed to remove unbound antigen.
- A specific antibody is added. The specific antibody binds to the antigen.
- The antigen is held between the antibodies. Enzyme-linked secondary antibodies are added as detection antibodies.
- They bind to the Fc region of specific antibodies.

Competitive ELISA

- In competitive ELISA, the sample antigen competes with the known antigen to bind the antibody.
- PROCEDURE
- Microtiter well is coated with HIV antigen.
- Blood is taken from a patient suffering from HIV infection and centrifuged to remove blood cells and large proteins.
- The remaining fluid is the antiserum for HIV. This is the primary antibody.
- The primary antibody is linked with sample antigen to form sample antigen-antibody complex. It is added to the HIV antigen coated on the microtiter well.
- The microtiter plate is incubated at 4°C for 12 hrs or at 37°C for 30 minutes.

- The more antigen present in the sample, the less free antibody will be available to bind to the antigen coated on the well. The microtiter well is thoroughly washed with a wash buffer to remove unbound antibodies in the HIV antigen.
- HIV is injected into a goat. After 4 hrs, Blood is taken and centrifuged to get HIV antiserum. This antiserum contains secondary antibodies.

  The enzyme horse radish peroxidase is linked to the secondary antibodies by mixing.
- This enzyme-linked secondary antibody is added to the microtiter well. It is incubated at 37°C for 30 minutes to facilitate the binding of secondary antibody to the primary antibody-antigen complex.

  The well is washed with a wash buffer.

  The substrate ortho-phenylene diamine dihydrochloride is added to the well and incubated for 30 minutes. Horse radish peroxidase reduces the substrate into a coloured compound.