BHARATHIDASAN UNIVERSITY

Tiruchirappalli- 620024,

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Programme: M.Sc., Biomedical Science

Course Code: 18BMS59C17

Course Title: Immune & Molecular Diagnostics

Unit-I

Basic Immunological Methods

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Unit I:

Basic Immunological Methods – Preparation of antigens, rising of antisera, routes of administration, doses for administration, purification of antibodies- methodology – IgG & IgA. Monoclonal antisera raising & Hybridoma technology. Types of conjugated antibodies, types of substrates and color detectors used in immunoassays. Immunofluorescence, Flow cytometry - clinical focus – Leukemia typing (immunophenotyping), ELISA and its variants- Principle and application, Surface Plasmon Resonance- principle and application- western blotting- principle and application

PRESENTATION: 3

Flow Cytometry

- Flow cytometry is a standard laser-based technology that is used in the detection and measurement of physical and chemical characteristics of cells or particles in a heterogeneous fluid mixture.
- The use of flow cytometry has increased over the years as it provides a rapid analysis of multiple characteristics (both qualitative and quantitative) of the cells.
- The properties that can be measured by this process include a particle's size, granularity or internal complexity, and fluorescence intensity.
- These characteristics are determined using an optical-to-electronic coupling system that detects the cells based on laser scattered by the cells.
- A flow cytometer, despite its name, does not necessarily deal with cells; it deals with cells quite often, but it can also deal with chromosomes or molecules or many other particles that can be suspended in a fluid.

Principle

- The flow cytometry runs on the principles of light scattering, excitation and emission.
- Fluorescencently 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.

Procedure

1.Fluidics:

1. **Sample Introduction**: A cell suspension is introduced into the flow cytometer. The fluidics system ensures that cells are aligned in a single file as they pass through the laser beam.
2. **Sheath Fluid**: The sample is injected into a stream of sheath fluid, which hydrodynamically focuses the cells into a narrow stream so they pass through the laser beam one at a time.

2.Optics:

1. **Laser Illumination**: As each cell passes through the laser beam, it scatters light and may emit fluorescence if stained with fluorescent markers.
2. **Light Scattering**: There are two types of light scattering:
    1. **Forward Scatter (FSC)**: Light scattered in the forward direction, which correlates with cell size.
    2. **Side Scatter (SSC)**: Light scattered at a 90-degree angle, which correlates with the internal complexity or granularity of the cell.

3. Fluorescence Detection:

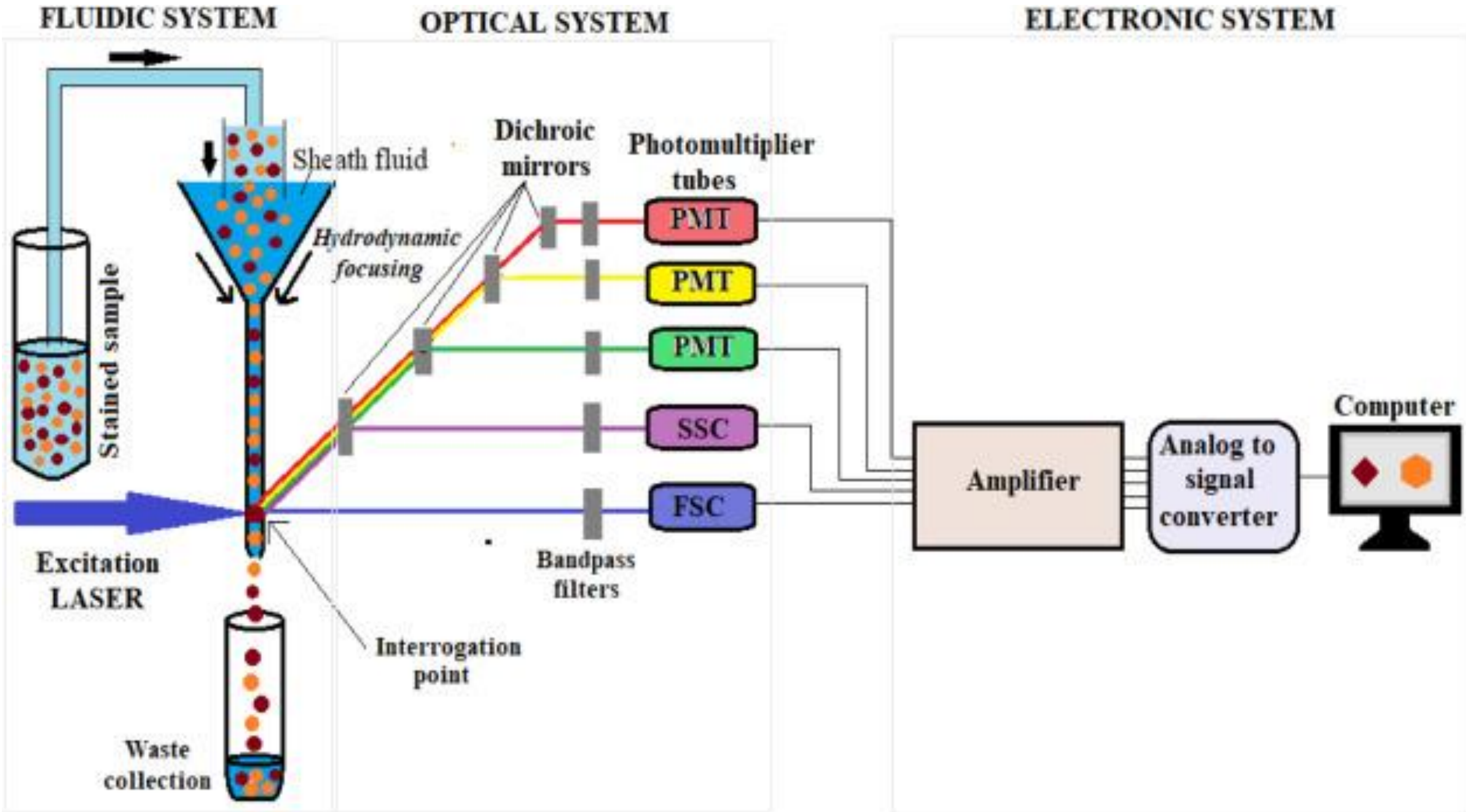
1. **Fluorochrome Staining**: Cells can be labeled with fluorescent antibodies that bind to specific cellular components (e.g., surface proteins, intracellular proteins, nucleic acids).
2. **Emission of Fluorescence**: When the laser excites these fluorochromes, they emit light at specific wavelengths.
3. **Optical Filters and Detectors**: The emitted fluorescence is separated by optical filters and detected by photomultiplier tubes (PMTs) or avalanche photodiodes (APDs). Different detectors measure different wavelengths of emitted light, corresponding to the various fluorochromes used.

4. Data Acquisition:

1. **Signal Processing**: The light signals (both scattered and fluorescent) are converted into electronic signals.
2. **Digital Conversion**: These electronic signals are digitized and sent to a computer for analysis.

5. Data Analysis:

1. **Multiparametric Analysis**: The data is analyzed to determine the size, complexity, and fluorescence intensity of each cell, allowing for the identification and quantification of various cell populations.
2. **Gating**: Cells are grouped based on specific characteristics (e.g., size, granularity, fluorescence intensity) using a process called gating, enabling detailed analysis of specific subpopulations.


FLUIDIC SYSTEM
OPTICAL SYSTEM
ELECTRONIC SYSTEM
Sheath fluid
Hydrodynamic focusing
Stained sample
Excitation LASER
Waste collection
Interrogation point
Dichroic mirrors
Photomultiplier tubes
PMT
PMT
PMT
SSC
FSC
Bandpass filters
Amplifier
Analog to signal converter
Computer

Steps

1. **Sample Preparation**: Cells are collected, processed, and stained with appropriate fluorochrome-conjugated antibodies or dyes.
2. **Staining**: Cells are incubated with fluorescently labeled antibodies that bind to specific markers of interest.
3. **Sample Loading**: The stained sample is loaded into the flow cytometer.
4. **Data Acquisition**: The flow cytometer collects data on thousands to millions of cells, measuring various parameters for each cell.
5. **Data Analysis**: The acquired data is analyzed using specialized software to identify and quantify different cell populations based on their size, complexity, and fluorescence characteristics.

Clinical Applications

1. **Hematology and Oncology**:
    1. **Leukemia and Lymphoma**: Flow cytometry is critical in diagnosing and classifying different types of leukemia and lymphoma. It identifies abnormal cells and determines their lineage (e.g., B-cell vs. T-cell).
    2. **Minimal Residual Disease (MRD) Detection**: It detects small numbers of cancer cells remaining after treatment, helping to monitor the effectiveness of therapy and predict relapse.
2. **Immunophenotyping**:
    1. **Immune Deficiency Disorders**: Flow cytometry can identify and quantify different subsets of lymphocytes (e.g., CD4+ T cells, CD8+ T cells, B cells), which is crucial for diagnosing conditions like HIV/AIDS and other immunodeficiencies.
    2. **Autoimmune Diseases**: It helps identify abnormal populations of immune cells in diseases like rheumatoid arthritis and systemic lupus erythematosus (SLE).

3. Transplantation:

1. **Graft vs. Host Disease (GVHD)**: Flow cytometry monitors immune cell populations in patients undergoing bone marrow or stem cell transplants, aiding in the early detection of GVHD.
2. **Organ Transplants**: It helps in the detection of donor-specific antibodies and monitoring of immune cell populations post-transplant.

4. Infectious Diseases:

1. **HIV Monitoring**: Flow cytometry is used to count CD4+ T cells, which are a critical marker of immune system health in HIV-infected patients.
2. **Other Infections**: It can help in the detection of specific pathogens and the immune response to infections.

5. Paroxysmal Nocturnal Hemoglobinuria (PNH): (Destruction of RBC by Complement system)

1. Flow cytometry detects the absence of specific surface proteins (e.g., CD55 and CD59) on red and white blood cells, which is characteristic of PNH.

6. Allergy and Asthma:

1. It is used to study the phenotypes and functions of eosinophils, basophils, and other immune cells involved in allergic reactions and asthma.

7. Research and Development:

1. **Drug Development**: Flow cytometry is used in clinical trials to monitor immune responses, evaluate the efficacy of new drugs, and study disease mechanisms.
2. **Biomarker Discovery**: It helps identify and validate new biomarkers for various diseases, which can lead to new diagnostic and therapeutic targets.

Advantages of Flow Cytometry in Clinical Settings

- **Speed**: Rapid analysis and real-time results are crucial for timely diagnosis and treatment decisions.
- **Multiparametric Analysis**: Ability to measure multiple characteristics (e.g., size, granularity, and expression of various markers) of individual cells simultaneously.
- **Sensitivity**: High sensitivity allows for the detection of rare cell populations., which is essential in MRD (Minimal Residual Disease) detection and other applications.
- **Quantitative**: Provides precise quantification of cell populations, which is important for monitoring disease progression and response to therapy.

Limitations

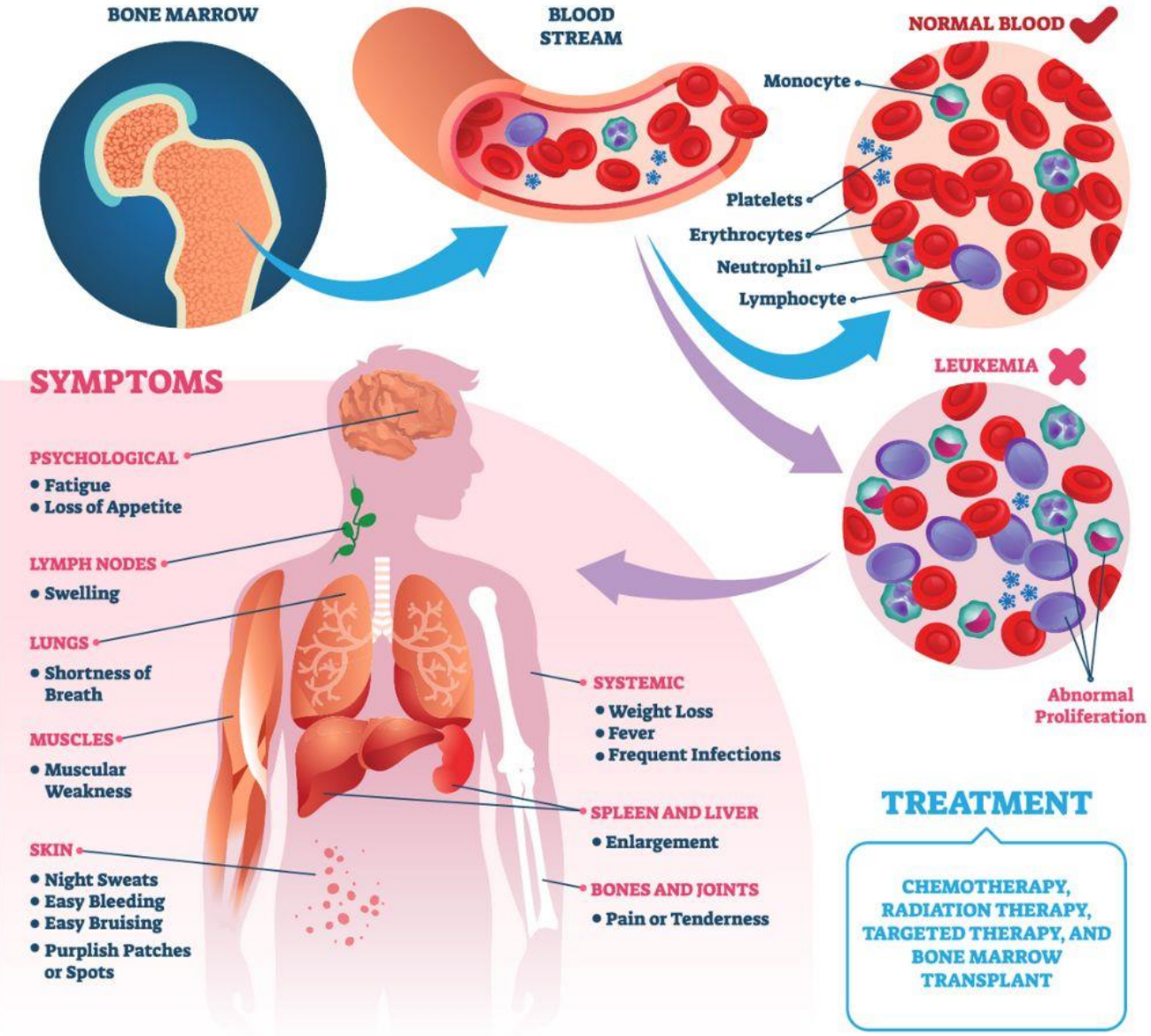
- This process doesn’t provide information on the **intracellular location or distribution of proteins.**
- Over time, debris is aggregated, which might result in **false results**.
- The pre-treatment associated with **sample preparation and staining is a time-consuming process.**
- Flow cytometry is an **expensive process** that requires highly qualified technicians.

Leukemia typing or leukemia immunophenotyping

- Leukemia is a type of cancer that originates in the body’s blood-forming tissues, primarily the bone marrow and lymphatic system.
- It results in the production of an excessive number of abnormal white blood cells, which do not function properly.
- These abnormal cells crowd out the normal blood cells, impairing the body's ability to fight infections, carry oxygen, and control bleeding.

LEUKEMIA

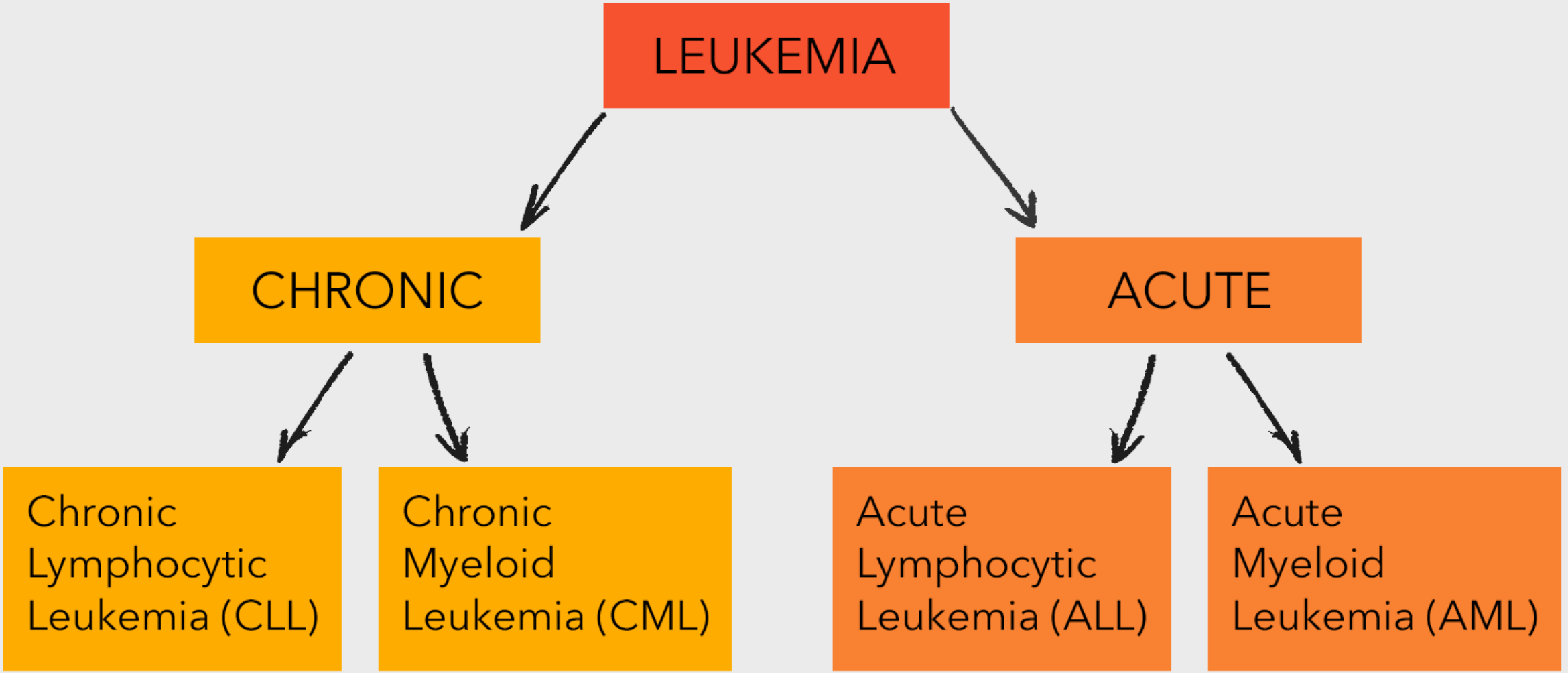
LEUKEMIA is a **GROUP OF BLOOD CANCERS** that Usually Begin in the Bone Marrow and Result in High Numbers of **ABNORMAL BLOOD CELLS**



TREATMENT

CHEMOTHERAPY, RADIATION THERAPY, TARGETED THERAPY, AND BONE MARROW TRANSPLANT

FOUR TYPES OF LEUKEMIA



Diagnosis

- **Blood Tests**: To check the levels of white blood cells, red blood cells, and platelets.
- **Bone Marrow Biopsy**: A sample of bone marrow is examined to identify leukemia cells.
- **Immunophenotyping**: Flow cytometry is used to classify the type of leukemia.
- **Cytogenetic Analysis**: Identifies specific genetic changes in the leukemia cells.

- Leukemia typing, also known as leukemia immunophenotyping, is the process of classifying leukemia into specific subtypes based on the presence of certain markers on the surface of cells.
- This is crucial for diagnosis, treatment planning, and prognosis.
- Flow cytometry is a key tool in this process due to its ability to analyze multiple cellular markers simultaneously.

Flow Cytometry in Leukemia Typing

Principle

Flow cytometry for leukemia typing involves labeling cells with fluorescent antibodies that bind to specific cell surface or intracellular markers. By analyzing these markers, clinicians can determine the lineage (e.g., myeloid or lymphoid) and maturation stage of the leukemia cells.

Procedure

1. Sample Collection:

1. Blood, bone marrow, or tissue samples are collected from the patient.

2. Cell Preparation:

1. Samples are processed to create a single-cell suspension.

3. Staining:

1. Cells are stained with a panel of fluorochrome-conjugated antibodies. The panel is selected based on the suspected type of leukemia.

4. Flow Cytometry Analysis:

1. Stained cells are run through the flow cytometer. The machine detects and measures the fluorescence intensity emitted by each cell, which corresponds to the presence of specific markers.

5. Data Analysis:

1. Data is analyzed to identify distinct cell populations based on their marker expression profiles. Specific gating strategies are used to isolate and characterize these populations.

Marker Panels

- Different types of leukemia are characterized by unique combinations of cell surface and intracellular markers. Here are some common markers used in leukemia typing:
- **Acute Lymphoblastic Leukemia (ALL)**:
  - **B-cell ALL**: CD19, CD10, CD20, CD22, TdT
  - **T-cell ALL**: CD3, CD7, CD4, CD8, TdT
- **Acute Myeloid Leukemia (AML)**:
  - CD13, CD33, CD34, CD117, MPO (myeloperoxidase)
- **Chronic Lymphocytic Leukemia (CLL)**:
  - CD5, CD19, CD20, CD23, kappa and lambda light chains
- **Chronic Myeloid Leukemia (CML)**:
  - Typically diagnosed through molecular methods like PCR for the BCR-ABL1 fusion gene rather than flow cytometry, but may show myeloid markers if flow cytometry is used.

Interpretation of Results

- **Lineage Determination**: Based on the presence of specific markers, cells are classified as myeloid or lymphoid in origin.
- **Subtype Classification**: Further classification into subtypes (e.g., B-cell vs. T-cell ALL) based on additional markers.
- **Aberrant Marker Expression**: Identification of abnormal marker expression patterns that are characteristic of leukemic cells.
- **Minimal Residual Disease (MRD) Monitoring**: Detection of low levels of leukemic cells during or after treatment to assess response and predict relapse.

Importance of Leukemia Typing

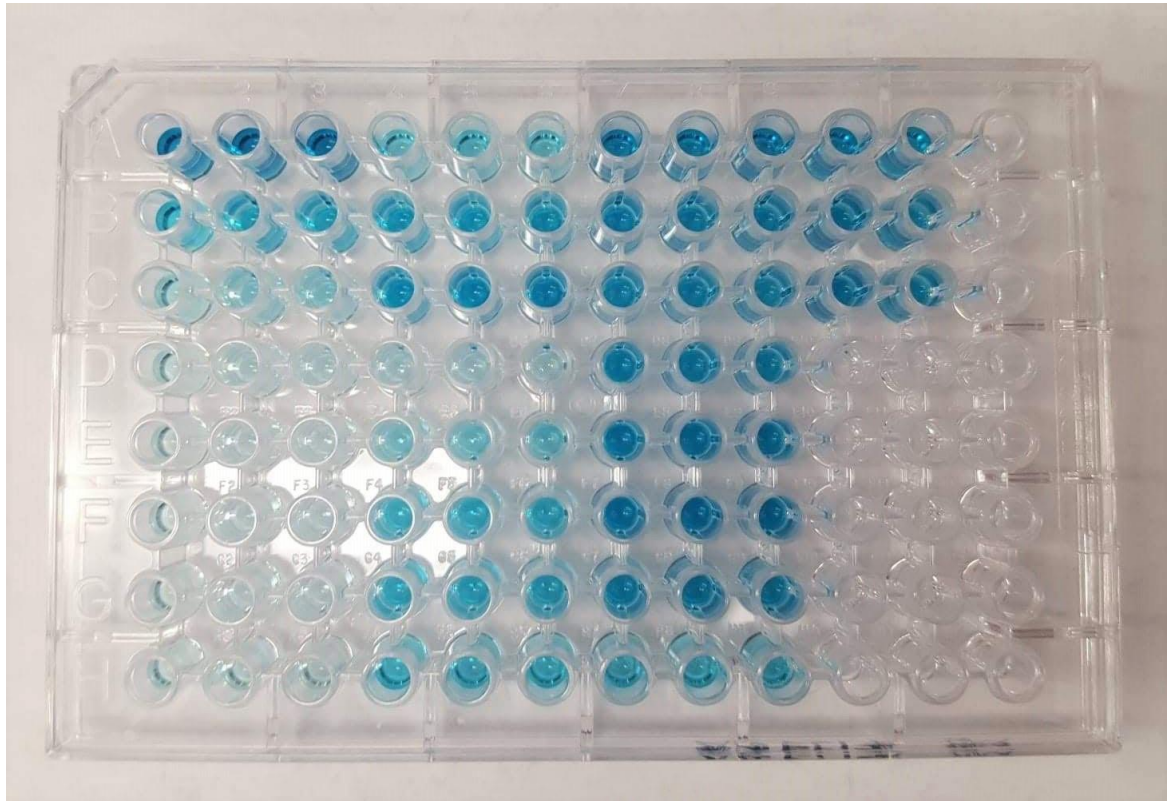
1. **Accurate Diagnosis**: Correctly identifying the type and subtype of leukemia is critical for determining the appropriate treatment strategy.
2. **Prognosis**: Certain subtypes of leukemia have distinct prognostic implications, influencing the expected course and outcome of the disease.
3. **Treatment Planning**: Specific therapies are tailored based on the type of leukemia. For example, targeted therapies may be used for subtypes with particular genetic mutations or marker expressions.
4. **Monitoring**: Flow cytometry can be used to monitor disease progression and response to treatment, as well as to detect minimal residual disease.

ELISA

- Enzyme-linked immunosorbent assay (ELISA) is a widely used analytical biochemistry assay that utilizes antibodies and color change to identify a substance.
- The **enzyme-linked immunosorbent assay (ELISA)** is an immunological assay commonly used to measure **antibodies, antigens, proteins** and **glycoproteins** in biological samples.
- Some examples include: diagnosis of HIV infection, pregnancy tests, and measurement of cytokines or soluble receptors in cell supernatant or serum.

- ELISA assays are generally carried out in 96 well plates, allowing multiple samples to be measured in a single experiment.
- These plates need to be special absorbant plates (e.g. NUNC Immuno plates) to ensure the antibody or antigen sticks to the surface.
- Each ELISA measures a specific antigen, and kits for a variety of antigens are widely available.

ELISA



Principle

1. **Antigen-Antibody Interaction**: ELISA relies on the specific interaction between an antigen and an antibody.

2. **Enzyme Conjugation**: An enzyme is linked to an antibody or antigen. This enzyme will produce a detectable signal, usually a color change, when it reacts with a substrate.

3. **Detection:** The presence and quantity of the antigen (or antibody) are determined by the intensity of the color change, which is measured using a spectrophotometer.

Steps

1. **Coating**: An antigen or antibody is attached to a solid surface, usually a microplate well.
2. **Blocking**: Any unbound sites on the plate are blocked with an irrelevant protein to prevent non-specific binding.
3. **Binding**: The sample containing the target antibody or antigen is added to the well and allowed to bind to the coated antigen or antibody.
4. **Detection**: A secondary antibody, which is enzyme-linked and specific to the target antibody or antigen, is added.
5. **Substrate Addition**: A substrate for the enzyme is added. The enzyme catalyzes a reaction that produces a detectable signal, typically a color change.
6. **Measurement**: The intensity of the color change is measured using a spectrophotometer, and it correlates with the amount of antigen or antibody present in the sample.

Types of ELISA

1.Direct ELISA:

1. **Principle**: The antigen is directly attached to the plate, and an enzyme-linked antibody specific to the antigen is added.
2. **Application**: Used for detecting high concentrations of antigens.

2.Indirect ELISA:

1. **Principle**: The antigen is attached to the plate, and a primary antibody specific to the antigen binds to it. Then, a secondary enzyme-linked antibody, which binds to the primary antibody, is added.
2. **Application**: Commonly used for detecting antibodies in a sample.

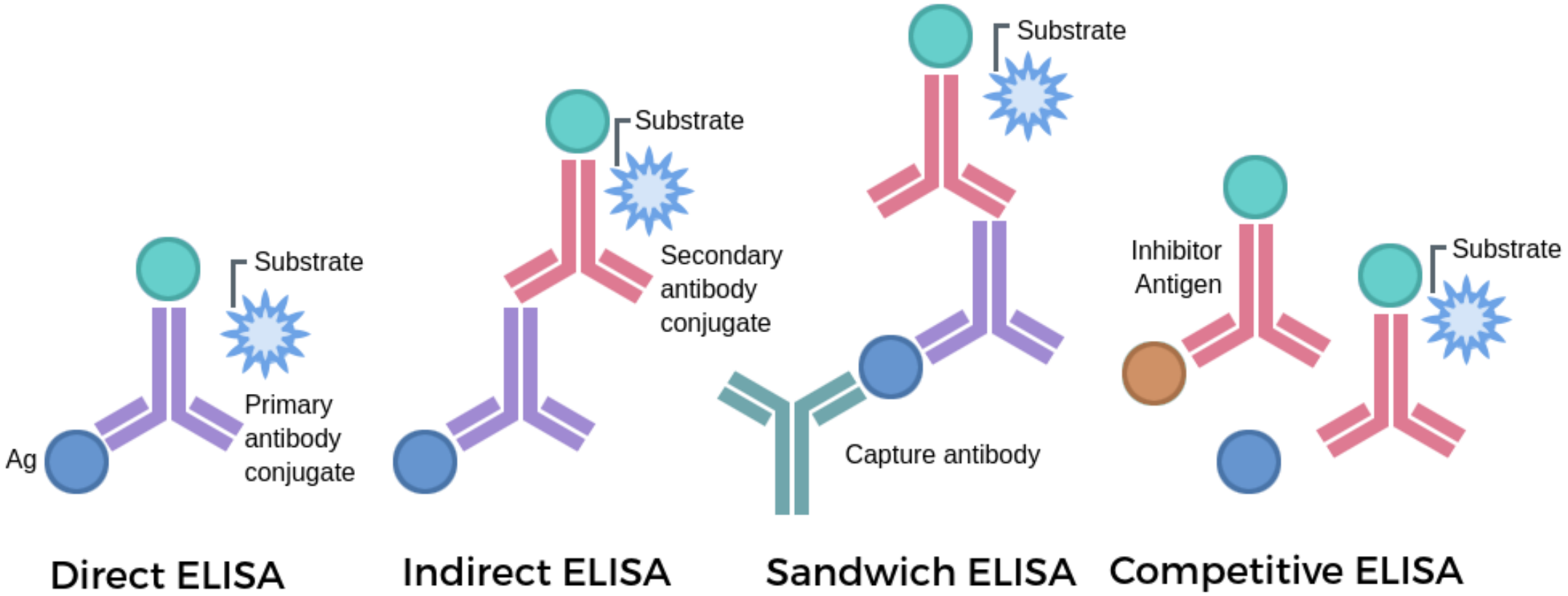
3. Sandwich ELISA:

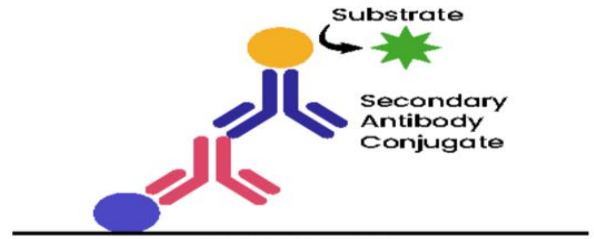
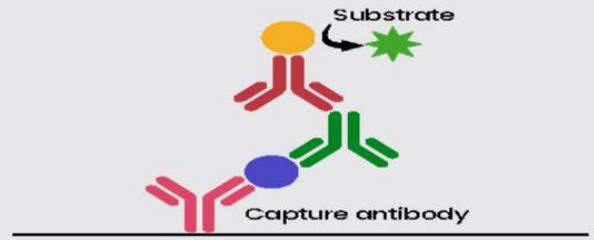
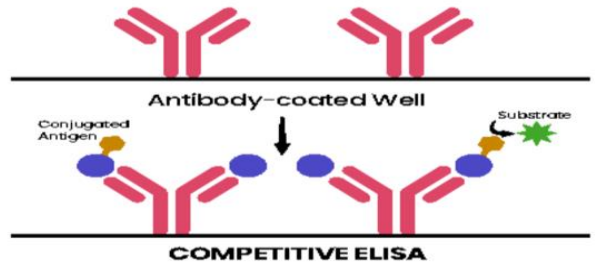
1. **Principle**: An antibody is attached to the plate, and the sample containing the antigen is added. Another antibody specific to the antigen, linked to an enzyme, is then added.
2. **Application**: Highly specific and sensitive, used for detecting antigens.

4. Competitive ELISA:

1. **Principle**: The sample antigen competes with a labeled antigen for binding to an antibody attached to the plate. The signal decreases with an increase in the sample antigen.
2. **Application**: Used for detecting small molecules and hormones.

Types of ELISA



Type	Key points	Advantages
 DIRECT ELISA	Binds antigens, including the desired target, in a sample directly to the plate. An enzyme-conjugated antibody is then added as a probe for the desired analyte.	Only one antibody is used, so cross-reactivity is not a concern Rapid
 INDIRECT ELISA	Binds antigens, including the desired target, in the sample to the plate. However, it involves two antibodies; a primary antibody and a secondary conjugated antibody.	High sensitivity
 SANDWICH ELISA	The target is bound between a capture antibody (for antigen detection) or capture protein (for antibody detection) and the conjugated detecting antibody, creating a "sandwich".	Highly sensitive and specific
 COMPETITIVE ELISA	Involves competition between the binding of the sample antigen and conjugated antigen to a specific amount of antibody. The more antigen in the sample, the less conjugated antigen binds and the lower the assay signal.	Rapid Requires little/ no sample pre-processing Useful for small targets that cannot easily be bound with two antibodies

Applications of ELISA

1.Medical Diagnostics:

1. **Disease Detection**: Used to detect infections (e.g., HIV, hepatitis), autoimmune diseases, and allergies.
2. **Hormone Levels**: Measures hormone levels (e.g., hCG in pregnancy tests).

2.Food Industry:

1. **Allergen Detection**: Identifies potential allergens in food products.
2. **Contaminants**: Detects contaminants such as pesticides and toxins.

3. Environmental Monitoring:

1. **Pollutants**: Used to detect environmental pollutants and toxins.

4. Pharmaceutical Industry:

1. **Drug Development**: Monitors the immune response during drug development.
2. **Quality Control**: Ensures the quality and safety of products.

5. Research:

1. **Protein Quantification**: Quantifies proteins and other biomolecules in research studies.
2. **Cell Signaling Studies**: Investigates cellular responses to various stimuli.

Variants of ELISA

1. Chemiluminescent ELISA:

1. **Principle**: Uses a chemiluminescent substrate that emits light upon reaction with the enzyme.
2. **Application**: Provides higher sensitivity and dynamic range compared to colorimetric ELISA.

2. Fluorescent ELISA:

1. **Principle**: Uses a fluorescent substrate or labeled antibodies.
2. **Application**: Allows multiplexing, where multiple targets can be detected simultaneously.

3. Magnetic ELISA:

1. **Principle**: Uses magnetic beads coated with antigens or antibodies.
2. **Application**: Facilitates automated and high-throughput screening.

4. ELISPOT (Enzyme-Linked ImmunoSpot):

1. **Principle**: Similar to ELISA but designed to detect and quantify individual cells secreting a particular cytokine or antibody.
2. **Application**: Used in immunology research to measure immune responses at the single-cell level.

Surface Plasmon Resonance (SPR)

- The strength and mode of intermolecular interactions are important indicators for evaluating candidate drugs in drug development.
- There are many methods for measuring the interaction strength between drug molecules and targets, including
- NMR (nuclear magnetic resonance),
- MST (microthermal mobility),
- ITC (isothermal titration calorimetry),
- fluorescence polarization and
- SPR (Surface plasmon resonance), which is essentially a physical optical phenomenon.

- **Surface plasmon resonance (SPR)** is a phenomenon where the oscillation of electrons on a metal surface is excited by incident light. This leads to the creation of a resonant wave known as a **surface plasmon**.
- SPR occurs at the interface between a metal and a dielectric material and is highly sensitive to changes in the refractive index of the dielectric layer, allowing it to be used as a sensing technique.
- In an SPR setup, light is directed at a metal film, such as gold or silver, that is covered with a thin dielectric layer. The incident light excites the electrons on the metal surface into a resonant wave.

- The angle at which this wave is created is related to the refractive index of the dielectric layer and changes in this refractive index result in changes in the angle at which the wave is created.
- This shift in angle can be detected by analyzing the reflected light from the metal surface.
- SPR is widely used in biosensing applications due to its high sensitivity, real-time measurement capability, and non-invasive nature. For example, it can be used to study the interaction between a molecule and a solid surface, monitor protein-protein interactions, and detect small changes in the concentration of biomolecules in solution.

SPR Instrumentation

1.Light Source:

1. Typically, a laser is used to provide a coherent and monochromatic light source.

2.Prism or Grating Coupler:

1. A prism or grating is used to direct the light onto the metal surface at the appropriate angle to excite surface plasmons.

3.Metal Film:

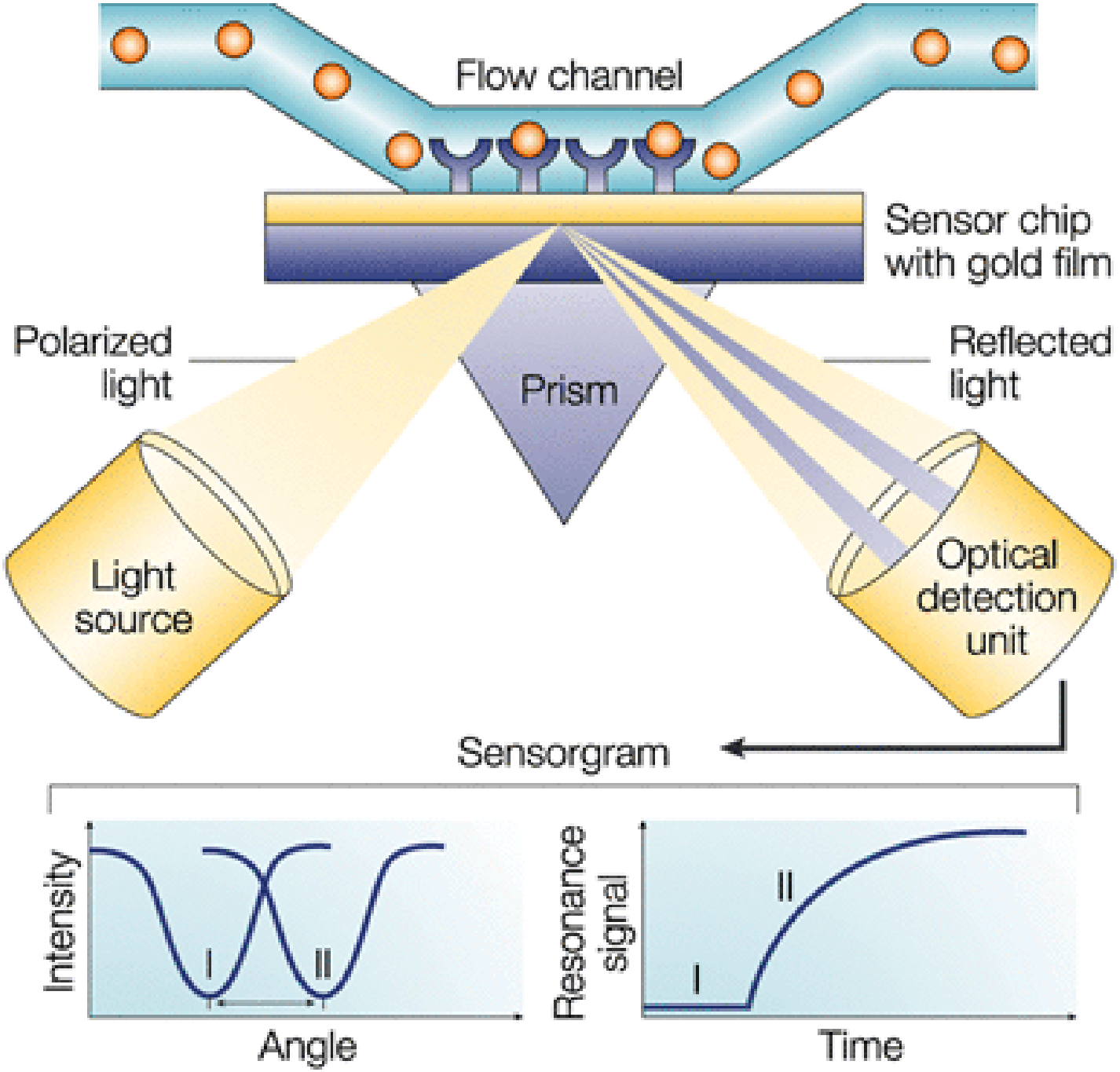
1. A thin metal film (usually gold) is deposited on a glass slide. This metal surface serves as the interface for the plasmon resonance.

4.Flow Cell:

1. A flow cell is placed on the metal film to allow the controlled introduction of analytes and reagents.

5.Detector:

1. A detector measures the intensity of reflected light as a function of angle or wavelength. The SPR signal is detected as a dip in the reflected light intensity, indicating resonance.


Flow channel
Sensor chip
with gold film
Polarized
light
Prism
Reflected
light
Light
source
Optical
detection
unit
Sensorgram
Intensity
I
II
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Resonance
signal
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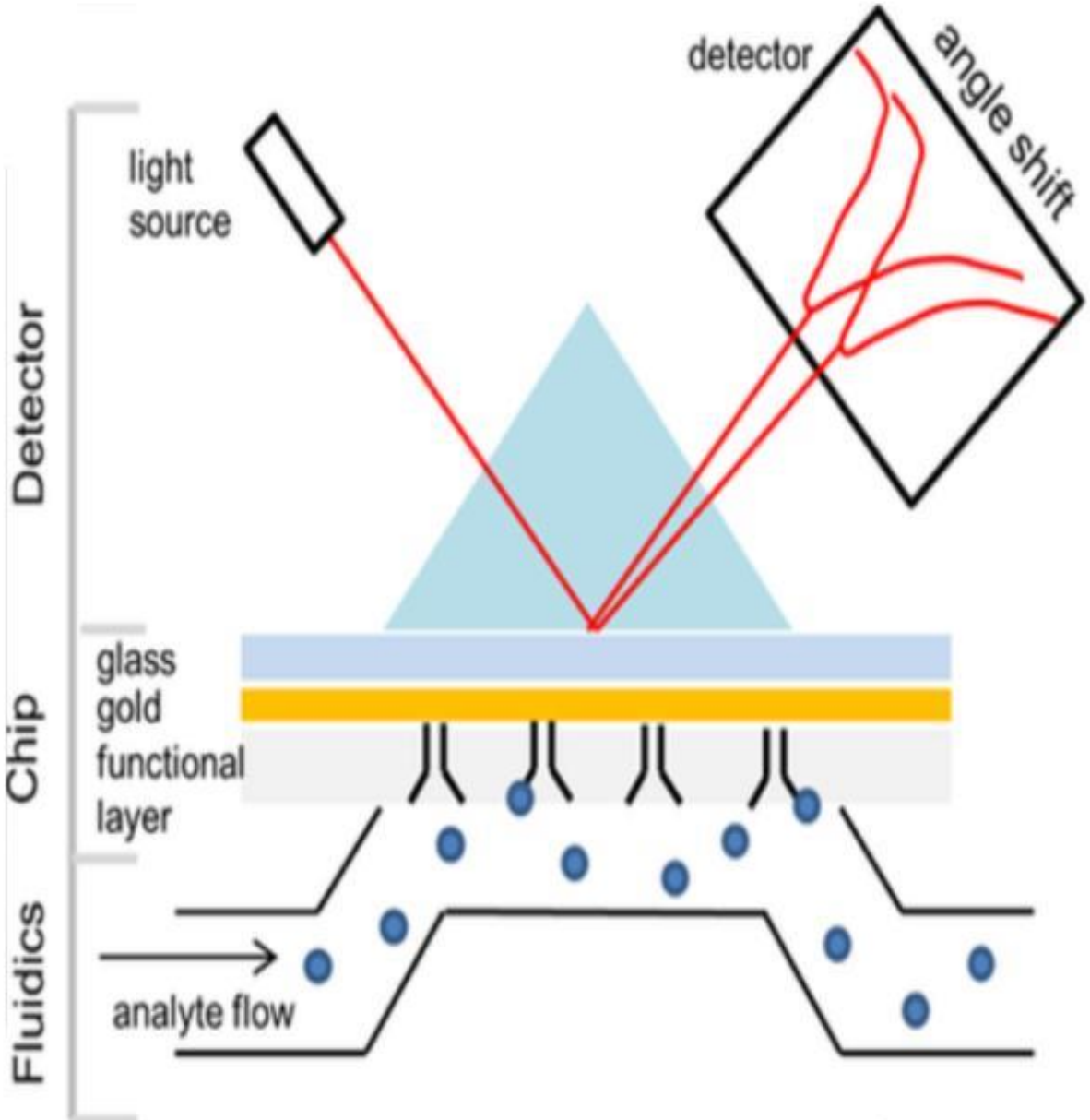
II
Time



Molecular interactions that spr is often used to analyze

- Protein-protein interactions
- Polymer-polymer interactions
- DNA-DNA interactions
- Protein-small molecule interactions
- Protein-carbohydrate interactions
- RNA-DNA interactions
- Protein-DNA interactions
- Antibody (protein)-bacterial interaction
- Antibody-Cell interaction
- Peptide-protein interactions

Applications

1.Biomolecular Interactions:

1. **Protein-Protein Interactions**: Studying binding kinetics and affinity of protein interactions.
2. **Protein-DNA Interactions**: Investigating how proteins interact with DNA sequences.
3. **Antibody-Antigen Interactions**: Analyzing the binding specificity and affinity of antibodies.

2.Drug Discovery:

1. **Ligand-Receptor Interactions**: Screening potential drug candidates by measuring their binding to target receptors.
2. **Kinetic Analysis**: Determining the association and dissociation rates of drug-receptor interactions.

3.Medical Diagnostics:

1. **Biomarker Detection**: Detecting biomarkers in biological samples for disease diagnosis.
2. **Pathogen Detection**: Identifying pathogens such as bacteria or viruses based on specific binding interactions.

4. Food Safety and Environmental Monitoring:

1. **Contaminant Detection**: Monitoring for contaminants such as pesticides, toxins, and pollutants in food and environmental samples.

5. Material Science:

1. **Surface Characterization**: Studying the properties of thin films, coatings, and surface modifications.
2. **Nanotechnology**: Investigating the interactions of nanoparticles with various substances.

6. Quality Control:

1. **Biopharmaceuticals**: Ensuring the quality and consistency of biopharmaceutical products by analyzing their molecular interactions.

Clinical Applications

1.Disease Diagnosis:

1. **Biomarker Detection**: SPR can detect biomarkers associated with various diseases, including cancer, cardiovascular diseases, infectious diseases, and autoimmune disorders. By identifying specific biomarker profiles, SPR aids in early diagnosis and monitoring disease progression.
2. **Pathogen Detection**: SPR can identify bacterial, viral, and fungal pathogens in clinical samples. This is crucial for diagnosing infectious diseases and determining the appropriate treatment.

2. Drug Development and Pharmacokinetics:

1. **Drug Screening**: SPR is used in the early stages of drug development to screen potential drug candidates by measuring their binding affinity to target proteins or receptors.
2. **Binding Kinetics**: It provides detailed information on the interaction kinetics between drugs and their targets, including association and dissociation rates. This data helps in understanding drug efficacy and optimizing drug candidates.
3. **ADME Studies**: SPR can be used to study the Adsorption, Distribution, Metabolism, and Excretion (ADME) of drugs, which are crucial for drug safety and efficacy.

3. Therapeutic Monitoring:

1. **Therapeutic Drug Monitoring (TDM)**: SPR can measure the concentration of therapeutic drugs in patient samples, helping to ensure that drug levels remain within the therapeutic range and avoiding toxicity or subtherapeutic dosing.
2. **Antibody Monitoring**: SPR can be used to monitor the levels of therapeutic antibodies in patients undergoing treatments such as monoclonal antibody therapy.

4. Allergy Testing:

1. **Allergen Identification**: SPR can identify specific allergens responsible for allergic reactions by detecting interactions between patient antibodies and potential allergens.
2. **Sensitivity Testing**: It helps in determining the sensitivity of patients to various allergens, guiding personalized treatment plans.

5. Autoimmune Disease Monitoring:

1. **Autoantibody Detection**: SPR can detect autoantibodies in patient samples, which are indicative of autoimmune diseases such as rheumatoid arthritis, lupus, and multiple sclerosis. Early detection and monitoring of autoantibody levels can help manage these conditions effectively.

6. Cancer Research and Monitoring:

1. **Cancer Biomarkers**: SPR is used to detect and quantify cancer biomarkers in blood or tissue samples, aiding in early cancer detection, prognosis, and monitoring response to therapy.
2. **Circulating Tumor Cells (CTCs)**: SPR can identify and analyze CTCs, which provide valuable information about the spread of cancer and the effectiveness of treatments.

7. Cardiovascular Disease:

1. **Biomarker Detection**: SPR can detect biomarkers associated with cardiovascular diseases, such as troponin, which is indicative of myocardial infarction. Early detection of these biomarkers can facilitate prompt intervention and treatment.

8. Personalized Medicine:

1. **Patient-Specific Treatments**: SPR can help in developing personalized treatment plans by analyzing patient-specific biomolecular interactions. This approach can optimize treatment efficacy and minimize adverse effects.

Advantages of SPR in Clinical Settings:

- **Label-Free Detection**: SPR eliminates the need for labels (like fluorescent label), reducing potential interference and preserving the native state of biomolecules.
- **Real-Time Monitoring:** Provides immediate feedback on molecular interactions, allowing for rapid decision-making.
- **Sensitivity and Specificity:** High sensitivity and specificity make SPR suitable for detecting low-abundance biomarkers and distinguishing between closely related biomolecules.
- **Quantitative Analysis:** Offers quantitative data on binding kinetics and affinities, which are crucial for understanding disease mechanisms and drug interactions.
- **Versatility:** Applicable to a wide range of biomolecules, including proteins, nucleic acids, lipids, and small molecules.

Western blotting

- Western blot was introduced by Towbin et al. in 1979, which is a commonly used method for protein analysis.
- It can be used for qualitative and semi-quantitative protein analysis.
- For the accomplishment of the western blot, there are three elements,
- Separation of proteins by size,
- Transferring proteins to a solid support, and
- Marking proteins by primary and secondary antibodies for visualization.

Principle of Western Blot

- SDS-PAGE allows protein samples to be separated and transferred to a solid support, such as nitrocellulose (NC) or polyvinylidene difluoride (PVDF) membrane.
- The solid support can absorb the protein and keep its biological activity unchanged.
- The transferred solid support membrane is called a blot and is treated with a protein solution to block the hydrophobic binding site on the membrane.

- The membrane is treated with the antibody (primary antibody) of the target proteins.
- Only the proteins to be studied can specifically bind to the primary antibody to form an antigen-antibody complex.
- After the primary antibody is washed and removed, only the position of the target protein binds to the primary antibody.
- The primary antibody-treated membranes are treated with a labeled secondary antibody after washing. After treatment, the labeled secondary antibody that binds to the primary antibody forms an antibody complex that can indicate the location of the primary antibody, both the location of the protein being studied.

There are six steps involved in western blot, including

- Sample preparation,
- Gel electrophoresis,
- Proteins transfer,
- Blocking,
- Antibody incubation, and
- Proteins detection and Visualization

Protocol

1.Sample Preparation:

1. **Protein Extraction**: Proteins are extracted from cells or tissues using lysis buffers.
2. **Protein Quantification**: The concentration of proteins is measured to ensure equal loading of samples.

2.SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis):

1. **Denaturation**: Proteins are denatured using SDS, a detergent that imparts a negative charge to the proteins, ensuring they migrate based on size.
2. **Gel Electrophoresis**: Proteins are separated by size on a polyacrylamide gel under an electric field. Smaller proteins migrate faster and further than larger ones.

3.Transfer:

1. **Blotting**: The separated proteins are transferred from the gel to a membrane (nitrocellulose or PVDF) using an electric field. This step makes the proteins accessible for antibody binding.

4. **Blocking**:

1. **Non-Specific Binding**: The membrane is blocked with a solution containing non-specific proteins (e.g., BSA or milk) to prevent non-specific binding of antibodies.

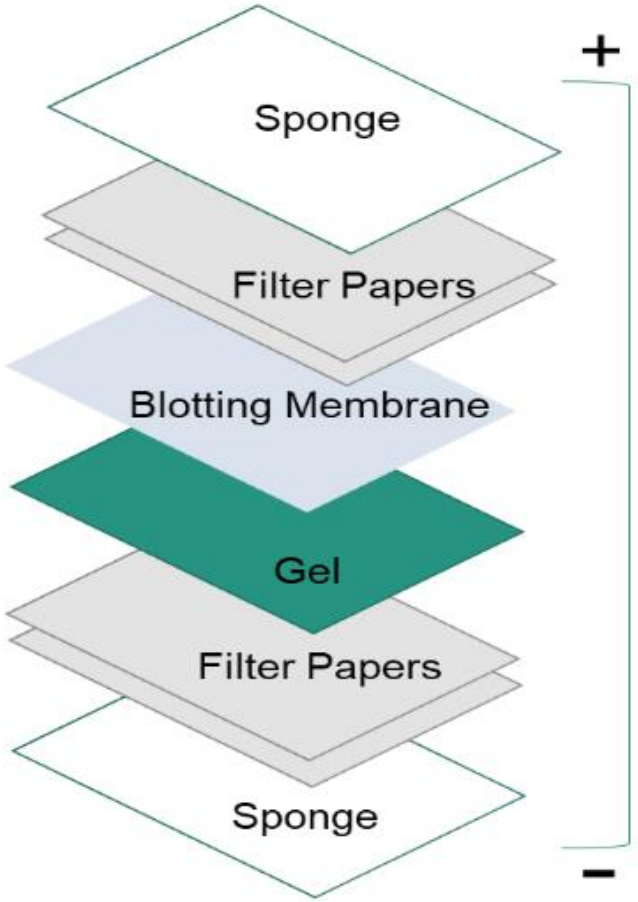
5. **Antibody Incubation**:

1. **Primary Antibody**: The membrane is incubated with a primary antibody that specifically binds to the target protein.
2. **Secondary Antibody**: After washing, the membrane is incubated with a secondary antibody that binds to the primary antibody. The secondary antibody is conjugated to an enzyme (e.g., HRP) or a fluorophore for detection.

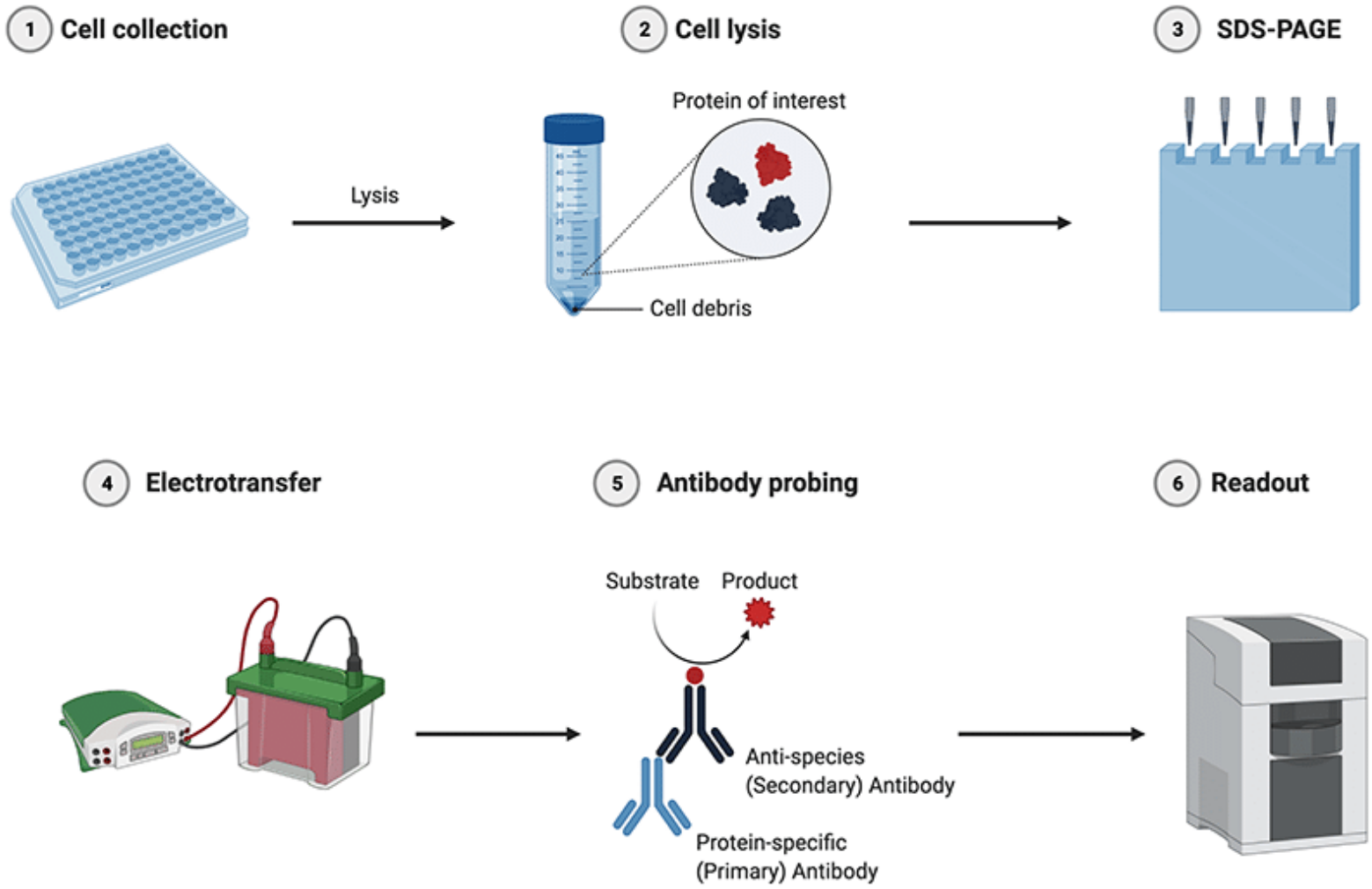
6. **Detection**:

1. **Substrate Addition**: A substrate for the enzyme linked to the secondary antibody is added. The enzyme catalyzes a reaction that produces a detectable signal (chemiluminescent, colorimetric, or fluorescent).
2. **Signal Detection**: The signal is detected using X-ray film, a digital imaging system, or a fluorescence scanner.

Western Blot Sandwich



Western Blot Protocol



Flow chart of Western blotting

Electrophoresing the protein sample

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Assembling the Western blot sandwich

↓

Transferring proteins from gel to nitrocellulose paper

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Staining of transferred proteins

↓

Blocking nonspecific antibody sites on the nitrocellulose paper

↓

Probing electroblotted proteins with primary antibody

↓

Washing away nonspecifically bound primary antibody

↓

Detecting bound antibody by horseradish peroxidase-anti-Ig conjugate and formation of a diaminobenzidine (DAB) precipitate

↓

Photographing the immunoblot

Applications

1.Protein Identification:

1. **Specific Protein Detection**: Identifies specific proteins in a complex mixture based on their size and immunoreactivity.
2. **Protein Isoforms**: Detects different isoforms of a protein that may have similar but distinct biological functions.

2.Quantification:

1. **Relative Quantification**: Compares the expression levels of a target protein between different samples or conditions.
2. **Absolute Quantification**: With the use of standards, absolute protein concentrations can be determined.

3.Post-Translational Modifications:

1. **Phosphorylation, Glycosylation, Ubiquitination**: Detects post-translational modifications that regulate protein activity and function.

4. Diagnostics:

1. **Disease Markers**: Identifies and quantifies biomarkers associated with diseases (e.g., viral proteins in HIV).
2. **Genetic Disorders**: Diagnoses genetic disorders by detecting abnormal protein expression or size (e.g., dystrophin in Duchenne muscular dystrophy).

5. Research:

1. **Signal Transduction Pathways**: Studies signaling pathways by detecting proteins and their modifications.
2. **Gene Expression Studies**: Investigates the effect of gene knockdown or overexpression on protein levels.
3. **Protein-Protein Interactions**: Confirms interactions by co-immunoprecipitation followed by western blotting.

6. Quality Control:

1. **Biotechnology and Pharmaceuticals**: Ensures the quality and consistency of recombinant proteins, antibodies, and vaccines.

Advantages:

- **Specificity**: High specificity due to the use of antibodies.
- **Versatility**: Can detect a wide range of proteins and modifications.
- **Quantitative**: Allows for both relative and absolute quantification.
- **Sensitivity**: Highly sensitive, capable of detecting low-abundance proteins.

Limitations:

- **Time-Consuming**: Multiple steps make it a lengthy process.
- **Requires High-Quality Antibodies**: The specificity and sensitivity depend on the quality of antibodies used.
- **Semi-Quantitative**: While it can provide quantitative data, it is often considered semi-quantitative compared to mass spectrometry.
- **Potential for Artifacts**: Non-specific binding and technical variations can introduce artifacts.

ACKNOWLEDGEMENT

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