REPORT:

FLOW CYTOMETRY WORKSHOP

ORGANIZED BY

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of Arts, Science & Commerce (Autonomous)

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This program has given me a wealth of information, insights, and skills that will definitely help me in my academic and professional endeavors. I would like to express our sincere appreciation to everyone who helped make this session a success once more. Your commitment has sparked an enthusiasm for study and research that will keep us striving for greatness in the bioinformatics discipline.

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CHAPTER 1 – BASICS OF FLOW CYTOMETRY

Flow Cytometry

Flow cytometry is an advanced analytical technique widely used in various fields, including immunology, molecular biology, and clinical diagnostics. It allows for the rapid multi-parametric analysis of single cells as they flow through a laser beam in a fluid stream.

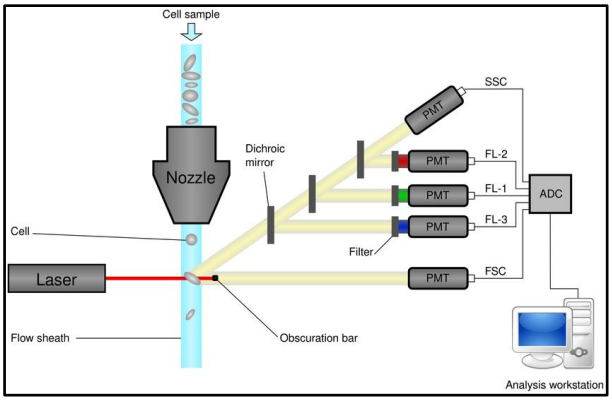

Figure 1: Flow Cytometer

Microscope vs. Flow Cytometry

Researchers examine cell populations using flow cytometry and microscopy, which gives them unique insights into the architecture and functions of the cells. The identification of individual cellular components is made possible by the thorough observation of cells and their architecture made possible by light microscopy in particular. Advanced kinds of microscopy, including fluorescence microscopy, employ fluorescent dyes to highlight components within cells, yielding higher-resolution insights.

Flow cytometry, on the other hand, objectively evaluates the physical and chemical features of cells as they flow via a laser beam, providing extensive information on cell size, granularity, and the presence of particular surface markers. This technology is particularly beneficial for immunological investigations, allowing researchers to study immune cell populations and their roles under varied situations, such as infections or malignancies.

Components of Flow Cytometer

1. **Laser System:** An essential part of Fluorescence Detection and Counting (FACS) is the laser system, which supplies the light source required to excite fluorescent dyes affixed to certain cell surface markers. Since different lasers produce light at different wavelengths, it is possible to detect numerous fluorescent markers at once. Typical laser wavelengths for FACS are 640 nm (red), 405 nm (violet), and 488 nm (blue). The selection of lasers improves the capacity of researchers to discriminate between different cell kinds and states by allowing them to assess a broad range of factors using various fluorescently tagged antibodies.

2. **Fluidics System:** The sample must be moved through the cytometer via the fluidics system. To concentrate the sample into a narrow stream, a sheath fluid—typically a buffered saline solution—is usually used. This makes sure that each cell or particle passes the laser beam separately, enabling precise measurement. A constant flow rate and pressure are maintained by the fluidics system's cooperating parts, which comprise pumps, tubing, and flow cells, guaranteeing peak performance throughout analysis. In order to guarantee that the single cell makes it past the laser by hydrodynamic focusing of the sample, the fluidics system is necessary.

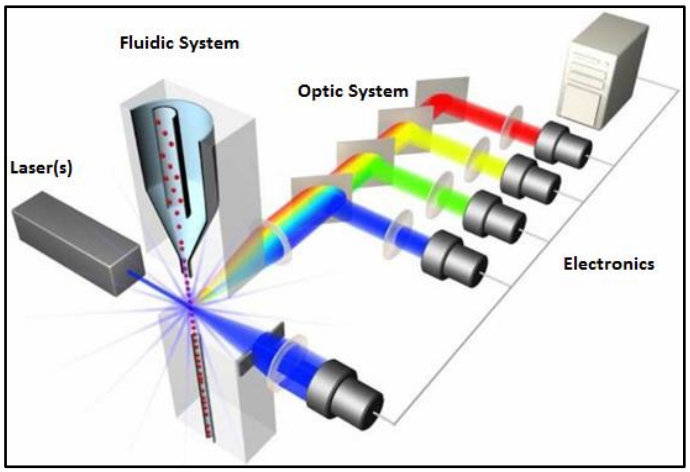

Figure 2: Components of a flow cytometer

3. **Optics System:** The optics component includes lasers, lenses, filters, and detectors. Lasers provide the excitation light needed to illuminate the fluorescently labeled cells. The emitted light is collected and filtered to isolate specific wavelengths corresponding to different fluorescent markers, allowing for detailed analysis of cell characteristics.

Type	Property	Description
Excitation Optics	Lasers	Emit light at specific wavelengths to excite fluorescent dyes on cells or particles.
	Positioning and Focusing	Lasers are precisely positioned and focused to illuminate the sample as it passes through the cytometer.
	Laser Coherence and Intensity	Essential for reliable fluorescence signals.
Collection Optics	Light Scattering and Fluorescence	Cells scatter light and emit fluorescence after interacting with the laser light.
	Collection of Emitted Light	Mirrors and lenses are used to collect this emitted light.
	Optical Filters	Separate different light wavelengths and direct specific signals to detectors.
	Detectors	Photomultiplier tubes (PMTs) or photodiodes are commonly used to detect the collected light signals.

4. Detectors

Detector Type	Description	Advantages	Disadvantages
Photomultiplier Tube (PMT)	Traditional detector with high sensitivity and low fluorescence detection capability. Converts light signals into electrical signals.	High sensitivity, low fluorescence detection	Bulky, high voltage requirements
Avalanche Photodiode (APD)	Solid-state detector with high sensitivity and linearity, especially for red fluorescence detection.	High sensitivity, linearity, solid-state	Higher noise than PMTs
Silicon Photodiode (SiPD)	Cost-effective solid-state detector for fluorescent signal detection.	Cost-effective, good performance	Lower sensitivity compared to PMTs and APDs
Imaging Detector (CCD/CMOS)	Captures images of cells for multi-parameter analysis at single-cell and population levels.	Provides detailed images, multi-parameter analysis	Higher data volume, slower acquisition
Spectral Detector	Measures the entire emission spectrum from each particle, enabling analysis of multiple fluorescent markers without spectral overlap.	Eliminates spectral overlap, enhances multi-parameter experiments	More complex data analysis

5. Visualization of Flow Cytometry Data

Key steps involved in data visualization and analysis for flow cytometry –

a. **Gating –** Use forward scatter (FSC) and side scatter (SSC) to identify cell populations based on size and granularity. Apply gates to exclude debris, dead cells, and doublets.

b. **Histograms –** Analyze single parameters to identify positive and negative populations for a specific marker. Overlay negative controls to accurately determine positive populations.

c. **Dot Plots –** Visualize two parameters simultaneously in a density plot. Identify distinct cell populations based on marker expression. Quantify relative proportions of different cell types.

d. **Compensation –** Account for spectral overlap between fluorochromes. Measure single-stained controls to generate a compensation matrix. Apply compensation to the data to correct for spillover.

e. **Automated Gating –** Use mathematical modeling to analyze high-dimensional data. Employ clustering algorithms like PCA, SPADE, and tSNE. Extract meaningful insights from complex datasets.

f. **Data Pre-processing –** Remove technical artifacts and low-quality data. Exclude doublets by plotting FSC-H vs FSC-A. Ensure proper compensation and gating before downstream analysis.

Fluorescence – Activated Cell Sorting (FACS)

A method used in flow cytometry called Fluorescence-Activated Cell Sorting (FACS) allows cells to be recognized and arranged according to their fluorescence properties. To identify different cell populations within a heterogeneous mixture, it binds to particular cell surface markers with antibodies that have been fluorescently tagged. Size, granularity, and protein expression are among the factors that are analyzed using the fluorescence and light scattering data that have been gathered.

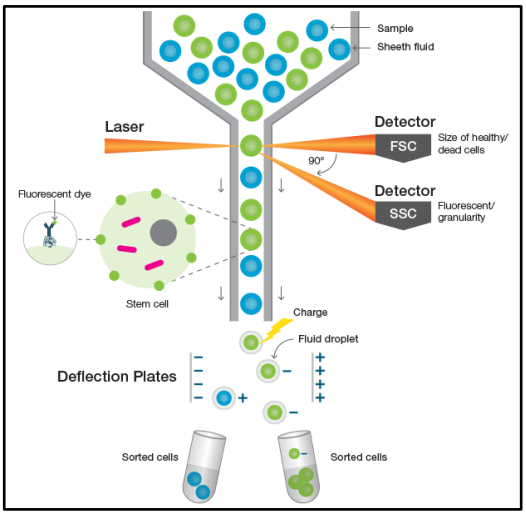

Figure 3: Workflow of FACS

Feature	Flow Cytometry	FACS
Primary Function	Analyzing cells without sorting	Analyzing and sorting cells
Cell Analysis	Measures cell size, granularity, and fluorescence	Measures cell size, granularity, and fluorescence
Sorting	Does not sort cells	Sorts cells based on fluorescence characteristics
Sample Fate	Typically discards samples after analysis	Collects sorted cells for further study
Applications	Immunology, cancer research, stem cell biology	Immunology, cancer research, stem cell biology
Key Difference	Focuses solely on analysis	Combines analysis with sorting

CHAPTER 2 – IMMUNE SYSTEM AND THE USE OF FLOW CYTOMETRY TECHNIQUES IN IMMUNOLOGY

Immune System

The immune system is a sophisticated network of cells and proteins that defends the body against pathogens, including viruses. It consists of two main components:

1. **Innate Immunity:** The first line of defense, providing immediate but non-specific protection against infections.
2. **Adaptive Immunity:** A more specialized response that develops over time, involving T cells, B cells, and memory cells that provide long-lasting immunity.

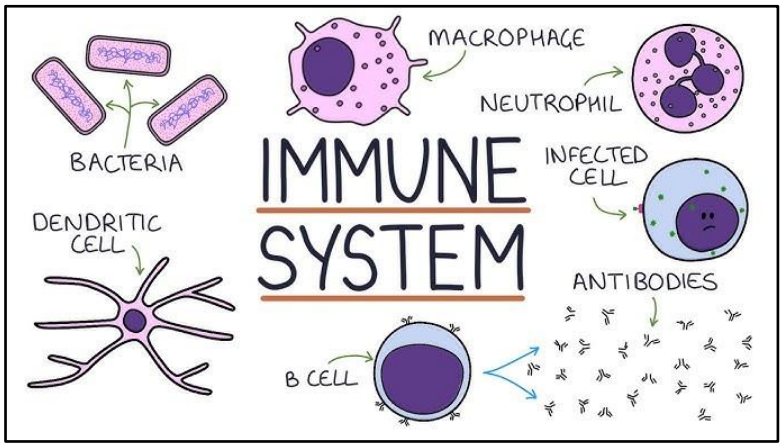

Figure 4: Immune system

T Cells

T cells play a pivotal role in the adaptive immune response and can be categorized into:

1. **CD4+ Helper T Cells:** These cells assist other immune cells by releasing cytokines and are crucial for orchestrating the immune response.
2. **CD8+ Cytotoxic T Cells:** They directly kill virus-infected cells by recognizing specific antigens presented by major histocompatibility complex (MHC) molecules.

Additionally, **memory T cells** are formed after an infection or vaccination, allowing for a faster and more robust response upon re-exposure to the same pathogen.

B Cells

B cells are responsible for producing antibodies that neutralize viruses. Their development occurs in two stages:

1. **Antigen-Independent Development:** Occurs in the bone marrow where B cells mature and express unique receptors.
2. **Antigen-Dependent Development:** When B cells encounter specific antigens, they proliferate and differentiate into plasma cells that produce antibodies or memory B cells that provide long-term immunity.

Natural Killer (NK) Cells

NK cells are part of the innate immune system and play a critical role in recognizing and destroying virus-infected cells without prior sensitization. They are characterized by their ability to identify stressed or infected cells through various receptors.

Lymphocyte Subset Analysis

The evaluation of lymphocyte subsets (T cells, B cells, NK cells) is essential for understanding immune responses during viral infections. Flow cytometry is a powerful tool used to enumerate these subsets based on specific surface markers:

1. **T Cells:** Identified using CD3 (pan-T cell marker), CD4 (helper), and CD8 (cytotoxic).
2. **B Cells:** Identified using CD19.
3. **NK Cells:** Identified using CD16 and CD56.

This analysis helps in diagnosing immunodeficiencies, monitoring infections, and evaluating responses to therapies.

Flow Cytometry Techniques

Flow cytometry is instrumental in viral immunopathogenesis research due to its ability to analyze individual cells rapidly. Several techniques enhance its utility:

1. **Use of Primary and Secondary Antibodies**
   In flow cytometry, primary antibodies bind specifically to target antigens on cell surfaces or within cells. Secondary antibodies, which are typically conjugated with fluorescent dyes, bind to primary antibodies to amplify the signal for detection. This dual-antibody approach enhances specificity and sensitivity in detecting viral proteins or immune cell markers.

2. **Multiplexed Assays**
   Multiplex assays allow simultaneous detection of multiple targets within a single sample using different fluorescently labeled antibodies. This capability is crucial for comprehensive profiling of immune responses during viral infections.

3. **Fluorescence Minus One (FMO) Controls**
   FMO controls are used to determine the background fluorescence level when analyzing flow cytometry data. By excluding one fluorochrome at a time from the panel, researchers can accurately set gates for positive populations without misclassifying negative events.

4. **Antibody Titration**
   Determining optimal antibody concentrations is essential for minimizing background noise while maximizing signal detection. Titration helps establish the best conditions for staining samples before flow cytometric analysis.

CHAPTER 3 – KEY STEPS IN SAMPLE PREPARATION

Sample Handling and Preparation

1. **Source of Sample and Transport**
   Samples for flow cytometry can be obtained from various sources, including blood or tissue biopsies. Proper transport conditions (e.g., temperature control) are critical to preserving cell viability and function until analysis.

2. **Cell Isolation**
   Isolating specific cell populations from mixed samples can be achieved using density gradient centrifugation or magnetic bead separation techniques. This step ensures that only the desired lymphocyte subsets are analyzed.

3. **Sample Preparation Techniques**
   Flow cytometry sample preparation can follow different protocols:
   a. **Single Platform:** Involves direct staining of whole blood or isolated cells before analysis.
   b. **Double Platform:** Involves initial isolation followed by staining.

Sample Preparation Protocols	Steps	Notes
Lyse-No Wash (LNW)	1. Add lysis buffer to whole blood. 2. Incubate. 3. Stain. 4. Analyze.	Simplifies sample prep, ideal for lymphocyte analysis in whole blood.
Tube Lysing or Stain-Lyse-Wash (SLW)	1. Stain cells. 2. Lyse. 3. Wash. 4. Analyze.	Suitable for isolated cells or whole blood, allows for removal of unbound antibodies.
Bulk Lysing or Lyse-Stain-Wash (LSW)	1. Lyse. 2. Wash. 3. Stain. 4. Wash. 5. Analyze.	Effective for larger sample volumes, ensures bulk lysis before staining.

Steps in Sample Preparation

STEP 1: Erythrocyte Lysis

- **Purpose:** To remove red blood cells (RBCs) from whole blood, enriching the lymphocyte population for analysis.

- **Procedure:**
  1. Prepare an erythrocyte lysis buffer (e.g., ammonium chloride buffer). A common formulation (10X stock) includes:

Ammonium chloride	82.6 g
EDTA	0.37 g
Potassium phosphate	10 g
Distilled water	1L

  2. Mix 2 mL of whole blood with 18 mL of 1X erythrocyte lysis buffer.
  3. Invert the tube gently 10 times to mix.
  4. Incubate at room temperature (20-25°C) for 2 minutes.
  5. Centrifuge at 400 x g for 5 minutes to pellet the white blood cells (WBCs).
  6. Carefully remove the supernatant to discard lysed RBCs, leaving a pellet of WBCs.

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STEP 2: Staining Tests

- **Purpose:** To label specific cell populations with fluorescently labeled antibodies.
- **Procedure:**
  1. Resuspend the WBC pellet in an appropriate buffer (e.g., PBS) at a concentration of approximately 1-10 million cells/mL.
  2. Add specific fluorescently labelled antibodies targeting cell surface markers (e.g., CD3, CD4, CD8, CD19).
  3. Incubate in the dark at 4°C for 30 minutes to 1 hour to allow binding.
  4. Ensure gentle mixing during incubation to maintain cell viability.

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STEP 3: Fixation and Permeabilization

- **Purpose:** To preserve cellular structures and allow access to intracellular targets.
- **Procedure:**
  1. After staining, wash cells with PBS to remove unbound antibodies by centrifuging at 400 x g for 5 minutes and discarding the supernatant.
  2. Fix cells using paraformaldehyde (typically at a final concentration of 1-4%) or another fixative for 10-20 minutes at room temperature.
  3. If analyzing intracellular proteins, permeabilize cells using a permeabilization buffer (e.g., saponin or Triton X-100) as per protocol requirements.

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STEP 4: Washing

- **Purpose:** To remove excess unbound antibodies and reduce background fluorescence.
- **Procedure:**
  1. Wash cells with PBS or flow cytometry buffer (e.g., FACS buffer containing BSA) by centrifuging at 400 x g for 5 minutes.
  2. Repeat this step 2-3 times to ensure thorough washing.

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STEP 5: Storage

- **Purpose:** To preserve samples until analysis can be performed.
- **Procedure:**
  1. Resuspend cells in TransFix solution or paraformaldehyde in PBS if immediate analysis is not possible.
  2. Store samples at 4°C for short-term storage (up to several days) or freeze at -80°C for long-term storage, depending on the specific requirements of the experiment.

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STEP 6: Acquisition

- **Purpose:** To run samples through a flow cytometer for data acquisition.
- **Procedure:**
  1. Ensure that the flow cytometer is calibrated and properly set up with appropriate laser settings and compensation controls before running samples.
  2. Acquire data from each sample, ensuring that enough events are collected for statistical validity (typically at least 10,000 events).

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STEP 7: Gating and Dot Plot Analysis

- **Purpose:** To analyze data and identify specific cell populations based on fluorescence intensity and scatter properties.
- **Procedure:**
  1. Use software tools associated with the flow cytometer to create dot plots based on forward scatter (FSC) vs. side scatter (SSC) to distinguish live cells from debris and dead cells.
  2. Apply gating strategies to isolate populations of interest based on fluorescent markers (e.g., T cells vs. B cells).
  3. Analyze results for specific markers, comparing populations across different conditions or treatments.

Troubleshooting and Compensation

During flow cytometry experiments, issues such as spectral overlap between fluorochromes may arise, necessitating compensation adjustments to ensure accurate data interpretation. Troubleshooting involves –

1. Checking instrument settings.
2. Verifying antibody specificity.
3. Ensuring proper sample preparation protocols were followed.
4. Ensuring that all reagents are prepared fresh and kept on ice when necessary to maintain cellular integrity.
5. Using sterile techniques throughout to prevent contamination.
6. Adjusting centrifugation speeds based on specific protocols or cell types being analyzed.

CHAPTER 4 – APPLICATIONS OF FLOW CYTOMTERY

Flow cytometry is a powerful analytical technique used in various fields, including immunology, clinical diagnostics, and microbiology. It allows for the rapid analysis and sorting of cells based on their physical and chemical characteristics. Below are detailed explanations of specific applications of flow cytometry.

Applications of Flow Cytometry

1. Cell Sorting

Cell sorting involves the physical separation of specific cell populations from a heterogeneous mixture. Specialized flow cytometers, known as fluorescence-activated cell sorters (FACS), can isolate cells based on predefined criteria such as size, granularity, and fluorescence intensity. This capability enables researchers to collect pure populations of cells for further study or cultivation. Modern cell sorters can achieve sorting speeds of up to 30,000 events per second with purities exceeding 95%. This technology is crucial for applications like genetic analysis and therapeutic cell preparations.

2. Detection of Apoptosis

Flow cytometry is instrumental in distinguishing between apoptosis (programmed cell death) and necrosis (cell death due to injury). By analyzing morphological changes and specific biochemical markers, such as phosphatidylserine exposure on the cell surface, researchers can accurately identify apoptotic cells. This differentiation is essential in cancer research and drug development, where understanding cell death mechanisms can inform therapeutic strategies.

3. Immunophenotyping

Immunophenotyping is a widely used application of flow cytometry that enables the identification and quantification of different cell types based on surface markers. It is particularly valuable in diagnosing hematological malignancies like lymphoma and leukemia. By using fluorescently labeled antibodies that bind to specific antigens, flow cytometers can provide detailed profiles of immune cell populations, aiding in disease classification and treatment planning.

4. Analysis of Cell Cycle

Flow cytometry allows for the assessment of the various phases of the cell cycle by measuring DNA content within individual cells. This analysis helps researchers understand cell proliferation rates and identify aneuploidy, which is associated with chromosomal abnormalities in cancer cells. By using DNA-binding dyes, flow cytometers can quantify the proportion of cells in G0/G1, S, and G2/M phases, providing insights into cellular growth dynamics.

5. Assays for Cell Proliferation

Cell proliferation assays using flow cytometry evaluate how cells respond to stimuli by measuring metabolic activity or proliferation markers such as Ki-67 or CFSE dye dilution. This application is crucial for studying immune responses, drug effects, and cellular interactions in various biological contexts.

6. Measurement of Intracellular Calcium Flux

Flow cytometry can monitor intracellular calcium levels, which are vital for numerous cellular signalling pathways. By using calcium-sensitive fluorescent dyes, researchers can track changes in calcium concentrations in response to external stimuli, providing insights into cellular activation processes such as neurotransmitter release or muscle contraction.

7. Applications in Microbiology

In microbiology, flow cytometry is employed to assess microbial viability and susceptibility to antibiotics. It also aids in detecting bacterial and viral infections by analyzing the physical properties of microorganisms or by using specific fluorescent probes that target microbial components. This rapid analysis is invaluable for clinical diagnostics and environmental monitoring.

8. Genetic Analysis

Flow cytometry plays a significant role in genetic analysis applications such as prenatal testing, carrier detection, and karyotyping. By analyzing DNA content and chromosomal structures within cells, this technique provides critical information for genetic counseling and disease prevention strategies.

9. Pharmacology

In pharmacology, flow cytometry assists in studying drug interactions with cells by evaluating changes in cellular responses upon drug treatment. This application helps researchers understand drug efficacy and mechanisms of action at the single-cell level, facilitating drug development processes.

Role of Flow Cytometry in Viral Immunopathogenesis

Viral immunopathogenesis is a critical field of study that focuses on understanding how viruses interact with the host immune system and the resulting pathological effects. It encompasses the processes by which viruses enter the body, replicate, spread, and ultimately cause tissue damage and disease. Understanding these mechanisms is crucial for developing effective treatments and preventive measures against viral infections.

1. **Single-Particle Analysis –** Flow cytometry allows for the detection and characterization of individual viral particles through a technique known as flow virometry. This capability enables researchers to analyze viral populations and their properties rapidly, which is crucial for understanding viral behavior and interactions with host cells.

2. **Immunophenotyping –** It provides detailed information about immune cell populations and their functional states. By assessing various markers on immune cells, researchers can determine how these cells respond to viral infections and vaccines.

3. **High Throughput –** Flow cytometers can analyze thousands of cells per second, allowing for robust statistical analyses that enhance our understanding of viral infections across diverse populations. This rapid data generation is vital for monitoring outbreaks and evaluating public health interventions.

4. **Versatility –** The technology can be adapted to measure a wide range of biological components, including proteins, RNA, and DNA from both viruses and host cells. This versatility makes flow cytometry invaluable in studying complex interactions within the host-pathogen dynamic.

In summary, viral immunopathogenesis is pivotal in addressing emerging viral threats like COVID-19, Monkeypox, and Nipah virus. Flow cytometry serves as a cornerstone technology in this field, enabling precise analysis of viral particles and host immune responses, ultimately contributing to better diagnostics, therapeutics, and vaccine development strategies.

CHAPTER 5 – REFERENCES

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