## Review Article

# "Flow Cytometry: A Comprehensive Review"

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**Abstract:**
Flow cytometry has revolutionized biomedical research by enabling rapid analysis and sorting of cells based on their physical and chemical properties. This review provides an overview of flow cytometry, its principles, and various applications. It illuminates how lasers and optics generate forward and side scatter signals, enabling size and granularity assessment. Fluorescence is central, using fluorophores to label specific cell populations. Moreover, the review explores the history of flow cytometry, highlighting milestones and contributors. It delves into the advancements in instrumentation, automation, and data analysis, resulting in improved accuracy, efficiency, and quality control. Different types of flow cytometry, including analytical, sorting, digital, imaging, and mass cytometry, are detailed, emphasizing their applications in cell analysis, diagnostics, and therapeutic development. The significance of fluorescence-activated cell sorting (FACS) in various fields is highlighted. Furthermore, the potential of AI and machine learning in data analysis is discussed. This comprehensive review showcases flow cytometry's transformative impact on diverse biomedical applications, emphasizing its potential for further innovation and discovery.
**Keywords:**Biomedical research, Applications, Automation, Fluorescence-activated cell sorting,

## 1. Introduction

Flow cytometry is a powerful tool for analysing the phenotype and characteristics of cells [1]. It is based on the light-scattering properties of cells, including fluorescence emissions [1]. The instrument illuminates' cells or particles individually in front of a light source and then detects and correlates the signals from those cells [2]. Fluidics is employed to guide the cells

individually past the illuminating beam [3]. Characteristics of cells suitable for flow cytometry, methods to illuminate cells, and the types of signals emitted by the cells and the detection of those signals are detailed [3]. Moreover, the technique involves the disciplines of electronics, computational data analysis, optics, and fluidics [4]. Flow cytometry allows for rapid, objective and quantitative computing, allowing calculations, correlations, and statistical conclusions from those few numbers derived from each of many cells [4]. It is also used to quantify the frequency of CD3+CD4+ T helper or CD3+CD8+ cytotoxic T-lymphocytes [5]. The levels of CD3, CD4, and CD8 are recorded for every leukocyte that passes through the flow cell [5]. The stained sample of leukocytes passes through a laser beam one at a time and the emitted spectrum is detected by sensors [5]. Furthermore, flow cytometry can be employed to sort cells [2]. The process of flow cytometry involves illuminating cells, detecting the signals emitted by
the cells, converting light signals to digital data, and using computers to analyze the data [2].
The history of flow cytometry dates back to the 1950s, when it was first used to count blood cells. The first fluorescence-based flow cytometry device was developed in 1968 by Wolfgang Göhde from the University of Münster, Germany, and first commercialized in 1968 [6]. In 1978, the name cytophotometry was changed to "flow cytometry" at the Conference of the American Engineering Foundation [3]. Since then, flow cytometry has become an essential tool in various fields, including immunology, hematology, microbiology, and cancer research.
Flow cytometry has a rich history (Table01)marked by significant milestones and contributions from visionary scientists.

| **Year** | **Milestone in Flow Cytometry** | **Scientist(s) / Contributor(s)** |
|---|---|---|
| 1934 | Invention of the Coulter Counter | Wallace H. Coulter |
| 1953 | Introduction of Electrozone Counting | Louis Kamentsky |
| 1965 | First Flow Cytometry Prototype | Wolfgang Göhde |
| 1968 | Dual-Beam Flow Cytometer Development | Mack Fulwyler |
| 1969 | Laser-Based Flow Cytometry Prototype | F. M. Siwek and F. A. H. M. van der Meer |
| 1970s | Introduction of Fluorescence Detection | Leonard Herzenberg |
| 1980s | Introduction of Immunophenotyping | Leonard Herzenberg |
| 1996 | Introduction of Flow Cytometry in DNA Cell Cycle Analysis | Wayne Cowan |
| 2000s | Introduction of High-Throughput and Multiparametric Analysis | Joe Trotter and Rui Gardner |
| 2010s | Advances in Single-Cell Analysis and Single-Cell RNA Sequencing | Aviv Regev and Rahul Satija |

**Table 01** provides a chronological overview of pivotal moments in the history of flow cytometry, highlighting the key scientists and their contributions to the evolution of this transformative analytical technique. From its beginnings as a particle counting method, flow cytometry has developed into a powerful tool for in-depth cellular analysis and high-dimensional data collection[8-16].

## 2. Basic Principle of Flowcytometry:

Flow cytometry is a technique that utilizes the principles of light scattering and fluorescence to analyze the physical and chemical characteristics of cells and particles [17]. When a sample is introduced into a flow cytometer, it is first subjected to a laser beam that produces both scattered and fluorescent light signals. Forward scatter, which is proportional to cell size, is used to determine the size of the cell or particle being analyzed [18]. Additionally, fluorescent molecules that are excited by laser light at specific wavelengths are used to identify and quantify specific cell types or biomolecules [19]. By utilizing these principles, flow cytometry can provide valuable insights into the characteristics of a sample.

The role of fluidics in flow cytometry is to deliver the sample containing suspended cells or particles to the flow cytometer [20]. The fluidics system consists of a flow cell where the sample fluid is injected, and sheath fluid that carries and aligns the cells or particles so that they pass through the laser beam individually [17] [19]. This ensures that each particle is analyzed individually, allowing for accurate and precise measurements of the sample.

The signals produced by the laser beam are detected and analyzed using electronic detectors. These detectors can measure both the scattered and fluorescent light signals produced by the sample [21]. The resulting data can be used to determine the size, shape, and other physical and chemical characteristics of the cells or particles in the sample. By utilizing the principles of fluid dynamics, optics, and electronics, flow cytometry has become a powerful tool for the analysis of cells and particles in a wide range of research and clinical applications [22].

## 3. Parts of flow cytometry

Flow cytometry, a transformative tool in cellular investigation, encompasses interconnected fluidics, optics, and electronics subsystems (as depicted in Table 02) that collectively enable the precise analysis of biological entities [17]. The fluidics subsystem ensures singular particle flow through hydrodynamic focusing, employing a sample injection port and sheath fluid propelled by air pressure [25][26]. Hydrodynamic focusing aligns cells into a singular stream, vital for accurate analysis [28]. The optics subsystem, employing lasers, lenses, and filters, detects emitted and scattered light from cells [30]. Detectors, including photomultiplier tubes, capture light signals and translate them into electrical signals [17]. These components collaboratively yield dependable cellular measurements [17]. Signal processing and data acquisition occur within the electronics subsystem, where emitted photons are transformed into electrons and then digitized, yielding data amenable for computer processing [15][34][35]. Key to flow cytometry are forward scatter, gauging size, and side scatter, assessing granularity, both synergizing with marker expression [18][25][40]. Fluorescence, fundamental in the technique, labels cell populations with fluorophores emitting detectable light [30]. Fluorescence-activated cell sorting (FACS), a technique within flow cytometry, offers versatile cell separation and profiling capabilities [33][47]. Applied in diverse fields, FACS contributes to research and clinical applications, including disease diagnosis and monitoring [41][52]. Overall, the integration of these components empowers flow cytometry to provide critical insights into cellular mechanisms and pathological conditions [17].

3.1 Fluidics System:a fundamental component of a flow cytometer, facilitates the movement of samples from the tube to the flow cell, through the laser, and into distinct populations. Comprising three main parts—sample injection, sheath fluid, and flow cell. The sample injection port (SIP) introduces the sample to the sheath fluid [25]. This fluid, present throughout the cytometer's process, envelops the sample stream, ensuring precise and consistent cell measurements [26] [27]. By using air pressure from a compressor, the sheath fluid is propelled through the flow chamber, simultaneously pushing the sample into the flow cell [26].Of notable importance is the sheath fluid's role in hydrodynamic focusing, a process that aligns cells into a singular stream, essential for precise cell analysis [28]. After achieving a smooth laminar flow, cells are injected into the fluid's core [27]. The flow cell marks the point of convergence between the sample stream and the laser beam, enabling the examination of individual cells [29].

3.2 Optical System:The optical system is responsible for producing both scattered and fluorescent light signals that are read by detectors [25]. It consists of three main components: light sources, filters, and detectors [30].

3.2.1Light source is typically a laser that passes through a focusing lens and is then directed through the flow cell [26]. On the other side of the flow chamber, a bar blocks the laser beam to prevent it from reaching the detectors when no cells are present.

3.2.2 Filtersare pieces of glass coated on both sides that allow light of a certain collection or band of wavelengths to pass through while absorbing or reflecting unwanted wavelengths [17]. The filters used in flow cytometry are typically bandpass filters, which allow light through within a specific range of wavelengths [31]. These filters are used to separate the signals generated by the fluorescent dyes from the scattered light signals produced by the cells [30].

3.2.3 Detectorsplay a crucial role in capturing light signals emitted by cells and converting them into electrical signals for computer analysis [17]. A flow cytometer usually incorporates multiple detectors, each specialized in sensing specific light wavelengths [17]. The predominant detectors in flow cytometry are photomultiplier tubes (PMTs), enhancing the electrical signals originating from light emissions [32]. Collectively, the optical components, including light sources, filters, and detectors, collaboratively yield precise and dependable measurements of cellular attributes.

## 3.3. Signal Processing and Data Acquisition

In flow cytometry, the electronics system is a pivotal element responsible for both signal processing and data acquisition. When a cell or particle intersects the laser beam, it scatters and emits photons that are subsequently captured by the detector, typically a photomultiplier tube (PMT) [33]. Within the electronics system, these photons undergo a transformation into electrons, followed by their digitization through an analog-to-digital converter (ADC) [15]. In this process, the ADC divides the PMT's analog current into discrete segments, referred to as "sampling" [34]. Through this digitization procedure, the analog signal is translated into distinct digital values, amenable for subsequent computer processing [35]. Additionally, the electronics system encompasses an electronic trigger mechanism that governs the timing of data acquisition [26].

3.5 Forward Scatter, designated as FSC, which provides essential insights into cellular physical characteristics. FSC measures the amount of light scattered forward by a cell, proportional to its diameter, enabling size-based cell differentiation[39][18]. Larger cells generate stronger FSC signals due to enhanced light scattering. FSC finds diverse applications in flow cytometry, discerning and quantifying various cell types in samples based on distinct FSC signals from cells of different sizes[25]. Moreover, FSC aids in estimating cell abundance and detecting changes in cell size or morphology that could indicate disease or physiological alterations[39]. Numerous factors influence FSC measurements, including cell refractive index, incident light angle, and the presence of particles or debris in the sample. Background noise or interferences can impact the forward scatter detector[17]. Despite potential variability sources, forward scatter remains valuable in flow cytometry, offering insights into the physical attributes of individual cells and their roles in intricate biological processes[17].

3.6 Side Scattera significant facet of flow cytometry, gauges cellular complexity or granularity [25]. It materializes when light is scattered at a 90-degree angle to the laser beam, influenced by cellular constituents like granules and nuclei [39]. The side scatter signal synergizes with forward scatter and marker expression to pinpoint cell populations sharing common traits [40]. Side scatter in flow cytometry finds diverse applications. Commonly, it is employed in immunophenotyping, where antibodies identify and quantify distinct cell types based on surface markers [19]. Additionally, it gauges cell viability, as compromised cells exhibit lower side scatter signals [41]. Moreover, side scatter aids the study of cellular processes like apoptosis and autophagy, as these processes alter cell granularity and complexity [30]. Various factors impact the side scatter signal in flow cytometry, including cell size, shape, presence of granules and nuclei, and the stability of the liquid path in the cytometer [18] [42] [43] [44]. Grasping these factors is vital for precise interpretation of side scatter data and effective gating strategies in flow cytometry analysis [25].

3.6 Fluorescenceis integral to flow cytometry, a technique for detecting and measuring physical and chemical attributes of cell or particle populations [33]. This phenomenon arises when a molecule, termed a fluorophore, absorbs and emits light at longer wavelengths [45]. Flow cytometry employs lasers as light sources to generate both scattered and fluorescent signals, detected by specific detectors [17]. Optical system components, including excitation light sources, lenses, and filters, manipulate light within the instrument [30]. Fluorochrome selection mandates understanding the excitation and emission properties of fluorescent compounds [46]. Fluorophores, absorbing specific wavelengths and emitting longer ones [30], label distinct cell populations or particles. Upon laser excitation, labelled cells or particles emit detectable fluorescent light [19], enabling quantitative analysis with unmatched sensitivity [33]. Fluorescence's applications in flow cytometry encompass specific cell quantification, intracellular signaling measurement, and protein/nucleic acid detection [25]. Additionally, flow cytometry finds use in cell sorting, immunophenotyping, and disease diagnosis [24][30]. In sum, fluorescence is pivotal in flow cytometry, facilitating the identification and quantification of cells and particles within samples.

3.7 FluorescenceActivated Cell Sorting (FACS), is a technique in flow cytometry that sorts and separates cells based on physical and chemical properties. It is commonly used in cell biology to analyze cells in heterogeneous liquid mixtures. FACS has several advantages such as separation of multifunctional cells beyond surface markers (e.g., size, metabolic state) (47), simultaneous analysis of different markers in mixed cell populations (48), and finds multiple applications including immunophenotyping, DNA cell cycle/tumor ploidy and membrane potential, ion flux, cell viability, and intracellular protein analysis [41]. FACS is of central importance in the fields of molecular biology, immunology, virology, infectious disease, and cancer biology [41], and its clinical applications include the diagnosis and monitoring of blood cancers, HIV, and other infectious diseases [52].

| Component | Description | Function/Importance |
|---|---|---|
| **Fluidics System** | Comprising three main parts: sample injection, sheath fluid, and flow cell | Facilitates controlled movement of samples, hydrodynamic focusing for accurate analysis |
| | **1. Sample Injection Port (SIP)** | Introduces sample to sheath fluid |
| | **2. Sheath Fluid** | Envelops sample stream, ensures consistent measurements and hydrodynamic focusing. |
| | **3. Flow Cell** | Marks convergence of sample stream and laser beam, enables individual cell examination |
| **Optical System** | Composed of light sources, filters, and detectors | Produces scattered and fluorescent light signals for detection |
| | **1. Light Source** | Typically, a laser that passes through focusing lens and flow cell |
| | **2. Filters** | Allow specific wavelengths of light to pass through, used to separate signals generated by fluorescent dyes and scattered light signals. |
| | **3. Detectors** | Capture emitted light signals, convert to electrical signals. |
| **Signal Processing and Data Acquisition** | Within electronics system, responsible for signal processing and data transformation | Converts emitted photons to electrons, digitizes via ADC |
| **Forward Scatter** | Measures light scattered forward by cells | Used for size-based cell differentiation, estimating cell abundance, and detecting size-related changes. |
| **Side Scatter** | Measures light scattered at 90-degree angle to laser beam | Gauges cellular complexity/granularity, identifies common traits |
| | Role | Immunophenotyping, cell viability assessment, studying cellular processes |
| **Fluorescence** | Arises from fluorophores absorbing and emitting light | Labels cell populations with detectable fluorescent light |
| | Role | Quantitative analysis, intracellular signaling measurement, protein/nucleic acid detection |
| **Fluorescence Activated Cell Sorting** | Technique in flow cytometry for cell sorting based on physical and chemical properties. | Immunophenotyping, DNA cell cycle/tumor ploidy, cell viability, protein analysis, clinical diagnosis |

Table 02: Overview of flowcytometry Parts.

## 4. Types of Flowcytometry:

Flow cytometry has evolved into various specialized techniques (as depicted in Table 03), each offering unique capabilities for analysing cell and particle populations. Analytical flow

cytometry employs lasers to excite fluorochromes, enabling multi-dimensional analysis of cellular components. This technique finds applications in cell counting, phenotyping, and DNA analysis, making it valuable for clinical diagnostics and research[17]. Sorting flow cytometry, on the other hand, focuses on isolating distinct cells from heterogeneous populations using specialized cell sorters[17][53]. Digital flow cytometry utilizes digital signal processing to rapidly quantify cells and supports DNA analysis and protein profiling[53][54]. Imaging flow cytometry merges traditional flow cytometry with microscopy, providing high-resolution images of individual cells alongside assessments of parameters like size and fluorescence[54][55]. Mass cytometry employs metal isotopes for cell labelling, allowing deep insights into protein expression and signaling pathways, with applications spanning immune function, cancer biology, and personalized medicine[17][53][55]. These diverse techniques collectively contribute to advancing cellular research and clinical diagnostics across various fields.

| Type | Description | Components | Applications |
|---|---|---|---|
| **Analytical Flow Cytometry** | Analyses physical and chemical characteristics of cells or particles in solution. | • Flow cell<br>• Measuring system<br>• Detector<br>• Amplificationsystem<br>• Computer for signal analysis | • Cell counting<br>• Phenotyping<br>• Cell cycle assessment<br>• Cell proliferation assays<br>• DNA analysis<br>• Clinical diagnostics<br>• Drug discovery |
| **Sorting Flow Cytometry** | Separates and isolates specific cells or particles from heterogeneous populations. | • Fluidics system<br>• Laser<br>• Collection system | • Immunophenotyping<br>• Cell cycle analysis<br>• Apoptosis - Cell proliferation assays<br>• Hematological malignancy diagnosis<br>• Cell-basedtherapy development |
| **Digital Flow Cytometry** | Utilizes digital signal processing to analyze and quantify cells or particles in a fluid sample. | • Fluidics system<br>• Optics system<br>• Electronics system | • Cell counting<br>• Cell sorting<br>• Cell characterization<br>• DNA analysis<br>• Protein characterization<br>• Immunology research<br>• Therapy development |
| **Imaging Flow Cytometry** | Combines flow cytometry with microscopy to provide high-resolution images of individual cells and analyze multiple parameters. | • Fluidics system<br>• Optical system<br>• Electronics system | • Cell morphology and function analysis<br>• Disease diagnosis<br>• Drug development<br>• Cancer and HIV detection<br>• Personalized medicine |
| **Mass Cytometry** | Uses metal isotopes instead of fluorescent | • Flow cell<br>• Measuring system | • Immune cell function study |

| | | | |
|---|---|---|---|
| | dyes to label cells, analysing protein expression on a single-cell level. | • Detector<br>• Amplification system<br>• Computer for data analysis | • Cancer biology<br>• Infectious disease research<br>• Development of targeted therapies<br>• RNA sequencing synergy |

Flow cytometry has evolved into specialized techniques (Table 03). Analytical flow cytometry uses lasers for multi-dimensional analysis, while sorting separates cells. Digital flow cytometry processes signals rapidly, imaging combines flow cytometry with microscopy, and mass cytometry uses metal isotopes. These techniques advance cellular research and diagnostics[17][30][53][54][55].

## 5. Advancements in Flow Cytometry

Recent advancements in flow cytometry focus on automation and instrumentation, leading to improved workflows and data quality.

### 5.1 Advancements in Instrumentation:

1. Laser Technology**:**Laser technology has significantly propelled the progress of flow cytometry, enabling the concurrent assessment of thousands of cells with the measurement of over 20 parameters in real-time [56]. This advancement has been made possible by the integration of multiple lasers, which has now become a widespread practice. The adoption of multiple lasers has not only expanded the analytical capabilities but has also provided increased adaptability for analysing diverse sample types [57].
2. Optics and Detectors: Improved optics and detectors allow for full spectral measurements on sub-millisecond time scales, enhancing accuracy.[58]
3. Signal Processing and Data Acquisition: Automated data acquisition and user-friendly software improve data collection, analysis, and reporting[59][60][61].
4. Robotics: Robotic automation instruments integrate sample processing, plate sampling, and fluidics, enhancing efficiency and reliability[62].
5. Artificial Intelligence (AI) and Machine Learning: AI and machine learning are explored for advanced data analysis and interpretation[63].

### 5.2 Automation in Sample Preparation:

Automated systems use liquid-handling robotics for end-to-end sample preparation, reducing errors, and hands-on time.Examples include Beckman CellMek, Sysmex PS-10, and BD FACSDuet™, streamlining clinical diagnostics and improving efficiency[64][65][66].

### 5.3 Automated Data Acquisition,

systems streamline the process, detect smaller particles with more sensitivity, and reduce human error[67][68].Integration with other automated tools creates fully automated workflows, enhancing overall efficiency. There are several examples of automated data acquisition systems available today like CytoFLEX CytoFLEX Flow

Cytometer can be directly integrated with BiomekI-Series Automated Workstations,[28], Genedata Screener® is another example of an automated data analysis tool that streamlines the analysis of flow assays and combines their results directly with information from large-molecule registration and workflow [69].

### 5.4 Automated Gating in Analysis:

Automated gating algorithms identify and refine cell populations based on fluorescence signals, improving efficiency and reproducibility.Automation overcomes limitations of manual gating, leading to faster and more accurate analysis.[70][71]

### 5.5 Automated Quality Control:

Automated systems enhance data quality and integrity by reducing human error and improving productivity.Real-time automated systems like ART-FCMs monitor bioreactor processes without intervention.[72 ][73]

### 5.6 Automated Reporting and Analysis:

Automated reporting systems analyze large datasets efficiently, reducing human error, and improving accuracy[67].Examples include FlowJo software package and robotic systems for clinical diagnostics.[5]

### 5.7 Integration of Automation and Instrumentation:

Integrated systems, like BD FACSDuet™ and Agilent cytometers, combine automation and instrumentation for improved accuracy and efficiency.[64][74]

### 5.8 Advancements in Nobel Fluorescent Probes and Dyes:

Recent advancements in fluorescent probes and dyes have been focused on improving resolution and sensitivity in cellular imaging and understanding the molecular mechanisms of biological events. Specifically, recent studies have focused on nucleus-targeted fluorescent probes to gain a better understanding of the pathogenesis of nuclei and related diseases. Small-molecule nucleus-targeted fluorescent probes have been developed in recent decades. However, these studies are currently immature, and there is a lack of general strategies for designing nucleus-targeted fluorescent probes [75]. Two common strategies for the design of nucleus-targeted fluorescent probes include utilizing commercial nucleus targeting dyes and nuclear localization signal (NLS) peptides [75]. Recent advancements in this field include the current general strategies for designing nucleus-targeted fluorescent probes and the applications of nucleus-targeted fluorescent probes in nuclear imaging, intranuclear biomolecular detection (e.g. DNA, RNA, Ca2+, H2O2, etc.), and cancer therapy [75]. Such fluorescence probes facilitate the study of intranuclear biomolecule functions during cancer diagnosis and treatment, helping researchers to understand the roles of the nucleus in physiological and pathological processes of cells [75]. Recent advancements have also been made in molecular design of pyridyl substituted fluorophores for developing fluorescent structures and materials for optical sensors, which have applications for analyte detection in real scenarios [76]. Furthermore, researchers have made advancements in neutralizing the negative charge on the surface of DNA strands to improve stability and cell permeability. Some of these advancements include modifying fluorescent DNA-Ag NCs with cationic polyelectrolytes via electrostatic force, leading to a threefold fluorescence intensity enhancement [77]. Additionally, DNA-templated silver nanoclusters (DNA-Ag NCs) are a

novel fluorescent imaging technology that has unique properties such as tunable fluorescence emission range, and researchers have developed a strategy to make gold nanoclusters with positive charge and silver nanoclusters with negative charge form aggregates by electrostatic interactions, resulting in an incredible 40-fold fluorescence intensity enhancement [77].

## 6. Application of Flow cytometry

- Immunophenotyping: identifies cell types based on surface markers
- Cell sorting, cycle analysis, apoptosis, and proliferation assays[78][79].
- In the analysis of chemokine receptor expression and function[80]
- In Early Drug Development and Discovery[81].
- Detection of Hematopoietic Stem Cell Proliferation[82]
- In Diagnosis of Primary Immunodeficiency Disease[83].
- Lymphoma and leukemia diagnosis and classification [84]
- Stem cell analysis for diagnosis and transplantation evaluation[85].
- Immunohematology: blood group antigens, antibodies[71] [86]
- Diagnosis and monitoring of hematological disorders.
- Minimal residual disease (MRD) detection
- Hemoglobinopathies diagnosis based on cell surface markers.
- Routine practice in haematological malignancies management.

## 7. Conclusion:

Flow cytometry, a transformative analytical method, has revolutionized biomedical research by enabling rapid cell analysis and sorting based on physical and chemical properties. Its principle of light scattering and fluorescence has evolved from simple cell counting to a sophisticated tool applied in immunology, hematology, microbiology, and cancer research. The technique's components, including fluidics, optics, detectors, and electronics, collaborate to assess cells individually. Forward and side scatter measurements offer size and granularity insights, while fluorescence enables specific cell population labeling. Advancements in lasers, optics, and automation have expanded capabilities, streamlining sample processing and data analysis. Specialized techniques like analytical, sorting, digital, imaging, and mass cytometry cater to various research needs. Although flow cytometry has limitations, its integration with AI, improved probes, and automation promises a bright future for innovative biomedical research, shaping discoveries and diagnostics across disciplines.

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