

Advances in Cellular and Molecular Biology Assays: A Review of Gold Standard Methods

Katiana Simões Lopes Kittelson^{1,2}; Allana Cristina Faustino Martins²;
Gizele Celante²; Arquimedes Gasparotto Júnior¹; Roberto Da Silva Gomes^{2*}

¹Laboratory of Cardiovascular Pharmacology (LaFaC), Faculty of Health Sciences,
Federal University of Grande Dourados (UFGD), Dourados, MS, Brazil

²Department of Pharmaceutical Sciences, College of Health and Human Sciences,
North Dakota State University (NDSU), Fargo, ND, United States.

Corresponding Author: Roberto Da Silva Gomes^{2*}

Publication Date: 2025/04/29

Abstract: Considering the number of available methods in molecular and cellular biology and the rapid development of new technologies, the need for an updated guide on what is currently applied and how to choose the best method for a specific goal has increased. Gold standard methods are known for their accuracy and reliable data under certain circumstances, allowing replicability. This perspective aims to characterize and define the current gold standard techniques from the last five years, enlighten their historical background, cite drawbacks and benefits, and delimit possible future aspects.

Keywords: *Molecular Medicine; Multiomics; Methodological Techniques.*

How to Cite: Katiana Simões Lopes Kittelson; Allana Cristina Faustino Martins; Gizele Celante; Arquimedes Gasparotto Júnior; Roberto Da Silva Gomes (2025). Advances in Cellular and Molecular Biology Assays: A Review of Gold Standard Methods. *International Journal of Innovative Science and Research Technology*, 10(3), 3307-3319.
<https://doi.org/10.38124/ijisrt/25mar736>

I. INTRODUCTION

To understand the characteristics of the cell, such as its physiology and interactions with the medium, several studies in molecular and cellular biology have been developed, utilizing techniques from other fields such as biochemistry and genetics¹. The enhancements and refinement in existing techniques are responsible for the progress in the field, and it has allowed for the extensive information we now have regarding cell mechanisms². Some of these techniques have shown to be accurate and reliable data throughout time, and for that, they are known as gold standard methods³. Overall, the main goal of this study is not to offer detailed descriptions or protocols, but a summary of the main applied techniques, background, possible advantages and disadvantages attached to these techniques, and perspectives.

II. HISTORICAL PERSPECTIVES

The advent of genomics in the 1990s through the genome sequencing program opened up the pathway to many advances in molecular biology. Moreover, technology also started to play a pivotal role in research once developments in bioinformatics allowed a great flow of data, the creation of databases, and the reinvention of data acquisition and analyses.⁴ The post-genomic era can be characterized by

studying mainly systems biology and epigenetics; however, the development of omics-based technology has been revolutionizing science: biotechnologies that describe genomics, transcriptomics, proteomics, and metabolomics^{4,5}.

The term "omics" cannot be fully defined, as "*omnis*", in Latin, means "*everything*" – it is intrinsically the investigation and analysis of all available data regarding the structure or functioning of any biological system at a particular chosen level^{6,7}. There is a great promise regarding integrating multiomics studies with artificial intelligence (AI) and machine learning (ML) in consideration of advanced diagnosis, prognosis, drug development, and delivery, as well as revealing deeper information about the pathophysiology of complex diseases⁵.

III. GOLD STANDARD METHODS

An ideal gold standard method displays sensitivity and specificity equal to 100%, which is not practically achievable⁸. Therefore, in research, the gold standard refers to the best standard test or experimental model available that presents accurate results, is thoroughly tested over time, and is under reasonable conditions or techniques known as reliable methods³. Gold standards may be a single test or a combination of preferred techniques and can vary according

to experiments and targeted results⁹. Aiming to enlighten current gold standard methods in cellular and molecular biology, the following tests were selected.

➤ Genetic Engineering

• CRISPR-Cas Gene Editing

The gene editing technology aims to modify deoxyribonucleic acid (DNA) sequences through an engineering system based on the DNA repair mechanisms originally existent in cells¹⁰ and can insert, delete, or replace nucleotides in the genome *in vivo*¹¹. Site-specific genetic or epigenetic alterations can be carried out through adaptable nucleases and regulatory proteins¹⁰.

Clustered, regularly interspaced short palindromic repeats or CRISPR were identified in DNA sequences of *Escherichia coli* in 1987¹², but its role as an evolved immune system has been discovered recently^{10,13}. The genes responsible for DNA repair and repairing proteins received Cas (CRISPR-associated) genes for the strict relationship with CRISPR¹³. The synergistic work between them is responsible for the acquired immune system in prokaryotes and archaea by integrating genetic elements into their genome¹¹. The latest classification for CRISPR-Cas systems includes Classes 1 and 2, based on the effector proteins, subdivided into six types according to their signature proteins^{11,13}. Currently, the best-studied multidomain effector protein is Cas9 nuclease, which is most used for medical purposes as a modifier of somatic cells¹⁴, followed by Cas12 gene regulation, which can be engineered for diverse applications. However, they need to be scientifically approached¹¹.

CRISPR-based gene-editing tools are flexible, efficient, and inexpensive, and for those, they have been widely applied¹⁵. CRISPR-Cas9 has been applied for genome screening and editing, agricultural applications, and the generation of animal or alternative research models¹⁶. Its clinical applications include cancer treatments, treating infectious diseases and addressing multidrug-resistant bacteria, point-of-care diagnosis, developing drug-delivery platforms, and human immunodeficiency virus (HIV) therapy^{10,17}.

As future outlooks, the potential of different Cas variants should be explored. CRISPR-based tools have the potential to act on HPV-related issues and blood disorders such as sickle cell disease (SCD), and in neuroscience, they address neurological, neurodegenerative, and neuromuscular disorders¹⁰.

• Cloning

Cloning is the deliberate asexual reproduction of identical copies of biological materials, such as genes, cells, or individuals, produced *in vitro* by two means: somatic cell nuclear transfer (SCNT) and embryo splitting, and they both require that the embryo is implanted in a uterus for gestation and birth^{18,19}.

In 1984, the first step into mammal cloning was taken by Solter and McGrath²⁰, but it was only in 1996, that Dolly, the sheep, was the first successfully cloned mammal²¹. Since then, new advances in genetic engineering have surfaced and allowed for the perfecting of cloning procedures²².

Cloning by SCNT procedure consists of replacing the chromosomes from an egg and adding the somatic cell nucleus of the genetic material donor (individual to be cloned). After stimulation and division, the egg will become a blastocyst – an embryo ready for implantation in a healthy uterus. If the implantation is successful, the gestation will take place and lead to the birth of a cloned individual^{18,19,22}. In comparison, cloning by embryo splitting starts with *in vivo* fertilization (IVF) of reproductive cells to generate a zygote or embryo that will go through division into identical cells. These identical cells can be separated into identical blastocysts ready for implantation^{18,19}. The latter procedure is the equivalent of the formation of monozygotic twins¹⁹.

The bioethics regarding human cloning can be controversial. Some arguments can advocate for its benefits in multiple medical applications, such as creating disease research models, treating infertility, and regenerative medicine^{18,23}, as well as considering it against human dignity^{18,22}. Some of the main considerations raised about cloning lie in the safety and well-being of individuals involved, the loss of personal identity and uniqueness when creating a clone, in addition to handling family dynamics and interpersonal relationships²². Thus, 'creating human embryos for research purposes' has been banned since 1998²⁴.

Despite the bioethical issues, the current main applications for cloning include agriculture, where genetically modified clones display enhanced qualities compared to the genome donor in creating livestock²⁵ and producing animal bioreactors, improving production and reducing production costs^{23,26}. As a perspective, xenotransplantation and investigating natural biosynthetic gene clusters (BGCs) might become valuable fields^{23,27}.

➤ Sequencing Technologies

• Next-Generation Sequencing

Aiming DNA sequencing, next-generation sequencing (NGS) technology, also named massively parallel or deep sequence, is currently a widely adopted method that refers to an enhanced data generator, which can run billions of Sanger sequencing experiments simultaneously, without physically separating the reactions^{28–30}. Thus, NGS can sequence entire genomes faster than other applied technologies, reducing financial costs and enabling precision³⁰.

Sanger's DNA sequencing protocol³¹ and Maxam-Gilbert methods³² were published in 1977, marking the first generation of sequencing. The second generation started in the early 2000s, also named NGS, whereas the third-generation sequences emerged in the 2010s. The so-called fourth generation encompasses the period starting from 2014³³.

The high-performing platforms for NGS are based on different technologies that usually follow similar steps but present different results and data. Among these tools, pyrosequencing technology, roche 454 sequencing, reversible terminator technology, illumine solexa genome analyzer (GA), sequencing by ligation technology, applied biosystems sequencing by oligonucleotide ligation and detection (ABI-SOLiD) system, compact personal genome machine (PGM) sequences are highlighted^{33,34}.

Some of the main applications for NGS include whole genome sequencing (WGS) or targeted genome sequencing, human microbiome, ribonucleic acid (RNA) sequencing, diagnosis of infectious diseases and determination of zoonotic transmission, and outbreak management.^{33,34} Whereas, the challenges related to NGS are often associated with data acquirement and management, storage, and analysis, as well as the cost of applying this technology on larger scales^{34,35}.

• *Single-Cell RNA Sequencing*

The single-cell RNA sequencing (scRNA-seq) method can be defined as a genomic path that can detect and analyze RNA molecules in biological samples to evaluate cellular responses.³⁶ The technique was first described by Tang et al. in 2009³⁷, and since then, upgraded versions of the technology have been created, bringing essential adjustments and advancements³⁸.

The scRNA-seq technique has been applied to assess cell heterogeneity, revealing rare and novel cell populations that other methods could not identify. It can also be used in the analysis of specific site cells, identify lineage and relationships among different tissue cells, and provide inherent characteristics of gene expression³⁶. scRNA-seq established a catalog of cells in all living organisms, also named atlas, that is a useful resource when understanding and treating especially cardiovascular, oncological, and degenerative diseases^{38,39}.

This technique can also distinguish healthy cells from cancer cells at different stages of tumors and identify the relations between healthy-tumor cells, providing insights for novel therapeutics⁴⁰. In drug development, scRNA-seq enhances biomarker identification targeting and novel mechanisms of action⁴¹. Moreover, enhancements in the technique, such as molecular spikes or RNA spike-ins, have helped with accurate RNA counting and improved estimate of total endogenous RNA in scRNA-seq⁴². As for future perspectives, new strategies, protocols, and platforms will remove any bias or errors and enhance data quality, and the atlas can become even more helpful in understanding the physiological processes in normal and pathological conditions and predicting or improving treatment effects.^{39,41}

➤ *Gene Expression and Regulation*

• *Real-Time PCR*

The polymerase chain reaction (PCR) can be defined as a nucleic acid amplification method of denaturation and renaturation of DNA or RNA sequences using DNA

polymerase I enzyme (Taq DNA)⁴³. It was initially developed to detect β -globin gene (HBB) gene mutations, responsible for sickle cell anemia, but later evolved for its applications in genetics, microbiology, and forensics^{44,45}. From PCR, many technologies have evolved, such as digital PCR (dPCR), droplet-based digital PCR (ddPCR), chip-based digital PCR (cdPCR), isothermal amplification, and real-time polymerase chain reaction (real-time PCR)⁴⁶.

Real-time PCR is a refined gold standard tool that monitors real-time PCR progress. Hence, the name provides a reliable quantification between the reaction ingredients and excellent sensitivity and does not need post-PCR processes, reducing bias risk.⁴⁴ It can be defined as a robust enzymatic method aiming to amplify a selected DNA segment and generate millions of copies of the segment^{44,47}. Real-time PCR has been applied in many research areas, including biomedicine, biotechnology, pharmacology, microbiology, agriculture, and veterinary sciences⁴⁴.

The most recent and important role of real-time PCR has been with pathogen detection and quantification as a point-of-care diagnostics, where the assay has been proven to be valuable, being able to identify bacteria that can cause pneumonia, and viruses, such as Chikungunya, Zika and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)^{44,48}.

• *RNA Interference*

Discovered in 1998, RNA interference (RNAi) or Post-Transcriptional Gene Silencing (PTGS) is a conserved biological mechanism that mediates resistance against pathogenic invasions and regulates gene expression through small interfering RNA (siRNA), microRNA (miRNA), and other molecules⁴⁹.

RNAi therapy uses the innate mechanisms of RNAi⁵⁰. In turn, RNAi has shown inherent advantages when compared to other molecular therapeutics. However, its clinical application is challenged by the delivery, intrinsic qualities such as stability and specificity, and the development of side effects. Therefore, many modifications have been made to optimize siRNA therapy over time⁴⁹. It was not until recently that the Food and Drugs Administration (FDA) approved the first siRNA drugs, targeting different pathologies and forging a path for novel, innovative therapies.^{50,51} RNAi technology can also be applied for prophylactic and therapeutical vaccines, protein replacement therapy, agricultural biotechnology, and evolutionary studies^{52,53}.

Moreover, miRNA suppresses gene expression by inhibiting protein translation and promoting mRNA cleavage. However, they also play a major role in intercellular exchanges, being renamed circulating miRNAs in these cases⁵⁴. The main applications of miRNA technologies include diagnosis and prognosis biomarkers, especially in cancer, anti-miR therapy (miRNA inhibitor), miRNA replacement therapy, and miRNA-based anticancer treatment^{50,54}.

Future research exploring RNAi and improvement of its characteristics are required for more insight into the mechanisms related to RNAi therapy and applications^{50,52,54}.

➤ *Protein Studies and Interactions*

• *Protein-Protein Interaction Techniques*

The primary components of biological function are proteins that carry out their biological and cellular functions via interacting with vital molecules such as DNA, RNA, and themselves⁵⁵. The term "protein-protein interaction" (PPI) refers to the interactions within a cell, which can result in protein complexes or other types of functional and physical interactions⁵⁶. The intricate network formed by PPIs is called interactome, which is pivotal in physiological and pathological processes and is proportional to the biological complexity of the organism associated. However, the biological information related to interactomes has not yet been completely elucidated.^{56,57} The identification of the interactions above may be pivotal for identifying disease prevalence, novel therapeutic targets, therapy development, and determining novel protein function⁵⁵⁻⁵⁷.

The techniques applied for PPI identification are usually divided into two different main groups: experimental methods, which are subdivided into a) biophysical methods based on structural information and b) direct or indirect high-throughput methods; and computational methods, which are subdivided into a) empirical predictions to infer in PPI and b) theoretical predictions to infer in PPI^{56,58}. For the computational methods, several PPI databases containing proteins' biological information, sequences, and structures are available^{55,59}. Proteomics and transcriptomics technologies can also help identify PPIs⁵⁸, especially if associated with mass spectrometry (MS) when appropriate^{59,60}. Among the main applications for PPIs, it is important to highlight the regulation of transcription factors and gene expression, protein identification, protein complexes and functional prediction of proteins, evolving interactomes, and viral-host PPIs⁶¹.

The main issue in targeting PPIs is their interfaces and binding sites, which can hamper finding matching molecules^{57,62}. The residues at the PPI interface responsible for the binding, the "hot spots", make the drug design possible. However, the additional large molecules often must compete with the native protein⁶². Therefore, developing smaller PPI drugs should manage the size while maintaining potency and selectivity, which is the major challenge in PPI drug development and could be pivotal for diverse biomedical areas, considering the abnormal PPI present in pathologies⁵⁵.

• *Western Blotting*

Western blotting (WB), or immunoblotting, is a simple, low-cost, and commonly used tool known as a gold standard methodology for molecular biology and proteomics to identify and quantify characteristics from proteins extracted from cells^{63,64}. The technique was first introduced in 1979,⁶⁵ and is based on proteins being separated by gel electrophoresis according to molecular weight transported to

a binding membrane, where specific antibodies identify the target protein and the binding provides informative data^{63,66}.

One of the main issues related to WB reproducibility is associated with the user's expertise and subjective choices, as any variations to the method may be the source of errors. Following standard protocols and checklists while maintaining accuracy and precision are valuable tools to ensure reliable data and quality⁶⁷.

The primary scientific applications of WB include detecting protein isoforms, post-translation modification, subcellular localization, identifying protein-DNA, protein-RNA, and protein-protein interactions, epitope mapping, and antibody development and characterization^{63,64}. Moreover, in terms of clinical applications, WB has a major role in the diagnosis of both infectious and non-infectious diseases⁶³. It is expected that automation in the WB process and more reliable antibodies may be developed and applied in the future⁶⁶, as well as improvements to the reagents, equipment, and technology, facilitating the WB method⁶⁴.

• *Enzyme-Linked Immunosorbent Assay*

Enzyme-linked immunosorbent assay (ELISA) is a flexible and easily applied method widely used for protein detection through enzymatic reaction.⁶⁸ In this assay, based on antigen-antibody reactions, nonspecific compounds are absorbed or covalently bound to a solid phase, which enables the separation of reactants and the identification of the antigen amount in a sample⁶⁹. Immunoassays have been used to study immune response after stimuli such as natural infection or vaccines once they're easy to apply and can provide results in a couple of hours⁷⁰.

Among the advantages and disadvantages of the assay listed by Sakamoto et al.⁷¹, it is highlighted that ELISA is a simple procedure, presenting high specificity, sensitivity, and efficiency, that can be analyzed simultaneously and is relatively low cost. On the other hand, the antibodies have to be functional and stable, which requires intensive and expensive techniques to obtain, and it may result in false negatives or positives^{68,71}.

Aiming to increase ELISA sensitivity, modifications to the assay have been made, such as digital ELISA, competitive ELISA, indirect ELISA, indirect competitive ELISA, sandwich and open sandwich ELISA, application of nanoparticles to ELISA, such as gold nanoparticles (AuNPs) and silver nanoparticles (AgNPs), and chemiluminescence ELISA^{68,71,72}. Moreover, the main applications for this assay are disease diagnosis, assessing hormone levels and drug usage, detecting biomarkers, evaluating pharmacokinetics and pharmacodynamics, and quality control.⁷¹⁻⁷³

Perspectives include the automation of procedures, decreased expenses related to the most technological versions, and integrating ELISA methods with developing transdisciplinary technologies, allowing for more development in diagnostic and research fields.^{72,74}

➤ *Advanced Imaging and Analysis*• *Advanced Microscopy Techniques*

The combination of emerging techniques and novel instruments to microscopy has opened the opportunity to provide qualitative information on cellular properties and report the relationship between the properties and respective biological structures while enabling these investigations at a macromolecular scale⁷⁵.

Advances made with fluorescence microscopy still left some limitations, which have been minimized by the emerging super-resolution microscopy (SRM), a group of different innovative methods of cellular imaging, such as fluctuation-based super-resolution microscopy, structured illumination microscopy (SIM), and stimulated emission depletion microscopy (STED)⁷⁶. SRM can be an effective tool for disease diagnosis and research^{77,78}.

Confocal microscopy is an improved method that allows capturing high-resolution images in thick issues while rejecting out-of-focus planes with no contribution to the original blur once it's a point-by-point scanning and capturing of the illuminated confocal field, and it has been largely applied in fluorescence imaging in biomedical sciences⁷⁹. Modifications have been made to address the method's weaknesses and aim to enhance it, such as multiview confocal microscopy.⁸⁰

Some of the techniques available include confocal laser scanning microscopy (CSLM), super-resolution microscopy (SRM), fluorescence correlation spectroscopy (FCS), fluorescence resonance energy transfer (FRET), micro spectrophotometry (MSP) and single-molecule localization microscopy (SMLM) to mention a few^{75,78,81,82}.

Aiming to improve imaging high-resolution, the future premise for microscopy techniques is the evolution of post-processing methods, such as learning algorithms, ML, and AI.⁷⁶

• *Fluorescence Resonance Energy Transfer*

Fluorescence or Förster resonance energy transfer (FRET) is the energy conveyed between excited fluorophore molecules.⁸³ The FRET transfer efficiency rate change indicates the distance between the fluorophores and the intermolecular interactions. At the same time, theory predicts that the transfer rate depends on the donor-acceptor separation distance.^{84,85}

There has been an inconsistency with the FRET acronym as the most appropriate name. In 1948, Förster developed the theory of energy transfer based on dipole-dipole interactions⁸⁵⁻⁸⁷, but recently, FRET has also been referring to "fluorescence" due to the usage of fluorescence probes in applications⁸⁶. However, once there is no emission of photons in FRET, Shrestha et al. affirm that the latter definition is incorrect⁸⁶.

FRET assay was first applied to describe protein-protein interactions⁸⁵, though nowadays, it has been used to supply

information and quantitative assessment on the structure of single biomolecules, such as conformational heterogeneity and dynamics⁸⁸. The assay also can characterize enzyme reactions, outline ligand-receptor interactions, and the movement of molecular motor proteins⁸⁵.

The integration of emerging technologies has enhanced the applicability of FRET assays, such as adding imaging tools, improving biosensors, and lab-on-a-chip devices^{89,90}. It extended its applications to more complex biological systems, aiming to enlighten cellular processes, such as functionality and stress conditions, and develop novel therapeutics⁹¹, as well as to fields other than biosciences and research, such as food and agriculture⁹².

• *Cryo-Electron Microscopy*

Cryo-electron microscopy (cryo-EM) is an alternate technique that enables the visualization, at atomic resolution, of a macromolecule's three-dimensional structure^{93,94}. It has been mainly applied to understand molecular and cellular characteristics of biological processes, pharmacokinetics, and pharmacodynamics of drugs and image processing⁹³. The method has been used with developments in cryo-electron microscopy tomography (cryo-ET) and single-particle analysis (cryo-EM SPA) to identify undetectable features using other techniques⁹⁵.

In cryo-EM, advances in detectors, transmission, and image processing have also been made to improve image acquisition⁹⁶. However, cryo-EM's main limitations are sample and EM grid preparation, image resolution, and processing.⁹⁶ Moreover, Cryo-EM has been applied to gene expression and regulation, protein synthesis and degradation, membrane protein dynamics and proteins from the immune system, and disease-related proteins.⁹⁵ Perspectives include automation, especially in image processing and algorithms, learning AI technologies for image recognition, enhancing efficiency and accessibility, and application in drug discovery and delivery^{93,95,97}.

• *Tissue Microarrays*

Tissue microarrays (TMAs) are formed by small-tissue cores extracted from paraffin donor blocks and later inserted into a recipient block. They can be simultaneously analyzed under exact experimental circumstances⁹⁸. Since their inception in 1999, TMAs have demonstrated numerous benefits over conventional histopathological techniques, including reduced expenses, less harm to donor blocks, reduced experimental error, and expedited processes^{99,100}.

Many modifications have been made to TMA construction to enhance the technique, making TMAs applicable to clinical and research, especially when identifying biomarkers and protein expression^{98,100}. Clinical TMAs can include multi-tumor microarrays, progression and prognosis microarrays, and cryomicroarrays¹⁰¹.

Aiming for a cost-efficient technique with reduced timing and effort, next-generation tissue microarrays, or ngTMAs, have emerged recently¹⁰². It has demonstrated high-throughput technology and time-efficiency features,

increased sensitivity, and large-scale detection while enabling annotations to be inserted on digital slides, guaranteeing accuracy without sacrificing data quality¹⁰². The current developments have been greatly beneficial for existing histological processes. However, there is still a need to enhance the technique, as TMAs can potentially improve routine diagnosis at the point-of-care.⁹⁸

➤ Cellular Analysis and Phenotyping

• Flow Cytometry

Flow cytometry evaluates cell suspensions or particles in flow within a cytometer using fluorescence and light scatter, which requires a device that allows cells to be sorted into multiple measurable qualities^{103,104}. The technique is the gold standard for analyzing single cells and can bestow information on cell physiology and pathological characteristics^{104,105}. Many advances have been made to instruments with sorting abilities, enabling more accuracy and efficiency in sorting different cell populations enhancing flow cytometry applications¹⁰³. The current method of identification of cells and their properties depends on the recognition of fluorescent signals emitted by probes¹⁰³.

The current advancements in flow cytometry have made it possible to apply the method in many contexts, such as spectral flow cytometry, which allows for a greater range of fluorochromes, mass cytometry (CyTOF) that enables cells to be targeted by metals, which increased assessed parameters, and alternate or hybrid detection methods for cell detection, such as surface plasmon resonance (SPR), imaging flow cytometers and electrical impedance^{103,106}.

Flow cytometry's main applications include several fields, such as immunology, drug discovery, and diagnostics, being heavily used for cell immunophenotyping, viability and cell cycle assays, cytokines analysis, and protein synthesis¹⁰³. In addition to the biomedical field, the technique has also been applied to biotechnology, veterinary medicine, and industrial applications¹⁰⁵.

Perspectives include enhanced data processing such as learning AIs, automation in cell identification aiming to reduce errors, second-generation spectral flow cytometry, enhancing probes, and integrating flow cytometry with omics data^{103,105}. Moreover, the association with next-generation instruments may play a significant role in the future aspects of flow cytometry¹⁰³.

• Cell Culture Techniques

Cell culture describes a range of techniques that enable the growth of cells in a controlled setting that mimics physiological circumstances, where cells can then be suspended or adhered to dishes^{107,108}. Hereafter, the use of cell culture may increase when replacing animal models in research¹⁰⁷. The 2-dimensional (2D) model is currently the most applied technique. However, developments in 3-dimensional (3D) cell culture models have been emerging.¹⁰⁹

The characteristic of 2D cultures is the growth of cells in an adherent monolayer in a culture flask or petri dish;

however, the approach can show drawbacks when considering the absence of a more complex and suitable environment observed *in vivo*^{108,109}. In light of the shortcomings of 2D techniques, 3D models seek to reproduce the physiological or pathological milieu unique to a given tissue, increase cell-to-cell interactions and cell-matrix interactions, and preserve or augment cellular features as seen *in vivo*^{110,111}. The advantages and disadvantages of 3D techniques have been listed in a previous study¹⁰⁷.

The spheroids culture system is an emerging 3D culture method, where spheroid structures enable cells to form several layers and maintain their properties, imitating features of pathophysiological tissues^{108,112}. Moreover, studies involving stem cells in 3D spheroid culture are carried out mainly in regenerative medicine and cancer studies^{108,110,112}.

Advancements in cell culture allowed for the rise of organoids. In personalized medicine, organoids are self-organized 3D tissues, usually derived from stem cells, that emulate a miniature organ in a 3D medium¹⁰⁷. However, the term now encompasses other techniques that comply with stem cells, which present similar functionality as the original harvested tissue¹¹¹. Organoid culture has been described for several organs and has been currently applied in drug discovery and precision therapy¹¹¹. Furthermore, organoids-based cancer tissue models, called tumoroids, aim to provide pivotal information about drug evaluation and development through cell culture from oncological patients' cells.^{111,113}

The 3D cell culture, paired with 3D bioprinting and fluidic chips, has enabled the advent of the organ-on-a-chip (OOAC) technology, which can be defined as systems containing engineered tissues inside microfluidic chips, where the latter control the microenvironment to emulate human physiology and maintain tissue functions^{114,115}. Multiple-organ systems on a chip have recently been designed and applied to pharmacological studies¹¹⁴.

➤ DNA and Protein Analysis

• Electrophoretic Techniques

Electrophoresis can be defined as a separation technique through the relocation of electrically charged molecules traveling in an electric field, and it is currently widely applied to separate, identify, and purify proteins and other biomolecules, such as DNA^{116,117}. The main principle was introduced in the 1930s, and it was not until the 1960s that gel electrophoresis methods were increased¹¹⁸.

When applying electrophoresis, many factors can affect the mobility of the biomolecules, those being related to inherent characteristics of the molecule or the method chosen once they use different buffers and mediums¹¹⁶. In this sense, there are two main types of electrophoresis: zone electrophoresis and moving boundary electrophoresis, each including a set of different techniques¹¹⁸. Gel and capillary electrophoresis can be highlighted among the widely applied methods.

Gel electrophoresis separates molecules and nanoparticles through an electric field, which will relocate the negatively charged molecules through a gel, often agarose gel, and it is majorly used for analytical purposes and diagnosis in biochemistry, molecular biology, and biomedical fields¹¹⁸. As a modification to the traditional method, 2D gel electrophoresis has emerged. It has been applied to proteomics, separating complex protein mixes into individual spots, which enables the identification of protein expression patterns¹¹⁹. Moreover, capillary electrophoresis (CE) uses electric field and chemical balance to separate molecules, can provide additional information to other techniques, and is mainly applied to analyze complex samples in chemistry, biomedical, and pharmaceutical fields¹²⁰. As an innovation, microchip electrophoresis (MCE) refers to a miniaturized technique, and it has been showing many advantages once it uses the smallest samples and offers faster results¹²¹. Perspectives regarding electrophoretic techniques development include instrument automation, implementation of portable devices, association with deep imaging strategies when appropriate, integration to emerging technologies and AI^{119,120}.

• Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) is an antibody-based technology used to identify the genomic loci related to a protein by isolating chromatin fragments and using the immunoselection technique¹²². ChIP is also considered a valuable tool in analyzing *in vivo* interactions between DNA and histones or proteins¹²³. The technique has been primarily applied in studies of cancer epigenome, but NGS allowed for ChIP to detail histone post-translational modifications (PTMs) and transcription factors (TF)¹²². Lately, ChIP technology has been modified into reverse chromatin immunoprecipitation (R-ChIP), sequential ChIP (ChIP-re-ChIP), ChIP-on-chip, and most importantly, ChIP followed by sequencing (ChIP-seq)¹²⁴.

ChIP-seq plays a pivotal role in epigenomic research once it allows the identification of specific epigenomic marks of a specific cell line. It can also recognize the relations between TPs and other regulatory elements, and gene expression in different stages^{125,126}. Advances made to NGS analysis technology have helped enlighten the epigenomics contribution to diseases at a cellular level, and nowadays, there are databases available with data around histone modifications¹²⁶. ChIP-seq has also been applied in drug response and discovery¹²⁷.

The difficulties faced in ChIP-seq are related to methodology, such as cell count from starting material, crosslinking between proteins and DNA, cell lysis, chromatin shearing, protein degradation, and antibody availability and quality during immunoprecipitation¹²⁵. Enhanced methods may be developed in the future, aiming to reduce errors and improve data, such as simultaneous identification of histone modifications and chromatin-binding proteins and localizing regulating factors within cells¹²⁶.

➤ Structural Biology Advances

• Biomolecular Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) spectroscopy is a physicochemical technique that identifies structural characteristics of molecules at an atomic level¹²⁸. NMR is characterized by the interaction of radiofrequency (in megahertz, MHz) electromagnetic radiation with the magnetic properties present in the nuclei of atoms in a strong magnetic field environment to identify molecular parameters¹²⁹. It can operate in liquid or solid states, obtain information from the intracellular space (in-cell) or at the cell surface (on-cell), and can be applied in one-dimensional (1D), 2D, or multidimensional (nD) experiments^{129,130}.

NMR is the gold standard method in compound identification, 3D structures, and molecular connectivity. It provides many advantages compared to other metabolomic platforms, such as being a nondestructive, noninvasive, easily automatable, and reproducible technique with a highly amenable quality that allows for living cell studies¹³¹.

Advances made to NRM instruments have made them more suitable for clinics once they are smaller, more affordable, and easily manageable, with enhanced software tools that enable better data acquisition. They have also allowed for an increase in applicability in solid-state experiments^{129,131}. NMR has been applied in biomolecules and nanoparticle characterization, molecular structure and interactions on-cell, drug and biomarkers discovery, medical testing, and diagnosis^{129–132}.

Future NRM innovations may include association with deep-learning AI that could autonomously operate NRM instruments and generate associated data. Enhancements on NRM signal-to-noise and data resolution can also allow for improved characterization of transient conformations in biomolecules^{133,134}. Moreover, applying NRM and MS in metabolomics increases the coverage of metabolite identification. Therefore, the techniques should be complementary and not individually applied¹³¹.

➤ Toxicity and Safety Assessment

• Cytotoxicity Assays

Cytotoxicity and cell viability assays are essential methods for *in vitro* toxicology or pharmacology studies to ascertain substances' safety profile since they will quantify the toxic damage or death of cells¹³⁵. Considering the measurement of end-points, the assays can be divided into a) dye exclusion assays (development of dye color in dead cells, otherwise excluded by viable cells); b) colorimetric assays (development of color in response of cell viability); c) fluorometric assays (development of fluorescence in response of cell viability, more sensitive than colorimetric assays); d) luminometric assays (development of glow signal after reagent addition); e) flow cytometric assays (quantitative single cell analysis, that may carry a fluorescent marker)^{136,137}. Each test presents its benefits and drawbacks. However, respectively, the main applied assays are the trypan blue stain assay, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide) assay, the resazurin (AlamarBlue) assay, the adenosine triphosphate (ATP) assay, and the membrane asymmetry assays^{135,137}.

Although many different assays are available, researchers must find the best "fit for purpose" alternative, considering that more than one assay should be applied throughout the evaluation *in vitro*¹³⁷. As future considerations, optimization of existing methods and current parameters may enhance precision in data acquittance¹³⁸ and develop new techniques that may directly assess measured parameters.

• Genotoxicity Assays

Genotoxicity testing includes the ability of a substance to permanently damage DNA or cellular components at a genomic level, as well as assessing adverse effects on the cell, including mutagenicity¹³⁹. Safety assessments are generally combined to evaluate different end-points such as gene mutation, clastogenicity, and aneuploidy^{139,140}. Assays can be *in vitro*, identifying underlying mechanisms and targeted toxicity, or *in vivo*, identifying secondary genotoxicity through alternate vias, such as inflammation¹⁴¹.

Among the most popular techniques are the Ames test, *in vitro* mammalian cell mutation (MCM), cell measurements with tests such as the comet assay, and cytogenetic assays such as micronucleus (MN) are included¹⁴². Although tests have been effective in hazard identification, some limitations exist in extrapolating the data for *in vivo* assessment, especially due to the genotoxicity qualitative approach¹⁴³.

Adding a determined reference point of dose-response and calculation of margin of exposure might enhance genotoxicity assessment¹⁴³. Moreover, using computational models, exploring alternate vias of administration or exposure, identifying genetic damage in multiple tissues, and more complex *in vitro* tests such as multi-organ-on-a-chip systems may improve the extrapolation of *in vitro* data and risk assessment^{140,143}.

IV. EMERGING TECHNOLOGIES

Unquestionably, science keeps evolving with each passing day, and as of now, many techniques rely on novel technologies to be applied. One of the leading emerging technologies responsible for scientific progress is AI application and deep learning-based models and algorithms, which have made several pivotal contributions to oncology, from diagnosis to personalized treatment¹⁴⁴. AI health interventions can also act on the patient risk assessment of morbidity and mortality, disease outbreaks and surveillance prediction, and health policies¹⁴⁵.

The rise of synthetic biology, an area focused on creating bio-components with beneficial bio-properties, has been observed recently, which allowed novel pathways for many biomedical applications such as cell therapies, development of therapeutic chemicals, nanotechnology, and nanomaterial production, and gene engineering¹⁴⁶. Moreover, some of the advances made with synthetic biology

contemplate cell-based technology and programming, cell-free biology and the creation of synthetic cells, applications of ML and automation, and novel biomaterials, including next-generation biomaterials such as 3D bioprinting¹⁴⁷.

Finally, the OOAC technology can be defined as a biomimetic system on a microfluidic chip, which means it is capable of mimicking structural and functional properties of human tissue and can provide information regarding response in the face of various stimuli, hence its vast application in physiological studies, especially in pharmacology and toxicology¹⁴⁸. Furthermore, multiorgan-on-a-chip (multi-OoC) approaches have been studied recently, modeling systematic diseases and leading to information about multiorgan interactions¹⁴⁹.

V. CHALLENGES AND LIMITATIONS

The main goal of this study was to highlight and define current gold standard methods and provide information on perspectives when appropriate, but the study has shown potential limitations. The limited access to data was one of the limitations found. The studies considered for the body of text were free to access and written in English, meaning studies that did not meet these criteria were not incorporated. The lack of current data on some topics was another limitation. In the hope of making this study as updated as possible, the research was delimited to five years. However, in some situations, finding quality data within the established time frame was impossible. To overcome the above limitations, it is suggested that a more in-depth systematic review should be performed once it can reduce limitations and overcome unintentional bias.

VI. CONCLUSION

Many advances in the biomedical field, including the search for and inclusion of gold standard methods, can be considered major challenges to be achieved. The developments in these methods can improve disease diagnosis and biomarkers, drug discovery and delivery, and their association with emerging technologies have created new tools that have enhanced society's quality of life. It is essential to highlight that even gold standard methods are subjected to errors and biases, especially during sample preparation and methodology. Therefore, it is pivotal that researchers oversee the newest protocols and emerging data when choosing appropriate tests.

LIST OF ABBREVIATIONS

1D - 1 dimensional; 2D - 2 dimensional; 3D - 3 dimensional; ABI-SOLiD - applied biosystems sequencing by oligonucleotide ligation and detection; AgNPs - silver nanoparticles; AI - artificial intelligence; ATP - adenosine triphosphate; AuNPs - gold nanoparticles; BGCs - biosynthetic gene clusters; Cas - DNA-repair gene; cdPCR - chip-based digital PCR; CE - capillary electrophoresis; ChIP - chromatin immunoprecipitation; ChIP-re-ChIP - sequential ChIP; ChIP-seq - ChIP followed by sequencing; CRISPR - clustered regularly interspaced short palindromic repeats;

cryo-EM - cryo-electron microscopy; cryo-EM SPA - cryo-electron microscopy single-particle analysis; cryo-ET - cryo-electron microscopy tomography; CSLM - confocal laser scanning microscopy; CyTOF - mass cytometry; ddPCR - droplet-based digital PCR; DNA - deoxyribonucleic acid; dPCR - digital PCR; ELISA - enzyme-linked immunosorbent assay; FCS - fluorescence correlation spectroscopy; FDA - Food and Drugs Administration; FRET - fluorescence or Förster resonance energy transfer; GA - genome analyzer; HBB - β -globin gene; HIV - human immunodeficiency virus; IVF - in vivo fertilization; MCE - microchip electrophoresis; MCM - mammalian cell mutation; MHz - megahertz; miRNA - microRNA; ML - machine learning; MN - micronucleus; MS - mass spectrometry; MSP - microspectrophotometry; MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; nD - multidimensional; NGS - next-generation sequencing; ngTMAs - next-generation tissue microarrays; NMR - nuclear magnetic resonance; OOAC - organ-on-a-chip; PCR - polymerase chain reaction; PGM - personal genome machine; PPI - protein-protein interaction; PTGS - post-transcriptional gene silencing; R-ChIP - reverse chromatin immunoprecipitation; RNA - ribonucleic acid; RNAi - RNA interference; SARS-CoV-2 - severe acute respiratory syndrome coronavirus 2; SCD - sickle cell disease; SCNT - somatic cell nuclear transfer; scRNA-seq - single-cell RNA sequencing; SIM - structured illumination microscopy; siRNA - small interfering RNA; SMLM - single-molecule localization microscopy; SPR - surface plasmon resonance; SRM - super-resolution microscopy; SRM - super-resolution microscopy; STED - stimulated emission depletion microscopy; Taq DNA - polymerase I enzyme; TMAs - tissue microarrays; WB - Western blotting; WGS - whole genome sequencing.

➤ *Data Availability*
Not applicable.

➤ *Author Contributions*

The authors confirm their contributions to this work: KSL and ACFM in conceptualization and original manuscript preparation, KSL in writing; RSG oversaw the review process and reviewed the final version. All authors have read and agreed to the published version of the manuscript.

➤ *Funding*

This project was supported by NIH-COBRE (1P20GM109024) and NDSU Start-up. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior- Brasil (CAPES)- Finance Code 001.

➤ *Conflicts of Interest*

The authors declare no conflict of interest.

REFERENCES

[1]. Blanco, A.; Blanco, G. The genetic information (I). in *Medical Biochemistry* 501–534 (Elsevier, 2022). doi:10.1016/B978-0-323-91599-1.00017-1.

[2]. Ronai, I. How molecular techniques are developed from natural systems. *Genetics* 224, (2023).

[3]. Cardoso, J. R., Pereira, L. M., Iversen, M. D. & Ramos, A. L. What is gold standard and what is ground truth? *Dental Press J Orthod* 19, 27–30 (2014).

[4]. Morange, M. The Historiography of Molecular Biology. in 1–20 (2018). doi:10.1007/978-3-319-74456-8_11-1.

[5]. D’Adamo, G. L., Widdop, J. T. & Giles, E. M. The future is now? Clinical and translational aspects of “Omics” technologies. *Immunol Cell Biol* 99, 168–176 (2021).

[6]. Dai, X. & Shen, L. Advances and Trends in Omics Technology Development. *Front Med (Lausanne)* 9, (2022).

[7]. Vogeser, M. & Bendt, A. K. From research cohorts to the patient – a role for “omics” in diagnostics and laboratory medicine? *Clinical Chemistry and Laboratory Medicine (CCLM)* 61, 974–980 (2023).

[8]. Habibzadeh, F. On determining the sensitivity and specificity of a new diagnostic test through comparing its results against a non-gold-standard test. *Biochem Med (Zagreb)* 33, 5–9 (2023).

[9]. Parikh, R., Mathai, A., Parikh, S., Chandra Sekhar, G. & Thomas, R. Understanding and using sensitivity, specificity and predictive values. *Indian J Ophthalmol* 56, 45 (2008).

[10]. Morshedzadeh, F. et al. An Update on the Application of CRISPR Technology in Clinical Practice. *Mol Biotechnol* 66, 179–197 (2024).

[11]. Paul, B. & Montoya, G. CRISPR-Cas12a: Functional overview and applications. *Biomed J* 43, 8–17 (2020).

[12]. Ishino, Y., Shinagawa, H., Makino, K., Amemura, M. & Nakata, A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. *J Bacteriol* 169, 5429–5433 (1987).

[13]. Ishino, Y., Krupovic, M. & Forterre, P. History of CRISPR-Cas from Encounter with a Mysterious Repeated Sequence to Genome Editing Technology. *J Bacteriol* 200, (2018).

[14]. Gostimskaya, I. CRISPR–Cas9: A History of Its Discovery and Ethical Considerations of Its Use in Genome Editing. *Biochemistry (Moscow)* 87, 777–788 (2022).

[15]. Liu, G., Lin, Q., Jin, S. & Gao, C. The CRISPR-Cas toolbox and gene editing technologies. *Mol Cell* 82, 333–347 (2022).

[16]. Hillary, V. E. & Ceasar, S. A. A Review on the Mechanism and Applications of CRISPR/Cas9/Cas12/Cas13/Cas14 Proteins Utilized for Genome Engineering. *Mol Biotechnol* 65, 311–325 (2023).

[17]. Khoshandam, M., Soltaninejad, H., Mousazadeh, M., Hamidieh, A. A. & Hosseinkhani, S. Clinical applications of the CRISPR/Cas9 genome-editing system: Delivery options and challenges in precision medicine. *Genes Dis* 11, 268–282 (2024).

[18]. Rahbaran, M. et al. Cloning and Embryo Splitting in Mammals: Brief History, Methods, and Achievements. *Stem Cells Int* 2021, 1–11 (2021).

- [19]. National Academy of Sciences (US), National Academy of Engineering (US), Institute of Medicine (US), National Research Council (US) & Committee on Science, E. and P. P. Scientific and Medical Aspects of Human Reproductive Cloning. (National Academies Press, Washington, D.C., 2002). doi:10.17226/10285.
- [20]. McGrath, J. & Solter, D. Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell* 37, 179–83 (1984).
- [21]. Campbell, K. H. S., McWhir, J., Ritchie, W. A. & Wilmut, I. Sheep cloned by nuclear transfer from a cultured cell line. *Nature* 380, 64–66 (1996).
- [22]. Bonetti, G. et al. Human Cloning: Biology, Ethics, and Social Implications. *Clin Ter* 174, 230–235 (2023).
- [23]. Tadesse, T., Muluneh, T. & Abraham, S. Agricultural and Biomedical Application of Animal Cloning: Review. *Journal of Natural Sciences Research* 8, (2018).
- [24]. Alkorta, I., Beriain, I. M. & Rodríguez-Arias, D. Cloning and the Oviedo Convention: The Socio-cultural Construction of Regulation. in *The Global Dynamics of Regenerative Medicine* 150–168 (Palgrave Macmillan UK, London, 2013). doi:10.1057/9781137026552_6.
- [25]. Kashim, M. I. A. M. et al. Animal cloning and consumption of its by-products: A scientific and Islamic perspectives. *Saudi J Biol Sci* 28, 2995–3000 (2021).
- [26]. Woodfint, R. M., Hamlin, E. & Lee, K. Avian Bioreactor Systems: A Review. *Mol Biotechnol* 60, 975–983 (2018).
- [27]. Lin, Z., Nielsen, J. & Liu, Z. Bioprospecting Through Cloning of Whole Natural Product Biosynthetic Gene Clusters. *Front Bioeng Biotechnol* 8, (2020).
- [28]. Behjati, S. & Tarpey, P. S. What is next generation sequencing? *Arch Dis Child Educ Pract Ed* 98, 236–238 (2013).
- [29]. McCombie, W. R., McPherson, J. D. & Mardis, E. R. Next-Generation Sequencing Technologies. *Cold Spring Harb Perspect Med* 9, a036798 (2019).
- [30]. Larson, N. B., Oberg, A. L., Adjei, A. A. & Wang, L. A Clinician's Guide to Bioinformatics for Next-Generation Sequencing. *Journal of Thoracic Oncology* 18, 143–157 (2023).
- [31]. Sanger, F., Nicklen, S. & Coulson, A. R. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences* 74, 5463–5467 (1977).
- [32]. Maxam, A. M. & Gilbert, W. A new method for sequencing DNA. *Proceedings of the National Academy of Sciences* 74, 560–564 (1977).
- [33]. Gupta, N. & Verma, V. K. Next-Generation Sequencing and Its Application: Empowering in Public Health Beyond Reality. in 313–341 (2019). doi:10.1007/978-981-13-8844-6_15.
- [34]. Mandlik, J. S., Patil, A. S. & Singh, S. Next-Generation Sequencing (NGS): Platforms and Applications. *J Pharm Bioallied Sci* 16, S41–S45 (2024).
- [35]. Hilt, E. E. & Ferrieri, P. Next Generation and Other Sequencing Technologies in Diagnostic Microbiology and Infectious Diseases. *Genes (Basel)* 13, 1566 (2022).
- [36]. Haque, A., Engel, J., Teichmann, S. A. & Lönnberg, T. A practical guide to single-cell RNA-sequencing for biomedical research and clinical applications. *Genome Med* 9, 75 (2017).
- [37]. Tang, F. et al. mRNA-Seq whole-transcriptome analysis of a single cell. *Nat Methods* 6, 377–382 (2009).
- [38]. Jovic, D. et al. Single-cell RNA sequencing technologies and applications: A brief overview. *Clin Transl Med* 12, (2022).
- [39]. Khozyainova, A. A. et al. Complex Analysis of Single-Cell RNA Sequencing Data. *Biochemistry (Moscow)* 88, 231–252 (2023).
- [40]. Zhang, Y. et al. Single-cell RNA sequencing in cancer research. *Journal of Experimental & Clinical Cancer Research* 40, 81 (2021).
- [41]. Van de Sande, B. et al. Applications of single-cell RNA sequencing in drug discovery and development. *Nat Rev Drug Discov* 22, 496–520 (2023).
- [42]. Ziegenhain, C., Hendriks, G.-J., Hagemann-Jensen, M. & Sandberg, R. Molecular spikes: a gold standard for single-cell RNA counting. *Nat Methods* 19, 560–566 (2022).
- [43]. Khehra, N., Padda, I. S. & Swift, C. J. Polymerase Chain Reaction (PCR). (2024).
- [44]. Artika, I. M., Dewi, Y. P., Nainggolan, I. M., Siregar, J. E. & Antonjaya, U. Real-Time Polymerase Chain Reaction: Current Techniques, Applications, and Role in COVID-19 Diagnosis. *Genes (Basel)* 13, 2387 (2022).
- [45]. Zeron-Medina, J., Ochoa de Olza, M., Braña, I. & Rodon, J. The Personalization of Therapy: Molecular Profiling Technologies and Their Application. *Semin Oncol* 42, 775–787 (2015).
- [46]. Zhu, H. et al. PCR Past, Present and Future. *Biotechniques* 69, 317–325 (2020).
- [47]. Harshitha, R. & Arunraj, D. R. Real-time quantitative <sc>PCR</sc>: A tool for absolute and relative quantification. *Biochemistry and Molecular Biology Education* 49, 800–812 (2021).
- [48]. Kadja, T., Liu, C., Sun, Y. & Chodavarapu, V. P. Low-Cost, Real-Time Polymerase Chain Reaction System for Point-of-Care Medical Diagnosis. *Sensors* 22, 2320 (2022).
- [49]. Hu, B. et al. Therapeutic siRNA: state of the art. *Signal Transduct Target Ther* 5, 101 (2020).
- [50]. Traber, G. M. & Yu, A.-M. RNAi-Based Therapeutics and Novel RNA Bioengineering Technologies. *Journal of Pharmacology and Experimental Therapeutics* 384, 133–154 (2023).
- [51]. Friedrich, M. & Aigner, A. Therapeutic siRNA: State-of-the-Art and Future Perspectives. *BioDrugs* 36, 549–571 (2022).
- [52]. Zhang, C. & Zhang, B. RNA therapeutics: updates and future potential. *Sci China Life Sci* 66, 12–30 (2023).

- [53]. Koeppe, S., Kawchuk, L. & Kalischuk, M. RNA Interference Past and Future Applications in Plants. *Int J Mol Sci* 24, 9755 (2023).
- [54]. Ho, P. T. B., Clark, I. M. & Le, L. T. T. MicroRNA-Based Diagnosis and Therapy. *Int J Mol Sci* 23, 7167 (2022).
- [55]. Soleymani, F., Paquet, E., Viktor, H., Michalowski, W. & Spinello, D. Protein–protein interaction prediction with deep learning: A comprehensive review. *Comput Struct Biotechnol J* 20, 5316–5341 (2022).
- [56]. Fionda, V. Networks in Biology. in *Encyclopedia of Bioinformatics and Computational Biology* 915–921 (Elsevier, 2019). doi:10.1016/B978-0-12-809633-8.20420-2.
- [57]. Lu, H. et al. Recent advances in the development of protein–protein interactions modulators: mechanisms and clinical trials. *Signal Transduct Target Ther* 5, 213 (2020).
- [58]. Armingol, E., Officer, A., Harismendy, O. & Lewis, N. E. Deciphering cell–cell interactions and communication from gene expression. *Nat Rev Genet* 22, 71–88 (2021).
- [59]. Roy, S., Manners, H. N., Elmsallati, A. & Kalita, J. K. Alignment of Protein-Protein Interaction Networks. in *Encyclopedia of Bioinformatics and Computational Biology* 997–1015 (Elsevier, 2019). doi:10.1016/B978-0-12-809633-8.20429-9.
- [60]. Jurkovic, C.-M. & Boisvert, F.-M. Evolution of techniques and tools for replication fork proteome and protein interaction studies. *Biochemistry and Cell Biology* 102, 135–144 (2024).
- [61]. Wang, S. et al. Protein-protein interaction networks as miners of biological discovery. *Proteomics* 22, (2022).
- [62]. Chan, A. M., Goodis, C. C., Pommier, E. G. & Fletcher, S. Recent applications of covalent chemistries in protein–protein interaction inhibitors. *RSC Med Chem* 13, 921–928 (2022).
- [63]. Meftahi, G. H., Bahari, Z., Zarei Mahmoudabadi, A., Iman, M. & Jangravi, Z. Applications of western blot technique: From bench to bedside. *Biochemistry and Molecular Biology Education* 49, 509–517 (2021).
- [64]. Begum, H., Murugesan, P. & Tangutur, A. D. Western Blotting: A Powerful Staple In Scientific and Biomedical Research. *Biotechniques* 73, 58–69 (2022).
- [65]. Towbin, H., Staehelin, T. & Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 76, 4350–4 (1979).
- [66]. Sule, R., Rivera, G. & Gomes, A. V. Western Blotting (immunoblotting): History, Theory, Uses, Protocol and Problems. *Biotechniques* 75, 99–114 (2023).
- [67]. Pillai-Kastoori, L., Schutz-Geschwender, A. R. & Harford, J. A. A systematic approach to quantitative Western blot analysis. *Anal Biochem* 593, 113608 (2020).
- [68]. Tsurusawa, N. et al. Modified ELISA for Ultrasensitive Diagnosis. *J Clin Med* 10, 5197 (2021).
- [69]. Alhajj, M., Zubair, M. & Farhana, A. Enzyme Linked Immunosorbent Assay. (2024).
- [70]. Portilho, A. I., Gimenes Lima, G. & De Gaspari, E. Enzyme-Linked Immunosorbent Assay: An Adaptable Methodology to Study SARS-CoV-2 Humoral and Cellular Immune Responses. *J Clin Med* 11, 1503 (2022).
- [71]. Sakamoto, S. et al. Enzyme-linked immunosorbent assay for the quantitative/qualitative analysis of plant secondary metabolites. *J Nat Med* 72, 32–42 (2018).
- [72]. Alhajj, M., Zubair, M. & Farhana, A. Enzyme Linked Immunosorbent Assay. (2024).
- [73]. Hayrapetyan, H., Tran, T., Tellez-Corrales, E. & Madiraju, C. Enzyme-Linked Immunosorbent Assay: Types and Applications. *Methods Mol Biol* 2612, 1–17 (2023).
- [74]. Peng, P. et al. Emerging ELISA derived technologies for in vitro diagnostics. *TrAC Trends in Analytical Chemistry* 152, 116605 (2022).
- [75]. Barsanti, L., Birindelli, L., Sbrana, F., Lombardi, G. & Gualtieri, P. Advanced Microscopy Techniques for Molecular Biophysics. *Int J Mol Sci* 24, 9973 (2023).
- [76]. Jacquemet, G., Carisey, A. F., Hamidi, H., Henriques, R. & Leterrier, C. The cell biologist’s guide to super-resolution microscopy. *J Cell Sci* 133, (2020).
- [77]. Siegerist, F., Drenic, V., Koppe, sssssssssssssssssssssT.-M., Telli, N. & Endlich, N. Super-Resolution Microscopy: A Technique to Revolutionize Research and Diagnosis of Glomerulopathies. *Glomerular Dis* 3, 19–28 (2022).
- [78]. Badawi, Y. & Nishimune, H. Super-resolution microscopy for analyzing neuromuscular junctions and synapses. *Neurosci Lett* 715, 134644 (2020).
- [79]. Elliott, A. D. Confocal Microscopy: Principles and Modern Practices. *Curr Protoc Cytom* 92, (2020).
- [80]. Wu, Y. et al. Multiview confocal super-resolution microscopy. *Nature* 600, 279–284 (2021).
- [81]. Mendes, A., Heil, H. S., Coelho, S., Leterrier, C. & Henriques, R. Mapping molecular complexes with super-resolution microscopy and single-particle analysis. *Open Biol* 12, (2022).
- [82]. Cardellini, J., Balestri, A., Montis, C. & Berti, D. Advanced Static and Dynamic Fluorescence Microscopy Techniques to Investigate Drug Delivery Systems. *Pharmaceutics* 13, 861 (2021).
- [83]. Kaur, A., Kaur, P. & Ahuja, S. Förster resonance energy transfer (FRET) and applications thereof. *Analytical Methods* 12, 5532–5550 (2020).
- [84]. Menaes, A. et al. Simplified Instrument Calibration for Wide-Field Fluorescence Resonance Energy Transfer (FRET) Measured by the Sensitized Emission Method. *Cytometry Part A* 99, 407–416 (2021).
- [85]. Wallace, B. & Atzberger, P. J. Förster resonance energy transfer: Role of diffusion of fluorophore orientation and separation in observed shifts of FRET efficiency. *PLoS One* 12, e0177122 (2017).
- [86]. Shrestha, D., Jenei, A., Nagy, P., Vereb, G. & Szöllősi, J. Understanding FRET as a Research Tool for Cellular Studies. *Int J Mol Sci* 16, 6718–6756 (2015).

- [87]. Förster, Th. Zwischenmolekulare Energiewanderung und Fluoreszenz. *Ann Phys* 437, 55–75 (1948).
- [88]. Agam, G. et al. Reliability and accuracy of single-molecule FRET studies for characterization of structural dynamics and distances in proteins. *Nat Methods* 20, 523–535 (2023).
- [89]. Hirata, E. & Kiyokawa, E. Future Perspective of Single-Molecule FRET Biosensors and Intravital FRET Microscopy. *Biophys J* 111, 1103–1111 (2016).
- [90]. Varghese, S. S., Zhu, Y., Davis, T. J. & Trowell, S. C. FRET for lab-on-a-chip devices — current trends and future prospects. *Lab Chip* 10, 1355 (2010).
- [91]. Lim, J., Petersen, M., Bunz, M., Simon, C. & Schindler, M. Flow cytometry based-FRET: basics, novel developments and future perspectives. *Cellular and Molecular Life Sciences* 79, 217 (2022).
- [92]. Shen, Y. et al. FRET-based innovative assays for precise detection of the residual heavy metals in food and agriculture-related matrices. *Coord Chem Rev* 469, 214676 (2022).
- [93]. Sorzano, C. O. S. & Carazo, J. M. Cryo-Electron Microscopy: The field of 1,000+ methods. *J Struct Biol* 214, 107861 (2022).
- [94]. Leung, M. R. & Zeev-Ben-Mordehai, T. Cryo-electron microscopy of cholinesterases, present and future. *J Neurochem* 158, 1236–1243 (2021).
- [95]. Benjin, X. & Ling, L. Developments, applications, and prospects of cryo-electron microscopy. *Protein Science* 29, 872–882 (2020).
- [96]. Renaud, J.-P. et al. Cryo-EM in drug discovery: achievements, limitations and prospects. *Nat Rev Drug Discov* 17, 471–492 (2018).
- [97]. Danev, R., Yanagisawa, H. & Kikkawa, M. Cryo-Electron Microscopy Methodology: Current Aspects and Future Directions. *Trends Biochem Sci* 44, 837–848 (2019).
- [98]. Ștefan, A. E. et al. Tissue microarrays – brief history, techniques and clinical future. *Romanian Journal of Morphology and Embryology* 61, 1077–1083 (2021).
- [99]. Kaur, R., A, N., Bansal, R., Saxena, S. & Rai, B. Tissue microarray- A review. *International Journal of Oral Health Dentistry* 4, 152–155 (2020).
- [100]. Zhang, W., Qiu, F., Jiang, Q., Liu, S. & Xiong, Z. Simple method for constructing and repairing tissue microarrays using simple equipment. *Journal of International Medical Research* 49, 030006052110001 (2021).
- [101]. Oyejide, L., Mendes, O. R. & Mikaelian, I. Molecular Pathology. in *A Comprehensive Guide to Toxicology in Nonclinical Drug Development* 407–445 (Elsevier, 2017). doi:10.1016/B978-0-12-803620-4.00016-5.
- [102]. Cui, M., Cheng, C. & Zhang, L. High-throughput proteomics: a methodological mini-review. *Laboratory Investigation* 102, 1170–1181 (2022).
- [103]. Robinson, J. P., Ostafe, R., Iyengar, S. N., Rajwa, B. & Fischer, R. Flow Cytometry: The Next Revolution. *Cells* 12, 1875 (2023).
- [104]. D'Amato Figueiredo, M. V., Alexiou, G. A., Vartholomatos, G. & Rehder, R. Advances in Intraoperative Flow Cytometry. *Int J Mol Sci* 23, 13430 (2022).
- [105]. Robinson, J. P. Flow Cytometry: Past and Future. *Biotechniques* 72, 159–169 (2022).
- [106]. Welsh, J. A. et al. A compendium of single extracellular vesicle flow cytometry. *J Extracell Vesicles* 12, (2023).
- [107]. Weiskirchen, S., Schröder, S. K., Buhl, E. M. & Weiskirchen, R. A Beginner's Guide to Cell Culture: Practical Advice for Preventing Needless Problems. *Cells* 12, 682 (2023).
- [108]. Kapałczyńska, M. et al. 2D and 3D cell cultures – a comparison of different types of cancer cell cultures. *Archives of Medical Science* (2016) doi:10.5114/aoms.2016.63743.
- [109]. Zuščíková, L., Greifová, H., Bažány, D., Lukáč, N. & Jambor, T. Current approaches and techniques of 3D cell culture systems: a review. *Archives of Ecotoxicology* 6, 22–27 (2024).
- [110]. Ylostalo, J. H. 3D Stem Cell Culture. *Cells* 9, 2178 (2020).
- [111]. Langhans, S. A. Three-Dimensional in Vitro Cell Culture Models in Drug Discovery and Drug Repositioning. *Front Pharmacol* 9, (2018).
- [112]. Ryu, N.-E., Lee, S.-H. & Park, H. Spheroid Culture System Methods and Applications for Mesenchymal Stem Cells. *Cells* 8, 1620 (2019).
- [113]. Tatullo, M. et al. Organoids in Translational Oncology. *J Clin Med* 9, 2774 (2020).
- [114]. Yan, J., Li, Z., Guo, J., Liu, S. & Guo, J. Organ-on-a-chip: A new tool for in vitro research. *Biosens Bioelectron* 216, 114626 (2022).
- [115]. Leung, C. M. et al. A guide to the organ-on-a-chip. *Nature Reviews Methods Primers* 2, 33 (2022).
- [116]. Sonagra, A. D. & Dholariya, S. J. Electrophoresis. (2024).
- [117]. Maity, A., Kesh, S. S., Palai, S. & Egbuna, C. Electrophoretic techniques. in *Analytical Techniques in Biosciences* 59–72 (Elsevier, 2022). doi:10.1016/B978-0-12-822654-4.00007-5.
- [118]. Gummadi, S. & Kandula, V. N. A review on electrophoresis, capillary electrophoresis and hyphenations. *Int J Pharm Sci Res* 11, 6038–6056 (2020).
- [119]. Lee, P. Y., Saraygord-Afshari, N. & Low, T. Y. The evolution of two-dimensional gel electrophoresis - from proteomics to emerging alternative applications. *J Chromatogr A* 1615, 460763 (2020).
- [120]. Li, Y., Miao, S., Tan, J., Zhang, Q. & Chen, D. D. Y. Capillary Electrophoresis: A Three-Year Literature Review. *Anal Chem* 96, 7799–7816 (2024).
- [121]. Hassan, S. Microchip Electrophoresis. *Encyclopedia* 1, 30–41 (2020).
- [122]. Amatori, S. & Fanelli, M. The Current State of Chromatin Immunoprecipitation (ChIP) from FFPE Tissues. *Int J Mol Sci* 23, 1103 (2022).
- [123]. Huang, X., Pan, Q., Lin, Y., Gu, T. & Li, Y. A native chromatin immunoprecipitation (ChIP) protocol for studying histone modifications in strawberry fruits. *Plant Methods* 16, 10 (2020).
- [124]. Saini, A., Rawat, Y., Jain, K. & Mani, I. State-of-the-art techniques to study epigenetics. in 23–50 (2023). doi:10.1016/bs.pmbts.2023.02.004.

- [125]. Yadav, M. P. & Kundra, V. Chromatin Immunoprecipitation and Library Preparation: a Powerful Tool to Unravel the Epigenome. *Biotechniques* 75, 7–10 (2023).
- [126]. Nakato, R. & Sakata, T. Methods for ChIP-seq analysis: A practical workflow and advanced applications. *Methods* 187, 44–53 (2021).
- [127]. Zou, Z., Iwata, M., Yamanishi, Y. & Oki, S. Epigenetic landscape of drug responses revealed through large-scale ChIP-seq data analyses. *BMC Bioinformatics* 23, 51 (2022).
- [128]. Anwar, F., Asrafuzzaman, Amin, K. F. & Hoque, M. E. Tools and techniques for characterizing sustainable hydrogels. in *Sustainable Hydrogels* 47–77 (Elsevier, 2023). doi:10.1016/B978-0-323-91753-7.00014-4.
- [129]. Kaliva, M. & Vamvakaki, M. Nanomaterials characterization. in *Polymer Science and Nanotechnology* 401–433 (Elsevier, 2020). doi:10.1016/B978-0-12-816806-6.00017-0.
- [130]. Pham, T. T. T. & Rainey, J. K. On-cell nuclear magnetic resonance spectroscopy to probe cell surface interactions. *Biochemistry and Cell Biology* 99, 683–692 (2021).
- [131]. Wishart, D. S. et al. NMR and Metabolomics—A Roadmap for the Future. *Metabolites* 12, 678 (2022).
- [132]. Joss, D. & Häussinger, D. Design and applications of lanthanide chelating tags for pseudocontact shift NMR spectroscopy with biomacromolecules. *Prog Nucl Magn Reson Spectrosc* 114–115, 284–312 (2019).
- [133]. Shukla, V. K., Heller, G. T. & Hansen, D. F. Biomolecular NMR spectroscopy in the era of artificial intelligence. *Structure* 31, 1360–1374 (2023).
- [134]. Pintér, G. et al. Real-time nuclear magnetic resonance spectroscopy in the study of biomolecular kinetics and dynamics. *Magnetic Resonance* 2, 291–320 (2021).
- [135]. Ude, A., Afi-Leslie, K., Okeke, K. & Ogbodo, E. Trypan Blue Exclusion Assay, Neutral Red, Acridine Orange and Propidium Iodide. in *Cytotoxicity - Understanding Cellular Damage and Response* (IntechOpen, 2023). doi:10.5772/intechopen.105699.
- [136]. Aslantürk, Ö. S. In Vitro Cytotoxicity and Cell Viability Assays: Principles, Advantages, and Disadvantages. in *Genotoxicity - A Predictable Risk to Our Actual World* (InTech, 2018). doi:10.5772/intechopen.71923.
- [137]. Kamiloglu, S., Sari, G., Ozdal, T. & Capanoglu, E. Guidelines for cell viability assays. *Food Front* 1, 332–349 (2020).
- [138]. Larsson, P. et al. Optimization of cell viability assays to improve replicability and reproducibility of cancer drug sensitivity screens. *Sci Rep* 10, 5798 (2020).
- [139]. World Health Organization & Food and Agriculture Organization of the United Nations. *Principles and Methods for the Risk Assessment of Chemicals in Food*. (2009).
- [140]. Beal, M. A. et al. Quantitative in vitro to in vivo extrapolation of genotoxicity data provides protective estimates of in vivo dose. *Environ Mol Mutagen* 64, 105–122 (2023).
- [141]. Dusinska, M., Rundén-Pran, E., Schneckeburger, J. & Kanno, J. Toxicity Tests: In Vitro and In Vivo. in *Adverse Effects of Engineered Nanomaterials* 51–82 (Elsevier, 2017). doi:10.1016/B978-0-12-809199-9.00003-3.
- [142]. Marchetti, F. et al. Error-corrected next generation sequencing – Promises and challenges for genotoxicity and cancer risk assessment. *Mutation Research/Reviews in Mutation Research* 792, 108466 (2023).
- [143]. Menz, J. et al. Genotoxicity assessment: opportunities, challenges and perspectives for quantitative evaluations of dose–response data. *Arch Toxicol* 97, 2303–2328 (2023).
- [144]. Bhinder, B., Gilvary, C., Madhukar, N. S. & Elemento, O. Artificial Intelligence in Cancer Research and Precision Medicine. *Cancer Discov* 11, 900–915 (2021).
- [145]. Schwalbe, N. & Wahl, B. Artificial intelligence and the future of global health. *The Lancet* 395, 1579–1586 (2020).
- [146]. Yan, X., Liu, X., Zhao, C. & Chen, G.-Q. Applications of synthetic biology in medical and pharmaceutical fields. *Signal Transduct Target Ther* 8, 199 (2023).
- [147]. Liu, A. P. et al. The living interface between synthetic biology and biomaterial design. *Nat Mater* 21, 390–397 (2022).
- [148]. Wu, Q. et al. Organ-on-a-chip: recent breakthroughs and future prospects. *Biomed Eng Online* 19, 9 (2020).
- [149]. Piccollet-D'hahan, N., Zuchowska, A., Lemeunier, I. & Le Gac, S. Multiorgan-on-a-Chip: A Systemic Approach To Model and Decipher Inter-Organ Communication. *Trends Biotechnol* 39, 788–810 (2021).