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

Katiana Simões Kittelson[1,2]; Allana Cristina Faustino Martins[2]; Gizele Celante[2]; Arquimedes Gasparotto Júnior[1]; Roberto Da Silva Gomes[2*]

[1]Laboratory of Cardiovascular Pharmacology (LaFaC), Faculty of Health Sciences, Federal University of Grande Dourados (UFGD), Dourados, MS, Brazil
[2]Department 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*]

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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.*

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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 genetics[1]. 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 mechanisms[2]. Some of these techniques have shown to be accurate and reliable data throughout time, and for that, they are known as gold standard methods[3]. 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 diseases[5].

III. GOLD STANDARD METHODS

An ideal gold standard method displays sensitivity and specificity equal to 100%, which is not practicably achievable[8]. 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 methods[3]. Gold standards may be a single test or a combination of preferred techniques and can vary according

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to experiments and targeted results[9]. 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 cells[10] 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 proteins[10].

Clustered, regularly interspaced short palindromic repeats or CRISPR were identified in DNA sequences of *Escherichia coli* in 1987[12], 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 CRISPR[13]. The synergistic work between them is responsible for the acquired immune system in prokaryotes and archaea by integrating genetic elements into their genome[11]. 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 cells[14], followed by Cas12 gene regulation, which can be engineered for diverse applications. However, they need to be scientifically approached[11].

CRISPR-based gene-editing tools are flexible, efficient, and inexpensive, and for those, they have been widely applied[15]. CRISPR-Cas9 has been applied for genome screening and editing, agricultural applications, and the generation of animal or alternative research models[16]. 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 disorders[10].

- *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 McGrath[20], but it was only in 1996, that Dolly, the sheep, was the first successfully cloned mammal[21]. Since then, new advances in genetic engineering have surfaced and allowed for the perfecting of cloning procedures[22].

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 twins[19].

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 relationships[22]. Thus, 'creating human embryos for research purposes' has been banned since 1998[24].

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 livestock[25] 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 precision[30].

Sanger's DNA sequencing protocol[31] and Maxam-Gilbert methods[32] 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 2014[33].

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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 2009[37], and since then, upgraded versions of the technology have been created, bringing essential adjustments and advancements[38].

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 expression[36]. 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 therapeutics[40]. In drug development, scRNA-seq enhances biomarker identification targeting and novel mechanisms of action[41]. 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-seq[42]. 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 sciences[44].

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 molecules[49].

RNAi therapy uses the innate mechanisms of RNAi[50]. 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 time[49]. 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 evolutional 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 cases[54]. 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].

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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 themselves[55]. 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 interactions[56]. 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 function[55–57].

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 PPIs[58], 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 PPIs[61].

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 protein[62]. 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 pathologies[55].

- *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 quality[67].

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 diseases[63]. It is expected that automation in the WB process and more reliable antibodies may be developed and applied in the future[66], as well as improvements to the reagents, equipment, and technology, facilitating the WB method[64].

- *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 sample[69]. 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 hours[70].

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]

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➢ *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 scale[75].

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 sciences[79]. 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 interactions[85–87], but recently, FRET has also been referring to "fluorescence" due to the usage of fluorescence probes in applications[86]. However, once there is no emission of photons in FRET, Shrestha et al. affirm that the latter definition is incorrect[86].

FRET assay was first applied to describe protein-protein interactions[85], though nowadays, it has been used to supply information and quantitative assessment on the structure of single biomolecules, such as conformational heterogeneity and dynamics[88]. The assay also can characterize enzyme reactions, outline ligand-receptor interactions, and the movement of molecular motor proteins[85].

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 therapeutics[91], as well as to fields other than biosciences and research, such as food and agriculture[92].

- *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 processing[93]. 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 techniques[95].

In cryo-EM, advances in detectors, transmission, and image processing have also been made to improve image acquisition[96]. 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 circumstances[98]. 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 cryomicroarrays[101].

Aiming for a cost-efficient technique with reduced timing and effort, next-generation tissue microarrays, or ngTMAs, have emerged recently[102]. It has demonstrated high-throughput technology and time-efficiency features,

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increased sensitivity, and large-scale detection while enabling annotations to be inserted on digital slides, guaranteeing accuracy without sacrificing data quality[102]. 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 applications[103]. The current method of identification of cells and their properties depends on the recognition of fluorescent signals emitted by probes[103].

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 synthesis[103]. In addition to the biomedical field, the technique has also been applied to biotechnology, veterinary medicine, and industrial applications[105].

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 cytometry[103].

- *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 research[107]. 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 study[107].

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 medium[107]. However, the term now encompasses other techniques that comply with stem cells, which present similar functionality as the original harvested tissue[111]. Organoid culture has been described for several organs and has been currently applied in drug discovery and precision therapy[111]. 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 studies[114].

➢ *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 increased[118].

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 mediums[116]. In this sense, there are two main types of electrophoresis: zone electrophoresis and moving boundary electrophoresis, each including a set of different techniques[118]. Gel and capillary electrophoresis can be highlighted among the widely applied methods.

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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 fields[118]. 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 patterns[119]. 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 fields[120]. 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 results[121]. 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 technique[122]. ChIP is also considered a valuable tool in analyzing *in vivo* interactions between DNA and histones or proteins[123]. 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 modifications[126]. ChIP-seq has also been applied in drug response and discovery[127].

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 immunoprecipitation[125]. 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 cells[126].

➢ *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 level[128]. 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 parameters[129]. 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 studies[131].

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 applied[131].

➢ *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 cells[135]. 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-

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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 acquittance[138] 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 mutagenicity[139]. 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 inflammation[141].

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 included[142]. 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 approach[143].

Adding a determined reference point of dose-response and calculation of margin of exposure might enhance genotoxicity assessment[143]. 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 treatment[144]. AI health interventions can also act on the patient risk assessment of morbidity and mortality, disease outbreaks and surveillance prediction, and health policies[145].

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 engineering[146]. 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 bioprinting[147].

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 toxicology[148]. Furthermore, multiorgan-on-a-chip (multi-OoC) approaches have been studied recently, modeling systematic diseases and leading to information about multiorgan interactions[149].

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;

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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.

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- *Conflicts of Interest*

The authors declare no conflict of interest.

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