UDC 577.3:602.9:57.085.23 https://doi.org/10.15407/biotech19.04.005

THREE-DIMENSIONAL CELL CULTURES IN BIOTECHNOLOGY: A CONTEMPORARY PERSPECTIVE

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Aim. To systematize and critically analyze contemporary three-dimensional (3D) cell culture platforms — spheroids, organoids, bioprinted constructs, and organs-on-chips — and to evaluate their potential as physiologically relevant alternatives to two-dimensional (2D) monolayer systems in biomedical research and preclinical drug evaluation.

Methods. A narrative review of current scientific literature was conducted, encompassing the principal categories of 3D cell culture technologies, their underlying biological and engineering principles, cultivation methodologies, extracellular matrix (ECM) composition, and available standardization platforms. Comparative analysis of 2D and 3D culture properties was performed, alongside evaluation of key analytical approaches — including Ki-67 immunolabeling, BrdU/EdU incorporation, metabolic assays, and confocal microscopy — adapted for three-dimensional structures.

Results. Three-dimensional cell cultures substantially surpass 2D monolayers in recapitulating cell–cell and cell–ECM interactions, diffusion gradients, and tissue-specific spatial organization. Each principal platform demonstrates distinct applicability: MCTS reproduces solid tumor zonal architecture for oncological and pharmacological studies; organoids enable tissue-specific self-organization for disease modeling and personalized medicine; bioprinting allows precise spatial deposition of cells and scaffolds; and organs-on-chips integrate microfluidic perfusion to model dynamic physiological barrier functions.

Conclusions. Three-dimensional cell culture systems represent a scientifically substantiated and ethically consistent alternative to animal models, advancing the implementation of the 3Rs principles in experimental biomedicine. Nevertheless, their broad translational application remains constrained by insufficient standardization, limited reproducibility, high operational costs, and the absence of functional vascularization. Overcoming these barriers requires harmonizing validated protocols and integrating them with high-throughput analytical platforms.

Keywords: three-dimensional cell cultures, spheroids, organoids, organs-on-chips, bioprinting, extracellular matrix, bioinks, oncology, pharmacology, regenerative medicine.

Citation: Trokhimenko, O. P., Mysko, Yu. P., Soloviov, S. O., Rossokha, Z .I., Smetiukh, M. P., Trokhymchuk, V. V., Pryputa, N. V., Rymar, M. V., Leseiko, T. O. (2026). Three-dimensional cell cultures in biotechnology: a contemporary perspective. *Biotechnologia Acta*, 19(4), 5–18. https://doi.org/10.15407/biotech19.04.005

Animal models remain the gold standard in biomedical research and regulatory assessment of drug safety and efficacy. However, modern biotechnology increasingly promotes alternative *in vitro* approaches aligned with the 3R principles (Replacement, Reduction, Refinement), aiming to enhance the physiological relevance of experimental systems. Three-dimensional (3D) cell cultures offer significant advantages over conventional two-dimensional monolayers by better recapitulating cell–cell and cell–extracellular matrix interactions as well as tissue-like spatial organization under in vivo-like conditions. A wide range of 3D platforms has been developed to date, including spheroids, organoids, bioprinted constructs, and organs-on-chips, which are finding application in pharmacology, oncology, toxicology, regenerative medicine, and embryology and developmental biology. Nevertheless, challenges related to standardization, reproducibility, automation, and cost still limit their broad industrial implementation. Further development of validated and high-throughput 3D culture systems is essential for advancing experimental design and fostering innovation in contemporary biotechnology.

In recent years, it has become evident that the complex architecture of animal and human tissues cannot be adequately represented by two-dimensional (2D) cell systems, which are widely used to model pathological processes and to evaluate drugs preclinically. As a result, three-dimensional (3D) cell cultures are attracting increasing attention from researchers. Since their first application in the 1970s as floating collagen gels, 3D cultures have evolved considerably and continue to be developed and refined, with the ultimate goal of reproducing native tissues and intact organs *in vitro*. Various scientific disciplines have contributed to the development of 3D culture tools and technologies, including medicine, biology, biochemistry and biophysics, bioengineering, materials science, microfluidics, bioprinting, digital technologies, and even artificial intelligence. The convergence of these efforts toward a common goal has led to the development of numerous 3D cell culture systems designed to meet specific scientific needs. The transition from monolayer (2D) cell cultures to 3D cultures is driven by the need to use cellular models that better mimic the functions of living tissues as integral structural units of the organism. Despite the considerable diversity of methods available today for their generation and maintenance, 2D cell cultures fail to capture important tissue properties, which limits their predictive potential for cellular responses under whole-organism conditions. At the same time, the integration of 3D cultures into Good Laboratory Practice (GLP) requires the development of standardized protocols, new cell lines, and quantitative analytical methods — including reliable approaches for three-dimensional identification, validation, and visualization. Under these conditions, 3D cell cultures are invaluable tools in embryology, cell biology, oncology, and pharmacy. In the preclinical evaluation of drugs in particular, the use of such cultures substantially reduces the use of laboratory animals in accordance with EU Directive 2010/63/EU, which invokes the 3Rs (Reduction, Refinement, and Replacement) — providing for the reduction, replacement, and improvement of animal use wherever possible, for example in the assessment of the toxicity of candidate drugs, as well as in personalized approaches to chemotherapy in oncology [1].

When working with 3D cultures, especially in pharmacy for preclinical drug studies, great attention is paid to standardizing research protocols and integrating them into the GLP system to ensure reproducibility of results. This task is even more difficult than with 2D cell cultures. To do this, a system of interconnected SOPs, acceptance criteria, and quality control (QC) must be developed and integrated into GLP, tailored to the specific cell culture and selected 3D model. It should be noted that there is currently no separate universal OECD/FDA protocol specifically for spheroids or organoids; instead, GLP, GIVIMP, and GCCP 2.0 set the boundaries within which a specific 3D model should be standardized and validated. This very large-scale methodological work goes beyond the scope of the presented literature review and is the subject of further research.

By providing a reproducible, controlled microenvironment that mimics in vivo conditions — including cell–cell and cell–extracellular matrix (ECM) interactions, as well as biochemical and oxygen gradients — 3D cell culture faithfully reproduces the appropriate structural architecture and differentiated function of normal tissues or tumors *in vitro*, emerging as an effective alternative to the use of animal models. Moreover, owing to the possibility of engineering designer microenvironments, 3D cultures allow investigation of questions that are difficult to address in the whole organism [2]. Thus, 3D

cell cultures realistically model the states and processes occurring in the tissues of an intact organism and reproduce responses to exogenous and endogenous stimuli.

Comparison of 2D and 3D Cell Culture Properties

The Table compares the principal properties of 2D and 3D cell culture formats [3].

For the cultivation of three-dimensional cell cultures, culture vessels made of low-adhesion polystyrene with hydrophobic surfaces are used, as are macroporous scaffolds — for example, meshes, foams, or fibrous structures based on polystyrene — which allow cells to be spatially distributed throughout the full thickness of the matrix. However, the cell–matrix interactions in these systems are closer to those of 2D cell cultures, and they are therefore referred to as semi-3D or 2.5D cultures [4]. Hydrogel substrates are also suitable for cultivation — for example, gel beads, injectable gels, and other synthetic materials that can be used as hydrogels: polyethylene glycol (PEG), polyvinyl alcohol (PVA), poly(2-hydroxyethyl methacrylate) (pHEMA), and polycaprolactone (PCL). Natural polymers and proteins capable of forming hydrogels are also suitable: alginate, collagen, chitosan, dextran, fibrin, as well as alginate–hyaluronan composites.

The most widely used techniques for culturing three-dimensional cell cultures are the hanging drop method — regarded as the classical approach — cell encapsulation in hydrogel matrices, and cultivation in microwell plates with either microstructured or non-microstructured hydrophobic surfaces [4–6].

The 3D cell culture models available today can be divided into four principal categories: (1) spheroids — clusters of randomly aggregated cells forming spherical structures; (2) organoids — tiny self-organized three-dimensional tissue cultures derived from stem cells (SCs); (3) 3D bioprinted models — more organized, artificially constructed structures combining cells and extracellular matrix (ECM); and (4) organs-on-chips — models that incorporate microfluidic circulation and are used to simulate blood flow, mechanical factors, mechanical stress, and to study interactions between different tissues.

Spheroids

Spheroids were the first 3D cell culture models. The use of spheroids was pioneered in the foundational studies of Sutherland and colleagues [7]. Johannes Holtfreter investigated tissue morphogenesis using spherical reaggregated cultures of embryonic or malignant cells [8]. Subsequently, multicellular tumor spheroids (MCTS) — also

Table. **Comparison of properties of 2D and 3D cell cultures**

2D culture	3D culture
Hydrophilic growth surface	Hydrophobic growth surface
Cells grow as a monolayer and spread across the growth surface	Cells form aggregates / spheroids; native cell architecture is preserved
Higher proliferation rate than in the natural environment; poorly differentiated	Proliferation and differentiation rates are cell line- or tissue-specific and depend on the 3D system used
Cell–cell contact is limited; cell–substrate surface interaction predominates	Cell–cell contact predominates
Contact with the extracellular matrix (ECM) occurs only at a single cell surface	Cells remain in contact with the extracellular matrix (ECM) across most of their surface area
No diffusion gradient between cells	Diffusion gradients of nutrients, metabolic products, oxygen, and test substances are present between cells
Co-culture systems have limited ability to recapitulate the microenvironment	Co-culture systems can simulate the microenvironment
No resistance to antitumor agents	Resistance to antitumor agents is present (mimicking tumor morphology)
Gene expression levels are lower compared to the 3D model	More physiologically relevant gene expression levels, closer to *in vivo* conditions
Relatively low cost	High cost

referred to as MTS — were introduced [9]. These are spherical, scaffold-free (stroma-free) self-organized aggregates of cancer cells that represent an intermediate between two-dimensional (2D) cell cultures *in vitro* and solid tumors in vivo, with which they share important characteristics. MCTS have also been used to study fundamental biological mechanisms, including the regulation of cell proliferation, differentiation, cell death, invasion, angiogenesis, and immune response. Early attempts to initiate spheroids directly from patient biopsy material for routine individualized drug sensitivity testing were unsuccessful, owing to the small number and considerable heterogeneity of such cells, and to the inability to reproduce the key stromal, immune, and matrix elements of a real tumor. For this reason, MCTS derived from stable cell lines were used as *in vitro* models to study the mechanisms governing drug penetration, binding, and action [10]. Today, the challenges of forming spheroids from limited patient biopsy material are being addressed using microfluidic platforms, organs-on-chips, and microstructured culture plates, which enable controlled, standardized spheroid formation for use in precision oncology [11].

Spheroids improve the relevance of *in vitro* results, serve as biological models of native tissues in tissue engineering, are used as building blocks for tissue formation, and — together with other aggregated cell formats — enable complex studies of the architectural organization of the resulting microtissues [11, 12].

The general principle of spheroid cultivation is to prevent cell attachment to the substrate surface. Many non-transformed and most malignant cells tend to form spherical aggregates when cultured in vessels with non-adhesive surfaces, which enables spheroid cultivation. A further characteristic of tumor spheroids is that, similarly to tumors in vivo, they contain both a surface-exposed, well-oxygenated outer cell layer and deeper-lying viable proliferating and non-proliferating cells, as well as a hypoxic core with necrotic cells — the latter being dependent on gradients of nutrients, oxygen, lactate, metabolites, pH, and other factors [13, 14]. These diffusion gradient characteristics, together with the absence of vascularization, account for the critical spheroid diameter of approximately 500 μm (ranging from 400 to 600 μm depending on cell type, microenvironment, and diffusion limitations). Exceeding this volume leads to hypoxia, necrosis, and zonal disorganization of spheroid cells [14].

Assessment of cell proliferative activity in spheroids

To assess cell proliferative activity in spheroids, a combination of molecular, immunohistochemical (IHC), metabolic, and imaging methods is employed — including light microscopy and confocal microscopy — adapted for 3D structures. Among these, the most informative are Ki-67 immunolabeling, particularly when combined with 3D imaging, and BrdU or EdU incorporation. The established "gold standard" for assessing cell proliferative activity is immunolabeling followed by flow cytometric quantification of the Ki-67 nuclear protein (MKI67). Ki-67 is expressed across all phases of the cell cycle (G1, S, G2, M), except G0; however, in spheroids, it is typically detected predominantly in cells located in the peripheral (proliferating) zone. BrdU (5-bromo-2′-deoxyuridine) or EdU (5-ethynyl-2′-deoxyuridine) incorporation enables investigation of DNA synthesis by measuring the intensity of nucleoside analog incorporation into DNA during the S phase of the cell cycle. This method allows spatial localization of proliferation and quantitative assessment of the proportion of cells in S phase. It has proven reliable for analyzing cell proliferation in organoids and spheroids. Metabolic assays — MTT, XTT, and resazurin/AlamarBlue® — are indirect methods and can be used to compare changes in proliferation in response to various factors or compounds. Quantitative morphometric approaches, such as changes in spheroid diameter or volume over time, or in growth rate, are simple and readily reproducible; however, the resulting parameters are non-specific because they are influenced by apoptosis and necrosis within the spheroids. [15, 16] Nevertheless, metabolic assays and morphometry are appropriate as complementary or screening methods and are most informative when used in combination with Ki-67 immunolabeling, 3D imaging, and BrdU or EdU incorporation.

In recent years, confocal microscopy has gained increasing importance in spheroid research. Native tumor tissues are heterogeneous, containing histologically distinct cell types: immune cells, fibroblasts, endothelial cells, glandular cells, and others. When using MCTS, this heterogeneity can be partially simulated *in vitro* through heterotypic spheroids composed of mixtures of patient-derived suspended cells — such as fibroblasts or monocytes — and cells from established tumor cell lines. MCTS combined with immune cells, such as monocytes or

cytotoxic lymphocytes, can be used to study immune cell infiltration and the cytostatic and/or cytotoxic effects of these cells on tumor cells [17]. Co-cultures of MCTS and fibroblasts are used to study tumor metastasis into connective tissue stroma [18]. Co-cultures of MCTS and endothelial cells represent a well-established model for studying angiogenesis and tumor invasion [19].

Organoids

Organoids are structures derived from clusters of stem cells (SCs) and engineered to precisely recapitulate the complex architecture and functions of human organs such as the lungs, liver, brain, and intestine. Organoids self-organize and self-renew *in vitro* through cell–cell and cell–extracellular matrix (ECM) interactions, mimicking organogenesis *in vivo*.

Organoids provide a more faithful representation of complex cellular responses and interactions in vivo than conventional two-dimensional (2D) cell cultures and spheroids. The principal cell sources for organoids are pluripotent stem cells (PSCs) — including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and organ-specific adult stem cells (ASCs). Organoids contain multiple cell types, reflect organizational and structural complexity, and recapitulate at least some aspects of tissue functionality. Organoids possess the capacity for cell self-organization *in vitro*, mediated by the complexity of the culture medium, control of cell–ECM interactions, and the presence of growth and differentiation factors [20, 21]. Organoids can also be generated by combining stem cells derived from iPSCs with mature tissues — such as adipocytes and bone marrow cells — and then differentiating them into various human cell types [21].

As microscale models of human tissues, organoids can be applied in oncology, neurobiology, pathophysiology, embryology and developmental biology, pharmacology, and related fields. When derived from stem cells, organoids can be differentiated into various tissue types, including liver, lung, brain, kidney, stomach, and intestine. Organoids derived from genetically modified cells allow investigation of the relationship between mutations and specific genetic disorders. Organoids can facilitate the study of viral infection pathogenesis at the tissue level [22]. In personalized medicine, organoids derived from patient tissue can be used for drug screening and assessment of drug toxicity and efficacy.

Working with patient material in personalized medicine to obtain organoids and organs-on-chips primarily requires procedures such as obtaining clinical material, isolating cells, cultivating them in media supplemented with growth factors and extracellular matrix, generating 3D cultures, and using them in preclinical studies. At each stage of the work, corresponding limitations arise: biological, technical, analytical, regulatory, organizational, and ethical. First, it should be noted that organoids and organs-on-a-chip are not complete copies of the patient's organ or tumor and do not reproduce the full complexity of native tissue architecture. The initial tumor may contain dozens of subclones of cells, and when cultivated, depending on the composition of the medium, extracellular matrix and growth factors, one or more of them may gain an advantage in accumulation: culture conditions may create a selective advantage for certain cells, therefore, it is necessary to experimentally confirm what part of the population cells is preserved during cultivation and, accordingly, which cells are expected to be affected by the drug under study. As the number of passages increases, the genotypic and phenotypic stability of the cell population may change. In addition, it is not possible to obtain a stable organoid from every patient sample; success depends on the type of cancer, tissue quality, method of cell preparation for cultivation, and the composition of the medium. Another limitation is that the pharmacokinetic parameters obtained from a 3D culture model do not always correspond to those in a whole organism; in particular, the effective concentration of the chemotherapy drug *in vitro* may be unattainable in the patient's target tissue. In this regard, the answer to the most difficult question — whether the selected 3D model predicts the real patient response — requires further validation and refinement. Ethical and organizational issues also impose certain restrictions on the use of patients' clinical material. Thus, organoids and organs-on-a-chip derived from clinical material from patients in preclinical drug studies, while offering undeniable advantages, have significant limitations that depend on the clinical material and the selected cultivation model.

General principles of organoid generation

The generation and analysis of organoids is a more complex and labor-intensive process than that of spheroids, and comprises five sequential steps:

Step 1. 2D pre-culture. Culture duration: 5–7 days or longer. Primary tissue stem cells, or iPSCs, capable of differentiating and self-assembling into a variety of tissue-specific organoids, are cultured in 2D format to expand the cell population.

Step 2. Formation of 3D organoids in Matrigel®. Cells are mixed with Matrigel® and dispensed as individual droplets into plate wells, where they self-assemble into three-dimensional structures.

Step 3. Organoid culture. Culture duration: 3 to 10 weeks. Organoids are cultured in tissue-specific media of varying composition that support the growth, differentiation, and spatial organization of the microtissues. The condition of organoids is monitored visually or under an inverted microscope at low magnification.

Step 4. Growth and development monitoring. Organoid development is monitored by light or fluorescence microscopy, with assessment of the size, shape, and structural complexity of the formed organoids. Before conducting primary studies, it is necessary to confirm that the organoids display appropriate tissue architecture and differentiation.

Step 5. Confocal imaging and 3D analysis. Confocal microscopy enables quantitative analysis of cellular composition, spatial organization, and the effects of test compounds in 3D; detection and quantification of organoids per unit volume or surface area; analysis of the cells composing them; and assessment of the effects of test compounds [23].

Extracellular Matrix (ECM)

Virtually all known human and animal cell types are amenable to cultivation in 3D format — including primary and passaged cell cultures, embryonic, somatic, and tumor cells, as well as stem cells and induced pluripotent stem cells (iPSCs). However, cultivation success depends not only on cell type and proliferative potential but also on the composition of the culture medium, the presence of appropriate growth factors, and the characteristics of the extracellular matrix (ECM), which should approximate those of the native environment.

The extracellular matrix (ECM) is produced by the cells themselves during cultivation and can also be engineered artificially. The native ECM contains fibrillar and non-fibrillar proteins — collagen types I, III, and IV, laminins, fibronectin, elastin, proteoglycans and glycosaminoglycans (GAGs), heparan sulfate, chondroitin sulfate, adhesion proteins, and others. These components interact with cell-surface integrins, providing cell–cell and cell–matrix adhesion, cell aggregation, and the mechanical stability of three-dimensional structures. In addition, the ECM plays a key role in regulating cell proliferation, differentiation, migration, and spatial positioning — and it is precisely this that underpins the possibility of modeling cell microenvironments *in vitro* in three-dimensional cell cultures [24, 25]. Synthetic extracellular matrices for organoids and spheroids are engineered with different biological objectives and therefore differ substantially in composition, mechanical properties, and degree of bioinstructiveness.

In spheroid cultivation, the ECM is typically minimal or secondary relative to cell–cell interactions. Most classical spheroids — particularly tumor MCTS — form under conditions of low or absent substrate adhesion, in which the key aggregation mechanism is cadherin-mediated cell–cell adhesion rather than cell–matrix interactions. The ECM may be absent during the initial stages of spheroid cultivation and is subsequently produced by the cells themselves during culture. Its composition is generally simplified and variable, containing small amounts of collagen types I and IV, fibronectin, laminin in peripheral zones, and some proteoglycans and glycosaminoglycans (GAGs). Functionally, the ECM in spheroids does not define tissue architecture and serves primarily mechanical and signaling roles — modulating cell migration, invasion, and response to therapy, and contributing to the formation of oxygen and nutrient gradients [26].

In organoid cultivation, the ECM is an obligatory and defining component without which organoid formation is practically impossible. Organoids are typically cultured within hydrogels or gel complexes based on exogenous matrices — most commonly Matrigel® and its analogs — that mimic the tissue basement membrane. The ECM of organoids is characterized by substantial structural and biochemical complexity and by the predominance of basement membrane components; it obligatorily contains laminins, collagen type IV, entactin/nidogen, heparan sulfate, and proteoglycans. This matrix provides cell polarization, maintains specialized stem cell niches in tissues, directs lineage-specific differentiation, and governs the spatial organization of the epithelium. Furthermore, the ECM in organoids actively

interacts with integrins and regulates the Wnt, Notch, and TGF- signaling pathways, determining morphogenesis, crypt-villus architecture, and the functional specialization of cells [27 29].

Thus, in organoid cultures, an attempt is made to reconstitute the stem cell niche *in vitro* through the use of Matrigel® or synthetic hydrogels, the addition of specific signaling factors, and control of the environment's mechanical properties. It is precisely for this reason that organoids are capable of self-organization and maintenance of tissue hierarchy — in contrast to spheroids, which are aggregates of heterogeneous or co-cultured cells.

Hydrogels, Hydrogel Matrices, and Standardization Challenges

In the cultivation of spheroids and organoids, techniques involving cell encapsulation in gels or gel complexes — typically of undefined composition — are employed. The most commonly used material is Matrigel® — a biological matrix gel derived from the basement membrane of the Engelbreth-Holm-Swarm (EHS) murine sarcoma, which reconstitutes the composition of the basement membrane in vivo. Matrigel® contains a heterogeneous mixture of growth factors (TGF- , EGF, IGF, and others) and ECM proteins (laminins, collagens, entactin/nidogen, and others) at elevated, non-physiological concentrations. Growth Factor-Reduced Matrigel® (GFR Matrigel®) variants exist in which the concentrations of these factors are substantially reduced. Due to their undefined composition, batch-to-batch variability, and extreme temperature sensitivity, these hydrogels require labor-intensive sample preparation steps and precautionary measures, rendering them unsuitable for standardized, high-throughput toxicological studies. At the same time, Matrigel® is incorporated into standard cultivation protocols for intestinal, hepatic, prostate, and breast tumor organoids, as well as in invasion and angiogenesis assays and in the maintenance of pluripotent and tissue-specific stem cells [29, 30].

Recently, synthetic hydrogels have been developed for 3D cultures that are more amenable to cultivation, contain no animal-derived proteins, and can substitute for native ECM by supporting organoid formation and 3D aggregation. However, these materials still require users to pre-mix cells and hydrogels before encapsulation [27, 31].

Today, to address the challenges outlined above in forming 3D cultures using hydrogel matrices, purpose-designed constructs are employed—TrueGel3D® HTS Hydrogel Plates (MilliporeSigma/Sigma-Aldrich). These are ready-to-use constructs based on fully synthetic, standardized polyethylene glycol (PEG)-based hydrogels, formatted as 96-well polystyrene plates with a transparent base on which synthetic functional hydrogels with a gradually increasing crosslink density — from top to bottom across the full depth of each well — have been pre-formed [32]. When using such plates, cell seeding occurs without any hydrogel preparation or encapsulation steps. Following seeding, cells gradually infiltrate the hydrogel and, over several days, establish a 3D culture. The hydrogel plates enable the sequential creation of co-culture systems that model human tumor tissue by inoculating different cell populations into the same well at different time points. The assembled hydrogel is optically transparent and compatible with imaging, including fluorescent dyes, as the 96-well black polystyrene plate has a glass base, allowing spheroid formation and growth to be monitored under an inverted microscope [33].

However, despite the advances achieved with TrueGel3D® HTS Hydrogel Plates for spheroid formation, standardization of shape, size, structure, and biomechanical properties remains a challenge. This can be addressed through the use of fully ready-to-use, standardized microstructured platforms — Sphericalplate 5D® (SP5D) (Kugelmeiers AG, Switzerland) — specialized Society for Biomolecular Sciences (SBS)-format culture plates compatible with standard laboratory equipment, including incubators, microscopes, and high-content analysis (HCA) systems. In these plates, each standard well contains a defined number of microwells, each designed to form a single spheroid. This enables the generation of uniform, standardized spheroids for scientific and preclinical research with high reproducibility and scalability. The base of the SP5D microwells features a convex/rounded geometry and low adhesion, which promotes the spontaneous formation of three-dimensional cell aggregates following inoculation of a cell suspension — without additional induction — and yields large numbers of spheroids of uniform size and morphology within a single plate. Control of spheroid size is achieved primarily by optimizing seeding cell density, followed by monitoring spheroid formation and growth under a bright-field inverted microscope,

thereby simplifying the protocol and reducing the risk of technical errors. This is critically important for comparable experiments investigating cytotoxicity and cell viability in preclinical and clinical drug studies, for predicting MCTS sensitivity to chemotherapy, for culturing stem cells, induced pluripotent stem cells (iPSCs), and organoids to study organogenesis and embryonic induction, differentiation, as well as for bioprinting and organ-on-a-chip development [34–37].

The use of the proposed platforms, in particular spheroids and organoids, is now both technically feasible and economically justified for widespread adoption in the pharmaceutical industry. When forming spheroids, the problem remains the standardization of their shape, size, structure, and biomechanical properties. It can be solved by using completely ready-to-use standardized microstructured platforms, Kugelmeier's Sphericalplate 5D (SP5D) — special culture plates of Society for Biomolecular Sciences (SBS) format, suitable for working with standard laboratory equipment — incubators, microscopes, and high-content analysis systems. The use of SP5D allows you to standardize and unify the methods of cultivating primary, transplantable, embryonic, stem, induced pluripotent stem, and, importantly, tumor cells obtained from biopsy material from patients, and therefore significantly accelerate and reduce the cost of preclinical studies of drugs, in particular in oncology, and in personalized medicine [34].

Nevertheless, despite significant innovations in the biomedical field enabled by 3D cell culture models, these systems still have limitations — most notably the absence of functional vascularization, owing to the complexity of culturing vascular endothelial cells, which frequently results in immature cell development, inefficient nutrient distribution, and the formation of necrotic zones in the central regions of the culture [38, 39].

These problems are quite effectively solved in the "Organs on a Chip" and "Bioprinting" technologies using spheroids and organoids. Thus, in microfluidic "Organs on a Chip" systems, various 3D cell cultures are supported in integrated systems that interact through microchannels and fluidic channels [50]. And in the bioprinting, to ensure proliferation, differentiation, and angiogenesis, vascular growth factors VEGF, TGF- , integrin-binding peptides, nanoparticles, and extracellular vesicles are introduced into the bioink, which, in a certain way, ensure the formation of vessels and vascularization of bioprinted tissues [46].

Although most spheroids and organoids may contain the relevant cell types, their structural organization does not fully reflect the architecture of organs in vivo. Furthermore, 3D methods are resource-intensive in terms of material requirements and cultivation time and do not allow full control over the physicochemical properties of the cellular microenvironment. This gives rise to the need to integrate 3D cell culture with microfluidic systems — known as organs-on-chips — and to use bioprinting. Although these systems show considerable promise, they are relatively new technologies that remain at early stages of development [40, 41].

3D Bioprinting

3D bioprinting technology involves the precise, layer-by-layer deposition of cells, biological scaffolds (stroma), and growth factors to create bioidentical tissues. However, before 3D bioprinting becomes clinically significant, numerous technical and biological challenges must be resolved.

The concept of 3D tissue bioprinting was first proposed by Charles Hull in 1986, who suggested that successive layers of biological material could be deposited one upon another using the principle of stereolithography (SLA) [42]. Modern 3D bioprinting integrates engineering, biomedicine, manufacturing, and digital technologies. Compared to spheroids, organoids, and organs-on-chips, this technology enables greater precision in the spatial relationships between individual elements of the target tissue. Three principal approaches to bioprinting are currently employed: biomimicry, autonomous self-assembly, and the microtissue-based approach.

The biomimicry approach seeks to recreate individual components of native tissue. Beyond the numerous cell types, signaling molecules, and structural stromal elements present within the tissue itself, all environmental factors — including pressure, temperature, and electrical forces — must be taken into account [43]. The advantage of biomimicry lies in the possibility of precise control at every stage of tissue construction, with high-accuracy cell positioning; however, accounting for all the factors that must be reproduced is extraordinarily complex.

In autonomous self-assembly, the aim is to reproduce the embryonic environment, thereby enabling autoregulation and self-renewal of the source elements, including stem cells (SCs)

and extracellular matrix (ECM). This approach enables rapid, efficient tissue growth at high cell density and does not require scaffolds; however, it does not permit precise positioning of individual cells or modification of the self-assembly process once initiated [44].

The microtissue-based approach is grounded in the concept that a typical complex tissue in vivo contains many simpler units — microtissues — whose collective structure must provide the principal functions characteristic of the target macrotissue. This approach allows rapid and efficient tissue scale-up, is amenable to automation, and offers sufficient controllability. Microtissues — for example, spheroids — are incorporated into bioinks, and their consolidation into a macrotissue occurs after printing. However, the fabrication of large-organ macrotissues by bioprinting, using spheroids as microtissues, is limited by the lack of methods for producing large numbers of uniform spheroids capable of containing multiple cell types. This problem can now be partially addressed using microfluidic systems and standardized culture plates — in particular, the Sphericalplate 5D® (SP5D) (Kugelmeiers AG, Switzerland) — as well as sorting platforms with appropriate software [45].

Bioprinting uses bioinks, which are deposited layer by layer via appropriate methods to form living tissue. The properties of a given bioink depend substantially on the printing method and the target tissue type (cartilage, vasculature, liver, tumor tissue, and others). Bioinks are multicomponent systems that must simultaneously ensure cell viability, biocompatibility, printability, and the stability of the printed tissue. Their composition typically includes cells, hydrogels, crosslinking systems, and bioactive and rheological modifiers.

The cells form the living tissue following bioprinting. These may be primary cells (epithelial cells, fibroblasts, chondrocytes, and others), stem cells (mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs)), organoid fragments, or spheroids—including multicomponent spheroids. Hydrogels, whether natural or synthetic, serve as potential bioscaffolds and must be cytocompatible, not elicit an immune response or inflammatory reaction, and not induce premature differentiation of stem cells. Natural polymers used include alginate, gelatin, collagen, fibrin, hyaluronic acid (HA), and Matrigel®. These provide cell adhesion but have variable composition and poor mechanical strength. In contrast, synthetic biodegradable thermoplastic polymers — polyethylene glycol (PEG), polylactide (PLA), and polycaprolactone (PCL) — offer controlled mechanical properties and composition but require modification to improve cell adhesion. To stabilize tissue shape following bioprinting, crosslinking agents are used: ionic crosslinkers (e.g., calcium chloride ($CaCl$) for alginate), photoinitiators (UV/visible light), enzymes — in particular, transglutaminase — and thermosensitive additives. To support cell proliferation, differentiation, and angiogenesis, growth factors such as VEGF and TGF-β, integrin-binding peptides, nanoparticles, and extracellular vesicles (EVs) are incorporated into bioink formulations [46].

It should be noted that each bioprinting method, and the mechanical phenomena arising during its execution — including stress, mechanical pressure, and shear forces — substantially affect the bioinks and, in particular, alter stem cell differentiation within the bioprinted macrotissue. Thus, increased mechanical pressure shifts the differentiation of mesenchymal stem cells (MSCs) toward cartilage or bone, while shear forces drive differentiation toward endothelial or bone tissue [47]. The physicochemical properties of bioscaffolds — notably their density and elasticity — can exert varied, sometimes opposing, effects on cell multipotency; consequently, the selection of optimal, compatible biopolymers is a critical consideration in bioprinting, and the optimization of their microarchitecture is an area of active investigation.

The creation of vascular networks represents the greatest challenge in bioprinting. Without appropriate channels for nutrient delivery and waste removal, even tissues of modest complexity will not survive. In vivo, a vascular network is required for tissues to grow beyond 100–200 μm—the diffusion limit for oxygen. In the absence of a vascular network, artificially constructed tissues will be subject to nutrient limitations. The creation of capillary networks is constrained by the technical capabilities of bioprinting resolution and speed; however, these limitations may be overcome by incorporating angiogenic growth factors into bioinks to stimulate host vascular ingrowth following implantation of the bioprinted tissue, or by engineering a synthetic vascular system from biodegradable polymers in combination with vascular myofibroblasts and endothelial cells [48, 49].

Thus, bioprinting technologies are today highly promising but require an interdisciplinary approach and considerable expertise. Although 3D bioprinting is a relatively new tissue engineering strategy, it holds great potential and will play a key role in personalized regenerative medicine in the future.

Organs-on-Chips

The 3D cell culture technology known as organ-on-a-chip (OoC) — which integrates biology and engineering — emerges as a promising approach to overcoming the limitations inherent in conventional 3D spheroids and organoids [50]. This technology is based on microfluidic systems in which various 3D cell cultures are maintained within integrated systems, interacting through microchannels and fluidic channels. These systems offer certain advantages over conventional spheroid and organoid cultures — notably the ability to control cell adhesion, provide mechanical stimulation, and ensure tissue perfusion, thereby enabling artificial vascularization and a uniform distribution of nutrients among the cultured cells. This enables the modeling of complex human physiological characteristics in a highly controlled *in vitro* environment [51, 52].

In such microfluidic devices, human cells — including patient-derived cells — are used to create personalized, physiologically relevant models of the liver, heart, skin, kidney, and even the brain [53, 54]. Polydimethylsiloxane (PDMS) is most commonly employed in microfluidic devices owing to its biocompatibility, flexibility, and gas permeability. Real-time monitoring of changes in culture parameters — including barrier integrity, metabolite levels, and oxygen levels — is achieved through optical imaging methods (microscopy) and integrated sensors.

Organ-on-a-chip technology is promising and is used in preclinical drug studies, toxicity assessment, disease modeling, and personalized medicine, while reducing the use of laboratory animals in accordance with the 3Rs. The convergence of different organ-on-a-chip models represents a significant advance in biomedical research, enabling the study of systemic interactions to investigate pathophysiological disease mechanisms and their pharmacological correction. However, the use of convergent models is associated with several technical challenges — particularly the engineering of microvessels and the simulation of endothelial barriers [55, 56]. Notwithstanding all the complexities and methodological challenges involved, organs-on-chips may prove especially valuable in preclinical drug studies, toxicology, and biomedicine.

Conclusions

Animal models continue to represent the “gold standard” in biomedical and biotechnological research — notably in the regulatory assessment of the safety and efficacy of drugs and biological products — as well as in regenerative and personalized medicine. At the same time, contemporary scientific development is increasingly directed toward the implementation of alternative approaches grounded in the principles of *in vitro* research and the interdisciplinary integration of biology, bioengineering, medicine, materials science, and digital technologies. This approach is consistent with the 3Rs concept (Replacement, Reduction, Refinement), as regulated by European normative documents, and implies a gradual paradigm shift in experimental design. In this context, three-dimensional (3D) cell cultures are regarded as a promising alternative to conventional two-dimensional (2D) monolayer systems, owing to their capacity to recapitulate cell–cell interactions, cell–extracellular matrix (ECM) contacts, and the spatial organization of tissues approaching in vivo conditions. Over recent years, a wide spectrum of 3D platforms has been developed — spheroids, organoids, bioprinted constructs, hydrogel-based systems, and organs-on-chips — which are being actively applied in pharmaceutical research, oncobiology, toxicology, stem cell research, and personalized medicine.

Despite their evident advantages, 3D models are not yet capable of fully reproducing the structural and functional complexity of tissues *in vivo*, and their broad implementation in biomedicine remains constrained by challenges related to standardization, validation of test systems, reproducibility, high cost, automation, and integration with high-throughput analytical methods. Further technological development, harmonization of protocols, and the introduction of validated methodologies are key prerequisites for the transition of 3D cell culture systems from academic research into applied biotechnology, biomedicine, and pharmacy. Thus, three-dimensional cell cultures constitute a new scientific approach to modeling biological

processes, contribute to the realization of the 3Rs principles, and open prospects for creating more physiologically relevant and ethically sound research platforms in the future.

Authors' Contributions

The authors contributed to the conception of the project and to the writing of the manuscript, and approved the final version.

Conflicts of Interest

The authors declare no conflict of interest. The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgements

The authors express their gratitude to the "Save Ukraine Together" foundation for supporting the project in the field of three-dimensional cell cultures. The funding sources did not participate in the analysis of the literature or in the decision to submit the review article for publication.

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ТРИВИМІРНІ КУЛЬТУРИ КЛІТИН В БІОТЕХНОЛОГІЇ: СУЧАСНИЙ ПОГЛЯД НА ПРОБЛЕМУ

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Мета. Систематизувати та критично проаналізувати сучасні платформи тривимірних (3D) клітинних культур — сфероїди, органоїди, біодруковані конструкти та органи-на-чіпі — й оцінити їхній потенціал як фізіологічно релевантних альтернатив двовимірним (2D) моношаровим системам у біомедичних дослідженнях і доклінічній оцінці лікарських засобів.

Матеріали й методи. Проведено огляд сучасної наукової літератури, що охоплює основні категорії технологій 3D-культур клітин, їхні біологічні та інженерні принципи, методи культивування, склад позаклітинного матриксу (ПКМ) та наявні платформи стандартизації. Здійснено порівняльний аналіз властивостей 2D- і 3D-культур, а також оцінку ключових аналітичних підходів — імунолейблінгу Ki-67, включення BrdU/EdU, метаболічних аналізів і конфокальної мікроскопії — адаптованих до тривимірних структур.

Результати. Тривимірні клітинні культури суттєво перевершують 2D-моношарі у відтворенні міжклітинних взаємодій, контакту з ПКМ, дифузійних градієнтів і тканиноспецифічної просторової організації. Кожна з основних платформ демонструє специфічну застосовність: MCTS відтворюють зональну архітектуру солідних пухлин для онкологічних і фармакологічних досліджень; органоїди забезпечують тканиноспецифічну самоорганізацію для моделювання захворювань і персоналізованої медицини; біодрук уможливлює точне просторове нанесення клітин і скаффолдів; органи-на-чіпі інтегрують мікрофлюїдну перфузію для динамічного моделювання фізіологічних бар'єрних функцій.

Висновки. Системи тривимірних клітинних культур є науково обґрунтованою та етично виваженою альтернативою тваринним моделям, що сприяє реалізації принципів 3R в експериментальній біомедицині. Водночас їхнє широке трансляційне застосування обмежується недостатньою стандартизацією, низькою відтворюваністю, високою вартістю та відсутністю функціональної васкуляризації; подолання цих бар'єрів потребує гармонізації валідованих протоколів та інтеграції з високопродуктивними аналітичними платформами.

Ключові слова: тривимірні клітинні культури, сфероїди, органоїди, органи-на-чіпі, біодрук, позаклітинний матрикс, біочорнила, онкологія, фармакологія, регенеративна медицина.

Received 2026/05/22
Revised 2026/06/17
Accepted 2026/08/31