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From single-layer cell cultures to 3D ecosystems: advancing therapeutics, pharmacology and toxicology through tumor spheroids

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Abstract

Two-dimensional monolayer cultures remain the most commonly used preclinical systems in anticancer drug discovery, yet their oversimplified structure fails to recapitulate the biochemical, mechanical, and architectural complexity of solid tumors. In 2D platforms, cells grow on rigid plastic surfaces under homogeneous conditions, with unrestricted access to nutrients, oxygen, and therapeutic agents. These artificial conditions distort key biological features such as proliferation dynamics, gene expression patterns, metabolic adaptation, extracellular matrix (ECM) signaling, and drug response heterogeneity. In contrast, three-dimensional multicellular tumor spheroids provide a physiologically relevant model that mimics essential characteristics of the tumor microenvironment, including oxygen and nutrient diffusion gradients, ECM remodeling, spatially stratified proliferation, and barriers to drug penetration. Rapid progress between 2021 and 2025 has expanded the technological landscape of spheroid generation—ranging from scaffold-free aggregation methods to ECM-rich scaffold-based systems, microfluidic tumor-on-a-chip platforms emulating vascular perfusion, and bioprinted architectures allowing precise spatial organization. This narrative review summarizes recent advances in spheroid generation, characterization, and application across therapeutics, pharmacology, and toxicology. We examine scaffold-free and scaffold-based approaches, microfluidic technologies, bioprinting strategies, and analytical readouts including high-content confocal microscopy, live imaging, and mass spectrometry imaging. We highlight the unique capacity of spheroids to provide realistic insights into drug penetration, chemotherapeutic efficacy, nanocarrier distribution, resistance mechanisms, and immunotherapy performance. Spheroids support organ-specific toxicity testing, long-term safety assessment, and alignment with the 3R principles by reducing animal use. Key limitations—variability, lack of vascularization, and analytical complexity—are critically assessed. Finally, we introduce the Pharmacological Relevance Index, a multidimensional descriptive framework that captures the conceptual and translational significance of spheroid models by integrating cellular complexity, ECM context, geometric control, analytical throughput, translational linkage, and toxicological breadth. Ultimately, tumor spheroids should be understood not merely as improved *in vitro* tools, but as customizable 3D micro-ecosystems capable of bridging early drug discovery and clinical translation.

Keywords: multicellular tumor spheroids, 3D cell culture models, tumor microenvironment, drug penetration and resistance, nanomedicine evaluation, microfluidic tumor-on-a-chip

Introduction

Cancer remains one of the leading global health burdens, and despite decades of progress in molecular oncology and drug development, a striking disconnect persists between preclinical success and clinical efficacy. Many agents that perform well in traditional preclinical models fail during late-stage clinical trials, revealing fundamental shortcomings in how tumor biology is modeled *in vitro*. Central to this translational gap is the heavy reliance on two-dimensional (2D) monolayer cultures, in which cells grow as flat sheets on rigid plastic surfaces and are exposed uniformly to nutrients, oxygen, and drugs.

These artificial conditions eliminate critical elements of tumor physiology. *In vivo*, cancer cells reside within intricate three-dimensional architectures characterized by cell–cell interactions, extracellular matrix (ECM) signaling, heterogeneous proliferation, and diffusion-limited microenvironments. By contrast, 2D cultures do not develop nutrient gradients, typically fail to recapitulate hypoxic microenvironments, and exhibit accelerated proliferation and altered gene expression patterns [1–4].

To overcome these limitations, a wide array of three-dimensional (3D) culture systems—including multicellular tumor spheroids (MCTS), organoids, tumoroids, and engineered ECM-based constructs—has been developed. Among them, spheroids stand out for their accessibility, scalability, reproducibility, and compatibility with high-throughput assays, making them particularly suitable for pharmacology and toxicology workflows [2, 4–9].

MCTS form through spontaneous aggregation, often accompanied by stromal or immune components, creating compact structures with distinct microenvironmental zones: an outer proliferative shell, intermediate quiescent layers, and a hypoxic or necrotic core [4, 5, 10]. This spatial heterogeneity generates physiologically relevant gradients that influence drug penetration, survival pathways, and adaptive resistance. Recent reviews published between 2021 and 2025 highlight the growing maturity of 3D culture systems and emphasize the need for their broader adoption in drug development [1–3, 6, 7, 9–12]. However, the rapid expansion of technical approaches—from low-adhesion plates to microwell arrays, hydrogels, microfluidic chips, and bioprinting—has produced a heterogeneous landscape in which "spheroid" can refer to markedly different model configurations. This diversity enriches translational opportunities but complicates experimental interpretation.

This review examines emerging spheroid technologies and their pharmacological, therapeutic, and toxicological applications. We position spheroids as engineerable 3D ecosystems whose architecture and complexity can be tuned to match specific research goals. We also introduce the Pharmacological Relevance Index (PRI), a conceptual tool designed to improve transparency, reproducibility, and interpretability across heterogeneous spheroid studies.

Technologies for spheroid generation and characterization

Scaffold-free approaches

Scaffold-free systems rely on minimizing cell–substrate adhesion, allowing cells to self-assemble via intrinsic cell–cell interactions. These methods dominate early-stage research due to their simplicity, cost efficiency, and compatibility with high-throughput workflows.

Hanging-drop and low-adhesion plates

The hanging-drop method is one of the most classical strategies for forming highly uniform spheroids. Cells suspended in microdroplets on an inverted lid aggregate at the bottom of the droplet under gravity, forming spheroids within 1–3 days [4, 6, 13]. This technique allows precise control of initial cell numbers and produces spheroids of reproducible diameter.

Similarly, ultra-low-attachment (ULA) plates are widely used in high-throughput settings. Their non-adhesive coating prevents cells from attaching to plastic surfaces, forcing them to cluster at the well's center [4, 6, 14]. These plates support automated liquid handling, microwell plate formats, and imaging systems, although spheroid morphology remains sensitive to seeding density and cell-line–specific behavior.

Microwell arrays for enhanced standardization

Microwell arrays—microfabricated platforms containing hundreds or thousands of small cavities—provide superior geometric control. Each microwell yields a single spheroid of defined size, significantly reducing variability across experiments [6, 15, 16]. Uniformity in spheroid diameter is crucial because size directly affects oxygen gradients, necrotic core formation, diffusion patterns, and drug penetration kinetics. Microwell-based platforms integrate seamlessly with robotic systems, automated confocal imaging, and high-content analysis pipelines, enabling reproducible, scalable experiments. Potential limitations include specialized manufacturing requirements and, in some designs, difficulties extracting spheroids from the wells without mechanical disruption.

Scaffold-based approaches

Scaffold-based systems introduce exogenous extracellular matrices (ECM), supplying biochemical cues and biomechanical properties more closely resembling *in vivo* tumor tissue.

Hydrogels and collagen matrices

Hydrogels composed of collagen I, fibrin, Matrigel, or synthetic polymers provide adjustable mechanical stiffness and ECM ligand density, influencing adhesion, migration, proliferation, and mechano-transduction. When spheroids are embedded within hydrogels, they experience physiologically relevant resistance and mechanical confinement that shape drug penetration and invasion dynamics [4, 7]. Collagen-embedded spheroids develop more realistic invasion patterns and drug distribution profiles than free-floating aggregates. ECM-mediated signaling can reveal resistance mechanisms that remain hidden in scaffold-free conditions.

Co-culture models: stromal, endothelial, and immune components

The tumor microenvironment (TME) includes fibroblasts, endothelial cells, immune cells, and pericytes—each influencing tumor progression and drug response. Incorporating these populations into spheroids yields models capable of recapitulating complex tumor–stroma interactions [2, 5, 8, 17, 18].

Examples include tumor–fibroblast spheroids, which promote ECM remodeling and drug resistance; tumor–endothelial spheroids, which can initiate primitive vascular-like networks and tumor–immune spheroids, useful for studying T-cell and natural killer (NK) cell infiltration, cytotoxicity, and immune evasion [5, 18].

Co-culture models are especially powerful for immunotherapy research, revealing infiltration barriers and resistance niches that are not apparent in 2D cultures.

Microfluidic tumor-on-a-chip systems

Microfluidic tumor-on-a-chip platforms represent one of the most significant technological advances in 3D tumor modeling. These systems incorporate micro-scaled perfusable channels, often lined with endothelial cells, that simulate key aspects of vascular physiology—including shear stress, directional flow, nutrient and oxygen delivery, and dynamic drug perfusion [9, 12, 19–21].

Unlike static spheroid cultures, microfluidic devices allow precise control of microenvironmental parameters. Drug gradients, oxygen levels, cytokine exposure, and interstitial flow can be adjusted in real time, generating pharmacokinetic-like (PK-like) profiles that more closely resemble *in vivo* conditions.

Key advantages include dynamic drug delivery, enabling assessment of treatment schedules and exposure kinetics; real-time imaging, as spheroids remain immobilized within transparent microchannels; co-culture integration, allowing the addition of endothelial barriers, stromal components, and immune cells and high spatiotemporal resolution, facilitating visualization of invasion fronts, necrosis progression, and immune cell migration.

Lipreri *et al.* demonstrated that tumor-on-a-chip systems reveal drug behaviors—including pulsatile penetration, endothelial retention, and delayed cytotoxicity—that cannot be captured in static spheroids [19]. Roman *et al.* further emphasized that microfluidic devices bridge the gap between spheroids and organ-on-a-chip platforms, offering a scalable route toward physiologically relevant drug modeling [20].

Despite these strengths, microfluidic platforms require specialized fabrication, fluid-handling expertise, and complex downstream analytics, which limit widespread adoption in standard laboratories.

Bioprinting and structured 3D architectures

Bioprinting technologies enable precise spatial patterning of cells, spheroids, ECM components, and biomaterials using additive manufacturing principles. Rather than relying on spontaneous aggregation alone, bioprinting can generate hierarchically organized tumor constructs, incorporating stromal compartments, perfusion channels, and stiffness gradients [1, 7, 21].

Advantages include: spatial compartmentalization, enabling separate but adjacent tumor, stromal, or immune regions; repeatable architecture, improving reproducibility and experimental comparability; integration of perfusable microchannels, enhancing nutrient transport and mimicking primitive vasculature and customization, allowing patient-specific tissue architectures for personalized oncology.

Ro *et al.* showed that bio-printed spheroid-based constructs can reproduce local invasion, heterogeneous drug penetration, and ECM remodeling at levels challenging to achieve with spontaneously formed spheroids [21].

Challenges include the need for specialized equipment, the optimization of printable bioinks, and the assurance of the viability and functionality of embedded spheroids. Nevertheless, bioprinting stands at the forefront of next-generation 3D cancer modeling.

Readouts and characterization techniques

A major strength of spheroid-based systems is the diversity of readouts that can be used to

quantify structural, metabolic, and pharmacological responses.

Viability and cytotoxicity assays

Conventional viability assays—such as MTT, resazurin reduction, and ATP luminescence assays—remain widely used but require careful interpretation in 3D systems. Diffusion barriers limit reagent penetration, and outer proliferative layers can mask inner cell death. To improve accuracy, viability assays are often paired with live/dead fluorescent staining, caspase-3/7 activity measurements, confocal z-stack imaging, and flow cytometry of dissociated spheroids [5, 14].

Abbas *et al.* demonstrated that conventional 2D assays overestimate drug potency by failing to account for quiescent or hypoxic subpopulations, reinforcing the need for multiparametric 3D readouts [14].

Advanced imaging and mass spectrometry imaging (MSI)

High-content imaging modalities enable detailed spatial analysis of spheroids, revealing structural and functional heterogeneity. Confocal microscopy provides high-resolution three-dimensional reconstructions, light-sheet fluorescence microscopy enables rapid volumetric imaging with minimal phototoxicity, and optical coherence tomography (OCT) offers label-free structural imaging at considerable depth [22]. Mass spectrometry imaging (MSI) has emerged as a breakthrough tool [23]. MSI provides label-free spatial mapping of drugs and metabolites, enabling correlation between drug distribution and biological response [10].

MSI is used to quantify differential accumulation of chemotherapeutics in spheroid layers, revealing that limited penetration—not intrinsic resistance—often drives treatment failure [10].

AI-driven analysis

Artificial intelligence (AI) and machine learning (ML) methods have transformed spheroid characterization. These approaches automate spheroid segmentation, morphological profiling, necrotic core quantification and prediction of treatment outcomes based on early imaging signatures [6, 15, 24, 25].

Kaur *et al.* showed that AI-assisted analysis enables phenotypic screening at unprecedented speed, allowing researchers to detect subtle morphological cues predictive of response before conventional assays register changes [25].

Pharmacological applications of tumor spheroids

Tumor spheroids have become central to modern pharmacology because they reproduce key microenvironmental features—diffusion gradients, cellular heterogeneity, ECM remodeling—that fundamentally alter drug response compared with 2D systems. Below, we examine their major contributions to drug screening, nanomedicine, resistance modeling, immuno-oncology, and precision medicine.

Dose–response relationships and drug screening

Dose–response studies conducted in 2D monolayers often lead to overestimated drug potency. In monolayers, all cells are equally exposed to drugs, oxygen, and nutrients, resulting in homogeneous killing patterns that fail to reflect the complexity of solid tumors. By contrast, spheroids produce oxygen gradients, proliferative and quiescent zones, diffusion-limited drug penetration, heterogeneous stress responses and resistant hypoxic cores [2, 3, 5, 11].

These features yield dose–response curves that better match *in vivo* outcomes. For example, doxorubicin, cisplatin, paclitaxel, and other chemotherapeutics often exhibit reduced efficacy in spheroids due to limited penetration and metabolic adaptation—an effect highly consistent with clinical resistance patterns [5].

High-throughput screening (HTS) is now widely compatible with spheroid systems. Lee and Kim showed that U-bottom plates and ultra-low-attachment platforms can generate hundreds of uniform spheroids per experiment, facilitating ATP-based assays and automated image-based quantification [6]. Sharma *et al.* emphasized that spheroid-based phenotypic screening is becoming essential for early drug discovery, providing more biologically relevant data than traditional monolayer assays [2].

Nanomedicine and drug delivery

Nanomedicine efficacy depends critically on the microenvironment through which nanoparticles travel. In 2D systems, nanoparticles interact freely with cells due to direct exposure. However, spheroids impose 3D barriers including ECM density, cell–cell junctions, hypoxia-driven changes in endocytosis, and pH gradients, all of which affect nanoparticle behavior [7]. Rossi and Blasi demonstrated that MCTS are indispensable for studying nanoparticle penetration, retention, ligand targeting, and payload release kinetics [7]. Yadav *et al.* used pancreatic cancer spheroids to reveal that nanoparticle size, surface charge, and coating chemistry drastically influence spatial drug distribution and therapeutic outcomes [9]. MSI has further revolutionized this area by mapping drug distribution without labeling. Wang and Hummon showed that varying intra-tumoral distribution

patterns correlate strongly with localized cytotoxicity, confirming MSI as an essential tool for nanomedicine optimization [10].

Modeling chemoresistance and tumor heterogeneity

One of the most powerful advantages of multicellular tumor spheroids (MCTS) is their ability to spontaneously develop structural and functional heterogeneity, a hallmark of real tumors. As spheroids mature, they establish distinct microenvironmental zones—including a proliferative periphery exposed to oxygen and nutrients, an intermediate quiescent region, and a hypoxic or necrotic core [4, 10]. These gradients generate diverse metabolic states and stress responses that profoundly influence therapeutic sensitivity. Peng *et al.* demonstrated that hypoxic regions in spheroids activate hypoxia-inducible factors (HIFs), altering glycolysis, mitochondrial respiration, and drug efflux transporter expression more robustly than in 2D cultures [3]. These adaptive responses contribute to chemoresistance, enabling survival under drug-induced stress. Mechanobiology also plays an important role. Mangani *et al.* showed that ECM stiffness alters intracellular tension, mechano-transduction pathways, and apoptotic thresholds, thereby modifying drug response [8]. Such biomechanical modulation cannot be captured in 2D and reinforces the need for physiologically structured models.

Co-culture tumor–stroma spheroids extend this complexity further. Interaction with fibroblasts enhances ECM deposition, promotes epithelial–mesenchymal transition (EMT)-like behavior, and increases resistance to targeted therapies. Immune cells incorporated into spheroids can either promote cytotoxicity or contribute to immune-mediated selection of resistant clones. Together, these models illuminate survival niches and residual disease behaviors that serve as precursors to clinical relapse.

Immuno-oncology applications

Spheroid-based immuno-oncology models provide mechanistic insight into how immune cells—such as T cells, macrophages, and natural killer (NK) cells—interact with spatially structured tumor environments. In 2D systems, immune cells contact tumor cells directly, resulting in an overestimation of cytotoxicity. By contrast, spheroids impose physical, biochemical, and immunosuppressive barriers that immune cells must overcome.

Srisantitham *et al.* quantified NK cell infiltration and cytotoxicity in 3D and found that NK cells often accumulate at the periphery, encountering difficulty penetrating deeper spheroid layers due to ECM density and cell–cell tight junctions [18]. This behavior mirrors *in vivo* findings, where NK cells frequently struggle to access poorly perfused tumor regions.

Similarly, tumor–T-cell co-culture spheroids enable investigation of checkpoint inhibitor dynamics, immune exhaustion, and antigen-dependent infiltration patterns. A robust T-cell infiltration model illustrates how spheroid architecture modulates T-cell migration trajectories, activation states, and cytotoxic efficiency [18].

Immuno-spheroid platforms provide valuable readouts for penetration depth and infiltration rate, immune cell persistence, cytokine-driven remodeling, immune escape mechanisms, and combinatorial strategies, such as pairing immunotherapy with ECM-modifying agents.

This makes spheroids indispensable for optimizing immunotherapies, CAR-T cell engineering, checkpoint blockade strategies, and oncolytic virotherapy.

Patient-derived tumor spheroids and precision medicine

Patient-derived tumor spheroids (PDTS) represent a rapidly advancing frontier in functional precision oncology, where treatment decisions are guided not only by genomic markers but also by direct *ex vivo* drug sensitivity testing.

PDTS preserves key attributes of the patient tumor such as genetic heterogeneity, epigenetic memory, patient-specific stromal interactions and differential drug response patterns [1, 5, 8].

Roman *et al.* demonstrated that PDTS, integrated with microfluidic perfusion and genomic profiling, predicts patient-specific drug sensitivity profiles and can identify non-intuitive therapeutic vulnerabilities [20]. This approach has the potential to reduce ineffective treatments and accelerate personalized therapy planning.

Despite promising results, challenges remain regarding variability in tissue quality and yield, the need for rapid processing workflows, standardization of assay duration and endpoints, and integration with clinical decision timelines.

However, advances in biobanking, automated imaging, and microfluidic culture promise to make PDTS a future clinical staple.

Toxicological applications and safety assessment

Spheroids extend far beyond oncology; they also provide physiologically robust models for toxicology and preclinical safety evaluation. Their 3D organization preserves long-term viability, multicellular communication, and differentiated function—attributes essential for assessing

drug-induced toxicity that 2D cultures cannot recapitulate [1–3].

Spheroids for organ-specific toxicity

Organotypic spheroids derived from hepatocytes, cardiomyocytes, renal epithelial cells, and neurons function as stable, long-lived microtissues suitable for toxicity screening. Marconi *et al.* demonstrated that liver spheroids maintain cytochrome P450 activity, albumin secretion, and metabolic integrity for extended periods, allowing repeated dosing regimens and modeling of drug-induced liver injury (DILI) [1].

Cardiac spheroids derived from cardiomyocytes retain essential electrophysiological properties, enabling measurement of beat rate, arrhythmogenic potential, and calcium handling. These models are particularly valuable for assessing cardiotoxicity associated with chemotherapeutics, tyrosine kinase inhibitors, and off-target nanoparticle accumulation.

Renal spheroids generated from epithelial or proximal tubule cells reproduce metabolite transport, filtration-like functions, and transporter-mediated drug clearance—key parameters in predicting nephrotoxicity. Neural spheroids, meanwhile, allow neurotoxicity assessment by preserving synaptic signaling and glial interactions absent in 2D systems.

Collectively, organ-specific spheroids allow long-term exposure studies under controlled conditions, capturing phenotypes such as fibrosis-like ECM accumulation, mitochondrial dysfunction, metabolic drift, and delayed apoptosis—attributes impossible to characterize in monolayers.

Tumor *vs.* non-tumor selectivity

One of the strengths of spheroid technology is the ability to directly compare therapeutic efficacy in tumor spheroids *versus* off-target toxicity in healthy tissue spheroids. This is critical for evaluating therapeutic windows and optimizing drug design.

Parallel testing can be performed using tumor spheroids to evaluate therapeutic efficacy, liver spheroids to assess metabolic toxicity, cardiac spheroids to examine electrophysiological safety, and renal spheroids to investigate clearance-related toxicity [5, 7, 8].

For example, antibody–drug conjugates or nanoparticle-based systems may accumulate differently in tumor *versus* hepatic spheroids due to tumor-specific uptake mechanisms or liver-specific clearance pathways. When combined with MSI or high-content imaging, researchers can map spatially resolved drug distribution across different organ spheroids, correlating drug localization with viability, apoptosis, and metabolic dysfunction.

This approach enhances preclinical safety assessment and may reduce reliance on early animal testing.

Off-target effects and long-term exposure

A major advantage of spheroid models over traditional 2D monolayers is their ability to sustain long-term culture while maintaining physiologically relevant phenotypes. This characteristic is crucial for studying sub-chronic or chronic toxicities, which often involve cumulative molecular damage, metabolic adaptation, and delayed-onset dysfunction that cannot be captured in short-term 2D assays.

Organ-specific spheroids demonstrate progressive mitochondrial stress, oxidative damage, extracellular matrix accumulation resembling fibrosis, gradual metabolic reprogramming, and delayed apoptosis [1–3]. Because spheroids mimic tissue-like organization, they allow researchers to observe subtle toxic effects in real time. These effects include low-level but persistent metabolic drift in hepatic spheroids, gradual impairment of electrical conduction in cardiac spheroids, or accumulation of toxic metabolites in renal models.

Mass spectrometry imaging (MSI) plays a crucial role in long-term exposure studies. MSI can map the spatial accumulation of chemotherapeutics and metabolites within spheroids, revealing localized toxic concentration hotspots undetectable in bulk assays [10]. By correlating MSI data with viability, apoptosis, and structural imaging, researchers can pinpoint early toxicity signatures that precede morphological deterioration.

This level of mechanistic insight is particularly valuable for evaluating drugs with narrow therapeutic windows, nanoparticle formulations prone to prolonged tissue retention, and combination therapies with synergistic toxicity profiles.

Contribution to the 3R principles

The 3R framework—replace, reduce, refine—aims to decrease animal use in biomedical research while improving scientific rigor. Spheroid systems directly support all three principles. *Replace*: Spheroids replicate many aspects of *in vivo* physiology, replacing early-stage animal experiments in screening for efficacy, toxicity, and mechanistic behavior [2, 6, 11]. *Reduce*: By filtering out ineffective or overtly toxic compounds before animal testing, spheroids reduce the total number of animals required for downstream validation. *Refine*: Spheroids enable more rational dosing and study design for subsequent *in vivo* work, reducing unnecessary harm by allowing researchers to optimize conditions beforehand. As regulatory agencies increasingly encourage integration of *in vitro* and in silico models in drug

development pipelines, spheroids are becoming an essential component of ethically responsible research programs.

Limitations and challenges

Despite their transformative potential, spheroid systems face several limitations that must be addressed to ensure their rigorous and reproducible use in pharmacology and toxicology.

Variability and lack of standardization

Variability in spheroid generation remains a major challenge. Different laboratories employ diverse methods—including hanging drops, low-adhesion plates, microwell arrays, hydrogels, or microfluidic devices—each producing spheroids with distinct structural and biochemical characteristics.

Critical parameters influencing experimental outcomes include the initial seeding density and aggregation kinetics, final spheroid diameter and compactness, extracellular matrix (ECM) content and stiffness, culture duration and oxygenation, as well as mechanical handling procedures [4, 11]. Without standardized reporting, comparing studies becomes difficult and sometimes misleading. Mitrakas *et al.* emphasized that inconsistent methodological details lead to irreproducibility and hinder integration of spheroid-derived data into translational and regulatory workflows [4]. Han *et al.* highlighted similar concerns, noting that even subtle differences in spheroid size can dramatically alter drug penetration and viability profiles [11].

To maximize utility, spheroid protocols should specify size distribution, culture conditions, ECM characteristics, and analytical methods with the same rigor expected of clinical or animal studies.

Absence of full vascularization and systemic context

Although microfluidic tumor-on-a-chip systems partially emulate vascular flow, most spheroids lack a fully functional vasculatureThis absence limits the ability to model pharmacokinetics (PK)—including absorption, distribution, metabolism, and excretion—as well as systemic immune interactions, hormonal signaling, and whole-body physiological feedback loops [1, 9]. Consequently, spheroids excel at modeling local pharmacodynamics (PD)—drug penetration, cytotoxicity, resistance, and microenvironmental adaptation—but cannot fully replace *in vivo* PK studies. Their optimal use is as intermediate models, bridging the gap between simple monolayers and complex animal systems.

Biological complexity *vs.* experimental tractability

Increasing biological complexity—through co-culture systems, extracellular matrix (ECM) integration, perfusion platforms, or bioprinting—enhances physiological relevance but introduces practical trade-offs, including reduced throughput, increased experimental variability, greater technical demands, and more challenging data interpretation [1–3, 8].

Accordingly, the choice between simple and complex spheroid models should be guided by the specific experimental objective. Simple monoculture spheroids are particularly well suited for high-throughput screening applications, whereas ECM-embedded or co-culture spheroids are more appropriate for mechanistic investigations and resistance studies. Microfluidic or bioprinted systems, in turn, are better aligned with translational research and pharmacokinetic/pharmacodynamic (PK/PD) modeling.

Balancing feasibility with biological accuracy remains a central challenge in spheroid research.

Technical demands and data interpretation

Advanced characterization techniques (*e.g.*, confocal imaging, light-sheet microscopy, OCT, MSI) require expensive instrumentation, specialized training, and sophisticated computational pipelines [10, 24, 25]. This barrier limits adoption in small laboratories and introduces variability in analytical depth across studies.

Furthermore, three-dimensional datasets present unique challenges, including the segmentation of irregular structures, quantification of spatial drug gradients, tracking of temporal changes across z-planes, and integration of multiple parameters. AI-driven solutions are improving this landscape, but the field still lacks standardized analytical benchmarks, contributing to inconsistency in data interpretation.

A proposal: the pharmacological relevance index (PRI)

The rapid expansion of spheroid technologies has generated a diverse array of models, each varying in biological realism, structural complexity, analytical compatibility, and translational relevance. To provide clarity, we propose the Pharmacological Relevance Index (PRI)—a multidimensional descriptive framework that allows researchers to transparently characterize the strengths and limitations of a given spheroid model.

Unlike numerical scoring systems, PRI is conceptual, focusing on narrative clarity rather than rigid ranking.

Rationale

The term "3D spheroid" encompasses a broad range of architectures, from simple cancer cell aggregates to extracellular matrix (ECM)-embedded co-cultures and perfused microfluidic constructs. These variations directly influence diffusion and drug penetration, the development of hypoxic zoning and metabolic adaptation, stromal signaling and immune interactions, as well as assay throughput and reproducibility [1–4, 11, 16]. Yet many publications do not describe these parameters in sufficient detail, making it difficult to determine whether a given model is appropriate for a specific pharmacological question.

The PRI framework encourages systematic reporting of key design features to promote transparent communication, improve reproducibility, and facilitate better alignment between model characteristics and research objectives.

Dimensions of the PRI

Below, we articulate each PRI dimension in narrative form, preserving clarity and conceptual integration.

Cellular complexity – refers to the diversity of cell types included in the spheroid. It spans from simple monocultures containing only cancer cells to sophisticated models incorporating fibroblasts, endothelial cells, macrophages, or lymphocytes. Increasing cellular diversity enhances biological realism by enabling paracrine signaling, immune modulation, and stromal remodeling—key determinants of therapeutic response [3, 4, 8].

Extracellular matrix (ECM) and biomechanics – the presence, composition, and stiffness of ECM profoundly influence cellular adhesion, migration, mechano-transduction, and drug penetration. ECM-rich spheroids, especially those embedded in hydrogels, reproduce mechanical resistance and ligand interactions that shape sensitivity to therapy [4, 7].

Geometric control and reproducibility – spheroid diameter, compaction, and circularity strongly affect diffusion gradients and metabolic zoning. Systems such as microwell arrays produce highly uniform spheroids, improving reproducibility and enabling robust dose–response comparisons [6, 15, 16].

Throughput and analytical compatibility – some platforms allow automated handling, high-content imaging, and AI-assisted analysis suitable for screening large drug libraries. Others focus on deep mechanistic insight at lower throughput [2, 6, 25].

Translational linkage – this dimension reflects how closely spheroid responses mirror clinical or *in vivo* outcomes. Patient-derived spheroids and microfluidic-perfused models often show higher translational congruence [5, 8].

Toxicological breadth – a spheroid model's ability to support multi-organ toxicological assessment—by integrating tumor spheroids with liver, heart, or kidney spheroids—expands its relevance for safety pharmacology [1–3].

PRI emphasizes whether a spheroid system supports such holistic evaluation.

Future directions

The field of three-dimensional multicellular tumor spheroids (MCTS) is rapidly evolving, driven by synergistic progress in microengineering, biomaterials, imaging technologies, and computational analysis. As drug development increasingly demands physiologically relevant and mechanistically informative models, spheroids are poised to become foundational platforms across pharmacology, toxicology, and personalized medicine. Below, we outline several key directions expected to shape the next generation of 3D tumor modeling.

Integration of microfluidics, bioprinting, and artificial intelligence

Three emerging technologies—microfluidics, bioprinting, and artificial intelligence (AI)—are converging to redefine spheroid-based systems.

Microfluidics: Microfluidic tumor-on-a-chip devices introduce physiologically relevant perfusion, shear stress, and drug delivery profiles that cannot be achieved in static cultures. These systems enable real-time monitoring of drug distribution, immune cell trafficking, and stromal interactions under controlled dynamic conditions [9, 12, 21]. Future platforms are expected to incorporate multi-tissue microenvironments, enhancing predictive validity for combination therapies and toxicity assessments.

Bioprinting: Advances in 3D bioprinting allow precise spatial patterning of tumor, stromal, and vascular elements, enabling construction of architecturally defined tumor microenvironments. Bio-printed constructs can integrate perfusable microchannels, variable ECM stiffness regions, and immune-competent compartments—components essential for modeling metastatic behavior and treatment escape [1, 7, 21]. Personalized tumor avatars, printed from patient-derived spheroids, represent a promising avenue for rapid therapy optimization.

Artificial Intelligence: AI-driven image analysis is revolutionizing phenotypic screening by extracting multidimensional features from high-content imaging. Machine learning models can detect subtle morphological, metabolic, or textural patterns predictive of therapeutic response, outperforming conventional viability assays [6, 15, 25].

Integration of AI with microfluidic and bioprinting systems will create closed-loop experimental platforms, where spheroid behavior continuously informs automated treatment adaptation.

Together, these technologies promise a transformative leap in the fidelity, scalability, and interpretability of spheroid-based cancer models.

Standardization, guidelines, and the potential role of the PRI

Despite the widespread adoption of spheroid models, standardization remains limited, hindering reproducibility and slowing progress toward regulatory acceptance. Several authors have advocated for structured reporting frameworks and benchmarking studies across laboratories [4, 11, 16]. As the field grows, consensus guidelines—akin to MIAME for microarray studies, CONSORT for clinical trials, or ARRIVE for animal research—will become increasingly essential.

The Pharmacological Relevance Index (PRI) provides a conceptual scaffold for such standardization efforts. By explicitly describing cellular composition, ECM context, geometric control, throughput, translational linkage, and toxicological breadth, PRI enables transparent comparison across studies. Although PRI does not impose numerical scoring, its narrative structure encourages authors to report relevant experimental details clearly and consistently.

Future applications may include the incorporation of PRI descriptors into journal submission checklists, the development of regulatory qualification frameworks for spheroid models, and the implementation of multi-laboratory benchmarking studies designed to evaluate reproducibility across PRI-defined categories. Ultimately, standardization will enhance comparability, increase confidence in spheroid-derived data, and accelerate integration into industrial and regulatory pipelines.

Functional precision oncology

Functional precision oncology aims to personalize cancer therapy by integrating molecular profiling with *ex vivo* drug sensitivity testing. Patient-derived tumor spheroids (PDTS) are ideally suited for such workflows because they maintain tumor heterogeneity and can be generated rapidly from biopsy material [1, 5, 8].

Recent studies have shown that patient-derived tumor spheroids (PDTS) can predict patient-specific therapeutic responses, identify drug resistance at an early stage, functionally validate genomic findings, evaluate therapeutic combinations that are not feasible to test directly in patients, and support treatment decisions within clinically actionable timeframes [20]. Ongoing challenges include standardization of PDTS preparation, integration with microfluidic perfusion, optimization of drug exposure protocols, and incorporation of immune components to model immunotherapy responses. As biobanking expands and analytical technologies evolve, PDTS-based screening is expected to become a routine component of personalized treatment planning, bridging the gap between genomic biomarkers and real-world therapeutic efficacy.

Conclusion

Multicellular tumor spheroids (MCTS) represent a critical advancement in preclinical cancer modeling, offering a middle ground between oversimplified 2D systems and resource-intensive *in vivo* studies. By recapitulating essential features of the tumor microenvironment—including hypoxia, nutrient gradients, ECM-dependent signaling, mechano-transduction, and cellular heterogeneity—spheroids provide a biologically meaningful platform for evaluating drug penetration, therapeutic efficacy, resistance mechanisms, and nanomedicine behavior.

Within toxicology, spheroids enable organ-specific safety assessment, long-term exposure studies, and multi-spheroid integration for therapeutic window characterization. Their contributions directly support the 3R principles, reducing reliance on animal testing while improving mechanistic insight. However, spheroid systems are not monolithic. Their diversity in biological complexity, ECM incorporation, geometric uniformity, throughput compatibility, and translational linkage necessitates thoughtful model selection and transparent reporting. The proposed Pharmacological Relevance Index (PRI) offers a structured, narrative-based framework for describing and evaluating spheroid models across these dimensions. PRI is not a ranking tool, but a communication tool—designed to improve clarity, reproducibility, and methodological rigor.

As microfluidics, bioprinting, and AI increasingly merge with spheroid technologies, the field is moving toward integrative platforms capable of modeling drug response, toxicity, and tumor evolution with unprecedented precision. Ultimately, spheroids should not be viewed simply as "better 2D models", but as engineered micro-ecosystems that hold the potential to transform early drug discovery, translational pharmacology, and personalized oncology.

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Conflict of interest

The authors declare no conflict of interest.

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