Review

Unlocking the Potential of Spheroids in Personalized Medicine: Systematic Review of Seeding Methodologies

Karolina Lonkwic *, Radosław Zajdel, Krzysztof Kaczka

Posted Date: 8 April 2025

doi: 10.20944/preprints202504.0640.v1

Keywords: spheroids; personalized medicine; drug screening; organoids; 3D models

 doi:10.20944/preprints202504.0640.v1

Review

Unlocking the Potential of Spheroids in Personalized Medicine: Systematic Review of Seeding Methodologies

Karolina Lonkwic [1,2,*], Radosław Zajdel [3,4] and Krzysztof Kaczka [5]

[1] Clinic of General and Oncological Surgery, Medical University of Lodz, 92-213 Lodz, Poland
[2] Mabion S.A., 95-050 Konstantynow Lodzki, Poland
[3] Department of Economic and Medical Informatics, University of Lodz, 90-214 Lodz, Poland
[4] Department of Medical Informatics and Statistics, Medical University of Lodz, 90-645 Lodz, Poland
[5] Clinic of General and Oncological Surgery, Medical University of Lodz, 92-213 Lodz, Poland
* Correspondence: karolina.lonkwic@stud.umed.lodz.pl or k.lonkwic@mabion.eu

Abstract: Three-dimensional (3D) spheroid models have revolutionized in vitro cancer research by offering more physiologically relevant alternatives to traditional two-dimensional (2D) cultures. This systematic review evaluates methods for spheroid formation and cellular sources used, highlighting diverse applications and preferences in the field. The five most investigated cancer origins for spheroid seeding are breast, colon, lung, ovary, and brain cancers, reflecting their clinical importance and research focus. Among seeding methodologies, forced-floating and scaffold-based methods predominate, demonstrating reliability and versatility in spheroid generation. Other techniques, including microfluidics, bioprinting, hanging drop and suspension culture also play significant roles, each with distinct advantages and limitations. This review underscores the growing adoption of spheroid models and the need for standardization in methodologies to enhance reproducibility and translational potential in cancer research.

Keywords: spheroids; personalized medicine; drug screening; organoids; 3D models

1. Introduction

In recent years, there has been a paradigm shift in biomedical research towards developing more clinically relevant models to study human physiology and diseases. Personalized medicine, aiming to tailor medical interventions to individual patient characteristics, has gained prominence in the quest for more effective and targeted treatments. Within this context, three-dimensional cell culture models, particularly spheroids, have emerged as valuable tools for bridging the gap between traditional two-dimensional cell cultures and in vivo studies. Spheroids, characterized by their spherically shaped cellular aggregates, exhibit enhanced physiological relevance by recreating cell-cell and cell-matrix interactions found in native tissues. This article aims to explore the developing field of spheroid research, focusing on their potential applications in personalized medicine and elucidating methodologies employed for spheroid seeding.

Traditional cell culture systems are often inadequate in reproducing the complexity of in vivo tissue structures and functions. Spheroids, formed through the self-assembly of cells, provide a closer representation of the native tissue microenvironment. This three-dimensional architecture facilitates cell-cell interactions, nutrient gradients, and spatial organization, closely mimicking the in vivo conditions. As a result, spheroids have gained prominence as an advanced in vitro model that bridges the gap between conventional cell cultures and in vivo studies.

1.1. Summary of Models for In Vitro Studies

 doi:10.20944/preprints202504.0640.v1

The concept of growing cells outside of the human body began in the early 20th century. In 1907, Ross Harrison obtained the first successful tissue culture by growing nerve fibers from frog embryos. This work paved the way for cell culture techniques that have since become foundational in biomedical research [1].

Furthermore, the establishment of the first continuous human cell line, HeLa cells, by George Otto Gey in 1951 marked a revolutionary step in cancer research. Derived from cervical cancer cells, HeLa provided researchers with an immortal cell line capable of indefinite growth under proper conditions. This breakthrough enabled consistent and reproducible experiments, fostering a deeper understanding of cancer progression and therapeutic responses [2].

The 2D monolayer culture technique dominated *in vitro* cancer research for much of the 20th century. 2D monolayer cultures have been the cornerstone of *in vitro* cancer research for decades due to their simplicity, cost-effectiveness, and adaptability to high-throughput screening. In these systems, cancer cells are grown on flat, rigid substrates, providing uniform exposure to nutrients, oxygen, and drugs. The standardized conditions in 2D cultures enable reproducible experiments and straightforward readouts, making them ideal for initial mechanistic studies and large-scale drug screening. Cells are grown on flat plastic or glass surfaces in a single layer, making it possible to study their morphology, growth, and responses to drugs in a controlled environment. These models were integral to early breakthroughs in cancer biology, such as identifying signaling pathways and testing chemotherapeutics. However, their limitations became increasingly apparent. The two-dimensional format failed to replicate the complexity of the tumor microenvironment, cell-cell interactions, and gradients of nutrients and oxygen present, and cellular architecture found *in vivo*. Cells grown in 2D often exhibit altered morphology, gene expression, and behavior compared to their *in vivo* counterparts [3].

The limitations of 2D models drifted the development of 3D models. In recent years, three-dimensional (3D) culture systems, including spheroids, organoids, and other, have emerged as advanced *in vitro* models that better mimic the *in vivo* tumor microenvironment. Spheroids, first described in the 1970s, allowed researchers to grow cells in a structure that mimics the spatial architecture of tumors. Spheroids, for instance, are self-organizing aggregates of cancer cells that develop gradients of oxygen, nutrients, and waste products, closely resembling the structural and functional characteristics of solid tumors. These models better simulate cell-cell and cell-matrix interactions, as well as the nutrient and oxygen gradients found in actual tumors. This advancement has significantly improved the study of tumor biology and drug resistance [4].

Today, 2D and 3D models are commonly used in tandem, each complementing the other's strengths. 2D cultures remain essential for high-throughput screening due to their simplicity and reproducibility. In contrast, 3D models provide a more physiologically relevant context, making them indispensable for studying tumor progression, metastasis, and resistance to therapy. Hybrid approaches combining 2D and 3D cultures, as well as emerging technologies like organ-on-a-chip and patient-derived organoids, are bridging the gap between *in vitro* and *in vivo* systems. These models aim to capture the advantages of both 2D simplicity and 3D physiological relevance, providing a more comprehensive understanding of tumor biology and therapeutic responses. While 2D cultures remain essential for preliminary studies due to their simplicity and efficiency, incorporating 3D models enhances the translational potential of *in vitro* research by mimicking critical aspects of tumor biology. The complementary use of both systems, along with advances in hybrid models, offers a robust framework for cancer research, drug discovery, and personalized medicine [5–7].

However, there is an emerging consensus that research should transition from reliance on traditional 2D models to the adoption of more physiologically relevant 3D models [8,9].

 doi:10.20944/preprints202504.0640.v1

1.2. Comparison of 2D and 3D Models

In vitro models are indispensable tools for studying cancer biology, screening therapeutic agents, and elucidating disease mechanisms. Among these, traditional two-dimensional (2D) cancer cell cultures and advanced three-dimensional (3D) models are widely utilized. Each model possesses distinct advantages and limitations. The most appropriate model selection should be made based on the specific context of the application. Both models also represent defined potentials. Table 1 highlights how each model is best suited to specific research objectives, emphasizing the importance of selecting the appropriate system based on the experimental needs [5–8,10–13].

Table 1. Advantages, limitations and potentials of 2D and 3D models.

	2D models	**3D models**
Advantages	• Simplicity and ease of use. • Cost-effective and widely available. • High-throughput capability.	• Closer mimicry of in vivo conditions. • Replicates tumor microenvironment and cellular interactions (e.g. cell-cell and cell-matrix interactions, hypoxic core). • Better simulation of drug penetration and resistance (drug diffusion barriers and heterogeneous cellular responses). • Represents gene and protein expression profile that reflects tumors.
Limitations	• Lack of physiological relevance. • Limited replication of cell-cell and cell-matrix interactions. • Fails to simulate gradients of oxygen, nutrients, and metabolites.	• Higher cost and technical complexity. • Longer culture time. • Scalability challenges. • Reproducibility issues in some systems.
Potentials	• Effective for initial drug screening and basic mechanistic studies. • Suitable for large-scale studies with consistent and reproducible outputs.	• Ideal for studying tumor progression, metastasis, and resistance mechanisms. • Promising for personalized medicine applications, including patient-derived models.

1.3. Spheroids in Personalized Medicine

Personalized medicine aims to customize healthcare interventions based on individual patient characteristics, and spheroids emerge as a powerful tool in this endeavor. The inherent heterogeneity within patient populations can be more accurately represented using spheroids, enabling the development of personalized therapeutic strategies. By incorporating patient-specific cells into spheroid models, researchers can assess drug responses and tailor treatment regimens for improved clinical outcomes [14].

Spheroids have emerged as a pivotal three-dimensional (3D) *in vitro* model in cancer research, offering a significant leap forward in replicating the complexity of *in vivo* tumor biology. Unlike traditional two-dimensional (2D) monolayer cultures, spheroids self-assemble into multicellular aggregates, creating a structurally and functionally relevant microenvironment that mimics many key aspects of solid tumors. This enhanced physiological relevance makes spheroids an invaluable tool for advancing our understanding of cancer biology and improving preclinical evaluations of therapeutic agents [15].

The defining characteristic of spheroids is their ability to form gradients of oxygen, carbon dioxide, nutrients, and metabolites. These gradients lead to the development of distinct cellular zones within the spheroid [15].

Cellular zones of spheroids are visualized and presented in Figure 1. Three cellular zones of spheroids are:

 doi:10.20944/preprints202504.0640.v1

- Proliferative outer layer: Consisting of actively dividing cells, with high accessibility to oxygen and nutrients.
- Quiescent intermediate layer: Consisting of quiescent and senescent cells with reduced metabolic activity due to limited nutrient and oxygen availability.
- Hypoxic apoptotic core: Consisting of cells in apoptotic state due to severe nutrient and oxygen deprivation. Core environment mimics what is observed in poorly vascularized tumor regions in vivo.

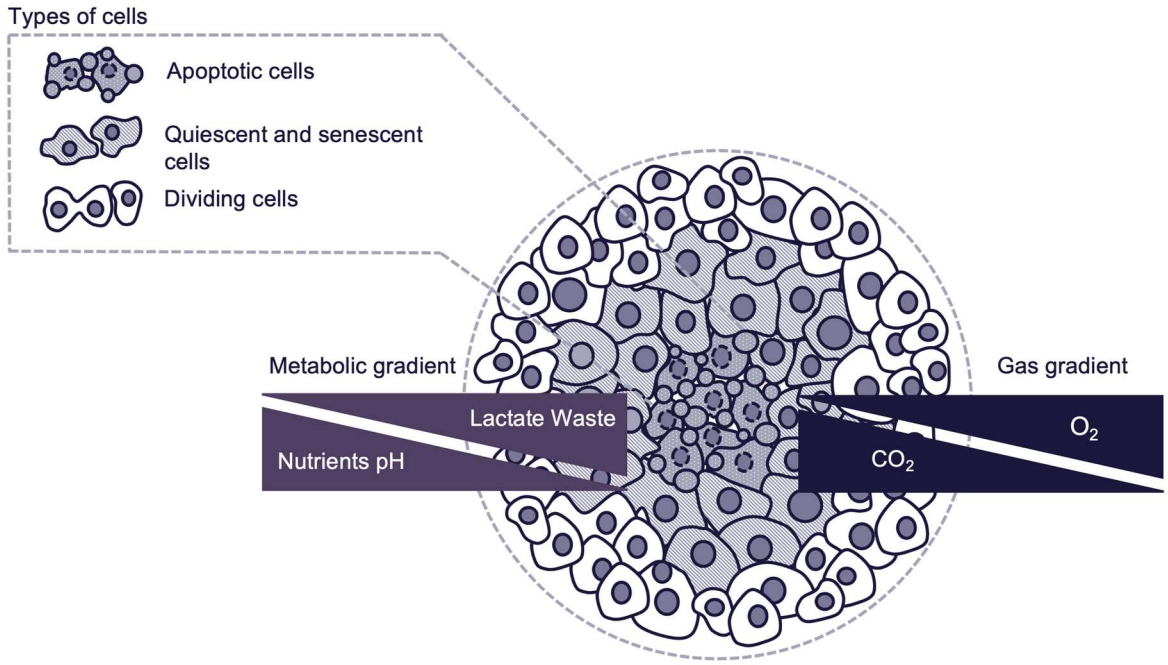

Figure 1. Cellular structure of spheroid.

This zonal architecture replicates the heterogeneous microenvironment of solid tumors, which is critical for studying tumor progression, metastasis, and resistance to therapies.

1.4. Applications of Spheroids Models

Spheroids are widely utilized in cancer research as robust models for examining critical biological processes in a three-dimensional context. They provide significant insights into tumor progression by enabling detailed investigation of invasion, metastasis, and interactions between the tumor and surrounding stroma. In the realm of therapeutic screening, spheroids facilitate the evaluation of anti-cancer drug efficacy, penetration dynamics within the tumor microenvironment, and mechanisms of drug resistance. They are also essential for modeling tumor responses to hypoxia and radiation therapy, offering a physiologically relevant system that closely mimics *in vivo* conditions. Additionally, spheroids play a pivotal role in immunotherapy research by supporting the study of immune cell-tumor interactions, thereby providing a more representative alternative to conventional two-dimensional culture models [15,16]

2. Materials and Methods

A comprehensive search was conducted in the PubMed database to identify relevant articles available until December 2024. The search utilized specific free words and Medical Subject Headings (MeSH) terms, including key terms such as 'spheroid,' 'cancer,' 'drug,' 'patient-derived,' and 'tumor.' Exclusion terms 'co-culture' and 'xenograft' were applied. Only articles published in English were considered for inclusion.

 doi:10.20944/preprints202504.0640.v1

Inclusion criteria encompassed studies involving spheroids formed from patient-derived cancer cells, while exclusion criteria covered articles that did not meet these inclusion criteria, reviews, case reports, and works focusing on the application or use of spheroids in areas other than drug screening.

Data extraction from included articles comprised information on authors, publication year, title, cancer type, cell aggregation protocol, culture conditions, spheroid measurements, *in vitro* functional properties, therapeutic effects *in vivo*, and study model employed.

The inclusion criterion for English-language publications may introduce potential bias, excluding relevant literature in other languages. Consequently, the findings should be interpreted cautiously, acknowledging the potential limitations associated with the language-based selection criteria.

3. Results

3.1. Database Screening

Following a screening of 190 articles retrieved through searches in PubMed pertaining to seeding of spheroids from human cancer cells for drug sensitivity screening, only 143 articles were evaluated for eligibility based on the search strategy outlined in the materials and methods section. The selection process is illustrated in Figure 2.

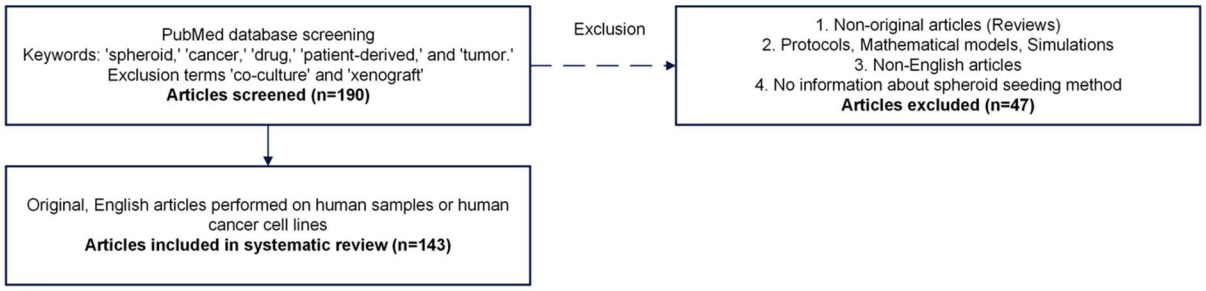

Figure 2. Selection Process.

3.2. Systematic Review

Table 2 displays the selected 143 articles along with their fundamental details, including: first author and publication year, cancer type by type of tissue in which cancer originates (histological type) and by primary site, spheroid seeding method.

Table 2. Articles selected for systematic review.

Reference	Cancer primary site (histological type)	Cell line	Spheroid formation method
Agastin S, 2011 [17]	Colon (adenocarcinoma), Breast (adenocarcinoma)	Colo205, MDA-MB-231	Hanging drop
Alhasan L, 2016 [18]	Breast (carcinoma)	BT-474	Scaffold-based methods
An HJ, 2020 [19]	Kidney (carcinoma)	A498	Scaffold-based methods
Árnadóttir SS, 2018 [20]	Colon	Patient-derived	Forced-floating (not defined)
Baek N, 2016 [21]	Prostate (carcinoma), Bone (neuroblastoma), Lung (carcinoma), Cervix (adenocarcinoma), Bone (osteosarcoma)	DU145, SH-SY5Y, A549, HeLa, HEp2, 0U-2OS	Forced-floating (agar coated plates)
Barone RM, 1981 [22]	Colon (adenocarcinoma)	HT-29	Suspension culture
Bartholomä P, 2005 [23]	Breast (carcinoma)	T-47D	Suspension culture
Boo L, 2020 [24]	Breast (adenocarcinoma)	MCF-7	Forced-floating (agar coated plates)

 doi:10.20944/preprints202504.0640.v1

Brooks EA, 2019 [25]	Ovary (adenocarcinoma)	Patient-derived, AU565, BT549, SKOV-3	Scaffold-based methods
Bruns J, 2022 [26]	Brain (glioblastoma)	Patient-derived, U87	Scaffold-based methods
Calori IR, 2022 [27]	Brain (glioblastoma, medulloblastoma)	U87, T98G, A172, UW473	Forced-floating (ULA)
Chang S, 2022 [28]	Breast (adenocarcinoma)	MCF-7	Recent advances
Chen G, 2022 [29]	Breast (adenocarcinoma)	MCF-7	Forced-floating (ULA)
Chen MC, 2010 [30]	Breast (melanoma)	LCC6/Her-2	Recent advances
Chen Z, 2021 [31]	Breast (adenocarcinoma)	MDA-MB-231	Forced-floating (ULA)
Cheng V, 2015 [32]	Brain (glioblastoma)	U87, U251	Forced-floating (ULA)
Close DA, 2022 [33]	Oral (squamous cell carcinoma)	Cal33, FaDu, UM-22B, OSC-19	Forced-floating (ULA)
Das T, 2013 [34]	Ovary (adenocarcinoma)	TOV112D	Hanging drop
Das V, 2016 [35]	Colon (carcinoma), Colon (adenocarcinoma), Bone (osteosarcoma), Cervix (adenocarcinoma), Colon (adenocarcinoma), Liver (carcinoma)	HCT116, HT29, U-2OS, HeLa, Caco-2, HepG2	Forced-floating (liquid overlay)
Das V, 2017 [36]	Colon (carcinoma)	HCT116	Forced-floating (liquid overlay)
De Angelis ML, 2018 [37]	Colon	Patient-derived	Forced-floating (ULA)
Dhamecha D, 2021 [38]	Lung (carcinoma), Bone (osteosarcoma)	A549, MG-63	Scaffold-based methods
Dias DR, 2016 [39]	Cervix (adenocarcinoma)	HeLa	Recent advances
Domenici G, 2021 [40]	Bone (sarcoma)	Patient-derived	Forced-floating (ULA)
Dufau I, 2012 [41]	Pancreas (adenocarcinoma)	Capan-2	Forced-floating (poly-HEMA)
Eetezadi S, 2018 [42]	Ovary (carcinoma), Ovary (adenocarcinoma)	UWB1.289, UWB1.289+BRCA1, OV-90, SKOV3, PEO1, PEO4, COV362	Forced-floating (ULA)
Eguchi H, 2022 [43]	Lung (carcinoma)	A549	Forced-floating (ULA)
Eimer S, 2012 [44]	Brain (glioblastoma)	Patient-derived	Forced-floating (ULA)
El-Sadek IA, 2021 [45]	Breast (adenocarcinoma)	MCF-7	Forced-floating (ULA)
Enmon RM Jr, 2001 [46]	Prostate (carcinoma)	DU 145	Forced-floating (agar plates)
Flørenes VA, 2019 [47]	Skin (Melanoma)	Patient-derived	Forced-floating (ULA)
Fu J, 2020 [48]	Liver (carcinoma), Prostate (carcinoma), Lung (carcinoma), Breast (adenocarcinoma)	HepG2, DU 145, A549, MCF-7, MDA-MB-231	Scaffold-based methods
Fu JJ, 2018 [49]	Prostate (carcinoma)	DU 145, LNCap	Scaffold-based methods
Gao Y, 2022 [50]	Lung (carcinoma)	A549	Recent advances
Gencoglu MF, 2018 [51]	Breast (adenocarcinoma), Breast (carcinoma), Prostate (carcinoma), Prostate (adenocarcinoma), Ovary (adenocarcinoma)	AU565, BT549, BT474, HCC 1419, HCC 1428, HCC 1806, HCC 1954, HCC 202, HCC 38, ZR75 1, HCC 70, LNCaPcol, PC3, SKOV3	Scaffold-based methods, Microwells, Suspension culture
Gendre DAJ, 2021 [52]	Lung (mesothelioma), Lung (adenocarcinoma)	H2052, H2052/484, H2452, LuCa1, LuCa61, LuCa62	Scaffold-based methods
Gheytanchi E, 2021 [53]	Colon (adenocarcinoma)	HT-29, Caco-2	Hanging drop, Forced-floating (Poly-HEMA)
Goisnard A, 2021 [54]	Breast (carcinoma), Breast (adenocarcinoma)	SUM1315, MDA-MB-231, HCC1937, SW527, DU4475	Forced-floating (ULA)
Guo X, 2019 [55]	Ovary (adenocarcinoma), Colon (adenocarcinoma),	OVCAR3, SW620, PANC-1, PC3	Scaffold-based methods

 doi:10.20944/preprints202504.0640.v1

	Pancreas (carcinoma), Prostate (adenocarcinoma)		
Hagemann J, 2017 [56]	Oral (carcinoma)	FaDu, Cal27, UPCI-SCC-154	Forced-floating (ULA), Hanging drop
Han S, 2022 [57]	Liver	Patient-derived	Forced-floating (ULA)
Harmer J, 2019 [58]	Brain (glioblastoma)	U251, KNS42	Scaffold-based methods
Herter S, 2017 [59]	Colon (adenocarcinoma)	LS174T, LoVo	Hanging drop
Ho WY, 2012 [60]	Breast (adenocarcinoma)	MCF-7	Forced-floating (liquid overlay)
Ho WY, 2021 [61]	Breast (adenocarcinoma)	MCF-7	Scaffold-based methods
Hofmann S, 2022 [62]	Breast	Patient-derived	Forced-floating (ULA)
Hornung A, 2016 [63]	Colon (adenocarcinoma)	HT-29	Scaffold-based methods
Huang Z, 2020 [64]	Breast (adenocarcinoma)	MDA-MB-231	Scaffold-based methods
Jove M, 2019 [65]	Breast (adenocarcinoma) Colorectal (adenocarcinoma)	MCF-7, DLD-1	Scaffold-based methods
Ju FN, 2023 [66]	Brain (glioblastoma)	U87	Recent advances
Karamikamkar S, 2018 [67]	Breast (adenocarcinoma)	MCF-7	Scaffold-based methods
Karlsson H, 2012 [68]	Colon (carcinoma)	HCT-116	Forced-floating (ULA)
Karshieva SS, 2022 [69]	Colon (carcinoma), Liver (carcinoma)	HCT-116, Huh7	Forced-floating (ULA)
Kato EE, 2021 [70]	Lung (carcinoma)	A549	Hanging drop
Kim CH, 2020 [71]	Liver (carcinoma)	HepG2	Recent advances
Ko J, 2019 [72]	Brain (glioblastoma)	U87	Scaffold-based methods
Kochanek SJ, 2019 [73]	Oral (carcinoma)	Cal33, Cal27, FaDu, UM-22B, BICR56, OSC-19, PCI-13, PCI-52, Detroit-562, UM-SCC-1, and SCC-9	Forced-floating (ULA)
Kochanek SJ, 2020 [74]	Oral (carcinoma)	Cal33, FaDu, UM-22B, BICR56, OSC-19	Forced-floating (ULA)
Koshkin V, 2016 [75]	Breast (adenocarcinoma)	MCF-7	Scaffold-based methods
Kroupová J, 2022 [76]	Colon (adenocarcinoma)	HT-29	Forced-floating (not defined)
Kudláčová J, 2020 [77]	Brain (glioblastoma)	U87	Forced-floating (ULA)
Kumari P, 2017 [78]	Cervix (adenocarcinoma), Lung (carcinoma)	HeLa, A549	Scaffold-based methods
Lal-Nag M, 2017 [79]	Ovary (adenocarcinoma)	Hey-A8–GFP	Forced-floating (ULA)
Lama R, 2013 [80]	Lung (carcinoma)	H292	Scaffold-based methods
Landgraf L, 2022 [81]	Prostate (adenocarcinoma), Brain (glioblastoma)	PC-3, U87	Forced-floating (liquid overlay)
Le VM, 2016 [82]	Lung (carcinoma), Colon (carcinoma), Brain (glioblastoma)	95-D, HCT-116, U87	Scaffold-based methods
Lee SW, 2019 [83]	Lung (carcinoma)	A549	Recent advances
Lee Y, 2022 [84]	Lung (carcinoma)	H460, A549	Forced-floating (ULA)
Lemmo S, 2014 [85]	Breast (adenocarcinoma)	MDA-MB-231	Scaffold-based methods
Li M, 2019 [86]	Cervix (carcinoma)	C-33-A, DoTC2 4510	Forced-floating (ULA)
Lim W, 2018 [87]	Colon (carcinoma), Brain (glioblastoma)	HCT-116, U87	Recent advances
Lin ZT, 2021 [88]	Breast (adenocarcinoma)	MDA-MB-436	Scaffold-based methods
Liu X, 2021 [89]	Sarcoma	HS-SY-II	Recent advances
Lorenzo C, 2011 [90]	Pancreas (adenocarcinoma)	Capan-2	Forced-floating (poly-HEMA)
Luan Q, 2022 [91]	Lung (adenocarcinoma), Lung (carcinoma)	HCC4006, H1975, A549	Scaffold-based methods, Forced-floating (ULA)

 doi:10.20944/preprints202504.0640.v1

Madsen NH, 2021 [92]	Breast (adenocarcinoma), Colon (adenocarcinoma), Pancreas (carcinoma)	MCF-7, HT-29, PANC-1, MIA PaCa-2	Forced-floating (ULA)
Marshall SK, 2022 [93]	Bone (osteosarcoma)	MG-63	Forced-floating (ULA)
Maruhashi R, 2018 [94]	Lung (carcinoma)	A549	Forced-floating (ULA)
Melnik D, 2020 [95]	Thyroid (carcinoma)	FTC-133	Suspension culture
Molyneaux K, 2021 [96]	Brain (glioblastoma)	LN229, U87, Gli36	Forced-floating (not defined)
Monazzam A, 2007 [97]	Breast (adenocarcinoma)	MCF-7	Forced-floating (agar plates)
Morimoto T, 2023 [98]	Gastric	Patient-derived	Scaffold-based methods
Mosaad EO, 2018 [99]	Prostate (cancer), Prostate (carcinoma)	C42B, LNCaP	Recent advances
Mueggler A, 2023 [100]	Lung	Patient-derived	Scaffold-based methods
Nashimoto Y, 2020 [101]	Breast (adenocarcinoma)	MCF-7	Recent advances
Nigjeh SE, 2018 [102]	Breast (adenocarcinoma)	MDA-MB-231	Forced-floating (agar plates), Forced-floating (ULA)
Nittayaboon K, 2022 [103]	Colon (carcinoma)	PMF-k014	Forced-floating (poly-HEMA)
Ohya S, 2021 [104]	Prostate (carcinoma)	LNCaP	Forced-floating (ULA)
Oliveira MS, 2016 [105]	Breast (adenocarcinoma), Ovary (adenocarcinoma)	MCF-7/Adr, NCI/Adr	Forced-floating (liquid overlay)
Ono K, 2022 [106]	Oral (carcinoma)	SAS, HSC-3, HSC-4, OSC-19	Forced-floating (ULA)
Pampaloni F, 2017 [107]	Brain (glioblastoma)	U343	Forced-floating (liquid overlay)
Park MC, 2016 [108]	Brain (glioblastoma)	Patient-derived and PC14PE6, PC14PE6_LvBr3, D54, LN428, LN751, U251E4, U87E4, SN-12C, SNU-119, SNU-216, SNU-668, SNU-719, HCC1171, HCC1195, HCC15, HCC1588, HCC2108, HCC44	Forced-floating (not defined)
Pattni BS, 2016 [109]	Ovary (adenocarcinoma)	NCI/ADR-RES	Forced-floating (liquid overlay)
Perche F, 2012 [110]	Ovary (adenocarcinoma)	NCI/ADR-RES	Forced-floating (liquid overlay)
Preda P, 2023 [111]	Breast (adenocarcinoma), Brain (glioblastoma)	MDA-MB-231, U87	Scaffold-based methods
Pulze L, 2020 [112]	Breast (adenocarcinoma)	MCF-7	Forced-floating (ULA)
Raghavan S, 2016 [113]	Breast (adenocarcinoma), Ovary (adenocarcinoma)	MCF-7, OVCAR8	Hanging drop, Forced-floating (liquid overlay)
Raghavan S, 2019 [114]	Ovary (adenocarcinoma)	A2780, OVCAR3	Hanging drop
Ralph ACL, 2020 [115]	Breast (adenocarcinoma), Breast (carcinoma)	MCF-7, MDA-MB-231, T47D	Hanging drop
Roering P, 2022 [116]	Ovary (adenocarcinoma)	Patient-derived, CAOV3, OVCAR8	Forced-floating (ULA)
Roudi R, 2016 [117]	Lung (carcinoma)	A549	Forced-floating (poly-HEMA)
Rouhani M, 2014 [118]	Breast (carcinoma)	T47D	Forced-floating (liquid overlay)
Sakumoto M, 2018 [119]	Sarcoma	Patient-derived	Forced-floating (ULA)
Salehi F, 2020 [120]	Breast (adenocarcinoma), Breast (carcinoma)	MDA-MB-231, T47D, MCF-7	Forced-floating (liquid overlay), Hanging drop
Sambi M, 2020 [121]	Breast (adenocarcinoma)	MDA-MB-231	Scaffold-based methods
Sankar S, 2021 [122]	Lung (carcinoma)	A549	Recent advances
Särchen V, 2022 [123]	Sarcoma	RH30	Forced-floating (ULA)
Sarıyar E, 2023 [124]	Liver (carcinoma)	Huh7	Hanging drop
Sauer SJ, 2017 [125]	Breast (carcinoma), Breast (adenocarcinoma)	SUM149, SUM190, T47D, MCF-7	Forced-floating (ULA)

 doi:10.20944/preprints202504.0640.v1

Shaheen S, 2016 [126]	Colon (carcinoma)	HCT-116	Forced-floating (not defined)
Shen K, 2014 [127]	Breast (adenocarcinoma)	MDA-MB-231	Scaffold-based methods
Sheth DB, 2019 [128]	Breast (adenocarcinoma)	MCF-7	Recent advances
Shortt RL, 2023 [129]	Colon (carcinoma)	HCT-116	Scaffold-based methods
Singh A, 2020 [130]	Breast (adenocarcinoma)	MCF-7	Scaffold-based methods
Suhito IR, 2021 [131]	Bone (neuroblastoma), Brain (glioblastoma)	SH-SY5Y, U-87	Recent advances
Tanenbaum LM, 2017 [132]	Ovary (adenocarcinoma)	UCI101, A2780	Forced-floating (not defined)
Tang S, 2017 [133]	Colon (adenocarcinoma), Ovary (adenocarcinoma)	HT-29, SKOV-3	Hanging drop
Taubenberger AV, 2019 [134]	Breast (adenocarcinoma)	MCF-7	Scaffold-based methods
Terrones M, 2024 [135]	Lung (adenocarcinoma)	HCC78	Forced-floating (ULA)
Tevis KM, 2017 [136]	Breast (adenocarcinoma)	MDA-MB-231	Scaffold-based methods
To HTN, 2022 [137]	Stomach (carcinoma)	SNU-216, SNU-484, SNU-601, SNU-638, SNU-668, and SNU-719	Forced-floating (ULA)
Torisawa YS, 2007 [138]	Breast (adenocarcinoma), Liver (carcinoma)	MCF-7, HepG2	Recent advances
Uematsu N, 2018 [139]	Breast (adenocarcinoma)	MCF-7	Recent advances
Varan G, 2021 [140]	Lung (carcinoma), Liver (carcinoma)	A549, HepG2	Forced-floating (poly-HEMA)
Vinci M, 2012 [141]	Brain (glioblastoma), Oral (carcinoma), Breast (adenocarcinoma)	U87, KNS42, LICR-LON-HN4, MDA-MB-231	Forced-floating (ULA), Agarose plates
Wan X, 2016 [142]	Colon (adenocarcinoma), Ovary (adenocarcinoma)	DLD-1, NCI/ADR	Scaffold-based methods
Wang Y, 2014 [143]	Cervix (adenocarcinoma)	HeLa	Scaffold-based methods
Ware MJ, 2016 [144]	Pancreas (carcinoma)	PANC-1, AsPc-1, BxPC-3, Capan-1, MIA PaCa-2 cells	Hanging drop
Wen Z, 2013 [145]	Pancreas (carcinoma)	MIAPaCa-2, PANC-1	Scaffold-based methods
Wenzel C, 2014 [146]	Breast (carcinoma)	T47D	Forced-floating (liquid overlay)
Weydert Z, 2020 [147]	Ovary (adenocarcinoma)	HEY, SKOV-3	Hanging drop
Wu G, 2019 [148]	Liver (carcinoma)	HepG2, Huh7	Scaffold-based methods
Wu KW, 2020 [149]	Bladder (carcinoma), Lung (carcinoma), Liver (carcinoma)	T24, A549, Huh-7	Recent advances
Xia H, 2020 [150]	Brain (glioblastoma)	LN229, U87	Scaffold-based methods
Xiong Q, 2023 [151]	Bladder	Patient-derived	Forced-floating (ULA)
Yamawaki K, 2021 [152]	Ovary	Patient-derived	Forced-floating (ULA)
Yoshida T, 2019 [153]	Bladder	Patient-derived	Scaffold-based methods
Yu L, 2015 [154]	Breast (adenocarcinoma)	MCF-7	Recent advances
Yu Q, 2021 [155]	Breast (adenocarcinoma)	MDA-MB-436, MDB-MB-231	Scaffold-based methods
Zhang JZ, 2012 [156]	Colon (adenocarcinoma), Ovary (teratocarcinoma)	DLD-1, PA-1 ovarian cancer cells	Forced-floating (liquid overlay)
Zhang JZ, 2012 [157]	Colon (adenocarcinoma)	DLD-1	Forced-floating (liquid overlay)
Zhang X, 2005 [158]	Breast (adenocarcinoma)	MCF-7	Recent advances
Zuchowska A, 2017 [159]	Liver (carcinoma)	HepG2	Recent advances

3.2.1. Source of Spheroids

Spheroids derived from various cancer types are extensively utilized in research to mimic in vivo tumor characteristics, providing insights into diverse cancer-specific processes. The ability to

 doi:10.20944/preprints202504.0640.v1

generate spheroids from a variety of cell sources, including patient-derived tumor cells, further enhances their value in studying personalized therapeutic responses. These models allow researchers to recreate the complexity of individual tumors and test specific treatment regimens in vitro, paving the way for advances in precision and personalized medicine. In the present systematic review, the most common cell sources of spheroids were identified. Five most investigated cancer origins associated with spheroids were breast (n=46, accounting for 25% of all investigated cancers), followed by colon (n=23, 12%), lung (n=21, 11%), ovary (n=19, 10%), and brain (n=18, 10%). A complete summary of systematic review results is presented in Figure 3 and Table 3.

Table 3. Summary of spheroid sources.

Spheroid formation method	Number of studies	Percentage of total number of studies
Forced-floating	70	31.8%
Scaffold-based	41	18.6%
Recent advances	21	9.5%
Hanging drop	14	6.4%
Suspension culture	4	1.8%
Magnetic levitation	0	0.0%

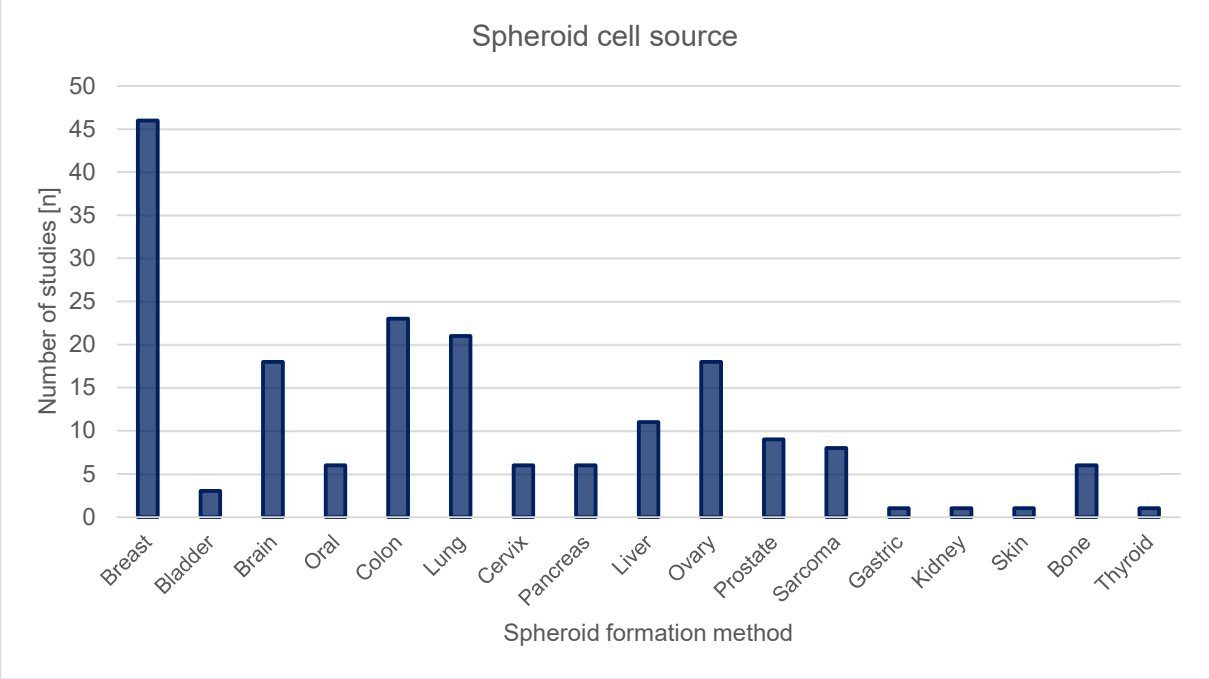

Figure 3. Summary of spheroid sources.

Breast Cancer

Spheroids derived from breast cancer cells represent one of the most extensively studied *in vitro* models, reflecting the prominence of breast cancer as both a clinical challenge and leading research focus. Systematic review of the literature revealed that breast cancer cell lines and patient-derived cells were the most frequently used sources for spheroid generation.

Breast cancer spheroid models facilitate the exploration of unique tumor characteristics (e.g. variations in growth dynamics, gene expression profiles, and interactions with the tumor microenvironment) and aspects of cancer biology (e.g. immortality, telomerase activation, antiapoptotic strategy) [20,28,48,156].

Moreover, this approach facilitates the exploration of therapies targeting estrogen-metabolizing enzymes and receptors, enabling the discovery of novel treatments that may prevent tumor initiation or inhibit cancer growth [26].

 doi:10.20944/preprints202504.0640.v1

Additionally, this methodology supports the development of patient-specific drugs, thereby aligning with the principles of precision medicine to optimize therapeutic outcomes for individual breast cancer patients [59].

Colon Cancer

Colon cancer is the second most common source of cells used for spheroid generation, reflecting its critical role in cancer research.

To enhance the utility of human 3D colorectal cancer spheroid models in preclinical drug assessment, there is the need for standardized and validated methodologies. While monoculture spheroids are useful for high-throughput drug screening due to their simplicity, spheroids provide deeper insights into tumor biology and chemoresistance mechanisms, offering a more accurate preclinical tool for evaluating therapeutic efficacy and developing new drug candidates [33,157].

3D cultures still face challenges in clinical implementation, and advancements in co-culture techniques, addressing tumor heterogeneity, and improving laboratory protocols are essential for enhancing reproducibility and drug testing reliability in colorectal cancer research [158]. Moreover, collecting cells during biopsy from different tumor sites might provide more comprehensive representation of tumor subclones, offering greater insight into the tumor's diverse properties and improving the accuracy of preclinical models for drug testing and personalized medicine [158].

Lung Cancer

Lung cancer represents the third most common source of cells for generating spheroids. Lung cancer-derived spheroids are utilized to investigate key aspects of tumor biology.

Lung cancer spheroids are particularly valuable for studying the progression of both non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), which differ significantly in their biological behavior and response to therapy [159,160].

Therapeutically, lung cancer spheroids serve as platforms for evaluating the efficacy of novel anti-cancer agents, including small molecules, biologics, and combination therapies. Their three-dimensional structure facilitates studies of drug delivery systems aimed at overcoming barriers such as limited penetration into solid tumors [40,47,49,67,77,119].

Ovarian Cancer

Ovarian cancer is the fourth most common source of cells used for spheroid generation. Spheroids derived from ovarian cancer cells are utilized in research to study processes central to ovarian cancer pathology, such as peritoneal metastasis, chemoresistance, and interactions with the tumor microenvironment. Given the propensity of ovarian cancer to spread via the peritoneal cavity through multicellular aggregates, spheroids serve as a physiologically relevant model to replicate these metastatic behaviors *in vitro* [31,52,76,106,107].

Moreover, patient-derived ovarian cancer spheroids are increasingly used for precision oncology, allowing for the evaluation of personalized therapeutic strategies tailored to the molecular profiles of individual tumors [113,148].

Brain Cancer

Brain cancers, including glioblastoma and other gliomas, rank as the fifth most common source of cells used for spheroid generation.

These three-dimensional models are pivotal for studying the unique microenvironment and invasive properties of brain tumors, which are characterized by their aggressive behavior and resistance to standard therapies. Brain cancer-derived spheroids closely mimic the *in vivo* conditions of brain tumors, providing insights into key processes such as tumor invasion, therapeutic resistance, and interactions with the extracellular matrix (ECM). Glioblastoma-derived spheroids are among the most studied in this category. They are particularly valuable for investigating the highly invasive

 doi:10.20944/preprints202504.0640.v1

nature of glioblastoma cells, which infiltrate surrounding healthy brain tissue, making complete surgical resection nearly impossible [24,63,74,93,104].

Recent advancements include patient-derived brain cancer spheroids, which preserve the genetic and phenotypic heterogeneity of primary tumors. These models are increasingly used for personalized medicine, enabling the testing of individualized therapeutic regimens in a controlled in vitro setting [30,64,147].

3.2.2. Spheroid Seeding Methods

The successful implementation of spheroids in personalized medicine relies on robust and reproducible methodologies for their generation. The generation of three-dimensional (3D) spheroids as in vitro models requires careful consideration of seeding methods to ensure reproducibility, scalability, and physiological relevance. A variety of techniques have been developed to create spheroids, ranging from traditional methods to advanced approaches incorporating cutting-edge technologies. An overview of the most common and recent seeding methods used for the seeding of spheroids from human cancer cells is presented below.

In conclusion, this article aims to provide a thorough understanding of the methodologies employed in spheroid seeding and highlights the manifold applications of spheroids in advancing personalized medicine.

Our systematic review revealed that, among spheroid seeding methodologies, the most common were forced floating (n=70), scaffold-based methods (n=41), recent advances (n = 20), hanging drop (n=14) and suspension culture (n=4). A summary of systematic review results is presented in Figure 4 and Table 4.

Table 4. Summary of spheroid seeding methods.

Spheroid formation method	Number of studies	Percentage of total number of studies
Forced-floating	70	31.8%
Scaffold-based	41	18.6%
Recent advances	21	9.5%
Hanging drop	14	6.4%
Suspension culture	4	1.8%
Magnetic levitation	0	0.0%

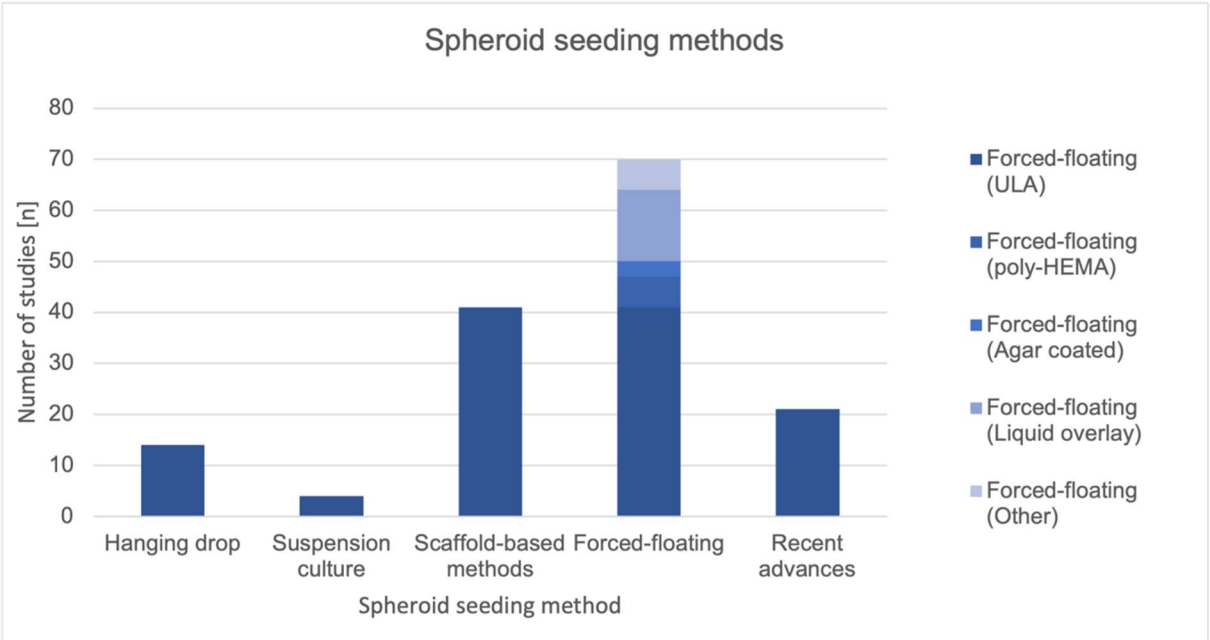

Figure 4. Summary of spheroid seeding methods.

 doi:10.20944/preprints202504.0640.v1

Based on the systematic review, a summary of the most common spheroid seeding methods is illustrated in Figure 5. The figure visualizes the most utilized spheroid seeding methods such as hanging drop (1), forced-floating (2), magnetic levitation (3), scaffold-based (4), suspension culture (5), and recent scientific advances (6) methods: microencapsulation (6a), bioprinting (6b), nanoparticles (6c), chips and microfluidics (6d).

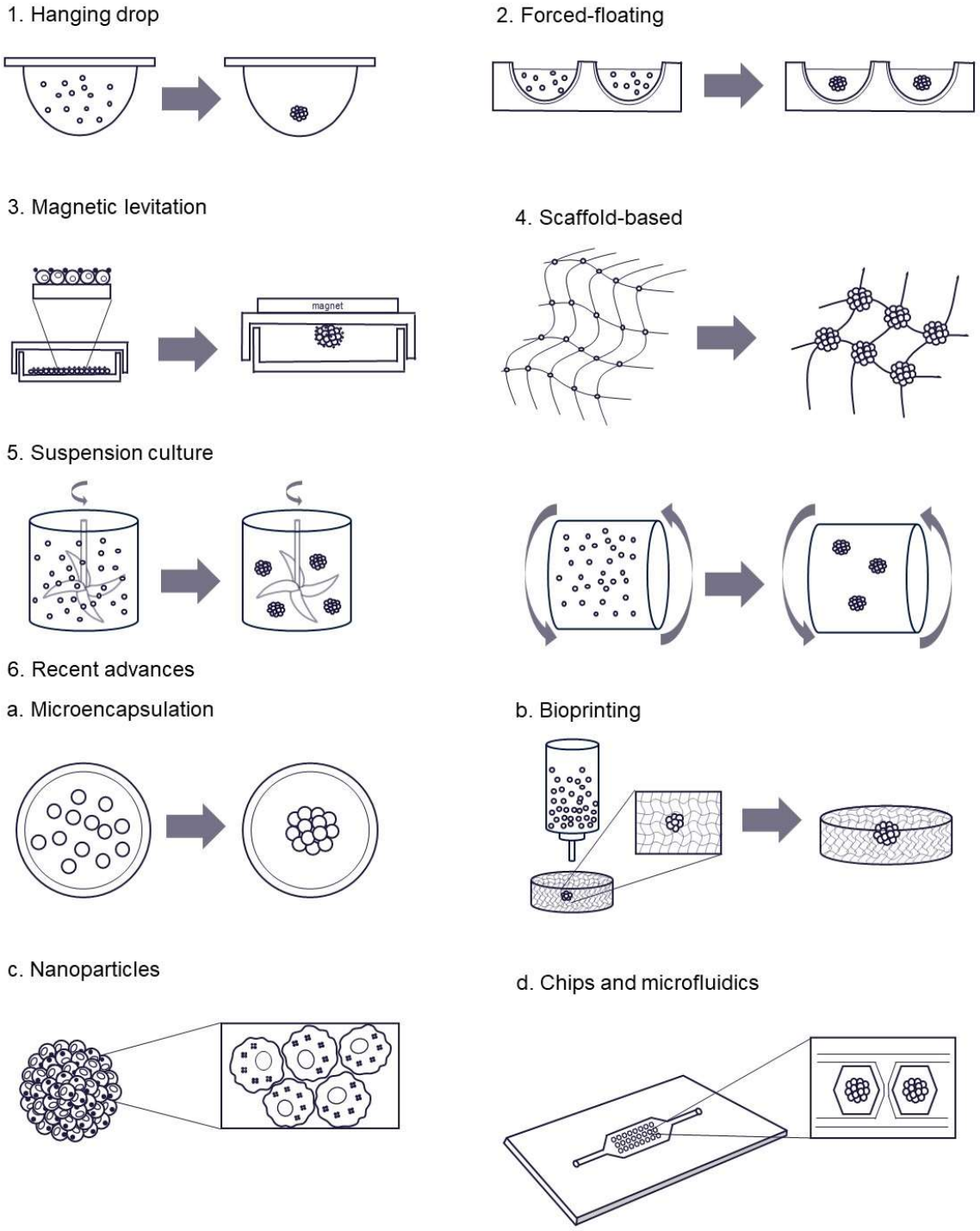

Figure 5. Techniques for creation of spheroids.

Forced Floating

The forced floating method is the most employed technique for spheroid formation due to its simplicity and scalability. This approach uses non-adhesive surfaces to prevent cell attachment,

 doi:10.20944/preprints202504.0640.v1

encouraging cells to aggregate and form three-dimensional (3D) spheroids. The technique involves seeding cells in multi-well plates that have been treated to inhibit surface adhesion, either through coating with low-attachment materials like poly-HEMA (Poly(2-hydroxyethyl methacrylate)) or using ultra-low attachment (ULA) culture plates designed specifically for this purpose. In the absence of adhesion sites, cells naturally aggregate in the medium, forming spheroids under the influence of gravity and intercellular interactions. The process begins with the preparation of a single-cell suspension of the desired cell density, typically ranging from 10^3 to 10^5 cells per well, depending on the type of cells and the intended spheroid size. The cell suspension is then distributed into the wells of the plate. Over the course of 24–96 hours, depending on the cell type and experimental conditions, the cells self-assemble into compact spheroids [22,100,138,163].

The forced floating method is particularly advantageous for producing uniform spheroids with consistent size and morphology, making it suitable for high-throughput applications such as drug screening and toxicity assays. Moreover, one of the significant benefits of the forced floating method is its compatibility with automated systems, allowing for large-scale spheroid generation and analysis. Additionally, this method does not require specialized equipment beyond the plates or coatings, making it accessible for most laboratories. However, the method has certain limitations. The reliance on non-adhesive surfaces can lead to variability in spheroid integrity and size if the cell density or culture conditions are not carefully optimized. Additionally, long-term culture may be constrained by limited nutrient and oxygen diffusion, necessitating periodic medium exchange or supplementation with perfusion systems [7,31 55,57,92,139,113].

Scaffold-Based

The scaffold-based method for spheroid seeding is the second most utilized method for generating three-dimensional (3D) cellular aggregates. This method uses biomaterials, known as scaffolds, that mimic the extracellular matrix (ECM) to provide the structural support and environment conducive to cell adhesion, proliferation, and aggregation into spheroids. Scaffolds can be fabricated from a wide range of materials, including natural polymers such as collagen, gelatin, and alginate, as well as synthetic polymers like PLGA (poly(lactic-co-glycolic acid)) and PEG (polyethylene glycol). The process typically begins with the preparation of the scaffold material, which may be in the form of hydrogels, porous matrices, or microcarriers. Cells are then seeded onto or encapsulated within the scaffold. Once seeded, the cells interact with the scaffold material and with one another, eventually forming spheroid structures. The scaffold not only facilitates cell aggregation, but it also supports nutrient and oxygen diffusion, which is crucial for maintaining cell viability and function in 3D cultures [140,165,166].

Scaffold-based methods offer significant advantages, including ability to recreate a more physiologically relevant microenvironment compared to non-adhesive-based techniques. The structural and biochemical properties of the scaffold can be engineered to closely mimic the *in vivo* ECM, supporting the growth and differentiation of specific cell types. This makes the method particularly suitable for modeling complex tissues and for co-culture systems involving multiple cell types. Additionally, scaffold-based systems are compatible with long-term culture, as the scaffold provides a sustained environment for nutrient and waste exchange. However, there are limitations to this approach. The use of scaffolds introduces variability in spheroid size and shape, depending on the uniformity of the material and the seeding protocol. The composition and mechanical properties of the scaffold can also influence cellular behavior, which may complicate the interpretation of results. Furthermore, the cost and complexity of scaffold fabrication, particularly for advanced synthetic materials, may be a barrier for some applications [7,165].

Hanging Drop

The hanging drop method is a robust and third most utilized technique for generating three-dimensional (3D) spheroids *in vitro*, particularly valued for its ability to produce uniform and physiologically relevant cellular aggregates. This method capitalizes on gravity-driven cellular self-

 doi:10.20944/preprints202504.0640.v1

assembly within droplets of culture medium, facilitating interactions that mimic those found *in vivo*. To implement this approach, a cell suspension of the desired density is prepared, often ranging from 10^2 to 10^4 cells per droplet. Droplets, typically 20–50 μL in volume, are then dispensed onto the inner surface of an inverted petri dish lid. The droplets are retained by surface tension, allowing them to remain suspended. This setup is placed over a dish containing a hydrating agent, such as phosphate-buffered saline (PBS) or water, to maintain humidity and prevent evaporation during incubation. Over 24 to 72 hours under standard culture conditions, the suspended cells settle at the bottom of the droplets and aggregate into spheroids through intercellular adhesion and natural cell-cell interactions [17,34,53,113,115,143].

The hanging drop method is particularly advantageous due to its simplicity, low cost, and minimal equipment requirements. This method offers significant benefits, including enhanced cellular aggregation and adhesion driven by gravitational forces, which minimize mechanical damage to spheroids. It also allows for precise control over the spheroid size and cell composition by adjusting the initial cell density and droplet volume. However, this technique has limitations, such as the potential disruption of spheroids during transfer to conventional culture plates due to mechanical stress. Moreover, it is labor-intensive and not easily scalable for high-throughput applications, as each droplet must be individually prepared and managed. Additionally, nutrient and waste exchange are limited by the small volume of medium, necessitating careful monitoring to maintain spheroid viability. It has also been adapted for co-culture systems to explore interactions between different cell types, such as tumor and stromal cells [7,53,58,113,167,132].

Suspension Culture

The suspension culture method is the fourth most utilized approach for generating spheroids in *in vitro* studies, particularly in cancer research, developmental biology, and drug discovery. This technique relies on culturing cells in a liquid medium without a solid substrate, allowing them to aggregate and form spheroids due to intercellular adhesion and natural aggregation tendencies. Typically, the suspension culture is conducted in low-attachment culture vessels, such as non-adherent plates or spinner flasks, to prevent cell adhesion to the container surface and promote spheroid formation [140,168].

The process begins with the preparation of a single-cell suspension at a defined density, which is a critical parameter for achieving uniform spheroid size and morphology. These vessels prevent cells from adhering to the surface and maintain them in suspension. Spinner flasks or bioreactors are dynamic systems, where gentle agitation or rotation keeps the cells suspended and evenly distributed, which can enhance the uniformity of spheroid formation and improve mass transport of nutrients and oxygen [23,140,168].

The suspension culture method offers several advantages. It is relatively simple and cost-effective, requiring minimal specialized equipment beyond vessels. The method is highly versatile, accommodating various cell types and allowing for easy incorporation of co-culture systems to model complex cell-cell interactions, such as those between tumor and stromal cells. Furthermore, the suspension culture can be adapted for high-throughput applications, making it suitable for large-scale drug screening and toxicity testing. Despite its strengths, the suspension culture method has limitations. Nutrient and oxygen diffusion can be inadequate in larger spheroids, leading to hypoxic or necrotic cores. This limitation necessitates careful control of spheroid size and medium composition to maintain viability. Additionally, while the method is relatively straightforward, achieving consistent spheroid size and morphology can be challenging without precise control over cell seeding density and culture conditions. Moreover, prolonged culture durations may require frequent medium changes or the use of perfusion systems to sustain spheroid health [7,168].

 doi:10.20944/preprints202504.0640.v1

Recent Advances in Spheroid Seeding Methods

Recent advances in spheroid seeding methods are represented by microencapsulation, bioprinting, nanoparticle-assisted techniques, microfluidics and lab-on-a-chip methods.

Microencapsulation encapsulates cells in biocompatible hydrogels like alginate, mimicking the extracellular matrix (ECM) and facilitating spheroid formation. It promotes intercellular interactions within a controlled microenvironment, protecting cells from shear stress and supporting co-culture systems. However, challenges include nutrient diffusion limitations and difficulties in spheroid retrieval [157,169].

Bioprinting is a method utilizing 3D printing technologies, allowing for the precise placement of cells and biomaterials to replicate native tissue architecture. It offers exceptional control over spheroid organization and is particularly effective for high-throughput applications and heterotypic co-cultures. Limitations include the need for bioink optimization and high costs of equipment [170,171].

Nanoparticle-assisted methods precisely guide cell aggregation into spheroids by leveraging functionalized nanoparticles. Magnetic nanoparticles enable external manipulation, while adhesive ligands support aggregation. It offers reproducibility and integration with imaging or therapeutic applications but raises concerns about nanoparticle cytotoxicity and scalability [39,66,130,172].

Microfluidics and lab-on-a-chip are technologies that provide highly controlled environments for spheroid formation, mimicking *in vivo* conditions through precise nutrient gradients and mechanical control. Microfluidics supports real-time monitoring and complex co-cultures, although challenges include high costs, technical expertise, and scalability [28,34,50,83,87,101,173].

Magnetic Levitation

The magnetic levitation method is a less frequently used approach to spheroid formation. This method utilizes leveraging magnetic fields to promote cellular aggregation and 3D structure development. This technique involves the use of magnetic nanoparticles that are internalized by cells through incubation. The nanoparticles are typically composed of biocompatible materials such as iron oxide and may be functionalized with extracellular matrix (ECM) proteins or other molecules to enhance cellular uptake and minimize toxicity. After the cells have internalized the nanoparticles, they are exposed to a magnetic field, which forces them to aggregate and suspend in the culture medium. The magnetic field enables the controlled formation of compact spheroids by facilitating cell-cell and cell-ECM interactions [168,174].

Magnetic levitation offers several advantages. It allows for rapid and reproducible spheroid generation and provides a high degree of control over spheroid size and structure. Additionally, this method supports the formation of co-culture spheroids by enabling the simultaneous aggregation of different cell types, which is particularly useful for modeling tumor-stroma or tumor-immune cell interactions. The technique also facilitates the incorporation of ECM components, improving the physiological relevance of the spheroid microenvironment. Furthermore, magnetic levitation is amenable to high-throughput applications and can be easily scaled up for drug screening or other large-scale studies. Despite its advantages, the method has limitations. The requirement for magnetic nanoparticles introduces potential concerns regarding biocompatibility and cellular toxicity, particularly for long-term studies. Additionally, the uniformity of nanoparticle uptake among cells can vary, potentially leading to inconsistencies in spheroid formation. The cost of magnetic nanoparticles and specialized magnetic devices may also be a barrier for some laboratories [7,168,174].

Summary of Spheroid Seeding Method

A summary of advantages and limitations of selected spheroid seeding methods is presented in Table 5.

 doi:10.20944/preprints202504.0640.v1

Table 5. Summary of advantages and limitations of different seeding methods.

Method	Advantages	Limitations
Forced floating method	• Simple and scalable • Produces uniform spheroids • Compatible with automation • Cost-effective	• Spheroid integrity varies with cell density • Limited nutrient and oxygen diffusion for large spheroids
Scaffold-based	• Mimics extracellular matrix (ECM) • Supports long-term cultures • Allows co-cultures • Highly physiological	• Variability in size and shape • High cost of scaffolds • Complexity in interpreting scaffold-induced effects
Hanging drop method	• Low cost • Produces uniform spheroids • Minimal mechanical damage • Control over spheroid size	• Labor-intensive • Limited scalability • Potential damage during transfer • Limited nutrient exchange
Suspension culture	• Versatile • Simple and cost-effective • Suitable for high-throughput • Closer mimicry of *in vivo* conditions	• Nutrient and oxygen limitations in large spheroids • Consistency challenges in spheroid size
Microencapsulation	• Provides controlled microenvironment • Supports co-cultures • Protects from shear stress	• Nutrient diffusion limitations • Challenges in retrieving spheroids
Bioprinting	• Precise spheroid placement • High control over architecture • Effective for high-throughput applications	• Requires optimization of bioinks • Expensive equipment
Nanoparticle-assisted techniques	• Enable precise aggregation • Integrate with imaging/therapeutics • Reproducible	• Potential cytotoxicity of nanoparticles • Scalability concerns
Microfluidics and lab-on-a-chip	• Controlled environment • Mimics *in vivo* gradients • Real-time monitoring • Supports co-cultures	• High cost • Requires technical expertise • Limited scalability
Magnetic levitation	• High control over size • Supports co-cultures • Possible to adapt to high-throughput • Rapid and reproducible	• Potential toxicity of nanoparticles • Inconsistencies in particle uptake • Equipment costs

4. Conclusions

The findings of this systematic review underscore the growing interest in 3D cancer cell models, particularly spheroids, for cancer research, therapeutic testing, and personalized medicine.

Breast cancer emerged as the most investigated cancer source for spheroid seeding, accounting for 25% of the studies included in this review, followed by colon, lung, ovary, and brain cancers. This distribution reflects the cancers' high clinical relevance, particularly in research into tumor progression, drug resistance, and the need for better preclinical models to predict patient responses to therapies.

Among the various methods for spheroid formation, forced floating and scaffold-based methods were the most employed, each used in 71 (32% of all methods) and 41 studies (19% of all methods),

 doi:10.20944/preprints202504.0640.v1

respectively. These approaches provide reproducible, easy-to-implement techniques for spheroid generation. Forced floating involves the aggregation of cells in suspension, which then self-assemble into spheroids. This technique has been adopted due to its simplicity and ability to consistently produce spheroids across different cancer types. The use of this method across a wide variety of cancer types underscores its applicability in generating tumor models for drug discovery and testing. Scaffold-based methods involve embedding cells in a 3D matrix, are well-established and widely adopted in cancer research. Both methods facilitate the development of spheroids that closely resemble in vivo tumor environments, with the ability to evaluate drug efficacy and tumor microenvironment interactions. In recent years, more advanced techniques have emerged, contributing to the versatility of spheroid culture. Notably, the hanging drop method is becoming increasingly popular, offering distinct advantages. Hanging drop, which involves placing small drops of cell suspension on an inverted culture dish lid, results in the spontaneous aggregation of cells into 3D spheroids due to gravity and surface tension. While this method is more labor-intensive than forced floating or scaffold-based methods, it offers more physiologically relevant 3D structures and is gaining traction for use in drug screening and tumor modeling.

Whereas these established methods have significantly advanced the field of cancer research, there are still several challenges and limitations in spheroid culture. One major issue is the inherent difficulty in achieving the necessary level of reproducibility and standardization for large-scale therapeutic testing. While methods like forced floating and scaffold-based systems provide relatively consistent spheroid formation, techniques such as hanging drop are more prone to variability depending on experimental conditions. Standardizing spheroid protocols, particularly for high-throughput drug screening, remains an ongoing challenge in the field.

Interestingly, the review also highlighted the increasing application of patient-derived spheroids, which offer a more clinically relevant model by capturing the genetic and phenotypic variability of individual tumors. The ability to generate spheroids from different regions of the same tumor has the potential to capture tumor heterogeneity, which could be critical for personalized medicine approaches. This is especially important for cancers like breast and colon cancer, where the tumor microenvironment and its interactions with various cell types play a key role in metastasis, drug resistance, and immune evasion.

In conclusion, spheroid culture techniques, particularly those employing forced floating, scaffold-based methods, have proven valuable in the study of cancer biology and the testing of novel therapies. However, to move towards more clinically translatable models, additional research is needed to refine the methods, particularly in terms of reproducibility, standardization, and incorporation of tumor heterogeneity. The adoption of patient-derived and co-culture spheroid models may significantly improve the predictive power of in vitro drug testing and lead to better-targeted therapies for cancer patients. This section is not mandatory but can be added to the manuscript if the discussion is unusually long or complex.

Author Contributions: All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Acknowledgments: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

 doi:10.20944/preprints202504.0640.v1

The following abbreviations are used in this manuscript:

2D	Two-dimensional
3D	Three-dimensional
ECM	Extracellular Matrix
NSCLC	Non-small cell lung lymphoma
PEG	Polyethylene glycol
Poly-HEMA	Poly(2-hydroxyethyl methacrylate
PLGA	poly(lactic-co-glycolic acid
SCLC	Small cell lung cancer
ULA	Ultra-low attachment

References

1. Harrison, R. G. The Outgrowth of the Nerve Fiber as a Mode of Protoplasmic Movement. *J Exp Zool* **1910**, *9* (4), 787–846. https://doi.org/10.1002/jez.1400090405.
2. Scherer, W. F.; Syverton, J. T.; Gey, G. O. Studies on the Propagation in Vitro of Poliomyelitis Viruses. IV. Viral Multiplication in a Stable Strain of Human Malignant Epithelial Cells (Strain HeLa) Derived from an Epidermoid Carcinoma of the Cervix. *J Exp Med* **1953**, *97* (5), 695–710. https://doi.org/10.1084/jem.97.5.695.
3. Liu, X.; Raju, P. 5.42 - In Vitro Cancer Model for Drug Testing. In *Comprehensive Biotechnology (Second Edition)*; Moo-Young, M., Ed.; Academic Press: Burlington, 2011; pp 543–549. https://doi.org/10.1016/B978-0-08-088504-9.00502-X.
4. Sutherland, R. M.; McCredie, J. A.; Inch, W. R. Growth of Multicell Spheroids in Tissue Culture as a Model of Nodular Carcinomas2. *JNCI: Journal of the National Cancer Institute* **1971**, *46* (1), 113–120. https://doi.org/10.1093/jnci/46.1.113.
5. Breslin, S.; O'Driscoll, L. The Relevance of Using 3D Cell Cultures, in Addition to 2D Monolayer Cultures, When Evaluating Breast Cancer Drug Sensitivity and Resistance. *Oncotarget; Vol 7, No 29* **2016**.
6. Hoarau-Véchot, J.; Rafii, A.; Touboul, C.; Pasquier, J. Halfway between 2D and Animal Models: Are 3D Cultures the Ideal Tool to Study Cancer-Microenvironment Interactions? *International Journal of Molecular Sciences* **2018**, *19* (1). https://doi.org/10.3390/ijms19010181.
7. Breslin, S.; O'Driscoll, L. Three-Dimensional Cell Culture: The Missing Link in Drug Discovery. *Drug Discovery Today* **2013**, *18* (5), 240–249. https://doi.org/10.1016/j.drudis.2012.10.003.
8. Jensen, C.; Teng, Y. Is It Time to Start Transitioning From 2D to 3D Cell Culture? *Front Mol Biosci.* **2020**, *7*. https://doi.org/10.3389/fmolb.2020.00033.
9. Goodbye, Flat Biology? *Nature* **2003**, *424* (6951), 861–861. https://doi.org/10.1038/424861b.
10. Fontoura, J. C.; Viezzer, C.; dos Santos, F. G.; Ligabue, R. A.; Weinlich, R.; Puga, R. D.; Antonow, D.; Severino, P.; Bonorino, C. Comparison of 2D and 3D Cell Culture Models for Cell Growth, Gene Expression and Drug Resistance. *Materials Science and Engineering: C* **2020**, *107*, 110264. https://doi.org/10.1016/j.msec.2019.110264.
11. Franchi-Mendes, T.; Eduardo, R.; Domenici, G.; Brito, C. 3D Cancer Models: Depicting Cellular Crosstalk within the Tumour Microenvironment. *Cancers* **2021**, *13* (18). https://doi.org/10.3390/cancers13184610.
12. Habanjar, O.; Diab-Assaf, M.; Caldefie-Chezet, F.; Delort, L. 3D Cell Culture Systems: Tumor Application, Advantages, and Disadvantages. *International Journal of Molecular Sciences* **2021**, *22* (22). https://doi.org/10.3390/ijms222212200.
13. Kamb, A. What's Wrong with Our Cancer Models? *Nature Reviews Drug Discovery* **2005**, *4* (2), 161–165. https://doi.org/10.1038/nrd1635.
14. Gilazieva, Z.; Ponomarev, A.; Rutland, C.; Rizvanov, A.; Solovyeva, V. Promising Applications of Tumor Spheroids and Organoids for Personalized Medicine. *Cancers* **2020**, *12* (10). https://doi.org/10.3390/cancers12102727.
15. Nayak, P.; Bentivoglio, V.; Varani, M.; Signore, A. Three-Dimensional In Vitro Tumor Spheroid Models for Evaluation of Anticancer Therapy: Recent Updates. *Cancers* **2023**, *15* (19). https://doi.org/10.3390/cancers15194846.

 doi:10.20944/preprints202504.0640.v1

16. El Harane, S.; Zidi, B.; El Harane, N.; Krause, K.-H.; Matthes, T.; Preynat-Seauve, O. Cancer Spheroids and Organoids as Novel Tools for Research and Therapy: State of the Art and Challenges to Guide Precision Medicine. *Cells* **2023**, *12* (7). https://doi.org/10.3390/cells12071001.
17. Agastin, S.; Giang, U.-B. T.; Geng, Y.; DeLouise, L. A.; King, M. R. Continuously Perfused Microbubble Array for 3D Tumor Spheroid Model. *Biomicrofluidics* **2011**, *5* (2), 024110. https://doi.org/10.1063/1.3596530.
18. Alhasan, L.; Qi, A.; Al-Abboodi, A.; Rezk, A.; Chan, P. P. Y.; Iliescu, C.; Yeo, L. Y. Rapid Enhancement of Cellular Spheroid Assembly by Acoustically Driven Microcentrifugation. *ACS Biomater. Sci. Eng* **2016**, *2* (6), 1013–1022. https://doi.org/10.1021/acsbiomaterials.6b00144.
19. An, H. J.; Kim, H. S.; Kwon, J. A.; Song, J.; Choi, I. Adjustable and Versatile 3D Tumor Spheroid Culture Platform with Interfacial Elastomeric Wells. *ACS Appl. Mater. Interfaces* **2020**, *12* (6), 6924–6932. https://doi.org/10.1021/acsami.9b21471.
20. Árnadóttir, S. S.; Jeppesen, M.; Lamy, P.; Bramsen, J. B.; Nordentoft, I.; Knudsen, M.; Vang, S.; Madsen, M. R.; Thastrup, O.; Thastrup, J.; L. Andersen, C. Characterization of Genetic Intratumor Heterogeneity in Colorectal Cancer and Matching Patient-Derived Spheroid Cultures. *Molecular Oncology* **2018**, *12* (1), 132–147. https://doi.org/10.1002/1878-0261.12156.
21. Baek, N.; Seo, O. W.; Lee, J.; Hulme, J.; An, S. S. A. Real-Time Monitoring of Cisplatin Cytotoxicity on Three-Dimensional Spheroid Tumor Cells. *Drug Des Devel Ther* **2016**, *10*, 2155–2165. https://doi.org/10.2147/DDDT.S108004.
22. Barone, R. M.; Calabro-Jones, P.; Thomas, T. N.; Sharp, T. R.; Byfield, J. E. Surgical Adjuvant Therapy in Colon Carcinoma: A Human Tumor Spheroid Model for Evaluating Radiation Sensitizing Agents. *Cancer* **1981**, *47* (10), 2349–2357. https://doi.org/10.1002/1097-0142(19810515)47:10<2349::AID-CNCR2820471005>3.0.CO;2-L.
23. Bartholomä, P.; Impidjati; Reininger-Mack, A.; Zhang, Z.; Thielecke, H.; Robitzki, A. A More Aggressive Breast Cancer Spheroid Model Coupled to an Electronic Capillary Sensor System for a High-Content Screening of Cytotoxic Agents in Cancer Therapy: 3-Dimensional in Vitro Tumor Spheroids as a Screening Model. *J Biomol Screen* **2005**, *10* (7), 705–714. https://doi.org/doi:10.1177/1087057105277841.
24. Boo, L.; Yeap, S. K.; Ali, N. M.; Ho, W. Y.; Ky, H.; Satharasinghe, D. A.; Liew, W. C.; Tan, S. W.; Wang, M.-L.; Cheong, S. K.; Ong, H. K. Phenotypic and microRNA Characterization of the Neglected CD24+ Cell Population in MCF-7 Breast Cancer 3-Dimensional Spheroid Culture. *Journal of the Chinese Medical Association* **2020**, *83* (1).
25. Brooks, E. A.; Gencoglu, M. F.; Corbett, D. C.; Stevens, K. R.; Peyton, S. R. An Omentum-Inspired 3D PEG Hydrogel for Identifying ECM-Drivers of Drug Resistant Ovarian Cancer. *APL Bioengineering* **2019**, *3* (2), 026106. https://doi.org/10.1063/1.5091713.
26. Bruns, J.; Egan, T.; Mercier, P.; Zustiak, S. P. Glioblastoma Spheroid Growth and Chemotherapeutic Responses in Single and Dual-Stiffness Hydrogels. *Acta Biomaterialia* **2023**, *163*, 400–414. https://doi.org/10.1016/j.actbio.2022.05.048.
27. Calori, I. R.; Alves, S. R.; Bi, H.; Tedesco, A. C. Type-I Collagen/Collagenase Modulates the 3D Structure and Behavior of Glioblastoma Spheroid Models. *ACS Appl. Bio Mater.* **2022**, *5* (2), 723–733. https://doi.org/10.1021/acsabm.1c01138.
28. Chang, S.; Wen, J.; Su, Y.; Ma, H. Microfluidic Platform for Studying the Anti-Cancer Effect of Ursolic Acid on Tumor Spheroid. *ELECTROPHORESIS* **2022**, *43* (13–14), 1466–1475. https://doi.org/10.1002/elps.202100382.
29. Chen, G.; Liu, W.; Yan, B. Breast Cancer MCF-7 Cell Spheroid Culture for Drug Discovery and Development. *J Cancer Ther.* **2022**, *13* (3), 117–130. https://doi.org/10.4236/jct.2022.133009.
30. Chen, M. C. W.; Gupta, M.; Cheung, K. C. Alginate-Based Microfluidic System for Tumor Spheroid Formation and Anticancer Agent Screening. *Biomedical Microdevices* **2010**, *12* (4), 647–654. https://doi.org/10.1007/s10544-010-9417-2.
31. Chen, Z.; Ma, N.; Sun, X.; Li, Q.; Zeng, Y.; Chen, F.; Sun, S.; Xu, J.; Zhang, J.; Ye, H.; Ge, J.; Zhang, Z.; Cui, X.; Leong, K.; Chen, Y.; Gu, Z. Automated Evaluation of Tumor Spheroid Behavior in 3D Culture Using Deep Learning-Based Recognition. *Biomaterials* **2021**, *272*, 120770. https://doi.org/10.1016/j.biomaterials.2021.120770.

 doi:10.20944/preprints202504.0640.v1

32. Cheng, V.; Esteves, F.; Chakrabarty, A.; Cockle, J.; Short, S.; Brüning-Richardson, A. High-Content Analysis of Tumour Cell Invasion in Three-Dimensional Spheroid Assays. *Oncoscience* *2* (6), 596–606. https://doi.org/10.18632/oncoscience.171.
33. Close, D. A.; Johnston, P. A. Detection and Impact of Hypoxic Regions in Multicellular Tumor Spheroid Cultures Formed by Head and Neck Squamous Cell Carcinoma Cells Lines. *SLAS Discovery* **2022**, *27* (1), 39–54. https://doi.org/10.1016/j.slasd.2021.10.008.
34. Das, T.; Meunier, L.; Barbe, L.; Provencher, D.; Guenat, O.; Gervais, T.; Mes-Masson, A.-M. Empirical Chemosensitivity Testing in a Spheroid Model of Ovarian Cancer Using a Microfluidics-Based Multiplex Platform. *Biomicrofluidics* **2013**, *7* (1), 011805. https://doi.org/10.1063/1.4774309.
35. Das, V.; Fürst, T.; Gurská, S.; Džubák, P.; Hajdúch, M. Evaporation-Reducing Culture Condition Increases the Reproducibility of Multicellular Spheroid Formation in Microtiter Plates. *JoVE* **2017**, No. 121, e55403. https://doi.org/10.3791/55403.
36. Das, V.; Fürst, T.; Gurská, S.; Džubák, P.; Hajdúch, M. Reproducibility of Uniform Spheroid Formation in 384-Well Plates: The Effect of Medium Evaporation. *SLAS Discovery* **2016**, *21* (9), 923–930. https://doi.org/10.1177/1087057116651867.
37. De Angelis, M. L.; Bruselles, A.; Francescangeli, F.; Pucilli, F.; Vitale, S.; Zeuner, A.; Tartaglia, M.; Baiocchi, M. Colorectal Cancer Spheroid Biobanks: Multi-Level Approaches to Drug Sensitivity Studies. *Cell Biology and Toxicology* **2018**, *34* (6), 459–469. https://doi.org/10.1007/s10565-018-9423-3.
38. Dhamecha, D.; Le, D.; Chakravarty, T.; Perera, K.; Dutta, A.; Menon, J. U. Fabrication of PNIPAm-Based Thermoresponsive Hydrogel Microwell Arrays for Tumor Spheroid Formation. *Materials Science and Engineering: C* **2021**, *125*, 112100. https://doi.org/10.1016/j.msec.2021.112100.
39. Dias, D. R.; Moreira, A. F.; Correia, I. J. The Effect of the Shape of Gold Core–Mesoporous Silica Shell Nanoparticles on the Cellular Behavior and Tumor Spheroid Penetration. *J. Mater. Chem. B* **2016**, *4* (47), 7630–7640. https://doi.org/10.1039/C6TB02668K.
40. Domenici, G.; Eduardo, R.; Castillo-Ecija, H.; Orive, G.; Montero Carcaboso, Á.; Brito, C. PDX-Derived Ewing's Sarcoma Cells Retain High Viability and Disease Phenotype in Alginate Encapsulated Spheroid Cultures. *Cancers* **2021**, *13* (4). https://doi.org/10.3390/cancers13040879.
41. Dufau, I.; Frongia, C.; Sicard, F.; Dedieu, L.; Cordelier, P.; Ausseil, F.; Ducommun, B.; Valette, A. Multicellular Tumor Spheroid Model to Evaluate Spatio-Temporal Dynamics Effect of Chemotherapeutics: Application to the Gemcitabine/CHK1 Inhibitor Combination in Pancreatic Cancer. *BMC Cancer* **2012**, *12* (1), 15. https://doi.org/10.1186/1471-2407-12-15.
42. Eetezadi, S.; Evans, J. C.; Shen, Y.-T.; de Souza, R.; Piquette-Miller, M.; Allen, C. Ratio-Dependent Synergism of a Doxorubicin and Olaparib Combination in 2D and Spheroid Models of Ovarian Cancer. *Mol. Pharmaceutics* *15* (2), 472–485. https://doi.org/10.1021/acs.molpharmaceut.7b00843.
43. Eguchi, H.; Kimura, R.; Onuma, S.; Ito, A.; Yu, Y.; Yoshino, Y.; Matsunaga, T.; Endo, S.; Ikari, A. Elevation of Anticancer Drug Toxicity by Caffeine in Spheroid Model of Human Lung Adenocarcinoma A549 Cells Mediated by Reduction in Claudin-2 and Nrf2 Expression. *International Journal of Molecular Sciences* **2022**, *23* (24). https://doi.org/10.3390/ijms232415447.
44. Eimer, S.; Dugay, F.; Airiau, K.; Avril, T.; Quillien, V.; Belaud-Rotureau, M.-A.; Belloc, F. Cyclopamine Cooperates with EGFR Inhibition to Deplete Stem-like Cancer Cells in Glioblastoma-Derived Spheroid Cultures. *Neuro-Oncology* **2012**, *14* (12), 1441–1451. https://doi.org/10.1093/neuonc/nos266.
45. El-Sadek, I. A.; Miyazawa, A.; Shen, L. T.-W.; Makita, S.; Mukherjee, P.; Lichtenegger, A.; Matsusaka, S.; Yasuno, Y. Three-Dimensional Dynamics Optical Coherence Tomography for Tumor Spheroid Evaluation. *Biomed. Opt. Express* **2021**, *12* (11), 6844–6863. https://doi.org/10.1364/BOE.440444.
46. Enmon, R. M.; O'Connor, K. C.; Lacks, D. J.; Schwartz, D. K.; Dotson, R. S. Dynamics of Spheroid Self-Assembly in Liquid-Overlay Culture of DU 145 Human Prostate Cancer Cells. *Biotechnol Bioeng* **2001**, *72* (6), 579–591.
47. Flørenes, V. A.; Flem-Karlsen, K.; McFadden, E.; Bergheim, I. R.; Nygaard, V.; Nygård, V.; Farstad, I. N.; Øy, G. F.; Emilsen, E.; Giller-Fleten, K.; Ree, A. H.; Flatmark, K.; Gullestad, H. P.; Hermann, R.; Ryder, T.; Wernhoff, P.; Mælandsmo, G. M. A Three-Dimensional Ex Vivo Viability Assay Reveals a Strong

 doi:10.20944/preprints202504.0640.v1

Correlation Between Response to Targeted Inhibitors and Mutation Status in Melanoma Lymph Node Metastases. *Translational Oncology* **2019**, *12* (7), 951–958. https://doi.org/10.1016/j.tranon.2019.04.001.

48. Fu, J.; Li, X. B.; Wang, L. X.; Lv, X. H.; Lu, Z.; Wang, F.; Xia, Q.; Yu, L.; Li, C. M. One-Step Dip-Coating-Fabricated Core–Shell Silk Fibroin Rice Paper Fibrous Scaffolds for 3D Tumor Spheroid Formation. *ACS Appl. Bio Mater.* **2020**, *3* (11), 7462–7471. https://doi.org/10.1021/acsabm.0c00679.
49. Fu, J. J.; Zhou, Y.; Shi, X. X.; Kang, Y. J.; Lu, Z. S.; Li, Y.; Li, C. M.; Yu, L. Spontaneous Formation of Tumor Spheroid on a Hydrophilic Filter Paper for Cancer Stem Cell Enrichment. *Colloids and Surfaces B: Biointerfaces* **2019**, *174*, 426–434. https://doi.org/10.1016/j.colsurfb.2018.11.038.
50. Gao, Y.; Wu, M.; Luan, Q.; Papautsky, I.; Xu, J. Acoustic Bubble for Spheroid Trapping, Rotation, and Culture: A Tumor-on-a-Chip Platform (ABSTRACT Platform). *Lab Chip* **2022**, *22* (4), 805–813. https://doi.org/10.1039/D1LC01012C.
51. Gencoglu, M. F.; Barney, L. E.; Hall, C. L.; Brooks, E. A.; Peyton, S. R. Comparative Study of Multicellular Tumor Spheroid Formation Methods and Implications for Drug Screening. *ACS Biomater Sci Eng.* **2018**, *4* (2), 410–420. https://doi.org/10.1021/acsbiomaterials.7b00069.
52. Gendre, D. A. J.; Ameti, E.; Karenovics, W.; Perriraz-Mayer, N.; Triponez, F.; Serre-Beinier, V. Optimization of Tumor Spheroid Model in Mesothelioma and Lung Cancers and Anti-Cancer Drug Testing in H2052/484 Spheroids. *Oncotarget; Vol 12, No 24* **2021**.
53. Gheytanchi, E.; Naseri, M.; Karimi-Busheri, F.; Atyabi, F.; Mirsharif, E. S.; Bozorgmehr, M.; Ghods, R.; Madjd, Z. Morphological and Molecular Characteristics of Spheroid Formation in HT-29 and Caco-2 Colorectal Cancer Cell Lines. *Cancer Cell International* **2021**, *21* (1), 204. https://doi.org/10.1186/s12935-021-01898-9.
54. Goisnard, A.; Daumar, P.; Dubois, C.; Aubel, C.; Roux, M.; Depresle, M.; Gauthier, J.; Vidalinc, B.; Penault-Llorca, F.; Mounetou, E.; Bamdad, M. LightSpot®-FL-1 Fluorescent Probe: An Innovative Tool for Cancer Drug Resistance Analysis by Direct Detection and Quantification of the P-Glycoprotein (P-Gp) on Monolayer Culture and Spheroid Triple Negative Breast Cancer Models. *Cancers* **2021**, *13* (16). https://doi.org/10.3390/cancers13164050.
55. Guo, X.; Chen, Y.; Ji, W.; Chen, X.; Li, C.; Ge, R. Enrichment of Cancer Stem Cells by Agarose Multi-Well Dishes and 3D Spheroid Culture. *Cell and Tissue Research* **2019**, *375* (2), 397–408. https://doi.org/10.1007/s00441-018-2920-0.
56. HAGEMANN, J.; JACOBI, C.; HAHN, M.; SCHMID, V.; WELZ, C.; SCHWENK-ZIEGER, S.; STAUBER, R.; BAUMEISTER, P.; BECKER, S. Spheroid-Based 3D Cell Cultures Enable Personalized Therapy Testing and Drug Discovery in Head and Neck Cancer. *Anticancer Res* **2017**, *37* (5), 2201.
57. Han, S.; Lim, J. Y.; Cho, K.; Lee, H. W.; Park, J. Y.; Ro, S. W.; Kim, K. S.; Seo, H. R.; Kim, D. Y. Anti-Cancer Effects of YAP Inhibitor (CA3) in Combination with Sorafenib against Hepatocellular Carcinoma (HCC) in Patient-Derived Multicellular Tumor Spheroid Models (MCTS). *Cancers* **2022**, *14* (11). https://doi.org/10.3390/cancers14112733.
58. Harmer, J.; Struve, N.; Brüning-Richardson, A. Characterization of the Effects of Migrastatic Inhibitors on 3D Tumor Spheroid Invasion by High-Resolution Confocal Microscopy. *JoVE* **2019**, No. 151, e60273. https://doi.org/10.3791/60273.
59. Herter, S.; Morra, L.; Schlenker, R.; Sulcova, J.; Fahrni, L.; Waldhauer, I.; Lehmann, S.; Reisländer, T.; Agarkova, I.; Kelm, J. M.; Klein, C.; Umana, P.; Bacac, M. A Novel Three-Dimensional Heterotypic Spheroid Model for the Assessment of the Activity of Cancer Immunotherapy Agents. *Cancer Immunology, Immunotherapy* **2017**, *66* (1), 129–140. https://doi.org/10.1007/s00262-016-1927-1.
60. Ho, W. Y.; Yeap, S. K.; Ho, C. L.; Rahim, R. A.; Alitheen, N. B. Development of Multicellular Tumor Spheroid (MCTS) Culture from Breast Cancer Cell and a High Throughput Screening Method Using the MTT Assay. *PLOS ONE* **2012**, *7* (9), e44640. https://doi.org/10.1371/journal.pone.0044640.
61. Ho, W. Y.; Liew, S. S.; Yeap, S. K.; Alitheen, N. B. Synergistic Cytotoxicity between Elephantopus Scaber and Tamoxifen on MCF-7-Derived Multicellular Tumor Spheroid. *Evidence-Based Complementary and Alternative Medicine* **2021**, *2021* (1), 6355236. https://doi.org/10.1155/2021/6355236.

 doi:10.20944/preprints202504.0640.v1

62. Hofmann, S.; Cohen-Harazi, R.; Maizels, Y.; Koman, I. Patient-Derived Tumor Spheroid Cultures as a Promising Tool to Assist Personalized Therapeutic Decisions in Breast Cancer. *Translational Cancer Research; Vol 11, No 1 (January 26, 2022): Translational Cancer Research* **2021**.
63. HORNUNG, A.; POETTLER, M.; FRIEDRICH, R. P.; WEIGEL, B.; DUERR, S.; ZALOGA, J.; CICHA, I.; ALEXIOU, C.; JANKO, C. Toxicity of Mitoxantrone-Loaded Superparamagnetic Iron Oxide Nanoparticles in a HT-29 Tumour Spheroid Model. *Anticancer Res* **2016**, *36* (6), 3093.
64. Huang, Z.; Yu, P.; Tang, J. Characterization of Triple-Negative Breast Cancer MDA-MB-231 Cell Spheroid Model. *Onco Targets Ther.* **2020**, *13*, 5395–5405. https://doi.org/10.2147/OTT.S249756.
65. Jove, M.; Spencer, J. A.; Hubbard, M. E.; Holden, E. C.; O'Dea, R. D.; Brook, B. S.; Phillips, R. M.; Smye, S. W.; Loadman, P. M.; Twelves, C. J. Cellular Uptake and Efflux of Palbociclib In Vitro in Single Cell and Spheroid Models. *J Pharmacol Exp Ther* **2019**, *370* (2), 242. https://doi.org/10.1124/jpet.119.256693.
66. Ju, F. N.; Kim, C.-H.; Lee, K.-H.; Kim, C.-D.; Lim, J.; Lee, T.; Park, C. G.; Kim, T.-H. Gold Nanostructure-Integrated Conductive Microwell Arrays for Uniform Cancer Spheroid Formation and Electrochemical Drug Screening. *Biosensors and Bioelectronics* **2023**, *222*, 115003. https://doi.org/10.1016/j.bios.2022.115003.
67. Karamikamkar, S.; Behzadfar, E.; Cheung, K. C. A Novel Approach to Producing Uniform 3-D Tumor Spheroid Constructs Using Ultrasound Treatment. *Biomedical Microdevices* **2018**, *20* (2), 27. https://doi.org/10.1007/s10544-018-0260-1.
68. Karlsson, H.; Fryknäs, M.; Larsson, R.; Nygren, P. Loss of Cancer Drug Activity in Colon Cancer HCT-116 Cells during Spheroid Formation in a New 3-D Spheroid Cell Culture System. *Experimental Cell Research* **2012**, *318* (13), 1577–1585. https://doi.org/10.1016/j.yexcr.2012.03.026.
69. Karshieva, S. Sh.; Glinskaya, E. G.; Dalina, A. A.; Akhlyustina, E. V.; Makarova, E. A.; Khesuani, Y. D.; Chmelyuk, N. S.; Abakumov, M. A.; Khochenkov, D. A.; Mironov, V. A.; Meerovich, G. A.; Kogan, E. A.; Koudan, E. V. Antitumor Activity of Photodynamic Therapy with Tetracationic Derivative of Synthetic Bacteriochlorin in Spheroid Culture of Liver and Colon Cancer Cells. *Photodiagnosis and Photodynamic Therapy* **2022**, *40*, 103202. https://doi.org/10.1016/j.pdpdt.2022.103202.
70. Kato, E. E.; Sampaio, S. C. Crotoxin Modulates Events Involved in Epithelial–Mesenchymal Transition in 3D Spheroid Model. *Toxins* **2021**, *13* (11). https://doi.org/10.3390/toxins13110830.
71. Kim, C.-H.; Suhito, I. R.; Angeline, N.; Han, Y.; Son, H.; Luo, Z.; Kim, T.-H. Vertically Coated Graphene Oxide Micro-Well Arrays for Highly Efficient Cancer Spheroid Formation and Drug Screening. *Advanced Healthcare Materials* **2020**, *9* (7), 1901751. https://doi.org/10.1002/adhm.201901751.
72. Ko, J.; Ahn, J.; Kim, S.; Lee, Y.; Lee, J.; Park, D.; Jeon, N. L. Tumor Spheroid-on-a-Chip: A Standardized Microfluidic Culture Platform for Investigating Tumor Angiogenesis. *Lab Chip* **2019**, *19* (17), 2822–2833. https://doi.org/10.1039/C9LC00140A.
73. Kochanek, S. J.; Close, D. A.; Johnston, P. A. High Content Screening Characterization of Head and Neck Squamous Cell Carcinoma Multicellular Tumor Spheroid Cultures Generated in 384-Well Ultra-Low Attachment Plates to Screen for Better Cancer Drug Leads. *ASSAY and Drug Development Technologies* **2019**, *17* (1), 17–36. https://doi.org/10.1089/adt.2018.896.
74. Kochanek, S. J.; Close, D. A.; Camarco, D. P.; Johnston, P. A. Maximizing the Value of Cancer Drug Screening in Multicellular Tumor Spheroid Cultures: A Case Study in Five Head and Neck Squamous Cell Carcinoma Cell Lines. *SLAS Discovery* **2020**, *25* (4), 329–349. https://doi.org/10.1177/2472555219896999.
75. Koshkin, V.; Ailles, L. E.; Liu, G.; Krylov, S. N. Metabolic Suppression of a Drug-Resistant Subpopulation in Cancer Spheroid Cells. *Journal of Cellular Biochemistry* **2016**, *117* (1), 59–65. https://doi.org/10.1002/jcb.25247.
76. Kroupová, J.; Hanuš, J.; Štěpánek, F. Surprising Efficacy Twist of Two Established Cytostatics Revealed by A-La-Carte 3D Cell Spheroid Preparation Protocol. *European Journal of Pharmaceutics and Biopharmaceutics* **2022**, *180*, 224–237. https://doi.org/10.1016/j.ejpb.2022.10.003.
77. Kudláčová, J.; Kotrchová, L.; Kostka, L.; Randárová, E.; Filipová, M.; Janoušková, O.; Fang, J.; Etrych, T. Structure-to-Efficacy Relationship of HPMA-Based Nanomedicines: The Tumor Spheroid Penetration Study. *Pharmaceutics* **2020**, *12* (12). https://doi.org/10.3390/pharmaceutics12121242.

 doi:10.20944/preprints202504.0640.v1

78. Kumari, P.; Jain, S.; Ghosh, B.; Zorin, V.; Biswas, S. Polylactide-Based Block Copolymeric Micelles Loaded with Chlorin E6 for Photodynamic Therapy: In Vitro Evaluation in Monolayer and 3D Spheroid Models. *Mol. Pharmaceutics* **2017**, *14* (11), 3789–3800. https://doi.org/10.1021/acs.molpharmaceut.7b00548.
79. Lal-Nag, M.; McGee, L.; Titus, S. A.; Brimacombe, K.; Michael, S.; Sittampalam, G.; Ferrer, M. Exploring Drug Dosing Regimens In Vitro Using Real-Time 3D Spheroid Tumor Growth Assays. *SLAS Discovery* **2017**, *22* (5), 537–546. https://doi.org/10.1177/2472555217698818.
80. Lama, R.; Zhang, L.; Naim, J. M.; Williams, J.; Zhou, A.; Su, B. Development, Validation and Pilot Screening of an in Vitro Multi-Cellular Three-Dimensional Cancer Spheroid Assay for Anti-Cancer Drug Testing. *Bioorganic & Medicinal Chemistry* **2013**, *21* (4), 922–931. https://doi.org/10.1016/j.bmc.2012.12.007.
81. Landgraf, L.; Kozlowski, A.; Zhang, X.; Fournelle, M.; Becker, F.-J.; Tretbar, S.; Melzer, A. Focused Ultrasound Treatment of a Spheroid In Vitro Tumour Model. *Cells* **2022**, *11* (9). https://doi.org/10.3390/cells11091518.
82. Le, V.-M.; Lang, M.-D.; Shi, W.-B.; Liu, J.-W. A Collagen-Based Multicellular Tumor Spheroid Model for Evaluation of the Efficiency of Nanoparticle Drug Delivery. *Artificial Cells, Nanomedicine, and Biotechnology* **2016**, *44* (2), 540–544. https://doi.org/10.3109/21691401.2014.968820.
83. Lee, S. W.; Hong, S.; Jung, B.; Jeong, S. Y.; Byeon, J. H.; Jeong, G. S.; Choi, J.; Hwang, C. In Vitro Lung Cancer Multicellular Tumor Spheroid Formation Using a Microfluidic Device. *Biotechnology and Bioengineering* **2019**, *116* (11), 3041–3052. https://doi.org/10.1002/bit.27114.
84. Lee, Y.; Chen, Z.; Lim, W.; Cho, H.; Park, S. High-Throughput Screening of Anti-Cancer Drugs Using a Microfluidic Spheroid Culture Device with a Concentration Gradient Generator. *Current Protocols* **2022**, *2* (9), e529. https://doi.org/10.1002/cpz1.529.
85. Lemmo, S.; Atefi, E.; Luker, G. D.; Tavana, H. Optimization of Aqueous Biphasic Tumor Spheroid Microtechnology for Anti-Cancer Drug Testing in 3D Culture. *Cellular and Molecular Bioengineering* **2014**, *7* (3), 344–354. https://doi.org/10.1007/s12195-014-0349-4.
86. Li, M.; Lu, B.; Dong, X.; Zhou, Y.; He, Y.; Zhang, T.; Bao, L. Enhancement of Cisplatin-Induced Cytotoxicity against Cervical Cancer Spheroid Cells by Targeting Long Non-Coding RNAs. *Pathology - Research and Practice* **2019**, *215* (11), 152653. https://doi.org/10.1016/j.prp.2019.152653.
87. Lim, W.; Park, S. A Microfluidic Spheroid Culture Device with a Concentration Gradient Generator for High-Throughput Screening of Drug Efficacy. *Molecules* **2018**, *23* (12). https://doi.org/10.3390/molecules23123355.
88. Lin, Z.-T.; Gu, J.; Wang, H.; Wu, A.; Sun, J.; Chen, S.; Li, Y.; Kong, Y.; Wu, M. X.; Wu, T. Thermosensitive and Conductive Hybrid Polymer for Real-Time Monitoring of Spheroid Growth and Drug Responses. *ACS Sens.* **2021**, *6* (6), 2147–2157. https://doi.org/10.1021/acssensors.0c02266.
89. Liu, X.; Lin, H.; Song, J.; Zhang, T.; Wang, X.; Huang, X.; Zheng, C. A Novel SimpleDrop Chip for 3D Spheroid Formation and Anti-Cancer Drug Assay. *Micromachines* **2021**, *12* (6). https://doi.org/10.3390/mi12060681.
90. Lorenzo, C.; Frongia, C.; Jorand, R.; Fehrenbach, J.; Weiss, P.; Maandhui, A.; Gay, G.; Ducommun, B.; Lobjois, V. Live Cell Division Dynamics Monitoring in 3D Large Spheroid Tumor Models Using Light Sheet Microscopy. *Cell Division* **2011**, *6* (1), 22. https://doi.org/10.1186/1747-1028-6-22.
91. Luan, Q.; Becker, J. H.; Macaraniag, C.; Massad, M. G.; Zhou, J.; Shimamura, T.; Papautsky, I. Non-Small Cell Lung Carcinoma Spheroid Models in Agarose Microwells for Drug Response Studies. *Lab Chip* **2022**, *22* (12), 2364–2375. https://doi.org/10.1039/D2LC00244B.
92. Madsen, N. H.; Nielsen, B. S.; Nhat, S. L.; Skov, S.; Gad, M.; Larsen, J. Monocyte Infiltration and Differentiation in 3D Multicellular Spheroid Cancer Models. *Pathogens* **2021**, *10* (8). https://doi.org/10.3390/pathogens10080969.
93. Marshall, S. K.; Saelim, B.; Taweesap, M.; Pachana, V.; Panrak, Y.; Makchuchit, N.; Jaroenpakdee, P. Anti-EGFR Targeted Multifunctional I-131 Radio-Nanotherapeutic for Treating Osteosarcoma: In Vitro 3D Tumor Spheroid Model. *Nanomaterials* **2022**, *12* (19). https://doi.org/10.3390/nano12193517.
94. Maruhashi, R.; Akizuki, R.; Sato, T.; Matsunaga, T.; Endo, S.; Yamaguchi, M.; Yamazaki, Y.; Sakai, H.; Ikari, A. Elevation of Sensitivity to Anticancer Agents of Human Lung Adenocarcinoma A549 Cells by

 doi:10.20944/preprints202504.0640.v1

Knockdown of Claudin-2 Expression in Monolayer and Spheroid Culture Models. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **2018**, *1865* (3), 470–479. https://doi.org/10.1016/j.bbamcr.2017.12.005.

95. Melnik, D.; Sahana, J.; Corydon, T. J.; Kopp, S.; Nassef, M. Z.; Wehland, M.; Infanger, M.; Grimm, D.; Krüger, M. Dexamethasone Inhibits Spheroid Formation of Thyroid Cancer Cells Exposed to Simulated Microgravity. *Cells* **2020**, *9* (2). https://doi.org/10.3390/cells9020367.
96. Molyneaux, K.; Wnek, M. D.; Craig, S. E. L.; Vincent, J.; Rucker, I.; Wnek, G. E.; Brady-Kalnay, S. M. Physically-Cross-Linked Poly(Vinyl Alcohol) Cell Culture Plate Coatings Facilitate Preservation of Cell–Cell Interactions, Spheroid Formation, and Stemness. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* **2021**, *109* (11), 1744–1753. https://doi.org/10.1002/jbm.b.34832.
97. Monazzam, A.; Josephsson, R.; Blomqvist, C.; Carlsson, J.; Långström, B.; Bergström, M. Application of the Multicellular Tumour Spheroid Model to Screen PET Tracers for Analysis of Early Response of Chemotherapy in Breast Cancer. *Breast Cancer Research* **2007**, *9* (4), R45. https://doi.org/10.1186/bcr1747.
98. Morimoto, T.; Takemura, Y.; Miura, T.; Yamamoto, T.; Kakizaki, F.; An, H.; Maekawa, H.; Yamaura, T.; Kawada, K.; Sakai, Y.; Yuba, Y.; Terajima, H.; Obama, K.; Taketo, M. M.; Miyoshi, H. Novel and Efficient Method for Culturing Patient-Derived Gastric Cancer Stem Cells. *Cancer Science* **2023**, *114* (8), 3259–3269. https://doi.org/10.1111/cas.15840.
99. Mosaad, E. O.; Chambers, K. F.; Futrega, K.; Clements, J. A.; Doran, M. R. The Microwell-Mesh: A High-Throughput 3D Prostate Cancer Spheroid and Drug-Testing Platform. *Scientific Reports* **2018**, *8* (1), 253. https://doi.org/10.1038/s41598-017-18050-1.
100. Mueggler, A.; Pilotto, E.; Perriraz-Mayer, N.; Jiang, S.; Addeo, A.; Bédat, B.; Karenovics, W.; Triponez, F.; Serre-Beinier, V. An Optimized Method to Culture Human Primary Lung Tumor Cell Spheroids. *Cancers* **2023**, *15* (23). https://doi.org/10.3390/cancers15235576.
101. Nashimoto, Y.; Okada, R.; Hanada, S.; Arima, Y.; Nishiyama, K.; Miura, T.; Yokokawa, R. Vascularized Cancer on a Chip: The Effect of Perfusion on Growth and Drug Delivery of Tumor Spheroid. *Biomaterials* **2020**, *229*, 119547. https://doi.org/10.1016/j.biomaterials.2019.119547.
102. Nigjeh, S. E.; Yeap, S. K.; Nordin, N.; Kamalideghan, B.; Ky, H.; Rosli, R. Citral Induced Apoptosis in MDA-MB-231 Spheroid Cells. *BMC Complementary and Alternative Medicine* **2018**, *18* (1), 56. https://doi.org/10.1186/s12906-018-2115-y.
103. Nittayaboon, K.; Leetanaporn, K.; Sangkhathat, S.; Roytrakul, S.; Navakanitworakul, R. Cytotoxic Effect of Metformin on Butyrate-Resistant PMF-K014 Colorectal Cancer Spheroid Cells. *Biomedicine & Pharmacotherapy* **2022**, *151*, 113214. https://doi.org/10.1016/j.biopha.2022.113214.
104. Ohya, S.; Kajikuri, J.; Endo, K.; Kito, H.; Matsui, M. KCa1.1 K+ Channel Inhibition Overcomes Resistance to Antiandrogens and Doxorubicin in a Human Prostate Cancer LNCaP Spheroid Model. *International Journal of Molecular Sciences* **2021**, *22* (24). https://doi.org/10.3390/ijms222413553.
105. Oliveira, M. S.; Aryasomayajula, B.; Pattni, B.; Mussi, S. V.; Ferreira, L. A. M.; Torchilin, V. P. Solid Lipid Nanoparticles Co-Loaded with Doxorubicin and α-Tocopherol Succinate Are Effective against Drug-Resistant Cancer Cells in Monolayer and 3-D Spheroid Cancer Cell Models. *International Journal of Pharmaceutics* **2016**, *512* (1), 292–300. https://doi.org/10.1016/j.ijpharm.2016.08.049.
106. Ono, K.; Sato, K.; Nakamura, T.; Yoshida, Y.; Murata, S.; Yoshida, K.; Kanemoto, H.; Umemori, K.; Kawai, H.; Obata, K.; Ryumon, S.; Hasegawa, K.; Kunisada, Y.; Okui, T.; Ibaragi, S.; Nagatsuka, H.; Sasaki, A. Reproduction of the Antitumor Effect of Cisplatin and Cetuximab Using a Three-Dimensional Spheroid Model in Oral Cancer. *Int J Med Sci* **2022**, *19* (8), 1320–1333. https://doi.org/10.7150/ijms.74109.
107. Pampaloni, F.; Mayer, B.; Kabat Vel-Job, K.; Ansari, N.; Hötte, K.; Kögel, D.; Stelzer, E. H. K. A Novel Cellular Spheroid-Based Autophagy Screen Applying Live Fluorescence Microscopy Identifies Nonactin as a Strong Inducer of Autophagosomal Turnover. *SLAS Discovery* **2017**, *22* (5), 558–570. https://doi.org/10.1177/2472555217696798.
108. Park, M. C.; Jeong, H.; Son, S. H.; Kim, Y.; Han, D.; Goughnour, P. C.; Kang, T.; Kwon, N. H.; Moon, H. E.; Paek, S. H.; Hwang, D.; Seol, H. J.; Nam, D.-H.; Kim, S. Novel Morphologic and Genetic Analysis of Cancer Cells in a 3D Microenvironment Identifies STAT3 as a Regulator of Tumor Permeability Barrier Function. *Cancer Research* **2016**, *76* (5), 1044–1054. https://doi.org/10.1158/0008-5472.CAN-14-2611.

 doi:10.20944/preprints202504.0640.v1

109. Pattni, B. S.; Nagelli, S. G.; Aryasomayajula, B.; Deshpande, P. P.; Kulkarni, A.; Hartner, W. C.; Thakur, G.; Degterev, A.; Torchilin, V. P. Targeting of Micelles and Liposomes Loaded with the Pro-Apoptotic Drug, NCL-240, into NCI/ADR-RES Cells in a 3D Spheroid Model. *Pharmaceutical Research* **2016**, *33* (10), 2540–2551. https://doi.org/10.1007/s11095-016-1978-1.
110. Perche, F.; Patel, N. R.; Torchilin, V. P. Accumulation and Toxicity of Antibody-Targeted Doxorubicin-Loaded PEG–PE Micelles in Ovarian Cancer Cell Spheroid Model. *Journal of Controlled Release* **2012**, *164* (1), 95–102. https://doi.org/10.1016/j.jconrel.2012.09.003.
111. Preda, P.; Enciu, A.-M.; Tanase, C.; Dudau, M.; Albulescu, L.; Maxim, M.-E.; Darie-Niță, R. N.; Brincoveanu, O.; Avram, M. Assessing Polysaccharides/Aloe Vera–Based Hydrogels for Tumor Spheroid Formation. *Gels* **2023**, *9* (1). https://doi.org/10.3390/gels9010051.
112. Pulze, L.; Congiu, T.; Brevini, T. A. L.; Grimaldi, A.; Tettamanti, G.; D'Antona, P.; Baranzini, N.; Acquati, F.; Ferraro, F.; de Eguileor, M. MCF7 Spheroid Development: New Insight about Spatio/Temporal Arrangements of TNTs, Amyloid Fibrils, Cell Connections, and Cellular Bridges. *International Journal of Molecular Sciences* **2020**, *21* (15). https://doi.org/10.3390/ijms21155400.
113. Raghavan, S.; Mehta, P.; Horst, E. N.; Ward, M. R.; Rowley, K. R.; Mehta, G. Comparative Analysis of Tumor Spheroid Generation Techniques for Differential in Vitro Drug Toxicity. *Oncotarget; Vol 7, No 13* **2016**.
114. Raghavan, S.; Mehta, P.; Xie, Y.; Lei, Y. L.; Mehta, G. Ovarian Cancer Stem Cells and Macrophages Reciprocally Interact through the WNT Pathway to Promote Pro-Tumoral and Malignant Phenotypes in 3D Engineered Microenvironments. *J Immunother Cancer* **2019**, *7* (1), 190. https://doi.org/10.1186/s40425-019-0666-1.
115. Ralph, A. C. L.; Valadão, I. C.; Cardoso, E. C.; Martins, V. R.; Oliveira, L. M. S.; Bevilacqua, E. M. A. F.; Geraldo, M. V.; Jaeger, R. G.; Goldberg, G. S.; Freitas, V. M. Environmental Control of Mammary Carcinoma Cell Expansion by Acidification and Spheroid Formation in Vitro. *Scientific Reports* **2020**, *10* (1), 21959. https://doi.org/10.1038/s41598-020-78989-6.
116. Roering, P.; Siddiqui, A.; Heuser, V. D.; Potdar, S.; Mikkonen, P.; Oikkonen, J.; Li, Y.; Pikkusaari, S.; Wennerberg, K.; Hynninen, J.; Grenman, S.; Huhtinen, K.; Auranen, A.; Carpén, O.; Kaipio, K. Effects of Wee1 Inhibitor Adavosertib on Patient-Derived High-Grade Serous Ovarian Cancer Cells Are Multiple and Independent of Homologous Recombination Status. *Frontiers in Oncology* **2022**, *12*.
117. Roudi, R.; Madjd, Z.; Ebrahimi, M.; Najafi, A.; Korourian, A.; Shariftabrizi, A.; Samadikuchaksaraei, A. Evidence for Embryonic Stem-like Signature and Epithelial-Mesenchymal Transition Features in the Spheroid Cells Derived from Lung Adenocarcinoma. *Tumor Biology* **2016**, *37* (9), 11843–11859. https://doi.org/10.1007/s13277-016-5041-y.
118. Rouhani, M.; Goliaei, B.; Khodagholi, F.; Nikoofar, A. Lithium Increases Radiosensitivity by Abrogating DNA Repair in Breast Cancer Spheroid Culture. *Arch Iran Med.* **2014**, *17* (5), 352–360.
119. Sakumoto, M.; Oyama, R.; Takahashi, M.; Takai, Y.; Kito, F.; Shiozawa, K.; Qiao, Z.; Endo, M.; Yoshida, A.; Kawai, A.; Kondo, T. Establishment and Proteomic Characterization of Patient-Derived Clear Cell Sarcoma Xenografts and Cell Lines. *In Vitro Cellular & Developmental Biology - Animal* **2018**, *54* (2), 163–176. https://doi.org/10.1007/s11626-017-0207-5.
120. Salehi, F.; Jamali, T.; Kavoosi, G.; Ardestani, S. K.; Vahdati, S. N. Stabilization of Zataria Essential Oil with Pectin-Based Nanoemulsion for Enhanced Cytotoxicity in Monolayer and Spheroid Drug-Resistant Breast Cancer Cell Cultures and Deciphering Its Binding Mode with gDNA. *International Journal of Biological Macromolecules* **2020**, *164*, 3645–3655. https://doi.org/10.1016/j.ijbiomac.2020.08.084.
121. Sambi, M.; Samuel, V.; Qorri, B.; Haq, S.; Burov, S. V.; Markvicheva, E.; Harless, W.; Szewczuk, M. R. A Triple Combination of Metformin, Acetylsalicylic Acid, and Oseltamivir Phosphate Impacts Tumour Spheroid Viability and Upends Chemoresistance in Triple-Negative Breast Cancer. *Drug Des Devel Ther* **2020**, *14*, 1995–2019. https://doi.org/10.2147/DDDT.S242514.
122. Sankar, S.; Mehta, V.; Ravi, S.; Sharma, C. S.; Rath, S. N. A Novel Design of Microfluidic Platform for Metronomic Combinatorial Chemotherapy Drug Screening Based on 3D Tumor Spheroid Model. *Biomedical Microdevices* **2021**, *23* (4), 50. https://doi.org/10.1007/s10544-021-00593-w.

 doi:10.20944/preprints202504.0640.v1

123. Särchen, V.; Shanmugalingam, S.; Kehr, S.; Reindl, L. M.; Greze, V.; Wiedemann, S.; Boedicker, C.; Jacob, M.; Bankov, K.; Becker, N.; Wehner, S.; Theilen, T. M.; Gretser, S.; Gradhand, E.; Kummerow, C.; Ullrich, E.; Vogler, M. Pediatric Multicellular Tumor Spheroid Models Illustrate a Therapeutic Potential by Combining BH3 Mimetics with Natural Killer (NK) Cell-Based Immunotherapy. *Cell Death Discovery* **2022**, *8* (1), 11. https://doi.org/10.1038/s41420-021-00812-6.
124. Sarıyar, E.; Karpat, O.; Sezan, S.; Baylan, S. M.; Kıpçak, A.; Guven, K.; Erdal, E.; Fırtına Karagonlar, Z. EGFR and Lyn Inhibition Augments Regorafenib Induced Cell Death in Sorafenib Resistant 3D Tumor Spheroid Model. *Cellular Signalling* **2023**, *105*, 110608. https://doi.org/10.1016/j.cellsig.2023.110608.
125. Sauer, S. J.; Tarpley, M.; Shah, I.; Save, A. V.; Lyerly, H. K.; Patierno, S. R.; Williams, K. P.; Devi, G. R. Bisphenol A Activates EGFR and ERK Promoting Proliferation, Tumor Spheroid Formation and Resistance to EGFR Pathway Inhibition in Estrogen Receptor-Negative Inflammatory Breast Cancer Cells. *Carcinogenesis* **2017**, *38* (3), bgx003. https://doi.org/10.1093/carcin/bgx003.
126. Shaheen, S.; Ahmed, M.; Lorenzi, F.; Nateri, A. S. Spheroid-Formation (Colonosphere) Assay for in Vitro Assessment and Expansion of Stem Cells in Colon Cancer. *Stem Cell Reviews and Reports* **2016**, *12* (4), 492–499. https://doi.org/10.1007/s12015-016-9664-6.
127. Shen, K.; Lee, J.; Yarmush, M. L.; Parekkadan, B. Microcavity Substrates Casted from Self-Assembled Microsphere Monolayers for Spheroid Cell Culture. *Biomedical Microdevices* **2014**, *16* (4), 609–615. https://doi.org/10.1007/s10544-014-9863-3.
128. Sheth, D. B.; Gratzl, M. Electrochemical Mapping of Oxygenation in the Three-Dimensional Multicellular Tumour Hemi-Spheroid. *Proceedings of the Royal Society A: Mathematical, Physical and Engineering Sciences* **2019**, *475* (2225), 20180647. https://doi.org/10.1098/rspa.2018.0647.
129. Shortt, R. L.; Wang, Y.; Hummon, A. B.; Jones, L. M. Development of Spheroid-FPOP: An In-Cell Protein Footprinting Method for 3D Tumor Spheroids. *J. Am. Soc. Mass Spectrom.* **2023**, *34* (3), 417–425. https://doi.org/10.1021/jasms.2c00307.
130. Singh, A.; Tayalia, P. Three-Dimensional Cryogel Matrix for Spheroid Formation and Anti-Cancer Drug Screening. *Journal of Biomedical Materials Research Part A* **2020**, *108* (2), 365–376. https://doi.org/10.1002/jbm.a.36822.
131. Suhito, I. R.; Angeline, N.; Lee, K.-H.; Kim, H.; Park, C. G.; Luo, Z.; Kim, T.-H. A Spheroid-Forming Hybrid Gold Nanostructure Platform That Electrochemically Detects Anticancer Effects of Curcumin in a Multicellular Brain Cancer Model. *Small* **2021**, *17* (15), 2002436. https://doi.org/10.1002/smll.202002436.
132. Tanenbaum, L. M.; Mantzavinou, A.; Subramanyam, K. S.; del Carmen, M. G.; Cima, M. J. Ovarian Cancer Spheroid Shrinkage Following Continuous Exposure to Cisplatin Is a Function of Spheroid Diameter. *Gynecologic Oncology* **2017**, *146* (1), 161–169. https://doi.org/10.1016/j.ygyno.2017.04.014.
133. Tang, S.; Hu, K.; Sun, J.; Li, Y.; Guo, Z.; Liu, M.; Liu, Q.; Zhang, F.; Gu, N. High Quality Multicellular Tumor Spheroid Induction Platform Based on Anisotropic Magnetic Hydrogel. *ACS Appl. Mater. Interfaces* **2017**, *9* (12), 10446–10452. https://doi.org/10.1021/acsami.6b15918.
134. Taubenberger, A. V.; Girardo, S.; Träber, N.; Fischer-Friedrich, E.; Kräter, M.; Wagner, K.; Kurth, T.; Richter, I.; Haller, B.; Binner, M.; Hahn, D.; Freudenberg, U.; Werner, C.; Guck, J. 3D Microenvironment Stiffness Regulates Tumor Spheroid Growth and Mechanics via P21 and ROCK. *Advanced Biosystems* **2019**, *3* (9), 1900128. https://doi.org/10.1002/adbi.201900128.
135. Terrones, M.; Deben, C.; Rodrigues-Fortes, F.; Schepers, A.; de Beeck, K. O.; Van Camp, G.; Vandeweyer, G. CRISPR/Cas9-Edited ROS1 + Non-Small Cell Lung Cancer Cell Lines Highlight Differential Drug Sensitivity in 2D vs 3D Cultures While Reflecting Established Resistance Profiles. *Journal of Translational Medicine* **2024**, *22* (1), 234. https://doi.org/10.1186/s12967-024-04988-0.
136. Tevis, K. M.; Cecchi, R. J.; Colson, Y. L.; Grinstaff, M. W. Mimicking the Tumor Microenvironment to Regulate Macrophage Phenotype and Assessing Chemotherapeutic Efficacy in Embedded Cancer Cell/Macrophage Spheroid Models. *Acta Biomaterialia* **2017**, *50*, 271–279. https://doi.org/10.1016/j.actbio.2016.12.037.
137. To, H. T.; Le, Q. A.; Bui, H. T.; Park, J.-H.; Kang, D. Modulation of Spheroid Forming Capacity and TRAIL Sensitivity by KLF4 and Nanog in Gastric Cancer Cells. *Current Issues in Molecular Biology* **2023**, *45* (1), 233–248. https://doi.org/10.3390/cimb45010018.

 doi:10.20944/preprints202504.0640.v1

138. Torisawa, Y.; Takagi, A.; Nashimoto, Y.; Yasukawa, T.; Shiku, H.; Matsue, T. A Multicellular Spheroid Array to Realize Spheroid Formation, Culture, and Viability Assay on a Chip. *Biomaterials* **2007**, *28* (3), 559–566. https://doi.org/10.1016/j.biomaterials.2006.08.054.
139. UEMATSU, N.; ZHAO, Y.; KIYOMI, A.; YUAN, B.; ONDA, K.; TANAKA, S.; SUGIYAMA, K.; SUGIURA, M.; TAKAGI, N.; HAYAKAWA, A.; HIRANO, T. Chemo-Sensitivity of Two-Dimensional Monolayer and Three-Dimensional Spheroid of Breast Cancer MCF-7 Cells to Daunorubicin, Docetaxel, and Arsenic Disulfide. *Anticancer Res* **2018**, *38* (4), 2101.
140. Varan, G.; Akkın, S.; Demirtürk, N.; Benito, J. M.; Bilensoy, E. Erlotinib Entrapped in Cholesterol-Depleting Cyclodextrin Nanoparticles Shows Improved Antitumoral Efficacy in 3D Spheroid Tumors of the Lung and the Liver. *Journal of Drug Targeting* **2021**, *29* (4), 439–453. https://doi.org/10.1080/1061186X.2020.1853743.
141. Vinci, M.; Gowan, S.; Boxall, F.; Patterson, L.; Zimmermann, M.; Court, W.; Lomas, C.; Mendiola, M.; Hardisson, D.; Eccles, S. A. Advances in Establishment and Analysis of Three-Dimensional Tumor Spheroid-Based Functional Assays for Target Validation and Drug Evaluation. *BMC Biology* **2012**, *10* (1), 29. https://doi.org/10.1186/1741-7007-10-29.
142. Wan, X.; Li, Z.; Ye, H.; Cui, Z. Three-Dimensional Perfused Tumour Spheroid Model for Anti-Cancer Drug Screening. *Biotechnology Letters* **2016**, *38* (8), 1389–1395. https://doi.org/10.1007/s10529-016-2035-1.
143. Wang, Y.; Wang, J. Mixed Hydrogel Bead-Based Tumor Spheroid Formation and Anticancer Drug Testing. *Analyst* **2014**, *139* (10), 2449–2458. https://doi.org/10.1039/C4AN00015C.
144. Ware, M. J.; Keshishian, V.; Law, J. J.; Ho, J. C.; Favela, C. A.; Rees, P.; Smith, B.; Mohammad, S.; Hwang, R. F.; Rajapakshe, K.; Coarfa, C.; Huang, S.; Edwards, D. P.; Corr, S. J.; Godin, B.; Curley, S. A. Generation of an in Vitro 3D PDAC Stroma Rich Spheroid Model. *Biomaterials* **2016**, *108*, 129–142. https://doi.org/10.1016/j.biomaterials.2016.08.041.
145. Wen, Z.; Liao, Q.; Hu, Y.; You, L.; Zhou, L.; Zhao, Y. A Spheroid-Based 3-D Culture Model for Pancreatic Cancer Drug Testing, Using the Acid Phosphatase Assay. *Braz J Med Biol Res.* **2013**, *46* (7), 634–642. https://doi.org/10.1590/1414-431X20132647.
146. Wenzel, C.; Riefke, B.; Gründemann, S.; Krebs, A.; Christian, S.; Prinz, F.; Osterland, M.; Golfier, S.; Räse, S.; Ansari, N.; Esner, M.; Bickle, M.; Pampaloni, F.; Mattheyer, C.; Stelzer, E. H.; Parczyk, K.; Prechtl, S.; Steigemann, P. 3D High-Content Screening for the Identification of Compounds That Target Cells in Dormant Tumor Spheroid Regions. *Experimental Cell Research* **2014**, *323* (1), 131–143. https://doi.org/10.1016/j.yexcr.2014.01.017.
147. Weydert, Z.; Lal-Nag, M.; Mathews-Greiner, L.; Thiel, C.; Cordes, H.; Küpfer, L.; Guye, P.; Kelm, J. M.; Ferrer, M. A 3D Heterotypic Multicellular Tumor Spheroid Assay Platform to Discriminate Drug Effects on Stroma versus Cancer Cells. *SLAS Discovery* **2020**, *25* (3), 265–276. https://doi.org/10.1177/2472555219880194.
148. Wu, G.; Zhan, S.; Rui, C.; Sho, E.; Shi, X.; Ding, Y. Microporous Cellulosic Scaffold as a Spheroid Culture System Modulates Chemotherapeutic Responses and Stemness in Hepatocellular Carcinoma. *J Cell Biochem.* **2019**, *120* (4), 5244–5255. https://doi.org/10.1002/jcb.27799.
149. Wu, K.-W.; Kuo, C.-T.; Tu, T.-Y. A Highly Reproducible Micro U-Well Array Plate Facilitating High-Throughput Tumor Spheroid Culture and Drug Assessment. *Global Challenges* **2021**, *5* (2), 2000056. https://doi.org/10.1002/gch2.202000056.
150. H. Xia; N. G. Avci; Y. Akay; Y. Esquenazi; L. H. Schmitt; N. Tandon; J. -J. Zhu; M. Akay. Temozolomide in Combination With NF-κB Inhibitor Significantly Disrupts the Glioblastoma Multiforme Spheroid Formation. *IEEE Open Journal of Engineering in Medicine and Biology* **2020**, *1*, 9–16. https://doi.org/10.1109/OJEMB.2019.2962801.
151. Xiong, Q.; Liu, T.; Ying, Y.; Yu, X.; Wang, Z.; Gao, H.; Lin, T.; Fan, W.; Zhang, Z.; Wei, Q.; Ge, Y.; Zeng, S.; Xu, C. Establishment of Bladder Cancer Spheroids and Cultured in Microfluidic Platform for Predicting Drug Response. *Bioengineering & Translational Medicine* **2024**, *9* (2), e10624. https://doi.org/10.1002/btm2.10624.
152. Yamawaki, K.; Mori, Y.; Sakai, H.; Kanda, Y.; Shiokawa, D.; Ueda, H.; Ishiguro, T.; Yoshihara, K.; Nagasaka, K.; Onda, T.; Kato, T.; Kondo, T.; Enomoto, T.; Okamoto, K. Integrative Analyses of Gene Expression and

 doi:10.20944/preprints202504.0640.v1

Chemosensitivity of Patient-Derived Ovarian Cancer Spheroids Link G6PD-Driven Redox Metabolism to Cisplatin Chemoresistance. *Cancer Letters* **2021**, *521*, 29–38. https://doi.org/10.1016/j.canlet.2021.08.018.

153. Yoshida, T.; Sopko, N., A.; Kates, M.; Liu, X.; Joice, G.; Mcconkey, D., J.; Bivalacqua, T., J. Impact of Spheroid Culture on Molecular and Functional Characteristics of Bladder Cancer Cell Lines. *Oncol Lett* **2019**, *18* (5), 4923–4929. https://doi.org/10.3892/ol.2019.10786.
154. Yu, L.; Ni, C.; Grist, S. M.; Bayly, C.; Cheung, K. C. Alginate Core-Shell Beads for Simplified Three-Dimensional Tumor Spheroid Culture and Drug Screening. *Biomedical Microdevices* **2015**, *17* (2), 33. https://doi.org/10.1007/s10544-014-9918-5.
155. Yu, Q.; Roberts, M. G.; Houdaihed, L.; Liu, Y.; Ho, K.; Walker, G.; Allen, C.; Reilly, R. M.; Manners, I.; Winnik, M. A. Investigating the Influence of Block Copolymer Micelle Length on Cellular Uptake and Penetration in a Multicellular Tumor Spheroid Model. *Nanoscale* **2021**, *13* (1), 280–291. https://doi.org/10.1039/D0NR08076D.
156. Zhang, J. Z.; Bryce, N. S.; Lanzirotti, A.; Chen, C. K. J.; Paterson, D.; de Jonge, M. D.; Howard, D. L.; Hambley, T. W. Getting to the Core of Platinum Drug Bio-Distributions: The Penetration of Anti-Cancer Platinum Complexes into Spheroid Tumour Models. *Metallomics* **2012**, *4* (11), 1209–1217. https://doi.org/10.1039/C2MT20168B.
157. Zhang, J. Z.; Bryce, N. S.; Siegele, R.; Carter, E. A.; Paterson, D.; de Jonge, M. D.; Howard, D. L.; Ryan, C. G.; Hambley, T. W. The Use of Spectroscopic Imaging and Mapping Techniques in the Characterisation and Study of DLD-1 Cell Spheroid Tumour Models. *Integr. Biol.* **2012**, *4* (9), 1072–1080. https://doi.org/10.1039/C2IB20121F.
158. Zhang, X.; Wang, W.; Yu, W.; Xie, Y.; Zhang, X.; Zhang, Y.; Ma, X. Development of an in Vitro Multicellular Tumor Spheroid Model Using Microencapsulation and Its Application in Anticancer Drug Screening and Testing. *Biotechnology Progress* **2005**, *21* (4), 1289–1296. https://doi.org/10.1021/bp050003l.
159. Zuchowska, A.; Kwapiszewska, K.; Chudy, M.; Dybko, A.; Brzozka, Z. Studies of Anticancer Drug Cytotoxicity Based on Long-Term HepG2 Spheroid Culture in a Microfluidic System. *ELECTROPHORESIS* **2017**, *38* (8), 1206–1216. https://doi.org/10.1002/elps.201600417.