

参赛学生姓名 Name of Participant: Catherine Fang, 杨郡竹

中学 School: Chongqing Yucai Secondary School

省方 Province/City: Chongqing

国家/地区 Country/State: China

指导教师姓名 Name of Supervisor: 陈沁萌

指导教师单位 Affiliation of Supervisor: Chongqing Yucai

Secondary School

论文题目 Title of Paper: When Spheroids Touch the Bottom:

Revealing a Hidden Variable in 3D Tumor Invasion Assays

When Spheroids Touch the Bottom: Revealing a Hidden Variable in 3D Tumor Invasion Assays

Junzhu Yang, Catherine Fang (Chongqing Yucai Secondary School)

Abstract

Three-dimensional tumor spheroid invasion assays are widely used to investigate tumor-cell motility and evaluate anti-invasive treatments. However, invasion morphologies can vary substantially, even when similar cell lines and hydrogel matrices are used. The basis of this variability remains insufficiently defined. Here, we hypothesized that the vertical position of a spheroid within the hydrogel, particularly unintended contact with the rigid culture substrate, is a critical determinant of the observed invasion phenotype. A structured analysis of 42 published spheroid-invasion studies showed that spheroid-substrate contact was either reported or could not be excluded in 78.6% of cases, indicating that this potentially influential experimental variable is frequently uncontrolled or insufficiently described. To address this issue, we developed a non-adherent PDMS microchamber with side-view imaging capability. The system reduced spheroid attachment to the culture substrate, and enabled direct verification of spheroid position. Comparison with standard culture dishes demonstrated that bottom-associated spheroids exhibited flattened, lateral spreading, whereas spheroids maintained within the hydrogel displayed matrix-associated three-dimensional outgrowth. The platform was further evaluated using alginate, Matrigel, and collagen-I. These hydrogels produced distinct spheroid behaviors that were consistent with differences in biochemical composition, cell-adhesive ligands, fibrillar architecture, and matrix remodeling potential. Finally, the system was applied to examine the effects of CK-666, blebbistatin, and GM6001 on collagen-I-based spheroid invasion. In standard culture dishes, inhibitor responses were difficult to interpret because substrate-associated spreading occurred simultaneously with hydrogel-directed outgrowth. In contrast, the non-adherent microchamber more clearly resolved inhibitor effects on three-dimensional invasion: CK-666 and blebbistatin nearly abolished outward invasion, whereas GM6001 produced partial inhibition, with fewer and shorter spindle-like protrusions remaining. These findings support the importance of distinguishing hydrogel-associated invasion from substrate-associated migration and demonstrate that spheroid vertical position is a fundamental, yet frequently overlooked, variable in invasion assays. The non-adherent PDMS microchamber provides a controllable platform for studying tumor-cell invasion, and anti-invasive therapies.

Keywords: tumor spheroid, 3D invasion, hydrogel, collagen type I, Matrigel, alginate, PDMS

2026 S.-T. Yau High School Science Award
仅用于2026丘成桐中学科学奖论文公示

Table of content

1. Introduction	- 1 -
2. Materials and Methods	- 3 -
2.1 Reagents and equipment.....	- 3 -
2.2 Generation and culture of 3D cell spheroid	- 4 -
2.3 Design and prepare microchamber for 3D spheroid invasion assay	- 4 -
2.4 Surface modification of microchamber to prevent spheroid attachment and spreading	- 4 -
2.5 Setup of observation of 3D spheroid's attachment and spreading	- 5 -
2.6 3D spheroid invasion assay in hydrogel.....	- 5 -
2.7 Respond of 3D spheroid to cell movement inhibitors in invasion assay ..	- 5 -
2.8 Image analysis and quantification	- 6 -
2.9 Statistical Analysis.....	- 6 -
3. Results and Discussion	- 6 -
3.1 Reporting of spheroid-substrate position is limited in published 3D spheroid invasion assays	- 6 -
3.2 Fabrication of non-adherent PDMS microchamber for hydrogel-based invasion assay.....	- 8 -
3.3 Establishment of a hydrogel-based spheroid invasion assay and in situ inspection of top-view and side-view morphology	- 10 -
3.4 Invasion potential of tumor spheroid in hydrogel with different biophysical properties.....	- 12 -
3.5 Cell movement inhibitor responses differ between assay geometries.....	17
4. Conclusion	19
Acknowledgement.....	20
References	21
Appendix: Hydrogel-based spheroid invasion assay - literature analysis.....	23
Table 1. Valid hydrogel-based spheroid invasion studies (n = 42).....	23
Reference for Table 1	30
Table 2. Excluded studies (n = 10)	33
Reference for Table 2	34

1. Introduction

Tumor metastasis is a significant challenge in cancer treatment. After tumor cells detach from the primary site, they need to pass through the surrounding extracellular matrix, enter the blood vessels or lymphatic system, and continue to grow in other tissues (Majidpoor & Mortezaee, 2021). Therefore, studying tumor cells migration and invasion in a three-dimensional environment is of great significance. Traditional two-dimensional culture usually places cells on the surface of a plastic culture dish. Two-dimensional culture is convenient to operate and the images are clear, but the cells mainly move in a plane, which is significantly different from the complex three-dimensional tissue environment in the body. In contrast, three-dimensional tumor spheroids are formed by the aggregation of multiple cells and have cell-cell connections, nutrient and oxygen gradients, and possible central hypoxic regions. Therefore, embedding spheroids in a hydrogel is often considered a better method to simulate the tumor microenvironment (Breslin & O'Driscoll, 2013).

While reading relevant literature, we found that the images of invasion in different articles vary greatly in terms of morphology. In some cases, cells or cell bundles extend outward in multiple directions, resembling three-dimensional radial invasion. In others, cells form a sheet-like region around the spheroid, resembling spreading along a flat plane. As shown in **Figure 1A**, spheroids of endothelial colony-forming cells (ECFCs) cultured in gelatin–dextran (Gel-Dex) hydrogel produced sprouts after two days of culture. Similar spike-like protrusions were observed from human fibrosarcoma HT1080 spheroids cultured in Matrigel (**Figure 1B**). In contrast, a dense cell sheet was observed around spheroids cultured in gelatin methacryloyl (GelMA) (Figure 1C) and in Matrigel (Figure 1D).

We also observed similar morphological differences in our own spheroid culture trials. The appearance of spike-like sprouts was not always consistent, and 2D-like cell spreading was sometimes observed.

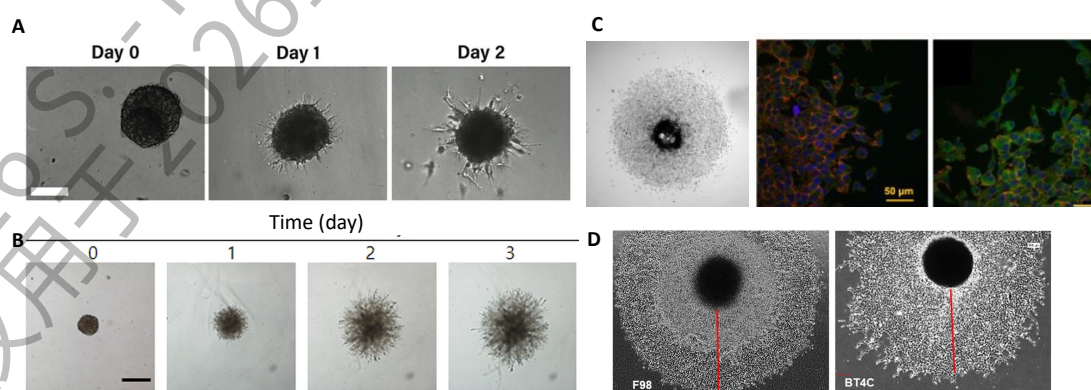


Figure. 1 Microscope images of 3D spheroid invasion assay in reported literature. (A) Representative brightfield images of endothelial spheroids encapsulated in Gel-Dex

(Ntekoumes et al., 2025); (B) Daily micrographs of HT1080 spheroid invasion (Lim et al., 2020); (C) The bright field image of cell invasion behavior over time in 5 % GelMA at Day 5 (Dogan et al., 2024); (D) glioma cell spheroid invasion into a Matrigel matrix on day 4 of culture (Chandekar et al., 2026).

We first considered whether these differences might be caused by variation in cell type, hydrogel type or culturing time. However, we also notice a detail that in conventional methods: spheroids are first added to a lower viscosity hydrogel precursor solution and waited for gelation. We hypothesize that during this period, the spheroids might sediment and come into contact with the bottom of the culture dish. Previous studies have shown that stiff surfaces promote tumor cell attachment and spreading (Fischer-Friedrich et al., 2014; Park et al., 2010). If the spheroids reach the rigid bottom surface before complete gelation, the cells might migrate along the culture dish base, potentially producing the sheet-like morphology and biasing the measured outcome. This reasoning led to our central question: in hydrogel-based invasion experiments, does the relative position of the spheroid to the culture base change the observed invasion results?

Previous studies have addressed the problem of bottom contact and presented some solutions, such as coating the bottom of the petri dish with P Polydimethylsiloxane (PDMS) or PDMS microwells (Goli et al., 2026). However, we believe that there is still room for improvement in terms of decreasing spheroid bottom contact. A previous study has shown that tumor cells show maximum proliferation and adhesion when cultured on modified PDMS with a certain critical surface energy. Adhesion decreases with increased deviation from this critical surface energy (Majhy et al., 2021).

To answer this question, we first did a literature analysis flowing meta-analysis protocol to review the experiment setting and outcome of spheroid invasion in hydrogels in hydrogel-based invasion assay. The focus was placed on spheroids generation method, ways of adding/mixing with hydrogel, culture vessel, spheroid invasion area etc. according to that information, the relative position between spheroid and culture vessel were categorized as non-contact, contact, and ambiguous. Then a PDMS microcavity was fabricated to hold the hydrogel for spheroids invasion, not only keep the position of hydrogel, also reduce the usage of the hydrogel. To enhance hydrogel spreading on the surface while prevent spheroid adhesion, the PDMS microcavity was modified with Pluronic F-127 triblock-polymer, making the originally hydrophobic surface hydrophilic). Moreover, a side-view mirror was applied to inspect the spheroid-substrate interaction/contacting. Tumor spheroids cultured for 3 days were

collected for hydrogel-based invasion assay. The morphological changes of tumor spheroids embedded in alginate, collagen-I, and Matrigel were compared to demonstrate the impact of contacting and attaching on the vessel substrate on spheroid movement or invading. Lastly, to further decipher the impact of vessel substrate contacting on spheroid movement, three small inhibitors targeting different pathways of cell motility were tested using hydrogel-based spheroid invasion assay.

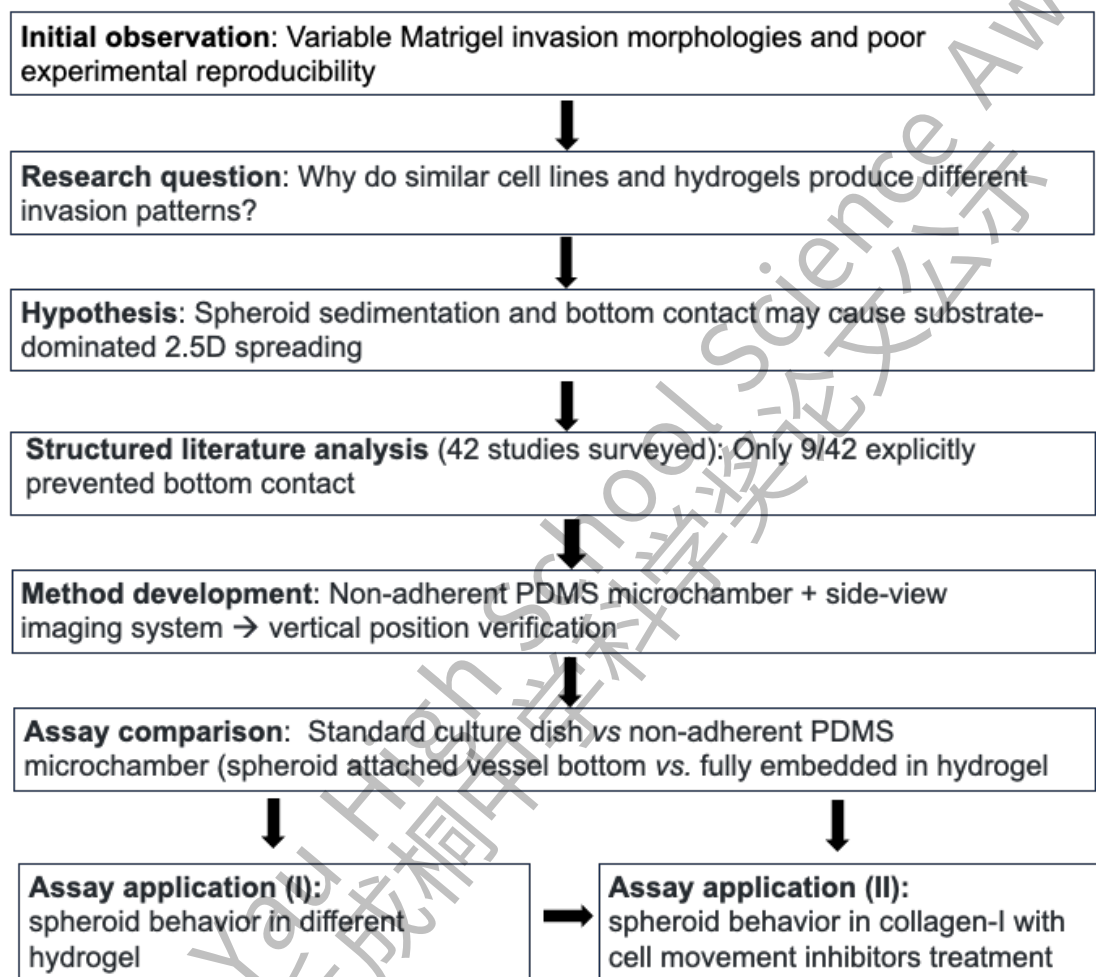


Figure 2. Research rationale and validation of a non-adherent, side-view-compatible platform for distinguishing substrate-associated spreading from three-dimensional spheroid invasion

2. Materials and Methods

2.1 Reagents and equipment

The human prostate cancer cell line DU145 was obtained from Procell Biotech (Wuhan, China). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Bio-Channel, Nanjing, China) supplemented with 10% fetal bovine serum (FBS; Bio-

Channel), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified incubator containing 5% CO₂.

Polydimethylsiloxane (PDMS) and the corresponding curing agent were purchased from Dow Corning Co., Ltd. (USA). Pluronic F-127 was obtained from Beyotime Biotechnology Co., Ltd. (Beijing, China). Rat collagen I was purchased from R&D Systems (USA), and Matrigel was obtained from Corning (USA). Agarose and sodium alginate were purchased from Aladdin Scientific (Shanghai, China), unless otherwise specified.

2.2 Generation and culture of 3D cell spheroid

Concave agarose microwell arrays were used to generate and culture 3D cell spheroid according to a previously described protocol (Zhang et al., 2018). First, each microwell was 2 mm deep, with an upper-opening radius of 3 mm. A total of 1×10^4 cells suspended in 8 µL of culture medium was pipetted into each agarose microwell and culture for 3 days at 37°C in a humidified atmosphere containing 5% CO₂. Spheroid formation and morphology were monitored daily an inverted microscopy (TS100-F, Nikon, Japan). The resulting cell spheroids were then collected by pipetting for subsequent invasion assay.

2.3 Design and prepare microchamber for 3D spheroid invasion assay

The PDMS microchamber was replicated by replicating a mold produced using Digital Light Synthesis (DLS) 3D printing (Form 3B+, Formlabs, USA). PDMS prepolymer and curing agent were thoroughly mixed at 10:1 weight ratio, poured into the 3D printed mold, and cured at 80°C for 2 h. Then cured PDMS microchamber was peeled away from the mold.

2.4 Surface modification of microchamber to prevent spheroid attachment and spreading

PDMS modification: The PDMS microchamber was treated by air-plasma for 1 minute, and then immersed in aqueous Pluronic F-127 for 2 h. The Pluronic F-127 treated PDMS microchamber was washed by sterile water, and dried at room temperature. Before cell culture experiment, the PDMS microchambers were sterilized with 75% ethanol and washed thoroughly to remove residual ethanol.

Water contact angle measurement: The water contact angle was measured to characterize the surface wettability of the modified PDMS. Contact angle measurement were performed using a contact angle goniometer (JC2000D, Shanghai Zhongchen Digital Technic Co. Ltd) to compare untreated and Pluronic F-127 modified PDMS.

Cell culture on modified PDMS surface: For cell attachment, DU 145 cell suspension was first added on PDMS surface (untreated, and Pluronic F-127 modified) and cultured for 24 h. The cell morphology was then recorded using microscope. Next, 3D spheroids cultured in agarose microwell for 3 days were collected and added on PDMS surface, and cultured for 24 h. Then spheroids top-view and side-view of spheroids were examined.

2.5 Setup of observation of 3D spheroid's attachment and spreading

The 3D spheroids were observed using an inverted microscopy. To enable observation from the top-view, representing the horizontal dimension, and the side-view, representing the vertical dimension, a dual observation experiment setup was adapted from previously published method (Ning et al., 2025). The side-view mirror was positioned at a 45° angle relative to the substrate using a 3D printed cassette which equipped with two magnetics. A corresponding 3D printed handle containing two magnetics was positioned on the opposite side of the Petri dish lid. The handle was located at outside and above the lid, whereas the cassette and mirror were positioned inside of the Petri dish. The distance between the spheroid and the mirror was adjusted by moving the handle.

2.6 3D spheroid invasion assay in hydrogel

Spheroid invasion assays were performed in three types of hydrogels: (1) Matrigel (4 mg/mL final concentration); (2) Type I Collagen (1.0 mg/mL); and (3) Sodium alginate (0.5 wt). For each assay, a 30 μ L droplet of liquid hydrogel containing a single spheroid was dispensed into the microchamber. Following gelation at 37°C for 30 min, 200 μ L culture medium was added. Top-view and side-view images were captured every 24 h for 96 h. The resulting time-dependent images were analyzed as described below.

2.7 Respond of 3D spheroid to cell movement inhibitors in invasion assay

Spheroid invasion assays in collagen-I were conducted in the presence of three cell-motility inhibitor: CK-666, which affects Arp2/3-dependent branched actin organization; blebbistatin, which reduces non-muscle myosin-II-mediated contractility; and GM6001, a broad-spectrum matrix metalloproteinase inhibitor. CK-666, blebbistatin, and GM6001 were incorporated into collagen-I (1.0 mg/mL) at final concentration of 10 μ M, 10 μ M, and 20 μ M, respectively. During the invasion assay, the medium was replaced every 48 h with fresh media containing corresponding inhibitors.

Hydrogel containing a spheroid was casted on a standard Petric dish, or on a modified PDMS surface to establish the 2.5 invasion and 3D invasion group, respectively.

Control groups received complete medium without inhibitors. Time-dependent images were collected using a microscope every 24 h.

2.8 Image analysis and quantification

Morphometric analysis was performed using ImageJ software (NIH, USA). The quantified parameters included projected area, solidity, maximum invasion distance, and total invasion area. For ImageJ analysis, microscopic images were first converted to 8-bit grayscale using the Image menu, followed by Type and 8-bit. The image scale was then calibrated by drawing a straight line along the scale bar and selecting Analyze, Set Scale. The corresponding scale-bar length and measurement unit were entered in the calibration window. To identify the invasion area, image edges were enhanced using Process, Find Edges. The threshold was subsequently adjusted through Image, Adjust, and Threshold until the invasion area was clearly distinguished from the background. The invasion boundary was then manually outlined, and the enclosed area was measured using Analyze and Measure.

2.9 Statistical Analysis

All experiments were performed in at replicate. Data are presented as Mean $\pm$ Standard Deviation (SD).

3. Results and Discussion

3.1 Reporting of spheroid-substrate position is limited in published 3D spheroid invasion assays

We identified 52 publications using combination of keys [(3D cell spheroid or tumor spheroid) and (spheroid invasion in hydrogel or Matrigel or collagen-I) and (3D invasion or hydrogel or in vitro)]. The included literatures were published between 2013 and 2026. Following the screening flowchart (**Figure 3A**), studies were excluded if they were reviews without original data ($n = 4$), used scaffold-free formats without hydrogel embedding ($n = 3$), used monolayer-based rather than 3D hydrogel-embedded invasion assays ($n = 2$), or focused on non-cancerous differentiation without invasion readouts ($n = 1$). After exclusion, 42 studies met the inclusion criteria.

For each included study, the following parameters were extracted from the full-text article and supplementary materials: (1) cell line(s) used; (2) spheroid formation method (hanging drop, ultra-low attachment plate, spinner flask, or other); (3) spheroid diameter at the time of embedding; (4) hydrogel type and concentration; (5) gelation mechanism (temperature, UV/photo-crosslinking, ionic, enzymatic, or other); (6) culture vessel format; (7) detailed spheroid placement protocol relative to the sol-to-gel

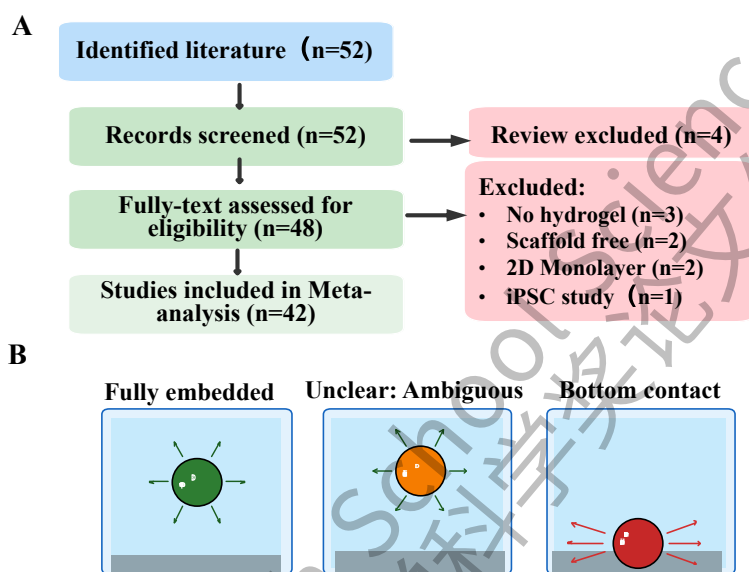
transition (**Appendix Table 1**). Based on this information, the relative vertical position between spheroid and culture vessel were categorized as Yes (fully embedded), NO (bottom-contact), and Unclear: ambiguous, as illustrated in **Figure 3B**.

For included studies, we also assigned a bottom-contact risk score for each study using the following criteria:

1= explicit anti-settling (ice, inversion, rapid crosslinking, sandwich layering);

2= mixed in precursor, no anti-settling mentioned;

3= placed on surface or settled to bottom.



Schematic Classification of Spheroid Position During Hydrogel Gelation

Figure 3. Systematic review flow diagram. (A) Flowchart of identification, screening, and inclusion of relevant studies; (B) Schematic of spheroids vertical position in hydrogel-based invasion assay

Although different types of hydrogels were used across these invasion assays, only 21.4% (9/42) of the included studies employed protocols that explicitly prevented spheroid-substrate contact and were classified as fully embedded (**Figure 4A**). In contrast, 35.7% (15/42) described conditions that would result in direct bottom contact (NO), and 42.9% (18/42) provided insufficient methodological detail to permit judgment (UNCLEAR). When stratified by bottom-contact risk score, low, moderate, and high risk accounted for 21.4% (9/42), 42.9% (18/42), and 35.7% (15/42), respectively (**Figure 4B**). Collectively, 78.6% (33/42) of the surveyed assays either confirmed or could not exclude potential spheroid-substrate contact.

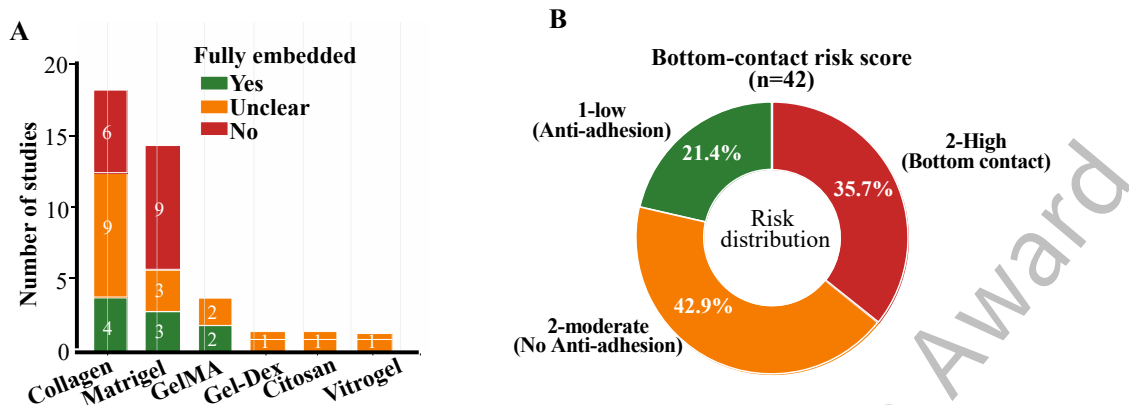


Figure 4. Analysis of the literature on spheroid invasion related to hydrogel. (A) Numbers of studies clarified the vertical position of spheroid in hydrogel; (B) Bottom-contact risk score of the reported works.

Overall, the literature analysis indicated that potential substrate-contact issues are common in 3D spheroid invasion assays, and that only a minority of published protocols explicitly prevent spheroid setting during gelation. The vertical position of spheroid within the hydrogel, whether it has settled onto the vessel substrate, appears to be largely overlooked. This finding supports our hypothesis that vertical position of spheroid should be inspected and controlled in order to distinguish authentic invasion from volume expansion or substrate-associated spreading.

3.2 Fabrication of non-adherent PDMS microchamber for hydrogel-based invasion assay

To reduce spheroids attaching bottom substrate and help maintain spheroids within the hydrogel bulk, we developed a non-adhesive PDMS microchamber (**Figure 5A**) designed to support hydrogel spreading over the substrate and to retain the hydrogel during the invasion assay. The microchamber incorporates a tapered geometry that mechanically retains the hydrogel and reduces gel displacement or detachment during medium exchange. To limit nonspecific protein adsorption and subsequent cell attachment, the hydrophobic PDMS spacers were treated with 2% Pluronic F-127 (**Figure 5B**). The hydrophobic segments of Pluronic F-127 adsorb onto PDMS through hydrophobic interactions, while the hydrophilic tails of the triblock polymer extend toward the medium and form a hydrophilic layer that reduces protein adsorption, cell anchorage, and attachment.

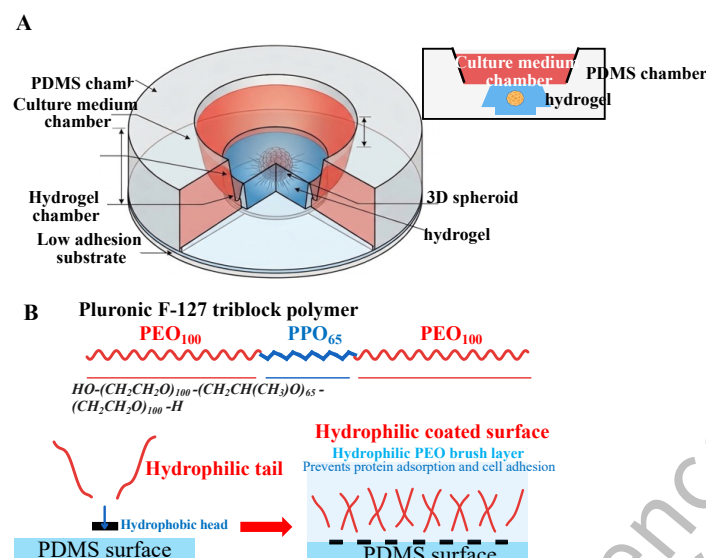


Figure 5. Preparation of non-adherent PDMS microchamber for hydrogel-based spheroid invasion assay. (A) Schematic of microchamber for hydrogel loading and spheroid invasion; (B) surface modification of PDMS by Pluronic F-127 polymer.

As shown in **Figure 6A**, the modification can significantly improve the hydrophilicity of PDMS, as the water contact angle decreased from $118^\circ \pm 4^\circ$ to $26^\circ \pm 2^\circ$. The increased hydrophilicity potentially repels serum proteins adsorption, and cell attachment. As shown in **Figure 6B**, cells spread on untreated PDMS surface, whereas cells cultured on the modified surface remained round and formed floating aggregates in the medium over the same culture period.

When 3D spheroids were added to untreated PDMS, cells migrated out of the spheroid after 48 h and formed a spreading monolayer sheet. In contrast, 3D spheroids added to modified PDMS remained floating, and the floating spheroids clustered and fused to form larger spheroid bodies (**Figure 6C**).

Together, the contact angle measurements and cell culture experiments indicated that Pluronic F-127 modification can reduce cell and spheroid attachment, providing a basis for maintaining spheroids fully embedded within the hydrogel in invasion assays.

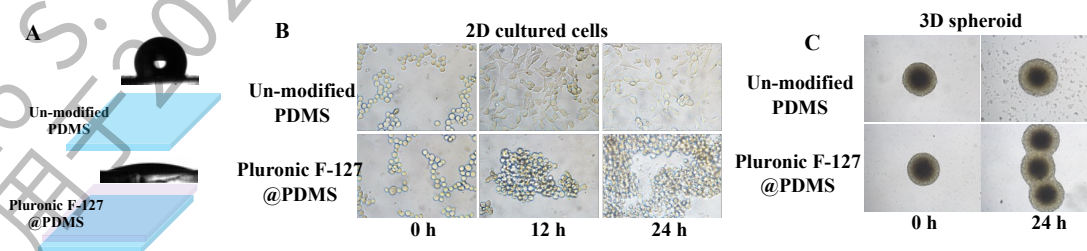


Figure 6. Reduced hydrophobicity and cell attachment on modified PDMS surface. (A) water contact angle measurement on PDMSs; (B) microscope image of cells culture on PDMS surface; (C) microscope images of 3D spheroids culture on PDMS surface.

3.3 Establishment of a hydrogel-based spheroid invasion assay and in situ inspection of top-view and side-view morphology

With standard inverted microscopy, the horizontal size of a spheroid can be measured from a top-view image, but the vertical dimension or thickness cannot be observed directly. Previous work has shown that a first-surface mirror placed at 45° relative to the substrate can provide a side-view image of the same sample (**Figure 7A**).

As illustrated in **Figure 7A**, the top-view and side-view of the same spheroid can be imaged by adjusting the mirror position without physically disturbing the sample. By moving the mirror with the stage micrometer, two optical pathways can be created. For top-view imaging, the sample is aligned vertically above the objective. For side-view imaging, the mirror is moved closer to the objective and positioned so that light from the sample is reflected into the objective, producing a side-view image. In this way, both views can be inspected without moving the spheroid. Because the vertical position of the spheroid can also be directly assessed, substrate-dominated migration can be distinguished from invasion into the hydrogel matrix.

As shown in **Figure 7D**, spheroids placed directly on the culture dish (without a hydrogel covering) allowed both the horizontal diameter and the vertical thickness to be measured. After 12 h of culture, cells had migrated out of the spheroid and spread on the culture dish surface, showing the typical morphology of monolayer-cultured cells.

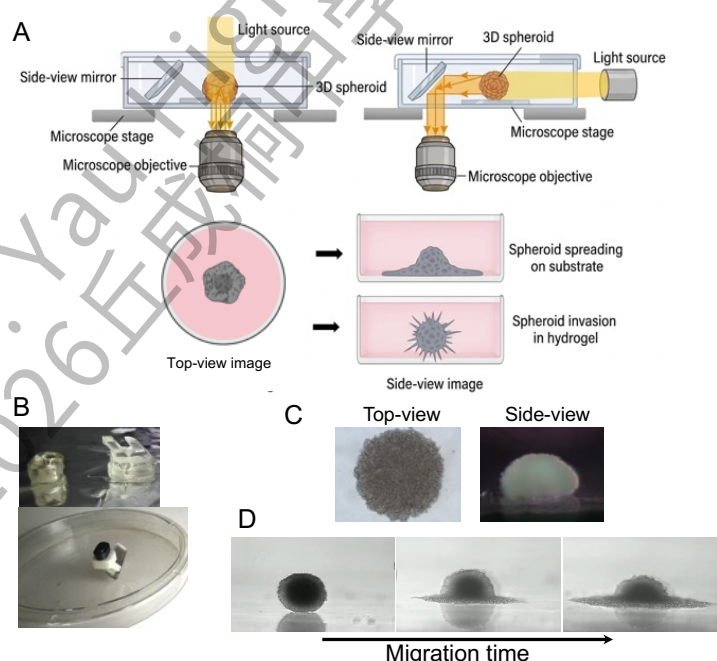


Figure 7. Observation of interaction of 3D spheroid and substrate during invasion process. (A) Schematic of observation of vertical/side-view image of spheroid; (B) side-view observation mirror; (C) Microscope image of horizontal (top-view) and

vertical (side-view) of spheroid; (D) Side-view microscope images of cells moving from spheroids along the substrate surface.

After establishing this side-view mirror strategy for inspecting both horizontal (top-view) and vertical (side-view) morphology, a preliminary hydrogel-based spheroid invasion assay was performed with alginate, collagen-I, and Matrigel. During the assay, hydrogel-encapsulated spheroids were either placed directly on the cell culture plate or within the non-adherent PDMS microchamber. After 24 h of culture, the top-view and side-view of the spheroids were examined under both conditions. As shown in **Figure 8**, spheroids covered in alginate, Matrigel, or collagen-I showed cell spreading along the bottom surface within 24 h. Cells on the substrate displayed flattened morphologies and broad lamellipodia-like extensions typical of 2D migration. Side-view imaging indicated that this spreading occurred primarily on the substrate, consistent with substrate-dominated cell movement. If only bottom-view imaging had been used, this substrate-associated spreading could readily have been misinterpreted as 3D invasion (**Figure 8**).

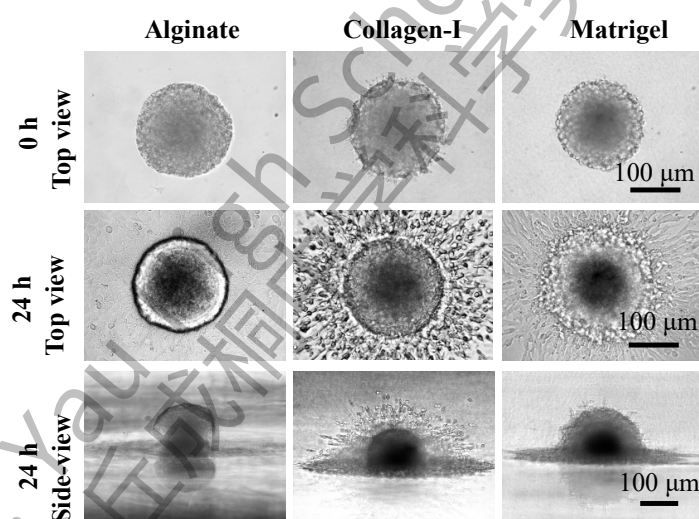


Figure 8. The mixture of 3D spheroids and hydrogels added on cell culture Petri dish.

In contrast, the non-adherent PDMS microchamber helped maintain spheroids within the hydrogel bulk. Side-view imaging showed a continuous hydrogel layer beneath the spheroids, with no apparent direct substrate contact (**Figure 9**). After 24 h of culture, spheroids in alginate remained largely compact and morphologically stable in both top-view and side-view. Spheroids in Matrigel showed relatively smooth and isotropic expansion in both views. In collagen-I, sharp spindle-like protrusions or invadopodia extended radially into the surrounding matrix (**Figure 9**). Thus, cell invasion only observed from collagen-I embedded spheroids.

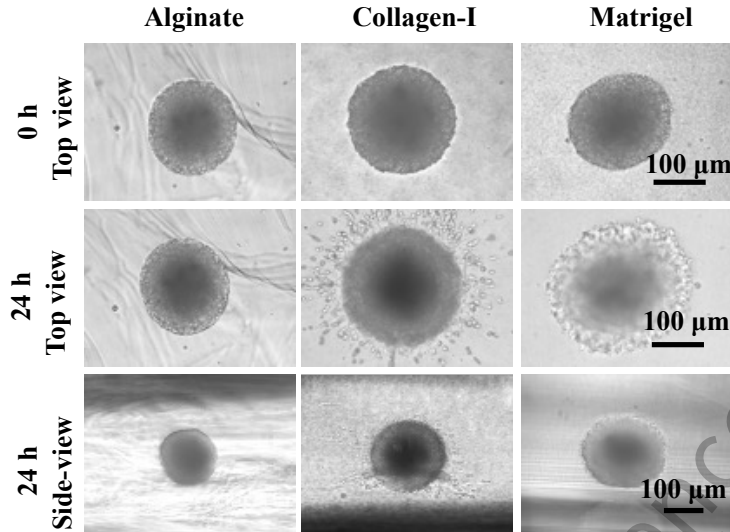


Figure 9. The mixture of 3D spheroids and hydrogels added in non-adherent PDMS microchambers.

Comparing the morphological changes in Figure 8 and 9 suggests that contact with the culture vessel bottom can promote cell migration along the substrate, and that such sedimentation-related effects can confound the distinction between 2D substrate migration and true 3D matrix invasion. In contrast, the non-adherent PDMS microchamber helps avoid direct spheroid–substrate contact and provides a more controlled environment for evaluating spheroid behavior within the hydrogel bulk. In the following experiments, this microchamber was therefore used to study invasion in different hydrogels and to investigate the underlying mechanisms of spheroid movement and invasion.

3.4 Invasion potential of tumor spheroid in hydrogel with different biophysical properties

3.4.1 tumor spheroid behavior in alginate hydrogel

Alginate is generally considered as a biologically inert hydrogel because it lacks intrinsic cell-adhesive ligands. Spheroids were mixed with the alginate precures, and subsequently transferred to non-adherent PDMS microchamber. Spheroid morphology was monitored by microscopy over 96 h, and morphological parameters were quantified using ImageJ. In 0.5% alginate hydrogels (mass ratio), spheroids maintained a highly circular, and compact morphology through the observation period. No obvious protrusive structures were observed at the spheroid boundary (**Figure 10A**). The spheroid area remained constant at approximately $3.1 \times 10^4 \mu\text{m}^2$ across all time points (**Figure 10B**), indicating minimal expansion in this matrix. Although the overall area

remained stable, the internal dark central region gradually expanded. The dark core area increased from approximately $0.7 \times 10^4 \mu\text{m}^2$ at 0 h to $1.4 \times 10^4 \mu\text{m}^2$ at 96h (**Figure 10C**). Accordingly, the ratio of dark core area to total spheroid area increased. According to literatures, this dark region may reflect reduced viability, increased cell density, hypoxia-associated changes. Moreover, solidity was calculated as the ratio of spheroid projected area to convex hull area, with lower values indicating a more irregular or protrusive boundary. The solidity index remained close to 1.0 (0.96–0.98) for all Alginate groups (**Figure 10D**), consisting with the absence of invasive protrusions morphology.

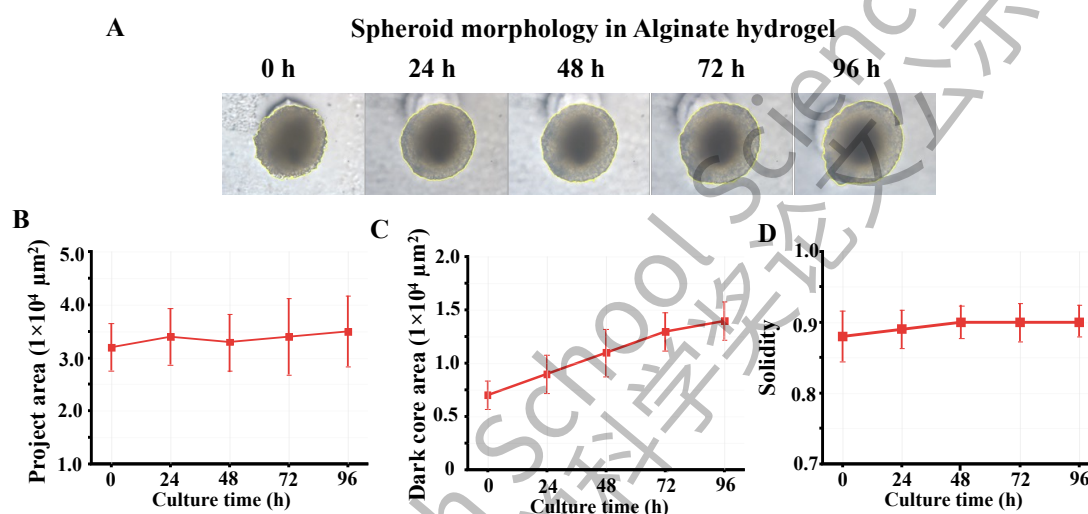


Figure 10. Spheroid behavior in alginate hydrogels. (A) Microscope images of spheroids embedded in alginate. (B) spheroid area and (C) dark core area over time. (D) Changes in spheroid solidity during culture. n=3

3.4.2 Tumor spheroid behavior in Matrigel

Matrigel supported spheroid expansion when cultured in non-adherent PDMS microchamber, and produced a biphasic morphological evolution (**Figure 11A**). During the first 24 h, the projected area increased from $\sim 2.8 \times 10^4 \mu\text{m}^2$ to $4.4 \times 10^4 \mu\text{m}^2$, after which it reached a plateau (**Figure 11B**). Although spheroid diameter continues to increase, the dark zone area did not show significant increase between 12 h and 96 h. At 12 h, transient spindle-like protrusions were observed at the spheroid periphery, causing an initial drop in solidity from 0.95 to 0.88 (**Figure 11C**). These structures may represent early exploratory protrusions; however, their identity cannot be confirmed from morphology alone. By 24 h, the protrusions had largely retracted, and a relatively uniform, translucent cellular layer developed around the central region. Consequently, the solidity index recovered to approximately 0.94 and remained relatively stable thereafter.

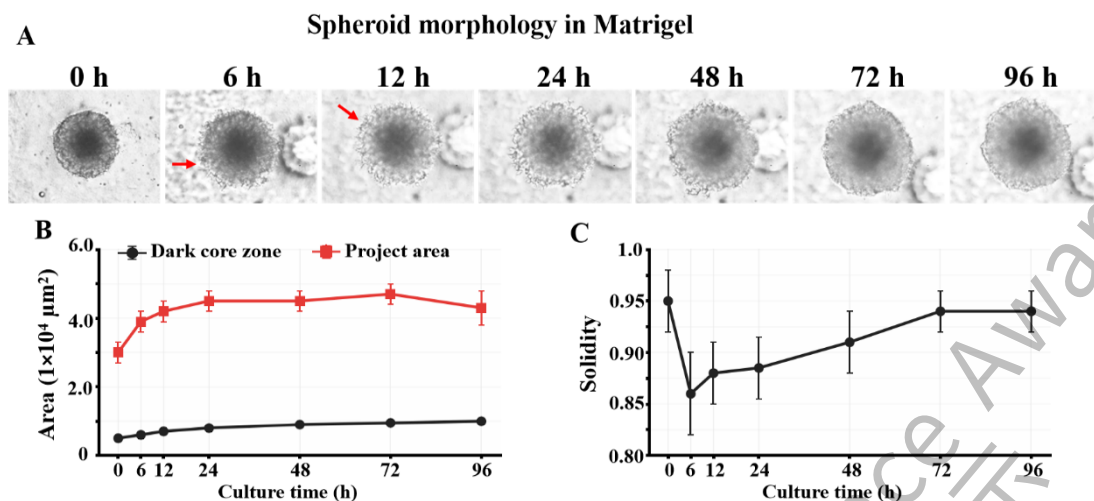


Figure 10 Spheroid behavior in Matrigel. (A) Microscope images of spheroids embedded in Matrigel. (B) spheroid area and dark core area over time. (C) Changes in spheroid solidity during culture. $n=3$

3.4.3 Tumor spheroid behavior in Collagen-I

Spheroids mixed with collagen-I and transferred to non-adherent PDMS microchamber for invasion assay. It was observed that spheroids in collagen-I developed sharp, spindle-like protrusions that extended into the surrounding fibrous matrix over 96 h (**Figure 12A**). In 1.0 mg/mL collagen-I, spheroids exhibited numerous elongated protrusions. The length and number of the protrusions increased with culture time. Quantitative analysis showed that in 1.0 mg/mL Collagen-I, the mean invasion distance reached approximately 150 μm by 96 h. The dark core area remained relatively smaller and more stable in Collagen-I than in alginate (**Figure 12B**).

Spheroid boundary irregularity was further quantified using the solidity index (**Figure 12D**). In 1.0 mg/mL Collagen-I, solidity followed decline trend: it decreased from approximately 0.96 to 0.77 during the first 24 h and then continued to decrease to 0.68 by 96 h, suggesting an invasion process through the observation period.

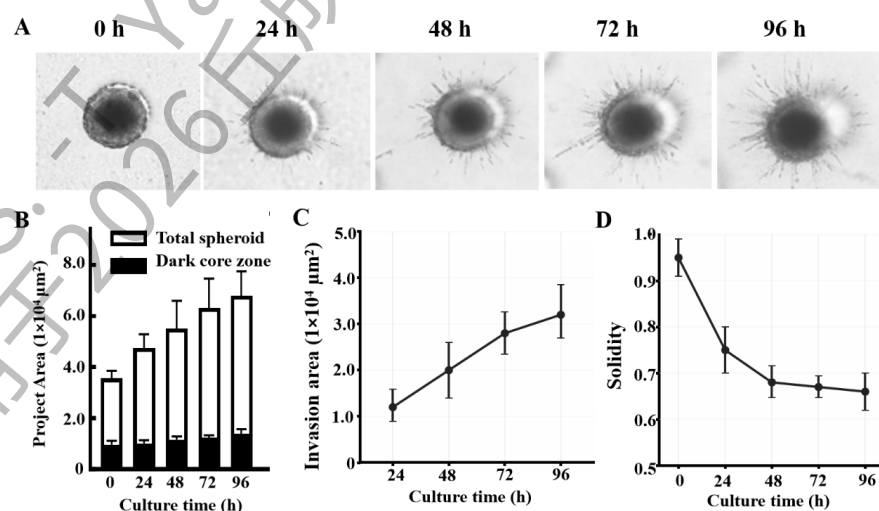


Figure 12. Spheroid behavior in collagen-I hydrogels. (A) Microscope images of spheroids embedded in collagen-I. (B) spheroid area and dark core area overtime. (C) Spheroid invasion area overtime (D) Changes in spheroid solidity during culture. n=3

3.4.4 Comparative analysis of matrix-dependent spheroid behavior and the importance of substrate isolation

The properties of alginate, Matrigel, and collagen-I differ substantially in terms of biological origin, cell-adhesive ligand presentation, fibrillar organization, degradability, and mechanical behavior (**Table 1**). These differences were associated with distinct spheroid phenotypes when spheroid–substrate contact was minimized using the non-adherent PDMS microchamber. In contrast, spheroids cultured in standard dishes frequently sedimented onto the rigid culture surface before complete gelation. Cells then spread laterally along the substrate, producing a shared monolayer morphology across the three hydrogel conditions. Therefore, the same apparent invasion phenotype may reflect different underlying processes depending on spheroid position: matrix-associated three-dimensional outgrowth when the spheroid remains suspended within the gel, or predominantly substrate-associated surface migration when the spheroid contacts the culture-dish bottom.

This comparison emphasizes that hydrogel composition alone is insufficient to interpret spheroid invasion. The vertical position of the spheroid must also be controlled and verified. The non-adherent PDMS microchamber helped maintain spheroids within the hydrogel bulk, while side-view imaging allowed direct assessment of the hydrogel layer beneath each spheroid. Together, these approaches enabled matrix-dependent three-dimensional responses to be distinguished from bottom-associated surface spreading.

Table 1. Comparison of spheroid behavior in different hydrogels

Hydrogel	Origin and key biophysical properties	Cell-adhesive ligands / structure	Standard culture dish	Non-adherent PDMS microchamber
Alginate	Algal polysaccharide; hydrated, non-fibrillar, and relatively biologically inert.	Lacks intrinsic mammalian cell-adhesive ligands; limited cell-mediated remodeling.	Spheroids settled onto the rigid substrate; cells spread laterally with flattened morphologies and broad extensions.	Spheroids remained compact and circular, with minimal expansion and no prominent protrusions
Matrigel	Basement-membrane extract containing laminin, collagen IV, entactin, proteoglycans, and growth-factor components.	Provides cell-adhesive ligands; relatively soft, homogeneous, and predominantly non-fibrillar.	Spheroids contacted the dish bottom and developed lateral, flattened cell spreading that could be mistaken for invasion.	Spheroids showed smooth, isotropic expansion. Early protrusions were transient and later retracted.
Collagen-I	Fibrillar interstitial extracellular-matrix protein.	Contains integrin-binding sites; supports cell adhesion, traction, and cell-mediated matrix remodeling.	Cells spread along the rigid substrate, producing predominantly lateral 2D migration and potentially confounding collagen-I invasion measurements.	Spheroids developed persistent radial, spindle-like protrusions that increased in length and number over time

3.5 Cell movement inhibitor responses differ between assay geometries

Tumor-cell migration and invasion are central processes in metastasis and important therapeutic targets for limiting tumor recurrence and metastatic dissemination. Based on the results in Sections 3.3 and 3.4, spheroid position within the hydrogel strongly influenced the observed phenotype. When spheroids contacted the rigid bottom of a standard culture dish, cells spread predominantly along the substrate, although outgrowth toward the collagen-I hydrogel could also occur. In contrast, the non-adherent PDMS microchamber minimized spheroid–substrate contact and promoted evaluation of hydrogel-associated three-dimensional invasion. We therefore used a collagen-I-based assay to examine the responses of spheroid outgrowth to inhibitors of cytoskeletal function and matrix remodeling.

From top-view and side-view observation, it was found that in standard culture dishes, untreated spheroids exhibited both lateral spreading along the rigid substrate and outgrowth into the overlying collagen-I hydrogel (**Figure 13A**). Blebbistatin strongly reduced both responses. CK-666 markedly inhibited outgrowth toward the hydrogel, whereas substrate-associated spreading remained evident. GM6001 partially reduced both types of outgrowths, but neither response was completely eliminated (**Figure 13B**).

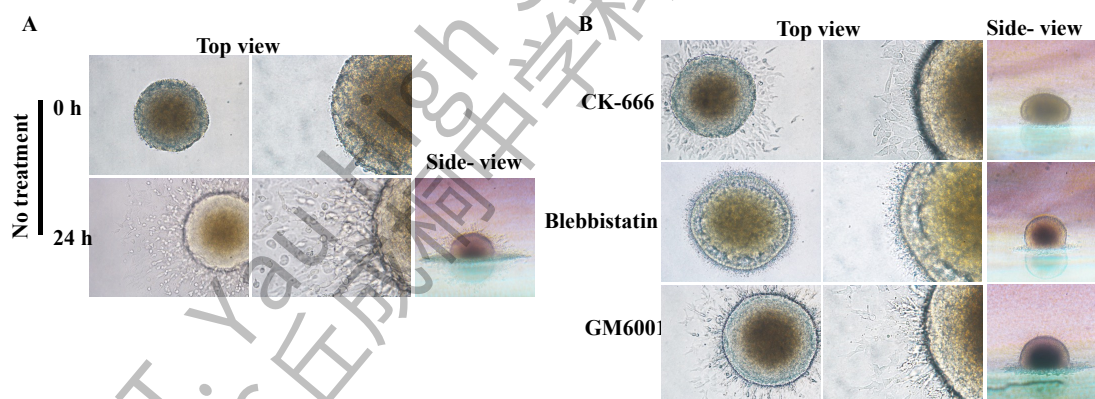


Figure 13. Spheroid behavior in collagen-I hydrogels with different cell movement inhibitors. Hydrogel mixed spheroids were added on cell culture plate (A) Microscope images of spheroids without inhibitors. (B) Microscope images of spheroids cultured with hydrogel with different inhibitors for 24 h.

In the non-adherent PDMS microchamber, direct spheroid–substrate contact was minimized and spheroid invade towards the collagen-I matrix without inhibitor treatment (**Figure 14A**), CK-666 and blebbistatin nearly abolished outward invasion into collagen-I. GM6001 produced partial inhibition; spindle-like protrusions remained visible but were fewer and shorter than those in untreated controls (**Figure 14 B**). These

findings are consistent with the reported activities of the inhibitors: CK-666 inhibits Arp2/3-dependent actin polymerization, blebbistatin inhibits myosin-II-dependent contractility, and GM6001 inhibits matrix metalloproteinases involved in extracellular-matrix remodeling. The persistence of some outgrowth after GM6001 treatment may reflect protease-independent migration, incomplete inhibition, or residual cell–matrix adhesion and contractility.

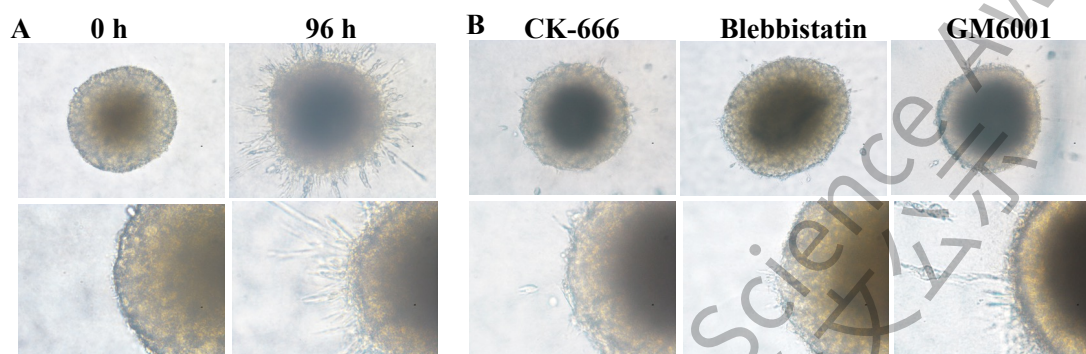


Figure 14. Spheroid behavior in collagen-I hydrogels with different cell movement inhibitors. Hydrogel mixed spheroids were added in non-adherent PDMS microchamber. (A) Microscope images of spheroids without inhibitors. (B) Microscope images of spheroids cultured with hydrogel with different inhibitors for 96 h.

The comparison between assay geometries demonstrates the importance of controlling spheroid position during invasion assays. In standard dishes, substrate-associated spreading persisted even when collagen-I invasion was strongly inhibited, potentially obscuring the specific effects of the inhibitors on three-dimensional invasion. The non-adherent PDMS microchamber reduced this confounding influence and enabled inhibitor effects on hydrogel-associated outgrowth to be evaluated more directly. Thus, fully embedded or suspended spheroids provide a more suitable model for distinguishing true three-dimensional invasion from migration along a rigid surface. The PDMS microchamber may be useful in future studies of matrix remodeling, cytoskeletal regulation, drug screening, and tumor-cell invasion under defined three-dimensional conditions. It could also be combined with live-cell imaging, fluorescent reporters, traction or matrix-deformation measurements, and molecular analyses to investigate invasion mechanisms. Nevertheless, substrate-associated 2.5D models remain useful when the objective is to study cell migration on or near a rigid interface. The two configurations should therefore be regarded as complementary models: the fully embedded format for evaluating matrix-associated three-dimensional invasion, and the 2.5D format for studying substrate-dependent spreading and migration.

Table 2

Inhibitor	Main target/process	Standard culture dish	Non-adherent PDMS microchamber	Interpretation
CK-666	Arp2/3-dependent branched-actin polymerization	Collagen-I invasion was strongly reduced, but lateral substrate spreading remained evident.	Outward invasion was nearly inhibited.	Arp2/3 activity contributes strongly to 3D protrusion formation, whereas residual 2.5D spreading may use alternative mechanisms.
Blebbistatin	Non-muscle myosin-II contractility	Both substrate spreading and collagen-I outgrowth were strongly reduced.	Outward invasion was nearly inhibited.	Myosin-II-dependent force generation is important for both matrix-associated invasion and substrate-associated migration.
GM6001	Matrix metalloproteinase activity	Both collagen-I outgrowth and substrate spreading were reduced but not completely abolished.	Spindle-like protrusions remained, but their number and length were significantly reduced.	MMP-dependent remodeling facilitates invasion, but residual movement may occur through protease-independent or incompletely inhibited mechanisms.

4. Conclusion

By reviewing published studies, this study found that the vertical position of the spheroid, especially whether it contacted the rigid culture surface, was often not clearly described. Then, a non-adherent PDMS microchamber treated with Pluronic F-127 was proposed. The modified microchamber reduced spheroid attachment to the culture

surface and allowed the spheroid position within the hydrogel. Compared with standard Petri-dish cultures, the microchamber reduced sheet-like, substrate-associated spreading. The non-adherent microchamber was applied for hydrogel-based spheroid invasion assay. The results showed that spheroids showed different behaviors in alginate, Matrigel, and collagen-I. Moreover, in the collagen-I based spheroid invasion assay, CK-666 and blebbistatin strongly inhibited outward invasion in the non-adherent microchamber, while GM6001 caused partial inhibition. These responses were easier to interpret when substrate-associated spreading was minimized. Overall, this study shows that a small experimental detail, the vertical position of a spheroid, can strongly affect the apparent invasion pattern and the interpretation of inhibitor responses. The non-adherent PDMS microchamber provides a simple and useful platform for studying three-dimensional tumor invasion more carefully. This project also demonstrates the importance of paying attention to overlooked details, questioning unexpected results, and designing experiments that directly test the proposed explanation.

Acknowledgement

This project began with the unexpected observation that spheroids in a Matrigel invasion assay did not always develop the same morphology, and that some results could not be reproduced consistently. We were frustrated because the same cell type and similar hydrogel conditions appeared to produce different invasion patterns. We asked that beyond treating such variations as experimental failure, was there the possibility for a small overlooked detail?

We reviewed published spheroid-invasion studies and found that the vertical position of the spheroid was often not clearly described. In particular, many studies did not explain whether spheroids remained suspended within the hydrogel or settled onto the rigid culture surface. This led us to propose the hypothesis that spheroid–substrate contact can change the apparent invasion pattern by promoting surface-associated spreading rather than true three-dimensional invasion.

To test this idea, we designed a non-adherent PDMS microchamber with side-view imaging. This system allowed us to reduce substrate attachment, maintain spheroids within the hydrogel, and directly observe their vertical position. We then compared spheroid behavior in standard culture dishes and microchambers using different hydrogels and invasion inhibitors.

This seemingly trivial experimental detail changed the interpretation of the entire essay. From this project, we learned to examine unexpected results carefully, question assumptions in published work, as well as recognize the fact that reliable science often depends on details that are easy to overlook.

In this project, Catherine Fang was responsible for literature gathering and statistical analyzation and the fabrication of Pluronic F-127@PDMS microcavity. Junzhu Yang was responsible for experimental designs. Both students collaborated to carry out the cellular experiments. Cathrine Fang was responsible for collecting images and Junzhu Yang was responsible for analyzing results and creating statistical graphs. For the paper, Catherine Fang was responsible for the methods and results and discussion and appendix sections. Junzhu Yang was responsible for the abstract, introduction, conclusion and reference sections.

We would like to express our sincere gratitude to Chongqing Yucai Secondary School for providing the experimental facilities and equipment for this project. We are especially grateful to Ms. Chen for providing valuable guidance throughout the research process. Her advice on academic writing and data analysis helped us overcome many difficulties encountered during the project. The experience not only supported the completion of this project but also allowed us to develop a deeper understanding of experimental design, scientific data analysis, and the importance of careful and reproducible research.

References

- Breslin, S., & O'Driscoll, L. (2013). Three-dimensional cell culture: The missing link in drug discovery. *Drug Discovery Today*, 18(5), 240–249. <https://doi.org/10.1016/j.drudis.2012.10.003>
- Chandekar, A., Laurel, S. R., Gupta, K., Lee, T., Wakida, N., & Hirschberg, H. (2026). Sonodynamic therapy inhibition of invasive glioma cells from tumor spheroids. *Photodiagnosis and Photodynamic Therapy*, 58, 105384. <https://doi.org/10.1016/j.pdpdt.2026.105384>
- Dogan, E., Galifi, C. A., Cecen, B., Shukla, R., Wood, T. L., & Miri, A. K. (2024). Extracellular matrix regulation of cell spheroid invasion in a 3D bioprinted solid tumor-on-a-chip. *Acta Biomaterialia*, 186, 156–166. <https://doi.org/10.1016/j.actbio.2024.07.040>
- Fischer-Friedrich, E., Hyman, A., Ju'licher, F., Mu'ller, D., & Helenius, J. (2014, August 29). Quantification of surface tension and internal pressure generated by single mitotic cells | *Scientific Reports*. <https://doi.org/10.1038/srep06213>
- Goli, S., Guduru, A. T., Karadagatla, S., Sreehari, E., Bhatnagar, I., Dandekar, M. P., Sharma, A., Bhatia, D., & Asthana, A. (2026). Generation of uniform-sized spheroids as 3D cancer models using simple and scalable PDMS-based microwell devices. *RSC Advances*, 16(6), 5680–5691. <https://doi.org/10.1039/d5ra07878d>
- Lim, G. J., Kang, S.-J., & Lee, J. Y. (2020). Novel invasion indices quantify the feed-forward facilitation of tumor invasion by macrophages. *Scientific Reports*, 10(1), 718. <https://doi.org/10.1038/s41598-020-57517-6>

- Majhy, B., Priyadarshini, P., & Sen, A. K. (2021). Effect of surface energy and roughness on cell adhesion and growth – facile surface modification for enhanced cell culture. *RSC Advances*, 11(25), 15467–15476. <https://doi.org/10.1039/d1ra02402g>
- Majidpoor, J., & Mortezaee, K. (2021). Steps in metastasis: An updated review. *Medical Oncology*, 38(1), 3. <https://doi.org/10.1007/s12032-020-01447-w>
- Ning, K., Xie, Y., Sun, W., Feng, L., Fang, C., Pan, R., Li, Y., & Yu, L. (2025). Non-destructive in situ monitoring of structural changes of 3D tumor spheroids during the formation, migration, and fusion process. *eLife*, 13, RP101886. <https://doi.org/10.7554/eLife.101886>
- Ntekoumes, D., Song, J., Liu, H., Amelung, C., Guan, Y., & Gerecht, S. (2025). Acute Three-Dimensional Hypoxia Regulates Angiogenesis. *Advanced Healthcare Materials*, 14(2), 2403860. <https://doi.org/10.1002/adhm.202403860>
- Park, J. Y., Yoo, S. J., Lee, E.-J., Lee, D. H., Kim, J. Y., & Lee, S.-H. (2010). Increased poly(dimethylsiloxane) stiffness improves viability and morphology of mouse fibroblast cells. *BioChip Journal*, 4(3), 230–236. <https://doi.org/10.1007/s13206-010-4311-9>
- Zhang, B., Li, Y., Wang, G., Jia, Z., Li, H., Peng, Q., & Gao, Y. (2018). Fabrication of agarose concave petridish for 3D-culture microarray method for spheroids formation of hepatic cells. *Journal of Materials Science: Materials in Medicine*, 29(5), 49. <https://doi.org/10.1007/s10856-018-6058-0>

Appendix: Hydrogel-based spheroid invasion assay - literature analysis

Table 1. Valid hydrogel-based spheroid invasion studies (n = 42)

Citation	Cell line	Spheroid formation method	Hydrogel (type / concentration)	Gelation mechanism	Culture vessel	Placement protocol	Fully embedded	Bottom-contact risk
Agrawal_2023	GFP-A549, SK-MES-1, HUVECs, normal human lung fibroblasts, human lung CAFs	U-bottom 96-well plate + 20% Methocel; centrifuged 350 rcf for 10 min	Collagen I 2 mg/mL (Layers 1–2); Fibrin (Layer 3: 6 mg/mL fibrinogen + thrombin)	Temperature (37°C)	4-well dish (Nunc, 10507591)	Layer 1 unpolymerised collagen gelled 30 min; spheroids in fresh unpolymerised collagen (Layer 2) poured over Layer 1 and gelled 60 min; Layer 3 (fibrin + stromal cells) poured on top	YES	1-Low
Ascione_2024	CT26 (mouse colon carcinoma)	Agarose-coated 48-well plate (non-adhesive concave surface, classical agarose protocol)	Collagen I, 2 mg/mL	Temperature (RT; 20 min to 50 % polymerization + 40 min complete)	Custom chemotaxis chamber (metal block on glass slide, porous membrane)	170 µL bottom collagen layer half-polymerized; 200 µL collagen containing ~10 spheroids poured on top; gravity sedimentation to interface between layers; complete polymerization 40 min	YES	1-Low
Breideband_2025	MCF-7, MDA-MB-231	Sphericalplate 5D (ULA microwell)	GelMA/PEGDA (3%, 5%, or 7% GelMA w/v + 3% PEGDA, 0.2% LAP, 0.003% tartrazine)	UV photopolymerization (SLA bioprinting, 405 nm)	96-well imaging plate (FEP-foil bottom, zell-kontakt) for bioprinting; modified 5 mL serological pipettes for 7-day culture	Spheroids resuspended in viscous, light-sensitive bioink, pipetted into 96-well vat, and crosslinked layer-by-layer via SLA; printed hydrogel constructs washed and transferred to clinostat vessels	YES	1-Low
Cai_2024	MCF10A, MDA-MB-231, MCF7, MCF10A shCDH1 KD	Inverse pyramidal PDMS microwells (AggreWell)	Collagen-alginate: Collagen I 3 mg/mL, alginate 0.25%	Temperature (37°C, 1 h for collagen); ionic (CaCl ₂ for alginate crosslinking)	6-well plate with central hole, glass slide adhered beneath	Harvested from microwells and "encapsulated" in collagen-alginate hydrogel; incubated at 37°C for 1 h for collagen polymerization (day 0)	UNCLEAR	2-Moderate

Citation	Cell line	Spheroid formation method	Hydrogel (type / concentration)	Gelation mechanism	Culture vessel	Placement protocol	Fully embedded	Bottom-contact risk
Cisneros Castillo_2016	U87MG (human glioblastoma)	Manual hanging drop; GravityPLUS hanging drop; or Spheroid Microplates (ULA 96-well flat-bottom, Corning)	PureCol bovine collagen type I, 2.2 mg/mL	Temperature (37°C, 20 min)	24-well plate	Spheroids implanted in center of well in 400 µL collagen mixture; incubated 20 min to solidify; no anti-settling step described	UNCLEAR	2-Moderate
Chandekar_2026	F98 (rat glioma), BT4C	Ultra-low attachment 96-well round-bottom plates + centrifugation (500 g, 10 min)	Matrigel (40 % v/v base layer; Fig. 1 indicates 20 % v/v top encapsulation layer)	Temperature (37 °C)	Petri dish (format not specified)	Base layer gelled 18 h; spheroids transferred onto pre-gelled base; incubated 2 h at 37 °C to allow initial adherence; top Matrigel layer pipetted over spheroids; gelled 4 h	NO	3-High
Chen_2022	SKOV-3	Agarose-coated honeycomb microframe patch (microfabricated support)	Type I collagen membrane (0.5 mg/mL) on ECM-patch (thin membrane, not bulk hydrogel)	Air drying at RT (drop-cast and dried)	Patch (1.3 cm diameter) transferred to culture dish	Pre-formed spheroids recovered and seeded onto the surface of a pre-formed ECM-patch coated with dried collagen membrane	NO	3-High
Cramer_2026	Panc10.05, hT231, WM983b, WM164, WM793, Human Dermal Fibroblasts (multiple lines)	Liquid overlay with agarose	Collagen I (rat tail) 1.5 mg/mL	Temperature (37°C)	24-well plate (Greiner Bio-One 662892)	Spheroids resuspended in 300 µL cold collagen mix, dispensed dropwise onto a pre-polymerized 300 µL collagen base layer per well; polymerized at 37°C for 15–45 min	UNCLEAR	2-Moderate
Crawford_2024	MDA-MB-231 (E-cad- and E-cad+)	Oil-in-water droplet microtechnology (collagen droplet gelled in oil)	Collagen I, 1–6 mg/mL	Temperature (37°C)	96-well glass bottom plate (Cellvis P96–1.5H–N)	Pre-gelled cores resuspended in liquid bulk collagen; 100 µL mixture plated into preheated wells and incubated at 37°C for 1 h to gel.	UNCLEAR	2-Moderate
Cui_2023	U87, LN229, primary human astrocytes (NHA)	ULA plate (Nunclon Sphera 96 U-well microplate)	Col-HA: Collagen I 1 mg/mL, hyaluronic acid 2 mg/mL	Temperature (37°C, 1 h)	96-well U-bottom plate (Nunclon Sphera)	Spheroids cultured in wells for 3 days; 150 µL medium removed, 50 µL Col-HA gel added; plate gently tapped to mix with residual medium; gelled 1 h at 37°C	NO	3-High

Citation	Cell line	Spheroid formation method	Hydrogel (type / concentration)	Gelation mechanism	Culture vessel	Placement protocol	Fully embedded	Bottom-contact risk
Dogan_2024	HT1080 (human fibrosarcoma)	ULA round-bottom 96-well plate (Corning) + centrifugation (2500 rpm/850×g, 5 min)	GelMA (gelatin methacryloyl), 5 %, 7 %, 10 % w/v	Thermal (5 min at 37°C) + UV (365 nm, 3 or 6 min, 10 mW/cm ²)	Custom bioprinted chip (sacrificial mold, ~1.5 mm thick, two parallel channels)	Spheroids mixed into GelMA bioink (50 spheroids/mL), injected into sacrificial mold, 5 min thermal gelation at 37°C, then UV crosslinking	YES	1-Low
Ducker_2020	Primary mouse RMS neuroblasts (P3–P5 CD1 WT)	ULA round-bottom plate (Greiner 650970, 96-well)	Matrigel 50% (v/v) in ice-cold NB-A+ medium (~4–5 mg/mL, estimated)	Temperature (37°C incubator)	96-well ULA round-bottom plate	Spheroids formed overnight at the well bottom; Matrigel (25 µL) trickled down the side of each well, plate returned to ice for Matrigel to settle to the bottom, warmed 3 min at RT, then moved to 37°C to solidify	NO	3-High
Feng_2025	DU 145, MCF-7, EMT-6	Agarose micro-well array (non-adherent; 2 mm diameter × 2 mm height)	Matrigel (1:10 dilution in DMEM)	Temperature (37°C, 0.5 h)	96-well flat-bottom microplate	Spheroids gently pipetted onto Matrigel-coated wells after Matrigel had gelled at 37°C for 0.5 h.	NO	3-High
Hey_2025	H1299-GFP (NSCLC); primary human macrophages	ULA plate (96-well ultra-low adhesion)	Collagen I 2.5 mg/mL (rat tail)	Temperature (37°C, 5% CO ₂ , 30 min)	15-well imaging chamber (glass bottom, inferred)	Spheroid transferred to the bottom of the imaging chamber by surface tension; residual DPBS removed; 40 µL collagen I/macrophage mix added swiftly on top; polymerized 30 min at 37°C	NO	3-High
Hirak_2023	MCF10A (LifeAct-GFP)	Inverse pyramidal PDMS microwells (AggreWell)	GelMA 5% w/v with DexVS fiber segments (1–3 vol%); LAP photoinitiator 0.15–0.3 mg/mL	UV (5 mW/cm ² , 60 s)	Not explicitly stated (glass coverslips/plates implied for microscopy)	Spheroids and fiber segments mixed into GelMA precursor, oxygen-purged 10 s, then photoencapsulated under UV light for 60 s	UNCLEAR	2-Moderate
Hong_2026	H2228 (ALK-rearranged NSCLC) and resistant derivatives H2228-CR1, H2228-CR2	ULA round-bottom 96-well plate (PrimeSurface 96U, Sumitomo Bakelite)	Matrigel (Corning, 356234) or Rat-tail collagen type I (3 mg/mL, Corning, 354236)	Temperature (standard culture conditions)	PrimeSurface 96U plate (formation); 3D embedding vessel not explicitly stated	Medium removed; spheroids “embedded in” Matrigel or collagen; exact timing/mixing not specified	UNCLEAR	2-Moderate

Citation	Cell line	Spheroid formation method	Hydrogel (type / concentration)	Gelation mechanism	Culture vessel	Placement protocol	Fully embedded	Bottom-contact risk
Iwata_2025	HEK293; MDA-MB-231; PANC-1	Hanging drop (preformed spheroids); single-cell suspension in Geltrex	Geltrex 10 mg/mL; Collagen I 3 mg/mL (rat tail)	Temperature (37°C; 30 min for Geltrex, 60 min for collagen)	24-well plate (Sigmacote-coated for Geltrex); 8-well chamber for single cells	Spheroids washed and resuspended in hydrogel precursor (1 spheroid/ μ L); 50 μ L droplet placed in well and incubated at 37°C to solidify; then 1 mL culture medium added	UNCLEAR	2-Moderate
Khawar_2018	Huh-7, LX-2	Liquid overlay in 96-well ultra-low-attachment (ULA) plate	Matrigel (100 μ L/well; concentration not stated) or Collagen I (2 mg/mL)	Temperature (37°C, 1 h)	96-well plate	5-day-old spheroids placed on the pre-gelled matrix surface.	NO	3-High
Kim_2022	Human adipose-derived stem cells (ASCs), HUVECs	Temperature-responsive hydrogel (Tetronic) with surface microwells; centrifuged 250 $\times$ g for 10 min	Matrigel 10 mg/mL (bottom and top layers)	Temperature (37°C, 30 min)	24-well plate	Bottom Matrigel layer pre-gelled; spheroids transferred from microwell hydrogel onto bottom layer; top Matrigel layer added and gelled	YES	1-Low
Kopanska_2016	CT26-GFP (murine colon carcinoma)	Agarose cushion protocol in 48-well plate; incubated 3 days	Collagen type I 2 mg/mL	Temperature (room temperature $\sim$ 22°C, 1 h)	Fluorodish chambers (35 mm glass bottom)	20 μ L collagen drop placed on dish; spheroid inserted into drop with 10 μ L additional collagen; dish flipped up and down several times to center spheroid; polymerized 1 h at RT	UNCLEAR	2-Moderate
Lim_2020	HT1080, LLC1, Raw264.7	Hanging drop (with 0.24% methylcellulose)	Matrigel (phenol red-free; concentration not reported)	Temperature (40 min first layer + 30 min second layer in incubator)	24-well plate with PDMS gasket (5 mm hole, 2.5 mm height)	Sandwich method: 25 μ L Matrigel cast and solidified; spheroid placed on first layer; 25 μ L Matrigel added on top; solidified 30 min	YES	1-Low
Liu_2018	MDA-MB-231, human mammary fibroblasts (HMF)	Low-adhesion 384-well plates (per cited protocol [34, 53])	Collagen/Alginate hybrid (Collagen I 3 mg/mL, Alginate 5 mg/mL, CaCl ₂ 7.5 mM)	Ionic (CaCl ₂ crosslinking of alginate) + temperature (37°C, 20 min)	Not explicitly stated (cylindrical gels 3 mm $\times$ 1 mm for characterization; multi-	Spheroids mixed into collagen/alginate/CaCl ₂ precursor and cured at 37°C; no anti-settling step described	UNCLEAR	2-Moderate

Citation	Cell line	Spheroid formation method	Hydrogel (type / concentration)	Gelation mechanism	Culture vessel	Placement protocol	Fully embedded	Bottom-contact risk
					well plate inferred for culture)			
Liu_2020	BLM, M14, MEL57, SW480, CACO2, LLC	Microcarrier beads (Cytodex) in round-bottom tubes/plates	Collagen I (1.4–2.3 mg/mL, typically 1.6 mg/mL), Matrigel (5 mg/mL), or Agar (0.3%)	Temperature (collagen/Matrigel at 37°C; agar at RT)	Multi-well plate (24-well or 96-well, not explicitly stated)	Spheroid suspension mixed with hydrogel precursor, dispensed into wells, and incubated until gelled; authors explicitly note that spheroids frequently settle to the well bottom before gelling	NO	3-High
Liu_2022	A549 (VIM RFP), MCF7, MDA-MB-231	Microbuckets (concave microchambers from PDMS microfluidic device)	dexMA (3.5 or 5 wt%) or gelMA (3 or 5 wt%)	UV (365 nm, 5–10 s)	8-well culture plate	Microbuckets placed in plate; cells loaded overnight; medium removed; 200 µL hydrogel precursor added to cover microbuckets; UV crosslinked 5–10 s	UNCLEAR	2-Moderate
Mehta_2026	MCF7, 19TT CAFs	ULA 96-well microcavity plates (separate spheroids); hanging drop (heterospheroids)	Collagen I, 3 mg/mL (rat-tail)	Temperature (handling on ice/4°C, then 37°C to gel)	ibidi 8-well glass-bottom slide	Thin collagen bed (100 µL) pre-gelled first; spheroids mixed with fresh collagen precursor and layered on top of the bed, then incubated to gel	UNCLEAR	2-Moderate
Monteiro_2020	hBM-MSC, MG-63, SaOS-2, A549	ULA 96-well round-bottom plate	PLMA 10/15/20 % (w/v); PEGDA 10% (w/v); Matrigel (undiluted)	UV photopolymerization (PLMA/PEGDA); 37 °C (Matrigel)	µ-Slide Angiogenesis (ibidi)	First gel layer (5 µL) UV-polymerized in well; spheroid deposited on top; second layer (5 µL) UV-polymerized over spheroid (sandwich)	YES	1-Low
Mungai_2024	Porcine VIC, NHF, rat SMC, human RPE-1, human PC9	Non-adherent agarose microwells (500 µm diameter)	Collagen I (rat tail), 2 mg/mL	Temperature (37°C, 30 min)	6-well tissue culture plate or CellScale MechanoCulture FX™ silicone well plate (8 mm × 8 mm wells)	Spheroids mixed into cooled neutralized collagen solution (~20 spheroids/mL), poured into well plates, and allowed to gel at 37°C for 30 min.	UNCLEAR	2-Moderate
Namaqi_2024	HCT-116-GFP	Agarose-coated 96-well plate (liquid overlay)	Collagen I 1.8 mg/mL; Matrigel 3 mg/mL	Temperature (RT 30 min for collagen; 37	Polycarbonate cultivation chamber (glass bottom,	Spheroids mixed into liquid collagen I (120 µL) and pipetted into chamber well; gelled at RT for 30 min	NO	3-High

Citation	Cell line	Spheroid formation method	Hydrogel (type / concentration)	Gelation mechanism	Culture vessel	Placement protocol	Fully embedded	Bottom-contact risk
				°C 30 min for Matrigel)	pHEMA-coated) or 6-well plate			
Nazari_2020	MCF10A, MDA-231BO	ULA plate (Costar ultra-low-attachment U-bottom 96-well)	Collagen I 4 mg/mL (rat tail); Matrigel 5% v/v for basement membrane assembly	Temperature (37°C, 30 min)	MatTek dish (35 mm, No.1.5 coverslip, 20-mm opening)	Spheroids washed with HBSS, aspirated into a cut pipette tip, expelled into ice-cold neutralized collagen, and 200 µL of the collagen-spheroid mixture transferred to a cooled MatTek dish; gel polymerized at 37°C for 30 min	UNCLEAR	2-Moderate
Ngo_2013	MDA-MB-231 (breast cancer); melanoma (supplementary)	Microwells (4-day culture)	Rat tail type I collagen (concentration not reported)	Not reported	Not reported (glass or PDMS substrate)	Spheroids generated in microwells, then "embedded within" collagen scaffolds over glass/PDMS substrates	NO	3-High
Ntekoumes_2025	ECFCs (endothelial colony forming cells)	Hanging droplet method	Gel-Dex: gelatin-ferulic acid (4 wt%) + aldehyde-modified dextran (0.5 wt%)	Chemical crosslinking (imine bonds), RT for 30 min	96-well plate	Spheroids mixed into Gel-Dex precursor (65 µL) and pipetted into wells; allowed to polymerize at RT for 30 min	UNCLEAR	2-Moderate
Pandey_2024	MDA-MB-231, MCF10A	Microwell array in agarose gel (soft lithography)	Collagen I 3.5 mg/mL	Temperature (37 °C 30 min, fast warming)	24-well plate with PDMS microwell membrane (115 µm deep) at base	Spheroid-embedded collagen (2.5 µL) pipetted into PDMS microwells and polymerized at 37 °C	NO	3-High
Piwocka_2025	BT474, T47D, MDA-MB-231, SK-BR-3 (+ CAFs, THP-1, Ea.hy926)	ULA 96-well plate	30 % Matrigel (v/v) in additive-free DMEM/F12	Temperature (37 °C 30 min)	ULA 96-well plate (same as formation)	Pre-formed spheroids at well bottom; medium removed; 100 µL 30 % Matrigel added on top	NO	3-High
Rizzo_2023	HCT116	ULA T75 flask	Chitosan-pectin (Ch/Pec) with β-glycerophosphate (concentration not reported)	Temperature (37°C, thermoresponsive sol-gel transition)	8-well chamber slide (Ibidi 80827) or 4-well (80427)	Spheroids and sensors dispersed in medium, mixed with Ch/Pec hydrogel (3:1.5 ratio) at RT; 30–50 µL deposited per well; gelled at 37°C for 20 min	UNCLEAR	2-Moderate

Citation	Cell line	Spheroid formation method	Hydrogel (type / concentration)	Gelation mechanism	Culture vessel	Placement protocol	Fully embedded	Bottom-contact risk
Rossi_2026	MG63, 143B, WS1 fibroblasts, BM-MSCs	ULA microcavity plate (Corning Elplasia 24-well)	VitroGel®RGD (diluted 1:1 with Dilution Solution Type I; concentration not reported)	Not reported	U-bottom 96-well plate	Spheroids + fibroblasts centrifuged, resuspended in medium (100 µL), mixed with VitroGel RGD (200 µL); dispensed dropwise into U-bottom 96-well plates containing medium; bead formation confirmed after 15 min	UNCLEAR	2-Moderate
Song_2023	12Z, iEc-ESC, iHUF, LP9, THP-1	Nonadherent 96-well agarose micro-molds (400 µm diameter, 1.5% agarose)	Matrigel (Corning 536232; concentration not stated) mixed 2:1 with LP9 cells	Temperature (37°C, 3–4 h)	96-well plate	Matrigel-LP9 layer pre-gelled and solidified; day 4 endometriotic spheroids harvested and seeded on top of the solidified layer	NO	3-High
Staneva_2018	CT26 (mouse intestinal adenocarcinoma)	Agarose-coated 48-well plate (0.01 g/mL agarose, 150 µL/well)	Rat tail collagen I, 2 mg/mL	Temperature (RT, 15 min polymerization before media addition)	Custom tissue culture plates (30 mm ²) with ~3–4 mm holes; 48-well plate for spheroid formation	Spheroids in 15-µL collagen drops positioned in holes; plate flipped every 30 s for 5 min to keep spheroids in center and prevent sedimentation to glass; polymerized 15 min at RT	YES	1-Low
van Bodegraven_2026	U251-MG	Low-adherent T25 culture flasks (spontaneous aggregation; alternative U-shaped 96-well plate)	Matrigel (undiluted; mg/mL not stated)	Temperature (37 °C, 45 min)	Glass-bottom 35 mm dish (Ibidi)	Spheroids mixed into Matrigel precursor (15 µL Matrigel + 5 µL spheroid suspension), pipetted as 20 µL dome, polymerized at 37 °C	UNCLEAR	2-Moderate
van der Net_2024	MV3 (melanoma), A549 (lung carcinoma)	Corning Elplasia 96-well ULA plate (500 cells/microwell)	Bovine collagen type I (2.4 mg/mL, 8 mg/mL) or GelMA (30 mg/mL, 50 mg/mL)	Collagen: temperature (37°C, 45 min); GelMA: UV (365 nm, 45 s)	m-Slide 8-well chambers (ibidi)	Collagen: sandwich protocol — 80 µL bottom layer polymerized, spheroids transferred onto it, 30 min attachment, 100 µL top layer added and polymerized. GelMA: spheroids mixed into 125 µL GelMA suspension and UV crosslinked.	YES	1-Low
Vinci_2015	U-87MG, CALS, CALR	ULA 96-well round-bottom plate	BMM (Matrigel), undiluted (concentration not specified)	Temperature (37 °C incubation to solidify)	ULA 96-well round-bottom plate (same as formation)	Pre-formed spheroids at well bottom; 100 µL BMM added; plate centrifuged at 300×g for 3 min if needed to center; gelled at 37 °C	NO	3-High

Citation	Cell line	Spheroid formation method	Hydrogel (type / concentration)	Gelation mechanism	Culture vessel	Placement protocol	Fully embedded	Bottom-contact risk
Zhao_2019	MCF-7, MDA-MB-231, HT-1080, HCT-116	3D-printed hanging drop dripper (3D-phd)	Collagen type I 3 mg/mL (for metastasis assay)	Temperature (37 °C, 2 h gelling)	96-well plate	For 3D metastasis: 60 µL collagen pipetted into device, spheroid-containing hanging drop falls into bottom well; plate incubated 2 h for gelling. For TEM: spheroids pipetted onto bottom endothelium layer with collagen, centrifuged 2000 rpm 5 min, gelled 2 h	NO	3-High
Zuo_2020	ECs, SMCs, EC-SMC co-culture	Round-bottom 96-well plate (Pluronic F-127 coated)	HA-MA/Fg composites: HA-MA 4 % (w/v) + Fg 15 % (w/v) at ratios 1:3, 1:1, 3:1	Temperature (37 °C 10 min for Fg/thrombin) then UV (365 nm, 2 min for HA-MA)	48-well plate	Spheroids mixed into Fg/HA-MA/thrombin precursor in well, stirred 1 min, gelled at 37 °C then UV	UNCLEAR	2-Moderate

Legend

GREEN = YES / 1-Low (anti-settling confirmed); YELLOW = UNCLEAR / 2-Moderate; RED = NO / 3-High (bottom contact confirmed)

Bottom-contact risk score: 1 = explicit anti-settling (ice, inversion, rapid crosslink, sandwich); 2 = mixed into precursor, no anti-settling mentioned; 3 = placed on surface or settled to bottom.

Reference for Table 1

- Agrawal, A., S. Lasli, Y. Javanmardi, D. Coursier, A. Micalet, S. Watson, S. Shahreza, B. Serwinski, B. Djordjevic, N. Szita, U. Cheema, S. Bertazzo, F. Calvo and E. Moeendarbary (2023). "Stromal cells regulate mechanics of tumour spheroid." Materials Today Bio **23**: 100821.
- Ascione, F., R. Ferraro, P. Dogra, V. Cristini, S. Guido and S. Caserta (2024). "Gradient-induced instability in tumour spheroids unveils the impact of microenvironmental nutrient changes." Scientific Reports **14**(1): 20837.
- Breideband, L., K. N. Wächtershäuser, R. Sarkar, M. Puspathasan, E. H. K. Stelzer and F. Pampaloni (2025). "Gravitational forces and matrix stiffness modulate the invasiveness of breast cancer cells in bioprinted spheroids." Materials Today Bio **31**: 101640.

- Cai, G., X. Li, S.-S. Lin, S. J. Chen, N. C. Rodgers, K. M. Koning, D. Bi and A. P. Liu (2024). "Matrix confinement modulates 3D spheroid sorting and burst-like collective migration." Acta Biomaterialia **179**: 192-206.
- Cisneros Castillo, L. R., A.-D. Oancea, C. Stüllein and A. Régnier-Vigouroux (2016). "Evaluation of Consistency in Spheroid Invasion Assays." Scientific Reports **6**(1): 28375.
- Chandekar, A., S. R. Laurel, K. Gupta, T. Lee, N. Wakida and H. Hirschberg (2026). "Sonodynamic therapy inhibition of invasive glioma cells from tumor spheroids." Photodiagnosis and Photodynamic Therapy **58**: 105384.
- Chen, C., Y. He, E. Lopez, F. Carreiras, A. Yamada, M.-C. Schanne-Klein, A. Lambert, Y. Chen and C. Aimé (2022). "High-throughput tuning of ovarian cancer spheroids for on-chip invasion assays." Micro and Nano Engineering **15**: 100138.
- Cramer, E. M., T. Lopez-Vidal, V. Wang, J. Johnson, D. R. Bergman, A. T. Weeraratna, E. J. Fertig, L. M. Heiser, Y. H. Chang and J. W. Zimmerman (2026). "Protocol for generation, time-course imaging, and automated quality control of 3D spheroid invasion using TRACEQC." STAR Protocols **7**(2).
- Crawford, A. J., C. Gomez-Cruz, G. C. Russo, W. Huang, I. Bhorkar, T. Roy, A. Muñoz-Barrutia, D. Wirtz and D. García-Gonzalez (2024). "Tumor proliferation and invasion are intrinsically coupled and unraveled through tunable spheroid and physics-based models." Acta Biomaterialia **175**: 170-185.
- Cui, Y., P. Lee, J. J. Reardon, A. Wang, S. Lynch, J. J. Otero, G. Sizemore and J. O. Winter (2023). "Evaluating glioblastoma tumour sphere growth and migration in interaction with astrocytes using 3D collagen-hyaluronic acid hydrogels." Journal of Materials Chemistry B **11**(24): 5442-5459.
- Dogan, E., C. A. Galifi, B. Cecen, R. Shukla, T. L. Wood and A. K. Miri (2024). "Extracellular matrix regulation of cell spheroid invasion in a 3D bioprinted solid tumor-on-a-chip." Acta Biomaterialia **186**: 156-166.
- Ducker, M., V. Millar, D. Ebner and F. G. Szele (2020). "A Semi-automated and Scalable 3D Spheroid Assay to Study Neuroblast Migration." Stem Cell Reports **15**(3): 789-802.
- Feng, L., R. Pan, K. Ning, W. Sun, Y. Chen, Y. Xie, M. Wang, Y. Li and L. Yu (2025). "The impact of 3D tumor spheroid maturity on cell migration and invasion dynamics." Biochemical Engineering Journal **213**: 109567.
- Hey, S. and S. Linder (2025). "A Macrophage-Tumor Spheroid Co-Invasion Assay." JoVE(215): e67374.
- Hiraki, H. L., D. L. Matera, W. Y. Wang, E. S. Prabhu, Z. Zhang, F. Midekssa, A. E. Argento, J. M. Buschhaus, B. A. Humphries, G. D. Luker, A. Pena-Francesch and B. M. Baker (2023). "Fiber density and matrix stiffness modulate distinct cell migration modes in a 3D stroma mimetic composite hydrogel." Acta Biomaterialia **163**: 378-391.
- Hong, D., H. J. Kwon, J. Jeong, S. Yoon, J.-H. Chung and S. Jeong (2026). "E-cadherin-driven adherens junction reinforcement promotes spheroid-mediated invasion and progression in ALK-rearranged lung cancer chemoresistance." Molecules and Cells **49**(4): 100329.
- Iwata, W., Y. Adachi, J. J. West, A. J. Ewald, Y. Zheng, L. D. Wood, R. A. Anders, H. Sesaki and M. Iijima (2025). "Context-dependent inhibitory roles of RhoA in 3D invasive cell migration within the extracellular matrix." Cell Reports **44**(12).
- Khawar, I. A., J. K. Park, E. S. Jung, M. A. Lee, S. Chang and H.-J. Kuh (2018). "Three Dimensional Mixed-Cell Spheroids Mimic Stroma-Mediated Chemoresistance and Invasive Migration in hepatocellular carcinoma." Neoplasia **20**(8): 800-812.
- Kim, S.-j., H. Byun, S. Lee, E. Kim, G. M. Lee, S. J. Huh, J. Joo and H. Shin (2022). "Spatially arranged encapsulation of stem cell spheroids within hydrogels for the regulation of spheroid fusion and cell migration." Acta Biomaterialia **142**: 60-72.

- Kopanska, K. S., Y. Alcheikh, R. Staneva, D. Vignjevic and T. Betz (2016). "Tensile Forces Originating from Cancer Spheroids Facilitate Tumor Invasion." PLOS ONE **11**(6): e0156442.
- Lim, G. J., S.-J. Kang and J. Y. Lee (2020). "Novel invasion indices quantify the feed-forward facilitation of tumor invasion by macrophages." Scientific Reports **10**(1): 718.
- Liu, C., D. Lewin Mejia, B. Chiang, K. E. Luker and G. D. Luker (2018). "Hybrid collagen alginate hydrogel as a platform for 3D tumor spheroid invasion." Acta Biomaterialia **75**: 213-225.
- Liu, H., T. Lu, G.-J. Kremers, A. L. B. Seynhaeve and T. L. M. ten Hagen (2020). "A microcarrier-based spheroid 3D invasion assay to monitor dynamic cell movement in extracellular matrix." Biological Procedures Online **22**(1): 3.
- Liu, Q., A. Muralidharan, A. Saatchi, Z. Ding, P. ten Dijke and P. E. Boukany (2022). "A Programmable Multifunctional 3D Cancer Cell Invasion Micro Platform." Small **18**(20): 2107757.
- Mehta, P., A. D. Bordoloi, C. Ravensbergen, M. K. H. David, W. Mesker, G. J. Liefers, P. ten Dijke and P. E. Boukany (2026). "Inter-spheroid proximity and matrix remodeling determine cancer associated fibroblast mediated cancer cell invasion." Acta Biomaterialia **209**: 350-361.
- Monteiro, C. F., S. C. Santos, C. A. Custódio and J. F. Mano (2020). "Human Platelet Lysates-Based Hydrogels: A Novel Personalized 3D Platform for Spheroid Invasion Assessment." Advanced Science **7**(7): 1902398.
- Mungai, R. W., R. J. Hartman, G. E. Jolin, K. W. Piskorowski and K. L. Billiar (2024). "Towards a more objective and high-throughput spheroid invasion assay quantification method." Scientific Reports **14**(1): 31007.
- Nazari, S. S. (2020). "Generation of 3D Tumor Spheroids with Encapsulating Basement Membranes for Invasion Studies." Current Protocols in Cell Biology **87**(1): e105.
- Namaqi, M., F. Moll, S. Wiedemeier, A. Grodrian and K. Lemke (2024). "Dynamic cell culture modulates colon cancer cell migration in a novel 3D cell culture system." Scientific Reports **14**(1): 18851.
- Nazari, S. S. (2020). "Generation of 3D Tumor Spheroids with Encapsulating Basement Membranes for Invasion Studies." Current Protocols in Cell Biology **87**(1): e105.
- Ngo, T. K. N., S. J. Yang, B.-H. Mao, T. K. M. Nguyen, Q. D. Ng, Y.-L. Kuo, J.-H. Tsai, S. N. Saw and T.-Y. Tu (2023). "A deep learning-based pipeline for analyzing the influences of interfacial mechanochemical microenvironments on spheroid invasion using differential interference contrast microscopic images." Materials Today Bio **23**: 100820.
- Ntekoumes, D., J. Song, H. Liu, C. Amelung, Y. Guan and S. Gerecht (2025). "Acute Three-Dimensional Hypoxia Regulates Angiogenesis." Advanced Healthcare Materials **14**(2): 2403860.
- Pandey, M., Y. J. Suh, M. Kim, H. J. Davis, J. E. Segall and M. Wu (2024). "Mechanical compression regulates tumor spheroid invasion into a 3D collagen matrix." Physical Biology **21**(3): 036003.
- Piwocka, O., K. Sterzyńska, A. Malińska, W. M. Suchorska and K. Kulcenty (2025). "Development of tetraculture spheroids as a versatile 3D model for personalized breast cancer research." Scientific Reports **15**(1): 27449.
- Rizzo, R., V. Onesto, G. Morello, H. Iuele, F. Scalera, S. Forciniti, G. Gigli, A. Polini, F. Gervaso and L. L. del Mercato (2023). "pH-sensing hybrid hydrogels for non-invasive metabolism monitoring in tumor spheroids." Materials Today Bio **20**: 100655.

- Rossi, M., L. Anconelli, M. M. Rydzyk, A. Crea, F. Rossi, E. Coschina, G. Ravegnini, G. Ulian, G. Valdrè, M. Montesi, D. Gardini, C. Cappadone, P. Blasi, S. Iotti and E. Malucelli (2026). "Modeling osteosarcoma invasion with fibroblast and tumor spheroids in functionalized hydrogels beads." *Biomedicine & Pharmacotherapy* **200**: 119485.
- Song, Y., G. W. Burns, N. R. Joshi, R. Arora, J. J. Kim and A. T. Fazleabas (2023). "Spheroids as a model for endometriotic lesions." *JCI Insight* **8**(11): e16081.
- Staneva, R., F. Burla, H. Koenderink Gijsje, S. Descroix, M. Vignjevic Danijela, Y. Attieh, M. Verhulsel and M. Théry (2018). "A new biomimetic assay reveals the temporal role of matrix stiffening in cancer cell invasion." *Molecular Biology of the Cell* **29**(25): 2979-2988.
- van Bodegraven, E. J., Y. Kesenci, J.-C. Olivo-Marin, A. Boquet-Pujadas and S. Etienne-Manneville (2026). "Protocol to quantify glioblastoma cell invasion and nuclear deformations in 3D hydrogels." *STAR Protocols* **7**(3): 104632.
- van der Net, A., Z. Rahman, A. D. Bordoloi, I. Muntz, P. ten Dijke, P. E. Boukany and G. H. Koenderink (2024). "EMT-related cell-matrix interactions are linked to states of cell unjamming in cancer spheroid invasion." *iScience* **27**(12):111424.
- Vinci, M., C. Box and S. A. Eccles (2015). "Three-Dimensional (3D) Tumor Spheroid Invasion Assay." *JoVE*(99): e52686.
- Zhao, L., J. Xiu, Y. Liu, T. Zhang, W. Pan, X. Zheng and X. Zhang (2019). "A 3D Printed Hanging Drop Dropper for Tumor Spheroids Analysis Without Recovery." *Scientific Reports* **9**(1): 19717.
- Zuo, X., H. Zhang, T. Zhou, Y. Duan, H. Shou, S. Yu and C. Gao (2020) "Spheroids of Endothelial Cells and Vascular Smooth Muscle Cells Promote Cell Migration in Hyaluronic Acid and Fibrinogen Composite Hydrogels." *Research* **2020**: 8970480

Table 2. Excluded studies (n = 10)

Citation	Cell line	Hydrogel (type / concentration)	Fully embedded	Batch	Exclusion reason
Du_2025	N/A (review article)	—	—	Batch_6	Systematic review
Grimm_2025	N/A (review article; discusses A549, MCF-7, PC-3, FTC-133, etc.)	—	—	Batch_6	Systematic review
Micalet_2021	N/A (systematic review)	—	—	Batch_1	Systematic review
Motta_2026	HCC1937; MDA-MB-231; A431; MLS; human dermal fibroblasts	N/A (scaffold-free spheroids, no hydrogel embedding)	—	Batch_2	No hydrogel / scaffold-free
Ninomiya_2024	MCF-7/GFP; HNDF; HUVEC	N/A (scaffold-free assembly on microneedles, no hydrogel)	—	Batch_2	No hydrogel / scaffold-free
Stellato_2025	Saos-2, human MSCs, 143B	N/A (spheroids cultured in suspension; Matrigel used only for single-cell volume assay, not spheroid invasion)	—	Batch_6	No hydrogel / scaffold-free

Citation	Cell line	Hydrogel (type / concentration)	Fully embedded	Batch	Exclusion reason
Sun_2026	MCF-7	N/A (no hydrogel)	NO	Batch_5	No hydrogel / scaffold-free
Tang_2026	AC-1M88 (trophoblast), Ishikawa (endometrial)	N/A (monolayer invasion into Ishikawa epithelium; no hydrogel)	NO	Batch_8	No hydrogel / scaffold-free
Tatsumi_2026	Mouse iPSC (mGF-iPS-3F-3)	—	—	Batch_7	No hydrogel invasion assay
Wu_2025	N/A (review)	—	—	Batch_5	Systematic review

Reference for Table 2

- Du, W., P. R. Nair, A. Forjaz, J. M. Phillip, P.-H. Wu and D. Wirtz (2026). "Selecting the optimal cell migration assay: fundamentals and practical guidelines." Nature Methods **23**(1): 30-42.
- Grimm, D., T. J. Corydon, J. Sahana, L. F. González-Torres, A. Kraus, S. Marchal, P. M. Wise, U. Simonsen and M. Krüger (2025). "Recent studies of the effects of microgravity on cancer cells and the development of 3D multicellular cancer spheroids." Stem Cells Translational Medicine **14**(3): szaf008.
- Micalet, A., E. Moeendarbary and U. Cheema (2021). "3D In Vitro Models for Investigating the Role of Stiffness in Cancer Invasion." ACS Biomaterials Science & Engineering **9**(7): 3729-3741.
- Motta, A., R. Daware, A. Nucci, S. Breuel, S. von Stillfried, J. Maurer, P. Boor, D. Jonigk, F. Kiessling, T. Lammers, A. M. Sofias and F. De Lorenzi (2026). "Bioengineering multicellular tumor spheroids with tunable extracellular matrix deposition." Acta Biomaterialia **212**: 357-372.
- Ninomiya, K. and T. Taniuchi (2024). "Assembly of a tumor microtissue by stacking normal and cancer spheroids on Kenzan using a bio-3D printer to monitor dynamic cancer cell invasion in the microtissue." Biochemical Engineering Journal **212**: 109536.
- Stellato, M., M. Malgorzata Rydzys, M. Pannella, F. Rossi, C. Cappadone, D. Remondini, J.-C. Pyun, N. Normanno, T. Ibrahim, G. Castellani, E. Malucelli, S. Iotti, E. Lucarelli and F. Piccinini (2025). "Radiomic analysis of 3D spheroids using 2D brightfield images." Biomedical Signal Processing and Control **103**: 107366.
- Sun, M., J. Zhang, F. Zhang, Z. Deng, T. Xuanyuan, X. Liu, D. Yu and W. Liu (2026). "Polydimethylsiloxane micropatterning-aided multi-size biomimetic tumor spheroid-on-a-chip for investigating chemotherapy and photodynamic therapy." Colloids and Surfaces B: Biointerfaces **259**: 115376.
- Tang, D., L. Izmaylova, A. Can, S. Ingebrandt, R. E. Leube and Z. Gao (2026). "Time-resolved monitoring and circuit modeling of trophoblast invasion in a model of human embryo implantation by electric cell-substrate impedance sensing." Biosensors and Bioelectronics: **X 31**: 100797.
- Tatsumi, H., H. Okawa, N. Vinaikosol, A. Moribayashi, H. Kayashima, H. Yatani and H. Egusa (2026). "Spheroid size-induced apoptosis enhances osteogenic differentiation of iPSC cells." Journal of Bioscience and Bioengineering **141**(3): 203-209.
- Wu, P.-H., J. M. Phillip, W. Du, A. Forjaz, P. R. Nair and D. Wirtz (2026). "Methods to analyze cell migration data: fundamentals and practical guidelines." Nature Methods **23**(1): 43-55