

RESEARCH ARTICLE

Monte Carlo simulation-guided design for size-tuned tumor spheroid formation in 3D printed microwells

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Abstract

Tumor spheroid models have garnered significant attention in recent years as they can efficiently mimic in vivo models, and in addition, they offer a more controlled and reproducible environment for evaluating the efficacy of cancer drugs. In this study, we present the design and fabrication of a micromold template to form multicellular spheroids in a high-throughput and controlled-sized fashion. Briefly, polydimethylsiloxane-based micromolds at varying sizes and geometry were fabricated via soft lithography using 3D-printed molds as negative templates. The efficiency of spheroid formation was assessed using GFP-expressing human embryonic kidney 293 cells (HEK-293). After 7 days of culturing, circularity and cell viability of spheroids were >0.8 and 90%, respectively. At 1500 cells/microwell of cell seeding concentration, the spheroids were $454 \pm 15 \mu\text{m}$, $459 \pm 7 \mu\text{m}$, and $451 \pm 18 \mu\text{m}$ when cultured in microwells with the diameters of 0.4, 0.6, and 0.8 μm , respectively. Moreover, the distance between each microwell and surfactant treatment before cell seeding notably impacted the uniform spheroid formation. The centrifugation was the key step to collect cells on the bottom of the microwells. Our findings were further verified using a commercial microplate. Furthermore, Monte Carlo simulation confirmed the seeding conditions where the spheroids could be formed. This study showed prominent steps in investigating spheroid formation, thereby leveraging the current know-how on the mechanism of tumor growth.

KEYWORDS

3D cell culture, 3D printing, microwell, Monte Carlo simulation, spheroids

1 | INTRODUCTION

The success rates of new drugs are considerably low due to the scarcity of an efficient biological system that accurately mimics in vivo systems.¹ Therefore, preclinical tests must employ more efficient techniques to elevate the precision of drug tests and their consequent

approval. Conventionally, preclinical drug tests have been performed using conventional 2D cell cultures, that is, where cells are cultured in a monolayer.² This type of cell culture is relatively cost-effective, and the results obtained can be compared to the existing data since the early 1940s.³ Regrettably, the behavior of cells in 2D culture can be predicted partially compared to in vivo studies. Therefore, systems

that mimic in vivo cultures can potentially increase the success of pre-clinical drug testing as 3D cell culture systems.

3D cell culture systems can be assembled using two different approaches, top-down and bottom-up.⁴ The “top-down” approach is employed by seeding cells into 3D porous scaffolds as a support material. The scaffold will degrade as cells divide; consequently, a tissue analog can be crafted. Despite the key benefits of this approach, the porous scaffold fails to mimic in vivo environment due to its simplified architecture.⁵ To tackle these challenges, the “bottom-up” approach emerged as an effective technique with increasing reports over the two decades.⁶ The bottom-up approach in tissue engineering comprises the formation of spheroids.⁷ These spheroids are formed by a series of complex cell-to-cell signaling processes, including activating transmembrane receptors (integrins) that facilitate cell-ECM binding and expression of type-1 transmembrane protein (cadherin).⁸ The expression of such signaling molecules leads to more cell interactions, linking a certain number of single cells together to assemble them into a spheroid.

Spheroids are intensively employed in cancer research as a highly dynamic micro-tumor model to evaluate the efficacy of new drug candidates.⁹ Spheroid models need to be carefully developed with optimal properties to evaluate the potential of drug formulations precisely. Under certain conditions, these cells organize themselves into spheroids in a self-assembly manner,¹⁰ promoted by cell-to-cell interactions. However, such self-assembly process relies on multiple parameters, mainly geometrical and chemical structures of the material on which cells grow. Interactions are forced when cells present low or no affinity for the surface of the material.⁷ New technologies have been developed to enable the self-assembly of spheroids, such as magnetic levitation,¹¹ hanging drop technique,¹² and suspension culture.¹³ Even though these platforms have been well-established and widely utilized, they possess several challenges including (i) tedious handling, (ii) frequent media exchange, and (iii) low throughput. Moreover, spheroids formed with these methods vary in size, hindering repeatability of the study and reducing the precision of drug testing.¹⁴ Therefore, spheroid formation in microwells is shown to address these unmet challenges in spheroid formation.¹⁵

Recent advances in 3D printing technology combined with polymer chemistry and materials science paved the way to fabricate high-quality architectures resistant to extreme conditions (e.g., high temperatures). Currently-available 3D printing technologies (e.g., stereolithography, SLA) allow rapid and facile fabrication of structures with desired geometry. If used directly in cell culture, the commercially available resins can present toxicity to cells. However, these materials are suitable to be used as molds for casting polydimethylsiloxane (PDMS), a silicon-based organic polymer.¹⁶ PDMS presents hydrophobicity, optical transparency, biocompatibility, and excellent gas permeability, making it convenient for cell culture studies. PDMS has great potential in designing easy and rapid prototypes for 3D cell culture devices. Many PDMS-based spheroid culture systems have been previously designed to produce spheroids. Despite the importance of 3D

tools, there is still a lack of systematic studies exploring the main parameters that play a key role in microwell design for spheroid formation.

In this contribution, we show an efficient and economically-viable spheroid formation process using PDMS micromolds (MM_{PDMS}) by exploring critical design parameters such as geometrical structure, microwell size, cell type, initial cell seeding concentration, and culture time. These molds were fabricated through a soft lithography technique, using 3D-printed negative molds as templates ($MM_{template}$). Enhanced Green Fluorescent Protein (GFP)-expressing human embryonic kidney 293 cells (HEK-293) were used in spheroid formation. The spheroids were characterized for circularity, size, geometrical shape, and cell viability over 7 days. Furthermore, spheroid formation was evaluated numerically using Monte Carlo simulation methods to explore the role of various factors such as cell diffusion, cell-surface adhesion, and gravity on spheroid formation. We have correlated the conditions in which the spheroids could be formed, as well as understanding the fundamentals of spheroid formation during cell organization and assembly, with the cell culture results. The methodology described in this study allows inexpensive, facile, and rapid fabrication of microwells that can be also applicable for nearly all basic cell culture laboratories.

2 | MATERIALS AND METHODS

2.1 | Materials

Enhanced Green Fluorescent Protein (eGFP)-expressing human embryonic kidney 293 cells (eGFP-HEK-293) was purchased from Cell Biolabs (San Diego, USA). Trypsin enzyme solution from bovine pancreas was purchased from Sigma-Aldrich (St. Louis, MO). FluoroBrite™ Dulbecco's Modified Eagle Medium (DMEM), a formulation with background fluorescence 90% lower than that emitted by standard phenol red-free, was purchased from Gibco (NY, USA). GlutaMAX™ supplement and Fetal Bovine Serum (FBS) were used as media supplements and purchased from Gibco and Thermo Fisher Scientific, respectively. Phosphate-buffered saline (PBS) was purchased from Gibco (NY, USA). Propidium iodide and Hoechst 33342 staining solutions were purchased from Chemometec (Denmark). The Sylgard™ 184 Silicone Elastomer Kit was purchased from Dow Corning (Auburn, MI, United States). NC-Slide A8™ was purchased from Chemometec (Denmark).

2.2 | Fabrication of microwells for spheroid formation

3D-printed negative micromold templates ($MM_{template}$) were designed in Inventor Professional 2019 (Autodesk, USA). Each $MM_{template}$ was designed in such a way as to accommodate a total of 109 MM_{PDMS} . The $MM_{template}$ were then printed in a stereolithography 3D printer (Form 2, FormLabs). Commercial photopolymer resins (high

temperature, clear, and white) were purchased from FormLabs and used to print the MM_{template} with a layer thickness of either 25 or 50 μm . High temperature, clear and white resins were initially selected to assess their ability to withstand high temperatures during PDMS curing, as well as to evaluate their effectiveness in facilitating precise pattern replication. The primary objective was to determine whether these resins could endure the elevated temperatures required for PDMS curing without compromising structural integrity. Additionally, we examined the ease of peeling PDMS off the cured resin surface, which is essential for achieving clean and precise micro-well structures. Evaluating the performance of each resin in terms of pattern replication allowed us to ensure that the fabricated MM_{template} maintained accurate geometries necessary for uniform spheroid formation. Non-processed resin residues after printing were removed by immersion of the print in isopropyl alcohol (99.9%) for 15 min. Curing was performed at 60°C for 30 min in a Form Cure (Formlabs). After the post-curing process, the border of the MM_{template} was covered with high temperature-resistant Kapton polyimide tape (Dupont, USA). We utilized Kapton tape because we required a material that could withstand high temperatures for further curing of PDMS. This tape was chosen specifically for its ability to resist the elevated temperatures necessary for our process. PDMS was prepared with a curing agent at a ratio of 10:1, thoroughly mixed, and degassed until a bubble-free polymer solution was obtained. After removing all air bubbles, the PDMS was poured over the MM_{template} . Afterward, the MM_{template} was placed in an oven for 2 hours at 80°C. The workflow for the MM_{PDMS} fabrication is

shown in Figure 1. After microfabrication, each PDMS micromold (MM_{PDMS}) was cleaned and autoclaved before spheroid culture.

2.3 | Animal cell culture and spheroid formation

HEK-293 cell line was cultured in DMEM containing 10% FBS and 4 mM GlutaMAX™ supplement. Cells were passaged every 2 or 3 days in a T25 cell culture flask with the Thermo Scientific™ Nunclon™ Delta cell culture surface (Thermo Fisher) and maintained in an incubator with 5% CO_2 at 37°C.

2.3.1 | Spheroid formation in PDMS micromolds

Spheroid formation was carried out in MM_{PDMS} . Before the cell seeding, MM_{PDMS} were placed in a 12-well plate under sterile conditions. The MM_{PDMS} were treated with 500 μL of Anti-Adherence Rinsing Solution (STEMCELL Technologies). The 12-well plate-containing MM_{PDMS} were centrifuged at 1000g for 5 min to ensure the rinsing solution was distributed equally in each MM_{PDMS} . After the centrifugation, MM_{PDMS} were rinsed with a culture medium. Afterward, the culture medium was aspirated from MM_{PDMS} using a pipette, and cell suspension at varying concentrations (500, 1000, and 1500 cells per MM_{PDMS}) was seeded into MM_{PDMS} . After seeding the cells, the 12-well plate containing the MM_{PDMS} was centrifuged at 100xg for 3 min to ensure the cells fell inside the MM_{PDMS} equally. The

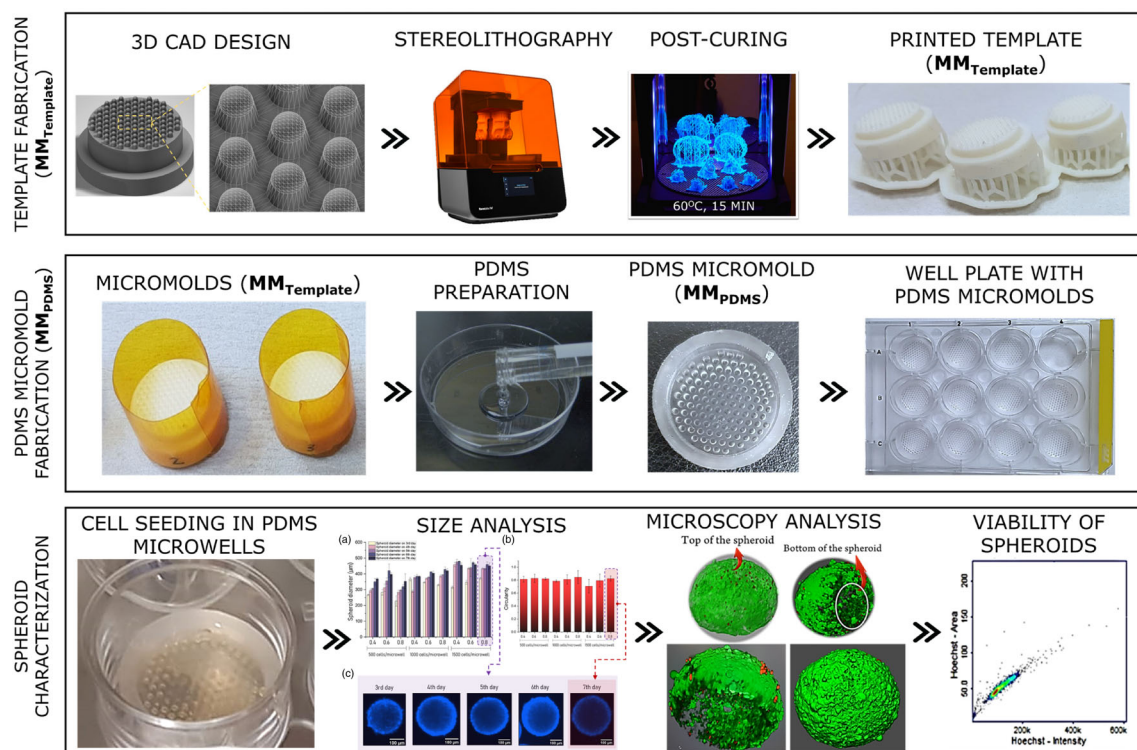


FIGURE 1 Experimental workflow of microwell fabrication for spheroid formation.

MM_{PDMs} were analyzed under the fluorescent microscope and placed in the incubator for spheroid formation (37°C, 5% CO₂).

2.3.2 | Spheroid formation in commercial platform

To confirm the spheroid formation of eGFP-HEK-293 cells, they were tested for their ability to form spheroids in a commercial 96-well clear round bottom TC-treated microplate (Corning, USA). Once 90% of cell confluency was achieved in the T25 flask, the cells were dissociated from the surface of the culture flask and counted using the NucleoCounter® NC-3000™ (Chemometec) using Via1-Cassette™ (containing acridine orange (AO) and DAPI fluorophores). For spheroid formation in a round bottom microplate, a highly-viscous culture media solution was prepared using 10% (vol/vol) of METHOCEL® cellulose ethers with 90% of FluoroBrite DMEM containing 10% (vol/vol) of FBS and Glutamax. Then 100 µL of methocel-containing culture media with a cell concentration of 1000, 2000, and 3000 cells was separately seeded into each microplate well. After three days of incubation (37°C, 5% CO₂), the microplates were analyzed under the microscope to evaluate the spheroid formation of each cell line.

2.4 | Microscopy analysis

The spheroid formation was visualized using EVOS™ FL Digital Inverted Fluorescence Microscope (Invitrogen™) using DAPI and GFP filters. Images were collected daily for 7 days. Leica SP8 confocal microscopy system and Multi-photon Microscope Leica TCS SP8 MP were used for advanced visualization studies.

2.5 | Spheroid analysis

2.5.1 | Morphological properties

Characteristics of spheroids (e.g., diameter, solidity, and circularity) were determined by analyzing the bright-field and fluorescent images using ImageJ. For the circularity measurement, the software “ImageJ” was launched, and the image containing the spheroids under analysis was loaded. Before conducting circularity measurements, the images were initially converted to binary format to appear in black and white. Subsequently, the circularity plugin (Author: Wayne Rasband (wsr@nih.gov)) integrated into ImageJ was utilized for the analysis. Then, the “Circularity” option was selected from the “Set Measurements” dialog in the “Analyze” menu. Each spheroid was outlined individually using the “Freehand selections” tool. Upon outlining a spheroid, the “Measure” option was chosen from the “Analyze” menu to compute its circularity. The plugin utilized the formula $\text{circularity} = 4\pi(\text{area}/\text{perimeter}^2)$ to calculate the circularity value of the outlined spheroid. This process was repeated for each spheroid in the image, and the recorded circularity values were subsequently used for further

analysis and interpretation. The images taken with multi-photon microscopy were evaluated with Leica proprietary software and the microscopy image analysis software Imaris.

2.5.2 | Spheroid density and viability

Approximately 50 spheroids were taken from each microwell using a pipette tip (diameter bigger than 600 µm) and placed in a suspension culture plate containing PBS. After counting the number of spheroids under EVOS™ inverted fluorescent microscope, they were placed into an Eppendorf tube (1.5 mL), and 500 µL of TrypLE™ Select Enzyme (10×) solution without phenol red (Thermo Fisher) was added into the suspension. The spheroids were gently mixed and incubated for ~3 min. Afterward, the solution was mixed vigorously using pipetting or a vortex to dissociate the spheroids. FBS-containing DMEM media was added immediately after mixing. The cell-containing solution was centrifuged at 1000 rpm for 3 min to remove the excess media and replaced with fresh media, followed by cell counting. Cell viability was determined using a Via1-Cassette™ with the NucleoCounter® NC-3000™.

2.6 | Monte Carlo simulations

Monte Carlo simulations were employed using MATLAB R2021b (MathWorks, Inc.). The microwell structure was designed directly in MATLAB as a 3D matrix represented by a $30 \times 30 \times 30$ lattice. In the matrix, the lattice sites recapitulating the boundaries and empty volume were assigned -1 and 0 , respectively, so the hemisphere structure, the conical upper part, and the empty volume could be defined. A total of 70 cells, corresponding to the number of cells in the experiments (1500 cells/microwell), were located during the simulation initialization and assigned to be 1 (Supplementary material S1). The simulations were initialized in three different configurations: (i) randomly distributed over the lattice, (ii) at the hemisphere base, and (iii) at the side of the hemisphere.

After the initialization step, an occupied lattice site (i.e., a cell) was chosen randomly. Subsequently, a neighbor lattice for this occupied lattice was determined. If the determined neighbor lattice was empty, the simulation created a random number Q_{random} using the built-in rand function on MATLAB. This function enabled drawing a random number from the uniformly distributed interval $(0, 1)$. The generated Q_{random} was, then, compared to p_{div} , which is the probability of division between $(0, 1)$. If the generated number Q_{random} was less than p_{div} , the cell was assumed to undergo division. After determining the action of a division, the value of the randomly chosen neighbor lattice was updated to 1.

When the randomly generated number Q_{random} was bigger than p_{div} , it was assumed that the cell could diffuse through the empty chosen neighbor. The final decision for the diffusion was made with the help of the four parameters ϵ_{cc} , ϵ_{cs} , ϵ_g , and ϵ_{cd} which mimic the effect of cell-cell adhesion, cell-surface adhesion, gravity, and cell-cell

interaction, respectively. In detail, the parameters of ϵ_{cc} and ϵ_{cs} were used for reflecting the aversion to diffusion when the cell was surrounded by other cells and boundary surfaces. The gravity and cell-cell interaction parameters (i.e., ϵ_g and ϵ_{cd}) provided the directionality for diffusion. In the case of diffusion, values of the occupied lattice and neighbor lattice were assigned to be 0 and 1, respectively. The values of ϵ_{cc} , ϵ_{cs} , ϵ_g were taken to be -1.2 , 0 , and -0.03 .¹⁷ The parameter sets for p_{div} and ϵ_{cd} were chosen to be $\{0.1, 0.01, 0.001\}$ and $\{-0.1, -0.2, -0.3, -0.4, -0.5, -0.6, -0.7, -0.8, -0.9, -1\}$ to evaluate their effects, respectively. The pseudocode that included the detailed decision control and execution mechanism of the algorithm was given in Supplementary Material S2.

The execution mechanism was performed until 45% of the microwell structure was occupied by cells. The reason for choosing this threshold was that a perfect sphere forming at the microwell base composed 45% of the total volume (Supplementary material S3). Reaching the threshold, the simulations were terminated. The matrices were saved as 25%, 50%, 75%, and 100% of the simulations were completed. Cells were visualized in Paraview 5.9.1 (Kitware Inc.) after obtaining the final cell coordinates using the Gaussian Point feature, in which the radius was chosen to be 0.5. Spheroids' visualizations were saved from five different camera positions: $-X$, $-Y$, $-Z$, $+X$, and $+Y$. Post-processing was performed using OpenCV in Python 3.8.5. During the image processing, the images underwent binary inverse thresholding, blurring, gray scaling, and binary thresholding in this order.

As a final step, the circularity values were examined with ImageJ 2.3.0. The circularity value for a spheroid was defined to be the average of the circularity values of recorded simulation results (i.e., 25%, 50%, 75%, and 100% completed simulation results) visualized in the mentioned camera positions. In the case of obtaining more than one contour, the entities of the contour having the largest area were used.

2.7 | Statistical analysis

Quantitative experiments were conducted at least in triplicates, and statistical analysis was performed using the Student's T-test SPSS Statistics 27. The pseudocode written on MATLAB was run three times, and the average of the three different images was considered.

3 | RESULTS AND DISCUSSION

3.1 | Microfabrication of microwells

We fabricated PDMS micromolds (MM_{PDMS}) using a combination of stereolithography and soft lithography techniques (Figure 1). The fabrication and process parameters were optimized for uniform and rapid spheroid formation in microwells. Our study examined sphere-like microwell structures of varied sizes for spheroid formation. The $MM_{template}$ to generate MM_{PDMS} was fabricated with different resin materials using stereolithography. Several resin

materials are commercially available for high-resolution rapid prototyping. Each resin has a different level of rigidity, light transmission, biocompatibility, and temperature resistance. Elevated temperatures are required to cure the PDMS once the cast-and-molding process is complete. Therefore, the resin must resist high temperatures without losing its physical (change in size, color, and volume) and/or chemical properties. We tested three different Formlabs resin materials (high temperature, clear, and white resins) to produce the $MM_{template}$. Briefly, the MM_{PDMS} were first fabricated using high temperature resin. The molds ($MM_{template}$ with PDMS) were left in the oven at temperatures varying from 50 to 80°C for 1 to 6 hours. We varied the temperature and curing time to observe how higher temperatures affect the resilience of the resin without altering its physical properties. Once the PDMS was completely cured, peeling off the cured MM_{PDMS} from the $MM_{template}$ without damaging the MM_{PDMS} was challenging. Some reasons that could be explored for the challenge of peeling off the cured MM_{PDMS} from the $MM_{template}$ without damaging the MM_{PDMS} include variations in the adhesive properties of the resin at different curing temperatures and durations, potential changes in the mechanical strength of the MM_{PDMS} due to prolonged exposure to higher temperatures, and the possibility of insufficient release agent application or inadequate surface preparation of the $MM_{template}$ before casting the MM_{PDMS} . However, when we tested either white or clear resin as a $MM_{template}$ to produce MM_{PDMS} , the resins could resist temperatures as high as 100°C without any visible physical alterations (i.e., color), and the PDMS could be easily peeled off from the resin. For these reasons, the white resin was used to fabricate the MM_{PDMS} . The $MM_{template}$ for spheroid formation in a static system was designed to be 20 mm in diameter (Figure 2); thus, each MM_{PDMS} would precisely fit into a single well of a 12-well plate. Previous MM designs made of PDMS had a diameter of 10 mm,¹⁸ 22 mm,¹⁹ and 35 mm²⁰ to fit into a well of a 24, 12, and 6-well plate, respectively. Additionally, we have evaluated three different diameters in MM_{PDMS} design: 400, 600, and 800 μm . The MM_{PDMS} with the width varying from 300 μm ²¹ to 2000 μm ²² have been previously investigated to form spheroids.

3.2 | Spheroid formation in PDMS microwells (MM_{PDMS})

We selected the HEK-293 cell line for spheroid formation studies. HEK cells, an type of immortalized cell line, have been previously explored in drug tests and toxicity analyses for their ability to generate spheroids^{23,24}; hence, further experiments were performed using eGFP-HEK-293 cell line to facilitate data acquisition in a fluorescent microscope.

Before seeding the cells into the MM_{PDMS} , the surface was treated with 200 μL of anti-adherence rinsing solution as a surfactant to reduce the surface tension in MM_{PDMS} and prevent cell adhesion. Using surfactant before seeding the cells into the system proved exceedingly crucial. Our results disclosed that the cells did not form

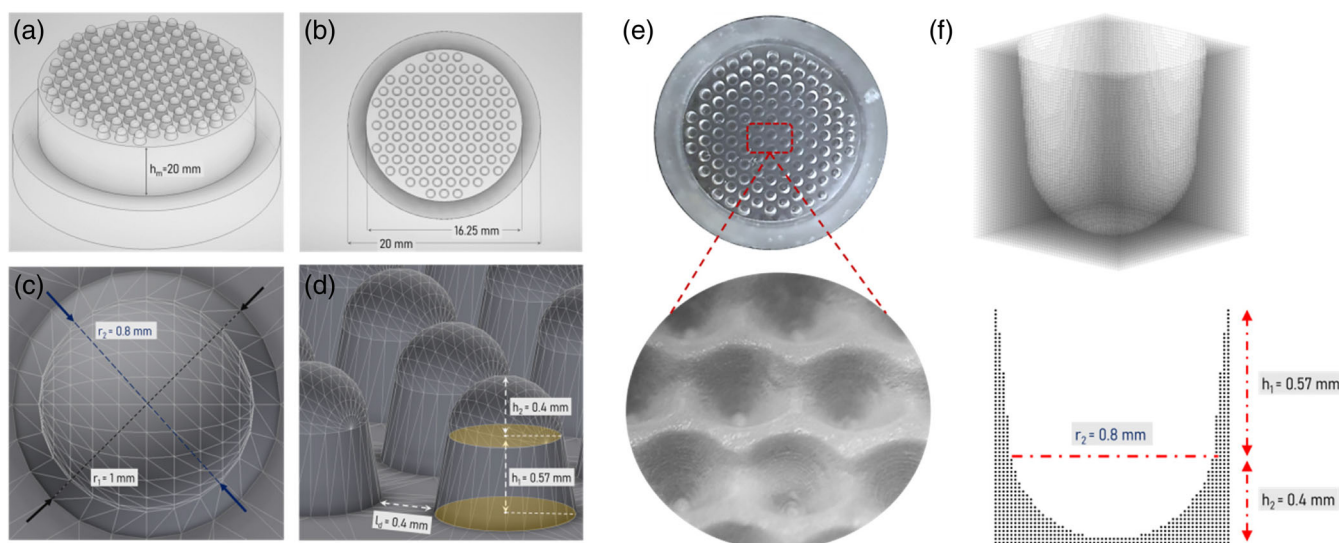


FIGURE 2 Optimized geometrical design parameters of the negative micromold and PDMS microwells for spheroid formation. The design of the static device was drawn in Autodesk Inventor 2019, and the molds were fabricated using stereolithography. (a and b) The illustration of the negative micromolds; (c and d) the illustration of sphere-like microwells; The distance between each microwell (l_d) was set to 0.4 mm. The total number of microwells per micromold was 109. (e) Morphology and geometrical structure of fabricated micromolds containing microwells. (f) Visualization of a single microwell presented in ParaView software from Matlab's data.

spheroids with desired characteristics without surfactant surface treatment. As a result, the cells formed aggregates with different cell populations, forming more than one spheroid with varying sizes in a single MM_{PDMS} (Figure S4). The circularity of the spheroids was less than 0.5 (a.u.). The tests in MM_{PDMS} without any surfactant pre-treatments led to forming of satellite-like structures around a large spheroid, probably decreasing future drug tests' success rate and repeatability. Hence, we observe that surface treatment before cell seeding is vital for homogeneous spheroid formation. In addition, the precision of the $MM_{template}$ is considered extremely important for the circularity of the spheroids. There is also a direct correlation between the $MM_{template}$'s precision and the MM_{PDMS} 's roughness. It was previously identified that the $MM_{template}$ with high precision resulted in a higher spheroid circularity value.²⁵

Besides the impact of surfactant pre-treatment on the MM_{PDMS} surface, it was noted that a centrifugal step was strictly required to induce the cells to fall into each MM_{PDMS} uniformly. The distance between each MM_{PDMS} on the MM_{PDMS} was optimized accordingly so that most seeded cells (>90%) could fall into MM_{PDMS} without significant loss after the centrifugal step. We tested different distance values, that is, 0.4, 0.8, and 1.2 mm, and verified their effects on the spheroid formation (Figure S5). Our evaluations with the eGFP-expressing HEK-293 cell line presented that the cells could easily fall into each MM_{PDMS} after a centrifugation step while using MM_{PDMS} with 0.4 mm of distance (Figure S5). More than this distance, cells did not fall into MM_{PDMS} even at higher centrifugal forces. Hence, the MM_{PDMS} with a 0.4 mm of distance between each MM_{PDMS} was defined as the optimum geometry for uniform and high-throughput spheroid formation to be used for further drug testing studies.

After defining the optimum distance between each MM_{PDMS} , we further analyzed the effect of geometry on spheroid formation. Spheroid formation relies highly on the geometrical properties of MM_{PDMS} (e.g., size and shape) where they are initially cultivated. These properties were shown to alter the outcome of spheroid formation significantly. Hence, we initially assessed different MM_{PDMS} diameters (400, 600, and 800 μm) to form spheroids in MM_{PDMS} (Figure S6A-C). The base diameter and volume of the conical frustum for each structure were fixed to 1 mm and 1.468 μL , respectively. On the other hand, D_s (diameter of the hemisphere on top) and h_{mw} (height of conical frustum) values were altered accordingly to keep the constant volume for each MM_{PDMS} (Figure S6-bottom images).

Spheroids were formed in MM_{PDMS} using different cell seeding concentrations and analyzed in size and circularity (Figure 3a,b). The change in spheroid size during 7 days of culture was presented in Figure 3c. Day 1 was defined as the time when cells were seeded into the MM_{PDMS} for the first time. On day 2, the cells initiated to form spheroids, possessing weak extracellular interactions. On day 3, the spheroids became more solid, and this might be possibly due to the involvement of more extracellular matrix compounds. Initial seeding concentration and MM_{PDMS} size greatly impacted the spheroid formation. A significant increase in spheroid size was observed for each condition throughout 7 days (Figure 3c). At the end of 7th day, the largest spheroid sizes were measured for the samples with a cell seeding concentration of 1500 cells/ MM_{PDMS} . Interestingly, spheroids had size values of $454 \pm 15 \mu m$, $459 \pm 7 \mu m$, and $451 \pm 18 \mu m$ when MM_{PDMS} with the diameter of 400, 600, and 800 μm were used, respectively.

According to our findings, the geometry and size of MM_{PDMS} , culture conditions, and particularly seeding density-to- MM_{PDMS} size ratio

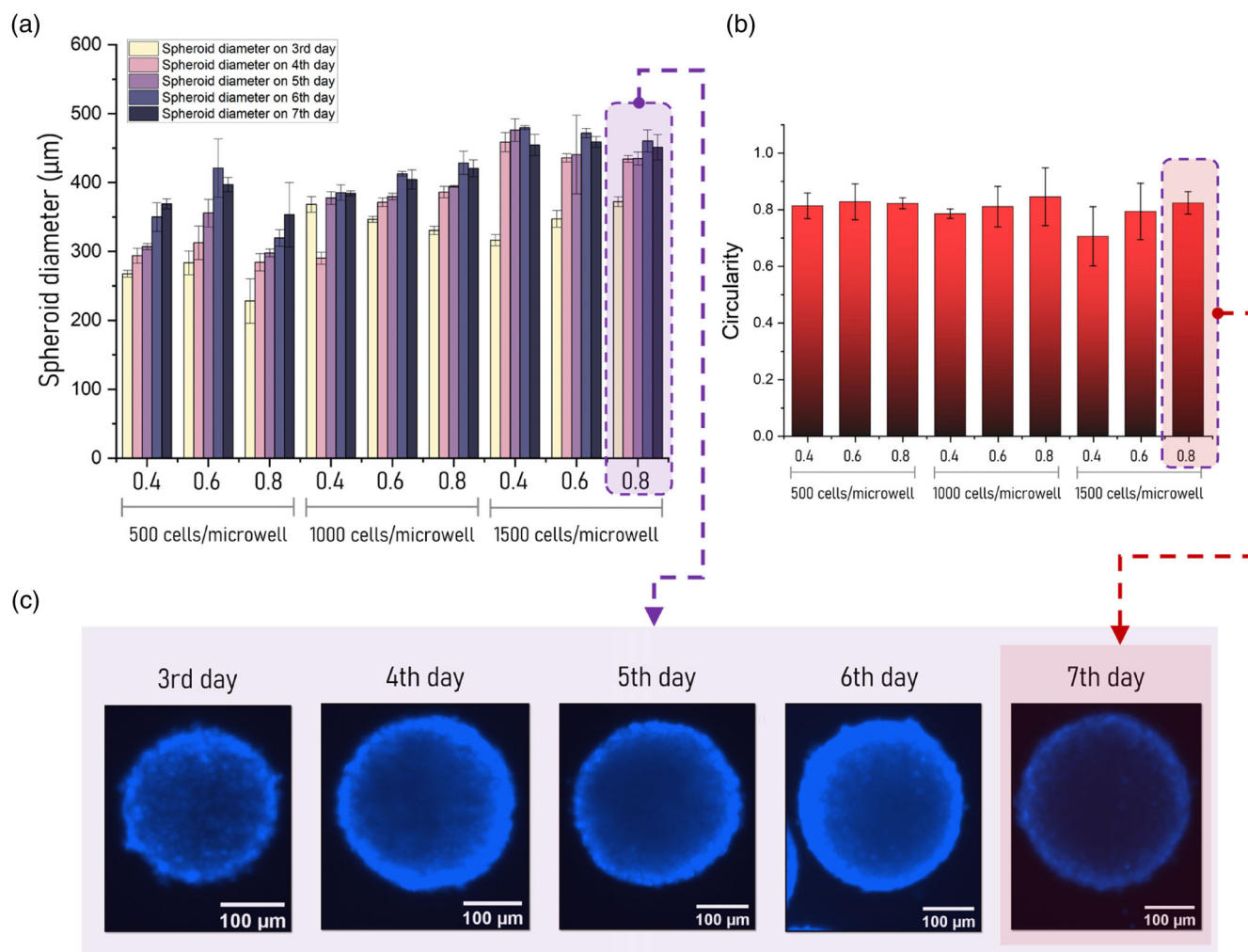


FIGURE 3 Spheroid analysis in PDMS microwells. (a) Change in spheroid size during 7 days of cultivation in microwells with varying microwell size (0.4, 0.6, and 0.8 mm) and initial cell seeding concentration (500, 1000, and 1500 cells/microwell). (b) The circularity of spheroids formed in PDMS microwells with varying sizes: 0.4, 0.6, and 0.8 mm and cell seeding concentrations: 500, 1000, and 1500 cells/microwell. (c) Fluorescent microscope images of spheroids generated in microwells with the size of 0.8 mm and seeding concentration of 1500 cells/microwell. The images were taken under DAPI filter on the 3rd, 4th, 5th, 6th, and 7th days. The scale bar indicates 100 µm in each figure. The spheroids shown in B were randomly selected from a microwell and did not represent the average data.

play a critical role in the outcome of spheroid formation. It should be noted that spheroid formation is highly cell-dependent. Each cell type possesses a unique ability to form spheroids. Numerous commercial and non-commercial microwell platforms have recently been employed for spheroid formation and drug testing. For instance, it was reported that the spheroids obtained from the HT-29 cell line in a conventional 96-well plate had irregular shapes with diameters varying between 60 and 100 µm.²⁶ However, when a round bottom platform with a microwell size of 600 µm and cell density of 3,000 cells/microwell was used, HT-29 spheroids with a size of 300 µm on 5th day could be effectively formed.²⁷ Recently, a microwell system with 800 µm diameter for spheroid formation with human embryonic stem cells has been proposed.²⁵ With an initial cell seeding density of 8000 cells/MM_{PDMS}, the spheroids had a size of approximately 700 µm after 7th day of culture. In another study, MM_{PDMS} size of 500 µm was used with a cell seeding density of 2300 cells/MM_{PDMS}. The

spheroids had a size of 275 µm on the 3rd day of culture. A comparison of the present MM_{PDMS} with the previously-designed MM_{PDMS} in terms of cell culture, spheroid size, and seeding cell density, MM_{PDMS} shape, MM_{PDMS} diameter, MM material, and culture time is shown in Table 1.

Conventionally, cell culturing (monolayer and 3D) is time-consuming; thus, the designed MM_{PDMS} are expected to generate spheroids more rapidly. In case of drug testing, time and precision are particularly essential, and using such a platform that allows the formation of spheroids as fast as possible with the desired spheroid size is a prerequisite so that the drugs can be immediately tested.²⁸ Additionally, it was shown that spatial confinement significantly provokes spheroid formation. If the diameter of a MM_{PDMS} is too large for the number of seeded cells, there is an increased risk of forming multiple spheroids in a single MM_{PDMS}. Hence, the number and the MM_{PDMS} size should be correlated carefully.²⁹ According to our findings, the MM_{PDMS} size

TABLE 1 Comparison of present micromold system with previously published microwell systems in terms of cell line, spheroid size, seeding cell density, microwell shape, microwell diameter, micromold material, and culture time.

Cell culture line	Spheroid size (μm)	Seeding cell density per microwell	Microwell shape	Microwell diameter (μm)	Material	Fabrication method	Culture time (days)	Ref
Panc-1	480	3000	Concave bottom	566.3	PDMS	Photolithography	5	27
HT-29	300	3000	Concave bottom	566.3	PDMS	Photolithography		27
hESC	700	8000	Concave bottom	800	PDMS	Stereolithography	7	25
HuH-7	~400	554	Concave bottom	500	SpheroFilm™	Commercial	10	17
SK-OV-3	275	816	Concave bottom	500	PDMS	A custom designed jig system	3	33
SK-OV-3	150	349	Flat bottom	200	Agarose	Not shown	10	34
MSC	350	10,000	V-shape bottom	800	Agarose	Not shown	2	35
MCF-7	360	2000	Concave bottom	800	Agarose	Not shown	7	36
MCF-7	470	8000	Concave bottom	800	Agarose	Not shown	7	36
HEK-293	~450	1500	Hemisphere	800	PDMS	Stereolithography	7	Present study

of 800 μm and cell seeding concentration of 1500 cells/MMPDMS is optimum for spheroid formation in MM_{PDMS}. The designed system is also expected to be high-throughput so that more data can be acquired for a more precise statistical analysis. In our design, each MM_{PDMS} contained 109 MM_{PDMS}. Considering that our MM_{PDMS} fits in a well of a 12-well plate, 1308 spheroids can be produced per plate as a future implementation of this strategy. This value is almost 14 times higher than a commercial 96-well transparent round-bottom microplate.

Tumor spheroid models have emerged as valuable tools in cancer research and drug discovery due to their ability to mimic the three-dimensional architecture and microenvironment of tumors more closely than traditional two-dimensional cell cultures. However, it is crucial to acknowledge and address potential limitations associated with the use of tumor spheroids as *in vitro* models. One significant limitation is the presence of diffusion constraints within the spheroids, which can affect the distribution of nutrients, oxygen, and therapeutic agents throughout the cellular mass. In traditional static microwell systems, cells at the core of the spheroid may experience limited nutrient and oxygen availability, leading to the formation of necrotic regions and altered cellular responses. This diffusion limitation also affects the penetration and distribution of drugs within the spheroid, potentially impacting the accuracy of drug efficacy assessments. Another limitation arises from the heterogeneity inherent in tumor spheroids, which can vary in size, shape, and cellular composition even within a single culture. This heterogeneity may introduce variability in experimental results and complicate the interpretation of findings. Furthermore, tumor spheroids may not fully recapitulate the complex tumor microenvironment found *in vivo*, including interactions with immune cells, stromal cells, and extracellular matrix components. Nevertheless, our developed technique for micromold template fabrication offers promise in addressing these limitations. By enabling precise control over spheroid size and geometry, our

method enhances uniformity and reproducibility in spheroid formation, mitigating variability in experimental outcomes and potentially improving the accuracy of drug efficacy assessments. Importantly, these microwells can be placed in a cell culture plate, keeping them away from contamination risks. Additionally, our device is miniature and can be optimized to possess more or fewer wells, allowing for the adjustment of spheroid number for desired experiments. Furthermore, our system allows reusability by autoclaving the PDMS mold again for the next culturing. This advancement shows the significance of our approach in overcoming challenges associated with traditional tumor spheroid models, thereby enhancing their utility in cancer research and drug discovery endeavors.

Additionally, our micromold template fabrication technique offers a flexible platform that can be adapted for various cell types and applications. For instance, by adjusting the dimensions and geometries of the microwells, researchers can tailor the template to accommodate different cell sizes and shapes, enabling the formation of spheroids from a wide range of cell types. Additionally, the ability to control seeding density and optimize culture conditions provides further flexibility for culturing diverse cell populations. Furthermore, our method's compatibility with different cell culture substrates and media formulations enhances its versatility for studying various cell behaviors and responses. Our device is a versatile tool for spheroid formation with broader applications across different cell types and research areas."

3.3 | Viability assay and spheroid density

Determining the number and viability of cells in a monolayer (2D) cell culture is easy; however, in more complex and native mimicking structures like 3D cell culture, it is remarkably difficult to determine these parameters, requiring sophisticated strategies. Before this process, spheroids must be dissociated with physical or enzymatic methods.

On the other hand, the method should not interfere with the viability of cells and dissociate the entire spheroid without leaving any cell aggregates. Dissociation of spheroids using only physical methods is challenging and most likely damages the cells and decreases their viability. Instead, enzymatic methods can be more effective for such dissociation. When trypsin was used, followed by vigorous pipetting and trypsin deactivation with serum, the viability of the cells was found to be below 50%, with several cell populations in aggregate form. Once the enzyme solution was tested, the viability of the cells was over 90%, with an insignificant number of cell aggregates. The high viability was also induced by continuously refreshing the spheroid culture with the culture medium (Figure S7).

Estimating the number of cells per spheroid is highly challenging as the growth of the spheroid in size is not linearly correlated with the number of cells due to the cell proliferation in different directions in an approximately spherical system. To determine the cell density of spheroids from 3rd to 5th day, we conducted a separate experiment using spheroids formed with non-fluorescent HEK-293 cells and dissociated them using an enzyme solution. The alterations in cell number with the spheroid size is shown in Table S1. According to this finding, the cell number increased drastically and almost doubled from ~8800 to ~15,300. At the same time, the spheroid diameter changed from 375 μm to 410 μm (~10% increase), pointing out that the cells in a spheroid might have attracted to each other and generated more tight connections due to the potential presence of integrins and cadherins, and causing this non-linear correlation between spheroid size and density.³⁰ This behavior also explains how aggressive and non-aggressive cell lines behave during spheroid formation. As stated before, non-aggressive cell lines such as HEK-293 form spheroid more densely and compactly, which confirms our findings (Table S1). Exploring such a correlation is vital, especially for drug testing studies, as it allows a precise estimation of the amount/concentration of drug to be administered into a tumor microtissue.

3.4 | Microscopy analysis of spheroids

Spheroid visualization holds some technical challenges through conventional fluorescent microscopy techniques since there must be a certain photon penetration to visualize the inner part of the 3D micro-tissues. Conventional confocal microscopes are also not very effective in penetrating the deep layers of spheroids, limiting them to less than 50 μm (Figure 4). Accordingly, using advanced microscopy techniques is especially essential to observe cells inside the spheroid. Advanced microscopy analysis would denote critical information about spheroids' proliferation, hypoxic, and necrotic zones. Moreover, it is also possible to obtain viable data about the transfection efficiency by employing different filters to acquire different fluorescent signals from each zone.

This study initially employed the confocal microscope (Leica SP8) to visualize the spheroids formed with non-GFP-expressing HEK-293 cell lines. Prior to the visualization, the cells were stained with Hoechst 33342. As shown in Figure 5, the spheroid's edge and shape

can be visualized; however, we could not penetrate inside the spheroid. Moreover, the spheroid shape was observed as oval, resulting from spheroids sedimentation on the microscope slide's surface. Considering that each spheroid used in this experiment had a size of ~450 μm and at least half of the spheroid (~200 micrometers) had to be visualized so the entire geometry of the spheroid could be estimated. Due to the penetration limitations of this confocal imaging system, a more effective imaging system was required. Hence, two-photon microscopy was employed to visualize the deeper regions of the spheroids. The GFP-expressing cells analyzed with two-photon microscopy can be seen in Figure 5. The dead cells stained with propidium iodide are shown in red. The spheroid was stained with propidium iodide to observe the position of the dead cells inside the spheroid. As expected, more penetration depth was achieved using this microscopy technique.

3.5 | Spheroid formation in commercial kits

Herein, we used a commercial kit to form spheroids using the HEK-293 cell line to compare our spheroid formation findings. The reason to select these microplates is that they are commercial systems to generate spheroids and are widely used in 3D culture studies. Compared to our micromold system, each well of the commercial plate has a height of 11.3 mm and width of 6.86 mm with a total volume of 330 μL . Culture media was prepared for each cell line using 10% of METHOCEL[®] cellulose ethers for spheroid formation in a microplate. Previous studies demonstrate that HEK-293 cell lines are considerably less aggressive,³¹ forming spheroids with exceptional circularity and rigidity features. The size of spheroids generated using the HEK-293 cell line was proportional ($R^2 = 0.988$) to the number of cells initially seeded into the microplate within the range of cell seeding density tested. At the end of 3rd day, the HEK-293 cell line formed spheroids with a size of $377 \pm 7 \mu\text{m}$, $433 \pm 8 \mu\text{m}$, and $471 \pm 24 \mu\text{m}$ when initially seeded with 1000, 2000, and 3000 cells per well, respectively (Figure S8). The spheroids formed in the commercial kit presented similar size and circularity to the spheroids formed in our system.

3.6 | Monte Carlo simulation

After confirming the formation of spheroids both in our MM_{PDMS} and commercial kit, we employed Monte Carlo simulation to acquire more information regarding the spheroid formation mechanism. Monte Carlo simulation was performed to demonstrate the significance of the centrifugal step, surfactant treatment, and the starting position of cells with different characteristics (e.g., diffusivity levels) in micro-wells for spheroid formation. To achieve that, the simulations were initialized with 70 cells in the microwell at different locations, as shown in Figure 6. The geometrical value of the microwell and, thereby, initial cell seeding density were adjusted to accelerate the process on the MATLAB, hence acquiring results faster.

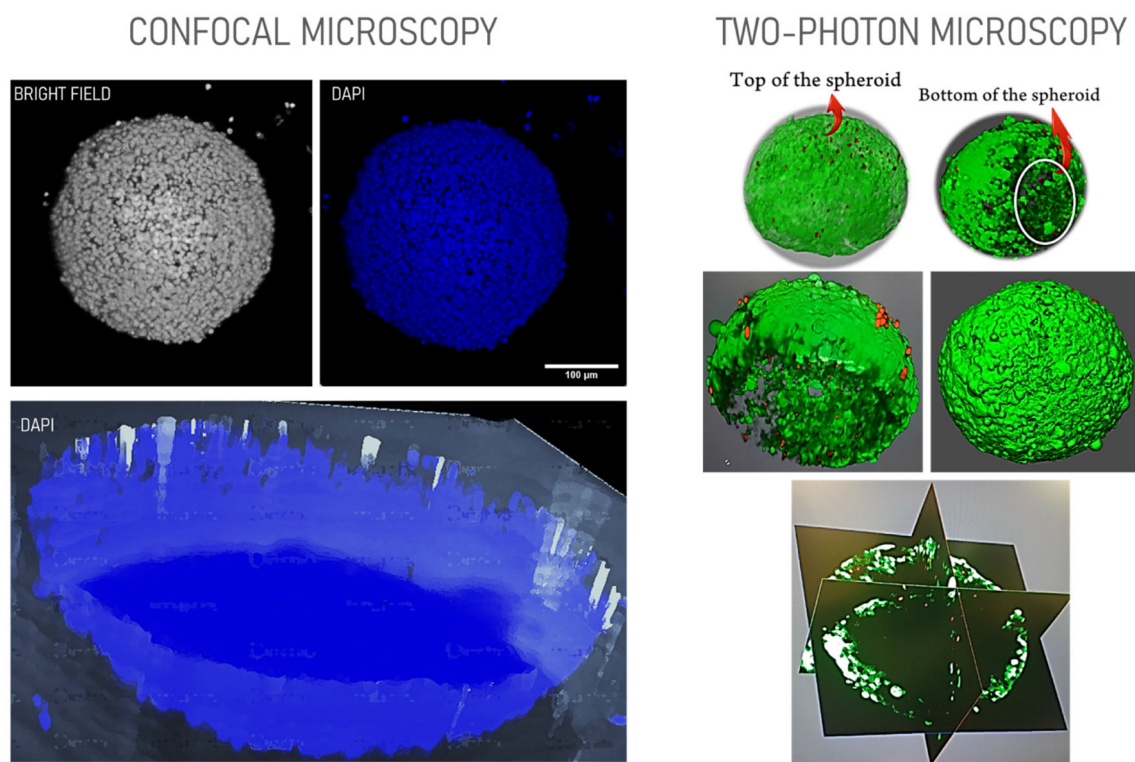


FIGURE 4 Empty microwell representation (a) and different cell starting positions during Monte Carlo simulations (b–e).

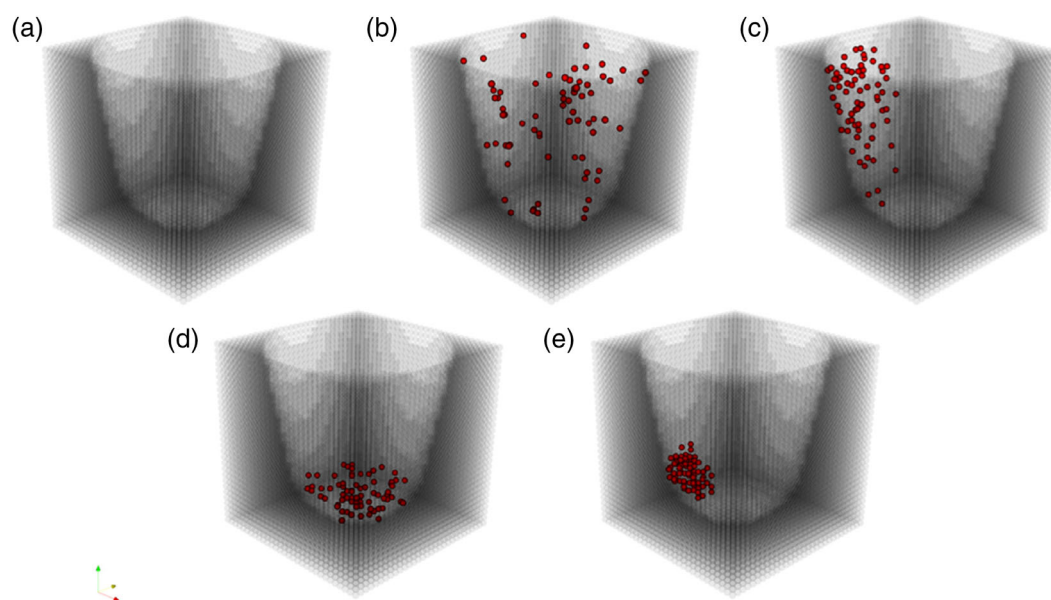


FIGURE 5 Confocal image of a spheroid visualized under confocal system. The spheroid was left for growth for 7 days. The two-photon microscopy system visualized fluorescence imaging of spheroids at the end of day 7. The red dots represent the dead cells that were stained with PI dye. The size of spheroids visualized using confocal and two-photon microscopy system was approximately 450 µm. The spheroids were formed using MM_{PDM5} with a diameter of 800 µm and an initial cell seeding concentration of 1500 cells/MM_{PDM5}.

The 70 cells/microwell was selected to use the same volumetric cell ratio as performed in the experiments. During experiments, 1500 cells were placed in a microwell having 0.653 mm³ volume. The volume ratio between the cells and the microwell volume was calculated

as 0.0029 after assuming a cell was a perfect sphere with a radius of 13 µm [4]. While calculating the microwell volume used in simulations (Figure S1), each lattice was similarly assumed to be spheres with a radius of 13 µm and boundaries excluded from the 30×30×30 matrix.

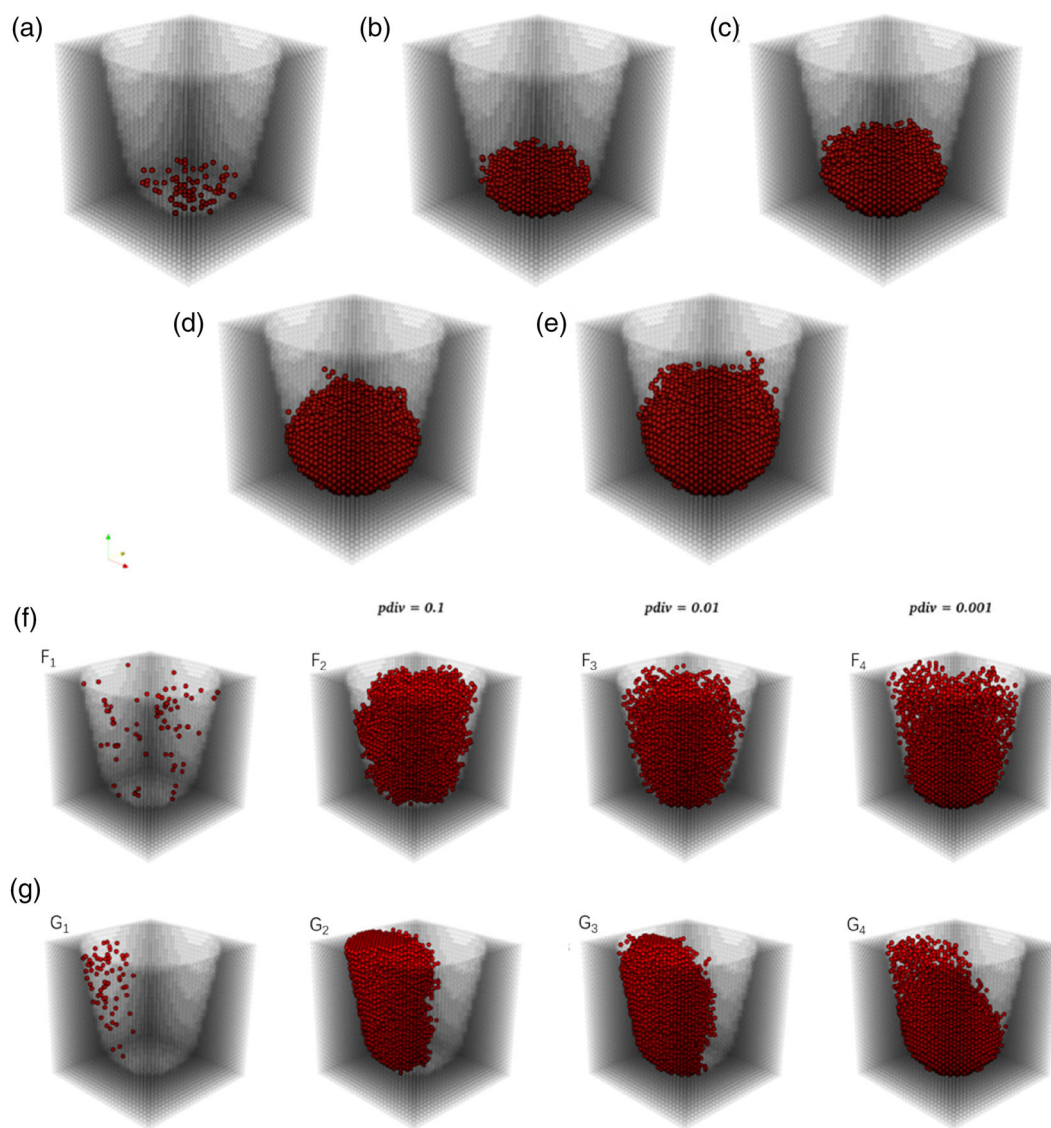


FIGURE 6 Monte Carlo numerical simulation for spheroid formation. Spheroid formation stages with parameters $pdiv = 0.1$ and $ecd = -0.1$: (a) Initial stage, (b) 25%, (c) 50%, (d) 75%, (e) Final form of spheroid. Final spheroids in the case of placing cells randomly (F_{1-4}) and at the side (G_{1-4}) with different $pdiv$ values.

As a result, it was decided to use 70 cells while initializing the simulations. The details about this calculation are given in Supplementary Material S1.

The main algorithm and parameters were employed during the simulations based on the previous work with modifications.¹⁷ In detail, the parameters of ϵ_{cc} and ϵ_g were taken to be -1.2 and -0.03 to illustrate cell-cell adhesion and the effect of gravity. The cell-surface dimensionless number ϵ_{cs} was chosen to be 0 since surfactant pretreatment was performed during the experiments. In addition to these parameters, a new parameter ϵ_{cd} was added to reflect the directionality of diffusion and cell-cell interaction during spheroid formation. In the case of diffusion, the cell chooses an empty neighbor lattice. If the neighbor lattice is not closer to the geometrical center of mass of the cells in the microwell, the probability of diffusion decreases by $\exp(\epsilon_{cd})$ such that the direction of cell diffusion can be directed toward

the geometrical center. The detailed algorithm of the simulations is provided in Supplementary Material S2.

3.6.1 | Validating our algorithm

When cells are located within a hemisphere structure, the resultant force toward the center leads to more compact spheroid formation.²⁹ Therefore, Monte Carlo simulations were performed by initially positioning the cells at the hemisphere bottom, as shown in Figure 6d, to verify whether the proposed algorithm allows spheroid formation. It was expected that the introduced parameter ϵ_{cd} could force cells to move towards the center. The stages of spheroid formation can be seen in Figure S9 when $pdiv$ and ϵ_{cd} were taken 0.1 and -0.1 , respectively. For all parameters, final spheroids are

given in Figure S10 when cells are initialized inside the bottom hemisphere.

3.6.2 | The effect of centrifugal step on spheroid formation

During the experiments, it was observed that centrifugation is a crucial step in forming spheroids. In the case of failure in the centrifuge step, the experiment was repeated. The requisite of the centrifugal step is demonstrated using Monte Carlo simulations. To achieve that, the simulations were performed by placing the cells randomly and close to the side of the wall in microwells, as shown in Figure 4. After positioning the cells in the microwells, the simulations were run up to 45% of the empty volume filled. The formed spheroids can be seen in Figure 6a–e.

As seen in Figure 6, putting cells inside a microwell without centrifugation did not lead to spheroid formation, complying with the experimental results. As the cell aggressiveness and diffusivity increase, which is indicated by smaller *pdiv* values (0.001), the cells start to accumulate at the bottom, and the chance to form spheroids increases (Figure 6 F4 and G4). Therefore, it can be inferred that the cells should not be placed in a microwell without centrifugation and surface treatment with surfactant. This finding was also observed experimentally, confirming the simulation. In addition to the centrifugal step, surfactant treatment was shown to provide a similar effect in our experiments as pre-treatment of the

microwells with surfactant facilitated the formation of spheroids with desired circularity.

3.6.3 | Analysis of cell diffusivity in spheroid formation

Each cell line possesses a different diffusivity that significantly impacts its ability to form spheroids. To evaluate the effect of cell diffusivity, various *pdiv* parameters {0.1, 0.01, 0.01} were used when ϵ_{cd} kept constant as -0.1 . Verifying spheroid formation with the proposed algorithm when the cells are located inside the hemisphere structure, Monte Carlo Simulations were performed by different *pdiv* and ϵ_{cd} values. Subsequently, the final spheroids were visualized in Paraview5.6, and the saved images were processed using OpenCV in Python 3.8.5, as discussed in the materials and method sections. Evaluating the images using ImageJ 2.3.0, the box plot of the circularity values for final spheroids is given in Figure 7.

Throughout the simulations, cells were chosen to undergo either division or diffusion after comparing the randomly generated number with the *pdiv* value. As a result, as the value of *pdiv* decreased, cells were more likely to diffuse. Therefore, smaller *pdiv* values indicate more diffusive cell lines. As seen in Figure 7, when comparably more diffusive cells were located inside the hemisphere during the start of the simulation, their circularity values decreased when compared to the less diffusive cells. This result suits the previous work in which phase-contrast images of invasive cells (more diffusive) and non-

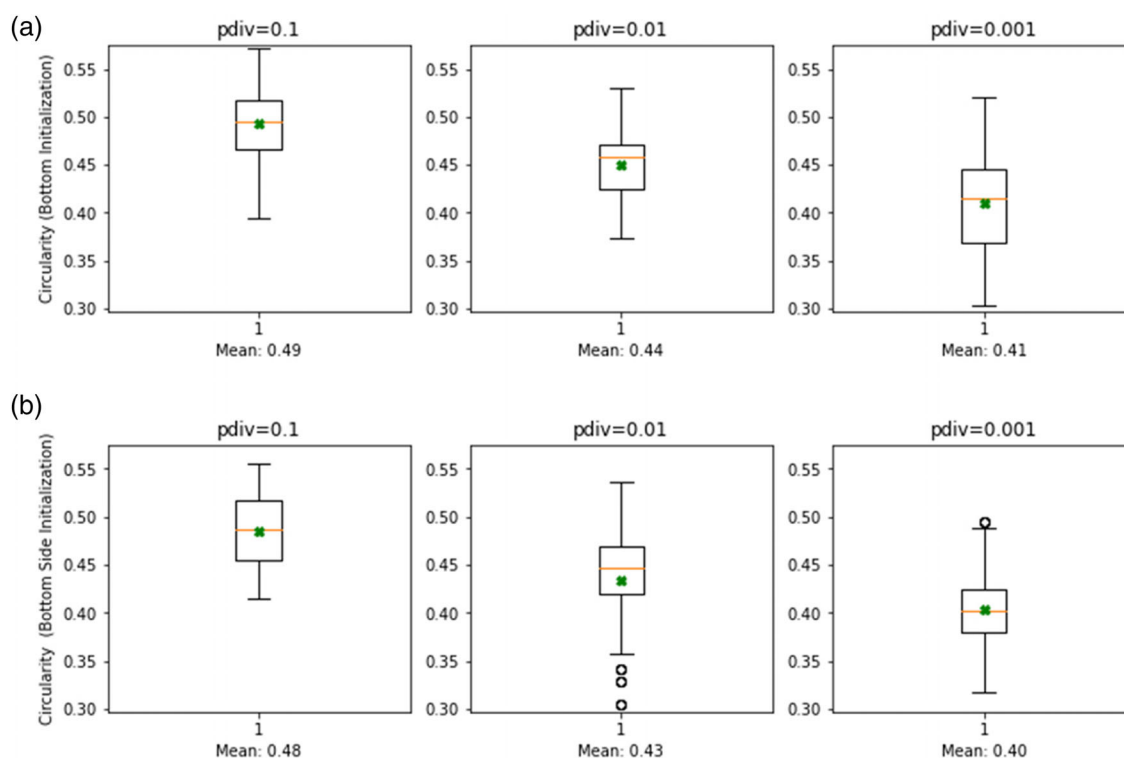


FIGURE 7 Comparison of the circularity values with different *pdiv* values when (a) the cells are initialized at the hemisphere bottom, (b) the cells are initialized at the side of the hemisphere.

invasive cells (less diffusive) during spheroid formation were examined and stated that non-invasive cells form a more compact structure.³²

After conducting the experiments, it was observed that placing cells similar to the configuration in Figure 6d leads to bigger and more uniform spheroid formation compared to the configuration in Figure 6e. However, this trend cannot be demonstrated in Figure 7. The reason could be that the parameter ϵ_{cs} recapitulating the cell surface adhesion was 0. Therefore, it can be concluded that there is a chance of adhesion between the cells and the surface, although surfactant pre-treatment is performed before the experiments.

4 | CONCLUSION

This article investigated several parameters in designing MM_{PDMS}, and subsequently optimized them for a high-throughput, size-tuned HEK-293 spheroid production. Optimizing specific parameters, such as spheroid size and microwell geometry, is crucial for more precise drug testing and less drug consumption. Initial cell seeding density, microwell geometry, the distance between each microwell positioned on a micromold, and surfactant pre-treatment were key parameters impacting the outcome of spheroid formation. The initial cell seeding density of 1500 cells/microwell and a sphere-like microwell geometry with a diameter of 0.8 mm were found to be effective for spheroid formation. These conditions were also selected to perform Monte Carlo simulation analysis.

The surfactant treatment and centrifugal steps played crucial roles in spheroid formation. It was found that the cells could not settle in the microwells without centrifugal forces. When centrifugation was employed without the surfactant treatment, we observed the formation of satellite-like spheroids of varying sizes in a single microwell. However, even when both the centrifugal step and the surface treatment were performed, further optimization of the distance between the microwells remained necessary as this distance had a different impact on uniform cell distribution in microwells. Our results showed that a distance over 0.4 mm between the wells prevented the homogeneous distribution of cells within each microwell. Conversely, when we tested the distances of 0.4 and 0.2 mm, all the cells settled and uniformly distributed inside the microwells. On the other hand, decreasing this distance from 0.4 mm to 0.2 mm led to a significant increase in microwell number per MM_{PDMS}, thus achieving more spheroids per batch.

The primary motive for designing such systems is to test drugs effectively. The scientific development of the current project involves testing lipid nanoparticles for the delivery of mRNA into spheroids and determining how deep a genetic-based drug can penetrate spheroids. Having said that, the current static microwell system faces prominent challenges regarding gene/drug delivery. For instance, being a static system, the delivery of drugs only depends on gravity due to the absence of a continuous flow. Once the drugs reach the proliferating layer of the spheroid and interact with the cells, there is a high chance that the penetration of the drug will face diffusion limitations. In such a system, the drugs are expected to penetrate the spheroid

only from the top, and the drug concentration will significantly decrease throughout the bottom of the spheroid. Such challenges can be tackled by using more advanced technological platforms. For instance, a dynamic microfluidic platform is a closed channel system with an inlet and outlet, with a continuous flow of culture media and drug/gene formulation. Generating spheroids and testing drugs in continuous microfluidic systems will provide a better mimicry of in vivo and precision for drug testing. Effectively, a hollow, coated, or dissolved microneedle technology can be carefully designed and directly applied in spheroids to deliver drugs in the necrotic zone of a spheroid. Overall, the methodology described in this study will allow the integration of a microfluidic perfusion culture effectively for drug screening studies as such a platform allows for precise control of nutrient supply, waste removal, and mechanical forces, facilitating the creation of more complex and functional cellular models.

AUTHOR CONTRIBUTIONS

Ismail Eş: Conceptualization; investigation; writing – original draft; methodology; validation; writing – review and editing; software; funding acquisition; visualization; data curation. **Ana-Maria Theodora Ionescu:** Methodology; writing – review and editing; software; data curation. **Burak M. Görmüş:** Software; writing – review and editing; methodology. **Fatih Inci:** Writing – review and editing; visualization; funding acquisition; software. **Marco Marques:** Funding acquisition; writing – original draft; writing – review and editing; validation; resources. **Nicolas Szita:** Funding acquisition; supervision; writing – review and editing; project administration; resources. **Lucimara Gaziola de la Torre:** Supervision; project administration; writing – original draft; writing – review and editing; funding acquisition; resources.

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CONFLICT OF INTEREST STATEMENT

No conflicts of interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Wong CH, Siah KW, Lo AW. Estimation of clinical trial success rates and related parameters. *Biostatistics*. 2019;20(2):273-286. doi:10.1093/biostatistics/kxx069
2. Kelm JM, Timmins NE, Brown CJ, Fussenegger M, Nielsen LK. Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types. *Biotechnol Bioeng*. 2003; 83(2):173-180. doi:10.1002/bit.10655
3. Jedrzejczak-Silicka M. History of cell culture. *New Insights Cell Cult Technol*. 2017;1-41. doi:10.5772/66905
4. Sultan S, Siqueira G, Zimmermann T, Mathew AP. 3D printing of nano-cellulosic biomaterials for medical applications. *Curr Opin Biomed Eng*. 2017;2:29-34. doi:10.1016/j.cobme.2017.06.002
5. Raphael B, Khalil T, Workman VL, et al. 3D cell bioprinting of self-assembling peptide-based hydrogels. *Mater Lett*. 2017;190:103-106. doi:10.1016/j.matlet.2016.12.127
6. Simian M, Bissell MJ. Organoids: a historical perspective of thinking in three dimensions. *J Cell Biol*. 2017;216(1):31-40. doi:10.1083/jcb.201610056
7. Laschke MW, Menger MD. Life is 3D: boosting spheroid function for tissue engineering. *Trends Biotechnol*. 2017;35(2):133-144. doi:10.1016/j.tibtech.2016.08.004
8. Klymenko Y, Kim O, Loughran E, et al. Cadherin composition and multicellular aggregate invasion in organotypic models of epithelial ovarian cancer intraperitoneal metastasis. *Oncogene*. 2017;36(42): 5840-5851. doi:10.1038/onc.2017.171
9. Mittler F, Obeid P, Rulina AV, Haguet V, Gidrol X, Balakirev MY. High-content monitoring of drug effects in a 3D spheroid model. *Front Oncol*. 2017;7:7. doi:10.3389/fonc.2017.00293
10. Whitesides GM, Grzybowski B. Self-assembly at all scales. *Science*. 2002;295(5564):2418-2421. doi:10.1126/science.1070821
11. Souza GR, Molina JR, Raphael RM, et al. Three-dimensional tissue culture based on magnetic cell levitation. *Nat Nanotechnol*. 2010;5(4): 291-296. doi:10.1038/nnano.2010.23
12. Lin B, Miao Y, Wang J, et al. Surface tension guided hanging-drop: producing controllable 3D spheroid of high-passaged human dermal papilla cells and forming inductive microtissues for hair-follicle regeneration. *ACS Appl Mater Interfaces*. 2016;8(9):5906-5916. doi:10.1021/acsami.6b00202
13. Hookway TA, Butts JC, Lee E, Tang H, McDevitt TC. Aggregate formation and suspension culture of human pluripotent stem cells and differentiated progeny. *Methods*. 2016;101:11-20. doi:10.1016/j.ymeth.2015.11.027
14. Benien P, Swami A. 3D tumor models: history, advances and future perspectives. *Future Oncol*. 2014;10(7):1311-1327. doi:10.2217/fon.13.274
15. Park SM, Lee SJ, Lim J, Kim BC, Han SJ, Kim DS. Versatile fabrication of size- and shape-controllable nanofibrous concave microwells for cell spheroid formation. *ACS Appl Mater Interfaces*. 2018;10(44): 37878-37885.
16. Shakeri A, Khan S, Didar TF. Conventional and emerging strategies for the fabrication and functionalization of PDMS-based microfluidic devices. *Lab Chip*. 2021;21(16):3053-3075.
17. Wang Y, Kim MH, Tabaei SR, et al. Spheroid formation of hepatocarcinoma cells in microwells: experiments and Monte Carlo simulations. *PLoS One*. 2016;11(8):1-13. doi:10.1371/journal.pone.0161915
18. Li Y, Wang Y, Shen C, Meng Q. Non-swellable F127-DA hydrogel with concave microwells for formation of uniform-sized vascular spheroids. *RSC Adv*. 2020;10(72):44494-44502.
19. Kuo CT, Wang JY, Lin YF, Wo AM, Chen BPC, Lee H. Three-dimensional spheroid culture targeting versatile tissue bioassays using a PDMS-based hanging drop array. *Sci Rep*. 2017;7(1):4363.
20. Osaki T, Kageyama T, Shimazu Y, et al. Flatbed epi relief-contrast cellular monitoring system for stable cell culture. *Sci Rep*. 2017;7(1): 1897.
21. Patra B, Chen YH, Peng CC, Lin SC, Lee CH, Tung YC. A microfluidic device for uniform-sized cell spheroids formation, culture, harvesting and flow cytometry analysis. *Biomicrofluidics*. 2013;7(5):54114.
22. Tang Y, Liu J, Chen Y. Agarose multi-wells for tumour spheroid formation and anti-cancer drug test. *Microelectron Eng*. 2016;158:41-45.
23. Ivascu A, Kubbies M. Rapid generation of single-tumor spheroids for high-throughput cell function and toxicity analysis. *SLAS Discov*. 2006;11(8):922-932.
24. Shin JY, Park J, Jang HK, et al. Efficient formation of cell spheroids using polymer nanofibers. *Biotechnol Lett*. 2012;34:795-803.
25. Chen C, Rengarajan V, Kjar A, Huang Y. A matrigel-free method to generate matured human cerebral organoids using 3D-printed microwell arrays. *Bioact Mater*. 2021;6(4):1130-1139. doi:10.1016/j.bioactmat.2020.10.003
26. Sargenti A, Musmeci F, Bacchi F, et al. Physical characterization of colorectal cancer spheroids and evaluation of NK cell infiltration through a flow-based analysis. *Front Immunol*. 2020;11(December):1-13. doi:10.3389/fimmu.2020.564887
27. Yeon SE, No DY, Lee SH, et al. Application of concave microwells to pancreatic tumor spheroids enabling anticancer drug evaluation in a clinically relevant drug resistance model. *PLoS One*. 2013;8(9):1-12. doi:10.1371/journal.pone.0073345
28. Chen Y, Gao D, Wang Y, Lin S, Jiang Y. A novel 3D breast-cancer-on-chip platform for therapeutic evaluation of drug delivery systems. *Anal Chim Acta*. 2018;1036:97-106.
29. Moshksayan K, Kashaninejad N, Warkiani ME, et al. Spheroids-on-a-chip: recent advances and design considerations in microfluidic platforms for spheroid formation and culture. *Sens Actuators B*. 2018; 263:151-176.
30. Berens EB, Holy JM, Riegel AT, Wellstein A. A cancer cell spheroid assay to assess invasion in a 3D setting. *Jove (J Vis Exp)*. 2015;105: e53409.
31. Jain R, Gahlyan P, Dwivedi S, et al. Design, synthesis and evaluation of 1H-1, 2, 3-Triazol-4-yl-methyl tethered 3-Pyrrolylisatins as potent anti-breast cancer agents. *ChemistrySelect*. 2018;3(19): 5263-5268.
32. Gayan S, Teli A, Dey T. Inherent aggressive character of invasive and non-invasive cells dictates the in vitro migration pattern of multicellular spheroid. *Sci Rep*. 2017;7(1):1-11.

33. Lee GH, Lee JS, Oh HJ, Lee SH. Reproducible construction of surface tension-mediated honeycomb concave microwell arrays for engineering of 3D microtissues with minimal cell loss. *PLoS One*. 2016;11(8):1-16. doi:[10.1371/journal.pone.0161026](https://doi.org/10.1371/journal.pone.0161026)
34. Demuyneck R, Efimova I, Lin A, Declercq H, Krysko DV. A 3D cell death assay to quantitatively determine ferroptosis in spheroids. *Cells*. 2020;9(3):703. doi:[10.3390/cells9030703](https://doi.org/10.3390/cells9030703)
35. Vorwald CE, Ho SS, Whitehead J, Leach JK. High-throughput formation of mesenchymal stem cell spheroids and entrapment in alginate hydrogels-biomaterials for tissue engineering: methods and protocols. *Methods Mol Biol*. 2018;1758:139-149.
36. Gong X, Lin C, Cheng J, et al. Generation of multicellular tumor spheroids with microwell-based agarose scaffolds for drug testing. *PLoS One*. 2015;10(6):1-18. doi:[10.1371/journal.pone.0130348](https://doi.org/10.1371/journal.pone.0130348)

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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