



High-efficiency 3D cell spheroid formation via the inertial focusing effect in rotating droplets

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The three-dimensional (3D) spheroid culture has been widely used as an important tool in biological research. Although several techniques have been established to prepare cell spheroids, fast and controllable production remains one of the major challenges. In this study, a simple but efficient method utilizing the inertial focusing effect in rotating hanging droplets is demonstrated for the rapid and controllable production of cell spheroids. This method enables the formation of compact cell clusters in rotating hanging droplets within 10 min and the production of compact spheroids within 12 h, a significant time reduction compared to conventional methods. The size and shape of cell cluster can be regulated by adjusting cell density and droplet shape, respectively, and the feasibility and versatility of our method are validated using fibroblast and liver tumor spheroids. Our strategy is further utilized to design and successfully produce three different co-culture models to mimic different cell growth environments. This highly efficient technique for the production of controllable 3D cell models could play a crucial role in advanced biomanufacturing technology for drug screening, tissue engineering, and biomedical research.

Introduction

Although widely used in biomedical research, the conventional two-dimensional monolayer cell culture in a Petri dish lacks several important tissue-specific properties (i.e., mechanical signals, tissue architecture, and cell interaction), resulting in experimental limitations [1, 2]. To mimic the *in vivo* environment of cell development in tissues, 3D cul-

ture models (e.g., multicellular 3D spheroids) have been designed for basic research and therapeutic studies preceding animal experiments [3]. First developed by Holtfreter and Moscona through cell self-assembly in a suspension, multicellular 3D spheroids exhibit many characteristics similar to natural tissues, such as internal structure, cellular heterogeneity, cell signaling pathways and gene expression [4]. It has been found that cell spheroids can secrete extracellular matrix (ECM) and mimic cell-to-cell and cell-to-ECM communication, reflecting the biological and mechanical signals in real physiological processes [4]. Compared to the monolayer culture, the close contact of cells in the spheroid model can better simulate the physical interactions and signal transduction pathways [5]. Additionally, due to the nonuniform nutrient distribution and lactate accumulation, cells in spheroids are arranged in metabolic and proliferative gradients along the radial direction, similar to those found in real tissues including solid tumors. In addition, as a result of glutamate consumption, ammonium also accumulates in the proliferative area of cell spheroids, which leads to higher proliferation rate and lower glucose utilization [6–8]. This heterogeneous microenvironment will lead to chronically acidic and hypoxic effects in solid tumors, producing many therapeutic issues including chemoresistance [3]. Therefore, multicellular 3D tumor spheroids are commonly used to mimic these effects, to facilitate exploring the resistance of chemotherapeutic drugs [9]. Similarly, different kinds of cell spheroids, including breast, liver, lung, colon, pancreas and skin, have been widely used to mimic various features of tissues *in vitro* [5, 10–12].

Several methods have been developed over the last decades to produce multicellular 3D spheroids, including the hanging drop method, liquid overlay culture, rotating bioreactor culture, and external force method (i.e., electric field force, magnetic force, and ultrasound) [2, 13–15]. To satisfy the general requirements for 3D models, methods to produce cell spheroids should feature easy preparation, high efficiency, high biocompatibility, and low cost, similar to the traditional monolayer culture system. However,

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some existing methods require at least two days to form compact multicellular cell spheroids. For the rotating bioreactor method, the time requirement could be as long as 15 days [16]. This long modeling time and the resulting low efficiency are limiting factors for many applications of 3D culture, which ideally targets the fast production of high-density spheroids [17]. In 3D bioprinting, for example, multicellular 3D spheroids as the building blocks need to be rapidly produced for their structural stability and viability [18–20]. Otherwise, the viability of cells will be affected by low nutrient levels in the core of spheroids during the long period of compact spheroid formation [19]. Other potential adverse effects (e.g., stress and toxicity) in some of the complicated preparation methods could also affect cell physical function and produce unreasonable outcomes. It has been reported that a rotating bioreactor could generate shear stresses that are continuously applied on cells, which may damage stress sensitive cells [4, 16]. In addition, the electrophoresis liquid or magnetic iron oxide directly contacts cells in the electric field, and magnetic force methods could also lead to impaired cell viability [16]. Recently, there have been indeed a number of efforts trying to reduce the production time of cell spheroids, but these usually involve complicated setups, including acoustic streaming and microfluidics [21].

In this work, we introduce a simple and highly efficient method for the rapid production of cell spheroids. The desired impact is achieved by introducing a concentrating flow inside the droplet that is generated by the inertial focusing effect, to the conventional hanging droplet method. By adding rotational motions via a table concentrator, the resulting concentrating flow can efficiently aggregate the cells in the hanging droplet. We studied the inertial effect formation in rotating hanging droplet by the simulation approach and optimized the procedure to form cell clusters. Despite the novelty of a simple modification, our approach significantly reduces the time requirement for cell aggregation, improves the productivity of cell spheroid culture upon microscale mixing, and has high biocompatibility. By changing the shape of droplets, we demonstrated the possibility of controlling the morphology of cell aggregates and the resulting cell spheroid. Using this technology, we also developed three common co-culture methods to mimic cell-to-cell and cell-to-ECM interactions. Finally, we verified the biological function of 3D tumor models produced with our method. When treated with the chemotherapeutic drug doxorubicin (DOX), the 3D tumor model showed similar behaviors in drug distribution and cell viability to those of real cancer tissues.

Workflow

Step I: Hanging droplet formation

In order to generate 3D cell spheroids, the hanging drop technique can be employed by utilizing liquid surface tension. Compared with other 3D culture methods, this technique has inherent advantages. First, no additional materials or supplements are required for spheroid formation. In addition, the method is easy to execute and requires no complicated skills. The hanging drop technique is biocompatible, allowing for further tissue biology studies. As shown in Fig. 1, hanging drops are prepared by placing cell suspension droplets on the lid surface of a Petri dish. After inverting the lid, hanging drops are formed due to liquid surface tension. Prior to use, the cell suspension is diluted to a certain concentration, which contains controllable cell numbers to adjust the diameter of cell spheroids, as shown in Fig. 2d.

Step II: Droplet rotation

A key step in our method is to provide a rotational motion to the hanging droplets to facilitate the aggregation of suspended cells. This can be achieved by placing the Petri dish in a table concentrator that generates circular motion at a fixed speed for a period of time. In this process, a hydrodynamic effect called the inertial focusing effect can be generated, which concentrates the suspended cells to the bottom of the hanging droplet. Here, the morphology of cell aggregates can be observed and checked by using a regular optical microscope. For the specific type and concentration of cells, the rotation speed and duration can be optimized to achieve the desirable assembly of cells at the end of this step. As an example, the morphological features of a fibroblast cell cluster after different rotation speeds and times are shown in Fig. S1 (Supplementary Information) and Fig. 3.

Step III: Cell incubation

After treatment in the table concentrator, the hanging droplets are incubated under the required conditions, e.g., 37 °C with 5% CO₂ for 12 to 24 h, without disturbance. To reduce the evaporation effect, the Petri dish with the hanging droplets should be placed inside another Petri dish containing sterile phosphate buffered saline (PBS) in the bottom part.

Step IV: Biological testing

The 3D samples can be collected and treated for biological analysis after being cultured in an incubator. For direct microscopic observations, such as the assessment of cell mor-

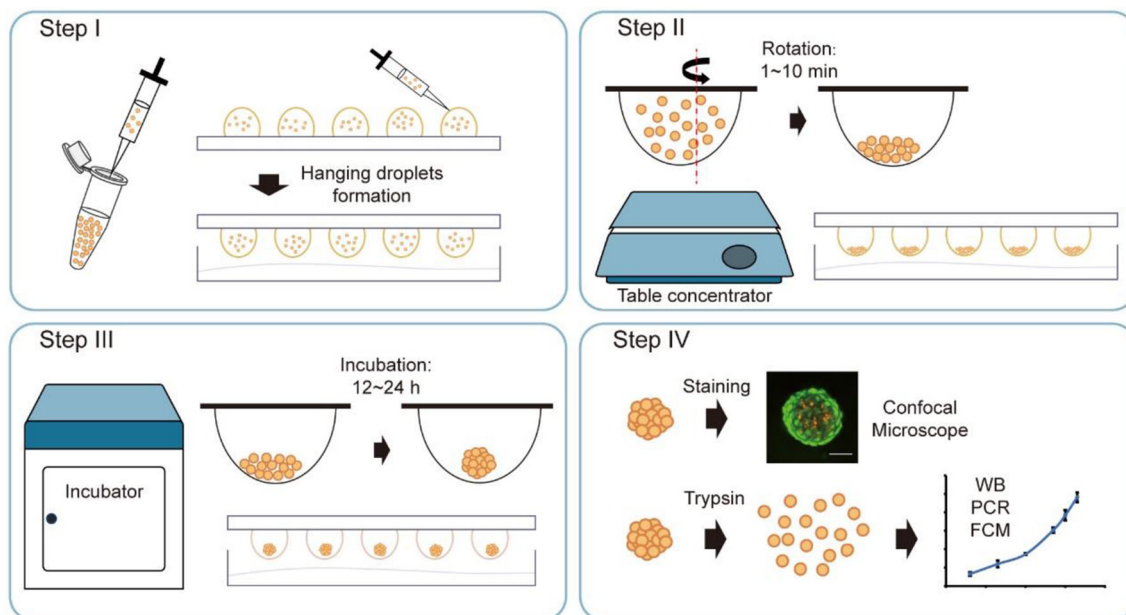


Fig. 1 Workflow sketch of an efficient way to achieve 3D cell spheroid formation using the inertial focusing effect in rotating droplets. WB: western blot; PCR: polymerase chain reaction; FCM: flow cytometry

phology, cell viability, and immunofluorescence, the 3D cell spheroids can be simply stained using specific fluorescent probes and observed using optical or confocal microscope. For other biological assays that require individual cells, such as flow cytometry, western blot, and polymerase chain reaction, 3D samples can also be dispersed using trypsin.

Examples

Experimental methods

In order to evaluate the feasibility of our approach, human skin fibroblasts (HSF) and hepatocellular cancer cells (HepG2) were cultured using the proposed method. HSF and HepG2 cells were cultured in Dulbecco's Modified Eagle Medium, high glucose (DMEM-H) cell culture medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin-amphotericin B. Prior to use, the HSF cell suspension was diluted to 4.0×10^3 , 2.0×10^4 , 1.0×10^5 , 5.0×10^5 , and 2.5×10^6 cells/mL. HepG2 cells were mixed with HSF cells in 1:1 ratio, and the cell number was adjusted to 1.0×10^5 per milliliter. To establish the cell spheroid, 10 μ L of cell suspension was placed on the surface of a 35-mm Petri dish (Corning, Cat No. 430165), which was then inverted to create hanging droplets of the cell suspension. Subsequently, the Petri dish was placed in a table concentrator that provided circular motion at 100 r/min for 10 min. The morphological characteristics of 3D cell culture models were obtained using an optical microscope and a confocal microscope.

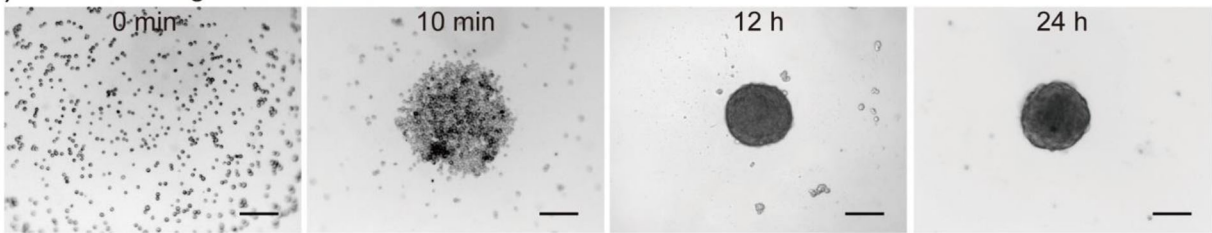
To determine the maturation of cell spheroids, laminin and E-cadherin were labeled by immunofluorescence staining. Anti-laminin (1:100, Proteintech) and anti-E-cadherin (1:100, Beyotime) as primary antibodies were incubated with the cell spheroids overnight at 4 °C. This was followed by incubation with secondary antibodies, including anti-mouse FITC (1:50, Proteintech) and anti-rabbit TRITC (1:50, Proteintech), for 1 h at 37 °C. Then, the cell nuclei were stained with DAPI. Immunofluorescence in the cell spheroids was photographed via a confocal microscope. The fluorescent signal intensity was quantified by ImageJ software.

In order to evaluate the anti-cancer drug treatment effect, the chemotherapy drug DOX was used to test the response of tumor spheroids. The tumor spheroids with HepG2 cells produced by the inertial effect were cultured with various doses of DOX for 4 h. Next, DOX distribution in the tumor spheroids was observed under a confocal microscope. After being cultured for 12 h, the tumor spheroids were washed with PBS twice and stained with calcein-AM/PI to assess cell viability. The cell spheroids were photographed via a confocal microscope. Finally, the fluorescent signal intensity was quantified using ImageJ software.

Experimental results

The procedure employed in this study to form cell spheroids through the inertial focusing effect is shown in Fig. 1. In an example shown in Fig. 2a, an aggregate of cells can be observed in the bottom of the droplet after 10 min of rotation at 100 r/min, and uniform cell spheroids are formed after 12

(a1) Inertial focusing effect method



(a2) Traditional hanging drop method

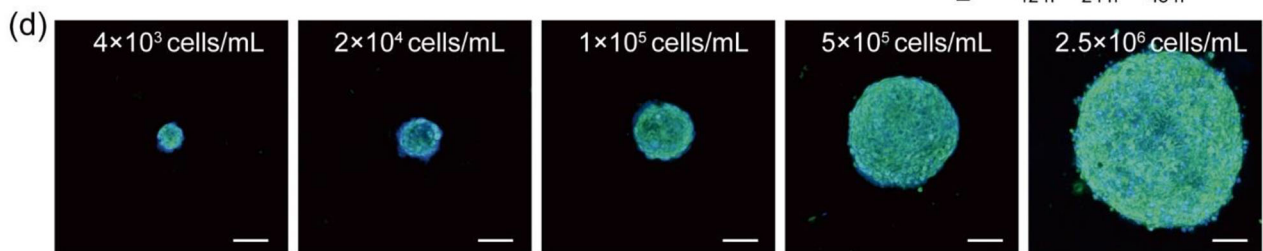
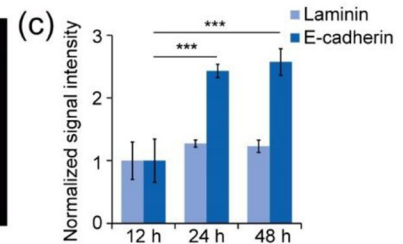
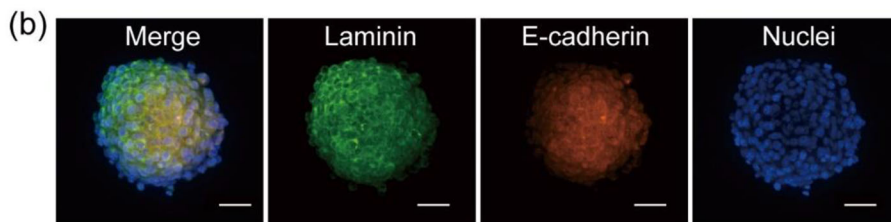
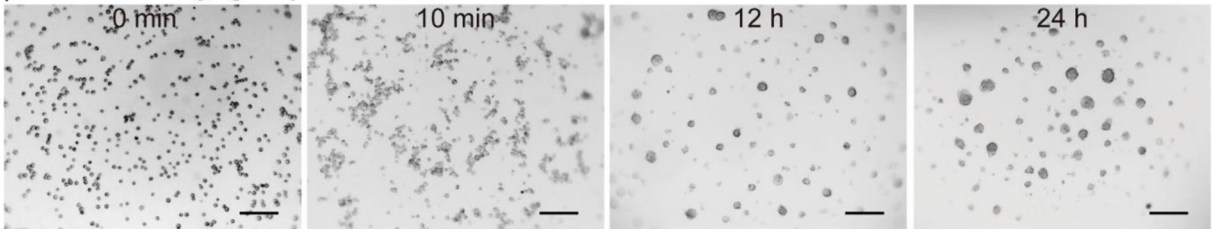


Fig. 2 The inertial focusing effect resulted in the rapid formation of cell spheroids. **a** Optical micrographs showing the morphology of cell aggregates using (a1) the inertial effect and (a2) the traditional hanging drop method at 0 min, 10 min, 12 h, and 24 h. Scale bars: 100 μm . **b** Immunofluorescence staining of laminin (green), E-cadherin (red), and nuclei (blue) in the cell spheroids produced by the inertial effect and cultured for 12 h. Scale bars: 50 μm . **c** Normalized signal intensities

of laminin and E-cadherin fluorescence in the cell spheroids cultured for 12, 24, and 48 h. **d** Fluorescent confocal images of cell spheroids of various sizes produced using different cell densities. Cell membranes were stained green by DIO and cell nuclei were stained blue by DAPI. Scale bars: 100 μm . The data were analyzed by unpaired Student's *t* test with statistical significance levels at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$

h in a cell incubator. By contrast, only small cell clusters are observed in the hanging droplets without rotation after 10 min, and several small cell spheroids with nonuniform size and shape are formed after either 12 or 24 h. This difference clearly demonstrates that the inertial effect method can more effectively produce cell spheroids with uniform size and shape, in comparison with the traditional hanging drop method. Moreover, cell spheroids with various diameters, as shown in Fig. 2d, were produced by using different cell densities (4.0×10^3 , 2.0×10^4 , 1.0×10^5 , 5.0×10^5 , and 2.5×10^6 cells/mL) in the hanging droplets. We found that the diameter of cell spheroids increases with the cell number in hanging

droplets, which indicates that the size of cell spheroids can be controlled accurately through varying the cell density in the hanging droplets.

In order to test the maturation of the cell spheroids produced using the inertial effect, laminin and E-cadherin were fluorescently labeled and observed under the confocal microscope after a culture period of 12, 24, and 48 h. As shown in Fig. 2b, the laminin and E-cadherin are clearly expressed in cell spheroids cultured for 12 h. For laminin, the amount of expression in cell spheroids cultured for 12, 24 or 48 h shows no significant difference, as seen in Fig. 2c. Meanwhile, the expression of E-cadherin is continuously enhanced from 12

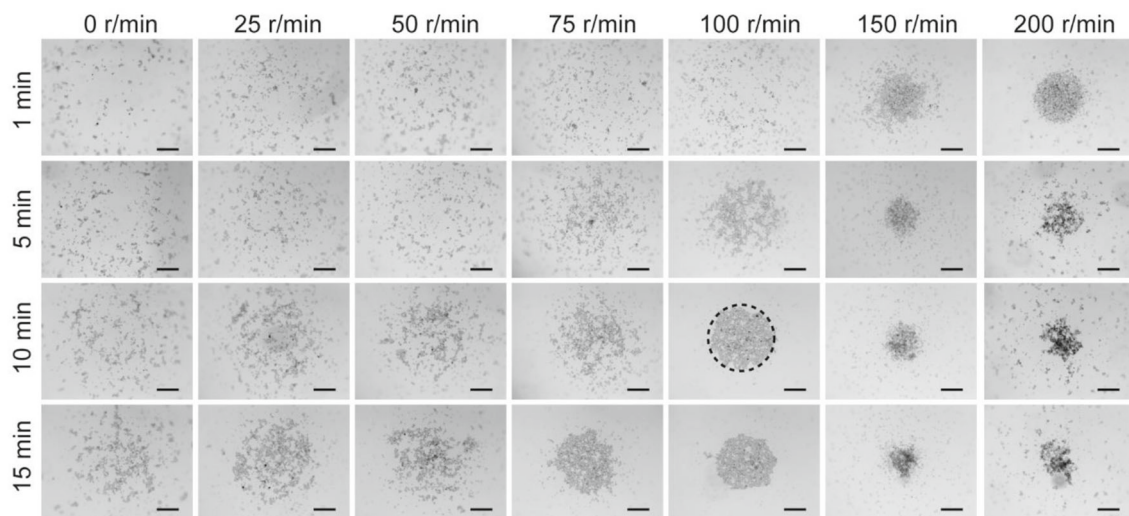


Fig. 3 Effect of rotating speed and time on cell cluster formation in droplets. Optical micrographs of the cell clusters formed at the bottom of droplets after various rotating speeds and times. Scale bars: 100 μm

to 24 h and then becomes stable, as shown in Fig. 2c. This result demonstrates the formation of extracellular matrix, as indicated by laminin, and its maturation after the 12 h culture period in the cell spheroids produced with our method. Moreover, abundant E-cadherin, a typical cell adhesive mediator, can be observed in the cell spheroids after the 12 h culture period, indicating the formation of compact cell spheroids with clear cell–cell junctions at this time, in which cells are adhered by E-cadherin. These junctions continue to be enhanced during 24 h culture. The distribution of laminin and E-cadherin in cell spheroids cultured for 24 and 48 h can be seen in the confocal micrographs (Fig. S2a in Supplementary Information). The micrographs showing the morphology of cell spheroids cultured for different periods of time (up to 7 days) are shown in Fig. S2b (Supplementary Information). Apparently, the cell spheroids remain relatively stable in shape and size after 12 h of culture and exhibit a slight increase in diameter with the incubation time.

Aiming to understand the efficient formation of cell clusters achieved by our improved method, we investigated the potential inertial focusing effect in the rotating hanging droplets. Using computational fluid dynamic software (COMSOL Multiphysics), we simulated the fluid flow inside the hanging droplet model with a diameter of 4 mm droplet subjected to rotational motion. The simulation results revealed a symmetrical fluid flow pattern on each side of the droplet, as shown in Fig. S3 (Supplementary Information). This flow pattern would result in an inertial focusing effect that is responsive for the migration and concentration of small particles, including cells, to the bottom of the droplet, thereby facilitating the aggregation of cells. By contrast, in hanging droplets without rotations, cells would have to rely on gravity

to form clusters. A detailed discussion regarding the inertial focusing effect can be found in Supplementary Information.

In the inertial focusing effect, flow speed can affect the intensity of inertial lift force and Dean drag force [22]. Therefore, we studied the effect of various rotating speeds (0, 25, 50, 75, 100, 150, and 200 r/min) on the cell cluster formation for different times (1, 5, 10, and 15 min), as shown in Fig. 3. The results demonstrated that the cells aggregated in the droplet center faster at higher rotating speeds. Especially, an optimal circular-disk-shaped cell cluster was formed at a rotation speed of 100 r/min for 10 min. With the increase in rotating speed, i.e., 150 and 200 r/min, although a similar cell cluster can be formed in a shorter period of time, many single cells are left out from the aggregate. For longer rotating time, cells tend to overlap, resulting in uncontrollable cell cluster shape. On the other hand, for lower rotating speeds, such as 25 and 50 r/min, no apparent cell cluster is observed even after the droplet is rotated for 15 min.

In addition, our method offers a potential to customize the shape of cell cluster by applying a hydrophilic pattern on the surface of the Petri dish and generate hanging droplets with different shapes. This additional step enables producing 3D cell models with non-spherical shapes. The detailed steps and results on the shape control of cell clusters can be found in Supplementary Information.

Moreover, 3D co-culture models can be produced based on the novel approach. By combining two types of cells using different procedures, three 3D co-culture models were developed using our method to mimic three different cell growth environments. These 3D co-culture models can be used to study cell-to-cell or cell-to-ECM interactions in biological tissues, which play important roles in the pathogenesis of several challenging conditions, including cancer and skin dis-

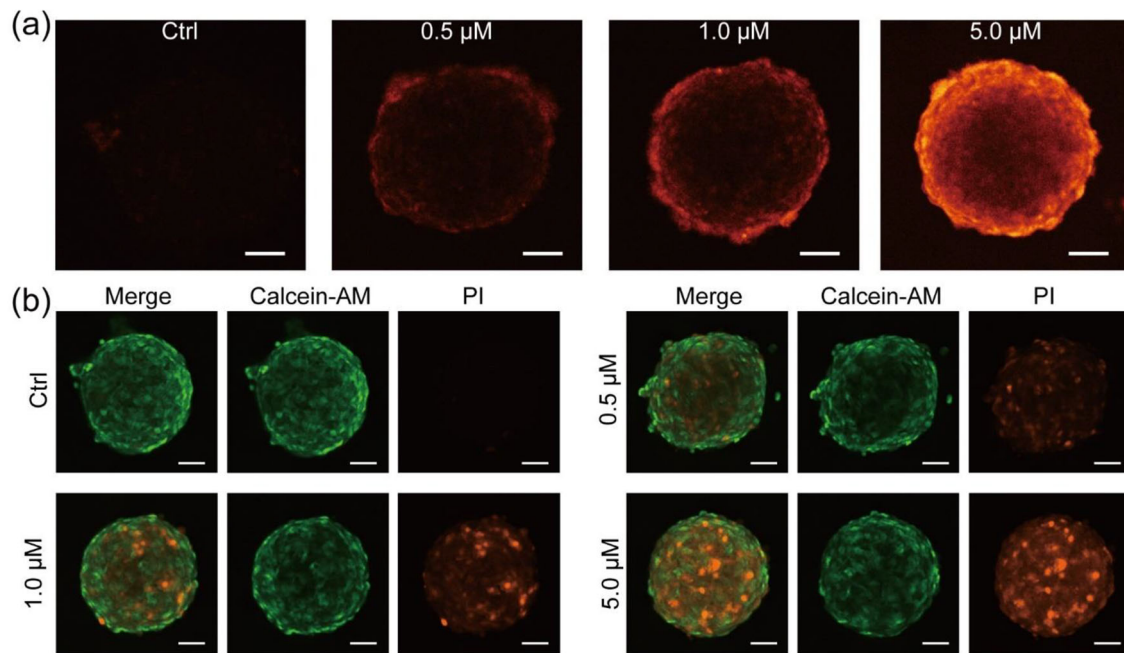


Fig. 4 Demonstration of the anti-cancer effect of DOX within tumor cell spheroids. **a** Confocal microscope images showing the diffusion gradient of DOX according to dosage (i.e., 0.5, 1.0, and 5.0 μM) applied

to tumor spheroids. **b** Fluorescent confocal micrographs of live cells (green) and dead cells (red) in the tumor spheroids after being treated with DOX. Scale bars: 50 μm

eases [23]. A detailed discussion about the co-culture model can be found in Supplementary Information.

To demonstrate the practicability of our approach in mimicking the behaviors of solid tumor tissue, we tested the responses of tumor spheroids produced by our method by subjecting them to treatment with the chemotherapy drug DOX. We focused on the gradient distribution of this drug and nutrients in the tumor spheroids, to represent the response of real tumor tissues [16]. As shown in Fig. 4a, the fluorescence signal of DOX in the cell spheroids indeed increased with the drug concentration applied to the spheroid. In addition, the fluorescence signal intensity was the highest at the outmost layer of the spheroids, and it gradually decreased inwards. The overall DOX fluorescent signal intensities are shown in Fig. S6a (Supplementary Information), showing an apparent increase with the rise of drug concentration. The spatial distribution of DOX signals of 5.0 μM DOX is also shown in Fig. S6b (Supplementary Information), which presents a gradient distribution from the shell layer to the center. These results suggest the occurrence of a slow diffusion of DOX into our cell spheroids, which is similar to the behavior of chemotherapy drugs applied to solid tumor tissues [24]. The effect of DOX was also confirmed by the viability tests of tumor cells in the spheroids after treatment with various dosages of DOX (i.e., 0.5, 1.0, and 5.0 μM). As shown in Fig. 4b, the red fluorescence, representing dead cells, increased with the dosage of DOX. The results revealed that the higher dosage of DOX,

the more dead cells were detected in the tumor spheroids. A similar phenomenon was shown by the quantification of red signal intensity, as shown in Fig. S6c (Supplementary Information). These results indicated that tumor spheroids produced using our approach are indeed capable of mimicking the response of tumor tissues subjected to chemotherapy drugs at different dosages.

We further demonstrated the applicability of our method by preparing hair follicle stem cell spheroids. Although hair follicle stem cells possess the potential to differentiate into hair follicle cell lineages after induced by hair follicle cells [25], the induction of hair follicle stem cells is faded in a monolayer culture method [26]. Therefore, the 3D culture of hair follicle stem cells is believed to be vital for the morphogenesis of hair follicle and sebocyte glands in hair regeneration engineering [26]. Using our method incorporating the inertial effect, hair follicle stem cell spheroids were successfully produced after 24 h, as shown in Fig. 5. Most importantly, the presence of α -smooth muscle actin, the marker of hair follicle dermis cell, was observed in hair follicle stem cell spheroids after 24 h, indicating that the hair follicle stem cell spheroids produced using our method are capable of differentiating into hair follicle dermis cells.

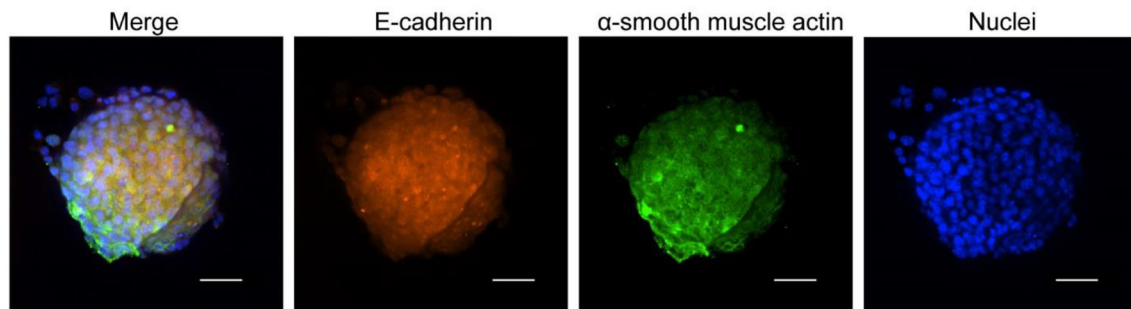


Fig. 5 Immunofluorescence staining of E-cadherin (red), α -smooth muscle actin (green), and nuclei (blue) in hair follicle stem cell spheroids produced by the inertial effect method. Scale bars: 50 μ m

Discussion

In this work, we developed and tested an inertial focusing effect based method that can efficiently produce 3D multicellular models in a controllable manner without using special equipment. This approach has the potential to overcome current challenges in the cell spheroid production process, such as long preparation time, low cell viability, and high cost. We demonstrated that, by utilizing the inertial focusing effect generated in rotating hanging droplets, dispersed cells can rapidly aggregate at the bottom of the droplet, facilitating the self-assembly process for cell spheroid formation. This non-intrusive feature of inertial focusing effect makes our method advantageous compared to other 3D culture methods that are developed based on external force to promote the close packing of cells [27]. For example, the traditional hanging drop method uses gravity to force cell aggregation [21]; however, this method requires at least 2 days to form cell spheroids. Further methods were developed to reduce the modeling time by convening cells by additional external forces, including magnetic force, electrophoretic force and ultrasound [13, 28, 29]. However, these apply additional materials such as superparamagnetic iron oxide and electrophoresis buffer, which may affect cell viability and bring potential negative side effects to the physical function of cells [30]. By contrast, our method only applies the inertial focusing effect to efficiently aggregate cells in a hanging drop without the addition of further materials, thus demonstrating better biocompatibility. By optimizing the processing parameters (i.e., rotating speed and time), uniform cell spheroids can be readily and reliably produced within 12 h, a significant decrease in preparation time compared to the 2–4 days requirement of conventional methods. More importantly, cell models with non-spheroid shapes can be achieved by controlling the shape of hanging droplets through patterned surfaces, which demonstrates the potential of our approach in regulating the shapes of cell aggregates for tissue engineering. Moreover, based on the proposed method, we constructed three co-culture models to mimic different cell growth environments. These mod-

els showcase the versatility of our method and its potential applications in diverse biomedical areas, including cancer, regenerative medicine, organoids, or drug discovery. Using our approach, a liver tumor spheroid model was successfully established, which exhibited similar drug diffusion to that in tumor tissue. In the future, other types of spheroids can be produced based on this strategy. It is worth noting that there are still a few limitations for our method. For example, this technique currently involves manual handling steps for droplets and spheroids. Therefore, a limited number of hanging droplets are generated for each batch, which results in the lack of high-throughput production ability of cell spheroids. These limitations can be potentially overcome by combining with other advanced processing methods, including photoetching, laser processing, and plasma treatment, to aim for the batch-production of hanging droplets and thus prepare cell spheroids in a high-throughput manner. We believe that our novel approach for the efficient production of diverse 3D cell models will promote the further development of biomanufacturing techniques for drug screening, tissue engineering, and basic biomedical research.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s42242-022-00211-5>.

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Author contributions TT, PZ, and YX conceived the study; TT and YX conducted the investigation and wrote the original draft; PZ, HJ, and YW contributed to data analysis and manuscript editing; all authors reviewed and commented on the manuscript.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval This study does not contain any studies with human or animal subjects performed by any of the authors.

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