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Using pipette tips to readily generate spheroids comprising single or multiple cell types

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Abstract: Three-dimensional (3D) cell culture methods have been validated that can replicate the tumor environment in vivo to a large extent, providing an effective tool for studying tumors. In this study, we demonstrated the use of standard laboratory pipette tips as micro vessels for generating 3D cell spheroids. No microfabrication or wet-chemistry surface modifications were involved in the procedure. Spheroids consisting of single or multiple cell types were generated within 24 h just by pipetting and incubating a cell suspension in pipette tips. Scanning electron microscope and optical microscope proved that the cells grew together tightly, and suggested that while gravity force might have initiated the sedimentation of cells at the bottom of the tip, the active aggregation of cells to form tight cell-cell interactions drove the formation of spheroids. Using common laboratory micropipettes and pipette tips, the rate of spheroid generation and the generation reproducibility was characterized from five boxes each with 80 tips. The ease of transferring reagents allowed modeling of the growth of microvascular endothelial cells in tumor spheroids. Moreover, the pairing and fusion of tumor spheroids could be manipulated in the pipette tips, suggesting the potential for building and assembling heterogeneous micro-tumor tissues in vitro to mimic solid tumors in vivo. This study demonstrated that spheroids can be readily and cost-effectively generated in standard biological laboratories in a timely manner using pipette tips.

Key words: Pipette tip; 3D cell culture; Tumor spheroids; Co-culture; In-situ observation

1 Introduction

Cancer is a leading cause of death and a huge barrier to increasing life expectancy worldwide (Bray et al., 2021). Though tremendous efforts have been made, the incidence and fatality rates of cancer are still viewed as one of humanity's most significant challenges. In addition to metastasis of cancer cells and the side effects of chemotherapy, cancer treatment faces considerable limitations due to the tumor environment in vivo, which is difficult to replicate in vitro using current technologies (Li et al., 2021). This makes the detailed study of tumors very difficult. Studying tissue structures and intercellular connections between cells has become essential for understanding the nature of tumors (Lee et al., 2021; Jin et al., 2022). However, to rebuild and replicate the tumor environment is extremely difficult. The most common cell culture method is 2D cell culture in a petri dish (Kadletz et al., 2015). 2D cell culture is used in many cell culture fields because of its convenience and cost-efficiency, but the cell morphology observed in 2D culture is very different from that of a tumor in vivo, which can lead to inconsistency in follow-up studies (Lee et al., 2018). To overcome this deficiency, 3D cell culture was proposed. The development of 3D cell culture is a great leap forward for cell culture technology. It soon became favored by many researchers because the in vivo tumor environment can be simulated to some certain extent (Nunes et al., 2019). Diverse 3D cell culture strategies have been proposed, including scaffold-based methods (Costa et al., 2016; Yang et al., 2019) and non-scaffold-based methods, such as liquid overlay technology (LOT) (Ivascu and Kubbies, 2006; Metzger et al., 2011; Costa

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et al., 2014), hanging drop cell culture (Tung et al., 2011; Shri et al., 2017), agitation-based technology (Han et al., 2006; Liu et al., 2006; Lee et al., 2011), microfluidics (Castiaux et al., 2019; Khot et al., 2020), and layer-by-layer (LbL) (Horiguchi and Sakai, 2015; Fukuda et al., 2018; Wang et al., 2018; Aljadi et al., 2022; Carvalho et al., 2022) methods. Though succeeding in generating tumor spheroids, these 3D culture methods face various challenges (Breslin and O'driscoll, 2013; Aljadi et al., 2022). For example, a challenge for scaffold-based 3D cell cultures is to find a scaffold which has not only enough mechanical strength but also low toxicity. As for LOT, repeatability is a challenge. One technical problem of hanging drop cell culture is the stability of the drops. Experimenters must be careful to avoid droplets falling or deforming. The size uniformity of spheroids produced by agitation-based technology is of concern. Also, stirring is an essential process for agitation-based technology, but it can cause a certain degree of damage to cells while maintaining them in suspension. Microfluidics need expensive instruments and sophisticated channel designs. A simple, fabrication-free, user-friendly, and high-throughput method is greatly needed for routine use in biological laboratories to reproducibly generate cell spheroids.

Here, we introduce a high-throughput, simple, and time- and cost-effective method to generate cell spheroids. We used pipette tips, the standard commercial and universal laboratory items, as unique cell culture vessels. Pipette tips offer several advantages. First, the pipetting action can precisely aspirate a defined volume of cells in suspension. Second, the defined shape and material of the tip head restrains cell adhesion and promotes cells to form spheroids. Third, a multichannel pipette facilitates the high-throughput generation of cell spheroids. The dynamics of cell spheroid formation at different cell densities was quantified. In addition, the potential for producing spheroids with multiple cell types, spheroid-spheroid pairing, and testing of drug sensitivity on tumor spheroids was demonstrated using the pipette-tip-based 3D culture platform.

2 Materials and methods

2.1 Materials and reagents

Human prostate cancer cells (DU145) and human umbilical vein endothelial cells (HUVECs) were bought from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Gaithersburg, USA) containing 10% fetal bovine serum (Gibco), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 ℃ in a 5% CO_2 atmosphere. The long-term fluorescence tracer Dio, Calcein-AM, and propidium iodide (PI) were purchased from Beyotime Biotechnology, China. Sterilized pipette tips (10 μL) and gel-loading pipette tips (1–200 μL) were purchased from Biosharp, China. Doxorubicin (DOX) used for drug testing was purchased from Aladdin, China.

2.2 Generation of spheroids of a single cell type in pipette tips

For single cell type spheroid formation, human prostate cancer cells (DU145) were harvested from the culture plate to prepare cell suspensions with different cell densities. The ready-to-go procedure for generating single cell type spheroids is detailed in Fig. 1a. First, a DU145 cell suspension with a cell density of 2×10^6 cells/mL was prepared. Then, 10-μL cell suspension was loaded into the tips. Next, the cell-loaded tips were unloaded and docked onto the tip box, which was filled with 50-mL sterilized deionized water to reduce evaporation of the medium inside the pipette tips. The tip box was placed in a cell incubator (37 ℃ and 5% CO_2). The cell culture medium was replaced every 24 h using sharp gel-loading pipette tips. The cell-loaded tips were placed under a microscope (TS100-F, Nikon, Japan), and cell images were directly captured. In addition, after 1 d of incubation, the spheroids were harvested by pipetting. The cell aggregates formed by the cell density of 2×10^6 cells/mL were collected in a 6-well microplate and images were captured using a desktop scanner (V800, Epson, Japan). The number and size of the spheroids were characterized using the particle analysis function of ImageJ software (NIH, USA).

2.3 Generation of spheroids with multiple cell types in pipette tips

Two procedures were conducted to generate spheroids consisting of multiple cell types. To facilitate the observation and tracing of cells under fluorescent microscope, the live cell fluorescent dye, Dio (10 μg/mL), was used to stain cells according to product protocols. In brief, Dio was added after cells were collected in a 15-mL centrifuge tube. The cells were incubated at

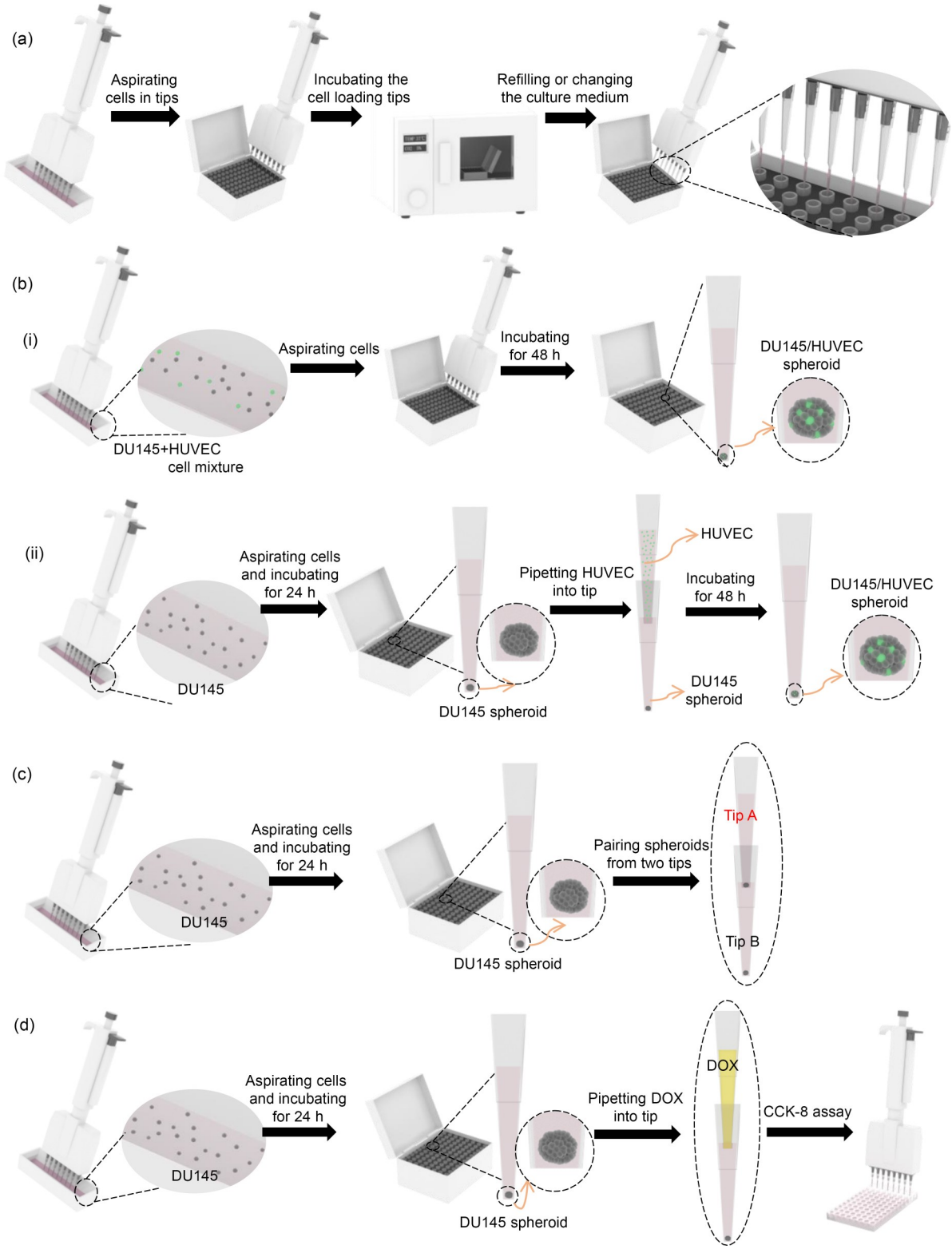

Fig. 1 Ready-to-generate cell spheroids in pipette tips: (a) generating spheroids of a single cell type in pipette tips; (b) generating spheroids with multiple cell types in pipette tips (one-step co-culture method (aspirating cell mixture) (b-i) and two-step co-culture method (adding HUVEC into a tip containing a DU145 spheroid) (b-ii)); (c) assembling and pairing of tumor spheroids; (d) evaluating drug efficacy using tumor spheroids in pipette tips

37 °C for 10 min and then at 4 °C for 15 min. Next, the stained cells were washed three times with phosphate buffered saline (PBS, pH 7.4). Dio-labeled HUVEC cells were adjusted to different cell densities, then two different manipulations were performed (Fig. 1b). (i) One-step co-culture method: DU145 and HUVEC cells were mixed in a 5∶1 ratio. The mixed cell suspension was pipetted into the tips simultaneously. In detail, 200-μL DU145 (1×10^6 cells/mL) and 200-μL HUVEC (2×10^5 cells/mL) were mixed in a container. Then 10-μL mixed cell suspension was aspirated into the tips which were placed in a tip box for cell culture. The process of spheroid formation was monitored under a microscope. (ii) Two-step co-culture method: DU145 (1×10^6 cells/mL) cells of 10 μL were loaded into the tips which were placed in a tip box for cell culture. After 24 h of culturing, 10-μL HUVEC (2×10^5 cells/mL) cells were added to the DU145-preloaded tips from the upper end of the tips and incubated continuously. The process of spheroid formation was monitored under the microscope.

2.4 Pairing and fusion of spheroids in pipette tips

The procedure for the pairing and fusion of two spheroids is shown in Fig. 1c. In brief, 10-μL cell suspension was loaded into the pipette tips (Tip A, Tip B) and incubated for 24 h. Then a cell spheroid formed in Tip A was gently pipetted into Tip B which contained another cell spheroid. The interaction of the two cell spheroids was tracked under the microscope.

2.5 Characterization of the cell spheroids generated in pipette tips

Scanning electron microscope (SEM) characterization: An SEM (SU3500, Hitachi, Japan) was used to examine the cell spheroids collected from the pipette tips. DU145 cell suspension (2.0×10^6 cells/mL) of 10 μL was loaded in the tips and incubated for 24 h in a cell culture incubator. Then, the cell spheroids were collected for fixation and dehydration. In brief, the cell spheroids were fixed in 4% paraformaldehyde solution and then dehydrated successively in a concentration gradient of ethanol solutions (50%, 60%, 70%, 80%, 90%, and 100%). Samples were dried overnight in air after the gradient dehydration was completed. Before SEM measurement, samples were sprayed with platinum for 360 s using an Auto Fine Coater (JEC-3000FC, JEOL, Japan) to facilitate SEM observation.

Live and dead cell staining: DU145 cell suspension (2.0×10^6 cells/mL) of 10 μL was loaded in the tips and cultured in a cell incubator. After 1, 3, or 5 d of incubation, the spheroids were pipetted into a 96-well plate for live and dead cell staining. In brief, a working solution containing 2-μmol/L Calcein-AM and 8-μmol/L PI was prepared in PBS (pH 7.4) for spheroid staining. After incubation for 1 h at 37 °C, the spheroids were washed with fresh PBS buffer three times to remove the excess dyes. Then, images of the spheroids were captured using an inverted fluorescence microscope at 645-nm excitation for PI and 530-nm excitation for Calcein-AM.

2.6 Three-dimensional tumor spheroids in pipette tips for drug testing

A concentration series of DOX was prepared to treat the cell spheroids. DU145 cell suspension (1.0×10^6 cells/mL) of 10 μL was loaded in the tips and incubated for 1 d. DOX solution of 10 μL was added to each tip. After 2 d of incubation, to quantify the antitumor effect of DOX, the percentage of live cells was measured using the CCK-8 assay (Fig. 1d). In brief, spheroids in the pipette tips were digested using Accutase™ stem cell dissociation reagent. Then, cells were suspended in 100-μL culture medium and placed in a 96-well plate. Next, CCK-8 reagent was added to the cells at a final concentration of 10% and incubated for 2 h at 37 °C. Finally, the absorbance was measured at 450 nm with a reference wavelength of 630 nm using an ELx800™ microplate reader (GENE, Hong Kong, China). The cell viability was calculated as the ratio of the percentage absorbance in DOX-treated cells and untreated cells.

2.7 Statistical analysis

All experiments were performed three times. Data are expressed as the mean±standard deviation. Data were analyzed with the Student's *t*-test using Origin Statistic software (OriginLab, USA). *P* values less than 0.05 were considered to be statistically significant.

3 Results

3.1 Cell aggregation and spheroid formation facilitated by ready-to-go universal pipette tips

Fig. 2a shows images of clear pipette tips (1–10 μL) that are most commonly used in chemistry, biology,

forensic, pharmaceutical, and drug discovery labs. The inner diameter of the tip head is 660 μm. The liquid in the tip will not drop out unless pushed (Fig. 2b). The liquid in the tip is much more stable than that of a hanging drop. The 10-μL liquid height in tips was 14 mm. There was space available above the liquid for adding additional cell suspension or cell culture medium from the upper end of the tip using a gel-loading pipette tip. The cells in the clear pipette tips could be observed from different angles and orientations under an optical microscope without perturbation or destruction of cell growth (Fig. 2c). Cells in the suspension gradually settled in the lower part of the tip because of the action of gravity (Fig. 2d). After 18 h of incubation, the shape of the cell aggregate gradually changed due to active cell interactions. Evaluation of cells in the pipette tip suggested that the force of gravity might initiate the settlement of the cells in the tip. However, cell-cell interactions were the driving force promoting cell aggregation and spheroid formation. The spheroid formation process finished after 24 h (Fig. 2d).

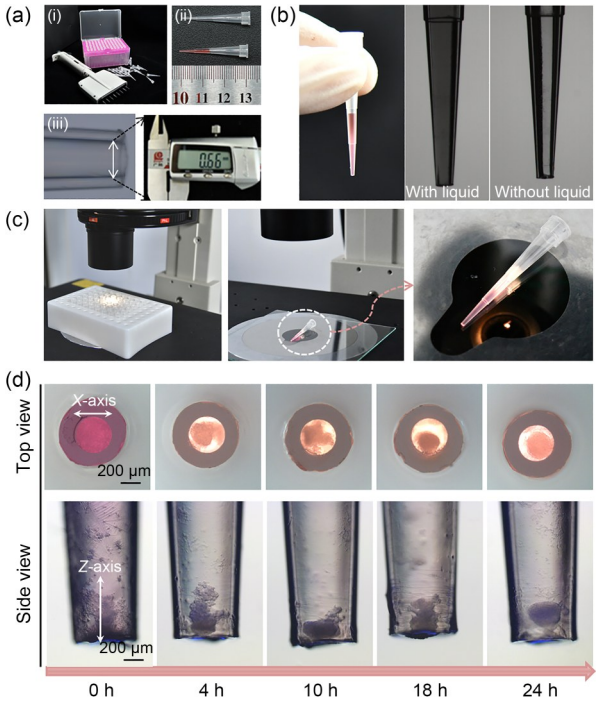

Fig. 2 Pipette tips and cell spheroids in the tips: (a) the features of pipette tips used for this experiment (the pipette and pipette tips (a-i), tips with and without 10-μL solution (a-ii), and micrograph of the tip head (inner diameter ~660 μm) (a-iii)); (b) photos of the 10-μL tip with and without liquid; (c) in-situ observation of the cells in the tip from different angles and orientations; (d) the process of cell spheroid formation (4×10^4 cells/tip)

Compared with previous methods, significantly less time was required to generate the spheroids (Table 1).

Table 1 Comparison of spheroid formation time for several 3D cell culture technologies

Technology	Spheroid formation time	Reference
LOT	Mostly >1 d	do Amaral et al., 2011; Costa et al., 2014; Kadletz et al., 2015; Lei et al., 2017
Hanging drop	>1 d, mostly >3 d	Kelm et al., 2003; Sodek et al., 2009; Shri et al., 2017; Cavo et al., 2020; Fu et al., 2021
Agitation-based	Ranging from 3 d to several weeks	Han et al., 2006; Rourou et al., 2007; Lee et al., 2011
Microfluidics	>1 d	Jeong et al., 2016; Dadgar et al., 2020; Khot et al., 2020
LbL	~3 d	Horiguchi and Sakai, 2015; Wang et al., 2018
Cells in pipette tip	<1 d	This study

Next, the impact of initial cell densities on spheroid formation was studied. With the increase in cell number from 1×10^4 to 4×10^4 cells/tip, the horizontal (X-axis) and vertical (Z-axis) dimensions of the cell aggregates enlarged proportionally (Fig. 3a). For instance, the ratio of the X-axis to the Z-axis for aggregates formed from 1×10^4 cells/tip, 2×10^4 cells/tip, and 4×10^4 cells/tip were 1.067, 1.145, and 1.029, respectively, suggesting a spheroid was formed. When the cell density was further increased to 8×10^4 cells/tip, the cell aggregate had no room to expand horizontally, but the Z-axis of the aggregate increased, forming a columnar cell structure. When the number of cells exceeded 4×10^4 cells/tip, the shape of the aggregate was no longer spheroid. The ratios of the X-axis to the Z-axis were 0.886 and 0.762 when the numbers of cells were 8×10^4 cells/tip and 1×10^5 cells/tip, respectively. The results indicate that the initial cell numbers determined the size and architecture of the cell aggregates. To prove that cell spheroid did not simply represent the stacking of cells because of gravitational action, but was the result of tight cell-cell interaction, the morphology of

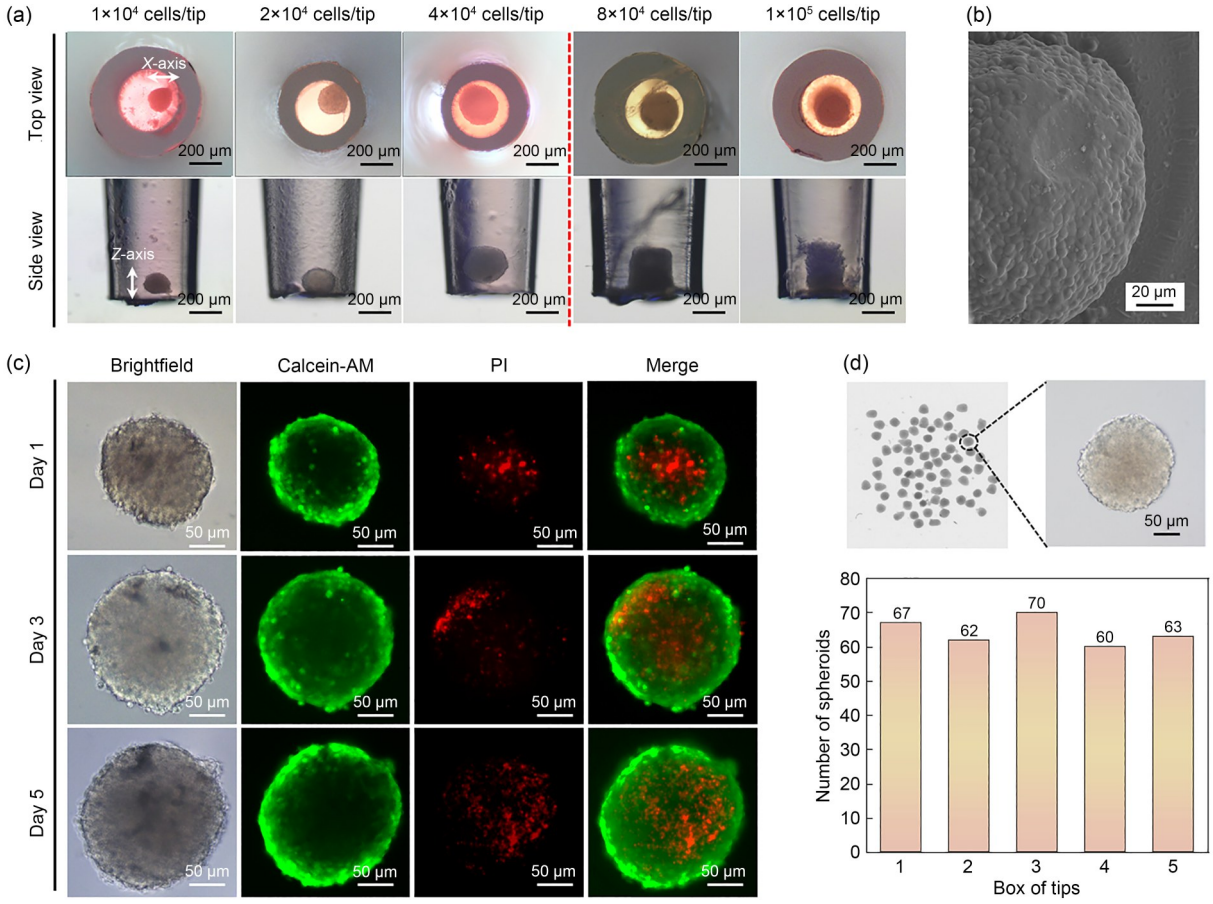

Fig. 3 Dynamics of tumor spheroid formation in pipette tips: (a) micrographs of cell aggregates formed from different initial cell numbers; (b) SEM image of a DU145 spheroid; (c) live/dead cell distribution in tumor spheroids stained using a Calcein-AM/PI kit; (d) scanner images of spheroids generated using a multichannel pipette

the cell spheroids was characterized. SEM characterization showed that cells were closely packed instead of being simply piled up (Fig. 3b). Cells were tightly connected because there were strong interactions between them. Brightfield microscopic images showed that the inner side of the spheroid was darker than the outer part, which is in line with the reported characteristics of spheroids (Fig. 3c). According to previous studies, cells in the inner part of spheroids suffer from nutrient depletion and oxygen scarcity (Costa et al., 2016). The Calcein-AM/PI staining result (Fig. 3c) indicated that the center of the tumor spheroids contained a higher proportion of dead cells, suggesting the formation of a necrotic core characteristic of solid tumors.

SEM and live/dead cell fluorescent staining showed that the proposed pipette tip method could be used to quickly generate tumor spheroids in ordinary laboratories. The high-throughput generation of reproducible spheroids is another advantage. We tested a multichannel pipette that can dock eight tips simultaneously to generate spheroids. For each box of tips, 80 tips were loaded with cells. After 1 d of incubation, cells were pipetted out of the 80 tips and transferred into a 6-well microplate. Particle analysis using ImageJ showed that 70 spheroids formed, and the formation rate was (87.5±4.2)% (Fig. 3d). The average area of the spheroids was (25492.31±2062.95) μm^2. Moreover, the reproducibility of the spheroids was characterized by testing five boxes of tips simultaneously. The spheroid formation ratio for the five boxes of tips was (80.5±5.0)%. The results highlighted the potential of the pipette tips for high-throughput generation of cell spheroids.

3.2 Formation of spheroids consisting of multiple cell types in pipette tips

Following the procedure illustrated in Fig. 1b-i, a cell suspension containing tumor cells of DU145 and HUVEC cells was loaded in the pipette tip. After

48 h of incubation, the mixed cells gathered and formed one spheroid (Fig. 4a). In addition, HUVEC cells labeled with the Dio green fluorescent dye were distributed throughout the whole spheroid. A higher density of green HUVEC cells was observed in the inner core of the tumor spheroid. Following the procedure illustrated in Fig. 1b-ii, tumor cells of DU145 were first loaded in the pipette tip to form a spheroid, then HUVEC cells were added to the same tip. After 48 h of co-culture, we found that HUVEC cells had infiltrated the existing DU145 tumor spheroid (Fig. 4b). Both methods proved that co-culture of different cell types could be achieved in the pipette tips.

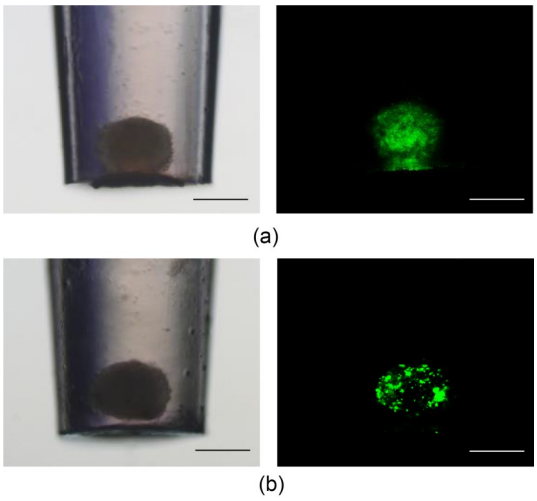

Fig. 4 Generation of spheroids consisting of multiple cell types: (a) one-step method (aspirating a cell mixture containing DU145 and HUVEC cells (green fluorescence) into a tip at the same time); (b) two-step method (aspirating DU145 cells into the tip to form a spheroid, and then adding HUVEC cells into the tip containing DU145 cell spheroids). References to color refer to the online version of this figure (scale bar= 200 μm)

3.3 Pairing and fusion of spheroids in pipette tips

Two tumor spheroids could be paired by pipetting one spheroid formed in a tip into another tip containing a spheroid (Fig. 1c). Initially, the newly added spheroid was in the upper part of the liquid inside the tip, which already had a spheroid at the bottom. The upper spheroid gradually migrated down until it met the spheroid at the bottom. The two spheroids began to merge and eventually formed a single bigger tumor spheroid. In addition to gravity, there were complex intercellular and extracellular interactions that made this process happen (Fig. 5a). Although occupied by two spheroids, there was still space available in the tip. Therefore, we conducted pairing experiments with more spheroids (Fig. 5b). All the procedures could be conducted using pipettes and pipette tips, highlighting the practical potential of assembling microtissues by pipetting.

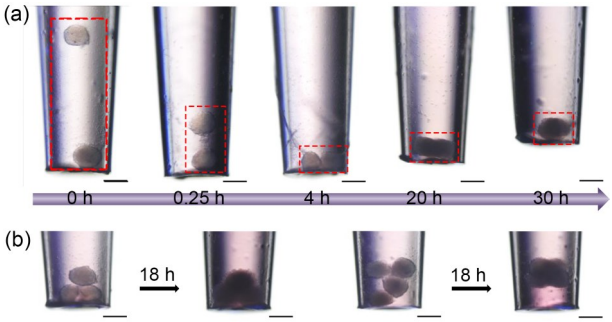

Fig. 5 Assembly of microtumor tissue by pairing spheroids in a pipette tip: (a) fusion of two spheroids; (b) fusion of multiple spheroids (scale bar=200 μm)

3.4 Evaluating anti-tumor efficacy in pipette tips

To verify the drug response of spheroids generated by this new culture platform, DOX was used as a model chemotherapy reagent to treat the spheroids. The morphology of the spheroids was impacted by the drug treatment (Fig. 6a). The CCK-8 results showed that with increasing DOX concentration, the percentage of live cells within the spheroids decreased. In addition, under the same DOX concentration, the cell survival rate of spheroids was higher than that of 2D cultured cells, indicating that cells in 2D culture were more sensitive to DOX treatment (Fig. 6b). The drug resistance characteristics of tumor spheroids generated by this new 3D culture platform were in line with the results of previous studies. The drug testing done in the pipette tips demonstrated that this platform can be used for screening drug efficacy.

4 Discussion

Tumor spheroids formed successfully in pipette tips, providing a new platform for 3D cell culture. Plenty of spheroids formed in a short period. The critical step in tumor spheroid formation is to make cell–cell connections produced by a wide variety of proteins stronger than cell–environment interaction forces (Białkowska et al., 2020). We hypothesized that cell adhesion into

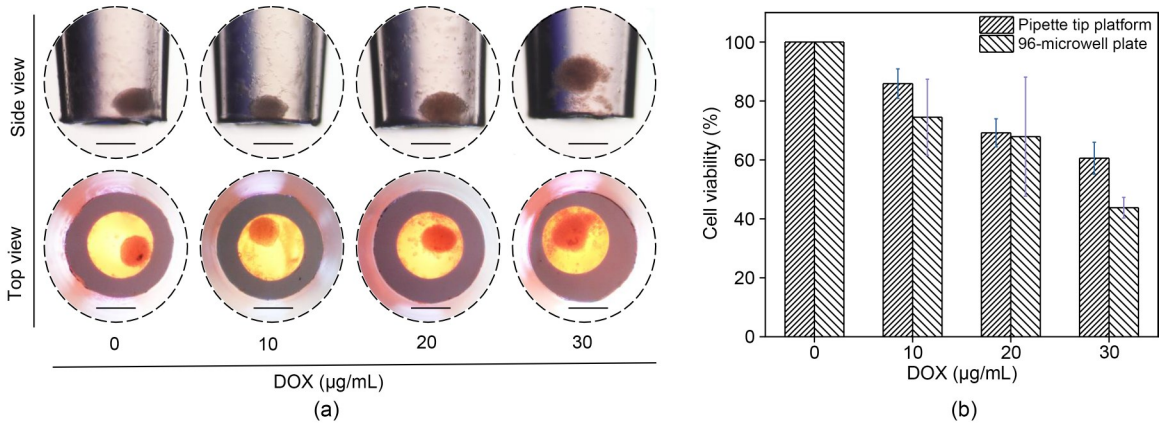

Fig. 6 Evaluating DOX anti-tumor efficacy in pipette tips: (a) images of tumor spheroids treated with different DOX concentrations (scale bar=200 μm); (b) the viability of DU145 cells cultured as a 3D spheroid in pipette tips and as a 2D monolayer in a 96-microwell plate

the vertical pristine polyethylene tips was constrained, and the geometrical conical shape of the tips promoted cell contact and even aggregation. The shape of cell aggregates could be adjusted by changing the initial cell densities. With increasing cell number, the shape of cell aggregates gradually changed from spherical to cylindrical because of the geometrical limitations of the tip. We proved that aspirating and incubating cells in a pipette tip could quickly generate tumor spheroids, even micro tumor tissue, to mimic in vivo 3D tissue structures in vitro.

Heterogeneity is one characteristic of cells in vivo (Altschuler and Wu, 2010). Cells in a living body are surrounded with other cells with different functions. They interact to sustain the activity of living organisms (Tang et al., 2020). Thus, the establishment of co-culture systems in vitro is one important strategy to replicate the microenvironment in vivo. For instance, angiogenesis is one of the critical steps in tumor growth and metastasis (Folkman, 1995; Quail and Joyce, 2013). The co-culture of tumor and blood vessel epithelial cells may be helpful for studying their interactions, including gene expression, proliferation rate, and cell functions. Previous studies suggested that the deficiencies in oxygenation in solid tumors stimulate the secretion of hypoxia-inducible factor (HIF)-1α, which controls the up-regulation of a number of factors necessary for solid tumor expansion, including the vascular endothelial growth factor (VEGF) (Ryan et al., 2000). The VEGF cytokine attracts vascular endothelial cells causing them to grow into the tumor tissue and form microvasculature (Hicklin and Ellis, 2005). For the co-culture system in pipette tips, HUVEC showed a preference for gathering in the inner part of the tumor spheroids, mimicking the angiogenesis process. For the one-step pipetting procedure, tumor cells, fibroblast cells, and stromal cells could be put together and aspirated into a tip to generate heterogeneous cell spheroids. The second procedure, involving sequentially introducing cells into an existing spheroid, could mimic the merging and infiltration functions of cells, such as vascular endothelial cells and lymphocytes penetrating solid tumors.

Pairing and fusion of tumor spheroids have been shown to be typical processes in cancer development (Sarkar et al., 2013), and are also two of the self-assembly processes crucial for multicellular architecture engineering (Kosheleva et al., 2020; Bustamante et al., 2021). Multicellular architectures are essential for modeling the construction of living systems, the occurrence of diseases, and the evaluation of therapeutic effects (Mueller et al., 2020; Cui et al., 2021). The fusion of spheroids is an efficient method of tissue reconstruction (Jakab et al., 2010). Cells undergo various changes during fusion and pairing which are important in cancer treatment studies (Cui et al., 2021). We performed a spheroid fusion experiment inside the tip. Pipetting a spheroid from one tip into another is a simple operation by which the fusion of two or more spheroids within one tip can be achieved. A drug testing experiment was also conducted using this platform. The drug was added directly into a tip containing a tumor spheroid. The killing process carried out in the tip could be directly observed under the microscope. The above

results show that the tip platform has more experimental versatility than other culture platforms. The whole experimental operation was simple, requiring few experimental skills for adding or changing the culture medium or transferring spheroids to other platforms for further analysis. It was extremely convenient without disrupting or disturbing the growth of the spheroids.

5 Conclusions

Tumor spheroid formation was demonstrated by aspirating cells in a pipette tip. A simple aspiration and incubation procedure was applied to generate spheroids consisting of a single or multiple cell types. In addition, pairing and fusion of spheroids to assemble larger cell aggregates were demonstrated, highlighting the potential for generating and culturing mini tumor tissues in the pipette tip. The pipette tip platform also can be applied to drug screening. Adding different DOX concentrations induced spheroid damage in the pipette tip. The small volume of the pipette tip significantly reduced the consumption of reagents. Since biological laboratories are commonly equipped with pipettes and pipette tips, the proposed pipette tip-based spheroid formation platform can eliminate the equipment barrier for resource-limited laboratories to conduct 3D cell culture and research.

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Author contributions

Conceptualization: Rong PAN and Ling YU. Data curation: Rong PAN, Xiaoyan YANG, Shiming WU, and Feng CHEN. Formal analysis: Rong PAN, Yuanyuan XIE, and Wei SUN. Methodology and investigation: Rong PAN, Ke NING, and Xiaoyan YANG. Project administration and supervision: Ling YU. Funding acquisition: Ling YU. Writing–original draft: Rong PAN. Writing–review and editing: Rong PAN, Ling YU, and Wei SUN.

Conflict of interest

Rong PAN, Xiaoyan YANG, Shiming WU, Yuanyuan XIE, Feng CHEN, Ke NING, Wei SUN, and Ling YU declare that they have no conflict of interest.

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