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Rapid formation of size-controllable multicellular spheroids *via* 3D acoustic tweezers

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Abstract

The multicellular spheroid is an important 3D cell culture model for drug screening, tissue engineering, and fundamental biological research. Although several spheroid formation methods have been reported, the field still lacks high-throughput and simple fabrication methods to accelerate its adoption in drug development industry. Surface acoustic wave (SAW) based cell manipulation methods, which are known to be non-invasive, flexible, and high-throughput, have not been successfully developed for fabricating 3D cell assemblies or spheroids, due to the limited understanding on SAW-based vertical levitation. In this work, we demonstrated the capability of fabricating multicellular spheroids in the 3D acoustic tweezers platform. Our method used drag force from microstreaming to levitate cells in the vertical direction, and used radiation force from Gor'kov potential to aggregate cells in the horizontal plane. After optimizing the device geometry and input power, we demonstrated the rapid and high-throughput nature of our method by continuously fabricating more than 150 size-controllable spheroids and transferring them to Petri dishes every 30 minutes. The spheroids fabricated by our 3D acoustic tweezers can be cultured for a week with good cell viability. We further demonstrated that spheroids fabricated by this method could be used for drug testing. Unlike the 2D monolayer model, HepG2 spheroids fabricated by the 3D acoustic tweezers manifested distinct drug resistance, which matched existing reports. The 3D acoustic tweezers based method can serve as a novel bio-manufacturing tool to fabricate complex 3D cell assemblies for biological research, tissue engineering, and drug development.

Graphic Content Entry

A 3D acoustic tweezers platform is developed to fabricate size-controllable multicellular spheroids in a rapid and high-throughput manner, utilizing the acoustic Gor'kov potential field and microstreaming.

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Introduction

During the last decade, increasing evidence has shown the limitations of 2D monolayer culture systems in mimicking cell behaviours observed in 3D *in vivo* conditions. Vital proteins in the matrix, cell-cell interaction, and structural support are lost in 2D cultures. For example, liver cells in a 2D environment quickly lose critical functions within a few days.^{1, 2} To mimic the *in vivo* microenvironment, various 3D cell/tissue culture models have been established and studied. These 3D cell cultures have become indispensable platforms for many biological studies including cell proliferation,³ apoptosis,⁴ tumour metastasis,⁵ toxicology,⁶ drug screening,⁷ and stem cells differentiation.⁸

The multicellular spheroid is one of the most characterized 3D culture models. Studies highlight the use of multicellular tumour spheroids (MCTS) as a bridge between cell-based assays and animal models for anti-cancer drug testing and therapy-oriented research.^{9, 10} For example, the oxygen/nutrient gradients inside MCTS are similar to poorly vascularized areas in solid tumours, which are obstacles in effective radiotherapy and chemotherapy.¹¹ Lead compounds which are screened by monolayer cultures lose their efficacy in 3D extracellular matrices (ECM). Analogous to the 2D case, MCTS expedites the pre-animal and pre-clinical studies during negative selection of drug candidates.

To use spheroids for accurate, efficient drug screening, they must be fabricated of identical structure, morphology, physiology and with sufficient quantity. Many of the existing spheroid-formation approaches, such as rotary cell culture system¹² and self-formation on non-adhesive surfaces¹³ and in scaffolds,¹⁴ are time-consuming and do not yield uniform spheroids. Other methods, including microfluidics,¹⁵ hanging droplets,¹⁶ dielectrophoresis,¹⁷ and magnetic-assisted assembly,¹⁸ are low-throughput and require medium modification, particle labelling, and/or complicated device design. Among the various spheroid formation techniques, approaches predicated on acoustics offers unique characteristics, such as excellent biocompatibility, flexibility, high throughput, and label-free nature.^{19, 20} Thus far, bulk acoustic wave (BAW) has been used for fabricating 3D spheroids.²¹ However, because BAW highly depends on complicated resonator structure design and a stable temperature, it is challenging to fabricate uniform spheroids in a high-throughput manner based on BAW devices.²³ Surface acoustic wave (SAW) is known to be more controllable and adjustable, and has been demonstrated to successfully manipulate cells in 2D plane for separation, focusing, and patterning.^{24–29} Most recently, we

demonstrated that acoustic radiation force and drag force from acoustic microstreaming have similar magnitude near the pressure nodes. As a result, it is possible to manipulate cells in vertical direction based on the balance of gravity, buoyant force, radiation force, and drag force.³⁰ However, in order to use surface acoustic waves to construct more *in vivo* like artificial tissue structures, the ability to manipulate multiple cells in 3D environment and aggregate them into uniform 3D assemblies is still badly needed.

Here, we report a 3D acoustic tweezers-based technique that can rapidly, high-throughput and reproducibly fabricate uniform, size-controllable 3D spheroids. In our 3D acoustic tweezers device, SAWs were guided into a microfluidic chamber for spheroid formation and patterning. The SAWs leaked into the chamber and formed Gor'kov potential field and microstreaming. Cells aggregated in the areas subject to the minimal Gor'kov potential in horizontal plane, and were levitated to the suspended trapping nodes in vertical direction by the balance of acoustic radiation force, microstreaming-induced drag force, gravity, and buoyant force, facilitating cell-cell communication in a geometrically confined space and forming cell spheroids within 30 min. More than 150 spheroids with similar sizes and compositions were formed every time, and around 50 mature spheroids each time were cultured in the Petri dishes without cell dissociation. Spheroids collected in Petri dishes were cultured for a whole week with good cell viability, and anti-cancer drug testing was performed to show the distinct responses between 3D spheroids and 2D monolayer cultures. By adjusting input cell type and density, spheroids with different sizes and compositions can also be fabricated.

Our method repeatedly and rapidly yields spheroids in a high-throughput manner. Compared with many other methods,^{14–18} the throughput was increased from ~100 spheroids/day to ~100 spheroids/hour. Taking advantages of label-free and non-invasive acoustic cell manipulation in 3D environment, this technique can potentially be used to fabricate multi-layer spheroids or other complex *in vivo*-like 3D tissue structures with different cell types. We believe this 3D acoustic tweezers based technique will benefit the fields of tissue engineering, cell-cell communication, and drug development.

Experiments

Fabrication of 3D acoustic tweezers based device

A 2 mm × 2 mm polydimethylsiloxane (PDMS) microchamber was bonded to a LiNbO₃ piezoelectric substrate, on which two orthogonal pairs of interdigital transducers (IDTs) were deposited (Fig. 1A). Each IDT had 40 pairs of electrodes of width 75 μm and periodic spacing 75 μm. We fabricated the PDMS chamber by standard soft-lithography and mould replica techniques. IDTs were formed by depositing double-layered metal (Cr/Au) on the LiNbO₃ substrate and removing a photoresist by the lift-off process. After plasma treatment, the PDMS chamber and the LiNbO₃ substrate were bonded and then heated in an oven for 24 h. The fabrication steps of the acoustic tweezers device were detailed in our previous work.²⁹

Cell preparation

HepG2 (ATCC, USA), a human hepatocellular carcinoma cell line, was cultured in Eagle's Minimum Essential Medium (ATCC, USA) with 10% fetal bovine serum (Sigma-Aldrich, USA) and 1% penicillin-streptomycin (Invitrogen, USA). HEK 293 (ATCC, USA), a cell line originally derived from human embryonic kidney cells, was cultured in Minimum Essential Medium (ATCC, USA) with 10% fetal bovine serum (Sigma-Aldrich, USA) and 1% penicillin-streptomycin (Invitrogen, USA). All the cells were incubated at 37°C, 5% CO₂ in humidified incubators. Cell suspensions for all the experiments were made by dissociation of cells with 0.25% trypsin-EDTA (Invitrogen, USA), centrifugation of dissociated cells at 1,000 rpm for 1 min at room temperature, and re-suspension in growth media. Cell density was estimated using a hemocytometer.

Spheroid formation and long-time culture

Before experiments, the 3D acoustic tweezers device was fixed with double-sided tape to a cooling plate in a biosafety hood and treated with UV light for 30 min. The cooling plate is to prevent the overheating of the device when acoustic field is applied. There were three steps to form multicellular spheroids. Firstly, we injected a cell suspension into a microfluidic chamber with a syringe. Secondly, two independent radiofrequency (RF) signals of frequencies 13.35 MHz and 13.45 MHz were applied to the IDTs, to generate standing SAWs and form arrays of pressure nodes on the substrate (Fig. 1A). Vibration of the substrate caused the formation of acoustic Gor'kov potential field and microstreaming inside the adjacent medium. A spheroidal cluster of cells was trapped in the areas of minimal Gor'kov potential in the horizontal plane. In the vertical plane, cells were levitated to the suspended trapping nodes by the combination of acoustic radiation force, microstreaming-induced drag force, gravity and buoyant force (Fig. 1B). Finally, we turned off the SAWs and re-injected the cell suspension into the chamber. Mature spheroids were washed through an outlet and collected in a Petri dish for further biological experiments or drug testing. In each experiment we fabricated more than 150 spheroids in the chamber. By repeating the three steps (*i.e.*, injecting, SAW for 20–30 min, and collecting), we can fabricate significant number of spheroids in a short period of time. After being fabricated, spheroids were collected and cultured in 35 mm sterile, non-attachable Petri dishes (VWR, USA). The growth medium was exchanged daily by removing 1 ml solution and adding 1 ml of fresh growth medium. Spheroid size and cellular viability were monitored every 24 h using a confocal fluorescence microscope. Living cells were labelled with green fluorescent using Calcein AM, and dead cells were labelled with red fluorescent using propidium iodide (Life Technologies, USA). The cellular viability was calculated by the ratio of green fluorescent area sizes over total spheroid area sizes measured in software Image J.

Anti-cancer drug testing

In an anticancer drug sensitivity test, spheroids were cultured for 72 h with 5-fluorouracil (5-FU) (Sigma-Aldrich, USA), which irreversibly inhibits thymidylate synthase. 5-FU solid particles were first absorbed in dimethyl sulphoxide (DMSO) (Sigma-Aldrich, USA) and then diluted in phosphate buffered saline (PBS) (Invitrogen, USA) at the ratio of 1:1000. Solutions of concentrations (1, 10, and 100 µM) were prepared before experiments. HepG2

spheroids were fabricated in our acoustic tweezers platform with input cell density of 8×10^6 cells/ml, then collected and cultured in Petri dishes. On day 1 of spheroid culture, 400 μ l of each concentration of 5-FU solution was added to each Petri dish containing 3,600 μ l of growth medium. For the control group, 5-FU drugs of same concentration were added to Petri dishes when cells were at 80% confluency. Cellular viability was measured under confocal microscope upon 24, 48, and 72 h of drug incubation using calcein AM and propidium iodide (Life Technologies, USA). The cellular viability was calculated by the ratio of green fluorescent area sizes over total spheroid area sizes measured in software Image J.

Results and discussion

Working mechanism of 3D acoustic tweezers based spheroid formation

3D acoustic tweezers-based spheroid formation technique (Fig. 2A and 2B) relies on establishing Gor'kov potential fields and microstreaming inside a microfluidic chamber, which levitates and concentrates cells into geometrically defined spaces. In our devices, standing SAWs were generated by applying RF signals to two orthogonal pairs of IDTs. The two orthogonal waves propagated on the surface of a LiNbO_3 substrate, forming a dotted array of transverse displacement nodes and anti-nodes (Fig. 2C). The surface vibrations were translated to the fluid at the substrate-liquid interface via angle θ_r , resulting in a Gor'kov potential distribution and a patterned microstreaming within the liquid medium. In horizontal plane, the distribution of Gor'kov potential in the medium was coincide with the distribution of displacement nodes and anti-nodes (pressure nodes and anti-pressure nodes) on the substrate surface (Fig. 2C). Horizontally, Cells aggregated in the regions where the Gor'kov potential is minimum. In vertical plane, cells were levitated to a stable position due to the balance of gravity, buoyant force, acoustic radiation force, and drag force from microstreaming. After establishing the Gor'kov potential field and microstreaming, we investigated the impact of parameters, such as channel height and input RF signal power, on the formation of uniform cell spheroids.

Fig. 2B is the simulated longitudinal section of the distributions of acoustic radiation force and drag force induced by microstreaming. A detailed description of the numerical model can be found in our previous papers.^{30, 34} Central ellipsoidal area A1 represents the region above a pressure node. In region A1, Gor'kov potential is small and acoustic radiation force F_R is comparable with the drag force F_D , gravity, and buoyant force in the vertical direction. In other regions, acoustic radiation force acting on a $\sim 15 \mu\text{m}$ cell is much larger than the drag force and dominates cells' movement.³⁵ After injecting cells into the channel, cells would distribute in different horizontal positions and different vertical layers of the channel randomly. Once RF signals were applied to the IDTs, standing SAWs were generated, forming Gor'kov potential field and microstreaming inside the medium. In the horizontal plane, the dominated acoustic radiation force would push cells to the areas above the pressure nodes. Once cells entered into area A1, acoustic radiation force, drag force, gravity, and buoyant force have similar magnitudes in the vertical direction. Based on the interplay between acoustic radiation force, drag force, gravity, and buoyant force, cells near the

substrate would be levitated and all the cells would be pushed towards a stable suspended trapping position and aggregate together to form a spheroid.

Chamber height should be large enough to provide space for spheroid formation. In our device, the microstreaming was a kind of boundary-driven acoustic streaming. In theory, it requires 20–30 μm for the streaming development.³³ The sizes of the spheroids were ~ 70 μm . Thus, the height of the chamber should be larger than 90–100 μm theoretically. Input acoustic power determined the magnitudes of acoustic radiation force and the drag force induced by microstreaming. The input power needs to be large enough to generate adequate streaming velocity aiming to effectively levitate or move cells. Meanwhile, the input power cannot be too large. In area A1, when a cell was near or rested on the substrate, the vertical direction of acoustic radiation force experienced by the cell was opposite to the vertical direction of microstreaming (Fig. 2A, Fig. S7). Too large input power would cause acoustic radiation force near the substrate dominating over the drag force. This would result in that cells near the substrate cannot be levitated above to form aggregates. In this case, there would form two layers of cell pattern, reducing the efficiency of forming spheroids.

Based on the working mechanism of our 3D acoustic tweezers, we optimized the chamber height and input signal power to find a stable area A1 for uniform spheroid integrity. We studied how different chamber heights (60 μm , 100 μm , and 120 μm) affected the formation of 3D cell aggregates. An optimized height was 100 ± 5 μm . Below 90 μm , the height of area A1 was less than a few cell diameters, so cells did not vertically “pile up” even under a high input power. Above 110 μm , cells easily separated into two layers in vertical direction, and there was no patterning for the bottom layer (Fig. S4). Then we studied how different input power would influence 3D cell aggregates in a 100 μm height channel. An optimized input power was around 30 dBm in a 100 μm height channel. A lower power was ineffective for vertically piling cells up, while a too large power generated two layers of cell patterning in vertical direction (Fig. S3). We also found that adding gel, such as type I collagen or alginate, of concentration less than 1% would stabilize cell aggregation when the spheroids were washed out and collected in Petri dishes. In the following experiments, however, we did not add gel because it would alter the microenvironment and bias drug testing.¹⁰

Controlling the size of cell spheroids

The size of the cellular spheroids influenced their physiological states, and thereby directly or indirectly influenced the accuracy of drug screening results. For example, as the size of the spheroids increased, central and secondary necrosis occurred to various degrees.^{31, 32} Therefore, controlling cell packing densities and the size of uniform cell spheroids is critical to minimizing variations in drug screenings. As discussed in the previous section, our system formed hundreds of same 3D suspended trapping nodes, so the spheroids generated by our system were of similar sizes.

Here we demonstrated that our method was also capable of producing cell spheroids of different sizes on demand. We controlled the final diameters of the spheroids by changing the densities of injected cell solutions. We tested HepG2 cell suspensions of various concentrations (2, 5, 8, 11, 14, 17×10^6 cells/ml). Fig. 3 shows the results of representative experiments. As expected, the average diameter of the spheroids was much smaller with an

initial seeding density of 2×10^6 cells/ml (Fig. 3A), while larger spheroids were fabricated by injecting a 1.7×10^7 cells/ml solution (Fig. 3B). Indeed, spheroids were formed from the cells initially trapped in 3D suspended trapping nodes, so the average size of the spheroids highly depended on the input cell density. The maximal spheroid diameter was determined by the size of the area A1 in Fig. 2B, and as a result was determined by the wavelength of standing SAWs. The wavelength used here was around 150 μm so that the maximal spheroid diameter was around 100 μm . By changing the distance between IDT fingers, we can change the wavelength and thereby change the maximum spheroid diameter. Fig. 3C shows that the average spheroid diameter increased as the input cell density was increased. To examine the biocompatibility of our method, we used Calcein AM and propidium iodide to determine the cell viability. Movie S1 shows cells labelled with Calcein AM were pushed to aggregate under acoustic wave. After applying SAWs to HEK 293 cells (Fig. S1) and HepG2 tumour cells (Fig. 3D) for more than 30 min, the live cell percentage was still greater than 95%.

Microfluidic-chip-free, long-term culture of spheroids

In the previous sections, we described the mechanism of manipulating cells in 3D environment and patterning cell aggregates of different sizes using 3D acoustic tweezers. For this 3D acoustic tweezers method to be applicable, it has to be amenable to biologists upon interfacing with commonly used platforms in cell biology such as Petri dish-based cell culture. In this regard, we tested the ability of our platform for microfluidic-chip-free, long-term culture of spheroids. After applying RF signals to generate an acoustic field and microstreaming inside the chamber, cells aggregated at the 3D suspended trapping nodes. After 20–30 min, strong connections formed between cells, allowing the mature spheroids to be transferred out of the microfluidic chamber. Different types of cells require different SAW durations to form strong connections, depending on their capabilities for cell-cell adhesion. We optimized the SAW duration for various types of cells. We tested 10 min, 20 min, and 30 min SAW duration times for different cell types, such as HEK 293, SH-FY5Y, HepG2, and HeLa cells. HEK 293, SH-SY5Y, and HepG2 cells required 20 min to form strong cell-cell adhesion; HeLa cells needed more than 30 min.

Recovery of mature spheroids from microfluidic devices is essential for the long-time culture and subsequent biological experiments. After forming mature spheroids, we injected a new medium into the chamber to wash them out and then collected them into a Petri dish. Each cycle, we could fabricate more than 150 mature spheroids and collect around 50 in a Petri dish. After washing out and culturing spheroids in Petri dishes for 30 min, the distinct cell-cell boundary started to disappear. After culturing for 1 day, the spheroids had formed into a smooth surface and were ready for drug testing or other experiments (Fig. 4A).

When spheroids were collected and cultured in a Petri dish, high cell viability was maintained for a whole week (Fig. 4B). We measured the average diameter of spheroids to monitor their growth. The spheroids kept growing due to the proliferation of individual cells (Fig. 4C). After seven days, a 40 μm spheroid grew nearly three times in diameter. We monitored viable cell percentage inside the spheroids using a confocal fluorescence microscope. After a whole week, cell viability was still higher than 90 percent (Movie S2). All the results indicate that our method forms intact and viable cell spheroids.

Anti-cancer drug sensitivity testing

The scalable, biocompatible, and rapid nature of our 3D acoustic tweezers-based spheroid fabrication method makes it convenient for fabricating cell spheroids and integrating with high-throughput drug screening. Here we used a classic anticancer drug 5-fluorouracil (5-FU, Sigma-Aldrich, USA), which inhibits cell proliferation, to compare the drug susceptibility of HepG2 cells under both monolayer and spheroid culture conditions. We used our 3D acoustic tweezers technique to fabricate spheroids of average diameter 70 μm , and cultured them in Petri dishes. For the control group, we added single cell suspension to Petri dishes and cultured it under the same conditions. After culturing in normal medium for 24 h, drugs with different concentrations were added to both spheroids and control groups. Fig. 5(A) shows live/dead staining results of HepG2 cells after adding drug for 72 h. As the concentration of 5-FU increased, the live cell percentage decreased rapidly in both 2D and 3D culture conditions. At the same 5-FU concentration, cell viability in spheroids was greater than 2D cultures, because drugs took longer time to diffuse and penetrate into spheroids. Particularly, at the concentration of 10 μM , cells close to the surface of the spheroids died and dissociated from spheroids, while cells in the inner layers of the spheroids were still alive (a more magnified image of central living spheroid is showed in Fig. S3). In a 2D monolayer culture, however, dead cells almost uniformly distributed across the entire Petri dish. Fig. 5(B) shows that 3D spheroids manifested higher resistance to the proliferation-inhibited drug than 2D monolayer culture, which agrees with previous reports.^{7, 10}

Conclusions

In this work, we developed a 3D acoustic tweezers-based method that rapidly formed size-controllable spheroids in a microfluidic chamber. Under acoustic field, cells were pushed to aggregate in the 3D suspended trapping nodes by the balance of acoustic radiation force, microstreaming-induced drag force, buoyant force and gravity. After optimizing parameters such as input signal power, chamber height, SAW duration time, and cell concentration, we achieved high-throughput fabrication of cell spheroids with identical structures. We further demonstrated that mature spheroids were able to be washed out of the chamber and cultured in Petri dishes for further studies and drug testing. Our platform can easily scale up to a larger chamber, and continuous spheroid generation can be achieved in the same device by repeating cell injection, acoustic patterning, and sample collection.

The major advantages of our acoustic tweezers-based spheroid formation technique are 1) label-free and non-invasive manipulation of cells in 3D environment; 2) rapid spheroid formation (*i.e.*, 20–30 min in our approach versus 1–4 days in traditional methods); 3) gel-free formation, avoiding “artificial” matrices influencing cell behaviours; 4) compatibility with Petri dish based cell culture, providing a more stable and controllable culture environment; 5) ease of integration with drug screening or other biological tests; and 6) high tunability (*i.e.* the ability to control spheroid size from several to hundreds of cells). With these advantages, our 3D acoustic tweezers based spheroid production and handling approach can be valuable for pharmacology, tissue engineering, and biological research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The concept of 3D acoustic tweezers-based spheroid formation system

(A) An image of the 3D acoustic tweezers device. (B) Schematic illustrates that longitudinal-mode leaky waves form Gor'kov potential field inside the chamber. Cells aggregate into spheroids in the 3D suspended trapping positions where acoustic radiation force, microstreaming induced drag force, gravity and buoyant force balance. (C) Spheroid formation consists of three steps: 1) injection of cell suspension, 2) 3D acoustic tweezers-based formation of spheroids, and 3) collection of spheroids in a Petri dish. By repeating the three steps, this platform can yield spheroids in a continuous fashion.

Figure 2. Spheroids were formed above pressure nodes via acoustic tweezers

(A) Schematic of the 3D acoustic tweezers-based spheroid formation mechanism (cross-sectional view) and an illustration of forces experienced by a cell floating inside the medium and a cell near the substrate. F_D represents drag force induced by microstreaming, F_R represents acoustic radiation force, F_B represents buoyant force, and G represents gravity. After applying acoustic waves, floating cells were horizontally pushed to aggregate by acoustic radiation force, while cells near the substrate were levitated by the balance of acoustic radiation force, drag force, buoyant force and gravity. (B) Simulation of the distribution of acoustic radiation force and microstreaming-induced drag force around a pressure node in the chamber's vertical plane. (C) Horizontal view of the Gor'kov potential arrays. Arrows indicate the acoustic radiation force vectors. (D) Cells aggregated in the arrays of suspended trapping nodes and formed into spheroids.

Figure 3. Formation of size-controllable spheroids by 3D acoustic tweezers. Optical image of patterned 3D spheroid arrays

HepG2 cells were added to microfluidic chambers at concentrations of (A) 2×10^6 cells/ml and (B) 1.7×10^7 cells/ml. (C) Diameters of spheroids measured at different cell densities (n = 30). (D) Percentages of viable cells in the spheroids after SAWs on for 30 min (n = 30).

Figure 4. Rapid formation and long-time culture of HepG2 spheroids

(A) Time-lapse images showing that spheroids were formed. After culturing for 30 min, cells started to merge into spheroids. After 24 h, the cell profile disappeared and mature spheroids were ready for drug testing. (B) Long-term culture of spheroids. Live cells were labelled with Calcein AM (green), and dead cells were labelled with propidium iodide (red). (C) The diameters of spheroids and (D) percentages of viable cells in the spheroids were measured each day (n = 30).

Figure 5. Anticancer drug testing of acoustic tweezers-generated HepG2 spheroids
 (A) Live/dead staining of HepG2 spheroids and 2D monolayer cell 72 h after adding anticancer drug 5-FU, respectively. Live and dead cells were stained with Calcein AM (green) and propidium iodide (red), respectively. (B) Bar graph of the live cell percentage 72 h after drug treatment. The statistical analysis showed a significant difference between the spheroids and 2D culture for different drug concentrations. * $p < 0.01$.