3D ACOUSTIC TWEEZERS-BASED BIO-FABRICATION

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by

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ABSTRACT

The ability to create 3D in vivo-like tissue models raises new possibility in studying complex physiology and pathophysiological process in vitro, and enabling the development of new therapy strategies for underlying diseases. Recent observations showed that gene expression in 3D cell assemblies was much closer to clinical expression profile than those in 2D cases, which generated hope in manufacturing artificial 3D tissue for therapy test platforms with a better prediction of clinical effects.

In this work, we developed a 3D acoustic tweezers-based method for rapid fabrication of multicellular cell spheroids and spheroid-based co-culture models. Our 3D acoustic tweezers method used drag force from acoustic microstreaming to levitate cells in vertical direction, and used acoustic radiation force from Gov’kov potential field to aggregate cells in horizontal plane. After optimizing the device geometry and input power, we demonstrated the rapid and high-throughput characteristics 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 whole 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. By co-culturing HepG2 spheroids with HMVEC cells, we found that spheroids tended to accelerate angiogenesis and tube formation process in Matrigel compared with isolated single cells. The 3D acoustic tweezers based method can serve as a novel bio-manufacturing tool to fabricate complex 3D cell models for tissue engineering, drug development, and fundamental biological research.
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CHAPTER 1: INTRODUCTION

1.1: In vitro model for drug testing

During the last decade, increasing evidence has shown the limitations of 2D monolayer culture systems in mimicking cell behaviors observed in 3D in vivo conditions. It is generally recognized that hard plastic Petri dishes or glass slides for cell attachment and culture cannot represent the 3D microenvironment inside our body. Vital proteins in the matrix, cell-cell interaction, and structural support are lost and gene expression is altered 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. 3D scaffold with different materials and structures fabricated by 3D printing technique is one of the most widely investigated 3D models (Fig. 1.1A). Cells cultured inside the 3D scaffold interacted with fibers inside the scaffold and showed similar biological functions as the in vivo conditions. Scaffolds were also widely used in regeneration of bone, neural and many other tissues.\(^3\) In recent years, due to the rapid development of microfluidics technology, people started to use different microfluidic channel structures to create 3D models for tumor metastasis research (Fig. 1.1B). In those models, gel was filled into the microfluidic channel. Cells were cultured inside the gel or cultured on the channel substrate and migrated into the gel. By controlling the chemical cues and mechanical cues applied to cells, researchers were able to investigate the underlying biological insight of tumor cell migration.\(^4\) Except for the tumor metastasis research, by using microfluidic device, researchers also constructed other 3D models to mimic the organ functions inside our body, which were called organ-on-a-chips.\(^5\) Fig 1.1C shows
the microfabricated lung-on-a-chip device, which used the deformation of the PDMS chamber to give cells mechanical stimulation and mimic the inhalation and exhalation conditions in a living lung. Cells cultured on the flexible membrane experiencing mechanical stretching and acquired similar immune responses inside the living human body. Although these microfluidic chip-based models were more accurate to recreate the tissue function, they were too complicated to be largely fabricated and applied to drug screening in industry. People came up with other simple but efficient 3D models for drug testing, such as the Transwell model (Fig. 1.1D) and multicellular spheroids (Fig. 1.1E). The Transwell model was widely used to investigate cell migration and tumor metastasis. In Transwell model, there was a thin polymer film with tiny pores in each well. One type of cells was cultured on the top of the polymer film and another type of cells was cultured on the bottom of the film. Two types of cells communicated with each other and some cells would migrate through the pores to reach to another type of cells. The multicellular spheroids were widely used in drug screening and negative selection of drug candidates. In multicellular spheroid model, one type or different types of cells were aggregate into spheroidal structure. Cells interacted with each other directly and grew into 3D tissue-like structures. By co-culturing cell spheroids with other types of cells in the 3D gel, deeper biological phenomenon such as tube formation and tumor migration was investigated. Overall, these 3D cell cultures have become indispensable platforms for many biological studies including cell proliferation, apoptosis, tumor metastasis, toxicology, drug screening, and stem cells differentiation.
Fig. 1.1. **3D cell culture models in tissue engineering.** (A) 3D scaffold with different materials and structures.\(^3\) (B) Microfluidics-based tumor metastasis model. Tumor cells and normal HMVEC were co-cultured inside channel and migrated into the collagen.\(^4\) (C) Organ on a chip model for mimicking the function of lung.\(^5\) (D) Transwell model to investigate coculture and migration phenomenon.\(^6\) (E) 3D Multicellular spheroid model.\(^7\)

The multicellular spheroid is one of the most-characterized 3D culture models, because it is simple compared with other 3D models, and it can recreate in-vivo like phenomenon such as ATP distribution and oxygen gradient (Fig. 1.2). Studies highlight the use of multicellular tumor spheroids (MCTS) as a bridge between cell-based assays and animal models for anti-cancer drug testing and therapy-oriented research.\(^{14-16}\) For example, the oxygen/nutrient gradients inside MCTS are similar to poorly vascularized areas in solid tumors, which are obstacles in effective radiotherapy and chemotherapy.\(^{17,18}\) 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 phases during negative selection of drug candidates.
1.2 Problem Statement

To use spheroids for accurate and 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 system and self-formation on non-adhesive surfaces and in scaffolds, are time-consuming and do not yield uniform spheroids. Other methods, including microfluidics, hanging droplets, dielectrophoresis, and magnetic-assisted assembly are low-throughput and require labeling particles, medium modification, and/or complicated device design. Among the various spheroid formation technique, approaches predicated on acoustics offers unique characteristics, such as excellent biocompatibility, flexibility, label-free nature, and high throughput. Thus far, bulk acoustic wave (BAW) has been used for 3D spheroids fabrication. However, because BAW highly depends on complicated
resonator structure design and temperature controller, it is really difficult to fabricate uniform spheroids in a high-throughput manner based on BAW devices. Surface acoustic wave is more flexible in engineering its wave front and is able to work with higher frequency.\textsuperscript{23-25} It has been demonstrated to successfully manipulate cells in 2D plane for separation and patterning. However, in order to use acoustic waves to construct more in vivo like artificial tissue structures, the ability to manipulate cells in 3D environment and aggregate cells into 3D assembles is still badly needed.

1.3: Objective

Here, we report a 3D acoustic tweezers-based technique that can rapidly and reproducibly fabricate uniform, size-controllable 3D spheroids. In this work, SAWs were guided into a microfluidic chamber for spheroid formation and patterning. The SAW leaked into the chamber and formed a 3D Gor’kov potential field. Cells aggregated in areas subject to the minimal Gor’kov potential, facilitating cell-cell communication 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. We also co-cultured HepG2 spheroids with HMVEC cells to investigate the acceleration effect of tumor spheroids on angiogenesis and tube formation process.

Our method repeatedly, rapidly yields spheroids in a high-throughput manner. Taking advantage of label-free and non-invasive acoustic manipulation of cells in 3D environment, this technique enables fabrication of multi-layer spheroids or other complex \textit{in vivo}-like 3D tissue structures with different cell types. Open-end culture system facilitates downstream spheroid
analysis with flexible image options. We believe this 3D acoustic tweezers based technique will benefit the fields of tissue engineering, cell-cell communication, and drug development.
CHAPTER 2: LITERATURE REVIEW

2.1: Conventional Cell Spheroids Fabrication Techniques

Multicellular spheroids recreate a microenvironment that has the similar micromilieu, volume growth kinetics and histomorphological features as the vascular and intervascular regions of large solid tumors. They are able to reestablish morphological and functional mass transport properties corresponding to the *in vivo* tumor tissues, due to the appropriate interactions between cells and extracellular matrix (ECM). For example, as the size of spheroids increased, oxygen and nutrient gradient can be observed inside the spheroids, affecting cellular RNA/protein expression, which was highly reminiscent of the poorly vascularized solid tumor structures inside human body. 26, 27 Thus far, multicellular spheroids have a great potential to serve as a tool for negative selection of drug candidates and would significantly contribute to the reduction of animal testing and the enhancement of the success in clinical trials. 28, 29

In order to fabricate uniform multicellular spheroids for advanced drug screening, numerous attempts have been made to develop spheroid fabrication, culture and analysis techniques. Back to 1952, Moscona et al. first demonstrated that multicellular spheroids could be fabricated based on self-assembly process. They found that the structure of culture vessel influenced the spheroid formation speed a lot. 30 The self-assembly process was simple, however, the efficiency of spheroid formation was really low because a large part of the cells attached on the substrate. In order to prevent cell attachment on the substrate and increasing the efficiency of spheroid formation in the self-assembly approach, chemical solutions, such as chitosan and poly (ethylene glycol) brushes, was used to improve the substrate surface properties, and nano-structures were fabricated on the substrate (Fig. 1A). 31-33 Cells were also cultured inside the gel-
based scaffold to mimic the 3D environment and self-aggregated into spheroids (Fig. 1E). But self-assembly technique is time-consuming, usually taking 2-4 days for cells aggregating together. Moreover, spheroids fabricated by this method display a broad size distribution, which also limits its wide spread in high-throughput standardized drug screening. NASA rotary system and spinner flask, which was designed to mimic the microgravity and maintained cells in suspension with low shear stress, greatly accelerated multicellular spheroid formation speed to several hours (Fig. 1C). But the rotary system and spinner flask are still hard to control spheroids size and fabricate standardized spheroids. Shuichi Takayama et al. developed 384-well format hanging droplet arrays for spheroid formation, cultivation and drug testing (Fig. 1F). Cell suspension droplets were formed under the balance of gravity and surface tension of the medium. Cells slowly aggregated in the center bottom of the droplets formed into spheroids. By controlling the cell density inside the medium and the size of the droplet, it was able to control the size of fabricated spheroids. Researchers has also used other external forces, such as magnetic force (Fig. 1B) and hydrodynamic force (Fig. 1D), to aggregate cells together to form spheroids. However, all those methods require labeling particles, medium modification, and/or complicated device design. Among the various spheroid formation technique, approaches predicated on acoustics offers unique characteristics, such as excellent biocompatibility, flexibility, label-free nature, and high throughput. Thus far, bulk acoustic wave (BAW) has been used for 3D spheroids fabrication. Nevertheless, because BAW highly depends on complicated resonator structure design and temperature controller, it is really difficult to fabricate uniform spheroids in a high-throughput manner based on BAW devices.
Fig. 2.1. Methods available for fabrication of 3D multicellular spheroids. (A) Self-assembly on the low-attachment substrate.30-32 (B) Magnetic force-based spheroid formation.37 (C) NASA rotary system.35 (D) Hydrodynamic force-based spheroid formation.38 (E) Self-assembly of spheroid in the scaffold.33, 34 (F) Hanging droplet for spheroid fabrication.36

2.2: 2D Acoustic Tweezers for Cell Manipulation

Surface acoustic wave is more flexible in engineering its wave front and is able to work with high frequency and manipulate cells precisely. It has been demonstrated to successfully manipulate cells in 2D plane for separation and patterning.39-41 In 2D acoustic tweezers device, 40 pairs of interdigital transducers (IDTs) were deposited on the 128° lithium niobate (LiNbO₃) substrate. A polydimethylsiloxane (PDMS) square chamber of 75 µm height was bounded in the middle of two orthogonal pairs of IDTs. After applying RF signals to IDTs, two series of orthogonal standing surface acoustic waves (SSAWs) were generated, forming periodical
distribution of pressure nodes (with minimal pressure potential) and anti-pressure nodes (with maximum pressure potential) on the substrate. When SSAWs encountering the adjacent medium inside the PDMS chamber, longitudinal-mode leaky waves were generated and caused fluctuation inside the medium. The particles and cells inside the medium were moved by the radiation force from the fluctuations in the medium. The radiation force can be expressed as

\[ F_r = -\left(\pi p_0^2 V_c \beta_w / 2\lambda\right) \cdot \phi(\beta, \rho) \cdot \sin(2kx) \]  
(2.1)

\[ \phi(\beta, \rho) = \frac{5\rho_c - 2\rho_w}{2\rho_c + \rho_w} - \frac{\beta_c}{\beta_w} \]  
(2.2)

Where \( p_0 \), \( \lambda \), \( V_c \) are the acoustic pressure, wavelength and the volume of the cell. \( \rho_c \) and \( \rho_w \) are the cell density and water density. \( \beta_c \) and \( \beta_w \) are the compressibility of the cell and water.

**Fig. 2.2. Working mechanism of 2D acoustic tweezers-based cell patterning.** SSAWs were generated by IDTs and formed pressure nodes and anti-pressure nodes inside the device chamber. By slightly changing the frequency of the SSAWs, different acoustic potential pattern can be formed. Cells were pushed to the area with minimal pressure potential, and formed different 2D cell patterning.

After turning on the RF signals and generating SSAWs, cells were pushed to the pressure nodes and formed 2D patterning. By changing the wavelength of the SSAW, it was able to change the pattern structure and form different kinds of 2D cell pattern showed in Fig. 2.2.
In order to use acoustic waves to construct more in vivo like artificial tissue structures, the ability to manipulate cells in 3D environment and aggregate cells into 3D assembles is badly needed. In the following sections, we further investigated the propagation of leaky acoustic wave in vertical direction and developed a 3D acoustic tweezers-based technique to rapidly and reproducibly fabricate uniform, size-controllable 3D cell assembles.
CHAPTER 3: METHODOLOGY

3.1: Experimental design

Fig. 3.1. 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 a 3D Gor’kov potential field inside the chamber. Cells aggregate into spheroids inside the well-like acoustic field. (C) Spheroid formation consists of three steps: 1) injection of cell suspension, 2) acoustic tweezers-based spheroid formation, and 3) collection of spheroids from a Petri dish. By repeating the three steps, this platform can yield spheroids in a semi-continuous fashion.

3D acoustic tweezers-based spheroid formation technique relies on establishing 3D Gor’kov potential fields and microstreaming inside a microfluidic chamber, which concentrates cells into geometrically defined spaces (Fig. 3.1 B). Fig. 3.1A is the image of the 3D acoustic tweezers device. 40 pairs of IDTs were deposited on the LiNbO₃ substrate. A PDMS chamber of
100 µm height was bounded on the substrate. At the beginning, cell suspension was injected into the PDMS chamber. Then, standing SAWs were generated by applying RF signals to two orthogonal pairs of interdigital transducers (IDTs). The two orthogonal waves propagated on the surface of LiNbO$_3$ substrate, forming a dotted array of transverse displacement nodes and antinodes. The surface vibrations were translated to the medium at the substrate-liquid interface, resulting in a Gor’kov potential distribution and a patterned microstreaming within the liquid medium. Cells formed aggregates in the regions where the Gor’kov potential is minimum in horizontal direction. After waiting for 30 minutes, mature spheroids were washed out from device, and new cell suspension was injected into the chamber. By repeating the fabrication procedures (injecting cell suspension-SSAWs based spheroid formation-washing spheroids out), we can continuously fabricate in a high throughput manner (Fig. 3.1C).

3.2: Working Mechanism of 3D Acoustic Tweezers

Fig. 3.2B 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 the Supporting Information. Cells in the central ellipsoidal area A1 (above the pressure node) were subject to minimal acoustic radiation force and drag force. In contrast, the areas near the substrate were subject to maximal drag force, and the areas above the anti-pressure nodes were subject to maximal acoustic radiation force. After injecting cells into the channel, cells would distribute in different layers of the channel randomly. Once RF signals are applied to the IDTs, standing SAWs were generated, forming Gor’kov potential field and microstreaming inside the medium. Floating cells (above the substrate) were pushed by acoustic radiation force to aggregate together in area A1. Cells rested on the substrate or near the substrate were levitated by
drag force induced by microstreaming in vertical direction, and then pushed to A1 area by acoustic radiation force (Fig. 2B). 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. The sizes of the spheroids were ~70 µm. Thus, the height of the chamber should be larger than 90–100 µm. 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 because in the areas near the substrate, the vertical direction of acoustic radiation force is opposite to the vertical direction of microstreaming (Fig. 2A). Too large input power will cause acoustic radiation force near the substrate dominating over the drag force, and results in that cells near the substrate cannot be levitated to form aggregates. In this case, there would form two layers of cell pattern, reducing the effectiveness of spheroid formation.

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 (75 µm, 90 µm, 100 µm, and 110 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, the cells easily separated into two layers in vertical direction, and there was no patterning for the bottom layer. 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 100µm 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. Incidentally we 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.  

Fig. 3.2. The working mechanism of 3D acoustic tweezers. (A) Schematic of the 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 pushed to aggregate by acoustic radiation force, while cells near the substrate were levitated by the drag force and then pushed to aggregate together to form spheroids. (B) Simulation of the distribution of acoustic radiation force and acoustic streaming force around pressure nodes 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 areas of minimal Gor’kov potential, forming patterned spheroid arrays.
3.3: Goals and Timeline

At the beginning of the project, a timeline was created, along with measures of success. The project was divided into three phases. The first phase (6 months) was dedicated to investigate the working mechanism of 3D acoustic tweezers for cell patterning, and demonstrating the feasibility of fabrication of 3D cell assemblies. The second phase (2 months) was dedicated to achieve rapid and high-throughput fabrication of cell spheroids based on 3D acoustic tweezers device. The third phase (4 months) was dedicated to demonstrate the multicellular spheroids fabricated by 3D acoustic tweezers device can function as well as spheroids fabricated with other methods, and can further be applied to anti-cancer drug testing.

The goals for the first two months of phase one was to use polystyrene beads to investigate the acoustic field and forces in vertical direction, and realize manipulating beads in 3D environment. In the month 3-5, we would work to investigate the optimized parameters (chamber height, input power, input frequency and chamber structure) for 3D cell manipulation. We used Hela cells for optimization experiments. The goal was to successfully manipulate cells in vertical direction and realize 3D cell aggregates formation. In months 4-6, we would work to build models to explain the underlying working mechanism of 3D cell manipulation, realizing the match between model and experimental results. The goals of phase 2 (months 7-8) were to fabricate standardized PDMS chips and realize stable experimental results of fabrication spheroids. We would also work to achieve fabrication of spheroids with different sizes by changing the input cell concentration. The goals of phase 3 (months 9-12) were to high-throughput fabricating size controllable spheroids and demonstrate the high live cell percentage and unchanged biological
properties of those spheroids. We would work to achieve long-term chip-free culture of spheroids and use the spheroids fabricated by our 3D acoustic tweezers method for anti-cancer drug testing.

In all of our experiments, videos of experiments were obtained with Nikon fluorescent microscope and confocal microscope, and analyzed with ImageJ 1.46 software.

3.4: Experimental Setup

3.4.1 Fabrication of acoustic tweezers device

A 2 mm × 2 mm polydimethylsiloxane (PDMS) microchamber was bonded to a LiNbO$_3$ 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 mold replica techniques. IDTs were formed by depositing double-layered metal (Cr/Au) on the LiNbO$_3$ substrate and removing photoresist by the lift-off process. After plasma treatment, the PDMS chamber and the LiNbO$_3$ substrate were bonded and then heated in an oven for 24 h. The fabrication steps of the acoustic tweezers device were detailed Appendix A and Appendix B.

3.4.2 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 1000 rpm for 1 min at room temperature, and re-suspension in growth media. Cell density was estimated using a hemocytometer.

3.4.3 Spheroid formation and long-time culture

![Experimental setup](image)

**Fig. 3.3 Experimental setup**

The experimental setup is showed in Fig. 3.3. It consists of one function generator, two amplifiers, one microscope and a cooling plate. Before experiments, the 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 was used 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 SAW and form arrays of pressure nodes on the substrate (Fig. 1A). Vibration of the substrate caused longitudinal-mode leakage waves and microstreaming inside the adjacent medium, establishing a 3D Gor’kov potential field above every pressure node (Fig. 1B). A spheroidal cluster of cells was trapped in the areas of zero Gor’kov potential by the combination of acoustic radiation force and microstreaming force. Finally, we turned off the SAW 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 steps (injecting — SAW for 20–30 min — 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 24h using a confocal fluorescence microscope. Living cells were labeled with green fluorescent using Calcein AM and dead cells were labeled with red fluorescent using propidium iodide (Life Technologies, USA). The cellular viability was calculated by the ratio of green fluorescent area over total spheroid area measured in software Image J.

### 3.4.4 Anticancer 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 3D acoustic tweezers platform with input cell density of $8 \times 10^6$ cells/ml, then collected and cultured in Petri dishes. On day 1 of spheroid culture, 400 µl of each concentration of 5-FU solution was added to each Petri dish containing 3600 µl 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 over total spheroid area measured in software Image J.
CHAPTER 4: RESULTS

4.1: Controlling Size of the 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.\textsuperscript{42, 43} Therefore, controlling cell packing densities and the size of cell spheroids is critical to minimizing variations in drug screenings. As discussed in the previous section, our system formed hundreds of same 3D Gor’kov potential area arrays, so the spheroids generated by our system were of similar sizes.

![Fig. 4.1. Formation of size controllable spheroids via 3D acoustic tweezers. Optical image of](image-url)
patterned 3D spheroid arrays. HepG2 cells were added to microfluidic chambers at concentrations of (A) $2 \times 10^6$ cells/ml and (B) $1.7 \times 10^7$ cells/ml. (C) Diameters of spheroids measured at different cell densities ($n \geq 30$). (D) Percentages of viable cells in the spheroids after turning on SAW for 30 min ($n \geq 30$).

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 ($2, 5, 8, 11, 14, 17 \times 10^6$ 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 $2 \times 10^6$ cells/ml (Fig. 3A), while larger spheroids were fabricated by injecting a $1.7 \times 10^7$ cells/ml solution (Fig. 3B). Indeed, spheroids were formed from the cells initially trapped in 3D Gor’kov potential arrays, 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 SAW. 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 labeled with Calcein AM were pushed to aggregate under acoustic wave. After applying SAW to HEK 293 cells (Fig. S1) and HepG2 tumor cells (Fig. 3D) for more than 30 min, the live cell percentage was still greater than 95%.
4.2: 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 based on input sample concentration. For this method to be applicable, it has to be amenable to biologists upon interfacing with commonly used platforms in cell biology such as Petri dishes-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 inside the chamber, cells aggregated at the areas of minimal Gor’kov potential. 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. 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).
Fig. 4.2. 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 labeled with calcein AM (green), and dead cells were labeled with propidium iodide (red). (C) The diameters of spheroids and (D) percentages of viable cells in the spheroids were measured each day (n ≥ 30).

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 larger 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.

### 4.3: Spheroids for Anti-cancer Drug Testing

The scalable, biocompatible, and rapid nature of our acoustic tweezers-based spheroid fabrication method makes it convenient for fabricating 3D 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 acoustic tweezers technique to fabricate spheroids of average diameter 70 µm, and cultured them in Petri dishes. For the control group, we added a single cell suspension to a Petri dish 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 take 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 magnified image is showed in Fig. S3 in the Supporting Information). 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.\textsuperscript{12,15}
Fig. 4.3. Anticancer drug testing of 3D acoustic tweezers-generated 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.

4.4: Spheroids based Coculture Model for Angiogenesis Investigation

Tumor angiogenesis is a complicated process involving tumor cells and endothelial cells communicating in the 3D microenvironment. During the angiogenesis, the development of blood vessels is critical for local tumor growth and distinct tumor metastasis. A better understanding about the individual steps of tumor angiogenesis and the formation of vascular tubes is critical for developing angiogenesis inhibitors and drugs for tumor metastasis treatment.47,48

During the past few years, researchers have found that human glioma cells (U87 cells) could induce an “activated” phenotype in endothelial cells and result in an increase of endothelial cell proliferation, migration and formation of vascular-like tube.49 Moreover, It is reported that the limitation of the nutrient and oxygen inside the tumor spheroids or solid tumors will stimulate tumor cells to express significant amount of angiogenesis factors to actively recruiting and
establishing blood vessel networks. However, until now, no models can recreate the complicated 3D U87 spheroids-endothelial cells interaction phenomenon and provide the insight of the mechanisms of solid tumor induced vascular formation and tumor metastasis inside the cancer patient body.

**Fig. 4.4. The procedures for developing 3D U87 spheroid-HMVEC cell coculture model**

Based on the tumor spheroids fabricated by the 3D acoustic tweezers device, we were able to construct a 3D U87 spheroid-HMVEC cell coculture model in Matrigel, and investigate the influence of tumor spheroid on tube formation process. In the experiments, we first used 3D acoustic tweezers device to fabricate size-controllable U87 spheroids. We transferred the mature spheroids into the Matrigel and mixed the gel with the spheroid uniformly. Next, we mixed the same amount of separated HMVEC cells with Matrigel uniformly. Then, we added the Matrigel with U87 spheroids in one position (position A) on the Petri dishes, and added the Matrigel with HMVEC cells in a different position (position B) on the Petri dishes. Finally, we used Matrigel without cells to connect position A and B together, ensuring the fluent chemical cues transmission between U87 spheroids and HMVEC cells (Fig. 4.4). We also did three control experiments. In the first control experiments, we cultured only HMVEC cell inside the gel. In the second control experiments, we uniformly mixed U87 spheroids and HMVEC cells together and cultured the cell-
spheroid mixture inside the Matrigel. In the third control experiments, we used separated U87 cells, instead of the U87 spheroid and repeat the procedures in Fig. 4.4 to build the 3D coculture model.

After building the 3D U87 spheroid-HMVEC cell coculture model and three control models, we put Petri dishes in the 37º incubator for 30 minutes, allowing the polymerization of the gel. Then we added cell culture medium inside the Petri dishes and put them in the incubator for long-term culture. Culture medium was changed every three days to provide sufficient nutrient and remove wastes. Cell growth conditions were monitored under the fluorescent microscope every day.

Fig. 4.5 shows the characterization of HMVEC-based tube formation in 3D Matrigel culture model after culturing for a whole week. While culturing HMVEC cells alone in the Matrigel, HMVEC cells tended to aggregate into spheroidal structure. Inside every spheroidal structure, there were lots of tiny micro-tubed formed. At this condition, no obvious cell proliferation was observed (Fig. 4.5A). In the second control experiments, HMVEC cells were mixed with U87 spheroids and cultured together inside the Matrigel. After culturing for a whole week, the cell morphology changed into thin and long. Discrete tubes were formed surrounding the U87 spheroids, but no network was observed (Fig. 4.5B). Fig. 4.5C shows the condition of co-culturing HMVEC cells and U87 cells separately. After a whole week, most cells remained in the original position. A small amount of cells formed discrete tubes in the center of the Petri dish between HMVEC cells and U87 cells.
Fig. 4.5. Characterization of HMVEC-based tube formation in 3D Matrigel model. (A) When only HMVEC cells were cultured in the Matrigel, cells tended to aggregate into spheroidal structure and formed tiny micro-tubes. (B) When HMVEC cells were uniformly mixed with U87 spheroids, the cell morphology elongated and discrete tubes were formed. (C) When HMVEC cells were co-cultured with U87 cells, discrete tubes were formed, but tube formation ratio was really low. (D) When HMVEC cells were co-cultured with U87 spheroids, a network of HMVEC tubes were formed with a high tube formation ratio. (Scale bar: 100 µm)

Fig. 4.5D shows the results of co-culturing HMVEC cells with U87 spheroids separately. In this condition, a tube network was formed in the whole Petri dish, not just in the center area between the two types of cells. The network connected all the U87 spheroids together and extended tubes into the areas with HMVEC cells. Obvious proliferation of HMVEC cells was observed and the volume of the U87 spheroids grew larger. Compared with other three control
experiments, we believe that the tumor spheroids have a stronger effect on increasing the expression of angiogenesis factors and accelerating tube formation.

**Fig. 4.6. The analysis of endothelial cells-based network formation in Matrigel.** (A) Co-culturing of U87 spheroids and HMVEC cells. (B) Co-culturing of U87 cells and HMVEC cells.

We also used the Angiogenesis Analyzer function in Image J to analysis of the pseudo vascular networks of endothelial cells in Matrigel. After using Image J to remove the small segments, which was the isolated cells, the binary map of extremities, nodes final binary pruned tree and elements of networks were showed in the color maps. And the master tree of the network was showed in the last phase contrast map. From the phase contrast map, a distinguished difference can be observed between the spheroid-cell coculture model and the cell-cell coculture model, which further demonstrated that tumor spheroids fabricated by our 3D Acoustic Tweezers can significantly enhance the pseudo vascular network formation of endothelial cells.
CHAPTER 5: CONCLUSION

5.1: Summary

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 areas where they were subject to minimal acoustic radiation force. After optimizing parameters such as input signal frequency, power, chamber height, SAW duration time, and cell concentration, we formed in the chamber large-scale cell spheroids of identical structures. We further demonstrated that mature spheroids were able to be washed out of the chamber and cultured in Petri dishes for biological studies and drug testing. We also used the U87 spheroids fabricated by 3D acoustic tweezers to build complicated 3D co-culture model in Matrigel and investigated the spheroid-based enhancement of angiogenesis. Our platform can easily scale up to a larger chamber, and continuous spheroid generation can be achieved in the same device by repeating injections, acoustic tweezers-based patterning, and collecting procedures.

The major advantages of our 3D 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 behaviors; 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 spheroids size from several to hundreds of cells). With these advantages, our acoustic
tweezers based spheroid production and handling approach can be valuable for pharmacology, tissue engineering, and biological research.

5.2: Future Work Suggestions

In the next phase of development, we should seek to fabricate more complex and *in vivo* like 3D cell assemblies to investigate 3D cell-cell communication and build more accurate models for drug screening. There are two directions for fabrication of complicated 3D artificial cell models. One is to adjust acoustic potential field, aggregating different types of cells into different structures, not just spheroidal structure. In order to adjust acoustic potential field, a deeper understanding of the underlying physics, such as the interaction of acoustic potential field and cells, is needed. IDT structures, micro-chamber structure and materials for micro-chamber should be designed carefully, taking advantages of various acoustic phenomenons, such as wave-guide and resonance. Another direction is using the spheroids fabricated by this 3D acoustic tweezers method to construct other co-culture models for deeper biological research. For example, co-culturing tumor spheroids with endothelial cells in gel to investigate how spheroids influence tube formation, mimicking the angiogenesis process inside the body. Overall, we believe our 3D acoustic tweezers technology provide a better way for bio-manufacturing, and we would expect this technology to fabricated more *in vivo* like tissue structures for tissue engineering and drug screening.
APPENDIX A

IDT Fabrication

Fig. A1(a) shows the IDT fabrication procedures. The IDTs, used to generate surface acoustic wave on the substrate, was fabricated through the standard lithography-deposition-lift of process. In order to couple efficient energy into medium, a Y+ 128 X-propagation lithium niobate (LiNbO3) wafer (500 mm thick) was used as the substrate for IDT deposition. At first, a layer of photoresist (SPR3012, MicroChem, Newton, MA) was uniformly coated on the LiNbO3 wafer. After exposure and develop, the pattern of IDTs was transferred from a plastic mask to photoresist. Then, a double metal layer (Cr/Au, 50 Å /500 Å) was deposited on the wafer via e-beam evaporator. At last, a lift-off process was used to remove the extra photoresist and metal, remaining only IDT structures on the substrate.  

Fig. A1. IDTs and PDMS chamber fabrication process.  

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APPENDIX B

PDMS Fabrication

Fig. A1(b) shows the PDMS fabrication procedures. The PDMS chamber was fabricated by a mold replica procedure. The mold for replica was a silicon wafer fabricated by lithography-deep reactive ion etching (DRIE) process. At first, a layer of photoresist (SPR955, MicroChem, Newton, MA) was uniformly coated on the silicon wafer. After exposure and develop, the chamber structure was transferred from a plastic mask to photoresist. Then, DRIE was performed on the pre-patterned silicon wafer and extra photoresist was removed by chemical solution Removal PG. Once the mold was fabricated, the PDMS chamber was made by simply filling the mold with PDMS gel (Sylgard184TM Silicone Elastomer base was mixed with curing agent with the ratio of 10:1), curing it in the 70°C oven for 30 minutes, and then removing it from the mold. The inlets and outlets of the PDMS chamber was fabricated by a 0.75 mm punch (Harris uni-core). At last, the LiNbO₃ substrate and PDMS chamber were treated with oxygen plasma. PDMS chamber was bonded on the substrate and cured in the oven overnight to form a sealed device.
APPENDIX C

Theoretical Framework and Model Setup

We have previously described the simulation model. Via perturbation theory, we asymptotically expanded the governing equations by a small parameter, and obtained first- & second-order equations. These two equations are solved numerically, together with a lossy-wall boundary condition (BC) and the vibrating displacement of the standing SAW, to model the reflection effects of the PDMS wall and the substrate actuation. The first-order solution describes the harmonic motion of acoustic particles, which contributes to the acoustic radiation force acting on cells and the sources of acoustic streaming. The second-order equation describes the dynamics of acoustic streaming.

The governing equations are the Navier-Stokes and continuity equations, given as:

\[ \rho \frac{\partial v}{\partial t} = -\nabla \cdot (\rho v) \]  \hspace{1cm} (S1)

\[ \rho \frac{\partial v}{\partial t} + \rho (v \cdot \nabla)v = -\nabla p + \mu \nabla^2 v + \left( \frac{1}{3} \mu + \mu_b \right) (\nabla \cdot v) \]  \hspace{1cm} (S2)

Here the fluid’s density is \( \rho \), velocity \( v \), pressure \( p \), shear viscosity \( \mu \), and bulk viscosity \( \mu_b \).

Per perturbation theory, the first-order harmonically time-dependent fields is:

\[ i \omega \rho_1 + \rho_0 \nabla \cdot (v_1) = 0 \]  \hspace{1cm} (S3)

\[ i \omega \rho_0 v_1 = -c_0^2 \nabla \rho_1 + \mu \nabla^2 v_1 + \left( \frac{1}{3} \mu + \mu_b \right) \nabla (\nabla \cdot v_1) \]  \hspace{1cm} (S4)
Here, \( \mathbf{v}_t \) is the velocity of acoustic particle; \( c_0 \) is the speed of sound of liquid; \( \rho_0 \) is the density of the quiescent liquid; \( \rho_1 \) is a small fluctuation of density and relates to acoustic pressure \( p_1 \) via the thermodynamic state equation \( \rho_1 = \frac{p_1}{c_0^2} \); and \( \omega \) is angular velocity.

The time-averaged second-order equation is

\[
\rho_0 \nabla \cdot \langle \mathbf{v}_2 \rangle = -\nabla \cdot \langle \rho_1 \mathbf{v}_1 \rangle \tag{S5}
\]

\[
\left\langle \rho_1 \frac{\partial \mathbf{v}_1}{\partial t} \right\rangle + \rho_0 \langle \mathbf{v}_1 \cdot \nabla \mathbf{v}_1 \rangle = -\nabla \langle p_2 \rangle + \mu \nabla^2 \langle \mathbf{v}_2 \rangle + (\mu_b + \frac{1}{3} \mu) \nabla (\nabla \cdot \langle \mathbf{v}_2 \rangle) \tag{S6}
\]

Here \( \langle \cdot \rangle \) is the time-averaged quantity over one oscillation period, and \( \langle \mathbf{v}_2 \rangle \) is the velocity of acoustic streaming.

To solve the first- and second- order equations, boundary conditions (BCs) representing the vibration of LiNbO\(_3\) substrate and the reflection of a lossy PDMS wall were included.

The BC of lossy PDMS wall is:

\[
n \cdot \nabla p_1 = i \frac{\omega \rho_0}{\rho_w c_w} p_1 \tag{S7}
\]

Here \( n \) is the normal vector, \( \rho_w \) is the density of PDMS wall, and \( c_w \) is the speed of sound of the PDMS wall.

The bottom boundary of the domain is LiNbO\(_3\) substrate vibration from standing SAW. The substrate’s vibration is described as the superposition of two oppositely propagating leaky SAWs:

\[
u_x(t,x) = \alpha u_0 e^{-c_x t} \sin\left(\frac{-2\pi x}{\lambda} + \omega t\right) + \alpha u_0 e^{-c_d(l_2-x)} \sin\left(\frac{-2\pi (l_2 - x)}{\lambda} + \omega t\right) \tag{S8}
\]

\[
u_z(t,x) = -u_0 e^{-c_x t} \cos\left(\frac{-2\pi x}{\lambda} + \omega t\right) - u_0 e^{-c_d(l_2-x)} \cos\left(\frac{-2\pi (l_2 - x)}{\lambda} + \omega t\right) \tag{S9}
\]
Here $u_x$ and $u_z$ are longitudinal and transverse displacements of the substrate, respectively; $C_d$ is the decay coefficient; $\alpha$ is the displacement ratio of transverse vibration to longitudinal vibration; and $l_z$ is the chamber’s width.

The acoustic radiation force is calculated based on the first-order fields, $F^{rad} = -\nabla U$, where $U$ is Gor’kov potential:

$$U = V_0 \left( \frac{\rho_0^2}{2\rho_0 c_0^2} f_1 - \frac{3\rho_0 v_1^2}{4} f_2 \right)$$  \hspace{1cm} (S10)

Here $V_0$ is the particle’s volume; the coefficients are $f_1 = 1 - \frac{\rho_0 c_0^2}{\rho_p c_p^2}$ and $f_2 = \frac{2(\rho_p - \rho_0)}{2\rho_p + \rho_0}$, where $\rho_p$ is the mass density of the particle; and $c_p$ is the speed of sound of the particle.

The Stokes drag force induced by the second-order effect of acoustic streaming is $f_d = -6\pi\mu R < \mathbf{v}_2 >$, where $R$ is the radius of the particle.

The finite element method-based software package COMSOL Multiphysics 4.4 was used to numerically solve the first- and second-order equations with the mentioned BCs. The acoustic radiation force and the acoustic streaming inside the fluid domain were calculated from first- & second-order fields. The parameters for the numerical solution are in Table S1.

**Fig. S1. The computational domain and boundary conditions (BCs).** (A) Schematic of the acoustic tweezers based patterning device: a PDMS chamber, the medium inside the chamber,
and a LiNbO$_3$ piezoelectric substrate. (B) The computational domain is the medium inside the microfluidic chamber. The chamber’s wall is modeled as an acoustically lossy wall BC, whereas the LiNbO$_3$ substrate is modeled as a vibrating BC with both longitudinal and transverse displacements.

<table>
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<th>PDMS chamber</th>
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<tbody>
<tr>
<td>Length ( (l_1) ): 6000 µm</td>
</tr>
<tr>
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<td>Density ( \rho_w )</td>
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<tr>
<td>Speed of sound ( c_0 )</td>
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<td>Bulk viscosity ( \mu_b )</td>
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<table>
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<th>LiNbO$_3$ substrate</th>
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<td>Density ( \rho_s )</td>
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<td>Speed of sound ( c_s )</td>
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<tr>
<td>Acoustic frequency ( f )</td>
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<tr>
<th>Cell</th>
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</thead>
<tbody>
<tr>
<td>Density ( \rho_p )</td>
</tr>
<tr>
<td>Speed of sound ( c_p )</td>
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</tbody>
</table>

**Table S1. Parameters for numerical solution**
APPENDIX D

Supplementary Results

1) Patterning polystyrene beads using acoustic tweezers

![Fig. S2. Patterning of 10 µm polystyrene beads using 3D acoustic tweezers. (A) When the SAW was off, the beads inside the PDMS chamber dispersed uniformly. (B) When the SAW was on, beads aggregated in the areas subjected to the lowest acoustic radiation force. The two photographs are different focuses at the same position: the beads are distributed in 3D environment.](image)

2) HEK 293 cell aggregates patterning and cell viability test

![Movie S1. Patterning of HEK 293 spheroids arrays using acoustic tweezers. HEK 293 cells](image)
were labeled with calcein AM to show the cell viability during acoustic tweezers-based patterning. Cells aggregated into spheroidal structure when subjected with the lowest acoustic potential. The movie is in real-time.

Fig. S3. The survival rate of HEK293 cells in spheroidal aggregates after turning on SAW for 20 min.

3) HepG2 cell aggregates under confocal microscope

Movie S2. Different layers in vertical direction of HepG2 spheroids under confocal fluorescent microscopy. The HepG2 spheroid was fabricated by the acoustic tweezers method and cultured for 4 days. HepG2 cells were labeled with calcein AM to show the cell viability.
4) Magnified images of HepG2 spheroids after anti-cancer drug testing

Fig. S4. Magnified images of HepG2 spheroids after anti-cancer drug testing.
REFERENCES


