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Micro-Ring Structures Stabilize Microdroplets to Enable Long Term Spheroid Culture in 384 Hanging Drop Array Plates

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Abstract

Using stereolithography, 20 different structural variations comprised of millimeter diameter holes surrounded by trenches, plateaus, or micro-ring structures were prepared and tested for their ability to stably hold arrays of microliter sized droplets within the structures over an extended period of time. The micro-ring structures were the most effective in stabilizing droplets against mechanical and chemical perturbations. After confirming the importance of micro-ring structures using rapid prototyping, we developed an injection molding tool for mass production of polystyrene 3D cell culture plates with an array of 384 such micro-ring surrounded through-hole structures. These newly designed and injection molded polystyrene 384 hanging drop array plates with micro-rings were stable and robust against mechanical perturbations as well as surface fouling-facilitated droplet spreading making them capable of long term cell spheroid culture of up to 22 days within the droplet array. This is a significant improvement over previously reported 384 hanging drop array plates which are susceptible to small mechanical shocks and could not reliably maintain hanging drops for longer than a few days. With enhanced droplet stability, the hanging drop array plates with micro-ring structures provide better platforms and open up new opportunities for high-throughput preparation of microscale 3D cell constructs for drug screening and cell analysis.

Keywords

micro-ring; spheroid; high-throughput; 3D culture; hanging drop; stereolithography

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Introduction

Three-dimensional (3D) cell cultures are widely recognized as crucial tools for tissue engineering, regenerative medicine, cancer research, stem cell biology, and drug discovery (Khademhosseini et al. 2006; Lin and Chang 2008; Kelm and Fussenegger 2004; Muelloer-Klieser 1997; Hirschhaeuser et al. 2010). Many of these applications require long term culture of cells in 3D, which is traditionally a challenging task. One of the best 3D cell culture models that is simple yet physiological is the spheroid, which is a spherical aggregate of cells typically formed spontaneously in environments where cell-cell interactions dominate over cell-substrate interactions (Lin and Chang 2008; Friedrich et al. 2009; Kunz-Schughart et al. 2004; Hirschhaeuser et al. 2010). However, conventional methods of spheroid formation that allow for long term culture such as the use of spinner flasks or NASA rotary system both require specialized equipments and have poor spheroid uniformity control (Lin and Chang 2008). Various other spheroid culture methods such as the hanging drop technique and sophisticated devices utilizing microscale technologies provide uniformity control over spheroid size, but cells often cannot be cultured in a long term manner (Lin and Chang 2008; Lee et al. 2010). The conventional hanging drop method is extremely difficult to exchange cell culture media without compromising the integrity of the droplets, which makes long term culture almost impossible. It is also challenging to treat microscale-based device surfaces non-adherent to cells for long term. As a result, such devices are often not suitable for 3D long term culture as cells eventually start attaching to device surfaces and thus becoming 2D cultures.

We have recently combined micro-technologies and the conventional hanging drop technique and successfully developed a 384 hanging drop array plate for high-throughput spheroid culture (Fig. 1(a), (c); Tung et al. 2011). This novel platform greatly simplifies laborious liquid handling processes involved in the conventional hanging drop method and offers high-throughput capabilities. We have already shown a number of valuable applications of the 384 hanging drop array plate including high-throughput drug testing and 3D cellular patterning (Tung et al. 2011). One limitation of this previously reported device was the lack of stability against droplet spreading triggered by mechanical shock and surface fouling. To further expand the versatility of the platform, the 384 hanging drop array plate must be able to culture spheroids in a long term manner to allow for more comprehensive biological studies and applications. Here, we address this issue by using rapid prototyping 3D printing technology to screen for different microstructures (plateaus, trenches, and micro-rings) that enhance stabilization of hanging droplets. A recent publication by Kalinin et al. (2008) offered particularly useful insights to enhance droplet stability using micro-topographical rings (Figure 1b, d).

Stereolithography is a rapid prototyping technology that has been a powerful tool in generating precise 3D structures by building components in a laminated fashion (Selvaraj et al. 2011). Stereolithography apparatus (SLA) typically utilizes ultraviolet (UV) laser beam to selectively induce chemical reactions that solidify photosensitive liquid resin into highly crosslinked polymers (Selvaraj et al. 2011; Chan et al. 2010). The fabrication process is directed by computer-aided design (CAD) with laser beam scanning layer by layer in three dimensions (Selvaraj et al. 2011). Stereolithography has been widely used in rapid prototyping of components with complex 3D geometries requiring resolutions beyond the limits of conventional machining (Selvaraj et al. 2011). Specifically in the biomedical field, 3D stereolithography has been widely adopted in the rapid prototyping and modeling of patient body parts such as the skull, mandible, temporal bone, and aorta (Bakhos et al. 2010; Fallahi et al. 1999; Kettner et al. 2011; Rudman et al. 2011; Melchels et al. 2010b). Such applications of the stereolithography technique have thus been very useful in the planning and training of craniofacial surgery as well as complex neurosurgery (Paiva et al. 2007;

Foroutan et al. 1998; Lopponen et al. 1997; Rudman et al. 2011; Melchels et al. 2010b). Stereolithography has also been used in the rapid prototyping and fabrication of 3D biocompatible polymers, implantable devices, and biodegradable scaffolds and cell-containing hydrogels for tissue engineering (Cooke et al. 2003; Lee et al. 2008; Gill and Claeysens 2011; Mapili et al. 2005; Shin et al. 2011; Chan et al. 2010; Melchels et al. 2010a; Melchels et al. 2010b).

Recently, fabrication of 3D nano- and microstructures has been achieved through microstereolithography and the implement of advanced techniques such as two-photon polymerization (Selvaraj et al. 2011; Lim et al. 2011; Park et al. 2009; Melchels et al. 2010b; Maruo and Ikuta 2002; Narayan et al. 2010). Sophisticated microdevices and parts such as optically driven micropumps (Maruo and Inoue 2006; Maruo et al. 2009) and micromachines (Maruo et al. 2003), 3D magnetic microstructures (Kobayashi and Ikuta 2008), 3D microfluidic chip prototype for biochemical applications (Kobayashi and Ikuta 2007), miniature flow sensors (Leigh et al. 2011), and even artistic structures such as the micro-bull (Kawata et al. 2001) have been fabricated. Such advances have become increasingly useful for application in microfluidic devices, microelectromechanical systems (MEMS), micro total analysis systems (microTAS), and rapid prototyping of microstructures. However, despite the increasing availability of stereolithography, the technique has yet to be applied to the development of biomedical microdevices more extensively. Here, we use stereolithography-based rapid 3D prototyping to screen various microscale features such as plateaus, trenches, and micro-rings for geometrical combinations that can stabilize microliter sized droplets against mechanical perturbations. We observed that micro-ring features were particularly helpful through this screening exercise and transferred this micro-ring structure into a mass manufacture-compatible format. The resulting injection molded polystyrene 384 hanging drop array plates with micro-ring features (Figure 1b, d) significantly enhanced robustness and user-friendliness enabling practical high-throughput manipulation and long term 3D hanging drop cell culture. We show that hanging drops formed in the newly modified plates are stable without the issue of droplets spreading outward for up to 22 days of incubator culture, and further demonstrated spheroid culture over a 14-day period; both presenting significant improvements over the previous model.

Materials and Methods

Micro-structures designed and tested

When making hanging drops using through-hole structures, liquid spreading can occur on the top side where liquid barely peaks out the top as well as on the bottom side where most of the droplet volume hangs (Figure 2a). To enhance the long term stability of the hanging drops in the 384 hanging drop plates, we considered three key features: plateaus, trenches, and micro-rings (Figure 2b, c). Plateaus are millimeter wide features that protrude 0.5 mm from the plate surface whereas trenches are millimeter features that indent 0.5 mm into the plate. Micro-ring structures for the initial structure screening were 0.25 mm wide and protruded to various heights. Table 1 summarizes various combinations of plateau, trench, and micro-ring features compared to stabilize droplets on the bottom side of the plate. Table 2 summarizes micro-structures tested for stabilization against liquid spreading on the top side.

Stereolithography

Rapid prototype plates each with multiple different design features and dimensions (Table 1 and Table 2) were fabricated by a stereolithography machine (SLA Viper si2, 3D Systems, Inc.). The different design features for stabilization of droplets on the bottom side of the

plate summarized in Table 1 were fabricated on a total of 4 separate 384 hanging drop array prototype plates. Each of the 4 prototype plates contains 4 to 6 different designs as illustrated in Table 1 (“Placement of Each Design” column). There are 64 through-holes for each design, with the exception of Design 1 and Design 11, where there are 192 through-holes for each. For the top side design features, all 8 designs were fabricated on the same 384 hanging drop array prototype plate as illustrated in Table 2 under the “Placement of Each Design” column. There are 48 through-holes for each design. The upper half of the prototype plate contains through-holes surrounded by extra micro-ring structures (Designs 1 to 4) at varying ring heights and widths, whereas the through-holes in the lower half of the prototype plate all contain indented trench structures (Designs 5 to 8) with varying heights and widths. All 5 prototype plates were designed in commercial software (SolidWorks) and fabricated on the stereolithography machine from a 25.4 cm cube build area using Accura® 25 liquid resin (3D Systems, Inc.). The X & Y resolution is within 25.4 μm, and the layering is 50.8 μm in the Z-axis.

Screening Different Microstructures for Droplet Stability

We performed two types of tests: A rapid test for stability of droplets on the bottom of the plate against an applied centripetal force, designed to test the acceleration effect of mechanical perturbations; and a long-term static test for liquid spreading on the top side of the plate, designed to test the resistance to surface fouling. In the rapid test, a piece of glass slide was attached to the bottom of each prototype plate (Figure 3a) to provide a flat surface for attachment of the prototype plates onto a spin coater. Droplets of 15 μL cell culture media (DMEM (Gibco 11995, Invitrogen Co.) + 10% v/v FBS (Gibco 10082, Invitrogen Co.) + 1% v/v Antibiotic-Antimycotic (Gibco 15240, Invitrogen Co.)) were formed in 2 to 4 hanging drop sites located at 2 different radii (R_1 and R_2) for each design. The rapid prototype plates were each subsequently placed on a spin coater (Cee 200X, Brewer Science, Inc., Rolla, MO) and spun at increasing speeds (with 4 seconds of ramp time and 20 seconds of total spin time), starting at 210 rpm with 30 rpm increments until the media droplets spread outward. The maximum speed at which each of the droplets spread outward on the bottom side of the plate was recorded to characterize the droplet stability of each design. The rationale is that depending on the distance (R_1 or R_2) of the droplets to the center of the spindle, each droplet will experience different centripetal acceleration. The best design is determined to be the one that is able to maintain the droplet stable at the highest centripetal acceleration. The centripetal acceleration is calculated using the following formula:

$$a_c = v^2/r = (\omega r)^2/r = \omega^2 r$$

where a_c is the centripetal acceleration, v is the linear velocity, r is the radius, and ω is the angular velocity.

For the static test, the main purpose was to test the ability of the modified microstructures in preventing droplets from merging with the media remnant left from repeated pipetting during media exchanges on the top of the plate. Droplets of 15 μL cell culture media (DMEM (Gibco 11995, Invitrogen Co.) + 10% v/v FBS (Gibco 10082, Invitrogen Co.) + 1% v/v Antibiotic-Antimycotic (Gibco 15240, Invitrogen Co.)) were formed by manually pipetting in 192 hanging drop sites at staggered positions. The plate was subsequently sandwiched between a 96-well plate lid and plate filled with distilled water, and incubated in a humidified incubator (37°C in an atmosphere of 5% CO₂) for a total of 8 days. Cell culture media was exchanged every other day using a multi-channel pipettor (remove 5 μL and add back 7 μL), and the number of droplets that spread outward on the top surface of the

plate was recorded for each design. The best design was determined to be the one with the least number of droplets spreading outward on the top surface.

Design and Testing of a 384 Hanging Drop Array Plate with Micro-Ring Structures Made by Injection Molding

Mass production by injection molding imposes design restrictions not present for stereolithography-based rapid prototyping. It was thus necessary to adapt the information obtained from the micro-structure screening studies into a design that is within the bounds of conventional injection molding tools. The main adjustment made was to increase the micro-ring feature width from 0.25 mm to 0.5 mm (Figure 2d). Also, to focus on the important role of micro-ring structures in droplet stabilization, we added the micro-ring structures to plateau rather than trench structures for easier comparison with our previous 384 hanging drop plates (Tung et al. 2011). This was also beneficial cost-wise as it allowed for modification of an original injection molding tool rather than preparing an entire new tool. This molding tool was used to perform a small production run of 500 plates by a local injection molding company.

Once the injection molded polystyrene 384 hanging drop array plate with micro-ring structures were obtained, we compared the hanging drop stability in both the original (Tung et al. 2011) and newly designed plates. For these tests, 15 μ L cell culture media only (DMEM (Gibco 11995, Invitrogen Co.) + 10% v/v FBS (Gibco 10082, Invitrogen Co.) + 1% v/v Antibiotic-Antimycotic (Gibco 15240, Invitrogen Co.)) hanging drops were formed in all 384 positions of both the original and new plates by a liquid handling robot (CyBi-Well, CyBio Inc.). The plates were not coated in 0.1% Pluronic F108 (BASF Co., Ludwigshafen, Germany) solution as described previously (Tung et al. 2011), but directly UV sterilized in a UV oven chamber for 30 minutes prior to use. After forming hanging drops, the plates were sandwiched between a 96-well plate lid and plate filled with distilled water, and incubated in a humidified incubator (37°C in an atmosphere of 5% CO₂). Cell culture media was exchanged every other day using the liquid handling robot (remove 5 μ L and add back 7 μ L to accommodate routine droplet evaporation). Directly after media exchange, each plate was then placed through 20 cycles of up and down, left and right motions on the liquid handling robot stage at the maximum speeds allowed by the liquid handler settings (horizontal speed = 57 mm/s; vertical speed = 40 mm/s). The number of droplets spreading out from both the top and bottom surfaces of the plate was recorded for each plate after the cycles.

General Cell Culture

PC-3 (ATCC, Rockville, MD) prostate cancer cells were originally isolated from vertebral metastases in prostate cancer patient. PC-3^{Luc} cells were constructed by stably transfecting PC-3 cells with luciferase construct using methods described previously (Kalikin et al. 2003). PC-3^{Luc} cells were further transfected with GFP lentivirus and subsequently enriched by FACS flow cytometry (BD FACSAria II Flow Cytometer, Becton Dickinson) for the brightest 10% of the population. The resulting cell line that stably express both luciferase and GFP is denoted as PC-3^{Luc GFP} cells. PC-3^{Luc GFP} cells were cultured in RPMI-1640 (Gibco 61870, Invitrogen Co.) with 10% v/v FBS (Gibco 10082, Invitrogen Co.) and 1% v/v antibiotic-antimycotic (Gibco 15240, Invitrogen Co.). MC3T3-E1 murine pre-osteoblasts (ATCC, Rockville, MD) were routinely cultured in α -MEM (Alpha Minimum Essential Medium; Gibco A10490, Invitrogen Co.) supplemented with 15% (v/v) FBS (Gibco 10082, Invitrogen Co.) and 1% v/v antibiotic-antimycotic (Gibco 15240, Invitrogen Co.). When cultured as spheroid, differentiation of MC3T3-E1 cells into osteoblasts was induced by the addition of 50 μ g/ml ascorbic acid (255564, Sigma-Aldrich Co.). HUVEC human umbilical vein endothelial cells (Lonza) passage number 2–6 were maintained in endothelial growth medium-2 (EGM-2, Lonza). All cell types were cultured in a humidified incubator at 37°C,

5% CO₂, and 100% humidity. PC-3^{Luc GFP} and MC3T3-E1 cell suspensions for the hanging drop experiments were made by dissociating cells with 0.25% trypsin-EDTA (Gibco 25200, Invitrogen Co.), followed by centrifugation of the dissociated cells at 1000 rpm for 5 minutes at room temperature. HUVECs were collected by washing and detaching with 0.25% trypsin-EDTA (Gibco 25200, Invitrogen Co.). Cells in trypsin solution were subsequently neutralized with 10% FBS (Gibco 10082, Invitrogen Co.) in DMEM (Gibco 11995, Invitrogen Co.), spun down with a centrifuge at 800 rpm for 5 minutes at 4°C, and re-suspended in EGM-2 (Lonza). The spin and re-suspension was repeated to ensure removal of trypsin. All cell pellets were re-suspended in their appropriate growth media. Cell density was estimated using a hemocytometer. In the prostate cancer co-culture spheroid experiment, the cells were cultured in co-culture media containing 1 part PC-3^{Luc GFP} media, 50 parts HUVEC media, and 50 parts MC3T3-E1 media (fractions of each cell type's growth media at the co-culture ratio) with 50 µg/ml ascorbic acid (255564, Sigma-Aldrich Co.).

Hanging Drop Formation and Spheroid Culture

To demonstrate actual long term culture of spheroids in the modified 384 hanging drop array plates, 4 modified plates were used to culture prostate cancer co-culture spheroids over a 14-day period. The general hanging drop formation method is described previously (Tung et al. 2011), with the exception that each hanging drop site in the modified plates now holds 20 µL of media and not the 15 µL used in the original plates. Prior to use for spheroid culture, the plates were coated in 0.1% Pluronic F108 (BASF Co., Ludwigshafen, Germany) solution for 1 hour and UV sterilized for 30 minutes. 192 20 µL hanging drops containing 1:50:50 PC-3^{Luc GFP}:HUVEC:MC3T3-E1 co-culture mixture of cells were formed in staggered positions (Figure 4a) in each of the 4 plates by a liquid handling robot (CyBi-Well, CyBio Inc.). The co-culture mixture of cells was prepared at 150 cells/µL to obtain a 3000-cell spheroid per hanging drop. All 4 hanging drop plates were sandwiched between a 96-well plate lid and plate filled with distilled water and maintained in a humidified incubator (37°C, 5% CO₂) for 14 days. Spheroids were routinely monitored by phase contrast and fluorescence microscopy (Nikon TE-300), and co-culture media was exchanged every other day using the liquid handling robot (remove 8 µL out and add back 10 µL in to accommodate routine droplet evaporation) for 14 days.

Results and Discussion

Optimization of the Bottom Features

In order to culture spheroids over long periods of time, the hanging drops in the 384 hanging drop array plate must be kept stable over extended periods and should be robust against sudden perturbations. In the original 384 hanging drop array plate (Tung et al. 2011), hanging drops tend to spread out from the bottom of the plate over time and upon experiencing sudden accelerations due to abrupt impulses (Figure 2a). Once a droplet spreads out, the droplet becomes much more vulnerable to coalesce with neighboring drops. Recent works by Kalinin et al. (2008) showed that micro-topographical ring structures greatly enhance the stability of droplets without spreading out. To identify the best type of topographical structure for droplet stabilization for the purpose of hanging drop cell culture, we used stereolithography-based rapid prototyping to design and test the ability of different plateau, trench, and micro-ring features (Figure 2c, Table 1) to resist droplet spreading due to centripetal acceleration. Figure 3b summarizes the result. A bottom trench design with extra micro-ring feature of 0.25 mm width and 0.50 mm height (Table 1, Design 20) was able to sustain the highest centripetal acceleration without droplet spreading. It is interesting to note that a bottom trench design alone without a micro-ring feature (Table 1, Design 11) is the worst in terms of preventing droplets from spreading out (Figure 3b). However, with

the addition of a micro-ring structure around the trench (Designs 12 to 20), the stability of the hanging drops was greatly enhanced. The combination of a trench design and micro-ring structure physically allows for a bigger volume of liquid to be held in the hanging drop and remain stable (as compared to the plateau design).

Optimization of the Top Features

In addition to stabilizing the hanging droplet on the bottom side of the plate, it is also necessary to prevent liquid spreading on the top side of the plate. To prevent liquid from spreading outward on the top surface of the plate (Figure 2a), extra micro-ring (extrusion) and trench (dent) design modifications around the top surface of the through-holes were rapid prototyped and tested (Figure 2b, Table 2). Upon routine cell culture media exchange over 8 days of incubation, some droplets will merge with undesired liquid remnants left by the pipette tips during media pipetting. Figure 3c shows the initial and final images of the actual test prototype plate. It is clear that the extra micro-ring designs perform better than the trench designs with no liquid spread on the top surface while the trench designs already have several droplets spreading outward from Day 0. It is also intuitive that since there is no physical barrier around the trench designs, liquid tends to merge easily with remnants on the neighboring top surface once the surface becomes hydrophilic with the wetting of the pipette tips. Whereas the extra micro-ring designs contrast with the trench designs by providing walls to guide pipette tips directly into the through-holes and preventing the hanging drops from merging with liquid remnants on the top surface. No significant difference was found between the different top side micro-ring dimensions.

Finalization of the Modified 384 Hanging Drop Array Plate Design Based on Stereolithography-derived Rapid Prototypes

As optimal design parameters were finalized based on stereolithography-derived rapid prototypes, we turned our attention to converting the insights obtained into designs compatible with mass manufacturing by conventional injection molding. For a platform to be broadly available, it is crucial to consider manufacturability. Due to limitations in injection molding tolerances, the final micro-ring dimensions had to be adjusted to be 0.5 mm rather than 0.25 mm in width (Figure 1d, 2d). It should also be noted that because the material used in the rapid prototypes is different from the clear polystyrene material used in the actual 384 hanging drop plates manufactured by injection molding, the surface property is slightly different (smoother for the injection molded plates). This could lead to minor differences in the test results from the rapid prototypes and the actual plate.

Despite the fact that the material used in the rapid prototypes was different from the actual plate material, the rapid and static tests performed on the prototype plates still provided relevant and useful insights that would otherwise be unattainable to optimize the final design features to be mass produced in the actual plates. It is extremely important to have an inexpensive and cost-effective rapid prototyping method for testing purposes before finalizing on the costly and often irreversible production of a master mold used for mass manufacturing. Here, we demonstrate a successful example of a stereolithography-based microstructure optimization process for biomedical microdevice development. The total cost of fabricating all 5 prototype plates using the stereolithography machine at the University of Michigan Medical Innovation Center was less than \$250. In addition, since multiple test designs could be placed on each prototype plate, it provides the flexibility for researchers to compare a wide variety of designs at a cost-effective manner. Alternatively, the total cost to simply modify an existing injection molding master mold was around \$10,000. It is therefore not practical to test multiple design prototypes manufactured by injection molding or to go through multiple design iterations.

3D stereolithography has already been widely applied to the rapid prototyping of macroscopic structures such as patient body parts (Kettner et al. 2011; Bakhos et al. 2010; Fallahi et al. 1999) as well as biocompatible polymers and scaffolds containing micro-features (Cooke et al. 2003; Lee et al. 2008; Gill and Claevssens 2011; Mapili et al. 2005; Shin et al. 2011; Chan et al. 2010) for tissue engineering. The technology has also been applied to the fabrication of microdevices and parts (Park et al. 2009; Lim et al. 2011; Selvaraj et al. 2011; Maruo and Inonue 2006; Maruo et al. 2009; Maruo et al. 2003; Kobayashi and Ikuta 2008; Kobayashi and Ikuta 2007; Leigh et al. 2011), but has not been widely used for biomedical applications. With the increasing complexity of sophisticated microdevices being developed in the biomedical community, it is becoming more common to require multiple iterations in design optimization to finalize reliable and robust devices. Since many universities and research laboratories now have access to stereolithography facilities, more researchers and scientists are encouraged to use such inexpensive rapid prototyping resources when developing biomedical microdevice. As 3D printing and stereolithography become more readily available, rapid microdevice prototyping are envisioned to be a crucial element in reducing the time and cost and therefore enhancing the development of biomedical microdevices.

Droplet Stability in the Modified 384 Hanging Drop Array Plate

The modified plate design with the added top and bottom micro-ring features was manufactured by injection molding using the same clear polystyrene material as the original plates (Tung et al. 2011). The original plate was compared to the modified plate with micro-ring structures for droplet stability during routine cell culture and media exchange procedures. Figure 3d shows a summary of the number of droplets spread out in each type of injection molded plate over time. The modified plate with micro-ring structures performs exceedingly better than the original plate in preventing droplets from spreading out. The modified plate has no droplets spreading out over the 22 days of culture, while the original plate already has 7 positions with liquid spreading out on the top surface during initial hanging drop formation on Day 0. This demonstrates that the modified new plates are significantly enhanced in preventing droplets from spreading out in both the top and bottom surfaces. It is also interesting to note that between Day 6 and Day 8, there is a sharp increase in the number of hanging droplets that have spread out in the original plate. This is most likely due to the coalescing of droplets leading to a chain reaction effect. Because the 384 hanging drops are so closely spaced together, once a droplet spreads out, it becomes very vulnerable to merge with neighboring drops, creating a larger droplet that is even more unstable. The modified plate with the extra ring structure around the bottom plateaus greatly prevents the initial drop from spreading out, thus enhancing the overall stability of the plate. The modified plate was subsequently shown to be stable with no droplets spreading until Day 24 when 33 drops spread outward.

Long Term Spheroid Culture in the Modified 384 Hanging Drop Array Plate

Four modified plates were used in actual 3D spheroid culture and were shown to be stable with no droplet spreading throughout the extent of the 14-day experiment. In addition to enhancing droplet stability, each through-hole in the modified plates also holds more liquid due to the extra volume generated from the extra top ring. This allows for bigger droplets to be more robust against physical perturbations, evaporation, and media osmolality shifts. Figure 4a shows an actual image of the modified 384 hanging drop array plate with 192 hanging drops containing prostate cancer co-culture spheroids cultured for 14 days. Enlarged images of the modified top and bottom sides of the plate highlighting the added micro-ring features with and without hanging drops are shown in Figure 4b and c, respectively. These hanging drops used for routine spheroid culture hang stably from the bottom side of the plate without spreading despite multiple fluid manipulation steps required

in routine media exchanges over the 14 days. All of the 768 hanging drops in the 4 modified plates used to culture prostate cancer co-culture spheroids stayed intact without spreading out by the end of the 14-day culture. Figure 4d shows the time-lapse images of a PC-3^{Luc GFP}:HUVEC:MC3T3-E1 (1:50:50 co-culture ratio) co-culture spheroid cultured in the modified 384 hanging drop array plate. As shown in both the phase and fluorescent images, PC-3^{Luc GFP} prostate cancer cells were able to be maintained and proliferate around the periphery of the spheroid in the modified hanging drop plate over the 14 days of culture.

Conclusion

Development of microdevices useful for the broad biomedical community requires multiple steps of design enhancement of initial research prototypes to produce robust and user-friendly products. Here we describe the technical process for efficiently realizing such improvements for a 3D hanging drop array cell culture platform. Based on initial user-feedback on the need for enhanced droplet stability together with a technical solution inspired by a previously published report on the ability of micro-topographical features to stabilize droplets, we used stereolithography-based rapid prototyping to fabricate and screen different micro-topographical features for their ability to stabilize microliter droplet arrays. The insights obtained by these prototype tests were used to design injection molding tools for large scale manufacturing of an improved 384 hanging drop array plate having micro-ring structures. As stereolithography becomes more accessible, the technology is anticipated to foster biomedical microdevice development by reducing the time and cost of fabricating initial prototypes. The modified polystyrene plate obtained allows for extended long term 3D spheroid culture in stable hanging drops, which opens up the opportunity to perform time-extensive cultures often required in tissue engineering, regenerative medicine, and developmental biology. As the obtained high-throughput 3D hanging drop culture platform is not only user-friendly but compatible with mass manufacturing by conventional injection molding, the platform is envisioned for wide adoption by researchers across multiple fields in academia, medical clinics, and the pharmaceutical/biotechnology industries. Our ultimate hope is that the resulting increase in the volume of data obtained by utilizing 3D cellular models will more accurately elucidate key biological processes, predict clinical efficacy, and expedite the drug discovery and therapeutics development processes.

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Figure 1.

(a) Cartoon of the original 384 hanging drop array plate and its cross-sectional view with a hanging drop schematic drawing. (b) Cartoon of the modified 384 hanging drop array plate and its cross-sectional view with a hanging drop schematic drawing. (c) Enlarged 3D schematic of the original 384 hanging drop array plate highlighting the key dimensions. (d) Enlarged 3D schematic of the modified 384 hanging drop array plate highlighting the key dimensions with the added extra rings (0.5 mm width and 0.5 mm height) on the top and bottom surfaces of the plate.

Figure 2.

(a) Illustration of a cross-sectional view of a hanging drop in a through-hole of the 384 hanging drop plate and how the droplet spreads outward from the top and bottom surfaces of the plate over time. (b) Cartoon showing the cross-sectional views of the proposed modifications to the top surface of the plate—an extra ring structure or a trench structure around the through-hole. (c) Cartoon showing the cross-sectional views of the proposed modifications to the bottom surface of the plate—an extra ring structure to the existing plateau, a new trench structure, or a new trench with an extra ring structure. (d) Cross-sectional schematics of the original design, the optimized design, and the final modified design of a hanging drop site in the 384 hanging drop array plate with the key dimensions shown in millimeters (mm).

Figure 3.

(a) Schematic of how the 384 hanging drop array prototype plates are tested on the spin coater with a glass slide (light blue area) attached to the underside of the plate using tape (white and transparent long rectangular areas on the two ends of the glass slide) to achieve a flat surface. Representative hanging drops (pink) and their corresponding radii to the center of the spindle are also illustrated. Hanging drops are placed in places outside the region where the glass slide is attached. (b) Bar graph summarizing the maximum centripetal force a hanging drop could withstand before spreading outward under different plate designs. (c) Day 0 and Day 8 (final day) photos of the top surface of the 384 hanging drop array prototype plate with different dimensions of the extra ring and trench designs. The enlarged Day 8 photo highlights droplets spreading out in the trench designs but not the ring designs. (d) Graph of cell culture media hanging drops spreading out condition in the original and the newly modified (additional top and bottom rings) 384 hanging drop array plate over time.

Figure 4.

(a) An actual picture of a modified 384 hanging drop array polystyrene plate with 192 hanging drops placed in staggered positions. (b) An enlarged picture of the top view of the modified hanging drop plate, highlighting the added micro-ring features around the through-holes with and without actual hanging drops. (c) An enlarged picture of the bottom view of the modified hanging drop plate showing the extra micro-ring features around existing plateaus with and without hanging drops. (d) Time-lapse phase and fluorescent images of a PC-3^{Luc GFP}:HUVEC:MC3T3-E1 (1:50:50 co-culture ratio on Day 0) co-culture spheroid formed and cultured in the modified 384 hanging drop array plate over a 14-day period. Scale bar is 200 μ m.

List of the specific dimensions of the different bottom surface structure designs in the 384 hanging drop array prototype plates

Table 1

Dimensions of the Bottom Structures in the 384 Hanging Drop Array Prototype Plates									
Plateau		Trench							
Plateau		Placement of Each Design (Top View)	Design		Ring Width (mm)	Ring Height (mm)			
			1 (Original)				Core Diameter (mm)	Ring Width (mm)	Ring Height (mm)
			2				3.00	0	0
			3				2.50	0.10	0.25
			4				2.50	0.25	0.25
			5				2.50	0.50	0.25
			6				2.75	0.10	0.25
			7				2.75	0.25	0.25
			8				2.75	0.50	0.25
			9				3.00	0.10	0.25
Trench		Placement of Each Design (Top View)	Design		Ring Width (mm)	Ring Height (mm)			
			10				3.00	0.25	0.25
			11				3.00	0.50	0.25
			12				3.00	0	0
			13				2.50	0.10	0.25
			14				2.50	0.25	0.25
			15				2.50	0.50	0.25
			16				2.75	0.10	0.25
			17				2.75	0.25	0.25
			18				2.75	0.50	0.25
			19				3.00	0.10	0.25
20	3.00	0.25	0.25						

Table 2

List of the specific dimensions of the different top surface structure designs in the 384 hanging drop array prototype plate

Dimensions of the Top Structures in the 384 Hanging Drop Array Prototype Plates				
Ring		Trench

Type	Placement of Each Design (Top View)	Design	Height (mm)	Width (mm)
Ring		1	0.25	0.25
		2	0.50	0.25
		3	0.25	0.50
		4	0.50	0.50
Trench		5	0.25	0.25
		6	0.50	0.25
		7	0.25	0.50
		8	0.50	0.50