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Authors
Aijian, Andrew P
Garrell, Robin L

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Digital Microfluidics for Automated Hanging Drop Cell Spheroid Culture

A.P. Aijian,[a] and R. L. Garrell[a,b,c,*]

[a] Department of Bioengineering, University of California, Los Angeles, CA 90095-1600, USA

[b] Department of Chemistry and Biochemistry, University of California, Los Angeles, CA 90095-1569, USA

[c] California NanoSystems Institute(CNSI), University of California, Los Angeles, CA 90095-7227, USA.

* Corresponding Author: E-mail: garrell@chem.ucla.edu; Tel: +1 310-825-4383

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Abstract

Cell spheroids are multicellular aggregates, grown in-vitro, that mimic the three-dimensional morphology of physiological tissues. While there are numerous benefits to using spheroids in cell-based assays, the adoption of spheroids in routine biomedical research has been limited, in part, by the tedious workflow associated with spheroid formation and analysis. Here we describe a digital microfluidic platform that has been developed to automate liquid handling protocols for the formation, maintenance, and analysis of multicellular spheroids in hanging drop culture. We show that droplets of liquid can be added to and extracted from through-holes, or ‘wells,’ fabricated in the bottom plate of a digital microfluidic device, enabling the formation and assaying of hanging drops. Using this digital microfluidic platform, spheroids of mouse mesenchymal stem cells were formed and maintained in-situ for 72 h, exhibiting good viability (>90%) and size uniformity (%CV <10%, intra-experiment, <20% inter-experiment). A proof-of-principle drug-screen was performed on human colorectal adenocarcinoma spheroids to demonstrate the ability to recapitulate physiologically relevant phenomena such as insulin-induced drug resistance. With automatable and flexible liquid handling, and a wide range of in-situ sample preparation and analysis capabilities, the digital microfluidic platform provides a viable tool for automating cell spheroid culture and analysis.

Introduction

Cell spheroids are multicellular compact aggregates, grown in-vitro, that have a three-dimensional (3D), spherical morphology. Unlike cells grown in two-dimensional adherent monolayers, cells grown in three dimensions possess a high degree of intercellular interactions and exhibit relatively complex nutrient and metabolic mass transport gradients. These lead to cellular heterogeneity within the 3D aggregate and to gene and protein expression patterns that more closely mimic in-vivo tissues.[1-6] The differential expression profiles result in significant differences in cellular behaviors such as drug sensitivity, differentiation capacity, malignancy, function, and viability. For example, hepatocellular carcinoma cells grown as spheroids exhibit more physiologically relevant levels of cytochrome P450 activity and albumin secretion compared to cells grown in monolayers.[7] Another example is mammary epithelial cells, which exhibit basement membrane-induced apoptosis resistance when grown in three dimensions, but are susceptible to apoptosis in monolayer culture.[8] Because of their enhanced physiological relevance compared to monolayer cell cultures, cell spheroids can provide more accurate models for cell-based assays and screens. Improved tissue and disease models not only enhance basic research, but can be extremely valuable in commercial research, particularly in the pharmaceutical industry, where the failure rates for drug candidates entering clinical trials are typically >80%.[9-13]

Despite the known advantages of 3D cell cultures, their use in cell-based assays and screens has been limited. It is estimated that <30% of cancer and molecular biologists

63 utilize 3D cell culture and that <20% of drug leads generated by the pharmaceutical
64 industry are developed through cell-based phenotypic assays.[14, 15] A major reason for
65 the relatively low adoption of 3D cell models is the limited number of user-friendly,
66 flexible, and automated methods for performing spheroid culture and analysis.[16, 17]
67 While a variety of technologies and methods are available for culturing 3D micro-
68 tissues, each approach has limitations that make it unsuitable for routine assays and
69 screens.[18] Non-automated spheroid culture methods, such as the manual hanging-drop
70 technique or the use of micro-molds, are inexpensive and relatively simple, but
71 interrogating individual spheroids requires manual transfer to a separate vessel.
72 Additionally, the non-automated methods often require a significant amount of manual
73 sample handling, which can be tedious, time-consuming, and prone to variability and
74 error.

75

76 A number of microfluidic techniques for spheroid culture have been developed that
77 can enable high-throughput and massively parallel spheroid formation, but which do
78 not support the interrogation of spheroids individually on the same device.[19-22] Rotary
79 vessels and spinner flasks can also be used to generate a large number of spheroids,
80 but provide limited control over spheroid size and also do not allow for in-situ
81 assaying of individual spheroids. Alternatively, specially engineered well plates, such
82 as those capable of supporting hanging drop culture[23] or those with non-adhesive
83 surfaces designed to induce cell aggregation,[24] are compatible with robotic liquid
84 handling equipment, enabling automation and high-throughput processing. However,
85 the ability to automate spheroid culture and analysis using these methods requires

86access to robotic liquid handling equipment, which can be prohibitively expensive to 87acquire, operate, and maintain for many research labs, particularly those in academic 88settings where the emphasis may not be on high-throughput experiments. 89Additionally, functionalities necessary for spheroid culture and analysis, such as in-90situ microscopy, mixing, and temperature control, require additional, often expensive, 91hardware to be added to the liquid handling instrument. Thus, there is a need for a 92spheroid culture and analysis technology that provides some of the advantages of 93automation, in a platform that is more accessible than current automated methods. We 94propose that digital microfluidics (DμF), a flexible and precise microfluidic liquid 95handling technology, can be used to automate cell spheroid culture and analysis as 96well as provide some unique benefits over existing automated techniques.

97

98Digital microfluidics is a type of microfluidic platform that enables the manipulation 99of discrete droplets of liquid in either an air or liquid ambient medium through the 100spatially and temporally-controlled application of electric fields.[25-27] The application 101of an electric potential across the solid-liquid contact line generates a combination of 102electrostatic and/or dielectrophoretic forces, depending on the frequency of the applied 103field and the relative permittivities of the liquid and ambient phases. Sequentially 104applying an electric potential to an array of planar electrodes can enable the translation 105of droplets across the array,[28] and can also be used to split, merge and mix droplets.

106

107Here we present a digital microfluidic device that enables the formation of hanging 108drops to allow in-situ cell spheroid culture. With the ability to automate liquid

109handling, and with a wide range of in-situ bioanalytical techniques developed for the

110DμF platform, DμF can ultimately provide a powerful tool for automation of spheroid-

111based assays and screens.

112

113**Materials and Methods**

114Bone marrow-derived mouse mesenchymal stem cells (ATCC® CRL-12424™) were

115generously donated by Prof. Tatiana Segura (UCLA). HT-29, human colorectal

116adenocarcinoma cells (ATCC® HTB-38™) and BJ, human foreskin fibroblasts (ATCC®

117CRL-2522™) were purchased from ATCC. Leibovitz L-15 cell culture medium,

118Dulbecco's Modified Eagle Medium (DMEM), penicillin-streptomycin (P/S) solution

119(10,000 U Pen., 10 mg Strep./mL), L-glutamine, fetal bovine serum (FBS), and the

120LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells were obtained from

121Life Technologies (Carlsbad, CA). Pluronic® F-68 was purchased from Sigma (St.

122Louis, MO). Cytop® (CTL-809M) and CYSOLV-180 were purchased from Bellex

123International Corporation (Wilmington, DE). Human recombinant insulin was

124purchased from R&D Systems, Inc. (Minneapolis, MN) and Irinotecan HCL was

125purchased from BIOTANG, Inc. (Lexington, MA).

126

127Device fabrication was conducted in the CNSI Integrated System Nanofabrication

128Cleanroom at UCLA. Briefly, water white glass substrates (LabScientific, Inc. CAT#

1297787) were coated with 1100 Å indium tin oxide (ITO) via sputtering and were

130patterned with electrodes via photolithography and reactive ion etching. For this

131work, the substrate with the patterned electrode array was used as the top-plate and an

132un-patterned ITO-coated slide was used as the bottom-plate. Prior to coating with the
133dielectric, through-holes were manually drilled into specific locations on the bottom-
134plate using a benchtop drill press and diamond-coated drill bits. Through-holes were
135also drilled into the footprint of the reservoir electrodes in the top-plate to provide a
136world-to-chip interface. The top-plates were then coated with 3–4 μm of dielectric
137polymer parylene-C (Specialty Coating Systems) by vapor deposition. The top and
138bottom-plates were rendered hydrophobic by spin coating ~300–400 nm of Cytop on
139each. Prior to use, the walls of the wells in the bottom-plate were gently scraped with
140a diamond-coated drill bit to remove the Cytop coating and expose the hydrophilic
141glass surface. A schematic of a DμF device assembly is shown in Figure 1.

142

143All microfluidic liquid handling was performed using a custom LabView application
144to control electrode actuation. Liquid handling was performed at 100–115 V_{pp} AC and
145at a frequency of 18.5 kHz. Analysis of hanging droplet liquid exchange was
146performed by measuring the absorption of a standardized solution of brilliant blue dye
147prepared in water before and after liquid exchange cycles using a Thermo Scientific
148NanoDrop 2000c UV-Vis spectrophotometer. The liquid exchange process is
149described in more detail below.

150

151For the preparation of cell solutions for use on the DμF device, cells were thawed and
152seeded in polystyrene dishes in growth medium (DMEM, 4 mM L-glutamine, 10%
153FBS, 100 U/mL P/S solution). Cells were grown to ~80% confluency, trypsinized, and
154re-suspended in spheroid growth medium (Leibovitz L-15 medium, 4 mM L-

155glutamine, 7.5% FBS, 100 U/mL P/S, 0.04% Pluronic® F-68) at cell densities ranging
156from ~7.5e5 – 1e6 cells/mL for culture on the device.
157
158Detailed schematics of the experimental setup, as used in this work, are shown in
159Figure S1 in the Electronic Supplemental Information. Prior to use, the devices were
160sterilized by dipping them in a 70% aqueous ethanol solution and gently drying with
161compressed air. For device operation, the bottom-plate was placed on an aluminum
162holding plate that contained a milled recess below the location of the wells to allow
163hanging drops to form beneath the device. The bottom-plate of the device was sealed
164to the aluminium plate using silicone grease (Dow Corning High Vacuum Grease).
165The bottom of the recess was enclosed with a glass slide to prevent exposure of the
166hanging drops to the laboratory environment during drop formation. To minimize
167evaporation, 1.5 µL of 10 cst silicone oil was pre-loaded into each well prior to the
168formation of hanging drops. Additionally, a small amount of water was placed in the
169enclosed recess to create a humidified environment. The top-plate was secured to
170another aluminum plate and was interfaced with the bottom-plate such that particular
171electrodes in the top-plate aligned with the location of the wells in the bottom-plate.
172The two plates were separated by a custom designed adhesive silicone spacer (Grace
173Biolabs, Bend, OR) to create a gap height of 300 µm and were secured using binder
174clips. Drops of cell-suspension were added to the reservoir electrodes via through-
175holes drilled into the top-plate.
176

177Hanging drop and spheroid formation were achieved by dispensing droplets of cell
178suspension from the reservoir and moving the droplets to the location of a well. Upon
179contact with the hydrophilic wall of the well, droplets were pulled into the well via
180capillary forces. Addition of multiple droplets to a well resulted in the formation of a
181hanging drop. Exchange of the medium within the hanging drop was achieved by
182performing the following sequence of steps one or more times per well: (1) delivering
183a drop of fresh medium to a well, (2) using electrowetting actuation to repeatedly pull
184out and release a liquid finger from the well to facilitate mixing of the liquid in the
185well, (3) extracting a drop from the well of twice the volume of the amount initially
186delivered, and (4) adding another drop of fresh medium to the well. Devices were
187kept in an incubator at 37 °C and 95% relative humidity at all times except during
188liquid handling.

189

190For confocal imaging, spheroids were stained with fluorescent markers by incubation
191in imaging medium for 2 h at 4 °C followed by 30 min at 37 °C to ensure enhanced
192staining of the interior of the spheroid.[29] The imaging medium consisted of 2 μM
193calcein-am and 4 μM ethidium homodimer-1 (Life Technologies, LIVE/DEAD®
194Viability/Cytotoxicity Kit, for mammalian cells) in Hank's Balanced Salt Solution
195(HBSS, Life Technologies) supplemented with 1 mg/mL ascorbic acid, 25 mM HEPES
196buffer solution – pH 7, 100 U/mL P/S, 100 μM non-essential amino acids, and 4 mM
197L-glutamine; 1N NaOH was used to adjust the pH to 7.2. Following staining with
198imaging medium, the spheroids were washed with HBSS containing 1 mg/mL ascorbic
199acid. A custom PDMS imaging chamber was secured beneath the bottom plate such

200that flooding the wells with the HBSS solution caused the hanging drops to detach 201from the device into individual wells within the PDMS chamber, enabling the 202spheroids to be imaged directly from the device. Figure S1 in the Electronic 203Supplemental Information shows a schematic of the device interfaced with the PDMS 204imaging chamber.

205

206Confocal imaging was performed using a Leica TCS SP2 confocal microscope. 207Spheroid images were constructed by creating a maximum projection of multiple z-208plane sections spaced 3–7 μm apart. The proportion of living cells within a spheroid 209was estimated by counting the number of live (green) and dead (red) cells in five 210different, equally spaced z-planes throughout the spheroid. ImageJ was used for all 211image analysis, which included hanging drop volume, as well as spheroid viability, 212diameter, and aspect ratio measurements.

213

214**Results and Discussion**

215***Device design and operation***

216To enable hanging drop formation, through-holes, or ‘wells,’ were fabricated into 217strategic locations in the bottom-plate of the device. The schematic in Figure 1 shows 218the basic principle of DμF hanging drop formation along with typical device 219dimensions. Hanging drops are formed when droplets of liquid are delivered to the 220location of a well and, upon making contact with the hydrophilic walls of the well, get 221pulled into the well spontaneously via capillary forces (Figure 2a). Adding multiple

222drops to a well results in the formation of a curved liquid-air interface that protrudes
223beneath the bottom-plate, similar to a hanging drop (Figure 2b). To ensure the
224formation of a hanging drop, the wells were designed such that the Bond number (Bo,
225a dimensionless parameter describing the ratio of gravitational to surface tension
226forces) of the system is greater than ~0.3, which is within the range where
227gravitational forces begin to influence the shape of the meniscus.[30-32] A Bo≥0.3
228requires a well diameter of ≥2.4 mm.

229

230To simplify device fabrication protocols, the top plate contained the actuating
231electrodes and the bottom-plate contained the ground electrode. While either the
232actuating or ground plate can be modified with through-holes and used as the bottom-
233plate to support hanging drop formation, we found that incorporating the wells into
234the plate containing the actuating electrodes was more difficult because the holes
235needed to be drilled precisely within the footprint of an electrode, which occasionally
236resulted in damaging the electrode. Additionally, decoupling the wells and actuating
237electrodes allows for the actuating top-plate to be removed and replaced in the case of
238dielectric breakdown, without disrupting the hanging drops in the wells in the bottom-
239plate.

240

241Dielectric breakdown occurs when the electric field across the dielectric layer exceeds
242the dielectric strength of the material, resulting in localized, physical destruction of
243the dielectric layer. Dielectric breakdown typically occurs on the actuating plate,
244where charges within the drop and electric field lines outside the drop concentrate

245near the droplet edge closest to the actuated electrode, creating a region of locally 246elevated electric field strength.[33-35] If the degree of dielectric breakdown is minor, 247droplets can still be transported normally across the location of the breakdown. 248Significant dielectric breakdown can cause electrolysis of aqueous solutions as a result 249of to current flow into the drop, and can also damage critical electronic connections on 250the device, thereby impeding droplet movement ("pinning"). For the top-plates used 251in this work, the entire spheroid culture process, which required ~800-1200 total 252electrode actuation steps for the culture of 6-8 spheroids, could typically be achieved 253without the occurrence of dielectric breakdown. Approximately one out of every four 254devices showed evidence of dielectric breakdown at some point during the culture 255protocol, typically during the 48 h medium exchange process, i.e., after the hanging 256drops had been formed and all the cell handling had been completed. Because the 257spheroids were maintained in hanging drops beneath the bottom plate and were 258relatively far from either of the interior surfaces of the top- and bottom-plates, 259dielectric breakdown did not disrupt or affect the spheroids within the hanging drops.

260

261To allow visualization of droplet handling, the actuating electrodes in the top plate 262were made from a transparent conductive material, indium tin oxide (ITO). Videos of 263liquid handling and hanging drop formation are provided in the Electronic 264Supplemental Information.

265

266The wells in the bottom plate contain a tapered opening on the top side to aid in the 267insertion of drops into the well. Droplets that reach the edge of a well can experience

268canthotaxis, or pinning at the intersection of two interfaces, due to both the change in
269geometry at the well edge and the difference in surface properties between the
270hydrophobic surface of the bottom plate and the hydrophilic interior of the well walls.
271By tapering the inside walls of the well to form an acute angle with the surface of the
272bottom plate, as opposed to a right angle formed by a cylindrical through-hole, the
273pinning effect on a drop of liquid at the edge of the well is reduced, facilitating droplet
274insertion into the well.

275

276The volume of a hanging drop is determined by the number of drops dispensed from a
277reservoir and added to a well. Thus, the volume and reproducibility of droplet
278dispensing from the reservoirs are critical to the volume and reproducibility of the
279hanging drops. To determine the variation in dispensed drop volumes, we used image
280analysis to measure the volumes of 144 drops of an aqueous surfactant solution
281(0.04% Pluronic® F-68) dispensed from different reservoirs across three different
282devices using a programmed dispensing sequence. An aqueous surfactant solution was
283used so that the surface tension of the liquid and, consequently, the volume of the
284dispensed drops, would be similar to that of the growth medium solution subsequently
285used in the cell culture experiments, which also contained 0.04% Pluronic® F-68. The
286dispensed droplet volume was determined by measuring the area of the drop in contact
287with the top-plate using device features of known dimensions as a scale, and
288multiplying by the known distance of the inter-plate gap. While in actuality the
289sidewalls of the droplet are curved, to simplify the volume measurements we used the
290straight-wall, cylindrical approximation to calculate droplet volumes, which is a

291reasonable approximation considering the relatively small droplet aspect ratio on our
292devices (h/w ~0.15) and the contact angle of approximately 100°.[36] The distribution of
293droplet volume measurements is shown in Figure 3. The average volume of a single
294dispensed drop was 1.75 ± 0.13 μL (7.7% CV; %CV = coefficient of variation). This
295degree of droplet volume variation is consistent with reproducibility values from other
296electrowetting devices that do not utilize capacitance metering to control dispensing
297volumes. Droplet dispensing reproducibility can be improved to low single-digit
298%CV by employing capacitance metering methods or by optimizing reservoir and
299dispensing electrode design.[37-39] The variation in the volumes of hanging drops was
300also determined (Figure 4). Hanging drops formed from 4, 5, and 6 dispensed drops
301had average volumes of 7.4 ± 0.5 μL, 8.8 ± 0.8 μL, and 10.2 ± 0.5 μL, respectively,
302corresponding to %CV range of 5–9%. This volume range was chosen because, for
303the devices used in this work, at least 4 drops are required to fill a well and form the
304curved surface necessary for cell aggregation.

305

306It should be noted that the volume and reproducibility data shown here represent
307results from a particular dispensing sequence and device arrangement (i.e., gap height
308= 300 μm). Various droplet volumes can be dispensed on a DμF device by simply
309altering the gap height and/or changing the dispensing sequence. The devices used in
310this work support the formation of hanging drops up to ~55 μL before the drops detach
311from the well due to their weight. We observed that hanging drops of larger volumes
312can be supported by varying the thickness of the bottom plate, the well geometry, or

313the surface tension of the liquid comprising the drop (determined experimentally, data
314not shown).
315
316Cell spheroids require ~50% medium exchange every 48 h for optimal growth.[40, 41]
317Thus, to enable long term hanging drop spheroid culture, protocols for in situ medium
318exchange using digital microfluidic liquid handling were developed. Medium
319exchange requires extracting the spent medium from a hanging drop and replacing the
320spent medium with fresh medium. Liquid can be extracted from a hanging drop by
321using the electrodes adjacent to a well to pull out a drop of liquid. Repeating the
322process of extracting and adding drops of medium to a well, as described in the
323Materials and Methods section, results in the exchange of the medium within the well.
324Assuming the hanging drop initially contains the volume of four dispensed drops, the
325medium exchange protocol theoretically allows for exchange of 40% and 64% of the
326initial drop volume after one and two exchange cycles, respectively (according to the
327dilution rate for this particular exchange protocol: $C = 0.6^n$, where C = the
328concentration of spent medium in the drop, and n = the number of exchange cycles).
329The video "Hanging Drop Liquid Exchange" in the Electronic Supplemental
330Information shows two cycles of the liquid exchange protocol. Using a hanging drop
331of a standardized brilliant blue dye solution to mimic spent medium and DI water to
332represent fresh medium, we assessed the degree of exchange by measuring the change
333in dye concentration of the hanging drop after successive exchange cycles by visible
334spectrophotometry. Figure 5 shows that the dye concentration calculated from UV-Vis
335absorption are consistent with the theoretical predictions, indicating that >50%

336medium exchange can be achieved with one or more exchange cycles. These data also
337indicate that DµF can provide precise control over the composition of the hanging
338drop, which is critical for performing cell-based assays and screens.
339
340***Cell Spheroid Culture***

341After establishing the ability to form a hanging drop and conduct medium exchange, a
342complete cell spheroid culture protocol was performed to demonstrate proof-of-
343principle for fully automated DµF cell spheroid culture. Droplets of mouse MSC
344suspension in growth medium were delivered to wells to form hanging drops of ~7-10
345µL (~5250-7500 cells/drop). Pluronic® F-68 was included in the growth medium to
346minimize the adsorption of proteins to the hydrophobic surface of the device, which
347can impede the movement of proteinaceous solutions.[42] At 0.04%, Pluronic® F-68 is
348known to be non-cytotoxic.[43] Leibovitz L-15 medium was used for spheroid culture
349because it is buffered by phosphates and free-base amino acids instead of sodium
350bicarbonate. This medium allows cell growth in the absence of a controlled CO_2
351atmosphere; our current digital microfluidic setup is operated outside of an incubator
352at ambient atmospheric conditions. During liquid handling, the microfluidic apparatus
353was kept at ~37 °C by placing a thin-film polyimide heater in contact with the
354aluminium device holder. After liquid handling, devices were transferred to an
355incubator at 37 °C and relative humidity of 95%. To prevent fluctuations in
356atmospheric conditions between the liquid-handling and incubation periods, the
357incubator was also maintained at ambient atmosphere (i.e., without CO_2 control).
358

359Medium exchange was performed once daily. During culture, the spheroid sits at the
360bottom of the hanging drop, which is ~1.8 mm below the top opening of the well
361(assuming a 7.4 µL drop in a 2.5-mm diameter well). Because liquid from the drop is
362extracted from the top opening of the well and medium exchange never requires
363extraction of more than 25% of the initial hanging drop volume, the spheroid remains
364settled within the hanging drop throughout the medium exchange protocol and does
365not get extracted from the well.

366

367Figure 6a shows confocal micrographs of typical spheroids of mouse mesenchymal
368stem cells cultured on the DµF device over the course of 72 h using automated sample
369handling protocols. The spheroids were stained with calcein-AM and ethidium
370homodimer-1 to indicate living (green) and dead (red) cells, respectively. Counting
371the number of living and dead cells at various z-planes within the spheroid indicated
372that the spheroids exhibited >90% cell viability. The spheroid diameter was measured
373at 24, 48 and 72 h (Figure 6b) following hanging drop formation using a USB-
374microscope (Dino-Light AD4013TL). A seeding density of 7.5e5 cells/mL produced
375spheroids of up to ~400 µm after 72 h in culture. The size and viability of the
376spheroids generated on the DµF platform are consistent with those obtained through
377other hanging drop techniques over the same timeframe using similar cell number
378conditions.[23] Intra-device spheroid diameter variation was ~8%; this is comparable to
379other hanging drop techniques, which exhibit a %CV range of ~3% (for robotic liquid
380handlers) to 15% (for manual methods), and is superior to spheroid generation on non-
381adhesive flat-bottom well plates, which show spheroid diameter variation of up to 40–

382 60%.[44, 45] Because the cell density is the same for each hanging drop, the intra-
383 experiment spheroid diameter variation is attributable to the variation in the volumes
384 of the hanging drops. The inter-device variation in spheroid diameter (i.e., for
385 spheroids grown on different devices) was 14%, 18%, and 18% for spheroids at 24,
386 48, and 72 h, respectively (Figure 6b). The relatively larger inter-experiment variation
387 compared to the intra-experiment results is likely due to variations in cell densities
388 between the different experiments. For this work, cell suspensions of ~7.5e5 cells/mL
389 were prepared based on hemocytometer measurements, which can exhibit variability
390 of 10-40% depending on cell concentration.[46, 47] More precise cell-density
391 measurement techniques, which can achieve a %CV of <3%,[48] would reduce the inter-
392 experiment spheroid diameter variability. Figure 6c shows the distribution in spheroid
393 aspect ratio (ratio of spheroid major axis to minor axis) for 77 spheroids of various cell
394 numbers and types. The average aspect ratio for the spheroids cultured on the DμF
395 platform was 1.15 ± 0.09, corresponding to a CV of ~8%. The spheroid aspect ratio
396 was measured after at least 48 h in culture to allow spheroid compaction to occur.
397 Table 1 summarizes performance characteristics for spheroid culture conducted on a
398 DμF device.
399
400 With the ability to initiate and maintain viable spheroids in culture as well as freely
401 add, mix, and extract liquid from a hanging drop, the DμF platform enables
402 automation of spheroid-based assays and screens. To demonstrate this capability, we
403 performed a proof-of-principle spheroid-based drug screen, using DμF to examine the
404 impact of insulin exposure on the chemosensitivity of colon cancer cells to treatment

405with the chemotherapeutic agent irinotecan. Insulin has been shown to cause
406resistance to chemotherapy in certain colon cancer cell lines via activation of the
407PI3K/Akt pathway.[49, 50] For the drug screening assay, hanging drops of HT-29 human
408colon adenocarcinoma cells were initiated and maintained on a DμF device for 48 h to
409allow for the formation of compact spheroids. After 48 h of culture, the medium for
410some spheroids was exchanged for medium containing 500 nM insulin, while the
411remaining spheroids received normal growth medium. The insulin-induced drug
412resistance effect has been observed in HT-29 cells in-vitro at insulin doses of 100-1000
413nM.[50, 51] Spheroids were allowed to incubate in their respective medium for 24 h after
414which the medium was exchanged for medium containing 100 μM irinotecan, or, for
415controls, normal growth medium. Previous studies had shown that HT-29 spheroids
416exhibit ~20-50% cell death upon exposure to 100 μM irinotecan.[40, 52, 53] The DμF drug
417screening assay workflow is depicted in Figure 7a. To evaluate drug toxicity, the
418diameter of each spheroid was measured at 48, 72, and 96 h. Figure 7b shows the
419average normalized diameter (the ratio of spheroid diameter at 96 h to the diameter at
42048 h) of the spheroids for the different assay conditions. Spheroids that received just
421the drug treatment exhibited a ~20% decrease in diameter, while those that were
422exposed to insulin prior to drug treatment did not exhibit any decrease in size. These
423results are consistent with the insulin-induced drug resistance effect observed in HT-29
424cells in-vitro and, to our knowledge, represent the first time this effect has been
425demonstrated using a three-dimensional HT-29 colon cancer model.

426

427Another interesting result of the colon cancer spheroid-based drug screen was the
428formation of ‘colonospheres’: spherical structures composed of several colonic
429mucosal epithelial cells that appear as rounded-off epithelial cysts.[54] The colonosphere
430morphology signifies a reorganization from a spherical aggregate into one that more
431closely mimics the morphology of the colon epithelium, which contains numerous
432glandular and crypt structures. Figure 8 shows examples of colon cancer spheroids
433exhibiting the colonosphere morphology. This phenotype is of particular interest in
434cancer research, as literature suggests that colonospheres exhibit a relatively high
435proportion of cells with a cancer-stem-cell phenotype, which is critical to tumor
436formation and growth.[55]

437

438The work presented here advances on previous DμF cell culture studies that
439established the ability to seed and maintain cells in adherent monolayer culture on a
440DμF device over an extended period of time.[56-59] Those studies confirmed that the
441electric fields used to drive droplet movement have negligible detrimental impact on
442cell viability, and developed protocols for the manipulation of complex biological
443solutions. Other work has demonstrated the ability to encapsulate a suspension of cells
444within hydrogel posts between the plates of a DμF device.[60-62] The encapsulation of
445cells within hydrogel posts provides a useful tool for modelling cell-matrix
446interactions, which are key to understanding the cellular microenvironment and
447important physiological processes such as the epithelial-mesenchymal transition.[63]
448However, while the use of hydrogel posts enables cell growth in three dimensions,
449there are certain limitations associated with these techniques. When using inter-plate

450gel posts, the thickness of the cell aggregates within the gel is limited to the thickness
451of the gap between the plates of the digital microfluidic device (typically ≤ ~300 μm).
452Additionally, when cells suspensions are encapsulated in gel-posts, the cells are
453randomly distributed throughout the gel, providing little control over the size and
454morphology of the aggregates that form. Lastly, these methods require the use of a
455scaffold or matrix to support 3D cell culture; in some cases, this can be
456disadvantageous, because the scaffold materials may require extra sample preparation
457steps, can be expensive, are susceptible to lot-to-lot variability, may consist of non-
458physiological materials, can complicate sample recovery/analysis, can restrict the
459movement of cells or nutrient transportation, can interfere with screening compounds,
460and may not allow recapitulation of processes that rely on a high degree of cell-cell
461interactions such as embryogenesis, morphogenesis, or tumorigenesis.[64-68] The
462platform described here allows scaffold-free three-dimensional cell culture. That said,
463because solutions can be freely added to or extracted from a hanging drop, cell
464suspensions or compact spheroids within a hanging drop could be encapsulated in a
465scaffold material if desired. This platform also enables the growth of spheroids that
466exceed the thickness of the inter-plate gap of a DμF device, allowing for the formation
467of spheroids that exhibit physiologically relevant morphologies specific to large
468aggregates, such as the development of a necrotic, hypoxic core that can occur within
469spheroids >400-500 μm in diameter.[18, 69-71] Because not all cell types form spheroids,
470and because the behavior of individual cells or small cell clusters encapsulated within
471an extracellular matrix can provide interesting physiological insights, the hanging-drop

472and gel-post techniques for three-dimensional cell culture on a DµF device are
473complementary.
474
475The platform described here also provides a number of unique advantages compared to
476existing spheroid culture techniques. The primary advantages of the DµF system are
477automation and the flexibility of the liquid handling protocols. By automating liquid
478handling, digital microfluidics can enable increased throughput and minimize hands-
479on time compared to manual spheroid culture methods, potentially reducing variability
480and human-error in spheroid culture and assay protocols. Digital microfluidics also
481allows droplets to be manipulated either sequentially or simultaneously and droplet
482handling can be pre-programmed for complete automation, or can be controlled in
483real-time allowing for assay flexibility and reconfigurability. Because DµF provides
484temporal and spatial control over the handling of discrete drops of liquid, any type of
485solution can be added to or extracted from any particular well at will. Thus, spheroids
486can be exposed to a wide variety of stimuli such as drug candidates, different cell
487types, differentiation factors, genetic modulators, and cell secretions in a highly
488controlled fashion. Additionally, because liquid movement on a DµF platform is not
489confined to channels, liquid can be freely exchanged from one hanging drop to
490another, allowing controlled communication between different spheroids on a device.
491The ability to extract solution from a well allows for in-situ or ex-situ analysis of
492secretions or extracellular conditions from distinct spheroids at any point throughout
493the spheroid culture. This precise control over the composition and analysis of the
494spheroid microenvironment is difficult or impossible to achieve using other spheroid

495culture techniques. For example, while flow-based microfluidic techniques are
496advantageous for massively parallel and/or high throughput spheroid culture protocols,
497such methods are non-ideal for assays that require flexible or reconfigurable liquid
498handling or precise and selective control over the microenvironment of individual
499spheroids. Likewise, microarray or micro-well techniques, in which cells passively
500aggregate in defined locations on a patterned substrate, allow for high-throughput and
501uniform spheroid formation, but do not allow for compartmentalization or
502interrogation of individual spheroids.

503

504While robotic liquid handling systems do allow for automation of spheroid culture and
505analysis, digital microfluidics enables unique liquid handling capabilities that are
506difficult or impossible to achieve using robotic liquid handling. For example, digital
507microfluidics allows for the interrogation of hanging drops either individually or in
508parallel, enables handling of very small volumes of liquid (pL–µL),[72, 73] allows for
509magnetic or dielectrophoretic sorting of cells or beads,[74-77] enables programmable and
510spatially controlled heating of individual or multiple locations,[78] supports rapidly
511sequential delivery of reagents to single or multiple locations,[79] allows for in-situ
512electrochemical detections,[80-82] and allows for the formation of hydrogels with
513controllable geometry and orientation.[61, 83, 84] Additionally, a wide range of
514bioanalytical capabilities including mass spectrometry sample preparation,[85] PCR,[86]
515qPCR,[87] immunoassays,[88] surface plasmon resonance imaging,[89] and fluorescence
516imaging,[90] have been developed for the DµF platform, providing in-situ analytical and

517multiplexing functionalities that could be challenging to incorporate into a robotic
518liquid handling spheroid-culture workflow.
519
520The DµF platform described here does have certain limitations compared to other
521automated spheroid culture techniques. The primary limitation of the system is the
522relatively low throughput compared to robotic liquid handling systems. Because all of
523the liquid handling in digital microfluidics is performed in the same two-dimensional
524plane, the device must accommodate both the wells and the transportation electrodes,
525which limits the number of wells that can be placed on a device. By contrast, the
526liquid handling path for robotic liquid handling systems usually occurs on a different
527plane than the well-plate, allowing the wells to be packed closer together. Thus digital
528microfluidics cannot achieve the same well density that is possible using hanging-drop
529well-plates, and is best suited for research environments in which medium-throughput
530processing is sufficient. While the prototype devices used in this work are limited to
53146 actuating electrodes, which enables the formation of up to eight hanging drops, a
532more advanced DµF setup, such as the DropBot,[91] an open source DµF hardware and
533software system which allows for hundreds of individually addressable electrodes,
534would enable >50 spheroids to be maintained and addressed on a single device.
535Although this well density is considerably lower than commercially available 96- or
536384 hanging drop well plates (3DBiomatrix Inc., InSphero), operating multiple DµF
537devices simultaneously would increase the throughput. Digital microfluidics also
538operates at lower working volumes than other automated spheroid culture methods (7–
53912 µL hanging drops on this DµF platform compared to 20–30 µL for a 384 well

540hanging drop plate[23]), which, while advantageous in some respects, can also present
541challenges. Specifically, smaller drops are more susceptible to evaporation, which can
542alter the composition of the hanging drop. Smaller hanging drops also require a higher
543cell density than larger hanging drops to achieve a spheroid of the same size. Working
544with higher cell densities requires more precise liquid handling as spheroid size is
545related directly to hanging drop volume and cell density. These challenges, however,
546can be mitigated by employing humidity controls and droplet dispensing monitoring
547and control systems. Lastly, DμF devices are susceptible to dielectric breakdown
548during prolonged operation, which can interfere with assay procedures. However,
549dielectric and hydrophobic material selection and deposition techniques are active
550areas of research in digital microfluidics and many design parameters, such as the
551materials, thicknesses, and organization of the dielectric and hydrophobic layers, as
552well as the ambient medium (i.e. air vs. oil) and operating voltage and frequency can
553be optimized to minimize the chance of dielectric breakdown.[92-95] The performance of
554optimized devices can support at least 25,000 droplet actuation steps without dielectric
555breakdown, which is sufficiently reliable for commercial applications.[96]
556
557The work presented here demonstrates that digital microfluidics, with highly flexible
558and automated liquid handling capabilities, and compatibility with a variety of in-situ
559analytical techniques, has the potential to serve as a powerful tool for automated cell
560spheroid culture. Ultimately, a digital microfluidic platform that facilitates cell
561spheroid culture and analysis may help increase adoption of three-dimensional cell-
562based assays and screens in routine biomedical research.

563

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764 Tables

Table 1. Performance characteristics of devices used in this work

Performance Characteristic	Value
Hanging drop volume reproducibility[a]	~8%
Maximum hanging drop volume[b]	~55 uL
Intra-experiment spheroid diameter variation[c]	~8%
Inter-experiment spheroid diameter variation[d]	~16%
Spheroid size (diameter) range[e]	50-700+ µm
Average spheroid aspect ratio[f]	1.15 ± 0.09

a. Average %CV of droplet volume for hanging drops.
b. Volume range specific to devices used for this work. Max volume = volume above which droplets detach from the device; determined experimentally.
c. Average %CV of spheroids formed on the same chip. N = 30 total spheroids across 8 different experiments.
d. Average %CV of spheroids formed on different chips. N = 30 total spheroids across 8 different experiments.
e. Values represent the smallest and largest spheroid diameters obtained in this work over a period of 24-72 h on the DµF devices. These values do not represent minimum or maximum possible sizes.
f. Aspect ratio = ratio of a spheroid's major axis to minor axis. N = 77 spheroids of different cell types

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782 **Figure Legends**

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784**Figure 1. Device schematic and dimensions.** Through-holes in the top plate allow for the 785addition of solutions to on-chip reservoirs, while through-holes, or 'wells,' in the bottom plate 786allow for the formation of hanging drops. Drops that are delivered to a well are drawn into 787the well spontaneously upon contact with the hydrophilic well wall. Addition of multiple 788drops to a well allows for the formation of a hanging drop with a curved air-liquid interface. 789Cells suspended in the drop can aggregate at this interface, forming a single spheroid within 790the drop.

791**Figure 2. Hanging drop formation on a digital microfluidic device.** (a) A series of 792images showing a top-down view of the insertion of drops of cell media (dyed blue for 793enhanced visualization, ~1.2 µL) into a well on the device. (b) A series of images showing a 794side-view of a well after the addition of multiple drops to the well. The drops insert 795spontaneously into the well and, after a sufficient volume has been added, form a hanging 796drop with the curved interface necessary to induce cell aggregation.

797**Figure 3. Distribution of dispensed drop volumes.** 144 drops were dispensed from 798different reservoirs across 3 different devices using a pre-programmed droplet 799dispensing sequence. The average drop volume was 1.75 µL and the %CV of the 800volume of all drops dispensed was ~8%.

801**Figure 4. Size and variation of hanging drop volumes.** Hanging drops comprised of 4, 5, 802and 6 dispensed drops had volumes of 7.4 ± 0.5 µL, 8.8 ± 0.8 µL, and 10.2 ± 0.5 µL, 803respectively (N = 8 hanging drops formed for each condition). For each condition, the 804variation in hanging drop volume was <10%. Hanging drops of volumes up to ~55 µL can

805be formed on the devices used in this work, however, only 7-10 µL is needed to form cell 806spheroids via the hanging drop technique.

807**Figure 5. Extent of liquid exchange, predicted and experimental results.** The extent of 808liquid exchange after one and two exchange cycles was monitored by measuring the change 809in absorbance of the dyed hanging drop solution and calculating the concentration from a 810standard curve. The dilution of a hanging drop after each cycle can be seen in the images 811above the plot. The agreement between the measured concentrations and the predicted 812values indicates that thorough mixing of the hanging drop is achieved during each exchange 813cycle and that DµF provides good control over the composition of the hanging drop. Error 814bars indicate the standard deviation of measurements from three different experiments.

815**Figure 6. Cell spheroids formed by Drmed by Dmages above the** (a) Representative 816images of spheroids of mouse mesenchymal stem cells (mMSC) grown on a digital 817microfluidic device after 24, 48, and 72h of in-situ incubation. Each image is of a different 818spheroid. Spheroids exhibit >90% viability during this time-frame as determined by staining 819with calcein-AM/ethidium homodimer-1 to visualize living (green) and dead (red) cells. (b) 820The spheroids of mMSC formed from cell suspensions of ~7.5e5 cells/mL had diameters of 821249±34 µm after 24 h (N=12 spheroids), 327±58 µm after 48 h (N=10 spheroids), and 822425±75 (N=8 spheroids) after 72 h in culture. The data here represent average spheroid sizes 823at each time point from 6 separate experiments. (c) The distribution and average aspect ratio 824of spheroids grown on the DµF platform (N=77 spheroids). The data represent 825measurements from HT-29 colorectal carcinoma spheroids, BJ fibroblast spheroids, and 826mouse MSC spheroids with diameters ranging from 100 100 h (N=12 solid line indicates

827the average aspect ratio value (1.15), the dashed lines indicate +/- one standard deviation 828(0.09).

829**Figure 7. Spheroid-based drug screening protocols and results.** (a) Diagram illustrating 830the workflow for a spheroid-based drug screen performed on human colorectal 831adenocarcinoma cells (ATCC HT-29). After 48 h of compaction, spheroids received either 832normal or insulin-containing medium. After another 24 h, the spheroids received either 833normal or drug-containing (irinotecan) medium, and were incubated for another 24 h. (b) 834Comparison of the normalized spheroid diameter (D_{96h}/D_{48h}) for spheroids exposed to the 835different drug screen assay conditions. The error bars indicate +/- one standard deviation 836from the average for each condition (medium + medium: N = 4, insulin + medium: N = 4, 837medium + drug: N = 6, insulin + drug: N = 8).

838**Figure 8. Colonosphere morphologies.** HT-29 colon adenocarcinoma spheroids exhibited 839colonosphere morphologies after 96 h of in-vitro hanging drop culture on a Dlture on a D6 h 840of in-vitro hanging drop culture on a D the average for each condition (medium + medium:pt-841like folds, similar to the morphology of the colon epithelium. Image (d) is a confocal cross-842section image taken at a z-plane ~75 µm into the spheroid interior. The image clearly shows 843the large lumen/crypt structure that is indicative of colonosphere morphology. The spheroids 844in these images are stained with calcein-AM/ethidium homodimer-1 to visualize living 845(green) and dead (red) cells. Scale bars correspond to 200 µm.

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