

Optimization of Aqueous Biphasic Tumor Spheroid Microtechnology for Anti-cancer Drug Testing in 3D Culture

STEPHANIE LEMMO,¹ EHSAN ATEFI,¹ GARY D. LUKER,^{2,3,4} and HOSSEIN TAVANA¹

¹Department of Biomedical Engineering, The University of Akron, Akron, OH 44325, USA; ²Department of Radiology, University of Michigan, Ann Arbor, MI 48109, USA; ³Department of Microbiology and Immunology, University of Michigan, Ann Arbor, MI 48109, USA; and ⁴Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI 48109, USA

(Received 15 February 2014; accepted 23 July 2014; published online 5 August 2014)

Associate Editor Cynthia A. Reinhart-King oversaw the review of this article.

Abstract—Tumor spheroids are three-dimensional clusters of cancer cells that exhibit characteristics of poorly perfused tumors and hence present a relevant model for testing the efficacy of anti-cancer compounds. The use of spheroids for drug screening is hindered by technological complexities for high throughput generation of consistent size spheroids individually addressable by drug compounds. Here we present and optimize a simple spheroid technology based on the use of an aqueous two-phase system. Cancer cells confined in a drop of the denser aqueous dextran phase are robotically dispensed into a microwell containing the immersion aqueous polyethylene glycol phase. Cells remain within the drop and form a viable spheroid, without a need for any external stimuli. The size of resulting spheroids is sensitive to volume variations of dispensed drops from the air displacement pipetting head of a commercial liquid handling robot. Therefore, we parametrically optimize the process of dispensing of dextran phase drops. For a given cell density, this optimization reproducibly generates consistent size spheroids in standard 96-well plates. In addition, we evaluate the use of a commercial biochemical assay to examine cellular viability of cancer cell spheroids. Spheroids

show a dose-dependent response to cisplatin similar to a monolayer culture. However unlike their two-dimensional counterpart, spheroids exhibit resistance to paclitaxel treatment. This technology, which uses only commercially-available reagents and equipment, can potentially expedite anti-cancer drug discovery. Although the use of robotics makes the ATPS spheroid technology particularly useful for drug screening applications, this approach is compatible with simpler liquid handling techniques such as manual micropipetting and offers a straightforward method of 3D cell culture in research laboratories.

Keywords—Aqueous two-phase system, Tumor spheroid, 3D (three-dimensional) culture, High throughput, Anti-cancer drug screening.

ABBREVIATIONS

ATPS	Aqueous two-phase system
3D	Three-dimensional
PEG	Polyethylene glycol
DEX	Dextran
CCS	Cancer cell spheroids

Address correspondence to Hossein Tavana, Department of Biomedical Engineering, The University of Akron, Akron, OH 44325, USA. Electronic mail: tavana@uakron.edu

This paper is part of the 2014 Young Innovators Issue.

Hossein Tavana received his PhD in Mechanical Engineering from University of Toronto. Subsequently, he received a prestigious postdoctoral fellowship award from NSERC Canada and joined the Biomedical Engineering department of the University of Michigan as a postdoctoral fellow. He has been an Assistant Professor of Biomedical Engineering at The University of Akron since 2010. Dr. Tavana's research is centered on developing novel microtechnologies to create biomimetic cellular models. His research spans engineering in vitro models for cancer, stem cells, and pulmonary research. In 2013, he was selected as one of top 20 Young Investigator Frontiers in Bioengineering. He has published over 40 peer-reviewed original research papers, review articles, and book chapters. His research is currently supported by federal and state grants from NIH, NSF, and Ohio Third Frontier. Dr. Tavana currently directs the Biofluid Microtechnology Laboratory at the Biomedical Engineering department of The University of Akron.



INTRODUCTION

Three-dimensional (3D) cell culture technologies have recently gained increasing recognition in cancer research.^{14,18,25} Under defined conditions, these approaches allow formation of a 3D compact cluster of cancer cells known as a spheroid.³⁴ Cancer cell spheroids (CCS) reproduce key features of avascular and poorly vascularized solid tumors and micrometastases in terms of morphology,⁴⁸ growth kinetics, restricted diffusion of nutrients and drugs and hypoxic core,²⁶ and gene expression profiles.²⁰ As such, they present a relevant model for testing the efficacy of conventional and novel chemical compounds against different phenotypes of cancer cells including proliferation, matrix invasion, and angiogenesis.^{15,31,43} Unlike traditional monolayer cultures, CCS are expected to result in more realistic prediction of cellular response to drugs and thus, expedite drug development and discovery and reduce associated costs by eliminating ineffective compounds from further consideration in animal models and pre-clinical tests.¹⁵ For drug screening applications, it is crucial to generate homogeneously sized CCS of similar baseline viability. This will ensure that changes in cell viability from this baseline are due to drug treatment and help reflect differential effects of concentrations of a drug or different drugs on cell viability of cellular spheroids.

Spheroid culture has been utilized in academic laboratories for several decades.³⁴ However, incorporation of CCS into mainstream drug development processes has been hampered by complex and expensive methodological requirements for the formation, maintenance, and drug screening of large numbers of consistently sized CCS.¹³ Traditional liquid overlay and spinner flask techniques generate spheroids by stirring or spinning of cell suspension; these techniques expose cells to non-physiologic shear forces and result in random size spheroids in one dish that cannot be used for drug screening.^{6,44} The original hanging drop method and its newer developments produce spheroids with minimal size variations.^{17,19,42} However, evaporation of media from drops containing CCS and drugs, difficulty of handling of plates during culture, adapting plates to robotic liquid handlers for automated addition of media and drugs, and the need for transfer of spheroids to a standard well plate for subsequent biochemical analyses remain major shortcomings. Microfabricated microwell arrays and microfluidic devices have also been used for 3D cell culture.^{22,27,39} These technologies are limited by expense and difficulties with exchange of media. More importantly, resulting spheroids are not individually addressable with drug compounds and downstream biochemical analyses reagents. Cell levitation by a

magnetic field also generates spheroids,³³ but the method is cumbersome and adapting it to a high throughput setting is complicated.

We have developed a robotically operated technology using a polymeric aqueous two-phase system (ATPS) to address these shortcomings. A drop of the denser aqueous phase containing cancer cells is robotically dispensed into a microwell containing the lighter immersion aqueous phase. During incubation, cells remain confined within the drop and aggregate to form a CCS. Our previous work with ATPS for 2D cell patterning and printing shows that ATPS is completely cell-friendly, retaining fully viable and functional cells.^{29,35-37} We have adapted this technology to 3D culture in standard 96-well plates to allow simultaneous formation of CCS individually addressable with drug compounds.³ This robotic add-on assay eliminates labor associated with tedious addition and removal of media and reagents and allows downstream analysis of CCS treated with drug compounds using standard biochemical assays and off-the-shelf equipment such as plate readers and liquid handling systems. Here we parametrically evaluate and optimize the dispensing process from a standard air displacement pipetting head of a liquid handler to generate consistent size and viable spheroids within and across microwell plates. We establish a culture condition for CCS and downstream analysis of cellular viability after drug treatment to enable integration of spheroids into routine drug testing. Treatment of CCS with known anti-cancer drugs shows anomalous cellular response compared to monolayer culture of cancer cells. This new technology is easy to implement and presents an enabling tool for high throughput compound screening against cancer cells with a physiologically relevant cellular model.

MATERIALS AND METHODS

Preparation of Aqueous Two-Phase System (ATPS)

We used polyethylene glycol (PEG, Mw: 35,000, Sigma-Aldrich) and dextran (DEX, Mw: 500,000, Pharmacosmos) as phase forming polymers. Stock solutions of PEG and DEX phases were prepared in complete growth medium at concentrations of 5.0% (w/w) PEG and 12.8% (w/w) DEX, respectively. To facilitate dissolution of polymers, solutions were vortexed and then kept in a 37 °C water bath for 1 h. Stock polymer solutions were passed through 0.2 μm pore size filters to remove small particles and impurities and then stored at 4 °C until use.

Cell Culture

The cell line used in this study for spheroid formation, optimization and anti-cancer drug testing was

triple negative breast cancer MDA-MB-157 (ATCC). Cells were cultured in complete growth medium containing phenol red Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 1% antibiotic (Life Technologies) and 1% glutamine (Life Technologies). Cells were grown in T75 flasks at 37 °C and 5% CO₂ until they were ~90% confluent. Then cells were dissociated using 3 mL of trypsin (Life Technologies) for 7 min. To neutralize trypsin, 6 mL of complete growth medium was added. The cell suspension was centrifuged at 1000 rpm for 5 min. After removing the supernatant, cells were resuspended in 1 mL of medium and counted with a hemocytometer.

Spheroid Formation and Culture

Wells of a non-adherent, round-bottom 96-well plate (destination plate) were coated with 100 µL of 1% (w/v) pluronic F108 co-block polymer (Mw: 14,600 and HLB > 24.0, Sigma-Aldrich) prior to experiments to prevent cell attachment during spheroid culture.^{9,41} Pluronic solution was added to each well, incubated for 24 h at 37 °C, and washed out with PBS. Then each well was loaded with 50 µL of 5.0% (w/w) aqueous PEG phase prepared with growth medium. The plate was placed onto the working surface of a liquid handling robot (SRT Bravo, Agilent).

To prepare CCS of 1.5×10^4 cell density, the 12.8% (w/w) aqueous DEX phase was mixed with 1×10^5 cells/µL at a ratio of 1:1 (v/v). Then 20 µL of this suspension was loaded into each well from one column of a flat-bottom 384-well plate (source plate) (Corning), which was subsequently placed onto the working surface of the liquid handler. The pipetting head of the liquid handler aspirated 0.3 µL from the suspension into each pipette and slowly dispensed it into each well of the destination plate containing the PEG phase. This was followed by dispensing of 0.9 µL air volume to ensure complete delivery of the DEX phase solution containing cells into each well. This process was performed column-by-column. Due to the large density of cells in the source plate, the liquid handler thoroughly mixed the suspension before each aspiration step to ensure a uniform distribution of cells. After 24 h, the polymeric ATPS was diluted out by adding 50 µL of fresh medium containing drugs to wells. This disrupted the concentration of polymers and resulted in a homogeneous, one-phase solution of growth medium containing trace amounts of polymers. Therefore, the ATPS was necessary and used only for formation of CCS and diluted out after 24 h of incubation at which point spheroids had formed. Diameter of each spheroid was measured by averaging the smallest and largest diameters.

Cellular Staining

To examine viability of cells in CCS generated after 24 h of incubation, the ATPS media was replaced by a dye solution containing 5 µM of Calcein AM (green) and ethidium homodimer-1 (red). Both dyes were obtained from Life Technologies. CCS were incubated for 2 h and the dye solution was carefully replaced with culture medium. Images were captured using an inverted fluorescent microscope (AxioVision, Zeiss) equipped with a high resolution camera (AxioCam MRm, Zeiss).

Preparation of Drugs

We used cisplatin (Spectrum Chemicals) and paclitaxel (Calbiochem) for drug experiments. Cisplatin was dissolved in ultrapure sterile water at 2 mg/mL and paclitaxel was dissolved in dimethylsulfoxide (DMSO, ATCC) at 10 mg/mL. Serial dilutions were made in growth medium using the stock solutions to result in desired working concentrations. Both drug solutions were protected from light when setting experiments. Stock solutions of cisplatin and paclitaxel were stored at room temperature and -20 °C, respectively.

Drug Testing With Monolayer of Cells (2D Culture)

MDA-MB-157 cells were seeded at a density of 1.5×10^4 cells in each well of a flat-bottom tissue culture treated 96-well plate (Corning) containing 50 µL of growth medium. Cells were allowed to attach and spread for 24 h. Then 50 µL of desired concentrations of each drug was added to wells, each concentration with at least one column of replicates ($n = 8$). Drug solutions were prepared in culture medium and at twice the desired final concentration before adding to an equal volume of culture medium in each well. Cells were cultured for 2 days in the presence of drugs; then drugs were renewed by the direct addition of a fresh dose in 50 µL of culture medium and experiments continued for an additional 2 days. The concentration of drugs during experiments was kept constant. Cell viability was analyzed with a PrestoBlue reagent as explained below. A nonlinear sigmoidal curve was fitted to cell viability data plotted vs. drug concentration (GraphPad Prism). Drug experiments with monolayer cultures were performed three times to ensure reproducibility.

Drug Testing with Spheroids (3D culture)

After formation of a single CCS of 1.5×10^4 cell density in each well using the ATPS, different

concentrations of each drug was added to wells in 50 μL of growth medium. Drugs were prepared in culture medium and at twice the desired final concentrations to account for the 50 μL of the aqueous PEG phase present in each well. This drug addition disrupted the initial polymer concentrations and diluted out the polymeric ATPS to a single phase.¹ Therefore, the two-phase system was not present throughout drug testing. Similar to drug experiments with monolayer cultures, CCS were cultured in the presence of each drug for 4 days. At day 2, 50 μL of culture medium containing a fresh dose of each drug was added to wells. As with drug tests with 2D cultures, plates were protected from light during incubation due to light sensitivity of drugs. For each concentration, CCS from two columns of a plate were used ($n = 16$). Cellular viability of spheroids was analyzed with PrestoBlue and a nonlinear sigmoidal curve was fitted to data plotted vs. drug concentration. Drug experiments with 3D cultures were performed three times to ensure reproducibility.

PrestoBlue Viability Assay

After incubation of cells with a drug, viability of cells in 2D monolayer and 3D spheroids was analyzed with a resazurin-based PrestoBlue reagent (Life Technologies) that reduces resazurin to resorufin in the presence of metabolically active, proliferating cells. The reduced form, resorufin, is fluorescent allowing the fluorescent intensity of wells to be measured. PrestoBlue was added to each well at 10% concentration of the total well volume and the plate was incubated while protected from light. Monolayer cultures were incubated for 15 min, as recommended by the manufacturer, whereas spheroids were incubated with PrestoBlue for 6 h, as determined from our optimization study. Then fluorescent intensity was detected with a standard plate reader (SpectraMax M2, Molecular Devices) at excitation and emission wavelengths of 560 and 590 nm, respectively. The average fluorescent intensity from wells containing drug-treated cells was normalized against that of control wells (non-treated cells) to determine percent viability of drug-treated cells.

RESULTS AND DISCUSSION

Spheroid Generation Using Aqueous Two-Phase System (ATPS) Technology

Aqueous solutions of PEG and DEX give rise to two immiscible phases above certain concentrations of the polymers (Fig. 1a).³⁵ With the molecular weights of polymers used in this study, 5.0% (w/w) PEG and

6.4% (w/w) DEX are the lowest concentrations of phase-forming polymers that result in an ATPS.³⁸ To generate spheroids, a small volume (0.3 μL) of the denser DEX phase containing cells is dispensed into a well containing the immersion PEG phase (Fig. 1b). The DEX phase forms a drop at the bottom of the well, confining the cells and keeping them in close proximity. Importantly, nutrients and metabolic waste products of cells can cross the interface between the drop phase and the immersion phase through diffusion, yet cells effectively partition to the DEX drop. During incubation, cells aggregate and form an individual CCS within the drop (Fig. 1b). Without ATPS, cells seeded on the same substrate only form loose aggregates; thus, using ATPS is crucial for CCS formation (Fig. SI-1). Cells of resulting spheroids are viable as demonstrated by fluorescent staining of live and dead cells (Fig. 1c). The staining also shows the limited diffusion of the green and red dyes into the core of the spheroid, mimicking diffusion limitations of certain drug compounds into solid tumors. The diameter of spheroids can be varied in two different ways: either changing the cell suspension density (number of cells/ μL) but using drops of the same volume (Figs. 1d, e), or keeping the same cell suspension density (number of cells/ μL) and changing the dispensed drop volume (Figs. 1d, f). This approach can thus be used to conveniently generate spheroids with pre-defined diameters.

Optimization of Dispensing Consistent Size DEX Phase Drops

The size of spheroids resulting from the ATPS assay critically depends on the volume of DEX drops containing cancer cells dispensed into wells. The high viscosity of the DEX phase solution makes it challenging to consistently dispense ultralow volumes of few hundreds of nanoliters using any dispensing mechanism including the air displacement pipetting system of standard commercial liquid handlers. We carried out a preliminary study and identified three parameters that can significantly affect the size of dispensed DEX drops: dispense flow rate of the DEX phase solution from tips, dispense height of tips from the well-bottom, and air volume above the DEX phase solution inside tips that is pipetted after dispensing the DEX phase. Then we performed an optimization study by varying each parameter within a defined range (Fig. 2a) while keeping the other two parameters constant. Each condition was tested with 32 replicates, i.e., four columns from a 96-well plate. To ensure the consistency of our experimental procedure, we used the same barrels of the pipetting head of the liquid handler to dispense DEX drops. For each experiment, we

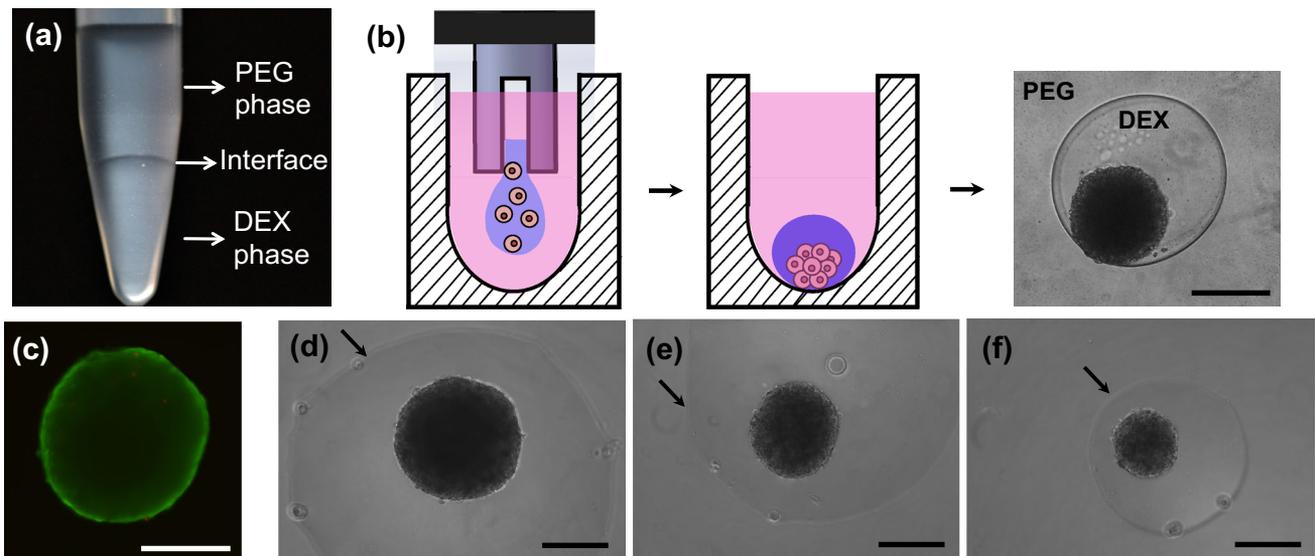


FIGURE 1. ATPS spheroid microtechnology. (a) Aqueous solutions of PEG and DEX separate and form two distinct phases with a visible interface, (b) pipetting tip of a liquid handler dispenses a DEX phase drop containing cells (blue) into a microwell containing the PEG phase (pink) to result in the formation of CCS within the drop during incubation (top view), (c) resulting CCS contain highly viable cells (green fluorescent color), (d–f) CCS size varies by either changing the cell suspension density (#cells/ μL) and keeping the drop volume the same (10×10^3 cells/ $0.4 \mu\text{L}$ in d to 5×10^3 cells/ $0.4 \mu\text{L}$ in e) or by keeping the cell suspension density (#cells/ μL) the same and changing the drop volume (10×10^3 cells/ $0.4 \mu\text{L}$ in d to 2.5×10^3 cells/ $0.1 \mu\text{L}$ in f). Arrows in the images indicate the periphery of DEX drops. Scale bar $250 \mu\text{m}$. Different colors in schematics of panel (b) are for clarity of presentation only.

captured top-view images of the drops and calculated their diameters. We ensured that data are normally distributed by constructing a quantile–quantile (q–q) plot (Matlab, MathWorks), which shows quantiles of acquired data vs. quantiles of a standard normal distribution. Figure 2b illustrates a typical result from experiments to evaluate the effect of air volume. Drop diameter data from experiments at air volumes of 0.3 – $1.5 \mu\text{L}$ follow a linear trend, indicating that data are normally distributed.^{24,40} Different slopes of the lines indicate differences between standard deviations of data from these air volumes.⁷ We confirmed that data obtained for drop diameter by varying each of the three parameters within the pre-defined range of Fig. 2a are all normally distributed.

Next we used one-way ANOVA with a post hoc tukey test to statistically evaluate the effect of each variable, within the studied range, on the diameter of dispensed drops. This analysis helped select the magnitude of the three variables that result in the most consistent drop size, i.e., the smallest standard deviation. The optimized values include a dispense height of 0.1 mm , a dispense flow rate of $0.5 \mu\text{L/s}$ for the DEX phase solution followed by dispensing an air volume of $0.9 \mu\text{L}$. To confirm that this optimized condition results in consistent size DEX drops, we printed drops in three 96-well plates and imaged and analyzed their diameters. Figure 2c shows the distribution of diameter of drops across these plates. These data return an average value of $730 \pm 31 \mu\text{m}$. The inset bar graph of Fig. 2c illustrates the distribution

frequency of drop diameters. This consistency is significant considering that the DEX phase is a viscous solution, drop volumes are very small, and the liquid handler is a standard commercial instrument designed for handling larger, microliter-scale liquid volumes. We emphasize that although the resulting optimized values of the three variables are only specific to the liquid handler used in this study, the principle of optimization remains the same and can be followed with other dispensing systems.

Evaluation of Consistency of Spheroid Generation and Growth Kinetics

We used the above protocol for spheroid generation with our ATPS technology. CCS of 1.5×10^4 MDA-MB-157 cells were formed in ten 96-well plates. After 24 h of incubation, the diameter of each spheroid was measured. Figures 3a, b show the distribution of average diameter of CCS from each plate and the frequency distribution of diameter of CCS across the ten plates. The average diameter across all plates is $305 \pm 19 \mu\text{m}$. We note that the observed variations of diameter are due to the limitations of the liquid handler to precisely deliver ultralow volumes ($0.3 \mu\text{L}$) of the viscous DEX phase solution containing cells. Most likely, further improving the consistency level requires using dispensing mechanisms more accurate at a nanoliter-volume scale such as pin tool or acoustic dispensing technologies.^{35,45}

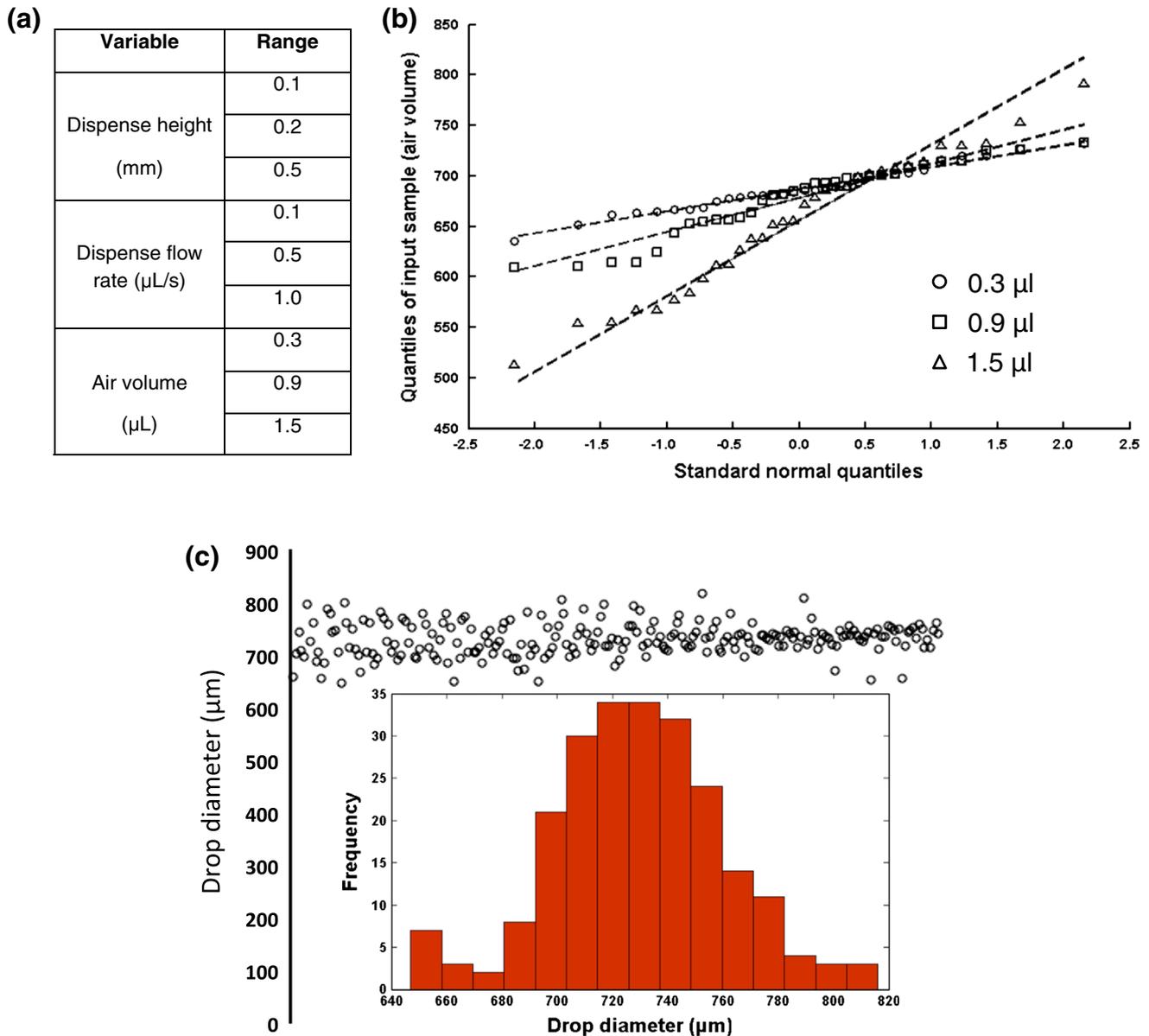


FIGURE 2. Optimization of dispensing DEX drops using air displacement pipetting system. (a) Three variables and their range selected for optimization of dispensing DEX drops, (b) quantiles of drop diameter obtained by varying air volume change linearly vs. quantiles of a standard normal indicating normal distribution of drop diameters, (c) frequency distribution of diameter of DEX drops from three 96-well plates obtained using optimized values of the three parameters in the table of panel (a).

Next we evaluated generating spheroids of different size using the first method described above, i.e. changing the cell suspension density (number of cells/ μL) and using the same DEX drop volume. DEX drops of $0.3 \mu\text{L}$ containing cell densities of 0.5×10^4 , 2.5×10^4 , 5.0×10^4 , and 10.0×10^4 were dispensed into wells containing the PEG phase ($n = 8$). Diameter of each spheroid was measured after 24 h of incubation. Figure 3c shows that increasing cell density within this range produces spheroids with 167 ± 19 – $517 \pm 31 \mu\text{m}$ in diameter.

To ensure that cells in spheroids proliferate, we evaluated the growth of MDA-MB-157 CCS over a 4-day period using the PrestoBlue assay that provides a measure of metabolic activity of cells. Monolayer cultures were used as control. Measurements of cell viability each day with CCS and monolayer cultures showed an increase in the fluorescent signal, indicating an increase in the number of metabolically active cells (Fig. 3d). The rate of increase was smaller with the 3D culture compared to the monolayer. This is likely due to limitations of nutrients and oxygen diffusion into

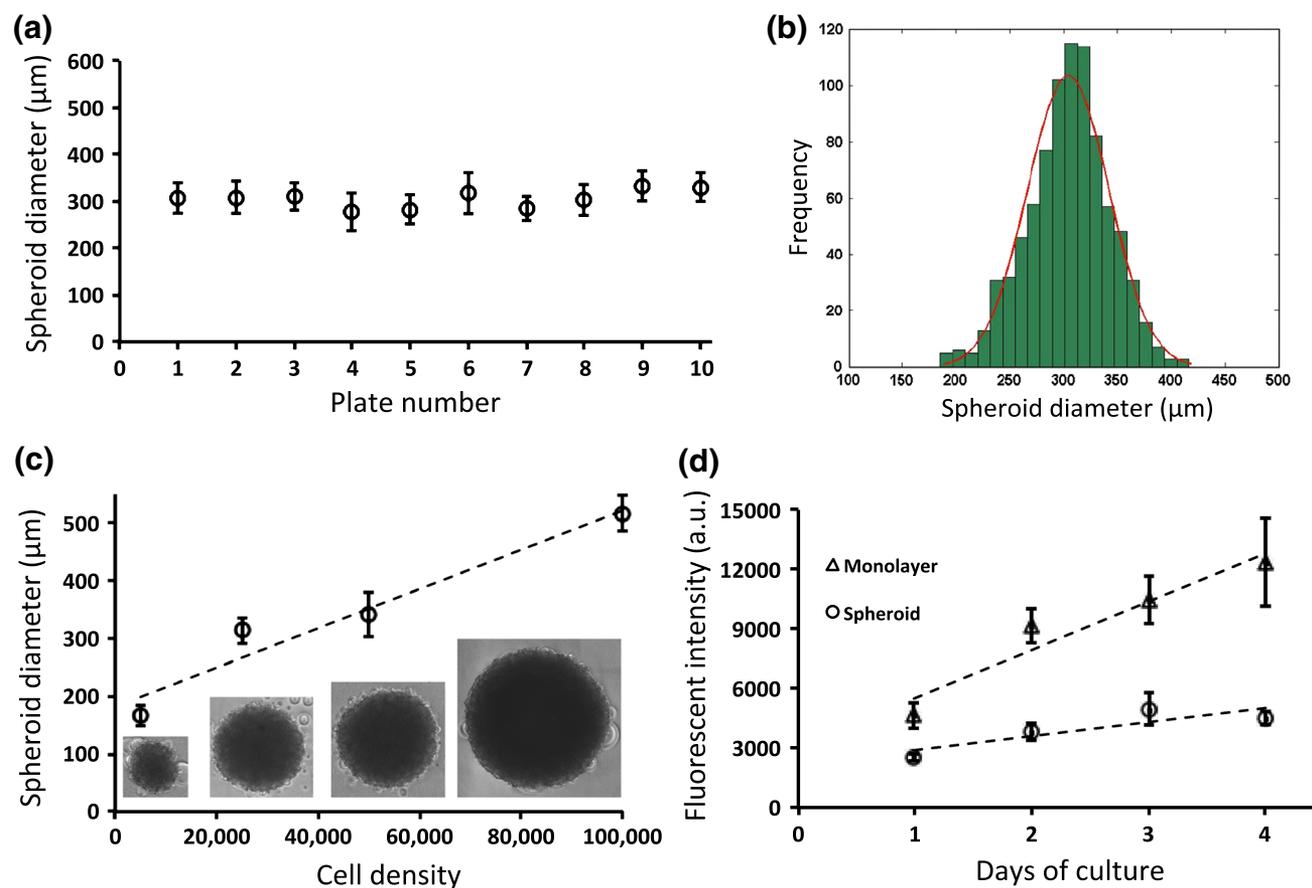


FIGURE 3. Consistency of size of spheroids. (a) Distribution of diameter of CCS of 1.5×10^4 cells generated using an air displacement pipetting head across ten 96-well plates. Each data point represents data from one plate, (b) Frequency distribution of diameter of CCS across the ten plates, (c) CCS of different diameters obtained using different cell suspension densities within $0.3 \mu\text{L}$ DEX drops ($R^2 = 0.95$), (d) metabolic activity of cells from both 2D (triangles) and 3D (circles) cultures increases over a 4-day culture period. Error bars in panels (a), (c), and (d) represent 95% confidence limits.

CCS and indicates that spheroids mimic a key property of solid tumors *in vivo*.

Optimization of Biochemical Analysis of Cellular Viability in Spheroid Culture

An important aspect of 3D cell cultures for drug screening studies is selecting an appropriate and sensitive end-point analytical assay. We surveyed existing assays and selected a PrestoBlue viability assay.¹¹ It is a very fast add-on assay and resolves cell viability in 2D cultures in 15 min; in addition, it allows analysis of viability in the same plate used for generating CCS and treating them with drugs, eliminating the need to transfer CCS to a special plate. To determine the sensitivity of this assay for 3D culture and an optimum incubation time, we generated CCS with four different cell densities of 0.1×10^4 , 1.0×10^4 , 6.0×10^4 , and 10.0×10^4 using our ATPS technology, each density in two columns of a 96-well plate ($n = 16$). Then we added the PrestoBlue reagent to each well, incubated

cells, and measured the fluorescent signal using a plate reader at four different time points. Figure 4 shows that the fluorescent signal from CCS of each cell density increases linearly within 6 h of incubation. The difference between metabolic activity of cells of CCS of different densities becomes statistically significant at 60 min of incubation (Fig. 4 and Table SI-1); this difference becomes more pronounced with longer incubation. In addition for CCS of the same cell density, measured fluorescent signal becomes significantly different with 120 min of incubation when all densities are considered collectively (Fig. 4 and Table SI-2). This characterization experiment suggests incubating CCS with PrestoBlue for at least 120 min. To increase the reliability of data, we selected an incubation time of 360 min that gives substantially different fluorescent intensity values and still yields a linear intensity change over time for CCS of different cell densities. Thus, we used 360 min incubation for subsequent studies of evaluating cellular viability of drug-treated CCS.

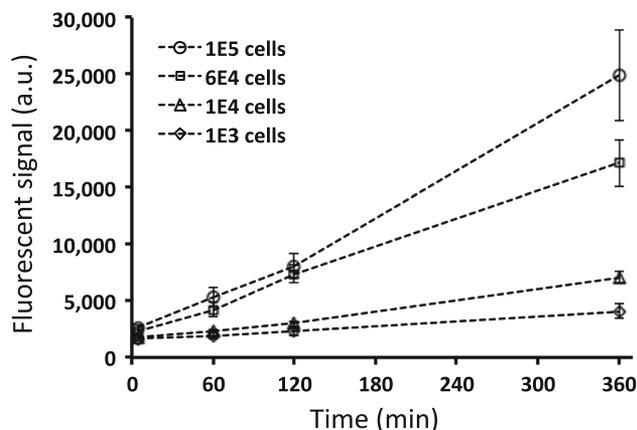


FIGURE 4. Optimization of PrestoBlue viability assay for spheroids. Time-dependent increase in the fluorescent signal (metabolic activity) of CCS of four different cell densities is linear over a 6-h time period. Difference between the signal from CCS of different densities becomes significantly different at 60 min ($p < 0.01$). Considering each cell density over time, a significant difference in the signal intensity is observed at 120 min of incubation, when all CCS are taken into account ($p < 0.01$). Two columns of a 96-well plate ($n = 16$) were used to generate CCS of each density.

Drug Response of Cancer Cell Spheroids

We evaluated the potential of the ATPS spheroid technology for anti-cancer drug screening by treating MDA-MB-157 CCS with two chemotherapeutic compounds, cisplatin and paclitaxel, which are clinically used for adenocarcinoma. Parallel drug treatment experiments were also set with monolayer culture of cells. Prior to spheroid formation, wells of a 96-well plate were coated with pluronic F108 to prevent attachment of spheroids to the substrate during the 5-day study (1 day for spheroid formation and 4 days of drug treatment and analysis of cellular response). CCS and monolayer cultures were treated with cisplatin at a concentration range of 0–200 μM for 4 days with a drug renewal after day 2. Experiments were repeated three times. Both culture conditions showed a dose-dependent decrease in cellular viability with similar LD50 values of $\sim 13 \mu\text{M}$ (Fig. 5a). Effective penetration of cisplatin into CCS and their disintegration is potentially responsible for identical response of 2D and 3D cultures to the drug. This is consistent with clinical observation of sensitivity of triple negative breast cancers (such as MDA-MB-157) to cisplatin.^{2,5,32}

CCS and 2D cultures were treated with paclitaxel at a 0–100 μM concentration range. The viability of cells of the monolayer decreased dose-dependently and resulted in an LD50 value of 8.04 nM (Fig. 5b). On the other hand, CCS were not sensitive to paclitaxel treatment at this concentration range and retained a high viability similar to non-treated, control spheroids

(Fig. 5b). Therefore an LD50 was not reached. We repeated these experiments three times and additionally confirmed the results with an established MTT viability assay. Although the underlying reason is not known, it is likely that MDA-MB-157 CCS acquire resistance when grown in 3D culture, consistent with clinical studies that suggest resistance of triple negative breast cancers to taxanes.^{2,4,30} The lack of drug sensitivity in CCS *in vitro* and solid tumors *in vivo* is proposed to be caused by close cell–cell contact in a 3D condition that results in mechanisms such as gap junctional-mediated “reciprocity” and cell shape-mediated alterations in gene expression.^{10,16,21,23,28} The effect was reversed in mouse mammary carcinoma spheroids treated with an anti-adhesive agent prior to drug treatment.²¹ The hypoxic microenvironment within CCS can also upregulate the MDR1-P glycoprotein drug transporter,^{8,46} resulting in the export of paclitaxel from cells.⁴⁷ In addition, paclitaxel targets mitotic cells; therefore reduced proliferation of cells in CCS compared to monolayer of cells (Fig. 3d) may render cells in CCS relatively drug resistant. Detailed molecular analysis combined with this 3D culture approach can elucidate the lack of sensitivity of MDA-MB-157 CCS to paclitaxel treatment, which may explain treatment failures in patients.

As explained above, the ATPS is solely used for spheroid formation. The phase-forming polymers are diluted out by addition of culture medium containing a drug during drug studies, converting the ATPS to a single aqueous phase. To ensure that presence of trace amounts of PEG and DEX does not interfere with drug diffusion through media and is not responsible for observed cellular response to drugs, we set a study using the LD50 = 8.04 nM concentration of paclitaxel with a 2D culture just containing media and a 2D culture containing trace amounts of PEG and DEX at concentrations present after a single phase forms during drug studies. Cell viability was measured after 4 days with PrestoBlue (Fig. SI-2). The result showed that cellular viability from these two conditions is identical ($p = 0.15$). Therefore presence of residual polymers does not interfere with drug diffusion through media, confirming the validity of cell viability data.

Similar response of CCS to cisplatin and paclitaxel has been observed before.¹² Monolayer and spheroid cultures of human ovarian cancer cells showed similar viability when treated with cisplatin. On the contrary, paclitaxel treatment of human ovarian CCS resulted in drug resistance whereas monolayer cultures showed dose-dependent response. Colony-forming efficiency of paclitaxel-treated CCS was suspected to be a potential cause for the drug resistance. Cells treated with paclitaxel in 3D cultures showed an increase in colony-

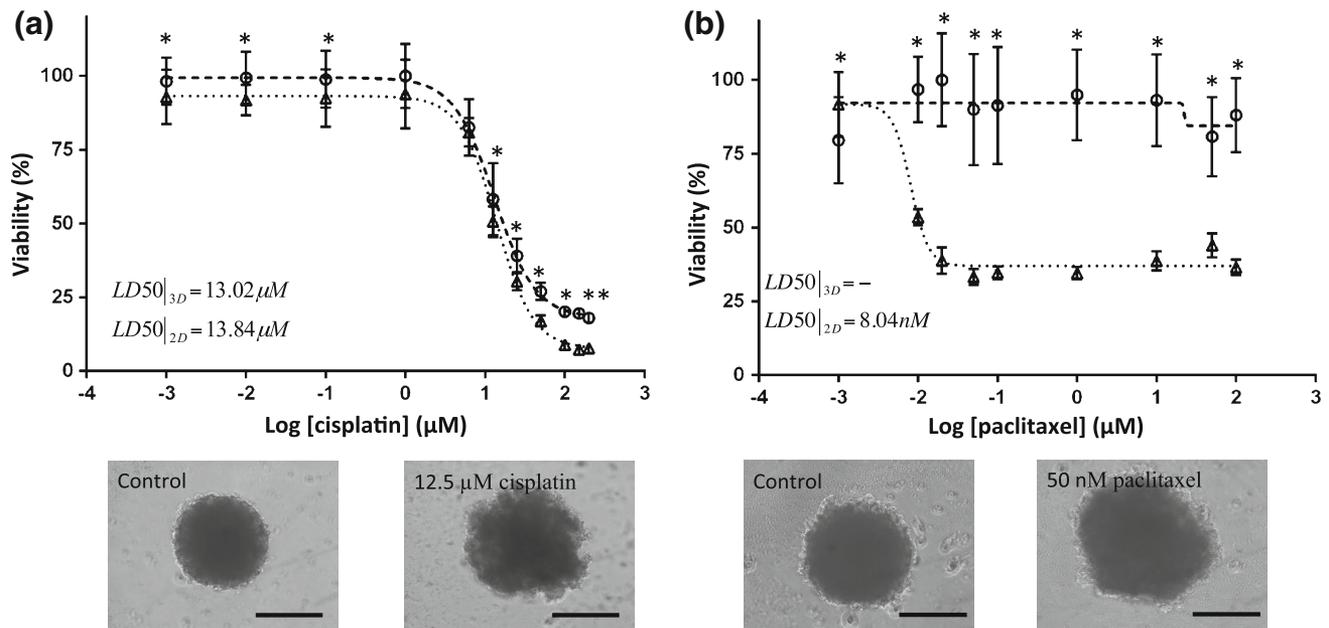


FIGURE 5. Drug response of MDA-MB-157 breast cancer cell spheroids. Spheroids (circles) and monolayer (triangles) cultures of cells were treated with (a) cisplatin (0–200 μM) and (b) paclitaxel (0–100 μM). All experiments were set three times. Cellular viability was measured 4 days post treatment with each drug both in 2D and 3D cultures with a fresh dose renewed after day 2. Dashed lines represent nonlinear sigmoidal curve fitting to experimental cell viability data. 3D and 2D culture experiments were set with 16 and 8 replicates, respectively. Asterisks denote statistically significant difference between viability of 2D and 3D cultures at each drug concentration ($p < 0.05$). Error bars are 95% confidence limits. Scale bar 200 μm.

forming efficiency whereas colony formation capability of cells of monolayer cultures significantly dropped. Further analysis confirmed apoptosis effects in monolayer cultures but not in CCS when treated with paclitaxel. This independent study validates our observations of drug response of MDA-MB-157 CCS. Altogether our study underlines the predictive potential of CCS and the importance of incorporating CCS in drug screening applications.

Broad Utility of the ATPS Spheroid Technology

While the use of liquid handling robotics makes the technology particularly useful for high throughput compound screening with tumor spheroids, the ease of working with ATPS renders it readily available for spheroid culture in laboratories without robotic tools. Our methods can be easily adapted to various liquid handling techniques including manual pipetting with sufficient precision at sub-microliter volumes to allow dispensing of cell-containing DEX drops into the PEG phase solution. This provides researchers who have a general knowledge of cell culture with an inexpensive and simple technique to perform 3D culture of cancer cells without a need for any special equipment. Rapid spheroid formation (within 24 h) in cell-friendly ATPS media, virtually with a variety of cell types in different

configurations of standard plates, and the ease of handling of spheroids during culture and analysis are important features of this technology that make it accessible to the research community.

CONCLUSIONS

We presented a technology to generate CCS in a high throughput, standard 96-well plate format. This approach utilized the immiscibility of two aqueous phases to rapidly form an individual spheroid within each well without the use of any external forces. Evaluation of several liquid handling parameters along with statistical analyses resulted in a set of pre-defined conditions to generate consistent size spheroids across standard 96-well plates. This approach eliminates the shortcomings of existing assays and results in viable spheroids that can individually be treated with drug compounds. Our proof-of-concept drug studies showed that MDA-MB-157 triple negative breast CCS treated with cisplatin disintegrated and displayed a dose-dependent drug response similar to 2D culture of cancer cells. However unlike their 2D counterpart, spheroids were not sensitive to paclitaxel treatment and showed resistance to the drug at a wide range of concentrations. Therefore this 3D culture technology can potentially elicit more realistic response from

cancer cells treated with chemotherapeutic compounds. The ease of handling of plates during culture, maintenance of culture, media exchange, addition of anti-cancer drugs and biochemical analysis reagents using robotic tools, and screening of spheroids with standard plate readers make this technology adaptable to centers with high throughput screening capabilities. Further developments of this technology to include different components of tumor microenvironment such as supporting stromal cells and extracellular matrix proteins will provide a physiologic tumor model that mimic complexity of solid tumors *in vivo* and allow high throughput screening of drug compounds not currently possible.

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (doi:10.1007/s12195-014-0349-4) contains supplementary material, which is available to authorized users.

ACKNOWLEDGEMENTS

This work is supported by a Grant from the National Institutes of Health R21CA182333.

CONFLICT OF INTEREST

Stephanie Lemmo, Ehsan Atefi, Gary D. Luker, and Hossein Tavana declare that they have no conflict of interest.

ETHICAL STANDARDS

This work involved no human subjects or animal studies.

REFERENCES

- Albertsson, P.-A., and F. Tjerneld. Phase diagrams. *Methods Enzymol.* 228:3–13, 1994.
- Andre, F., and C. C. Zielinski. Optimal strategies for the treatment of metastatic triple-negative breast cancer with currently approved agents. *Ann. Oncol.* 23(Suppl 6):46–51, 2012.
- Atefi, E., S. Lemmo, D. Fyffe, G. D. Luker, and H. Tavana. High throughput, polymeric aqueous two-phase printing of tumor spheroids. *Adv. Func. Mater.* 2014, in press (doi:10.1002/adfm.201401302).
- Blum, J. L., V. Dieras, P. M. Lo Russo, J. Horton, O. Rutman, *et al.* Multicenter, phase II study of capecitabine in taxane-pretreated metastatic breast carcinoma patients. *Cancer* 92:1759–1768, 2001.
- Byrski, T., T. Huzarski, R. Dent, J. Gronwald, D. Zuziak, *et al.* Response to neoadjuvant therapy with cisplatin in BRCA1-positive breast cancer patients. *Breast Cancer Res. Treat.* 115:359–363, 2009.
- Carlsson, J., and J. M. Yuhas. Liquid-overlay culture of cellular spheroids. *Rec. Results Cancer Res.* 95:1–23, 1984.
- Chen, H., S. A. Quandt, J. G. Grzywacz, and T. A. Arcury. A distribution-based multiple imputation method for handling bivariate pesticide data with values below the limit of detection. *Environ. Health Persp.* 119:351–356, 2011.
- Comerford, K. M., T. J. Wallace, J. Karhausen, N. A. Louis, M. C. Montalto, and S. P. Colgan. Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. *Cancer Res.* 62:3387–3394, 2002.
- Corey, J. M., C. C. Gertz, T. J. Sutton, Q. Chen, K. B. Mycek, *et al.* Patterning N-type and S-type neuroblastoma cells with Pluronic F108 and ECM proteins. *J. Biomed. Mater. Res. A* 93:673–686, 2010.
- Desoize, B., and J. Jardillier. Multicellular resistance: a paradigm for clinical resistance? *Crit. Rev. Oncol. Hematol.* 36:193–207, 2000.
- Desplats, P., B. Spencer, L. Crews, P. Pathel, D. Morvinski-Friedmann, *et al.* Alpha-synuclein induces alterations in adult neurogenesis in Parkinson disease models via p53-mediated repression of Notch1. *J. Biol. Chem.* 287:31691–31702, 2012.
- Frankel, A., R. Buckman, and R. S. Kerbel. Abrogation of taxol-induced G2-M arrest and apoptosis in human ovarian cancer cells grown as multicellular tumor spheroids. *Cancer Res.* 57:2388–2393, 1997.
- Friedrich, J., C. Seidel, R. Ebner, and L. A. Kunz-Schughart. Spheroid-based drug screen: considerations and practical approach. *Nat. Protoc.* 4:309–324, 2009.
- Ghajar, C. M., and M. J. Bissell. Tumor engineering: the other face of tissue engineering. *Tissue Eng. A* 16:2153–2156, 2010.
- Hirschhaeuser, F., H. Menne, C. Dittfeld, J. West, W. Mueller-Klieser, and L. A. Kunz-Schughart. Multicellular tumor spheroids: an underestimated tool is catching up again. *J. Biotechnol.* 148:3–15, 2010.
- Holder, J. W., E. Elmore, and J. C. Barrett. Gap junction function and cancer. *Cancer Res.* 53:3475–3485, 1993.
- Hsiao, A. Y., Y. C. Tung, C. H. Kuo, B. Mosadegh, R. Bedenis, *et al.* Micro-ring structures stabilize microdroplets to enable long term spheroid culture in 384 hanging drop array plates. *Biomed. Microdev.* 14:313–323, 2012.
- Hutmacher, D. W., D. Loessner, S. Rizzi, D. L. Kaplan, D. J. Mooney, and J. A. Clements. Can tissue engineering concepts advance tumor biology research? *Trends Biotechnol.* 28:125–133, 2010.
- Kelm, J. M., N. E. Timmins, C. J. Brown, M. Fussenegger, and L. K. Nielsen. Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types. *Biotechnol. Bioeng.* 83:173–180, 2003.
- Kenny, P. A., G. Y. Lee, C. A. Myers, R. M. Neve, J. R. Semeiks, *et al.* The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. *Mol. Oncol.* 1:84–96, 2007.
- Kerbel, R. S., B. St Croix, V. A. Florenes, and J. Rak. Induction and reversal of cell adhesion-dependent multicellular drug resistance in solid breast tumors. *Hum. Cell* 9:257–264, 1996.
- Kim, T., I. Doh, and Y. H. Cho. On-chip three-dimensional tumor spheroid formation and pump-less perfusion culture using gravity-driven cell aggregation and balanced droplet dispensing. *Biomicrofluidics* 6:34107, 2012.

- ²³Kobayashi, H., S. Man, C. H. Graham, S. J. Kapitan, B. A. Teicher, and R. S. Kerbel. Acquired multicellular-mediated resistance to alkylating agents in cancer. *PNAS* 90:3294–3298, 1993.
- ²⁴Lemmo, S., S. Nasrollahi, and H. Tavana. Aqueous biphasic cancer cell migration assay enables robust, high-throughput screening of anti-cancer compounds. *Biotechnol. J.* 9:426–434, 2014.
- ²⁵Mehta, G., A. Y. Hsiao, M. Ingram, G. D. Luker, and S. Takayama. Opportunities and challenges for use of tumor spheroids as models to test drug delivery and efficacy. *J. Control. Release* 164:192–204, 2012.
- ²⁶Mueller-Klieser, W. Three-dimensional cell cultures: from molecular mechanisms to clinical applications. *Am. J. Physiol.* 273:C1109–C1123, 1997.
- ²⁷Napolitano, A. P., D. M. Dean, A. J. Man, J. Youssef, D. N. Ho, *et al.* Scaffold-free three-dimensional cell culture utilizing micromolded nonadhesive hydrogels. *BioTechniques* 43(494):96–500, 2007.
- ²⁸Olive, P. L., and R. E. Durand. Drug and radiation resistance in spheroids: cell contact and kinetics. *Cancer Metast. Rev.* 13:121–138, 1994.
- ²⁹Petrak, D., E. Atefi, L. Yin, W. Chilian, and H. Tavana. Automated, spatio-temporally controlled cell microprinting with polymeric aqueous biphasic system. *Biotechnol. Bioeng.* 11:404–412, 2014.
- ³⁰Rivera, E., and H. Gomez. Chemotherapy resistance in metastatic breast cancer: the evolving role of ixabepilone. *Breast Cancer Res.* 12(Suppl 2):S2, 2010.
- ³¹Seano, G., G. Chiaverina, P. A. Gagliardi, L. di Blasio, R. Sessa, *et al.* Modeling human tumor angiogenesis in a three-dimensional culture system. *Blood* 121:e129–e137, 2013.
- ³²Silver, D. P., A. L. Richardson, A. C. Eklund, Z. C. Wang, Z. Szallasi, *et al.* Efficacy of neoadjuvant cisplatin in triple-negative breast cancer. *J. Clin. Oncol.* 28:1145–1153, 2010.
- ³³Souza, G. R., J. R. Molina, R. M. Raphael, M. G. Ozawa, D. J. Stark, *et al.* Three-dimensional tissue culture based on magnetic cell levitation. *Nat. Nanotechnol.* 5:291–296, 2010.
- ³⁴Sutherland, R. M., J. A. McCredie, and W. R. Inch. Growth of multicell spheroids in tissue culture as a model of nodular carcinomas. *J. Nat. Cancer Inst.* 46:113–120, 1971.
- ³⁵Tavana, H., A. Jovic, B. Mosadegh, Q. Y. Lee, X. Liu, *et al.* Nanolitre liquid patterning in aqueous environments for spatially defined reagent delivery to mammalian cells. *Nat. Mater.* 8:736–741, 2009.
- ³⁶Tavana, H., B. Mosadegh, and S. Takayama. Polymeric aqueous biphasic systems for non-contact cell printing on cells: engineering heterocellular embryonic stem cell niches. *Adv. Mater.* 22:2628–2631, 2010.
- ³⁷Tavana, H., B. Mosadegh, P. Zamankhan, J. B. Grotberg, and S. Takayama. Microprinted feeder cells guide embryonic stem cell fate. *Biotechnol. Bioeng.* 108:2509–2516, 2011.
- ³⁸Tavana, H., K. Kaylan, T. Bersano-Begey, K. E. Luker, G. D. Luker, and S. Takayama. Polymeric aqueous biphasic system rehydration facilitates high throughput cell exclusion patterning for cell migration studies. *Adv. Funct. Mater.* 21:2920–2926, 2011.
- ³⁹Tekin, H., M. Anaya, M. D. Brigham, C. Nauman, R. Langer, and A. Khademhosseini. Stimuli-responsive microcavities for formation and retrieval of cell aggregates. *Lab Chip* 10:2411–2418, 2010.
- ⁴⁰Thode, H. C. Testing for normality. New York: CRC Press, 2002.
- ⁴¹Torisawa, Y. S., B. Mosadegh, G. D. Luker, M. Morell, K. S. O’Shea, and S. Takayama. Microfluidic hydrodynamic cellular patterning for systematic formation of co-culture spheroids. *Integr. Biol.* 1:649–654, 2009.
- ⁴²Tung, Y. C., A. Y. Hsiao, S. G. Allen, Y. S. Torisawa, M. Ho, and S. Takayama. High-throughput 3D spheroid culture and drug testing using a 384 hanging drop array. *Analyst* 136:473–478, 2011.
- ⁴³Vinci, M., S. Gowan, F. Boxall, L. Patterson, M. Zimmermann, *et al.* Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. *BMC Biol.* 10:29, 2012.
- ⁴⁴Wartenberg, M., F. Donmez, F. C. Ling, H. Acker, J. Hescheler, and H. Sauer. Tumor-induced angiogenesis studied in confrontation cultures of multicellular tumor spheroids and embryoid bodies grown from pluripotent embryonic stem cells. *FASEB J.* 15:995–1005, 2001.
- ⁴⁵Wong, E. Y., and S. L. Diamond. Advancing microarray assembly with acoustic dispensing technology. *Anal. Chem.* 81:509–514, 2009.
- ⁴⁶Xie, J., D. W. Li, X. W. Chen, F. Wang, and P. Dong. Expression and significance of hypoxia-inducible factor-1alpha and MDR1/P-glycoprotein in laryngeal carcinoma tissue and hypoxic Hep-2 cells. *Oncol. Lett.* 6:232–238, 2013.
- ⁴⁷Xie, Z., L. Cao, and J. Zhang. miR-21 modulates paclitaxel sensitivity and hypoxia-inducible factor-1alpha expression in human ovarian cancer cells. *Oncol. Lett.* 6:795–800, 2013.
- ⁴⁸Yamada, K. M., and E. Cukierman. Modeling tissue morphogenesis and cancer in 3D. *Cell* 130:601–610, 2007.