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Tumor Spheroid Formation Using the I.DOT

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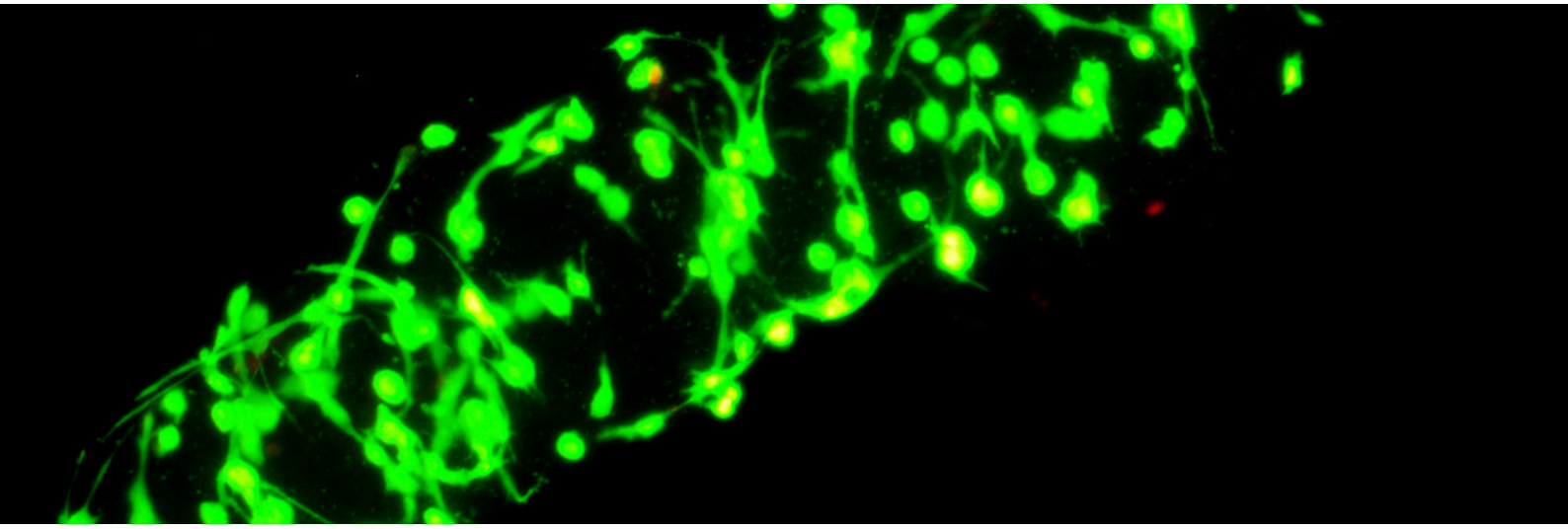

Abstract

This study used the I.DOT (Immediate Drop-on-demand Technology), a low-volume noncontact liquid dispensing device, in the liquid overlay technique (LOT) to form spheroids. LOT inhibits cell attachment to seeded surfaces in order for cells to form spheroids. There are several techniques for spheroid formation, but LOT has the potential to meet researchers' demand for a scalable and reproducible technique that results in spheroids with uniform shape and consistent size. In this study, we used the I.DOT to dispense human lung cancer cell line A549, which aggregated into spheroids after a few days. Cells were monitored from Day 0 to Day 14, and the diameter and area of the spheroids were observed. The results show that the I.DOT dispensed the target number of cells, which then formed cell clusters that were more uniform in shape than those formed when cells were dispensed using manual pipetting. In addition, the I.DOT system dispensed up to 10 times faster than manual pipetting.

Introduction

Although 2D monolayer cancer models have helped us gain understanding of carcinogenesis in vitro, researchers are starting to realize that there are limitations to studying a complex disease using flat models. Therefore, it is important to evaluate more accurate in vitro cancer models to gain knowledge efficiently and find alternative methods of treatment. 3D models mimic in vivo cell behavior more closely than 2D models, making drug screening assays more predictive (Zanoni et al., 2016).

Spheroid formation (Figure 1) is the most common liquid-based method for 3D cell culture. Multicellular tumor spheroids are formed either through self-assembly or forced to grow together from single cell suspensions (Yamada and Cukierman, 2007). Although there are multiple spheroid-forming techniques, a technique must be scalable and reproducible for preclinical drug screening (Costa et al., 2018).

LOT has been the most explored method due to its low cost, easy handling and high potential for scalability and reproducibility. It has the potential to inhibit cells from adhering to the super hydrophilic surface they have been seeded on (Costa et al., 2018; Carvalho et al., 2017). Cell-to-cell interactions are more prominent when cells are inhibited from interacting with the surface, enhancing the aggregation that leads to spheroid formation in 1 to 3 days for most cell lines (Costa et al., 2018).

This study sought to determine whether the I.DOT (Figure 2), a low-volume noncontact liquid dispensing system that uses automation and optimized protocols, could be combined with the LOT technique to reliably dispense into a target well plate droplets (nanoliters to several microliters) of A549 cells that then formed lung cancer spheroids with high throughput and volume reproducibility, while preserving cell viability.

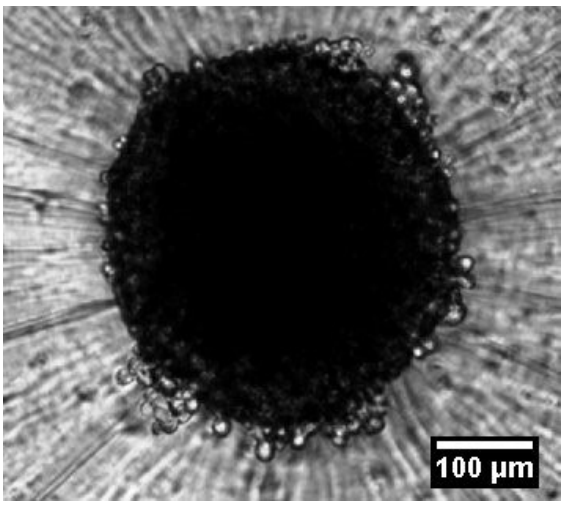

Figure 1. *Representative image of a A549 spheroid formed through liquid overlay technique (LOT). Scale bar 100 µm.*

Figure 2. *The I.DOT, a low-volume noncontact dispensing device, was used to perform the liquid overlay technique (LOT).*

Materials and Methods

Cell culture

Human lung cancer A549 (Figure 3) cell line was cultured in Rosewell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher, MA, U.S.) that contained 10% fetal bovine serum (Thermo Fisher, MA, U.S.), 100 U/mL penicillin, and 100 µg/mL streptomycin (Thermo Fisher, MA, U.S.) at 37°C in a 5% CO2 environment. Cells were cultured until confluency reached 85% ± 5% before being detached.

Cell detachment

Cell culture medium (RPMI) was removed, washed with phosphate-buffered saline solution (Thermo Fisher, MA, U.S.) and incubated with TrypLE (Thermo Fisher, MA, U.S.) at 37°C in a 5% CO2 environment for 5 minutes. RPMI medium was used to wash the flask to collect any lingering cells on the culture surface and neutralize the TrypLE. The cell solution containing RPMI medium and TrypLE was pipetted into a centrifuge tube and centrifuged for 3 minutes at 180 relative centrifugal force (RCF). The supernatant was removed, and the cell pellet was resuspended in RPMI medium. The cell density of the single-cell suspension was determined by combining 10 µL of the single-cell suspension and 10 µL Trypan Blue (Thermo Fisher, MA, U.S.), mixing thoroughly, loading 10 µL of the sample mixture into a Countess Cell Counting Chamber Slide (Thermo Fisher, MA, U.S.), and inserting the slide into the Countess II Automated Cell Counter (Thermo Fisher, MA, U.S.).

I.DOT calibration

The liquid class calibration option in the I.DOT Assay Studio software was used to calibrate the various liquids used in the study. When indicated, 20 µL solution was added to a source well, then the I.DOT (Dispendix, Germany) was prompted to dispense. The I.DOT calculated the droplet size based on the known volume, the pressure applied and the feedback from the sensor underneath the source well. Five data points were measured for one solution to calibrate the I.DOT. The liquids calibrated were RPMI medium and A549 cancer cells in RPMI medium.

A549 spheroid formation with the I.DOT

The I.DOT Studio Assay generated a protocol in which one source well with A549 cells in RPMI medium dispensed 30 µL each into two wells of a target 384 round bottom ultra-low attachment (ULA) microwell plate (Corning, NY, U.S.). The A549 single-cell suspension was prepared with a cell density of 40,000 cells/mL using RPMI medium. The single-cell suspension was loaded into the source well and the I.DOT was used to dispense into empty target wells in the ULA plate. For comparison, 30 µL of single-cell suspension was manually pipetted into other empty wells in the ULA plate using an Eppendorf Research Plus single-channel pipette (Eppendorf AG, Germany). The ULA plate was centrifuged at 180 RCF for 3 minutes and cultured for 2 weeks at 37°C in a 5% CO2 environment. Images were procured every day for each well with brightfield microscopy at 4 times magnification.

Measuring spheroid area

The acquired images from the spheroid formation experiments were analyzed using ImageJ to determine the diameter of the spheroids. The X and Y diameter of the A549 spheroids were measured on Day 4 and averaged to find the overall diameter. The line tool was used to draw a line across the spheroids in either the X-direction or Y-direction, and the measure tool was used to calculate the X and Y diameters of the spheroids in pixels. The diameters were converted into µm with the relationship 0.62 pixel equals to 1 µm. The overall diameter of a spheroid was calculated by averaging the X and Y diameters of spheroids. After the diameters were converted into µm, radii were calculated. The radii were then used to calculate the spheroid area.

Results and Discussion

Cell viability after I-DOT dispensing

Using the I.DOT, we demonstrated that cell viability was not greatly affected after dispensing. The mean cell viability for all the wells after I.DOT dispensing was 94% ± 5%, a representable image shown in Figure 4. The cell suspension placed in the source well had a cell viability of 96% ± 2%.

A549 spheroid formation with the I.DOT in comparison with manual pipetting

The I.DOT successfully dispensed A549 cancer cells in a round bottom 384-well ULA plate that lead to tumor spheroid formation. These spheroids were monitored every day from Day 0 to Day 14 to see if they would retain the spheroid shape (Figure 5). In comparison with manual pipetting, these spheroids were more compact and uniform in shape as seen in the images below (Figure 6). Using the I.DOT, it took 3 minutes 25 seconds to dispense 20 µL of medium with cells in a full 384-well plate, whereas it took approximately 32 minutes with manual pipetting.

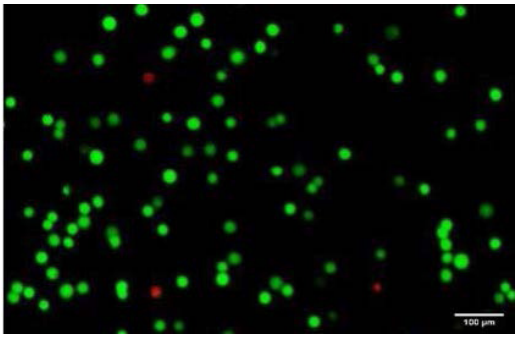

Figure 4. *Representative fluorescent image depicting viable cells (green) and non-viable cells (red). 10x magnification, scale bar 100 µm.*

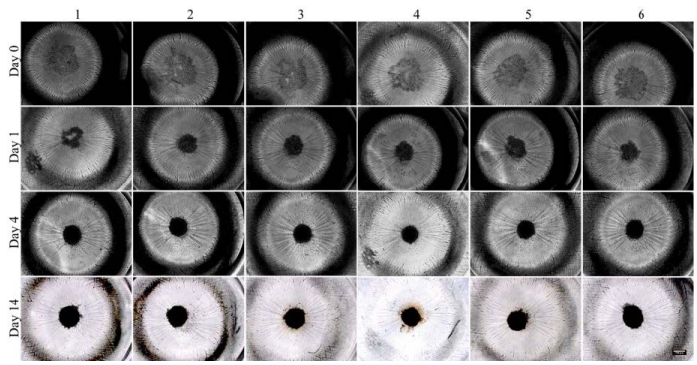

Figure 5. *Spheroid formation of A549 cells in 384-well ULA plate using the I-DOT. A549 cancer cells (6 replicates) were monitored every day from Day 0 to Day 14. Brightfield images of A549 cancer cells were taken after dispensing on Days 0, 1, 4 and 14. 4x magnification, scale bar 200 µm.*

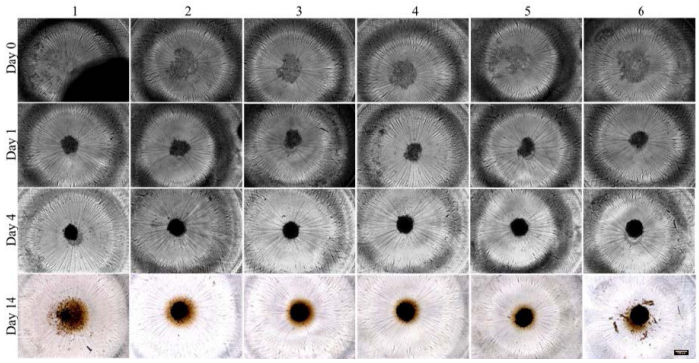

Figure 6. *Spheroid formation of A549 cells in 384-well ULA plate using manual pipetting. A549 cancer cells (6 replicates) were monitored every day from Day 0 to Day 14. Brightfield images of A549 cancer cells were taken after dispensing on Days 0, 1, 4 and 14. 4x magnification, scale bar 200 µm.*

Diameter and area of spheroids

A549 cancer cells were dispensed in 6 wells of a 384-well plate using the I.DOT and manual pipetting. After 4 days, the diameter and area of these spheroids were measured to see if these spheroids were uniform in shape across all wells. As shown (Table 1) below, the diameter and area of the spheroids measured on Day 4 were similar in shape and size across all wells using the I.DOT, whereas the manual pipetting was inconsistent.

Table 1. *Summary of the spheroid's diameter and area based on six replicates measured on Day 4.*

Replicates	A549 manually pipetted		A549 I-DOT dispensed	
	Diameter (µm)	Area (µm^2)	Diameter (µm)	Area (µm^2)
1	281	70 392	353	101 573
2	381	104 153	363	108 953
3	348	94 003	368	109 939
4	334	98 968	365	115 440
5	366	102 964	382	119 121
6	361	100 688	365	110 822
Average ± SD	345 ± 32	95 194 ± 12 662	366 ± 9	110 975 ± 5 998

Conclusion

This study provides many insights into the I.DOT's capability to be used for spheroid formation. In conclusion, our data supports that the I.DOT:

- can dispense cells with high cell viability, indicating that the automated dispensing does not affect cell viability.
- is approximately 10 times faster at dispensing compared to manual pipetting.
- can be used to form spheroids that are more uniform in size and shape than those formed with manual pipetting.

References

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