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APPLICATION NOTE

TECAN.

Automated spheroid production by magnetic cell assembly for screening applications

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1/ INTRODUCTION

Three-dimensional (3D) cell culture systems have emerged as valuable tools in biomedical research, offering closer mimicry of native tissue microenvironments compared to traditional two-dimensional (2D) cultures. 3D spheroids – compact aggregates of cells – have drawn particular attention for their ability to replicate in vivo cellular behaviors, including proliferation, differentiation, and genetic or metabolic responses to stimuli. As the use of these 3D models grows, there is an increasing need for methods that ensure the reproducibility, scalability and efficiency of screening assays. This requires methods that are compatible with automated liquid handling, using microplate geometries that enable accurate and reproducible imaging-based evaluation. Magnetic 3D cell culture (M3D) offers a way to address some of the current challenges in 3D cell culture, allowing quick and consistent generation of spheroids. This method involves magnetizing cells using nanoparticles that electrostatically adhere to the cell membrane, then placing them in cell-repellent microplates positioned on top of a magnet array to accelerate the formation of uniform-sized spheroids **(Figure 1)**.

This application note demonstrates how this approach can be automated using the Fluent Automation Workstation. To illustrate the efficacy of the resulting spheroids in compound screening, a D300e Digital Dispenser was used to create low volume concentration gradients across a microplate containing magnetized spheroids. The combination of the Fluent workstation, D300e dispenser, and M3D significantly accelerates the workflow, reducing time and material consumption. In addition, a Spark Cyto multimode reader was used to conduct imaging-based quality control throughout the growth phase – to ensure reliable screening outcomes – and to analyze dose-response effects, by performing luminescence-based cell viability assessment **(Figure 2)**.

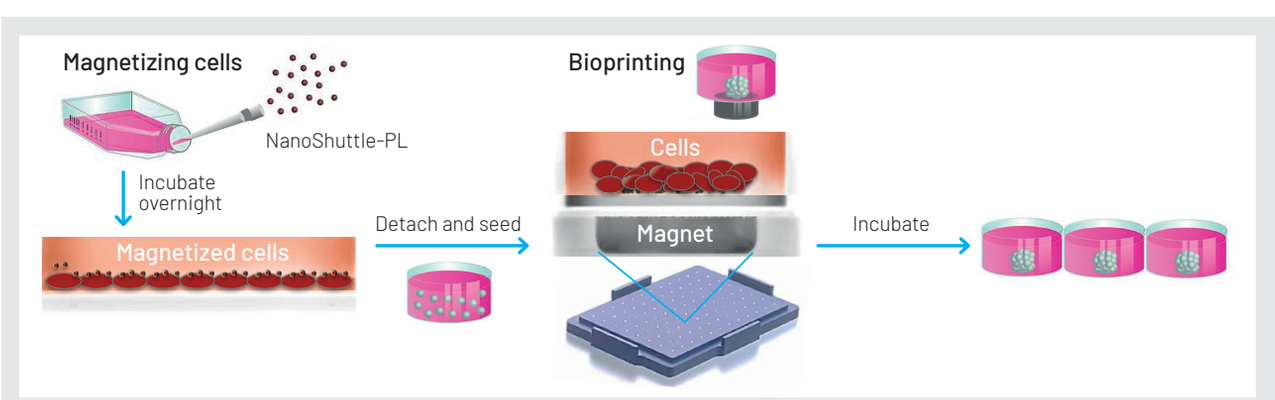

Figure 1: Magnetic 3D cell culture protocol. Cells are magnetized overnight with NanoShuttle, enzymatically detached, and resuspended in media before being distributed into a microplate. The microplate is then placed on top of a magnetic drive, which aggregates cells into spheroids at the bottom of the well within a few hours.[1,2]

Figure 2: Schematic overview illustrating a drug screening workflow using the Fluent Automation Workstation, D300e Digital Dispenser and Spark Cyto multimode reader.

2/ MATERIAL AND METHODS

2.1/ AUTOMATION WORKSTATION

Experiments were conducted on a Fluent 780 workstation with an integrated Multiple Channel Arm™ (MCA) and Robotic Gripper Arm™ (RGA) **(Figure 3A)**. The use of a MCA COMBO Adapter in 384-channel format allowed parallel pipetting of up to 125 µl per tip. An active carrier module was placed on the Dynamic Deck™, enabling pick-up of 384 tips at once.

A Cytomat™ 2 C450-LiN Automated Incubator (Thermo Fisher Scientific) with a water bath equipped with two 21-position stackers **(Figure 3B)** was integrated with the system, enabling the storage and incubation of cells in microplates throughout the workflow. The automated incubator was set to 37 °C and 5 % CO_2 to provide an optimal, stable environment for cell spheroid growth and maintenance. The ContraCon™ automated decontamination routine on the Cytomat 2 C450-LiN offers simplified cleaning and eliminates variability in disinfection without using harmful gases.

Figure 3: A Fluent Automation Workstation **(A)** was integrated with a Cytomat 2 C450-LiN Automated Incubator **(B)** to automate the preparation and storage of the spheroid microplate. The D300e Digital Dispenser **(C)** was used for drug dispensing, while imaging and measurement of the chemiluminescent signal were performed with a Spark Cyto 600 **(D)**.

The Fluent system was equipped with a vertical laminar flow HEPA hood with UV light (Bigneat) to ensure a clean environment for sterile liquid handling. Cell seeding was conducted with the MCA using 125 µl filtered disposable tips. The cell suspension was kept in sterile 300 ml troughs (Integra, #6327), before pipetting the cells into sterile, cell-repellent, flat-bottom, 384-well microplates (Greiner Bio-One, #781976). Scripts for cell seeding with the MCA, labware handling with the RGA, and incubation in the automated incubator were developed using FluentControl™ software. The D300e Digital Dispenser **(Figure 3C)** was used with T8+ Dispense-head cassettes to directly dispense drug stock solutions into wells containing the spheroids to generate the dose-response curve. Brightfield imaging and luminescence measurements were performed with the Spark Cyto 600 multimode reader **(Figure 3D)**.

2.2/ CELL CULTURE

HeLa cells (DSMZ, #ACC 57) were thawed at passage 12 following the manufacturer's protocol. Cells were seeded into T75 flasks at 500,000 cells/flask, and expanded in Dulbecco's modified Eagle's medium (DMEM), high glucose, proliferation media (Merck, #D6429) supplemented with 10 % fetal bovine serum (FBS; Gibco, #10270-106). Cells were maintained in a humidified environment (37 °C, 5 % CO_2), and were split once before use.

2.3/ CELL MAGNETIZATION AND HARVEST

Nanoshuttle-PL (Greiner Bio-One, #657852) for cell magnetization was added to a T75

flask at 70 % confluency. The culture media was removed, and 20 ml of fresh media was mixed with 1.2 ml Nanoshuttle reagent before it was added to the culture flask. After overnight incubation, the cells were harvested enzymatically with TrypLE™ Express Enzyme (Gibco, #12605010) and counted with a Vi-CELL™ XR cell counter (Beckman Coulter).

3/ SPHEROID FORMATION

3.1/ MANUAL

The cell seeding density for the M3D approach was optimized manually before automation on the Fluent. To achieve this, HeLa cells were seeded in 100 µl volumes in a flat bottom 96-microplate (Greiner Bio-One, #655976) in different concentrations – 2,000, 5,000, 10,000 and 15,000 cells per well – to identify suitable seeding densities. Cell numbers of 50, 100, 200, 1,000, 2,000 and 4,000 per well in 40 µl volumes were tested for the 384-well microplate (Greiner Bio-One, #781976). After cell seeding, the microplates were placed onto their respective magnetic drives (Greiner Bio One, #655830 and #781830) for 1 hour at room temperature, before being incubated at 37 °C, 5 % CO_2 and high humidity in a standard incubator.

3.2/ AUTOMATED

A flat-bottom 384-well microplate (Greiner Bio-One, #781976) was placed onto the matching magnetic drive, and the lid was removed using the RGA. The MCA was then used to seed all wells at once with 4,000 cells in 40 µl volume per well. After cell seeding, the lid was replaced, and the microplate was incubated for 1 hour at room temperature on the magnetic drive, before being transferred to the Cytomat 2 C450-LiN and incubated at 37 °C, 5 % CO_2, with relative humidity above 90 % for 24 hours to allow the spheroid formation.

3.3/ IMAGING

For manual cell density optimization, brightfield imaging with the 4x objective on the Spark Cyto was performed 24 hours after seeding in 96-well microplates. The spheroid sizes were determined by calculating the area with GraphPad Prism (n=22-24 spheroids per cell concentration). In addition, brightfield imaging using the 10x objective was performed 24 hours after automated seeding in 384-well microplates with the Fluent, to ensure successful spheroid formation before the drug addition.

3.4/ DRUG TREATMENT

Newly formed spheroids underwent compound treatment 24 hours after automated seeding. The D300e dispenser was placed into a standard laminar flow biosafety cabinet, and drug stock solutions (10 mM in DMSO) were applied to the assay plate with T8+ cassettes in different concentrations. Drug responses were assessed for a variety of drugs (all supplied by Selleckchem), including:

- **Gemcitabine (S1714)**
- **Doxorubicin (S1208)**
- **Topotecan (S1231)**
- **Panobinostat (S1030)**
- **Dexamethasone (S1322)**
- **Mitoxantrone (S2485)**
- **Idarubicin (S1228)**
- **Mitomycin (S8146)**
- **Vincristine (S1241).**

Duplicate testing of 10 different concentrations was performed for each compound, covering a logarithmically distributed range from 0.4 µM to 10 µM. Wells without any drug, and with 40 nl of pure DMSO – equivalent to the dispensed volume of the highest drug concentrations – were also created as controls.

3.5/ CELL VIABILITY ASSESSMENT

The CellTiter-Glo® 3D Cell Viability Assay (Promega, #G9681) was used to assess the spheroid viability in the 384-well microplate, 72 hours after drug treatment. The microplate and reagents were equilibrated to room temperature for 30 minutes, then 40 µl of CellTiter-Glo 3D reagent was added to each well before the microplate was placed on an orbital plate shaker at 1000 rpm for 5 min to facilitate cell lysis. The microplate was then transferred to the Spark Cyto for 25 minutes before measurement to allow the signal to stabilize. The luminescent signals were measured with an integration time of 100 ms, using the automatic OD filter to attenuate high intensity light.

3.6/ ANALYSIS

Area calculation for spheroid size determination (n=22-24 spheroids per condition), as well as analysis and plotting drug response data, was conducted using GraphPad Prism. A 4-point non-linear regression curve was fitted to the data points, and IC50 values were calculated. The goodness of fit of the curve was assessed by determining the R2 values for each compound. The Z' prime factor was calculated using the formula

$$Z' = 1 - \frac{3x\,(SD_{positive} + SD_{negative})}{[\,mean_{positive} - mean_{negative}\,]}$$

where SD represents the standard deviation of the positive and negative controls.

4/ RESULTS

4.1/ MANUAL

Imaging 24 hours after seeding confirmed the formation of single, compact and uniformly sized HeLa spheroids in flat-bottom 96-well microplates for all tested seeding concentrations **(Figure 4)**. Between 22 and 24 spheroids were acquired for each condition, all of which could be captured within a single field of view using the 4x objective of the Spark Cyto.

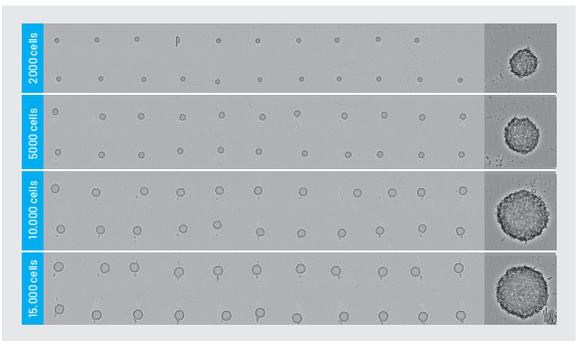

Figure 4: Brightfield imaging (4x) showed the formation of compact spheroids 24 hours after seeding for all cell concentrations.

The size of each spheroid was quantified, revealing uniform spheroid sizes within each category and a strong correlation between cell concentration and occupied area per spheroid **(Figure 5)**, as demonstrated by the narrow error bars.

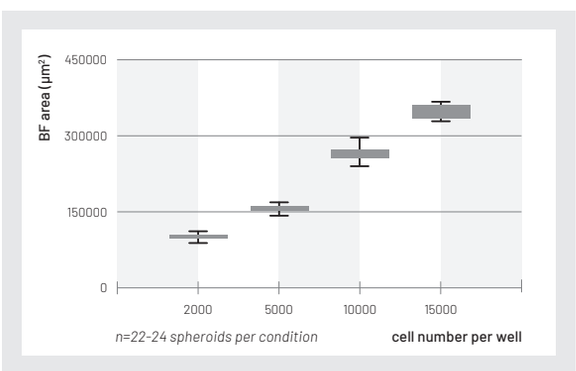

Figure 5: Variation in HeLa spheroid size distribution based on the initial seeding concentration.

The most uniform and tightly packed spheroids in the 384-well microplate resulted from initial concentrations of 2,000 and 4,000 cells per well (data not shown). A seeding concentration of 4,000 cells per well was therefore selected for automated seeding in 384-well microplates.

4.2/ AUTOMATED

An entire 384-well microplate could be simultaneously seeded with HeLa cells in less than one minute with the MCA. Before the drug treatment, brightfield imaging was performed with the Spark Cyto to confirm the formation of spheroids **(Figure 6).**

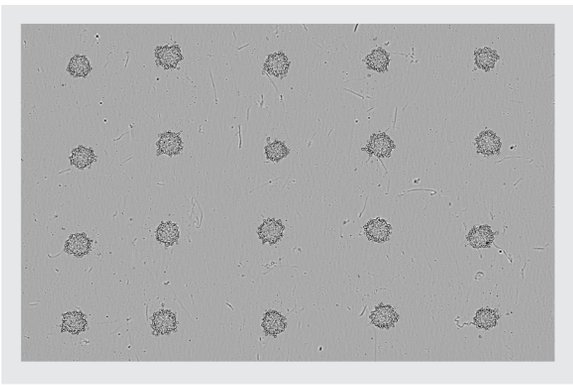

Figure 6: Stitched brightfield images of spheroids in 384-well microplates 24 h after cell seeding, acquired with the 10x objective of the Spark Cyto.

The viability assay identified significant effects on HeLa spheroid viability for most of the compounds tested, including vincristine, staurosporine, and panobinostat **(Figure 7A)**.

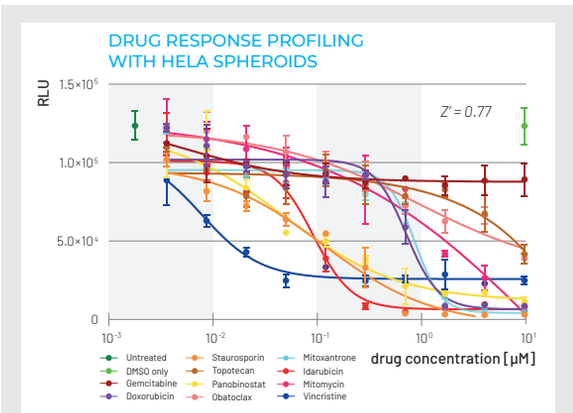

Figure 7A: Drug effects on HeLa spheroid viability 3 days after compound addition, determined by the CellTiter-Glo 3D viability assay. 10 different concentrations were tested in duplicate for each compound. Untreated and DMSO only controls are shown in green.

In contrast, notable tolerance was observed to even elevated concentrations of gemcitabine, topotecan, and obatoclax, as evidenced by elevated or indeterminable IC50 values **(Figure 7B)**.

Drug	IC_{50} [µM]	R^2
Gemcitabine	na	na
Doxorubicin	0.78	0.94
Staurosporine	0.11	0.96
Topotecan	na	0.74
Panobinostat	0.06	0.94
Obatoclax	2.42	0.92
Mitoxantrone	0.92	0.93
Idarubicin	0.10	0.95
Mitomycin	0.92	0.94
Vincristine	0.01	0.93

Figure 7B: Table summarizing IC50 and R2 values.

R2 values above 0.9 were calculated for all relevant compounds, indicating that the linear regression model fits the data well. As expected, untreated and spheroids treated with solvent only (DMSO) showed the highest cell viabilities. A Z' factor of 0.77 further indicates a robust assay with a high level of differentiation between the positive and negative controls.

5/ CONCLUSION

This application note presents an automated workflow for M3D cell culture, using the Fluent workstation to produce uniformly sized spheroids in flat-bottomed microplates suitable for downstream high throughput screening applications. Imaging-based evaluation using the Spark Cyto allowed growth monitoring and viability assessment to be performed with a single device, improving data acquisition efficiency and increasing the meaningfulness of the data through multiplexing. The use of a 384-well microplate expanded throughput, allowing simultaneous analysis of a larger number of samples while reducing time and material consumption. Combining controlled spheroid formation with automated liquid handling and high quality imaging enhances both the throughput and reproducibility of drug screening workflows using 3D cell cultures, with applications in drug discovery and biomedical research.

6/ REFERENCES

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