High-Throughput, 3D Assay Development on Spheroids in 1536 Well Plates

Introduction

3D cell models are often a better representation of the in vivo environment than traditional 2D methodologies. Accordingly, spheroid and other 3D models are increasingly being used for drug discovery. A high throughput system is essential to meet the 3D cell screening and 3D cell culture demands of most largescale drug discovery programs. An automated method has been established for seeding, dosing and assaying spheroids in Corning 1536-well spheroid microplates using INTEGRA Biosciences' VIAFILL reagent dispenser and VIAFLO 384 handheld electronic pipette. With a maximum volume of 14 µl per well, accurate liquid handling is essential for the generation of quality data. The method was proven to generate robust, high quality data for assay development and high throughput screenings.

Key benefits:

- The Corning 1536-well spheroid microplate can be used to generate 1536 uniform, single spheroids that can be assayed by imaging, fluorescence or luminescence measurements directly in the microplate.
- The VIAFILL is designed for rapid bulk liquid dispensing of volumes as low as 0.5 µl. It can precisely seed cells in a 1536 well microplate in less than 1 minute using the 16 channel dispensing cassette.
- Pairing the VIAFILL with a plate stacker increases the system capacity to up to 50 plates in a row.
- The adjustable spring loaded plate holders with slide function and 1536 well offset offer optimal alignment of the VIAFLO 384 pipetting head with the target 1536 well plate.
- The 12.5 µl SHORT GripTip pipette tips are specifically designed for 1536 well pipetting, and enable precise targeting of small wells.
- Setting the Tip Align strength to 3 and using the VIAFLO 384's z height function makes it easy and accurate to pipette into a 1536 well plate.

Step-by-step procedure:

Experimental set-up

DU 145 (ATCC[®] HTB-81[™]) and PANC-1 (ATCC CRL-1469[™]) cells were routinely cultured in DMEM containing 10 % FBS. Cells were seeded into Corning 1536-well spheroid microplates at 1000 cells per well, in a volume of 5 µl per well, using the VIAFILL with the 16 channel dispensing cassette. The pipetting steps were performed using a VIAFLO 384 together with a 384 pipetting head (0.5-12.5 µl), an adjustable spring loaded plate holder with 1536 offset, and 12.5 µl SHORT, Sterile, Filter GripTips.

Corning 1536-well Spheroid Microplate



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The experiment is divided into three steps.

Overview of the steps:

Step 1: Spheroid formationStep 2: Homogenous cell-based assayStep 3: High-content imaging assay

1. Spheroid formation

STEP: Cell seeding into the Corning 1536-well spheroid microplates using the VIAFILL reagent dispenser. **HOW TO:** Accurate handling of the cell suspension during seeding is critical to ensure that the spheroids are of uniform size across the entire microplate. For rapid cell seeding, use the VIAFILL reagent dispenser.

Install the 16 channel dispensing cassette, and place the Corning 1536-well Spheroid Microplate on the plate sledge of the VIAFILL reagent dispenser. If using the VIAFILL with a 16 channel dispensing cassette and a 1536 well plate for the first time, adjust the stage alignment and save the settings. The stage alignment only has to be done once. Use the Repeat Dispense function of the VIAFILL to quickly fill the wells at 1000 cells per well, in a volume of 5 μ I per well, with a touch off. Incubate the plates overnight at 37 °C in a humidified CO2 incubator to allow for spheroid formation.

Experimental results (Corning) showed the formation of uniform, single spheroids in each well across an entire microplate (**Figure 1**).

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Figure 1: Uniform, single spheroids formed in each well across the entire spheroid microplate. Representative image of single uniform DU 145 spheroids formed in the Corning 1536-well spheroid microplate with one well digitally zoomed in. Images were taken with Thermo Fisher CellInsight CX7 high-content screening platform using a 4X objective.

Tips	
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- Remove the dispensing cassette crash guards to dispense the cells into the 1536 well plate with a touch off.
- Using the VIAFILL reagent dispenser allows accurate, automatic seeding in less than 1 minute per plate.
- The VIAFILL can be paired with the plate stacker for an increased capacity of up to 50 plates at a time.

2. Homogenous cell-based assay

STEP: Spheroid treatment with staurosporine to assess the uniformity of signal, signalto-background ratio (S/B) and calculated Z' values, and demonstrate the utility of this experimental design for 3D cell screening. **HOW TO:** Uniformity of signal was demonstrated by treating sections of the microplate with a single concentration of buffer (control) or staurosporine to induce cell death. Spheroid viability was assessed with a homogenous luminescence cell viability assay.

After DU 145 spheroid formation, dispense 1 μ l of 6 μ M staurosporine or buffer into each well. Use the VIAFLO 384 with a 384 channel pipetting head (0.5-12.5 μ l) and 12.5 μ l SHORT Sterile, Filter GripTips. Insert the Corning 1536-well Spheroid Microplate into an adjustable spring loaded plate holder B with 1536 offset placed on **Position B**. If using for the first time, adjust the plate holder to the Corning 1536-well Spheroid Microplate for a perfect tip alignment. Set the Tip Align strength to 3 to easily and rapidly access the center of the tiny wells of the 1536 well microplate. Use the slide function of the plate holder to move the 1536 well plate and access all four quadrants with the 384 channel pipetting head. Incubate the plates overnight at 37 °C in a humidified CO₂ incubator.

The next day, transfer 6 µl of CellTiter-Glo[®] 3D reagent from a 150 ml automation friendly reagent reservoir placed on **Position A** to each well, and incubate at room temperature for 1 hour. The luminescence signal is then read using a microplate reader (Tecan Infinite[®] M200).

Corning's experimental results showed good signal uniformity regardless of a well's location in the microplate (**Figure 2**). The mean S/B ratio from this assay was greater than 20 for 3 independent studies (**Figure 3 A**). Additionally, Z' values were consistently above 0.7, indicating the robustness of the viability assay in the Corning 1536-well Spheroid Microplate (**Figure 3 B**).



Figure 2: Uniform signal across Corning spheroid microplate. Representative image of color-scaled luminescence signal generated from an assay where DU 145 spheroids were exposed to a final concentration of buffer (green) or 1 µM staurosporine (red). Green corresponds to high luminescence values, high viability. Red corresponds to low luminescence values, low viability.



Figure 3: Large, robust luminescence assay window. (A) Mean \pm SD luminescence of DU 145 spheroids exposed to buffer or 1 μ M staurosporine, resulting in S/B of 20.2. p<0.0001 with unpaired t-test. (B) Excellent Z' values above 0.7 in 3 independent studies. N = 2304 wells in 3 independent studies. (A) Mean \pm SD luminescence of DU 145 spheroids exposed to buffer or 1 μ M staurosporine, resulting in S/B of 20.2. p<0.0001 with unpaired t-test. (B) Excellent Z' values above 0.7 in three independent studies. N = 2304 wells in S/B of 20.2. p<0.0001 with unpaired t-test. (B) Excellent Z' values above 0.7 in three independent studies. N = 2304 wells.

		 Tips: For optimal alignment of the pipetting head with the target 1536 well plate, the spring loaded plate holder with 1536 offset is adjustable forwards and backwards. The easy-to-use slide function of the plate holder shifts the 1536 well plate in order to access all four quadrants with a 384 channel pipetting head. 12.5 μl SHORT GripTips have been developed to enhance 1536 well pipetting performance. These tips are specifically designed for 1536 well pipetting, and enable precise targeting of small wells. They are available in both standard and Low Retention Sterile, Filter versions. By setting the Tip Align strength of the VIAFLO 384 to 3, pipetting into the center of the tiny wells of a 1536 well plate is as easy and accurate as pipetting into a reagent reservoir. By defining the z heights of the VIAFLO 384, you can ensure optimal tip immersion that prevents accidental aspiration, scratching of the pipette tips on the bottom of the reservoir or damage to the spheroids.
3. High-content imaging assay	STEP: Proof of concept assay. Addition of cisplatin to assess the cytotoxicity.	HOW TO: Use Position A for the 150 ml automation friendly reagent reservoirs needed for this step and Position B for the Corning 1536-well spheroid microplates placed in the adjustable spring loaded plate holder B with 1536 offset.
		After formation, both DU 145 and PANC-1 spheroids were exposed to various concentrations of cisplatin by adding 1 μ I of solution to each well using the VIAFLO 384 with 12.5 μ I SHORT Sterile, Filter GripTips, followed by overnight incubation.
		To assess cytotoxicity, 6 µl per well of 40 µg/ml Hoechst 34580 and 16 µg/ml propidium iodide (PI) in PBS were added to each well for 1 hour. Microplates were than imaged on the Thermo Fisher CellInsight high-content screening platform.
		Representative images were captured directly without the need to transfer the spheroids to another microplate, demonstrating the utility of culture and subsequent imaging of spheroids in the spheroid microplate (Figure 4). Image analysis of propidium iodide staining revealed typical concentration-response curves for cisplatin cytotoxicity (Figure 5).



Figure 4: Confocal imaging of spheroids within Corning 1536-well Spheroid Microplates. Representative z-stack images of DU 145 (top) and PANC-1 (bottom) spheroids exposed to 0, 0.025 and 83 mM cisplatin (left, middle, right, respectively) for 24 hours. Spheroids were stained with Hoechst (blue) and PI (red) to assess cell viability. Spheroids were imaged directly in the spheroid microplate with the Thermo Fisher CellInsight CX7 high-content screening platform using a 10x objective. Scale bar = 100 µm.



Cisplatin Cytotoxicity

Figure 5: Concentration-dependent cisplatin cytotoxicity. Mean \pm SD PI fluorescence of DU 145 and PANC-1 spheroids following 24 hour exposure to varying concentrations of cisplatin. N = 96 wells.

Tips:

- When using the Repeat Dispense mode of the VIAFLO 384, define a pre- and post-dispense volume to be discarded. These two dispenses contain the largest volume errors. It is especially important to discard the last dispense as it contains the accumulated error of all previous dispenses.
- The SureFlo[™] array located on the flat bottom of the automation friendly reagent reservoir prevents pipette tip seal off. This allows users to rest the pipette tips directly on the bottom of the reservoir, offering an incredibly low dead volume of less than 3 ml.

Conclusion

- Using the VIAFILL and VIAFLO 384 instruments for liquid handling in Corning 1536-well spheroid microplates generated robust quality data for assay development and high throughput screens.
- Corning 1536-well spheroid microplates allow for the formation of uniform, single spheroids in each well that are ideally suited for high throughput 3D drug screening. The black sidewalls and clear bottom design of these microplates are ideal for imaging, luminescence and fluorescence based cellular assays.
- The combination of the VIAFILL and VIAFLO 384 instruments with the Corning 1536-well Spheroid Microplate resulted in good signal-to-background ratios, with excellent and consistent *z*' values.
- The VIAFILL reagent dispenser with the 16 channel dispensing cassette allows accurate, automatic plate seeding in less than 1 minute per plate. For high throughput, it can be paired with a plate stacker to automatically fill up to 50 plates.
- The VIAFLO 384 handheld electronic pipette, together with the 384 channel pipetting head (0.5-12.5 μ I), adjustable spring loaded plate holder with 1536 offset, and 12.5 μ I SHORT GripTips allows easy, accurate and rapid pipetting into 1536 well microplates, even those containing spheroids.

Materials

Manufacturer	Part Number	Description	Link
INTEGRA Biosciences	5600	VIAFILL reagent dispenser	https://www.integra-biosciences.com/global/en/ microplate-dispensers/viafill#parts-and-numbers
INTEGRA Biosciences	5742	16 channel dispensing cassette, small bore	https://www.integra-biosciences.com/global/en/ microplate-dispensers/viafill
INTEGRA Biosciences	6031	VIAFLO 384 handheld electronic pipette (base unit)	https://www.integra-biosciences.com/global/en/ electronic-pipettes/viaflo-96384#parts-and-numbers
INTEGRA Biosciences	6131	384 channel pipetting head 12.5 μl	https://www.integra-biosciences.com/global/en/ electronic-pipettes/viaflo-96384
INTEGRA Biosciences	6225, 6226	Adjustable spring loaded plate holder A/B for 24, 96, 384 and 1536 well plates, with slide function (1536 offset)	https://www.integra-biosciences.com/global/en/ electronic-pipettes/viaflo-96384#parts-and-numbers

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Manufacturer	Part Number	Description	Link
INTEGRA Biosciences	6475	12.5 µl SHORT, Sterile, Filter GripTips	https://www.integra-biosciences.com/global/en/ griptip-selector-guide
INTEGRA Biosciences	6303, 6317, 6318	150 ml automation friendly reagent reservoir	https://www.integra-biosciences.com/global/en/ reagent-reservoirs/automation-friendly-reagent- reservoirs
Corning	4527, 4637	Corning 1536-well Black/ Clear Round Bottom Ultra-low Attachment Spheroid Microplate, with Lid, Sterile	https://ecatalog.corning.com/life-sciences/b2c/ US/en/Microplates/Assay-Microplates/1536-Well- Microplates/Corning%C2%AE-1536-well-Spheroid- Microplates/p/4527
Millipore Sigma	569397	InSolution Staurosporine, Streptomyces sp.	https://www.sigmaaldrich.com/catalog/product/ mm/569396?lang=en®ion=US
Promega	G9683	CellTiter-Glo 3D Cell Viability Assay	https://www.promega.com/products/cell-health- assays/cell-viability-and-cytotoxicity-assays/celltiter_ glo-3d-cell-viability-assay/?catNum=G9681&cs=y
Millipore Sigma	1134357	Cisplatin	https://www.sigmaaldrich.com/catalog/product/ usp/1134357?lang=en®ion=US
Thermo Fisher Scientific	H21486	Hoechst 34580	https://www.thermofisher.com/order/catalog/product/ H21486#/H21486
AnaSpec	AS-83215	Propidium iodide, 1.0 mg/ml solution in water	https://www.anaspec.com/products/product. asp?id=29637
Corning	21-030-CM	Corning Dulbecco's Phosphate- Buffered Saline, 1X with calcium and magnesium	https://ecatalog.corning.com/life-sciences/ b2c/US/en/Media,-Sera,-and-Reagents/ Buffered-Salt-Solutions/Dulbecco's-Phosphate- Buffered-Saline-(DPBS)/Corning%C2%AE- DPBS-(Dulbecco%E2%80%99s-Phosphate- Buffered-Saline)/p/21-030-CM

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