TECHNICAL NOTE

Automated high-content assay using GrowDex-embedded spheroids dispensed on JANUS G3 automated liquid handling workstation.

Key features

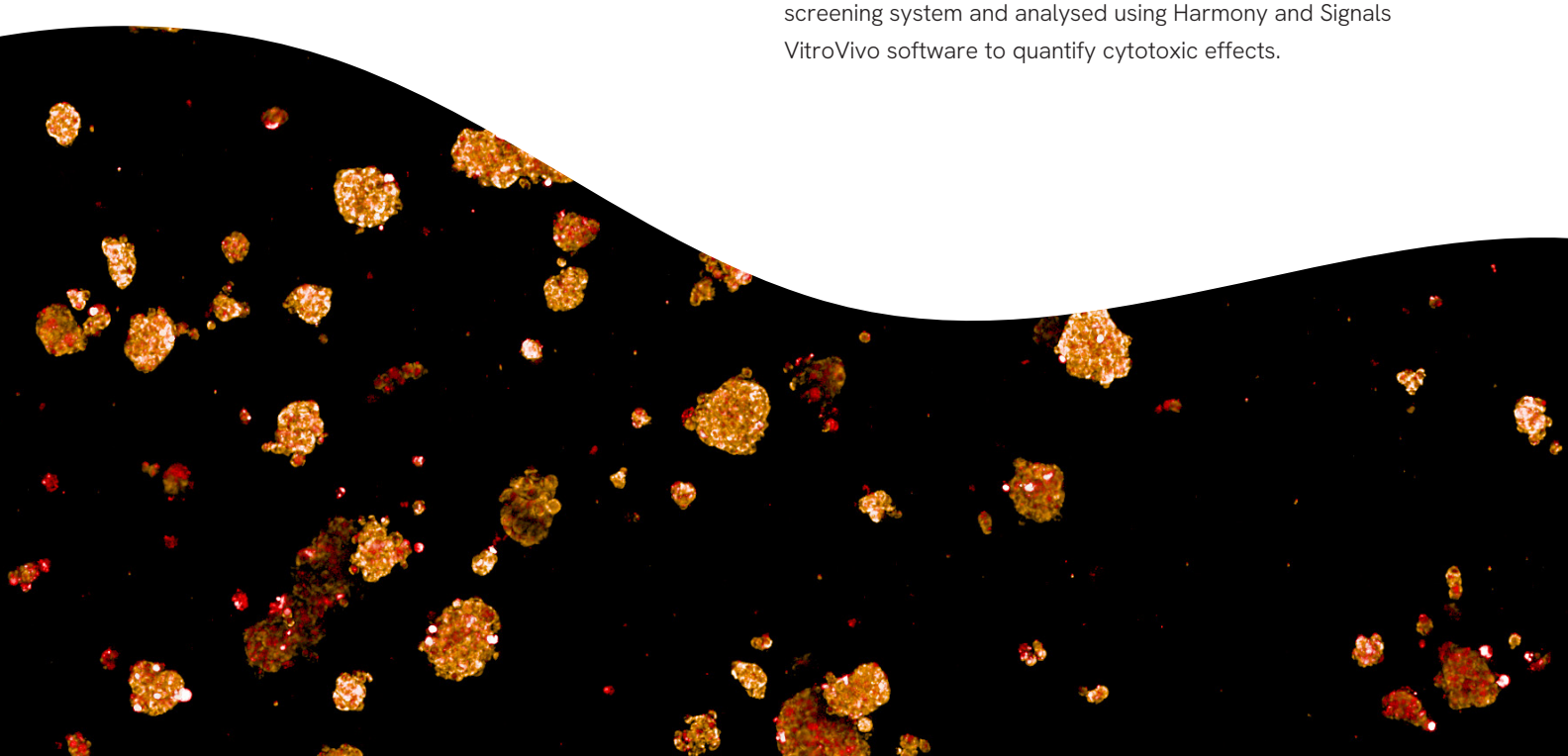
- Automated liquid handling of GrowDex® using the JANUS® G3 automated liquid handling workstation.
- Automated high-content toxicity assay with GrowDex-embedded HepG2 spheroids.
- Room temperature compatible, animal-free hydrogel for homogenous, scalable, and reproducible spheroid growth.

Introduction

Physiologically more relevant 3D cell models such as spheroids and hydrogel-embedded organoids have been successfully established in recent years, resulting in the demand to robustly automate and scale up 3D assays for high throughput drug discovery.

A typical challenge when working with 3D cell models is high heterogeneity between samples. Heterogeneity can be either biological heterogeneity, which is caused by the cells used to build the 3D model, or technical heterogeneity. This may be caused by variations in media compositions, growth factors, hydrogel batch-to-batch variations, pipetting variations, or human error. Automated liquid handling helps to reduce some of this technical heterogeneity as it reliably pipets the defined volumes. It also provides defined pipetting speed and heights, thereby avoiding losing hydrogel during the multiple pipetting steps or lifting the hydrogel off from the plate bottom.

Here, we demonstrate how the JANUS G3 liquid handling workstation can be used to improve throughput as well as assay statistics by automating the liquid handling steps of a 384-well, hydrogel-based high-content toxicity assay. For this, HepG2 spheroids were grown in automation compatible GrowDex hydrogel made of nanofibrillar cellulose and treated with 3 different compounds known to affect cell health. Changes in the mitochondrial membrane potential were analyzed to assess cell health. Fluorescently stained, GrowDex-embedded HepG2 spheroids were imaged on an Opera Phenix® Plus high-content screening system and analysed using Harmony and Signals VitroVivo software to quantify cytotoxic effects.



Materials and methods

Table 1: List of materials, instruments, and software.

Cells	HepG2 cells (hepatocellular carcinoma cells)
Growth medium	DMEM high glucose w/o phenol red (Capricorn Scientific, # HXRXA) + 10% FCS (Sigma Aldrich, # F9665) + 2 mM L-Glutamine (Capricorn Scientific, # GLN-B) + 1x penicillin/streptomycin (Capricorn Scientific, # PS-B)
GrowDex	1.5% GrowDex® (Revvity, # 100103005)
Microplates	PhenoPlate™ 384-well ULA coated (Revvity, # 6057800) StorPlate-384 deep well, V-bottom, 384-well polypropylene plate (Revvity, # 6008690)
Automated liquid handling platform	JANUS® G3 automated liquid handling workstation with I30/384 MDT disposable tip head (Revvity) Disposable 30 µL filter tips, sterile (Revvity, # 6001620)
Compounds	Acetaminophen (Sigma-Aldrich, # A5000) FCCP (Sigma-Aldrich, # C2920) DMSO (Sigma-Aldrich, # D2650)
Mitochondrial and nuclear stain	PhenoVue™ 551 mitochondrial stain (Revvity, # CP301) PhenoVue™ DRAQ5™ (Revvity, # CP163)
Image acquisition and analysis	Opera Phenix® Plus high content screening system (Revvity, # HH14001000) Harmony® high-content imaging and analysis software (Revvity)
Secondary analysis	Signals™ VitroVivo (Revvity Signals)

Cell culture

HepG2 cells were cultured in DMEM high glucose medium w/o phenol red supplemented with 10% FCS, 2 mM L-glutamine and 1x concentrated penicillin/streptomycin in a humidified atmosphere at 37 °C and 5% CO_2. Sub-confluent cells were trypsinized, counted and the required amount embedded in GrowDex hydrogel.

Embedding HepG2 cells in GrowDex hydrogel

To seed 3000 HepG2 cells in 25 µL 0.5% GrowDex hydrogel per 384-plate-well, first 14 mL of 1.5% GrowDex stock were diluted and mixed with 24.5 mL growth medium according to GrowDex instructions under a sterile workbench. Then, 3.5 mL of a 1.44 E6/mL HepG2 cell suspension were gently suspended into this mix to reach a cell concentration of 1.2 E5/mL and a final volume of 42 mL GrowDex-cell mix.

Automated dispensing of GrowDex-cell-mix into 384 well plates

42 mL of GrowDex-cell-medium suspension were transferred into a sterile, single well, deep well reservoir and placed onto a HEPA-enclosed JANUS G3 automated workstation equipped with a 384-MDT pipetting module I30 head, fitted with 30 µL sterile filter tips. 25 µL of GrowDex-cell-mix were dispensed into three PhenoPlate 384-well ULA coated microplates according to layout (Figure 1): 180 wells per 384-well plate were filled with GrowDex-cell-suspension. The speed for aspiration and dispensing was set to 5 µL/s for this and all following steps.

Next, all 384 wells were overlayed with a total of 50 µL growth medium by transferring 2x 25 µL from a growth medium-filled StorPlate-384 deep well V-bottom polypropylene plate using the “tip-touch” mode. With this mode, the gel surface remains untouched: medium is dispensed in reverse pipetting mode 3 mm above the well bottom followed by a short movement of the tip to contact the side wall of the wells thereby completely releasing the liquid into the well. This way, potential assay variation due to satellite drop formation is reduced. Empty wells were filled with medium as well to minimize evaporation effects.

Contact-free reverse pipetting and tip-touch mode were used for all following liquid additions as well.

384 well plates were placed into a cell culture incubator at 37 °C and 5% CO_2 for 72 hours to grow HepG2 spheroids.

Dispensing compound dilutions

After 72 hours incubation time, 2x 25 µL medium were aspirated from all GrowDex-filled wells in a safe height of 2 mm above the well bottom using the JANUS G3 I30/384 MDT workstation. 25 µL of 2x concentrated compound dilutions (diluted in growth medium) were then dispensed from a compound-filled StorPlate-384 deep well plate according to layout (Figure 1). Plates were centrifuged for 4 minutes at 400x g and placed back into the cell culture incubator for another 72 hours.

Automated addition of fluorescent staining mix

25 µL of compound dilution were aspirated on the JANUS G3 workstation followed by adding 25 µL of a 2x concentrated staining mix containing 300 nM PhenoVue 551 mitochondrial stain and 2 µM PhenoVue DRAQ5 from a pre-filled StorPlate-384 deep well plate into spheroid-containing wells. Plates were centrifuged for 4 minutes at 400x g and placed back into the cell culture incubator for 30 minutes.

Image acquisition and analysis

Images of PhenoVue 551 mitochondrial stain and PhenoVue DRAQ5 channels were captured on the Opera Phenix Plus high-content screening system equilibrated to 37 °C and 5% CO_2 using a 10x objective in confocal mode. 4 fields of view (FOV) with 2% overlap and a stack with a z-distance of 20 µm were acquired. The number of planes were 25 covering an overall height of 480 µm.

Image analysis was performed using the Harmony high-content imaging and analysis software: A maximum intensity projection of a montage of the 4 FOVs (called global image) was created from all planes. The PhenoVue DRAQ5 channel was used to segment the spheroids, after which the intensity of the PhenoVue 551 mitochondrial stain was quantified within segmented spheroids.

Downstream analysis

Using Signals™ VitroVivo software, from each plate the mean fluorescence intensities per well and compound concentration were plotted against the compound concentration and normalized to the intensity of the respective control. IC_{50} and R^2 were calculated based on the normalized data.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								
B				DMSO [%]		Acetaminophen [mM]			FCCP [µM]						DMSO [%]		Acetaminophen [mM]			FCCP [µM]				
C				0	0	0	0	0	0	0	0				0	0	0	0	0	0	0	0		
D				0	0	0	0	0	0	0	0				0	0	0	0	0	0	0	0		
E				0.1	3	0.03	0.03	0.03	0.03	0.03	0.03				0.1	3	0.03	0.03	0.03	0.03	0.03	0.03		
F				0.1	3	0.1	0.1	0.1	0.1	0.1	0,1				0.1	3	0.1	0.1	0.1	0.1	0.1	0,1		
G				0.1	5	0.3	0.3	0.3	0.3	0.3	0.3				0.1	5	0.3	0.3	0.3	0.3	0.3	0.3		
H				0.3	5	1	1	1	1	1	1				0.3	5	1	1	1	1	1	1		
I				0.3	5	3	3	3	3	3	3				0.3	5	3	3	3	3	3	3		
J				0.3	10	10	10	10	10	10	10				0.3	10	10	10	10	10	10	10		
K				1	10	30	30	30	30	30	30				1	10	30	30	30	30	30	30		
L				1	10	100	100	100	100	100	100				1	10	100	100	100	100	100	100		
M				1	0	-	-	-	300	300	300				1	0	-	-	-	300	300	300		
N				3	0	-	-	-	500	500	500				3	0	-	-	-	500	500	500		
O																								
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Figure 1: Layout of dose-response titration for 3 independent 384-well plates: a total of 180 wells per 384-well plate were filled with 3000 HepG2 cells per well in 0.5% GrowDex in a duplicated layout. Compound treatment started on day 3 post seeding: 6 concentrations of DMSO, 8 of Acetaminophen and 10 concentrations of FFCP were applied onto the spheroids in 6 repetitions per concentration (n=6) on each plate.

Results and discussion

Using the workflow shown in Figure 2, we were able to automate the liquid handling steps of a 384-well high-content toxicity assay with GrowDex-embedded, 3D-distributed HepG2-spheroids. In total, 3 plates with GrowDex embedded HepG2 spheroids were generated.

After the initial seeding, the plates were incubated for 3 days in a cell culture incubator followed by a 3-day incubation with different concentrations of DMSO, Acetaminophen or FCCP. Compounds were administered to 6 wells per concentration and plate. 30 minutes before imaging, spheroids were stained with PhenoVue 551 mitochondrial stain and PhenoVue DRAQ5. Images were acquired on an Opera Phenix Plus. Example images of the dose response treatments are shown in Figure 3, showing a decrease in the PhenoVue 551 mitochondrial stain (orange) intensity with increasing compound concentration.

The images were analyzed using Harmony software. A maximum intensity projection of all acquired planes was created, the spheroids segmented, and the mean intensity of PhenoVue 551 mitochondrial stain was calculated within the segmented area.

Individual dose response curves for each compound and plate are shown in Figure 4. The resulting IC_{50} values were highly reproducible across the three plates. This clearly shows that this automated spheroid formation workflow delivers highly reproducible 3D models.

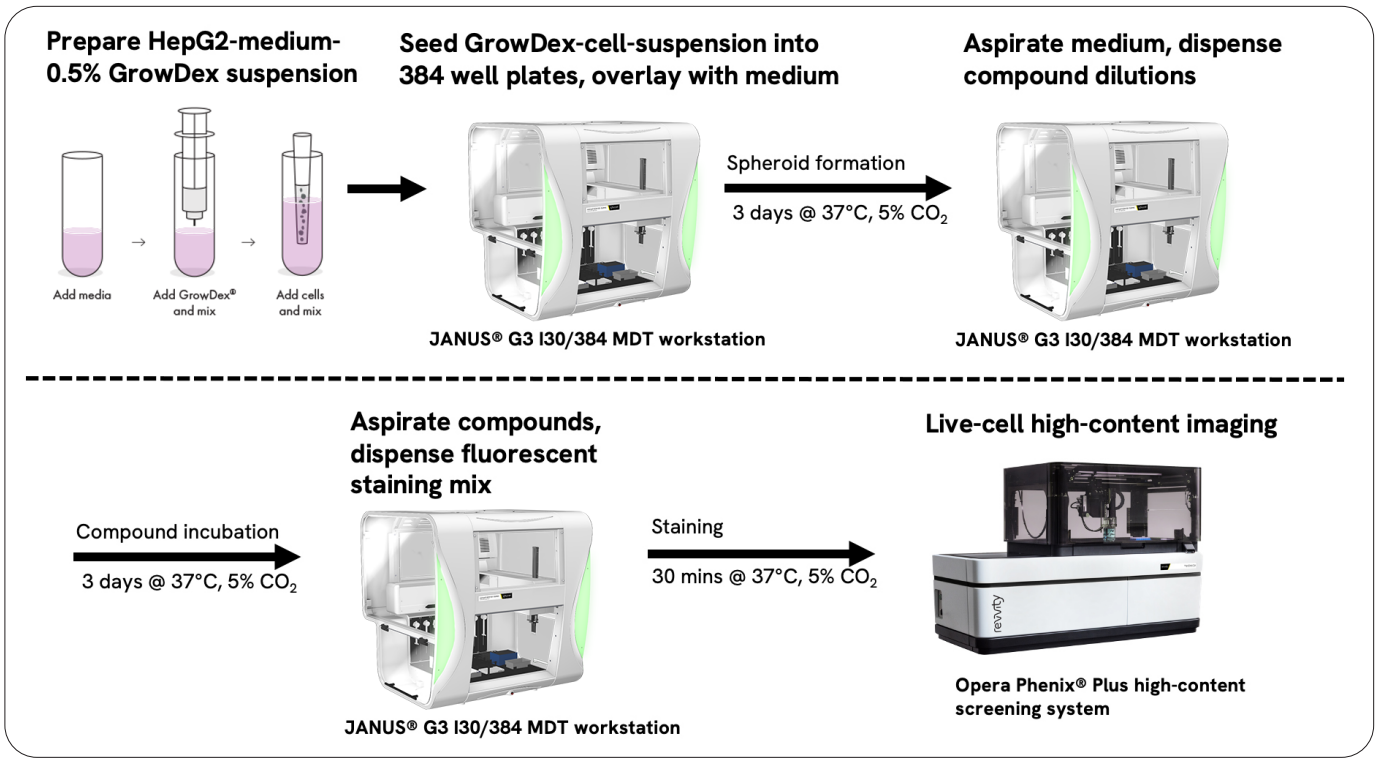

Figure 2: Workflow for an automated high-content toxicity assay with compound-treated, GrowDex-embedded HepG2 spheroids using the JANUS G3 I30/384 MDT workstation. To grow spheroids, HepG2 cells were manually suspended in medium-diluted 0.5% GrowDex hydrogel under a sterile workbench. The GrowDex-cell-medium suspension was transferred into a sterile, single well, deep well reservoir and placed onto an enclosed JANUS workstation equipped with a HEPA filter and the 384-MDT pipetting head fitted with 30 µL sterile filter tips. 25 µL of GrowDex-cell-mix were dispensed into three PhenoPlate 384-well ULA coated microplates. Next, all 384 wells were overlayed with a total of 50 µL growth medium by transferring 2x 25 µL from a growth medium-filled StorPlate-384 plate in a safe distance to the gel surface using the "tip-touch" mode. Spheroids were then allowed to form for 3 days in a standard cell culture incubator. After 3 days, medium was aspirated from the wells and 25 µL compound dilutions dispensed on top of the gel layer using the JANUS liquid handler. Compounds were incubated for another 3 days in a cell culture incubator. Last, compound dilutions were aspirated and 25 µL of 2x concentrated fluorescent of 2x concentrated fluorescent staining mix containing 300 nM PhenoVue 551 Mitochondrial stain and 2 µM PhenoVue DRAQ5 dispensed onto the hydrogel layer. After 30 min incubation at 37 °C, 5% CO2, spheroids were imaged in the Opera Phenix Plus.

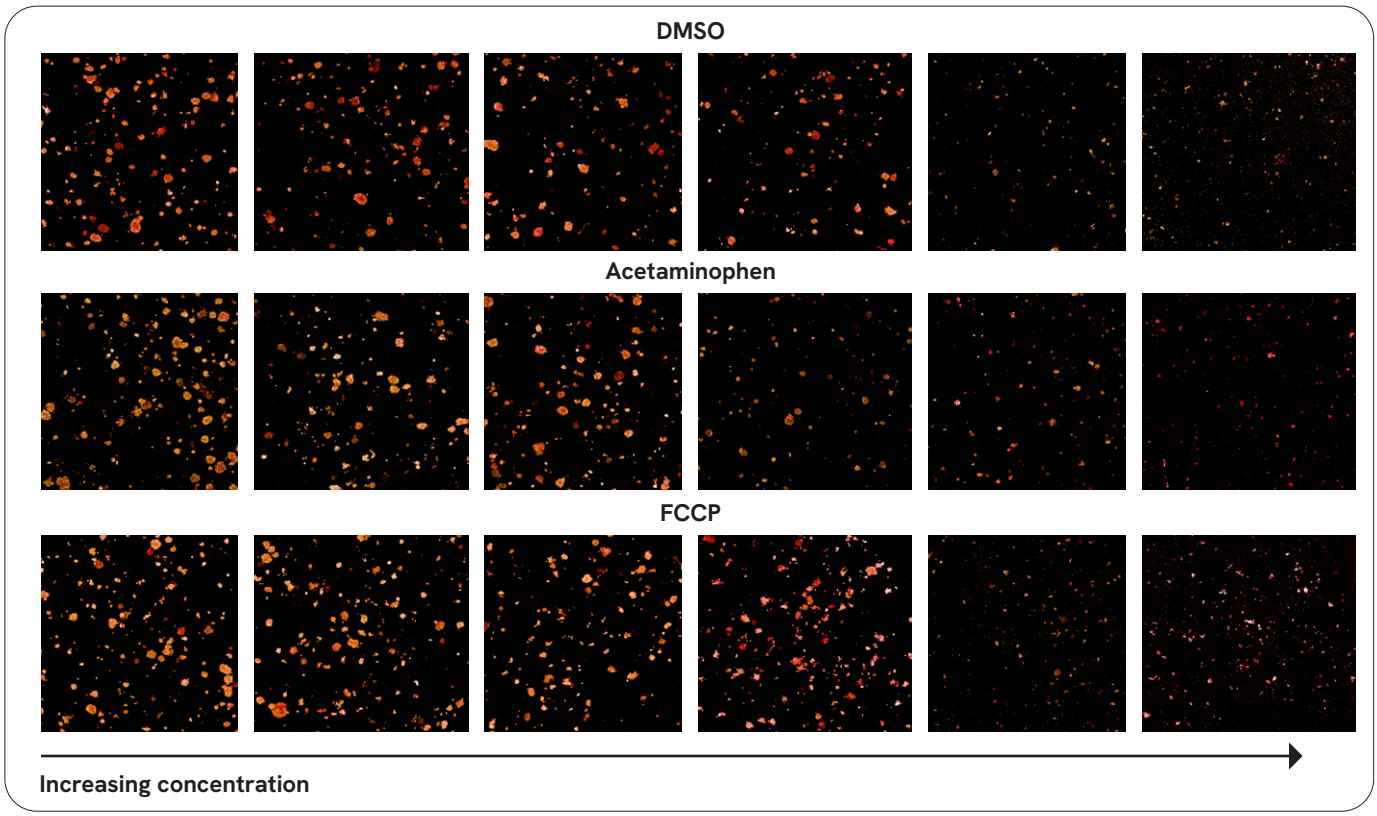

Figure 3: Acetaminophen, DMSO and FCCP decrease PhenoVue 551 mitochondrial stain in a dose dependent manner. Higher dosages of compounds resulted in a decreased mitochondrial staining intensity as well as smaller spheroid sizes. Images are maximum intensity projections of 480 µm z-height and a montage of the 4 fields of view (covering 90% of a well) acquired confocally with a 10x objective on Opera Phenix Plus system. Nuclei are stained with 1 µm PhenoVue DRAQ5 and mitochondria stained with 150 nM PhenoVue 551 mitochondrial stain. For the montage, auto contrast was turned off.

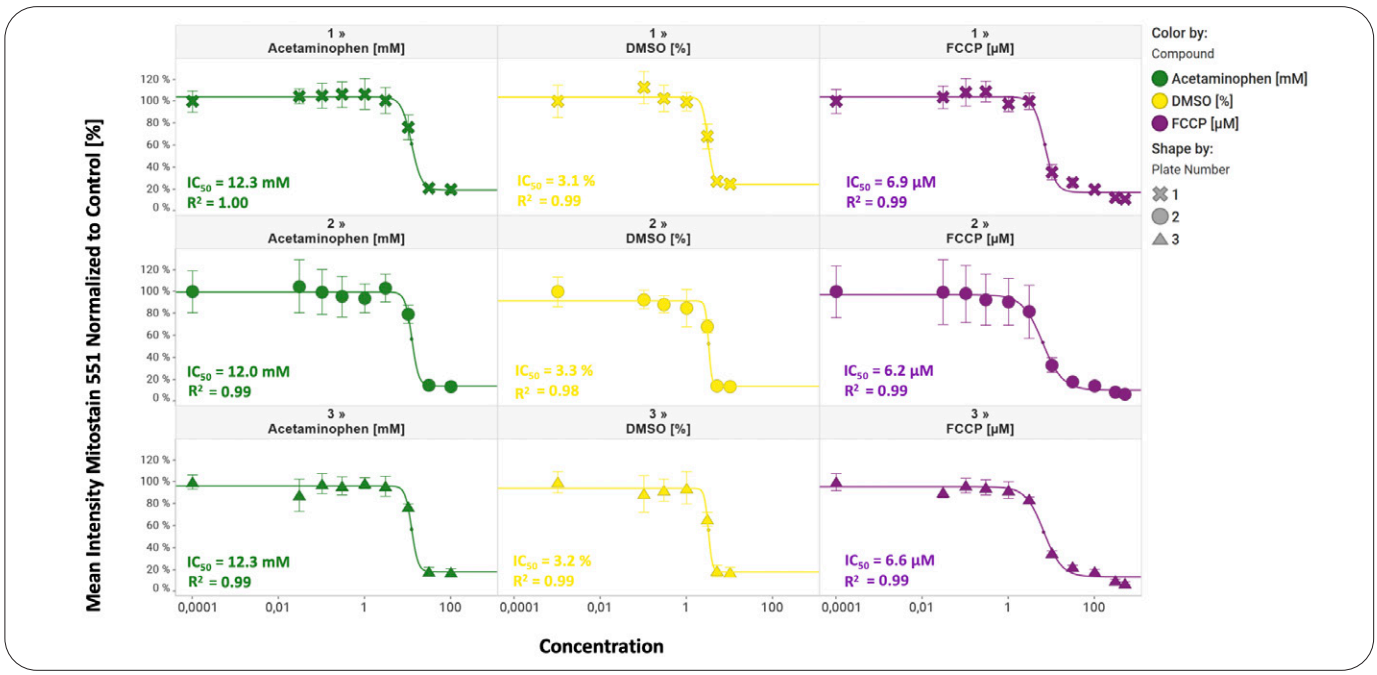

Figure 4: Automated generation of HepG2 spheroids using the JANUS liquid handling platform delivers reproducible results across different plates. JANUS was used to seed HepG2 cells into three different plates, and dose-dependent loss of mitochondrial staining intensity was analyzed using the Opera Phenix Plus. As shown, increasing compound concentrations lead to a decrease in PhenoVue 551 mitochondrial stain intensity and similar to identical IC_{50} -values across the three independent plates (with n=6 per data point). Additionally, the reproducible R^2 values indicate high quality curve fits.

Conclusions

In this work, we established a proof-of-concept assay showing the use of the JANUS G3 liquid handling workstation for automated liquid handling of GrowDex hydrogel. Critical liquid handling parameters were identified and optimized to allow simpler adoption of GrowDex in automated protocols. Using the workflow described, reproducible 3D spheroid models were generated across different plates, as shown by the highly reproducible IC_{50} values for Acetaminophen, DMSO and FCCP. Compared to manual preparation of 3D spheroid models, the main advantages of this automated workflow are scalability to produce as many plates as needed with minimal plate to plate variation and ease of media exchange (for example for compound addition or staining and washing). The risk of touching the gel surface and thus destroying the 3D spheroids is minimized.

In addition to the presented automation setup, the workflow can be further automated by using the JANUS inside a HEPA filtered environment (i.e., explorer™ G3 workstation, as shown in Figure 5).

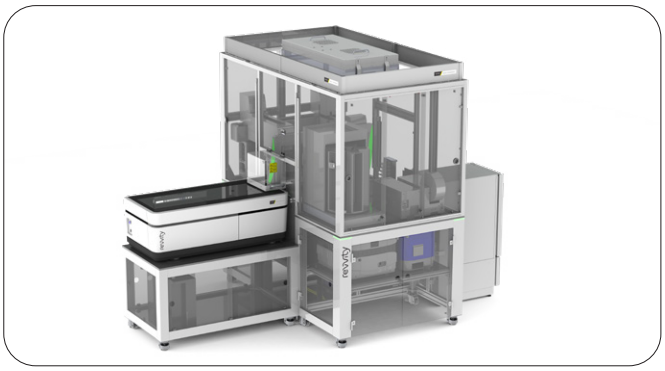

Figure 5: Example for an explorer™ G3 workstation for an integrated and fully automated workflow for a complete hands-free and standardized high-content toxicity assay with GrowDex-embedded HepG2 spheroids described in here. This automation solution includes for example a JANUS G3 automated liquid handler, cell culture incubator, centrifuge, sealer/peeler, Opera Phenix Plus and Celigo® image cytometer.

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Additionally, a multi-mode plate reader for biochemical assays like ATP-based luminescence or HTRF and AlphaLISA® assays can be integrated to allow orthogonal assays and readout technologies.

Tips and tricks for GrowDex-based cell assays

- Spheroid formation and growth depend on the optimal GrowDex concentration and should be optimized for each cell line during assay development: Typically, concentrations between 0.1% and 1% are tested.
- For dispensing cell-GrowDex suspensions, different options can be used: for JANUS, we recommend 96- or 384-well MDT heads.
- Fontus™ automated liquid handling workstation, a next-generation liquid handler, additionally offers partial loading of 96 or 384 tips.
- For all liquid handling cassettes, especially for higher GrowDex concentrations, a mild and short shaking of the reservoir during or after aspiration is recommended. Strong or prolonged shaking of the reservoir could be harmful to certain cell types and should be carefully optimized.
- Additionally, a lateral offset between aspiration positions can be tested.
- To minimize dead volume of Growdex solution, we recommend to use half-height reservoirs with pyramid bottom (for example https://www.analytical-sales.com/product/clear-pp-reservoir-170ml-half-height-pyramid-bottom-384-well-38455/).

Authors

Angelika Foitzik
Joern Linkner
Karin Boettcher
Alexander Schreiner
Revvity Inc., Hamburg, Germany
Piia Mikkonen, UPM Biomedicals, Helsinki, Finland

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com

For a complete listing of our global offices, visit www.revvity.com