TECHNICAL NOTE

A scalable and reproducible workflow for high-content analysis of cytotoxic effects in RASTRUM™ 3D cell cultures

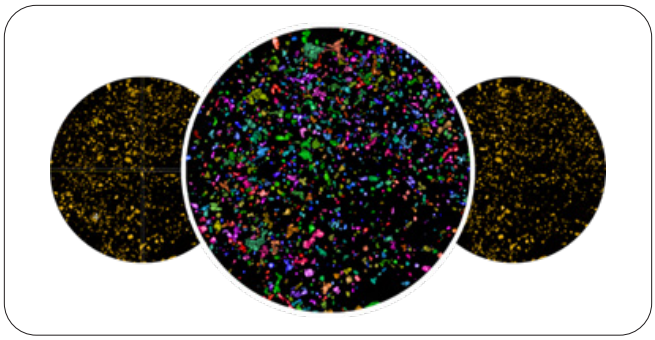
Key features

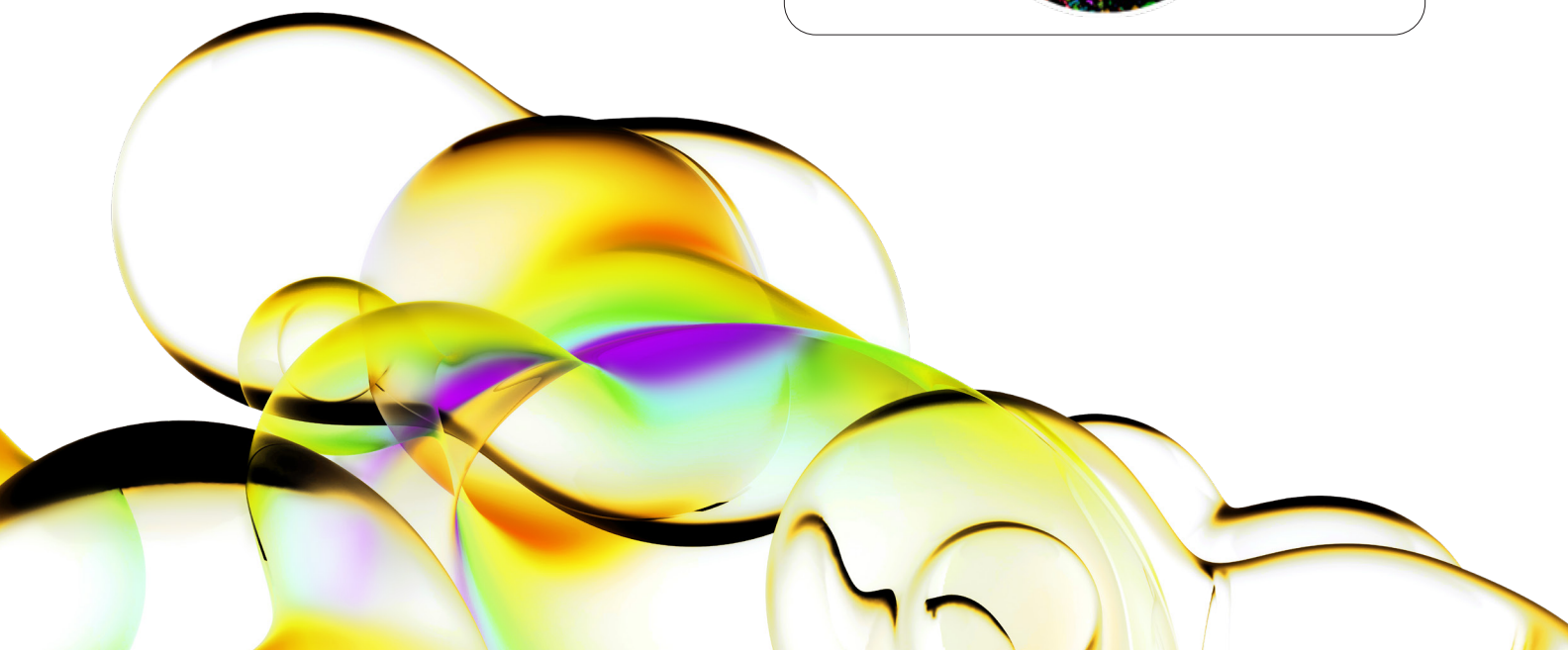
- Easy and reproducible creation of 3D models
- Consistent 3D cell culture placement for fast high-content image acquisition
- Mitochondrial Stain for analysis of cytotoxic substances

Introduction

Advanced cell models have the potential to be more physiologically relevant by better recapitulating the *in vivo* tissue environment. This is achieved by tailoring the microenvironment through matrix stiffness and biofunctionalization with peptide or protein additives. These 3D models can also help improve drug-induced cytotoxicity predictions to eliminate cytotoxic drug candidates as early as possible. However, generating complex cell models is frequently accompanied by more challenging workflows. First, the 3D cell culture models need to be created and then once successful, the reproducibility and scalability of endpoint analysis methods such as high-content imaging are limited.

Here, we demonstrate how to create reproducible 3D matrix cultures of HepG2 cells using the RASTRUM™ platform. The cultures are easy to image and analyze due to their precise and consistent location in the microwell plate and the absence of autofluorescence from the RASTRUM™ matrix. Cytotoxic effects can be studied by high-content imaging on the Opera Phenix® system and analyzed with Harmony® software.





Here we describe how to address three major challenges along the workflow:

Reproducibility and scalability of 3D culture creation: The RASTRUM™ platform is a drop-on-demand bioprinter that can deposit cells and matrix components with high precision into microplates resulting in reproducible 3D cultures with consistent dimensions, independent of cell type or culture condition.

3D signal attenuation/loss: Imaging in 3D cell cultures often suffers from signal attenuation or loss due to absorption and light scattering in deeper layers. As shown previously, this can be reduced by using water immersion objectives, applying optical clearing, or reducing the volume that needs to be imaged.[1,2]

Acquisition time and data volume: Imaging of 3D models is usually performed in z-stacks, which is time-consuming and creates large data volumes. The number of required images and thus also the volume of data can be decreased if the 3D models are in a defined position and only distributed over a small volume. This precise positioning is possible using the RASTRUM™ instrument.

Materials and Methods

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

Cells	HepG2 cells (hepatocellular carcinoma cells)
Growth medium	DMEM high glucose (Capricorn Scientific, #HXRXA) +10% FBS (Sigma-Aldrich, #F9665) + 2mM L-Glutamine (Sigma-Aldrich, #G7513) + Pen/Strep (Sigma-Aldrich, #P4333)
Compounds	Acetaminophen (Sigma-Aldrich, #A5000) FCCP (Carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazone) (Sigma-Aldrich, #C2920) DMSO (Sigma-Aldrich, #D2650)
Advanced cell model creation	RASTRUM™ Platform and Software (Inventia Life Science) RASTRUM™ Base cost (Inventia Life Science, #PR.BC) RASTRUM™ HTP model (Inventia Life Science, #PR.HTP.R) RASTRUM™ Imaging model (Inventia Life Science, #PR.IM.R) RASTRUM™ Matrix Px02.40
Microplates	PhenoPlate™ 96-well (Revvity, #6055300) PhenoPlate™ 384-well (Revvity, #6057300)
Mitochondrial stain	PhenoVue™ 551 Mitochondrial stain (Revvity, #CP301)
Image acquisition and analysis	Opera Phenix® Plus High Content Screening System (Revvity, #HH14001000) Harmony® High-Content Imaging and Analysis Software (Revvity)

Cell culture

HepG2 cells were cultured in DMEM high glucose supplemented with 10% FBS, 2mM L-glutamine, and 1x Pen/Strep at 37°C and 5% CO2. For cell preparation, cells were trypsinized, counted, and the required amount was resuspended in RASTRUM™ activator according to the manufacturer's protocol.

Advanced cell model creation

Advanced cell models were created using the RASTRUM™ platform, utilizing synthetic PEG-based matrices, which can be modified in stiffness as well as biofunctionalized. First, an inert base is printed preventing cell attachment to the well bottom and formation of a monolayer culture instead of 3D growth. Second, the advanced cell model is printed within a matrix. The RASTRUM™ matrix comprises two components: a bioink and an activator. The cells are resuspended in an activator and gelated in the well after contact with bioink resulting in cells encapsulated in a 3D formation. The RASTRUM™ matrix is transparent, does not autofluoresce, and is stable at room temperature.

For the PhenoPlate 384-well plate, $1.96x10^4$ cells were encapsulated in RASTRUM™ matrix Px02.40 (1.1 kPa in stiffness and biofunctionalized with peptides RGD, GFOGER, and hyaluronic acid) using the HTP model setting (Figure 1). The last two rows of this plate contained the inert base plus cells only as a 2D control. Cells were incubated for 48 hours before staining and imaging.

For compound treatment in PhenoPlate 96-well microplate, $1.5x10^7$ HepG2 cells were encapsulated in RASTRUM™ matrix Px02.40 using the imaging model shown in Figure 1. Cells were grown for a total of nine days, with medium renewal on day 2, compound addition on day 7, and staining and imaging on day 9.

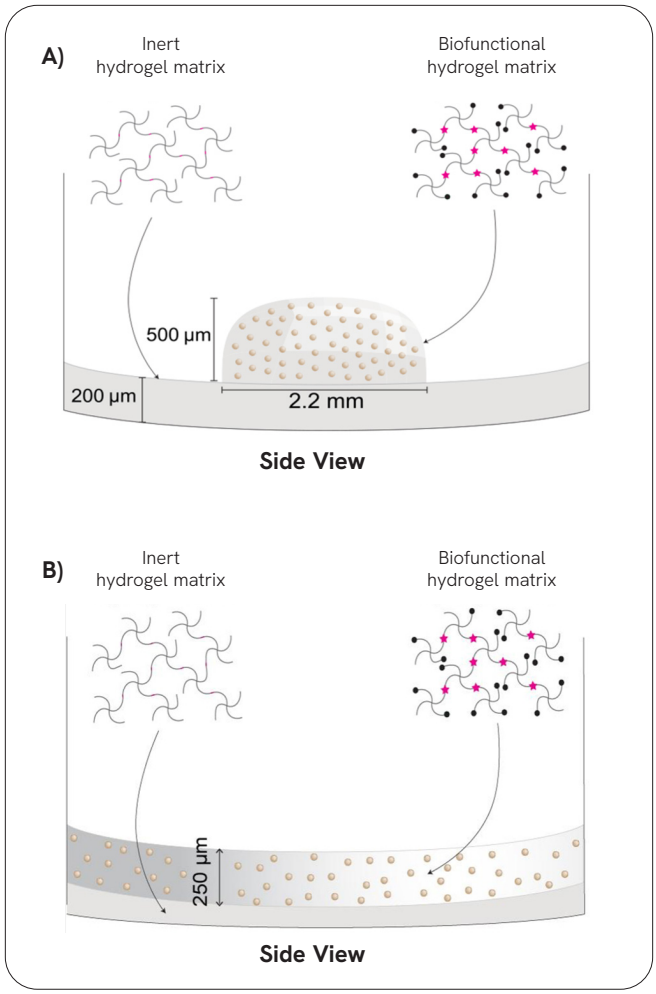


Figure 1: RASTRUM model graphic outlining the inert base structure height at the bottom of the well and the model structure height and width above. A) Imaging model graphic of an individual well in a 96-well plate. B) HTP model graphic of an individual well in a 384-well plate. The height of the inert base is the same for both models.

Image acquisition and analysis

Images of the PhenoVue 551 Mitochondrial Stain channel were captured on the Opera Phenix Plus using a 10x objective in confocal mode. Four fields with 2% overlap and a z-distance of 35 µm were acquired. The number of planes was 15 for the PhenoPlate 384-well plate and 17 for the PhenoPlate 96-well plate.

Image analysis was performed using Harmony software. A maximum intensity projection of a montage of the four fields (global image) was created from all planes.

The PhenoVue 551 Mitochondrial Stain channel was used to segment the 3D structures and the number, area and z-distribution of the 3D structures as well as the intensity of the mitochondrial stain were analyzed.

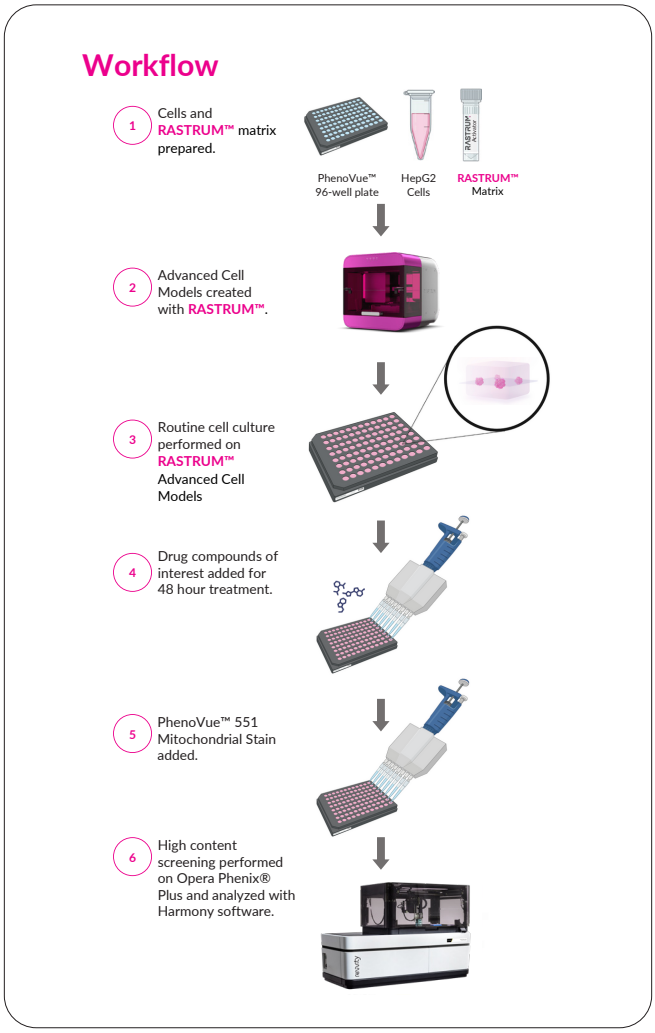


Figure 2: Experimental workflow for generation of 3D cell cultures with the RASTRUM™ platform and analysis of compound cytotoxicity with the Opera Phenix Plus in 96-well microplates. The RASTRUM™ imaging model structure is used in 96-well plates for easier image positioning.

Results and Discussion

Analysis of 3D culture reproducibility in 384-well format

First, images were acquired from a complete 384-well plate. The number, size, and z-distribution of the 3D structures were analyzed to determine the level of reproducibility of the 3D structures in all wells of the plate. For the analysis, a maximum intensity projection of a montage of the four fields, called global image, was used. In such a projection, the brightest pixel for one x/y position within a z-stack is kept and "projected" into a new image. To extract z-distribution information from such a single plane projection image, advantage was taken of the fact that it is known from which plane a given pixel was projected and this height is stored in a new channel called Plane Map. From this Plane Map channel, the z-height of the object could be extracted. An example image of one well is shown in Figure 3.

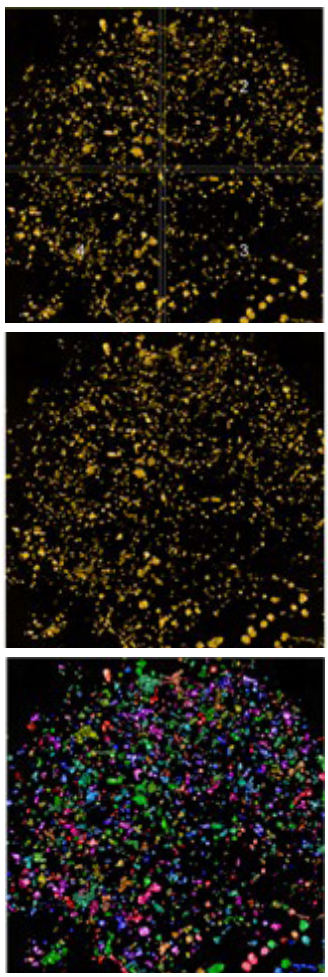


Figure 3: Global image with the four fields marked (top), the global image without field marking (middle), and the segmentation of the 3D objects (bottom)

As shown in Figure 4, indicated by the small error bars, the mean number of objects, the area, and z-distribution are highly reproducible from well to well.

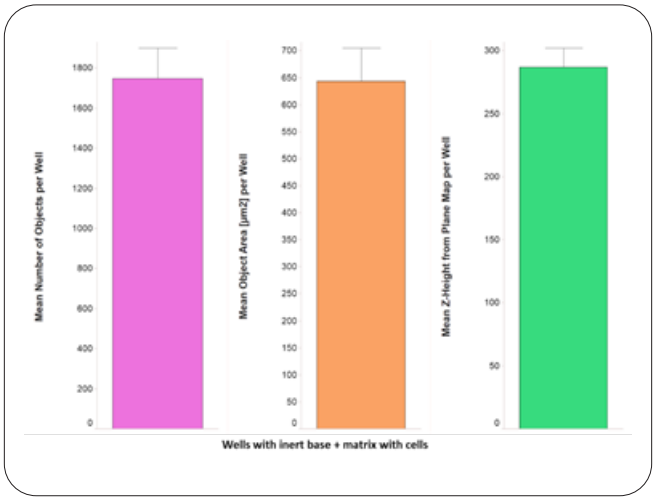


Figure 4: Image analysis shows reproducible creation of 3D objects across the 384-well plate. Shown are the results from the wells containing the inert base plus biofunctional matrix with non-treated cells 48 hours post-printing. Error bars indicate standard deviation, n = 336.

Analysis of compound cytotoxicity in 96-well format

Next, we used RASTRUM™ advanced cell models created in a 96-well plate format to analyze the effect of compound treatment on mitochondrial membrane potential as an indicator for cell health. The workflow is shown in Figure 2.

The RASTRUM™ models were incubated with either DMSO, Acetaminophen, or FCCP in a dose-response setting with six different concentrations per compound. Untreated wells served as controls. Similar to the 384-well plate, z-stacks of four fields per well were acquired on an Opera Phenix Plus using a 10x objective in confocal mode. Figure 5 shows an overview of the PhenoVue 551 Mitochondrial Stain. The last row of the plate contained untreated inert base controls and is not shown here.

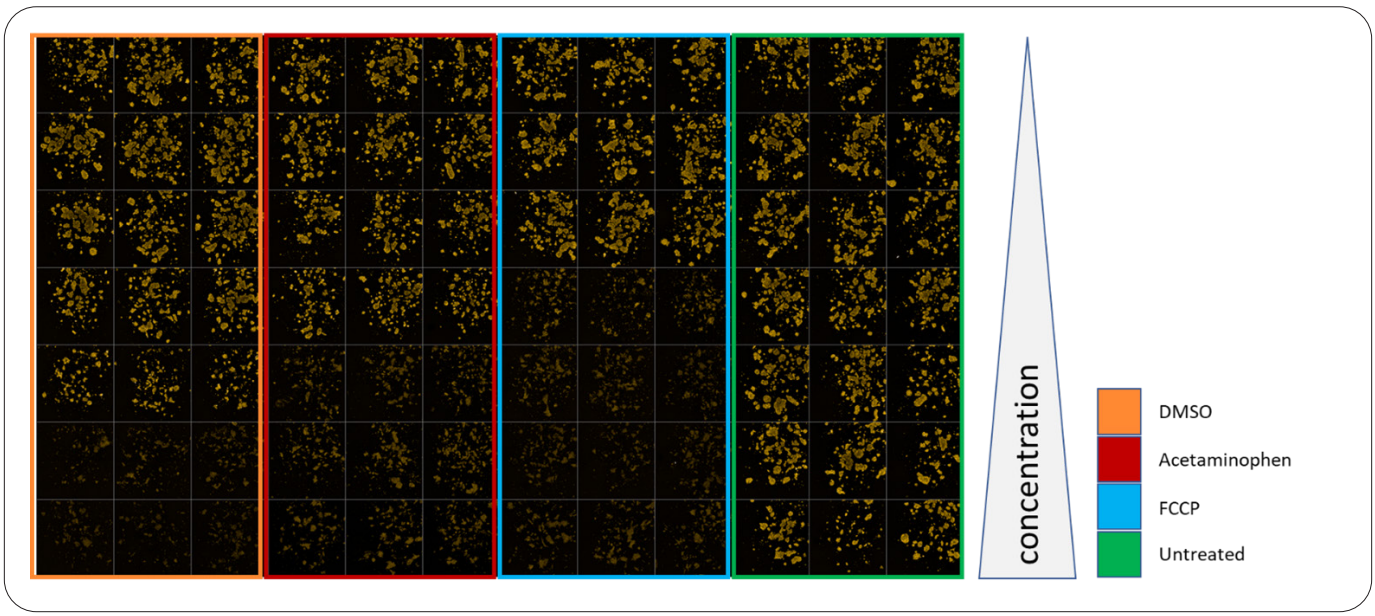


Figure 5: Compound treatment decreases mitochondrial staining intensity indicating cytotoxic effects.

To quantify the decrease in mitochondrial staining, the 3D objects were segmented and the PhenoVue 551 Mitochondrial Stain intensity was calculated. Figure 6 shows the dose-dependent decrease of staining intensity, with FCCP having the strongest effect, followed by Acetaminophen, and then DMSO.

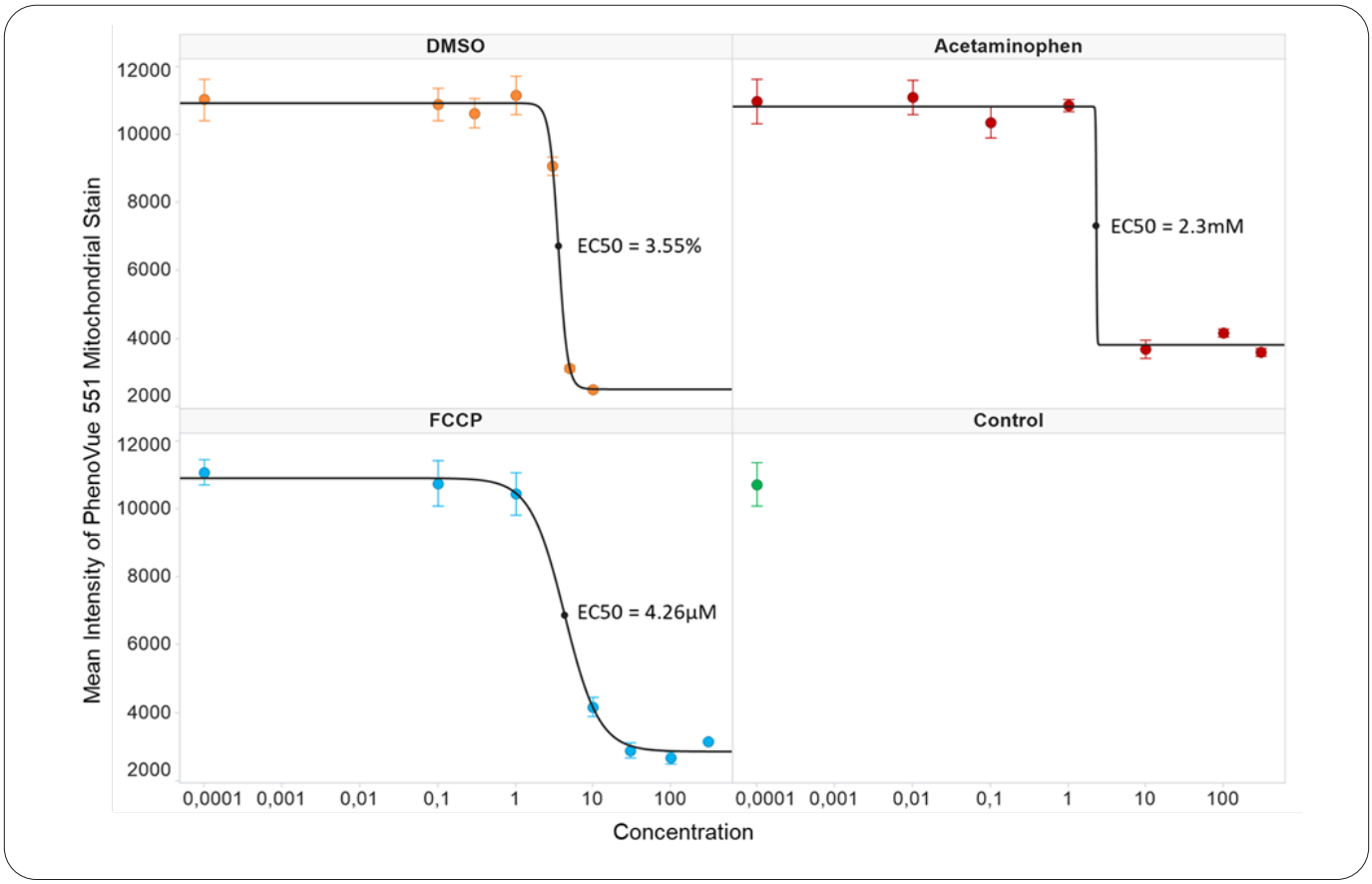


Figure 6: Compounds affect mitochondrial stain intensity in a dose-dependent manner. Shown are EC_{50} curves for changes in the intensity of the mitochondrial stain. N=3 for each concentration of DMSO, Acetaminophen, and FCCP, and n=21 for untreated wells.

Reducing acquisition time and data volume

The above analysis used all planes for the maximum intensity projection. However, for optimization of acquisition time and data volume, only the minimum number of planes that are needed for a robust read-out should be imaged. A commonly used method to determine assay quality is the z-prime (Z′) factor. Z′ determines the accuracy and sensitivity of an assay including sample and imaging conditions as well as image analysis sequence. The maximal value is 1 with values above 0.4 considered sufficient for cellular models. To identify the minimum number of planes required for a robust assay, the plane number for the maximum projection can be reduced and then the dataset is reanalyzed with the reduced plane number of projected images. Revvity's Harmony software provides automated Z′ calculations by simply assigning positive and negative control wells.

The Z′ value was calculated for the mean PhenoVue 551 Mitochondrial Stain intensity using all planes or every 2nd, 3rd, and 4th for the maximum intensity projection. In total, 17 planes at a 35 µm distance covering 560 µm were acquired. As a negative control, wells with no compound treatment, and as a positive control, wells treated with the highest concentration were selected. The results of the Z′ calculation are shown in Table 2. Up to a plane distance of 105 µm the Z′ prime values for all compounds are well above 0.4. This means that for this particular assay, it would be sufficient to acquire every 3rd plane only, decreasing both acquisition time and data volume.

Table 2: Correlation of plane distance and Z' value for 3D cytotoxicity assay.

Planes	Plane distance [µm]	DMSO	Acetaminophen	FCCP
All	35	0.75	0.70	0.74
Every 2nd	70	0.62	0.58	0.60
Every 3rd	105	0.50	0.45	0.49
Every 4th	140	0.43	0.26	0.37

Conclusions

Advanced 3D cell models are increasingly used in drug discovery, which requires robust and scalable assay workflows. Here, we demonstrated how to seamlessly integrate advanced cell models into existing workflows and downstream analyses for cytotoxicity studies. The 3D cell models made with the RASTRUM™ platform can be created simply, consistently, and reproducibly in a high-throughput 96- or 384-well format. They can also be easily imaged and analyzed on Revvity's high-content imaging platforms. The RASTRUM™ bioprinter allows both whole well filling and hydrogel dome printing within the well. The precise dome positioning helps reduce image acquisition time and data volume.

For more physiologically relevant cytotoxic responses, the method can also be easily adapted to co-cultures or primary cell types.

References

1. Boettcher, K., & Schreiner, A. (2017). The Benefits of Automated Water Immersion Lenses for High-Content Screening [Technical Note]. Revvity, Inc.

2. Letzsch, S., Boettcher, K., & Schreiner, A. (2018). Clearing Strategies for 3D Spheroids [Technical Note]. Revvity, Inc

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