Multi-parametric high-throughput analysis of hepatotoxicity using a 3D primary liver cell culture model.

Key features

- Reproducible and consistent primary 3D liver models created on RASTRUM™ platform
- A simple workflow for 3D hepatotoxicity screening using primary hepatocytes
- Flexibility to analyze data by immunoassays or high-content imaging

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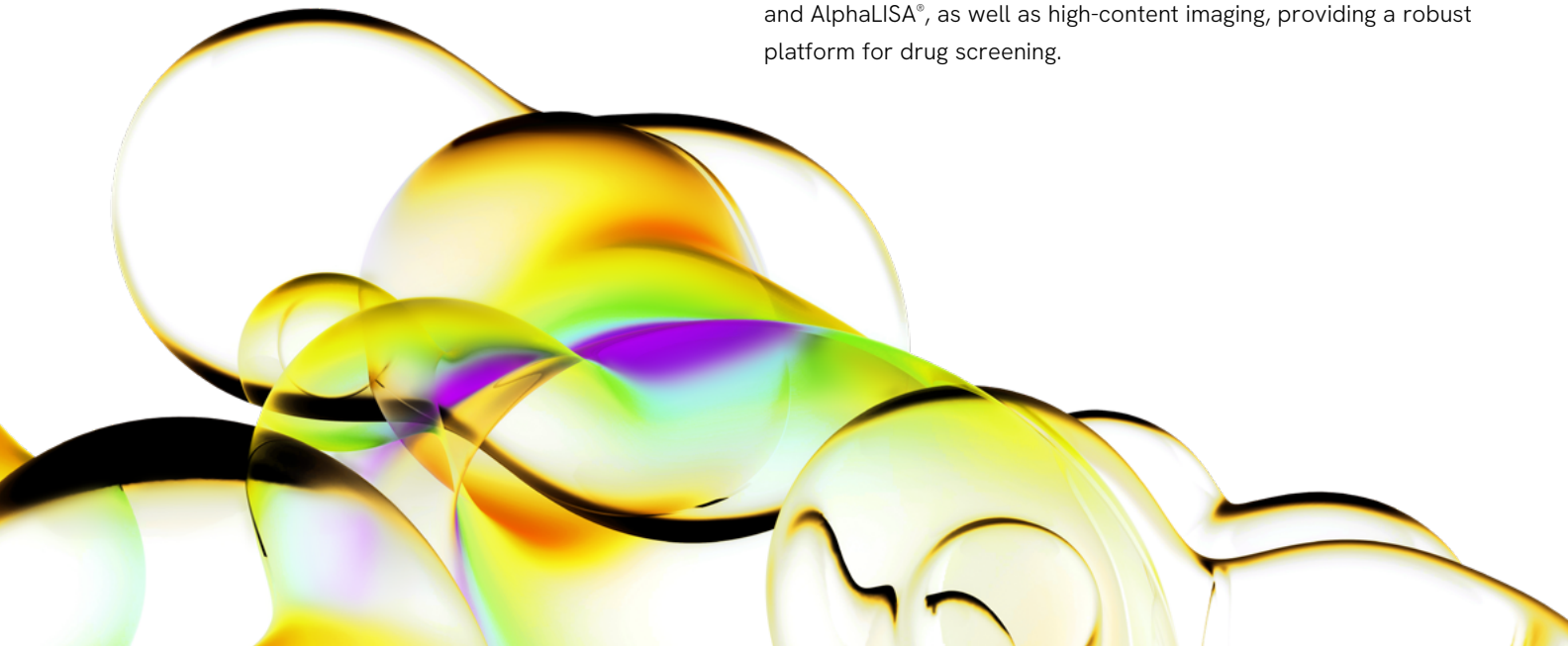
Introduction

3D cell cultures using human cells or tissues represent a more physiological relevant option for hepatotoxicology testing than 2D cultures due to the inclusion of extracellular tissue factors that can influence drug response[1]. There are currently only a few high-throughput screening (HTS) 3D systems that have high consistency and allow easy integration with downstream analysis methods.

In this application note, we demonstrate the creation of an advanced 3D primary human hepatocyte (PHH) model using the RASTRUM™ platform[2] that seamlessly integrates with Revvity's HTS technologies. 3D liver cell models were created in a high-throughput 384-well plate format using RASTRUM with high reproducibility and consistency. We show maintenance of PHH viability in synthetic, xeno-free RASTRUM matrices over an extended culture window of 14 days. Using this PHH model, we assess the hepatotoxicity of acetaminophen in a 384-well plate format, demonstrating compatibility of the model with multiple drug hepatotoxicity assays, including viability, albumin secretion and human aspartate transaminases (hAST). These readouts were performed from the same well within a single plate, demonstrating flexibility with multiplexing assays, allowing for more comprehensive and reliable experiments. Additionally, we show that the PHH model is compatible with high-content imaging and analysis.

The combination of reproducible RASTRUM models with Revvity's readout methods offers a simple approach to hepatotoxicity testing in a high-throughput context with minimal changes to current workflows. The proposed workflow supports multiple combined readouts, including biochemical assays such as HTRF® and AlphaLISA®, as well as high-content imaging, providing a robust platform for drug screening.



Workflow

Materials and methods

Table 1: List of materials, instruments and software.

Cells	HepG2 cells (human hepatoma cell line) Cryopreserved primary human hepatocytes (Lonza, #HUCPI)
Culture medium	HepG2: DMEM high glucose (Gibco, #11-965-118) + 10% FBS (Hyclone, #SH30084.03) + Pen/Strep (Gibco, #15-140-122) PHH: Hepatocyte Culture Media (Lonza, #CC-3198) + 20% FBS (Hyclone, #SH30084.03) + 25mM HEPES (Gibco, #15630106)
Compounds	Acetaminophen (Sigma, #A5000) Y-27632 dihydrochloride (STEMCELL Technologies, #72302) DMSO (Sigma-Aldrich, #D2650) Rifampicin (Cayman Chemical, #14423)
Advanced cell model creation	RASTRUM™ Platform and Software (Inventia Life Science) RASTRUM™ Base cost (Inventia Life Science, #PR.BC) RASTRUM™ Imaging model (Inventia Life Science, #PR.IM.R) RASTRUM™ HTP model (Inventia Life Science, #PR.HTP.R) HepG2: RASTRUM™ Matrix Px02.0 PHH: RASTRUM™ Matrix Px02.29PH
Microplates	PhenoPlate™ 96-well (Revvity, #6055300) PhenoPlate™ 384-well (Revvity, #6057300) OptiPlate™ 384-well (Revvity, #6007290)
Viability assays	ATPlite™ 1step 3D (Revvity, #6066736) Live/dead viability assay (Biotium, #30002) Hoechst 33342 (Invitrogen, #H3570)
Immunoassays	Albumin (human) AlphaLISA® detection kit (Revvity, #AL294C) Human Serum Albumin (HSA) kit HTRF® (Revvity, #6FHSAPEG) Human Aspartate Transaminase (AST) AlphaLISA® detection kit (Revvity, #AL3078C) P450-Glo CYP3A4 Assay with Luciferin-IPA (Promega™, #V9002)
Microplate readers	VICTOR® Nivo multimode microplate reader (Revvity, #HH35000500)
Image acquisition and analysis	Opera Phenix® Plus high-content screening system (Revvity, #HH14001000) Harmony® high-content imaging and analysis software (Revvity)

Advanced cell model creation

Two cell models were printed:

- 1 x 10^6 cells/mL of HepG2 (human hepatoma cell line) with matrix Px02.00 (1.1 kPa stiffness)
- 6.25 x 10^6 cells/mL of PHH (primary human hepatocytes) with matrix Px02.29PH (1.1 kPa stiffness, containing RGD, GFOGER, DYIGSR and Hyaluronic acid)

3D liver cell model consistency study

HepG2 cell line was printed as RASTRUM high-throughput (HTP) model in PhenoPlate 384-well, black-walled, clear-bottom plates. ATPlite™ 1step 3D was used to assess consistency between wells, as per manufacturer's instructions. Luminescence was read using a VICTOR Nivo plate reader. Data were visualised using ggplot2 and ggplate packages in R.

3D PHH model characterisation

PHH were printed as an Imaging model in PhenoPlate 96-well, black-walled, clear-bottom plates. After printing, Hepatocyte Culture Media (HCM) with 10 µM Y-27632 dihydrochloride was added and refreshed every 2–3 days for up to 14 days post-printing. Calcein AM staining and ATP-monitoring assays were used for viability assessment. Secreted albumin in supernatant was measured using Albumin (human) AlphaLISA and 2 µL of supernatant. CYP3A4 activity was determined using P450-Glo assay as per manufacturer's instructions.

3D PHH model in 384-well format

PHH were printed as an HTP model in PhenoPlate 384-well, black-walled, clear-bottom plates. After printing, 50 µL of HCM with 10 µM Y-27632 dihydrochloride was added to each well for 48 hours and replaced with 50 µL of HCM (without Y-27632) 24 hours prior to drug treatment. PHH were treated with acetaminophen (0.43–37.5 mM) or 0.75% DMSO vehicle control for 48 hours. At the endpoint (after 2 days of drug treatment), the cell model supernatant was collected and stored at -80°C. An ATP-monitoring luminescence assay was used as the endpoint viability measurement. For hAST AlphaLISA and HSA HTRF immunoassays, the manufacturers' instructions were followed using a 2 µL sample volume per well in triplicate. IC_{50} values were calculated in Graphpad Prism v9.0.

Image acquisition and analysis

Images of the Calcein AM and Hoechst nuclear stain channel were acquired on an Opera Phenix Plus using a 10x objective in confocal mode. Per well, 4 fields with 5% overlap and a z-distance of 20 µm were acquired. The number of planes was 30 for plate 1 and 35 for plates 2 and 3, respectively. Image analysis was performed using Harmony software on a maximum intensity projection of a montage of the 4 fields (global image) created from all planes. The Hoechst nuclear stain channel was used to segment the nuclei and the intensity of Calcein AM was calculated in the segmented region.

Results

RASTRUM platform creates reproducible and scalable 3D cell models for high-throughput applications

To firstly establish the 3D cell culture performance, eight 384-well HepG2 culture plates were created in one day. The overall coefficient of variation (CV%) within a plate was 5.51%, based on metabolic activity (Figure 1A). Inter-plate CV% between corresponding wells of each 384-well plate was 11.89% (Figure 1B).

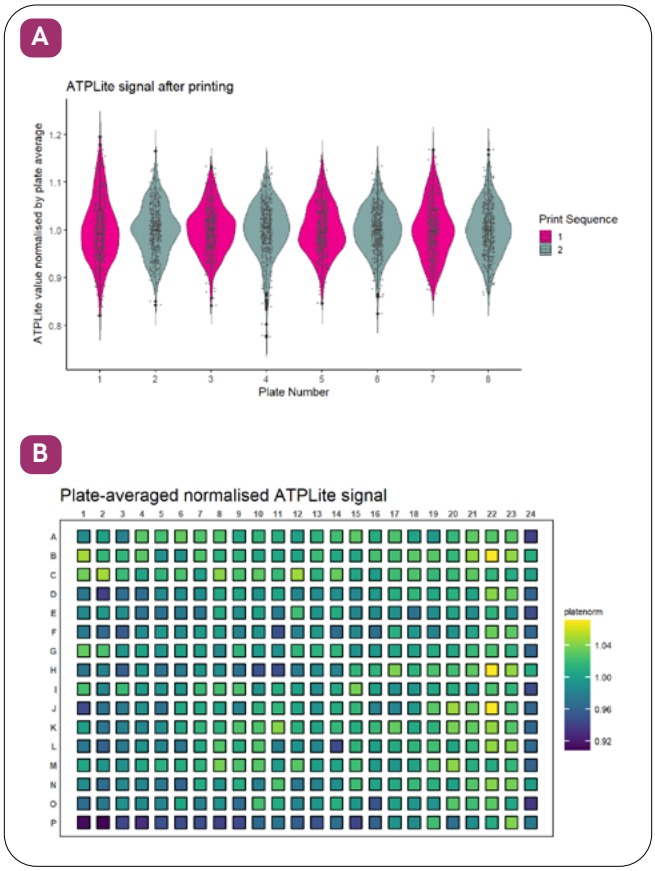


Figure 1: RASTRUM created reproducible and consistent advanced 3D cell models in a 384-well high-throughput format. (A) Low intra-plate variability (CV = ~5%) within each 384-well plate in eight consecutively printed well plates. (B) Low variability per well across eight 384-well plates (CV = ~11%). Viability determined by ATPlite™ 1step 3D reagent immediately post-printing, with values normalised to plate average and visualised by color scale.

Primary human hepatocytes in RASTRUM matrices are a physiologically relevant liver 3D model

RASTRUM-printed PHH show characteristic round to cuboid morphology with a high viability immediately post-printing (Figure 2) and stable metabolic activity for two weeks (Figure 3).

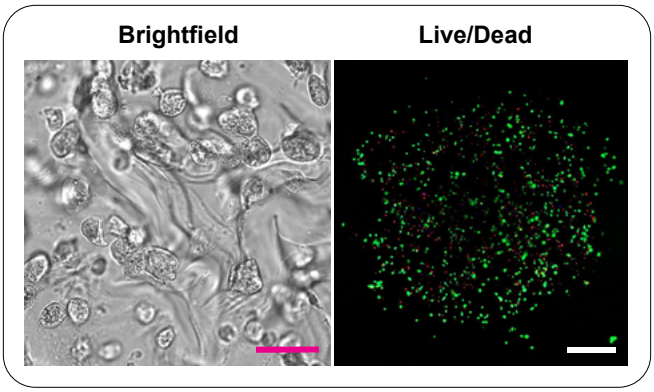


Figure 2: Primary hepatocytes show characteristic morphology and high cell viability 1 day after encapsulation in RASTRUM matrix. Brightfield image taken using a 20x objective and live/dead image taken using a 5x objective 1 day after printing. Green = live, Red = dead. Scale bars are 50 µm (left) and 500 µm (right).

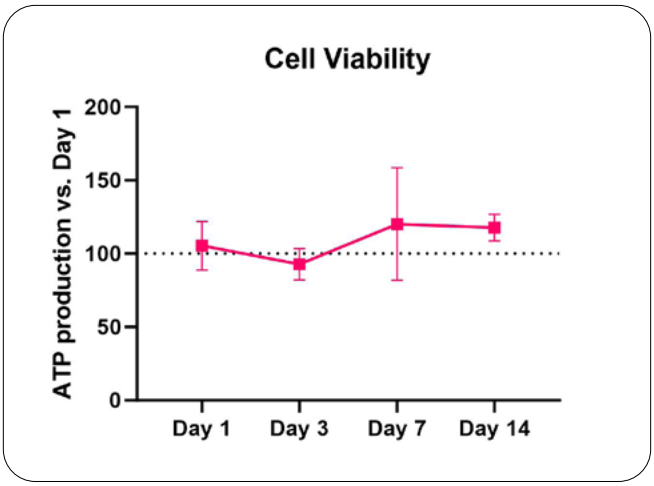


Figure 3: Long-term PHH viability is maintained over 14 days as a 3D culture with Y-27632 addition to the media. Viability did not change over time, as determined by ATP-monitoring assays (data normalised to Day 1). Note: Lack of proliferation is expected from hepatocytes. Data expressed as mean ± SD.

Functional characterization of the RASTRUM PHH model demonstrated that secreted albumin in the supernatant – a gold-standard indicator of in vitro hepatocyte function[3] – was detectable by human serum albumin AlphaLISA over 14 days (Figure 4A). The drug metabolism capability of the model was tested by measuring CYP3A4 enzyme activity with rifampicin, a known enzyme inducer, resulting in a ~4-fold increase of its activity and confirming the maintenance of this function.

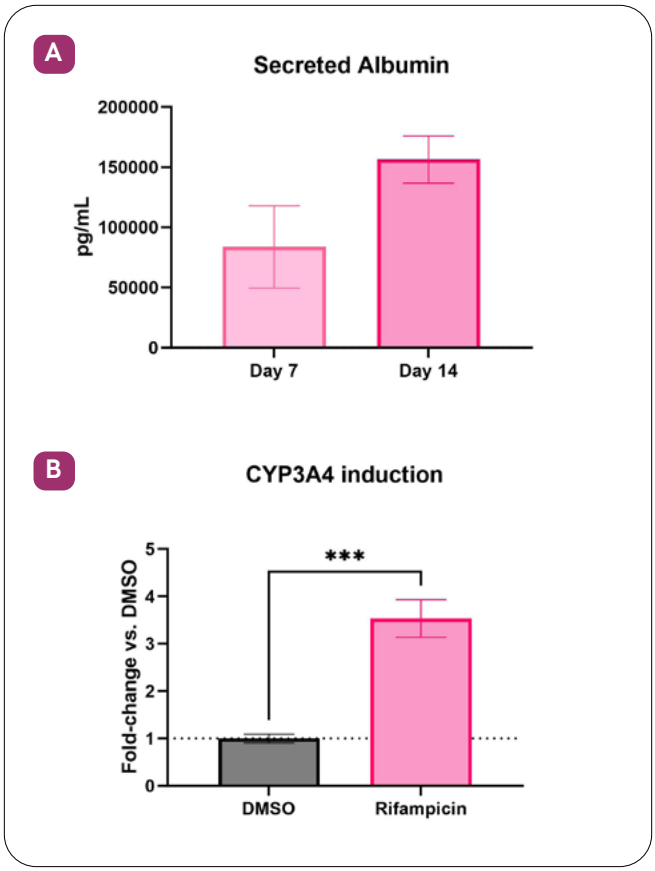


Figure 4: RASTRUM-printed PHH exhibited characteristic functions of hepatocytes. (A) Albumin secretion by PHH was detectable up to 14 days post-printing in RASTRUM matrices. (B) CYP3A4 enzyme activity could be induced by rifampicin treatment for 48 hours. Data expressed as mean ± SD. *** $p < 0.001$ vs DMSO control.

Assessment of hepatotoxicity in RASTRUM-printed PHH using high-sensitivity immunoassays

To demonstrate the assessment of toxicological response, the RASTRUM 3D PHH model was printed into a 384-well plate and treated with acetaminophen for 48 hours. Measurement of human aspartate transaminases (hAST), typically measured in clinical liver function tests for cellular injury, showed an expected trending increase with higher acetaminophen doses (Figure 5).

ATP as an indicator of cell viability (Figure 6A) and albumin secretion (Figure 6B) are accepted methods of liver toxicity assessment. While both methods achieved similar IC_{50} levels, secreted albumin measurement from the culture supernatant achieved a more reliable IC_{50} value based on sigmoidal curve shape.

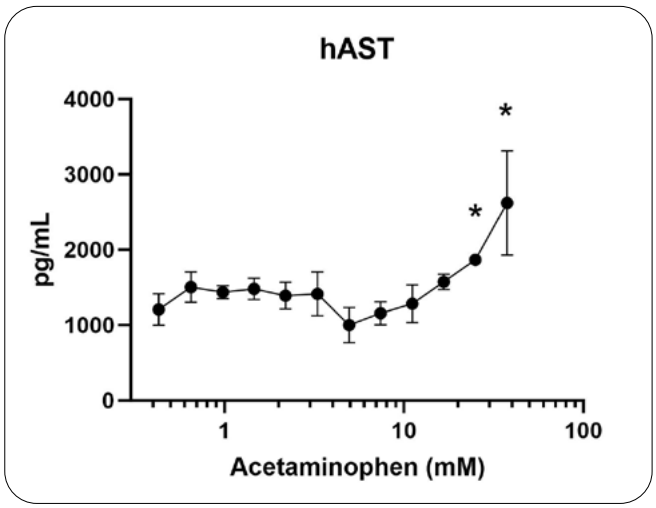


Figure 5: Human AST (hAST) from cell model media as determined by AlphaLISA. Significant cellular injury measured by hAST leak into the media was indicated at doses > 25 mM, * $p < 0.05$ vs. DMSO control. Data expressed as mean ± SD.

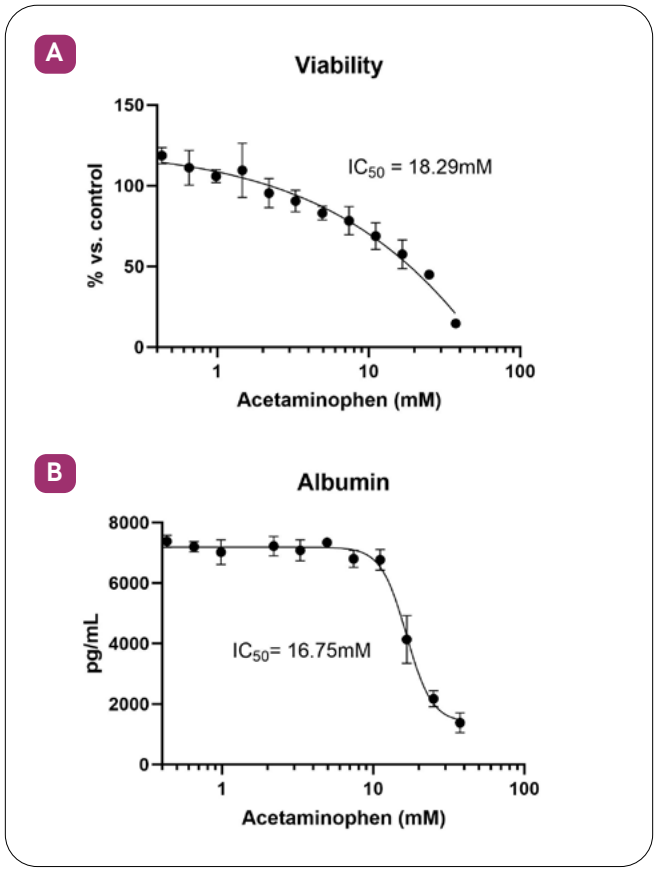


Figure 6: Quantification of hepatotoxicity of acetaminophen (2 days) in RASTRUM-printed PHH. (A) ATP measurement indicated a dose-dependent change in cell viability. (B) Secreted albumin from cell model media as determined by HTRF detection kit. Albumin secretion as a measure of hepatocyte function presented a typical sigmoidal curve compared to that for ATP, and therefore may be a more robust method for detection of hepatotoxicity in PHH. Data expressed as mean ± SD, corrected to DMSO control.

RASTRUM PHH model is compatible with Opera Phenix Plus for high-content imaging in 3D

The RASTRUM 3D PHH model was printed into a 384-well plate and incubated for 72 hours prior to drug treatment. The 3D PHH models were then treated with either DMSO or acetaminophen for 48 hours and subsequently stained with Calcein AM (2 µM) and Hoechst nuclear stain (10 µg/mL).

Imaging of RASTRUM-printed 3D PHH models using an Opera Phenix® Plus high-content screening system enabled single-cell analysis of drug response based on Calcein AM (live cells) and Hoechst (all cells) staining. Acetaminophen induced a dose-dependent reduction in mean Calcein AM intensity (Figure 7A). This was consistently observed in three individual printed plates of PHH from different donors (Figure 7B) and resulted in an IC50 value similar to what we have observed using conventional immunoassay and ATP measurement. These results demonstrate the reproducibility of RASTRUM-printed 3D PHH models and support the use of toxicological data collection from imaging-based methods, conventional immunoassays or ATP-based assays.

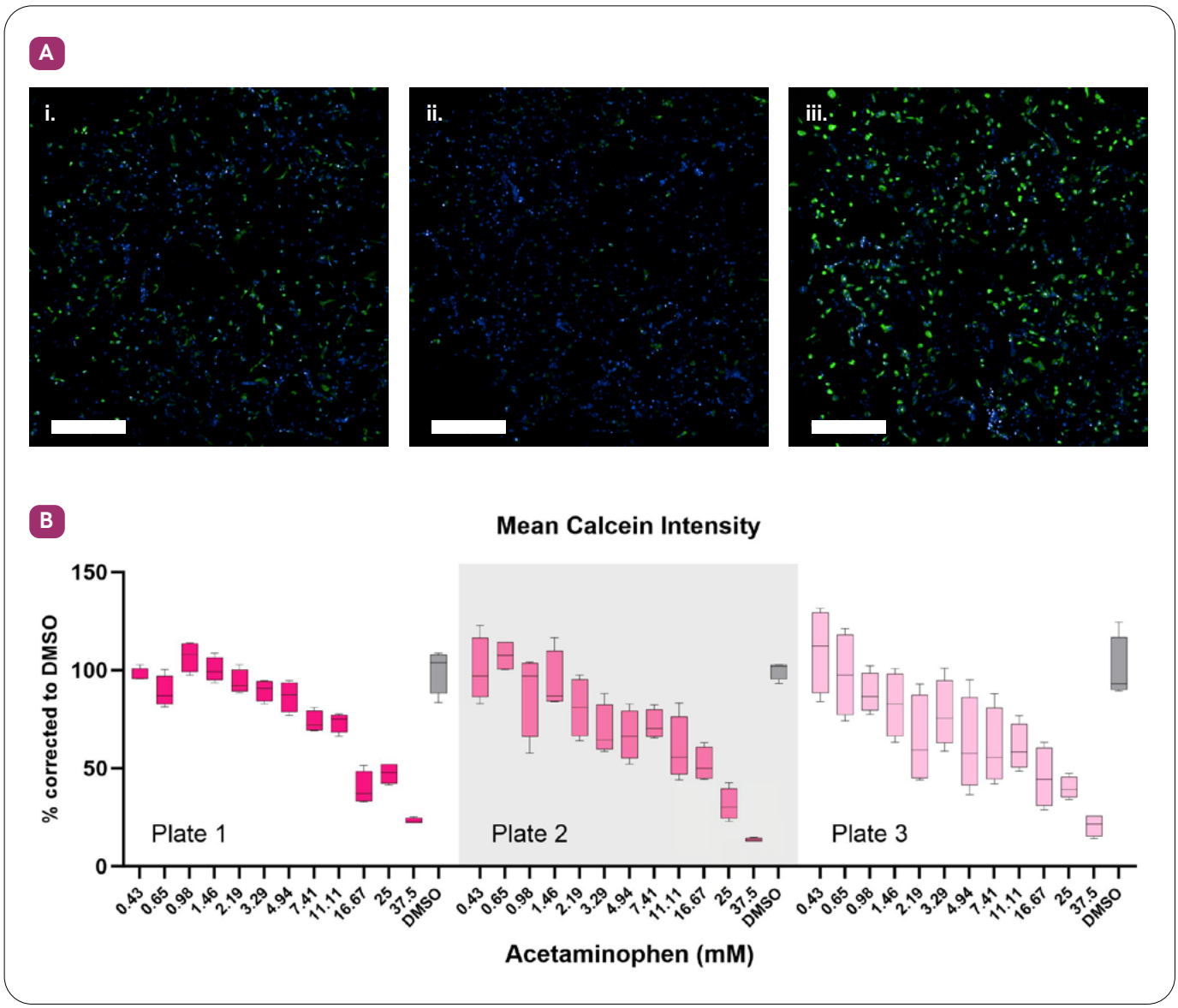


Figure 7. (A) High-content imaging of PHH stained with Calcein AM (green) and Hoechst 33342 (blue) showed a dose-dependent reduction in Calcein staining with increasing acetaminophen concentration: (i) 16.67 mM, (ii) 37.5 mM, (iii) DMSO control. Scale bar = 500 µm. (B) Mean intensity of calcein-stained RASTRUM-printed 3D PHH, as analyzed in Harmony® software, were reproducible across multiple plates and donors. Collective IC50 values (Average: 14.35 mM) were similar to those acquired using immunoassays for secreted albumin and ATP-based assay.

Summary

- RASTRUM is able to create 3D cell models in high-throughput formats, enabling reproducible advanced cell models for drug screening applications. Furthermore, RASTRUM uses xeno-free matrix, enabling more consistency batch-to-batch as compared to animal-derived matrices.
- Using Opera Phenix Plus for high-content imaging and Harmony software for feature extraction, toxicity of acetaminophen could be detected similarly to traditional ATP-based metabolism assay. These findings were further supported by immunoassays from cell model supernatants.
- Consistency of the viability data extracted from either ATP-based or imaging-based methods enables flexibility in choosing methods to determine drug response.
- The combination of RASTRUM-printed primary hepatocytes, Revvity immunoassays and Opera Phenix Plus for high-content imaging could be used to extract more in-depth toxicological data in a biologically relevant model, which may enable better translation of in vitro drug responses to clinical applications.

References

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