High-throughput Screening for Mitochondrial Hepatotoxicants in 3D HepG2 C3A Spheroid Cultures

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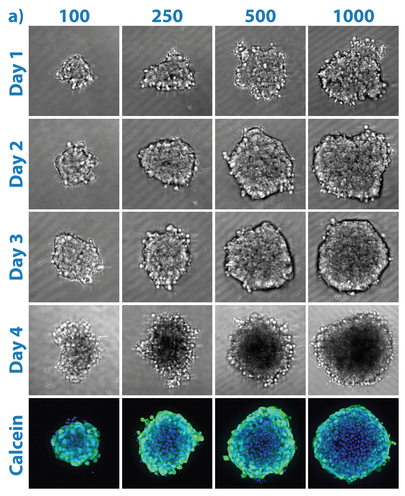
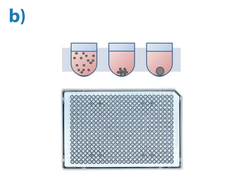
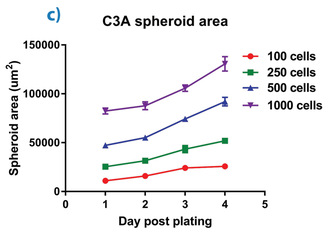
Introduction

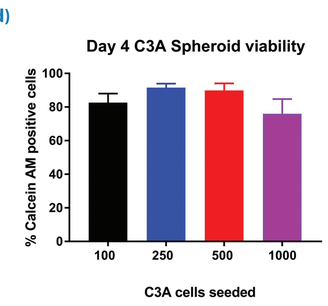
Effective early prediction of drug induced liver injury (DILI) induced by compounds exhibiting cytotoxic and mitochondrial liabilities is a key challenge in the reduction of drug attrition rates and the selection of desirable lead candidates. 3D culture systems are increasingly used as screening tools to predict hepatic toxicity due to their ability to maintain hepatocyte function *in vitro,* and more accurately than 2D cultures to reflect *in vivo* hepatic responses. A high throughput and automated hepatocyte spheroid screening platform is described. Scaffold-free HepG2 C3A spheroid cultures were subject to repeat and long term compound exposure using the Biomek® NXP liquid handler with quantitation of mitochondrial toxicity by TMRE (tetramethylrhodamine, ethyl ester) and cytoplasmic membrane disruption (a late stage cytotoxicity) by TOTO-3 using the ImageXpress Micro Confocal (IXM-C). The predictive capacity of this multi-parametric assay platform to screen for mitochondrial hepatotoxicants and potential DILI risk is demonstrated and forms part of our expanding Discovery Toxicology capability.

HepG2 C3A Spheroid Formation and Growth





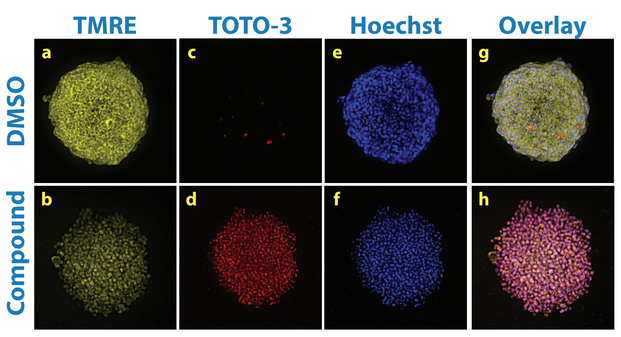




	100 cells	250 cells	500 cells	1000 cells
Shape factor	0.85	0.87	0.86	0.86
Area (µm²)	44444	76398	130475	186984
Width (µm)	248	308	421	501

Figure 1: HepG2 C3A cells were plated into Nexcelom 384 well ultra low attachment (ULA) plates, cultured for up to 4 days and imaged at 10x using transmitted light (a, b). Spheroid area was quantified using MetaXpress 6 and shown to increase at all cell densities over the 4 day period (c). Spheroid viability was >75% at day 4 as determined by Calcein AM staining (d). Increases in spheroid size and shape showed a high degree of uniformity at day 4 (table).

High Content Imaging of Mitochondrial Toxicity



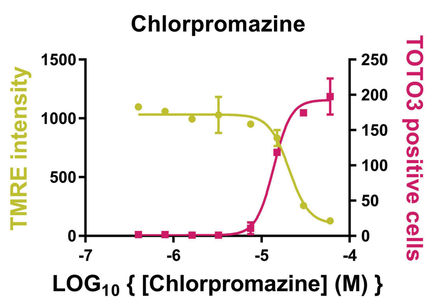


Figure 2: Multiplexed imaging of 48 hr cultured HepG2 C3A spheroids treated for 24 hr with increasing concentrations of the mitochondrial toxicant Chlorpromazine. Mitochondrial membrane depolarisation was detected by a decrease in TMRE staining (a and b) and cytoplasmic membrane disruption by an increase in TOTO-3 positive cells (c and d). Hoechst was used to determine nuclei count and to assess spheroid size (e and f). Images were acquired using IXM-C and are shown as a 2D maximum projection. TMRE intensity and TOTO-3 positive cells can be quantified using MetaXpress 6 and IC_{50} values determined by non-parametric curve fitting (right graph).

HepG2 C3A Spheroid Uniformity

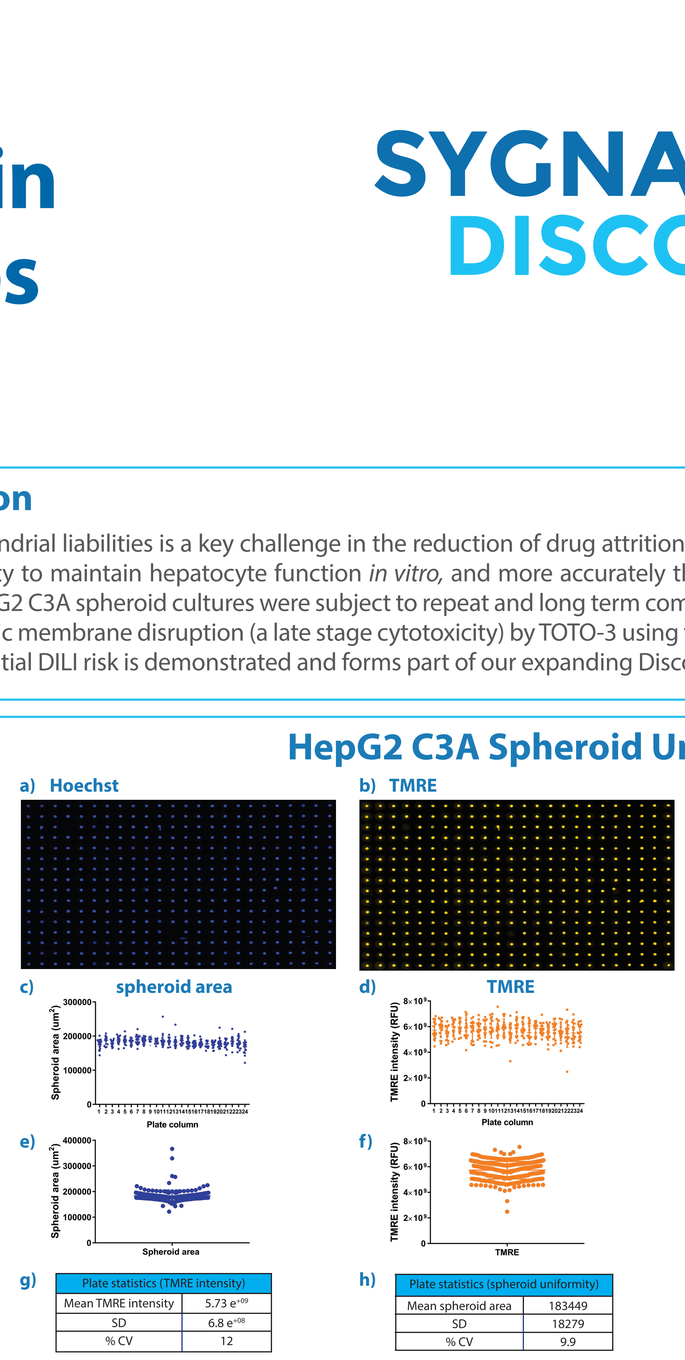
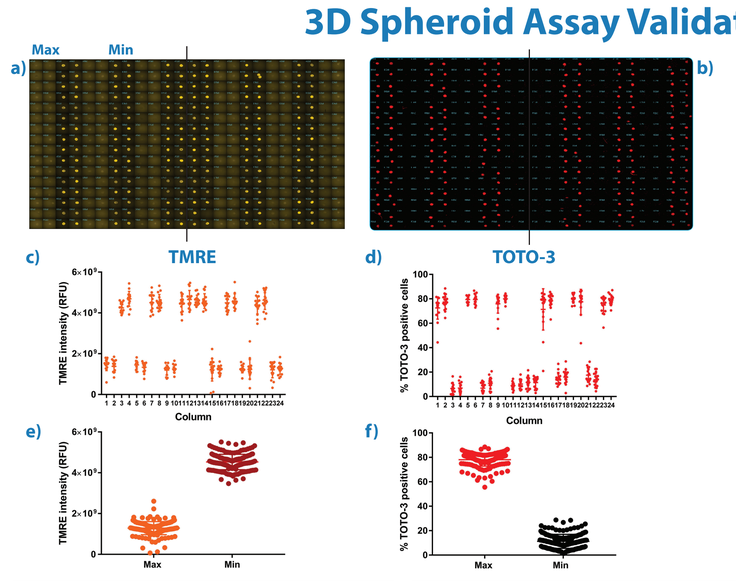


Plate statistics (TMRE intensity)	
Mean TMRE intensity	5.73 e^{+09}
SD	6.8 e^{+08}
% CV	12

Plate statistics (spheroid uniformity)	
Mean spheroid area	183449
SD	18279
% CV	9.9

Figure 3: Spheroid uniformity assessment was performed with optimised assay conditions on HepG2 C3A spheroids formed from 500 cells/well seeding density and cultured for 3 days prior to delivery of vehicle (0.3% DMSO) using the Biomek® NXP liquid handler. Spheroids were stained 24 hr later with TMRE and counterstained with Hoechst (a and b). Column data (c and d) indicates no significant edge effects in spheroid unformity or TMRE intensity. Both parameters are highly uniform across the assay plate (e and f) resulting in coefficients of variation (% CV) of <15 (g and h).

3D Spheroid Assay Validation



Assay validation (TMRE)	Max (IC_{90} chlorpromazine)	Min (0.3% DMSO)
Mean intensity	1.28e^{+09}	4.53e^{+09}
SD	3.21e^{+08}	3.79e^{+08}
% CV	19.9	8.4
S:B	3.5	

Assay validation (TOTO3)	Max (IC_{90} chlorpromazine)	Min (0.3% DMSO)
Mean % positive	77.1	11.3
SD	8.1	5.8
% CV	10.4	51.2
S:B	6.8	

Figure 4: Spheroid assay validation of max and min signal across the assay plate. HepG2 C3A spheroids were formed as previously and alternate columns treated with an IC_{90} dose of Chlorpromazine (max) or vehicle (0.3% DMSO; min) using the Biomek® NXP liquid handler. Staining with TMRE and TOTO-3 after 24 hr was used to determine assay performance (a,b). TMRE signal was highly reproducible across plate columns with % CV <20 for both max and min groups (c,e). TOTO-3 was less reprodicible, reflecting the effect of processing assay plates for compound delivery (d,f).

Mitochondrial Toxicity Screening and Prediction of Drug Induced Liver Injury (DILI)

Assay Workflow

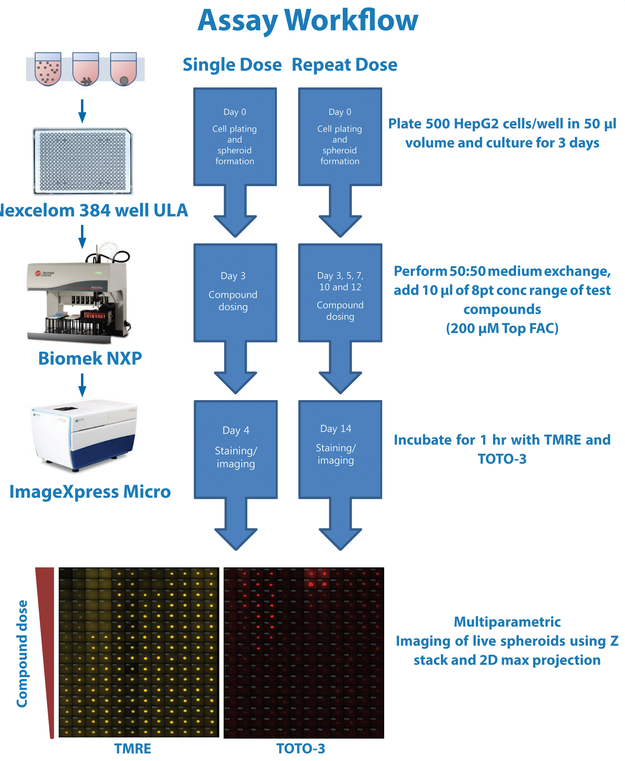
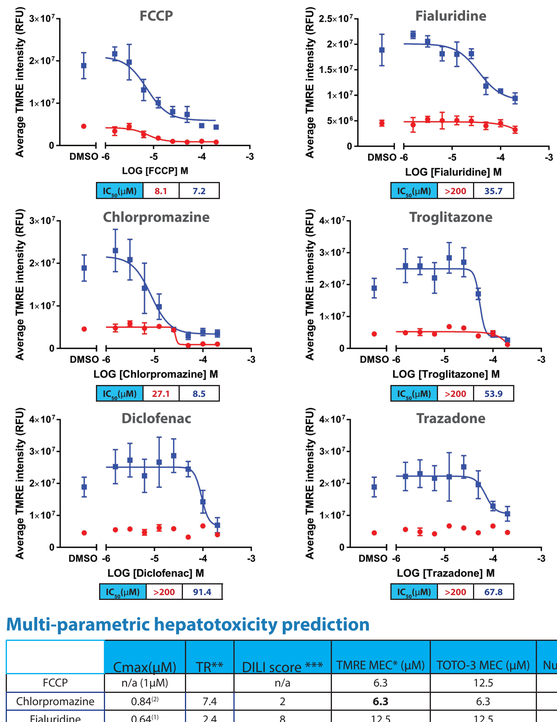
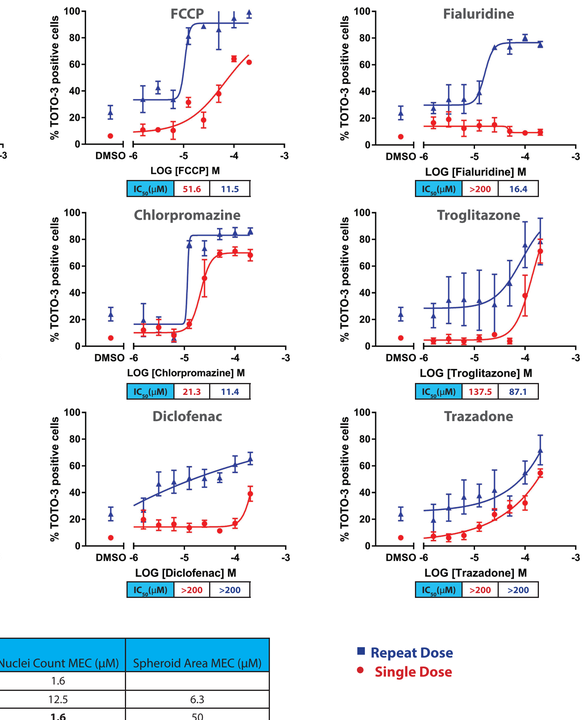


Figure 5: Spheroid assay workflow incorporating HepG2 C3A spheroid culture in 384 well ULA format with single or repeat delivery of test compounds using the Biomek NXP and quantitative multi-parametric imaging of spheroid staining using the IXM-C. Single dose protocols are run for 4 days, repeat dose protocols run for 14 days.

TMRE



TOTO-3



Multi-parametric hepatotoxicity prediction

	Cmax(µM)	TR**	DILI score ***	TMRE MEC* (µM)	TOTO-3 MEC (µM)	Nuclei Count MEC (µM)	Spheroid Area MEC (µM)
FCCP	n/a (1µM)		n/a	6.3	12.5	1.6	
Chlorpromazine	0.84[2]	7.4	2	**6.3**	6.3	12.5	6.3
Fialuridine	0.64[1]	2.4	8	12.5	12.5	**1.6**	50
Troglitazone	6.39[1]	2.0	8	50	100	25	**12.5**
Diclofenac	7.99[1]	0.2	8	100	>200	50	**1.6**
Trazadone	5.05[2]	0.5	5	NS	>200	25	**3.1**

■ Repeat Dose
● Single Dose

*MEC; minimum effective concentration calculated using one-way ANOVA with Dunnett's multiple comparison test
**TR; toxicity risk calculated as a ratio of MEC to Cmax (minimum effective concentration) from the most sensitive parameter (bold)
***DILI score; DILI score according to the FDA Liver Toxicity Knowledge Base classification system

Figure 6: Mitochondrial toxicity screening and prediction of DILI using multiparametric imaging of TMRE and TOTO-3. Spheroids were treated with a range of known hepatotoxic compounds in a single dose or repeat dose regime every 48 hr. TMRE intensity (left panel) as a marker of mitochondrial depolarisation or TOTO-3 positive cells (right panel) as a marker of cytoplasmic membrane disruption is plotted against compound concentration and IC_{50} determined by non parametric curve fitting (n = 4 spheroids per compound dose). Comparable day 14 vehicle controls of 0.3% DMSO are shown to indicate the effect of the dosing procedure alone. Increased sensitivity to hepatotoxicants is demonstrated by a shift in IC_{50} when exposed to a repeat dose and is also indicated by a reduction in nuclei count and spheroid area. Toxicity risk calculated from the ratio of minimum effective conc (MEC) obtained from multi-parametric imaging to Cmax successfully ranks DILI risk in line with established DILI scores.

Summary:

- High throughput validated HepG2 C3A spheroid assay provides multi-parametric assessment of mitochondrial toxicity, cytotoxicity and DILI risk.
- Repeat Biomek NXP delivery of compounds permits long term drug exposure and more accurately replicates clinical effects versus single dose
- High content image acquisition and analysis using ImageXpress demonstrates a high degree of spheroid uniformity and delivers robust quantitative spheroid data

Acknowledgements:
We would like to thank Sarah Rowe at Molecular Devices for valuable discussion and assistance in setting up spheroid image acquisition and analysis protocols in MetaXpress6

References:
1. Gaskill H et al Toxicol Res 2016 5:1053 2. Xu et al 2008 Toxicol Sci 107 1: 97-105