Phenotypic Cell-Based Screening of a High Content Imaging Cell Health Assay using the IN Cell Analyzer 2000

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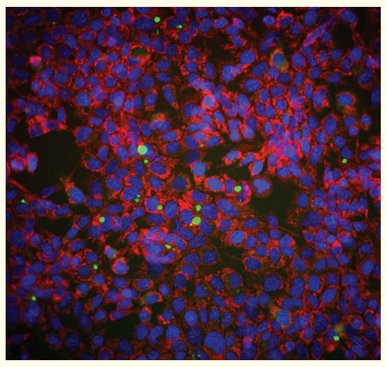
1. Introduction

- Cell Health assays play a key role in early drug discovery process as they study the effect of chemical substances or biotherapeutic agents on cells and provide a crucial means of ranking novel lead hits during screening.
- They help to gain a better understanding of changes at the cellular level in normal vs. disease pathologies as they now commonly employ a multi-parametric approach enabling the capture of both phenotypic and mechanistic information of the cells.
- Recently, the emergence of High Content Screening (HCS) has allowed the integration of cell based imaging with multiple readouts in relevant cells in a high-throughput format. This has allowed *in vitro* cell health assays to be developed into High Content Imaging screening assays, using automated fluorescence imaging to simultaneously analyse multi-parametric indicators of cellular toxicity that alter the phenotype of the cells.
- High Content Screening of cell health assays is now commonly being used as an early predictive toxicity assay to evaluate compound toxicity, in particular hepatotoxicity.
- We describe the assay development and establishment of an in-house cell health assay for high content phenotypic screening in the human hepatocellular carcinoma cell line, HepG2 cells (Figure 1) using the IN Cell Analyzer 2000 and Genedata Screener analysis software. Validation of the assay is shown using tool compounds and initial screening of the FDA set.

Figure 1: Human Hepatocellular Carcinoma Cell Line, HepG2 cells



Normal HepG2 cells stained with four live-staining fluorescent dyes (Invitrogen) and imaged on IN Cell Analyzer 2000. Nuclei- Hoechst (blue); Mitochondria- TMRM (red); Cellular Calcium- Fluo-4 AM (green); Dead nuclei- TOTO-3 (dark red). 20x objective.

2. Materials and Methods

The assay development included miniaturising from 96 to 384-well format; optimising compound addition, incubation time and dyes addition; setting up acquisition and analysis protocols on the IN Cell Analyzer and optimising handling and visualisation of data. This led to a final method that was amenable for High Content Screening.

Cell culture: HepG2 cells (EACC origin) were cultured a week before assay in EMEM inc. NEAA (Lonza) with 10% Fetal Bovine Serum and 2mM GlutaMAX. 1% Penicillin/Streptomycin was added to media during cell plating.

Cell Health Assay: HepG2 cells were plated out in 25ul as 2000 cells per well into 384-well PDL coated; black µClear plates (Grenier Bio-one) and incubated overnight at 37°C. After 24h, compound addition onto assay plates was done on Biomek robot and incubated for 72h at 37°C. End-point imaging was performed using four live staining fluorescent dyes (Hoechst 33342, TMRM, TOTO-3 Iodide; Fluo-4 AM (Invitrogen) (Table 1), added onto assay plates using Biomek robot and incubated 1h at 37°C. Imaged on IN Cell Analyzer 2000 using four wavelength channels followed by analysis on IN Cell Workstation software using Multi-target analysis. Data (images + cell measurements) imported into Genedata Screener analysis software, High Content Extension for the handling and visualisation of data.

Table 1: Live Staining Fluorescent Dyes

Dye name	Final conc (uM)	Excitation/ Emission peaks (nm)	Channel	Output image	Objects identified
Hoechst 33342	0.8	365/515	1	DAPI- Blue channel	All nuclei
Fluo-4 AM	0.02	475/515	2	FITC- Green channel	Cellular calcium
TMRM	4	549/600	3	Texas Red- Orange/Red channel	Mitochondria
TOTO-3	5	655/730	4	Cy5-Red channel	Dead cells nuclei

Details on the four live staining fluorescent dyes used for end-point imaging in the Cell Health High Content Screening assay.

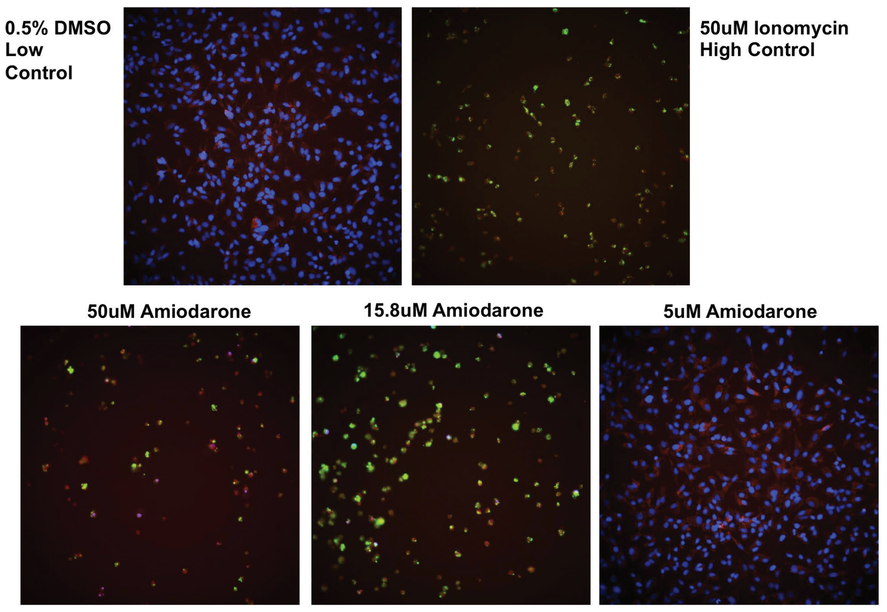
References

O'Brien PJ, et al. (2006). High concordance of drug-induced human hepatotoxicity with in vitro cytotoxicity measured in a novel cell-based model using high content screening. Arch Toxicol. 80(9):580-604.

3. Validation of the Cell Health Assay

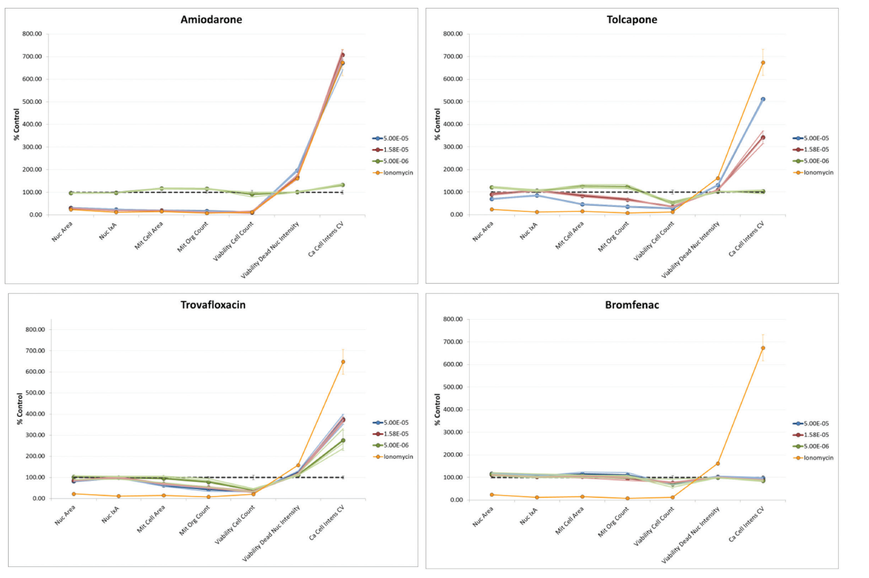
Tool compounds consisting of drugs withdrawn from the market or known to cause hepatotoxicity (MRCT Annotated set) was used to optimise and validate the Cell Health assay and the handling and visualisation of data.
Exemplar images acquired on the IN Cell Analyzer 2000 is shown in figure 2. Profile plots generated in an Excel spreadsheet template and dose-response curves generated in Genedata Screener analysis software for representative tool compounds are shown in figures 3 and 4.

Figure 2: Exemplar Images Acquired on IN Cell Analyzer 2000



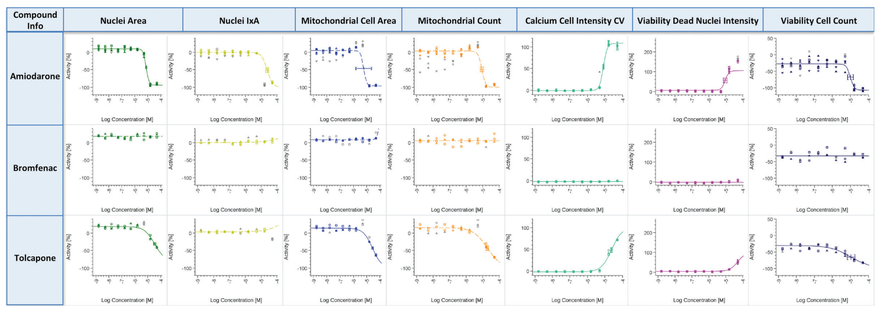
HepG2 cells treated +/- with compound for 72h then stained with fluorescent dyes and imaged on IN Cell Analyzer 2000. Nuclei- Hoechst (blue); Mitochondria- TMRM (red); Cellular Calcium- Fluo-4 AM (green); Dead nuclei- TOTO-3 (dark red). 20x objective.
HepG2 cells treated with low control (media + 0.5% DMSO) shows well defined normal nuclei and healthy mitochondria with no presence of cellular calcium or dead nuclei. In contrast, high control (50µM Ionomycin) significantly affects the phenotype of HepG2 cells with no viable nuclei or healthy mitochondria present, only dead nuclei is seen with an increase in cellular calcium, indicating cellular toxicity. Amiodarone, a tool compound shows a dose-dependent response on HepG2 cells with changes in cell phenotype signifying toxicity at 50µM and 15.8µM; however cells are healthy and viable at 5µM.

Figure 3: Profile Plots of Exemplar Tool Compounds



HepG2 cells treated +/- with compound for 72h and imaged on IN Cell Analyzer 2000. Profile plots generated in an Excel spreadsheet template is shown for representative tool compounds, 7 selected IN Cell analysis cell measurements (Nuclei area, nuclei IxA, mitochondrial cell area, mitochondrial count, viability cell count, viability dead nuclei intensity and calcium cell intensity CV) are normalised as a % against low control. Any cell parameter that deviates from 100% baseline represents a change in cell phenotype and cellular toxicity. Varying degrees from none to a dose-dependent compound effect on each cell parameter is seen. Each profile plot displays a baseline at 100% (dotted line), high control 50uM Ionomycin (orange line) and compound at 3 concentrations- 50µM (blue line), 15.8µM (red line) and 5µM (green line) (mean ± SEM, triplicate curves).

Figure 4: Dose Response-Curves of Exemplar Tool Compounds



HepG2 cells treated +/- with compound for 72h and imaged on IN Cell Analyzer 2000. DR-curves generated in Genedata Screener analysis software is shown for representative tool compounds against the 7 selected cell parameters. A range of activities and potencies are seen at each cell parameter for each compound, Amiodarone has the most effect with dose-dependent responses at all cell parameters indicating cell toxicity, Tolcapone shows partial effects and Bromfenac has no effect at any cell parameter. Data is expressed as % activity against controls (mean ± SEM, triplicate curves).

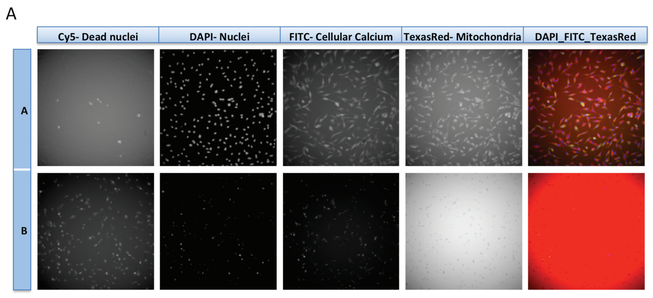
4. High Content Screening in the Cell Health Assay

An initial High Content Screen of the FDA set (960 compounds) was carried out in the Cell Health assay, this consisted of 4 runs (2 runs at 50µM and 2 runs at 25µM single shot final concentrations) with low (media + 0.5% DMSO) and high (50µM Ionomycin) controls on each plate. Figure 5 illustrates exemplar results from one run generated by Genedata Screener analysis software with a comparison of the 2 runs at 25µM shown in Figure 6. Genedata Screener generated compound hit lists for each run based on filter criteria that would show a change in cell phenotype at each cell parameter. 180 compounds were hits in both 50µM and 25µM runs, now triaged to 56 compounds based also on chemical structures and similarities.

Figure 5: High Content Screen Results Generated by Genedata Screener

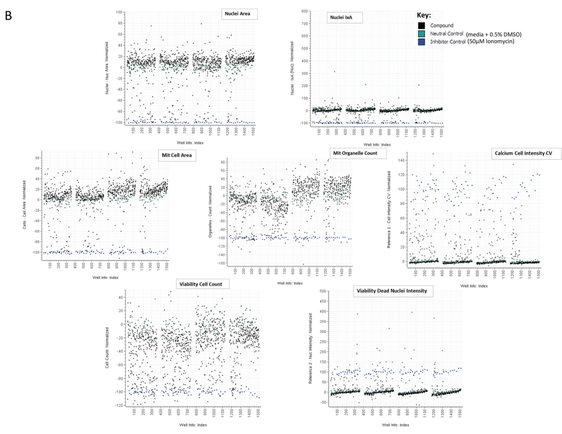
HepG2 cells treated +/- with FDA set compounds for 72h, imaged on IN Cell Analyzer 2000 and analysed in IN Cell Workstation and Genedata Screener analysis software. Exemplar results from one run generated in Genedata Screener are shown.

A



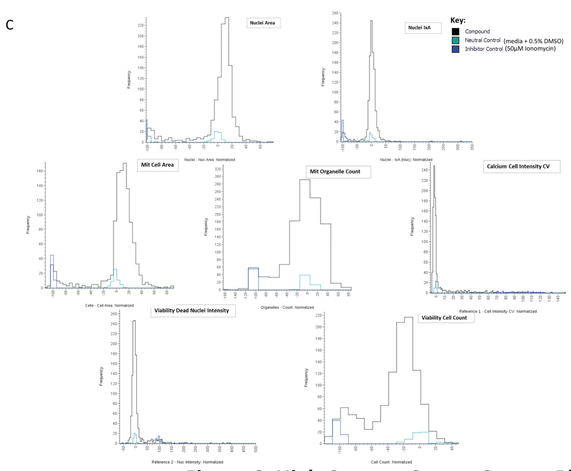
A) Exemplar images acquired on the IN Cell Analyzer for 2 compounds from the screen. Images from each of the 4 wavelength channels are shown- Cy5 (dead nuclei); DAPI (nuclei); FITC (Cellular Calcium); Texas Red (Mitochondria) and fused images of DAPI_FITC_Texas Red. Compound A displays minimal effect on the cell phenotype, indicating little cellular toxicity. In contrast, compound B appears toxic by altering the cell phenotype- affecting viable nuclei and healthy mitochondria by increasing dead nuclei and cellular calcium.

B



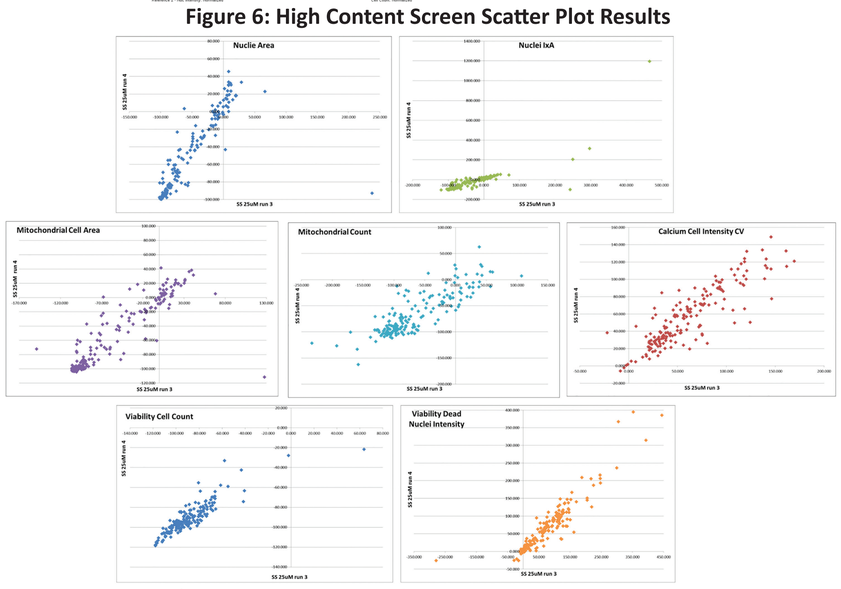
B) Scatter plots showing the effect of compounds and controls from the screen against the normalised activity of the 7 cell parameters. A spread of activities is seen with compounds at each cell parameter.

C



C) Frequency distribution plots showing the distribution of compounds and controls against the normalised activity of the 7 cell parameters. A range of activities and distributions are seen with compounds at each cell parameter; however a high frequency is seen in the low control range.

Figure 6: High Content Screen Scatter Plot Results



HepG2 cells treated +/- with FDA set compounds for 72h, imaged on IN Cell Analyzer 2000 and analysed in IN Cell Workstation and Genedata Screener analysis software. Comparison of the compound hit lists generated in Genedata Screener for the 2 runs at 25µM is illustrated as scatter plots for each cell parameter. A good correlation between the 2 runs is seen at each cell parameter, indicating reproducible and robust results with a range of activities.

5. Summary

- High Content Screening of Cell Health assays are increasingly being used in drug discovery to help gain a better understanding of changes in cell phenotype and as an early predictive compound toxicity assay.
- We have successfully developed and established in-house a High Content Imaging Cell Health Assay in HepG2 cells using the IN Cell Analyzer 2000 and Genedata Screener.
- Validation of the assay was done using tool compounds followed by a successful initial screen of the FDA set, full curves confirmation studies with a triaged compound hit list is now to be carried out. Exploring other relevant cells (e.g. stem-cell derived primary Hepatocytes) is also being investigated.
- The aim is to use this assay for Phenotypic cell-based High Content Screening of further subsets of the MRCT compound libraries and in-house compounds allowing us to gain much more detailed phenotypic information related to cell health and indications of cellular toxicity.