High-Throughput Phenotypic Profiling (HTPP) of HepaRG Cells Using the Cell Painting Assay

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Background

Cell Painting is an assay in which cells are labeled with six fluorescent dyes, imaged and analyzed to create a phenotypic profile. This assay has been identified as a promising candidate for high throughput hazard screening and has the potential to aid in the reduction of animal usage in toxicity testing. To date, most Cell Painting studies have been conducted in U-2 OS osteosarcoma cells, a human cancer-derived model with no appreciable metabolic activity. The absence of metabolic competence in the U-2 OS model precludes evaluating the toxicity of metabolites generated by biotransformation of parent chemical. However, the Cell Painting assay is extensible to other cell models, including those which are metabolically competent. In this study, we adapted our existing laboratory, imaging and bioinformatics workflows for Cell Painting to the HepaRG cell model. HepaRG cells can be cultured in a two-dimensional configuration (a requirement for the Cell Painting assay) and differentiation to mature hepatic state resembles human primary hepatocytes and cholangiocytes and is metabolically active. As a first step in establishing the HepaRG Cell Painting assay, we tested a set of 20 candidate phenotypic reference chemical to characterize their effects on HepaRG cells and identify a subset of chemicals that could be used to track assay performance in larger screening campaigns. A chemical with a defined and distinct Cell Painting phenotypic profile can be used to ensure replicability of assay results and can be incorporated into the data analysis workflow in order to serve as an assay quality control check. *This poster does not necessarily reflect USEPA policy.*

Cell Painting Assay Modifications

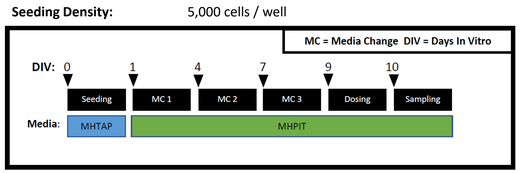
	Cell Painting "Standard" Workflow	HepaRG Workflow
Acquisition Mode	Confocal	Confocal
Imaging Objective	20X Water Immersion	63X Water Immersion
# Fields / Well	5	4
# Channels / Field	Hoechst (Nucleus) A488_Offset1 (ER) A488_Offset2 (Nucleolus) A568 (Golgi / Cytoskeleton / Plasma Membrane) A647 (Mitochondria)	Hoechst (Nucleus) A488 (ER & Nucleolus) A568 (Golgi / Cytoskeleton / Plasma Membrane) A647 (Mitochondria)
# Z-planes	1 / channel	7 / channel
Z-height / Z-spacing	Z-height optimized for each channel Spacing = N/A	9.6 µM overall height w / 1.6 µM spacing
# of Images / 384-well plate	9,600	43,008
Acquisition Time	~1 hr	~2 hr
File Size	~32 GB	~95 GB
Image Segmentation	Composite Image	Maximum projection composite image
Compartments	Cell Nucleus Cytoplasm Ring Plasma Membrane	Cell Nucleus Cytoplasm Ring Plasma Membrane

Table 1. The cell painting assay was previously optimized for adherent cultures, not cultures grown in suspension. As a result, considerations were made to capture the different levels of height along the Z-plane while still keeping data to a manageable size. Four fields of the well are measured instead of the standard five, and at a higher magnification to analyze a smaller area. Seven z-planes are imaged as opposed to one optimized plane to analyze the height dimension of the cells. This produces drastically more images compared to an adherent culture, but gathers the same level of detail as other adherent cultures. A noticeable drawback is a dramatic increase in file size.

Conclusions

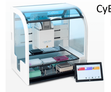
- The Cell Painting assay is compatible with HepaRG cells with minor modifications and produces similar results across technical and biological replicates.
- Of the twenty potential reference chemicals, seven produced a significant Cell Painting response well below the threshold for cytotoxicity. Of these seven, three are recommended for further use as reference chemicals: Bafilomycin A1, Cycloheximide, and Amiodarone hydrochloride.
- Reference chemicals which have been shown to be inactive in non-proliferating cells are also inactive in HepaRG cells.

Cell Culture Conditions and Equipment



Culture Vessel:
CellCarrier-384 Ultra,
Collagen-I coated (6057700)

Cell Seeding
Gyger Certus Flex
Selenoid Microdispenser



Cell Maintenance
CyBio Felix Automated
Pipetting station

Figure 1. Cell Culture Overview. HepaRG cells were thawed and seeded at 5,000 cells per well into PerkinElmer CellCarrier-384 Ultra plates using a Certus Flex Microdispenser and maintained for 10 days in vitro (DIV). Plates were kept in an incubator at 37°C with 5% CO_2, with media changes performed on DIV 1, 4, and 7. Chemical dosing occurred on DIV9, and was completed using a Labcyte Echo 550 acoustic liquid handler. Three individual cultures were created, and each culture contained four technical replicate wells for each treatment for a total of 12 technical replicates / treatment.

Pathway Altering Concentrations

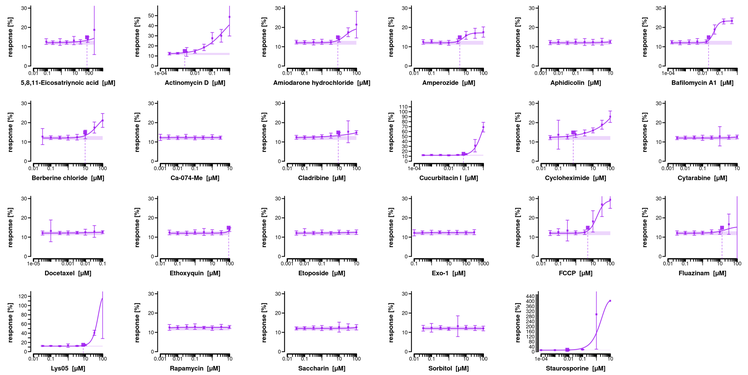


Figure 2. Concentration-Response Modeling to Determine Phenotype Altering Concentration (PACs) for 22 Chemicals. Phenotypic responses to test chemicals were evaluated using a global Mahalanobis distance modeling approach. Vehicle normalized and scaled phenotypic features (n = 1208) were used as input and single latent variable was output for concentration-response modeling. Panels are overall response magnitude (response) plotted against the $\log_{10}$ tested concentrations of each chemical in µM. X and Y axes are not equivalently scaled across panels, but instead scaled to individual chemical-level data. Cytotoxic doses are not excluded from each plot. Benchmark doses (BMDs) were calculated and curves were fit using the R package *tcplfit2*, and BMDs are indicated with a vertical dashed line. The set includes 20 test chemicals, two negative control chemicals (Saccharin and Sorbitol), and a cell viability positive control (Staurosporine).

A BMD could be determined for 13 of the 20 test chemicals (5,8,11-Eicosatriynoic acid, Actinomycin D, Amiodarone hydrochloride, Amperozide, Bafilomycin A1, Berberine chloride, Cladribine, Cucurbitacin I, Cycloheximide, Ethoxyquin, FCCP, Fluazinam, and Lys05.) at or below the highest tested concentration for the cell painting assay. Several chemicals which were classified as having no statistically significant altered phenotype are identified as cellular replication disruptors, notable due to the non-proliferative nature of HepaRG cells cultured as described above.

Reference Chemical Selection

The ideal reference chemical produces a distinctive, robust phenotypic profile with a PAC significantly below any cytotoxic dose. Tested chemicals which had detectable phenotypic changes were compared against these criteria as well as against each other, and the three most promising chemicals were: Bafilomycin A1, Cycloheximide, and Amiodarone hydrochloride.

Bafilomycin A1

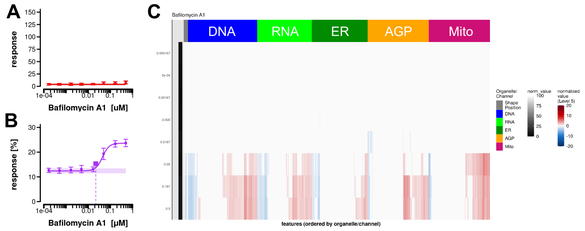


Figure 3. Phenotypic Profile for Bafilomycin A1. A: Cell Viability Plot; B: Cell Painting Plot, C: Heatmap depicting full range of tested concentrations on the y-axis, and extracted features on the x-axis grouped by category.

Cytotoxicity was not observed for Bafilomycin A1, and the calculated BMD is well within the tested concentration range. Feature responses are consistent across dose levels, and the profile produced is largely unique compared to phenotypic profiles produced by other test chemicals.

Cycloheximide

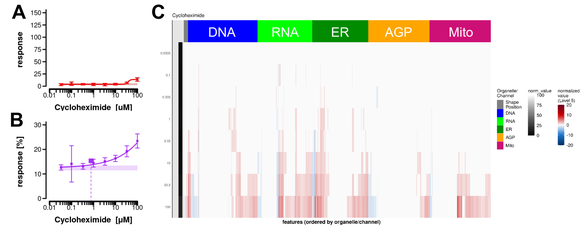


Figure 4. Phenotypic Profile for Cycloheximide. A: Cell Viability Plot; B: Cell Painting Plot, C: Heatmap depicting full range of tested concentrations on the y-axis, and extracted features on the x-axis grouped by category.

No cytotoxic dose for Cycloheximide was observed, although a slight uptick in response at the highest tested concentration implies potential cytotoxicity at higher doses. The Cell Painting BMD is nearly two orders of magnitude lower than the highest tested dose.

Amiodarone hydrochloride

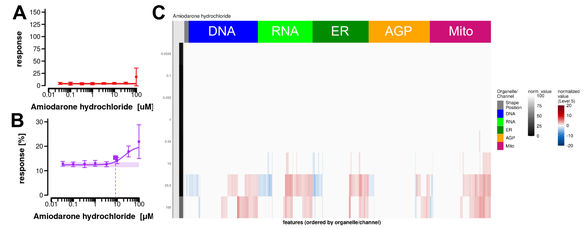


Figure 5. Phenotypic Profile for Amiodarone hydrochloride. A: Cell Viability Plot; B: Cell Painting Plot, C: Heatmap depicting full range of tested concentrations on the y-axis, and extracted features on the x-axis grouped by category.

No cytotoxic dose for Amiodarone hydrochloride was observed, although variability in the highest dose indicates potential cytotoxicity at higher doses.