BENCHMARK DOSE (BMD) MODELING OF IMAGE-BASED PHENOTYPIC PROFILING DATA YIELDS MORE POTENT ESTIMATES OF CHEMICAL BIOACTIVITY COMPARED TO CELL VIABILITY AND APOPTOSIS ASSAYS

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Abstract

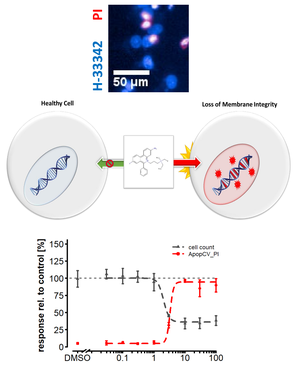
High-throughput imaging-based phenotypic profiling (HTPP) is a high-throughput chemical screening method that combines automated microscopy and image analysis to measure a large variety of morphological features at the single cell level. Here we describe workflows for concentration-response screening and image analysis using an HTPP assay that quantitatively evaluates changes in organelle morphology (i.e. Cell Painting) and calculation of in vitro points-of-departure (POD_{HTPP}) using high-throughput concentration-response modeling with the BMDExpress 2.2 software package. A set of 16 reference chemicals were tested in six human cell lines (U-2 OS, MCF-7, HTB-9, A549, ARPE-19, HepG2). Cell were plated in 384-well plates and after 24 h treated with 7 concentrations (semi-log spacing, n = 3/plate, 3 cultures) in a randomized pattern. After 48 h, cells were live labeled with MitoTracker (mitochondria), fixed, permeabilized and labeled with Hoechst-33342 (nuclei), SYTO14 (nucleoli) and fluorescent conjugates of concanavalin A (ER), phalloidin (cytoskeleton), and wheat germ agglutinin (Golgi/plasma membrane). A multiplexed cell viability (CV) and apoptosis (AP) assay was run in parallel. Confocal images were acquired using an Opera Phenix HCS system and analyzed using Harmony software, yielding ~1300 features per cell. Cell-level data were median absolute deviation (MAD) normalized to DMSO controls. BMD modeling was performed on well-level median values. Most chemicals (n=14) affected cell morphology. Distinct patterns of affected cellular features were observed across the chemical set and, in most cases, were consistent with observations from the literature. In general, the chemicals produced similar patterns, with highly correlated potency estimates, across the six different cell lines. For all compounds, Cell Painting BMDs (BMD_{CP}) were at least as sensitive as Cell Viability BMDs (BMD_{CV}). In some cell lines, BMD_{CP} were > 10x lower that BMD_{CV}. Screening of a larger set of chemicals (n=420) also demonstrated marked differences in BMD_{CP} and BMD_{CV} potency estimates. In summary, testing of diverse compounds yielded distinct patterns of affected features below the threshold for cytotoxicity, indicating that this profiling method could be used to derive *in vitro* potency estimates for screening level risk assessments. *This abstract does not necessarily reflect USEPA policy.*

Background and Experimental Design

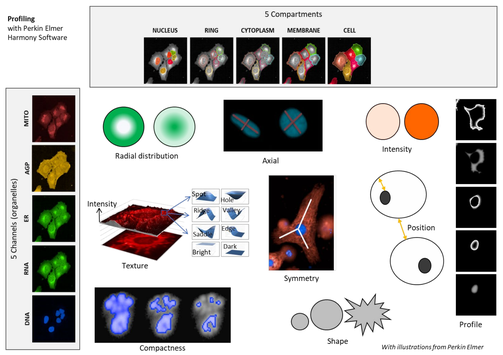
- HTPP is a chemical screening method that measures a large variety of morphological features of individual cells in *in vitro* cultures.
- Successfully used for functional genomic studies and in the pharmaceutical industry for compound efficacy and toxicity screening.
- May be used as an efficient and cost-effective method for evaluating the bioactivity of environmental chemicals.
- May be used to determine effect thresholds (i.e. *in vitro* point-of-departure, POD) for chemical bioactivity.

Experimental Design			
Cell Types	U-2 OS, MCF-7, A549, HepG2, HTB-9, ARPE-19	**Solvent controls / plate**	3
Exposure Duration	48 hours	**Replicates / plate**	24
# Chemicals	14 phenotypic reference chemicals; 2 negatives	**# Independent experiments**	3
# Concentrations	7 (1/2 log_{10} spacing)		

Assay 1: Cell Viability



Assay 2: High Throughput Phenotypic Profiling (i.e. Cell Painting)



Methods

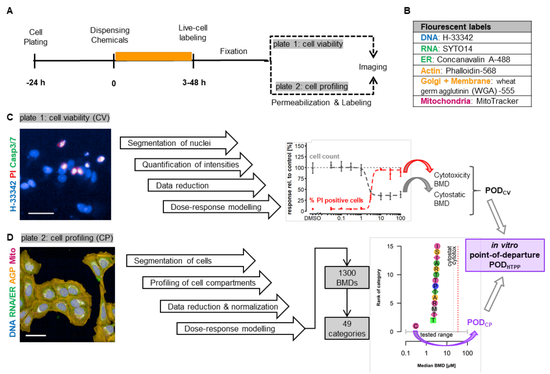
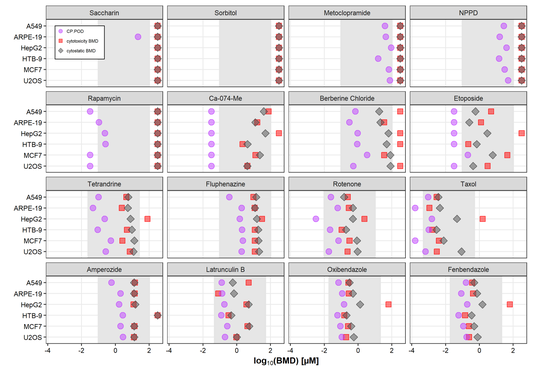


Figure 1: Assay and Data Analysis Workflows. (A) Diagram illustrating plate processing for both assay formats. (B) Table listing organelles labeled using the Cell Painting assay and the corresponding fluorescent probes. (C and D) Example images of labeled U-2 OS cells for both assays along with the image analysis steps for each. Cell Viability POD (POD_{CV}) values were calculated using the minimum of the cytostatic and cytotoxicity BMD values. Cell Painting POD (CP_{POD}) values were obtained from the median BMD of the most sensitive ontology. The in vitro point-of-departure (PODHTPP) was defined as the minimum of POD_{CV} and POD_{CP}.

Results: Reference Chemical PODs



Cell Line	Description
A549	Human lung adenocarcinoma epithelial cells
ARPE-19	Human retinal pigment epithelial cell line
HepG2	Human liver carcinoma cells
HTB-9	Human urinary bladder epithelial cells
MCF-7	Human breast adenocarcinoma cell line
U-2 OS	Human bone osteosarcoma epithelial cells

Figure 4: The 16 reference chemicals were tested in 6 cell types. The PODs for the Cell Painting assay (POD_{CP}), cytotoxicity BMD and cytostatic BMD were compared. In all cases, POD_{CP} was less than the cytotoxicity or cytostatic PODs. In many cases, the POD_{CP} was left-shifted as compared to the cytotoxicity or cytostatic PODs by several orders of magnitude. For each reference chemical, the Cell Painting and cytotoxicity / cell count PODs were remarkably consistent across cell lines.

⇨ **Cell Painting PODs < Cytotoxicity / Cytostatic PODs**

⇨ **PODs vary less than 2 order of magnitude across cell types.**

Results: Reference Chemical Phenotypes

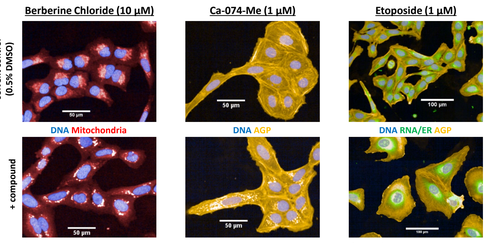


Figure 2: Examples of chemical-specific phenotypes. U-2 OS cells were treated for 48 h with reference compounds before cells were live-labeled for mitochondria, fixed, permeabilized and remaining labels applied. Images were acquired with a 20x water immersion objective on a Perkin Elmer Opera Phenix HCS System. Only selected channels are shown to highlight the resultant phenotypes. Affected endpoints are mentioned below the images.

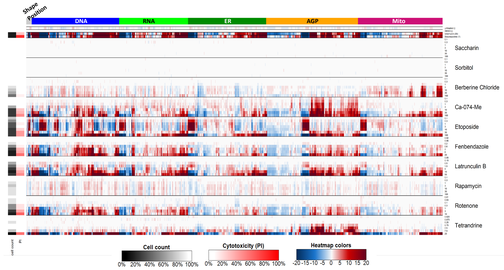


Figure 3: Examples of chemical-specific profiles. Standardized well-level data of U-2 OS cells were averaged across 3 technical and 3 biological replicates. Endpoints are ordered on the y-axis according to the corresponding channel / organelle. For each chemical, treatments are ordered from lowest to highest concentration tested. The coloring to the left indicates decreases in cell count (gray) and increases in cytotoxicity (red), respectively. Effects on cell morphology were observed at concentrations below the threshold for cytotoxicity.

Results: Chemical Screening

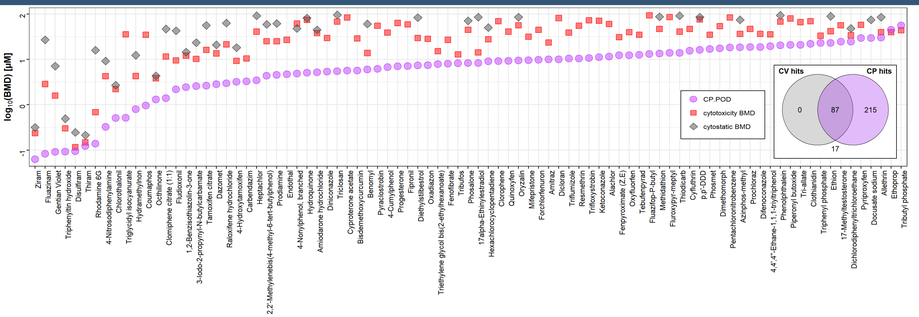


Figure 5: Comparison of PODs for Cell Painting (CP) and Cell Viability (CV) Assays. U-2 OS cells were screened in conc.-response mode using a 24 hour exposure duration. Chemicals were defined as a CV hit, if they their cytostatic EC_{50} or cytotoxic BMD_{320} was below the highest tested conc. Compounds were defined as a CP hit, if they had a POD below the highest tested conc (i.e. if for at least one ontology ≥ 30% of endpoints had a BMD below the highest tested conc). The inset compares the number of hits for the two assays. Chemicals with PODs for both CP and CV are displayed in the dot plot (n = 87). There is clear separation between the threshold for morphological effects and cytotoxicity in most cases. A majority of chemicals (215 / 319 = 67%) were active in the CP assay in the absence of cytotoxicity.

Conclusions and Future Directions

⇨ **A set of phenotypic reference chemicals with distinct response profiles and comparable potency across multiple cell types was identified.**

⇨ **The morphological phenotypes of the reference chemicals were consistent with those reported in the scientific literature for the Cell Painting assay.**

⇨ **The HTPP assay was able to replicate the morphological phenotypes for a set of phenotypic reference chemicals identified from the scientific literature.**

⇨ **In a screen of several hundred chemicals, HTPP detected concentration-dependent changes in cell morphology with a hit rate of ~95%.**

⇨ **Future directions include expanding the number of chemicals, and the number of cell types, screened using HTPP and incorporating the resulting information into existing approaches for hazard binning and prioritization using *in vitro* screening data.**