Characterizing Responses to Reference Chemicals in An Animal Component-Free System for High Throughput Phenotypic Profiling of Human Neural Progenitor Cells

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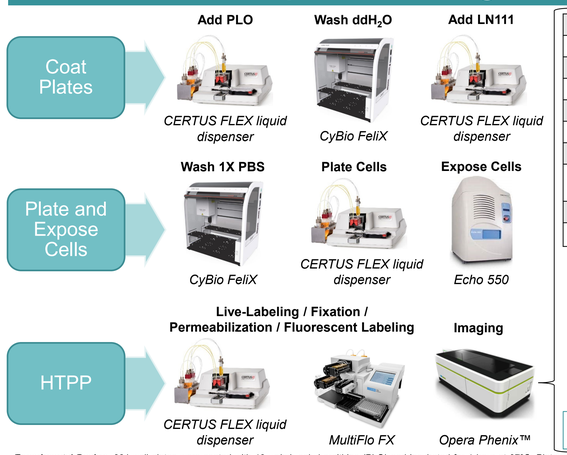
Background

- Traditional *in vivo* studies are too resource-intensive to address the large population of chemicals requiring hazard assessment, necessitating the development of *in vitro* high-throughput new approach methods (NAMs) that also serve to replace or reduce the use of animals in toxicity testing.
- High-throughput phenotypic profiling (HTPP) is a NAM that uses cell viability and Cell Painting assays to quantify morphological features, allowing for the derivation of chemical-specific phenotypic profiles and phenotype altering concentrations (PACs).
- Our laboratory has optimized HTPP for use with human neural progenitor (hNP1) cells to evaluate the potential for developmental neurotoxicity hazard (Culbreth et al., 2022; doi: 10.3389/ftox.2021.803987), but this approach relies on mouse-derived laminin (MDL) as a growth substrate.
- Seeking to replace the MDL with a non-animal product, our laboratory performed further work to identify BioLamina LN111 human recombinant laminin (HRL) as a suitable substitute growth substrate.

HTPP was used to screen 20 phenotypic reference chemicals, 2 negative controls (saccharin and sorbitol) and 1 cell viability control (staurosporine) in hNP1 cells seeded on HRL LN111, mirroring a previous screen performed with hNP1 cells seeded on MDL.

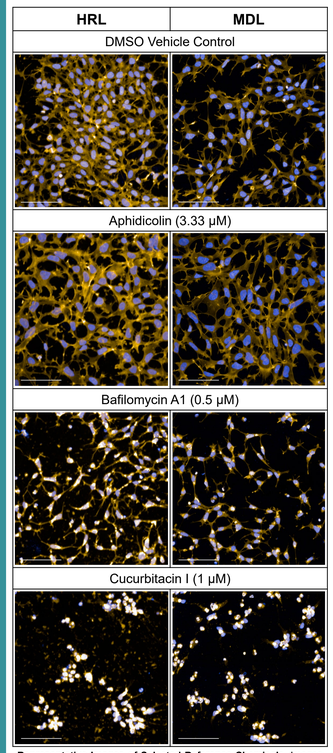
Chemical-specific phenotypic profiles were developed and compared across both screens.

Experimental Design

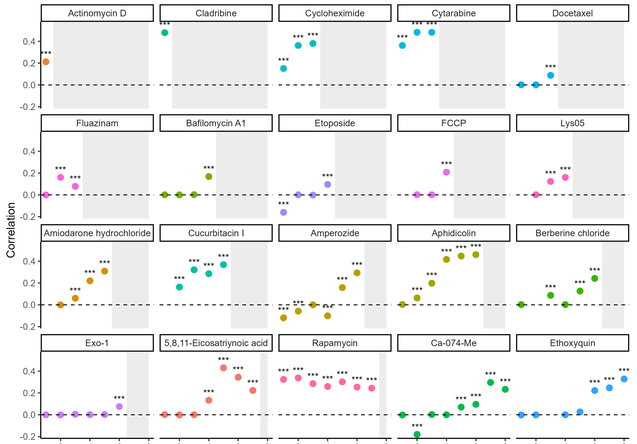


Experimental Design. 384-well plates were coated with 10 µg/mL poly-l-ornithine (PLO) and incubated for 1 hour at 37°C. Plates were then washed once with double-distilled water (ddH_2O) and coated with 5 µg/mL LN111. Plates were stored at 4°C for approximately 96 hours then washed once with 1X PBS before hNP1 cells were seeded at 6,000 cells/well. Cells were allowed to attach and grow for 24 hours prior to dosing. Test plates were exposed in unique randomized patterns of the 23 chemicals in 8-point concentration response format, including 24 DMSO vehicle control wells. All chemicals had two technical replicates per plate, apart from staurosporine, which had one. Cells were live-labeled with MitoTracker Deep Red for Cell Painting or Hoechst-33342 and propidium iodide for cell viability, then fixed 24 hours following exposure. Plates were stained for Cell Painting as applicable and stored at 4°C until imaging. Following imaging, endpoints in each cell compartment were analyzed in PerkinElmer Harmony Software. The collected data from this analysis was then reduced and normalized for concentration response modeling and phenotypic profile derivation. Each of the four biological replicates consisted of one cell viability plate and one cell painting plate. **Abbreviations:** actin cytoskeleton, Golgi Apparatus, and plasma membrane (AGP), endoplasmic reticulum (ER), Concanavalin-Alexa Fluor™ 488 (Concanavalin A-488), Wheat Germ Agglutinin-Alexa Fluor™ 555 (Wheat Germ Agglutinin-555), Alexa Fluor™ 568 Phalloidin (Phalloidin-568).

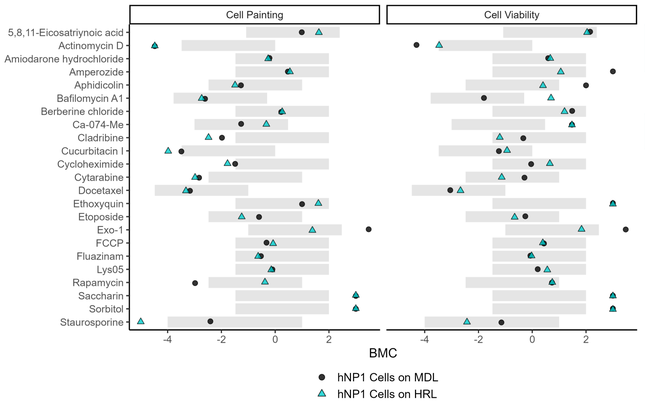
Significant Correlations Found Between Phenotypic Profiles of hNP1 Cells Seeded on Human Recombinant Laminin and Mouse Derived Laminin



Representative Images of Selected Reference Chemicals. Images were produced under identical conditions using the Opera Phenix™ High Content Imaging System. DMSO vehicle control wells represent the typical, untreated morphology of hNP1 cells on both MDL and HRL coating types. The seventh (Aphidicolin) or eighth (Bafilomycin A1 and Cucurbitacin I) highest concentration is shown for each chemical treatment to highlight the chemical-specific phenotypes. The two most affected channels across the three selected reference chemicals are shown, with nuclei visualized in the DNA channel (blue) and the actin cytoskeleton, Golgi Apparatus, and plasma membrane visualized in the AGP channel (yellow). Aphidicolin, Bafilomycin A1, and Cucurbitacin are shown because they were selected as reference chemicals to be used in future hNP1 screens on each laminin type. Scale bars are 100 µm.



Correlation of treatments between hNP1 cells seeded on HRL or MDL. The points in each plot represent the Kendall correlation (y-axis) between profiles generated for the given treatment in each screen. Each plot represents the data for a single chemical, with dose level (1-8) specified on the x-axis. Only non-cytotoxic doses were eligible for comparison, so light grey boxes span the cytotoxic doses with a lack of correlation data. Dose is designated as being between level 1 and level 8 as each chemical has a unique dose range. Black dashed lines indicate a Kendall correlation value of zero. Three asterisks above a point designate that the correlation had an FDR adjusted p-value of < .001, as determined via t-test of the individual correlations.



Cell Painting and cell viability potencies for hNP1 cells seeded on HRL or MDL. Each point represents the Cell Painting (left) or cell viability (right) log-scaled BMC (x-axis) for a given chemical (y-axis). Light blue triangles indicate the BMC for hNP1 cells seeded on HRL. Black circles indicate the BMC for hNP1 cells seeded on MDL. Light grey boxes indicate the dose range for a given chemical. **Abbreviations:** benchmark concentration (BMC).

1300 features/cell output by PerkinElmer Harmony Software

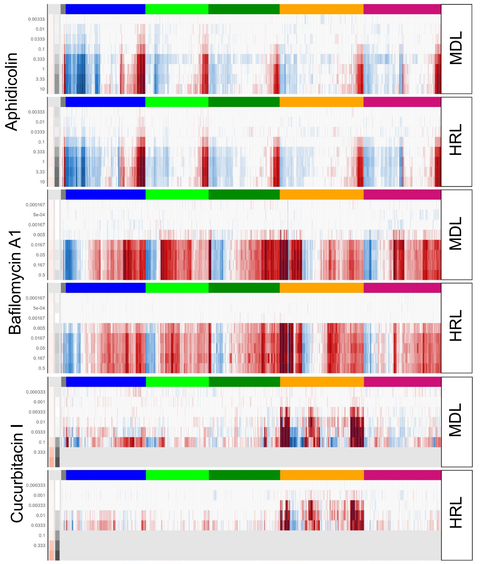
Each feature summarized to well level (median value/well) and normalized to DMSO vehicle control wells

Summarize to median across technical and biological replicates

Feature selection to remove redundant and uninformative features

Determine Kendall correlations between resultant profiles

Phenotypic Profiles for Selected Reference Chemicals



Full Phenotypic Profiles for Selected Reference Chemicals. Full profiles of all 1300 features for the three reference chemicals that were independently selected for use in later screens with both MDL- and HRL-seeded hNP1. For each chemical, the profiles derived from data for hNP1 cells seeded on MDL (Culbreth et al., 2022; doi: 10.3389/ftox.2021.803987) is shown directly above the profiles derived from data for hNP1 cells seeded on HRL. Profiles were derived as described in the outlined panel to the left, excluding the feature selection process. Features are ordered by organelle/channel along the x-axis and each section represents the lowest to highest dose in descending order, with individual rows corresponding to each dose level. Rows that are colored light grey met the cytotoxicity threshold that precluded profile generation. The coloring of the tiles indicates if the feature value is decreased (blue) or increased (red) relative to DMSO vehicle control, with darker coloration indicating a larger effect size. Annotation to the left of the heatmap represents cell viability data, with darker colors indicative of decreasing cell count (grey) or increasing propidium iodide response (red) relative to DMSO vehicle control. **Abbreviations:** propidium iodide (PI), mouse derived laminin (MDL), human recombinant laminin (HRL), actin cytoskeleton, Golgi Apparatus, and plasma membrane (AGP).

Conclusions and Future Directions

- LN111 HRL is a comparable growth substrate to MDL.
- Most chemicals had cell viability and Cell Painting derived BMCs within one order of magnitude for LN111 HRL compared to MDL.
- For most chemicals, phenotypic profile similarity across laminin types increased as a function of dose and was positively correlated.
- Aphidicolin, Bafilomycin A1, and Cucurbitacin I were selected as reference chemicals across both laminin types.
- More than 1,500 chemicals are now being screened in hNP1 cells seeded on LN111 across two different chemical sets: 282 developmental neurotoxicology-relevant chemicals and 1,280 pharmacologically active chemicals.

United States Environmental Protection Agency

References

Culbreth M, Nyffeler J, Willis C and Harrill JA (2022) Optimization of Human Neural Progenitor Cells for an Imaging-Based High-Throughput Phenotypic Profiling Assay for Developmental Neurotoxicity Screening. *Front. Toxicology* 3:803987. doi: 10.3389/ftox.2021.803987