Optimization of human neural progenitor cells for an imaging-based high-throughput phenotypic profiling assay for developmental neurotoxicity screening

Megan Culbreth[1], Clinton Willis[1], Johanna Nyffeler[1,2], Rick Brockway[1,3], Joshua Harrill[1]

[1]CCTE, ORD, U.S. EPA, Durham, NC; [2] ORISE, Oak Ridge, TN; [3]ORAU, Oak Ridge, TN

Megan Culbreth | culbreth.megan@epa.gov | 919-541-1193

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Section I. Introduction

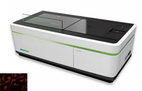
- There is a need to develop efficient and reliable *in vitro* assays for developmental neurotoxicity
- As such, our laboratory has utilized the hNP1 human neural progenitor cell line in a previously established high-throughput phenotypic profiling (HTPP) assay
- HTPP allows for simultaneous visualization of multiple cellular organelles
- Potential chemical-induced perturbation of organelle morphology can be measured at the cell-level and data aggregated for concentration-response modeling
- HTPP requires cell seeding in 384-well plate format, however, the hNP1 cells have yet to be seeded in this format in our laboratory
- Furthermore, the hNP1 cells require pre-coating of the cell growth surface and are typically cultured in antibiotic-free conditions, a liability for high-throughput applications as inspection of thousands of assay wells is not feasible
- Thus, optimization of 3 laboratory procedures was required prior to chemical screening
  - (1) cell seeding density in 384-well plates
  - (2) cell surface coating
  - (3) cell growth and maintenance protocols to prevent contamination
- Each process utilized a combination of laboratory automation and microfluidics to promote reproducible and efficient preparation of test cultures

Section II. Experimental Design

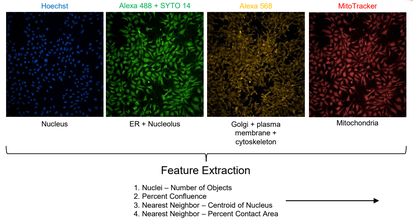
Step 1. Pre-coated 384-well plates
- Added 40 µL/well poly-l-ornithine (10 µg/mL; PLO) in ddH_2O
- Incubated at 4° C for 24-96 hours
- Washed 1X ddH_2O
- Performed on CyBio Felix

Step 2. Seeded hNP1 cells in 384-well plates
- Diluted laminin (20 µg/mL; unless otherwise stated) in cell culture suspension
- Seeded passage 10 cells at 40 µL/well with the CERTUS FLEX

Step 3. Fixed and immunostained cells 48 hours after plating
- 48 hours allows for 24 hours of cell attachment and growth, and 24 hours for subsequent chemical exposure
- Added MitoTracker to live cells with CERTUS FLEX
- Fixed (PFA), washed (1X PBS), and stained cells with combination of MultiFlo FX and CERTUS FLEX



Step 4. Imaged and analyzed cells on the Opera Phenix High-Content Screening System



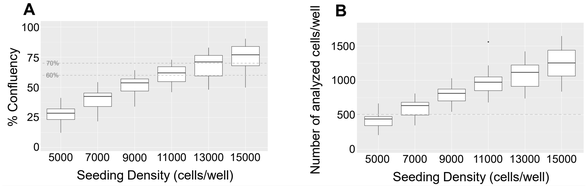
Step 5. Data analysis in RStudio
Tidyverse (ggplot2) package

Section III. 10000 cells/well selected as optimal seeding density for hNP1 cells in 384-well plate format

Table 1. Extrapolation of hNP1 seeding density to 384-well plate format

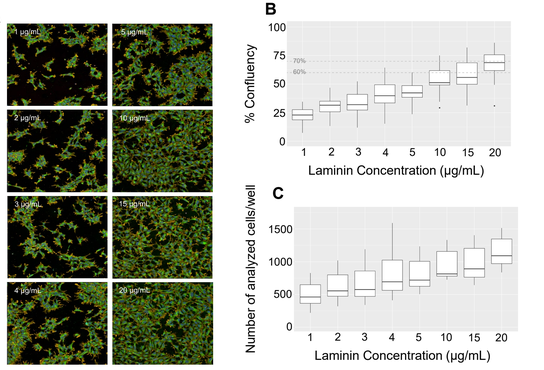
	96-well	384-well
well diameter	6.4 mm	3.26 mm (width)
area	32.17 mm^2	10.63 mm^2
cells/well	15000	5000

hNP1 cells have previously been seeded at 15000 cells/well in 96-well plate format for high-content image analysis at the U.S. EPA. Relative to the area of an individual well, this would extrapolate to approximately 5000 cells/well in 384-well plate format. 5000 cells/well was the lower limit assessed in our seeding density experiments.



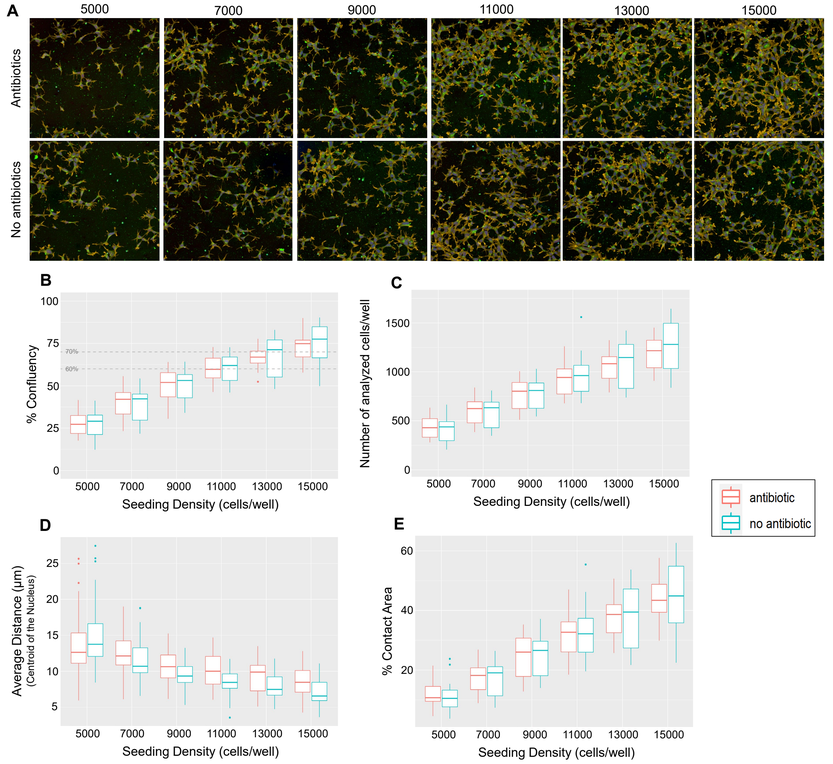
Section III. Determination of optimal seeding density for hNP1 cells in 384-well format. For the HTPP assay, our laboratory targets a confluency of 60-70% and a minimum of 500 cells analyzed in 5 fields-of-view. This allows for appropriate cell segmentation and subsequent modeling of extracted cell-level features. hNP1 cells were seeded at respective seeding densities. Percent (%) confluency was calculated as (total area of the cell bodies/total area imaged) x 100 (A). Number of analyzed cells/well is the number of intact Hoechst-positive cells within the 5 fields-of-view (B). Cells seeded at 9000-11000 cells/well resulted in approximately 60-70% confluence and facilitated the measurement of roughly 800-1000 cells/well. Therefore, 10000 cells/well was selected as the appropriate seeding density for hNP1 cells in the HTPP assay.

Section IV. 20 µg/mL laminin required for appropriate hNP1 cell attachment and growth



Section IV. Evaluation of minimum laminin concentration required for appropriate hNP1 cell attachment and growth. hNP1 cells are typically grown on 20 µg/mL laminin coated surfaces. This is a relatively high concentration and will be expensive to implement in high-throughput applications. Therefore, we wanted to determine whether lower laminin concentrations would yield a similar cell growth pattern as 20 µg/mL. Cells were seeded at 10000 cells/well at the respective laminin concentrations. A representative image for each laminin concentration is displayed (A). Percent (%) confluency (B) and number of analyzed cells/well (C) were quantified as in section III. Data represent three biological replicates. All concentrations examined were significantly different from 20 µg/mL by one-way analysis of variance (ANOVA). Based on these data and practical considerations we decided to proceed with 20 µg/mL laminin in subsequent experiments.

Section V. Antibiotic addition in the hNP1 cell culture media does not alter cell growth or cell-cell proximity phenotypes



Section V. Assessment of potential antibiotic effects on hNP1 cell growth and cell-cell proximity phenotypes. hNP1 cells are typically grown in antibiotic-free conditions, not ideal for high-throughput automated applications. We want to evaluated whether antibiotics would alter the cell growth pattern. hNP1 cells were seeded at respective seeding densities in the presence (antibiotic) or absence (no antibiotic) of antibiotics. A representative image for each seeding density with or without antibiotics is displayed (A). Percent (%) confluency (B) and number of analyzed cells/well (C) were quantified as in section III. There was no significant effect on % confluency or number of analyzed cells/well by two-way ANOVA (seeding density x ± antibiotics). Average distance measured in microns (µm), a measure of the distance from the centroid of the cell nucleus to its nearest neighbor centroid of the cell nucleus (D) was significantly different by two-way ANOVA ($p = 0.017$). However, there was no main effect of antibiotic (one-way ANOVA). % Contact area measures the percent contact area of a cell mask with its nearest neighbor cell mask (E); there was no significant effect on this endpoint by two-way ANOVA. Data represent three biological replicates. Based on these data, antibiotics will be included in the cell culture media for subsequent experiments.

VI. Conclusions

This work demonstrates the optimization of hNP1 human neural progenitor cells for the high-throughput phenotypic profiling (HTPP) assay by establishing cell seeding density in 384-well plate format (10000 cells/well), a minimum laminin concentration required for cell attachment and growth (20 µg/mL), and addition of antibiotics to the cell culture media without alteration of cell phenotype. Appropriate cell and assay specific chemical controls can now be identified, and the hNP1 cell model utilized in developmental neurotoxicity chemical screening.

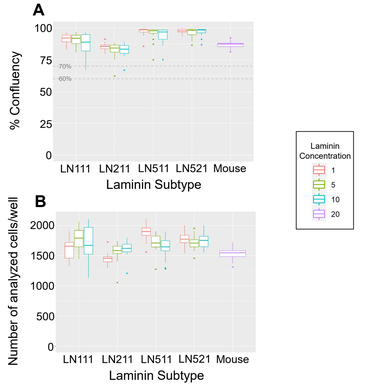
VII. Future Directions

A. Evaluate a set of reference chemicals to identify hNP1 cell HTPP assay specific controls. The reference chemical list includes compounds with established phenotypic profiles, as well as negative and cell viability controls.

Chemical Name	DTXSID	Type	Top Concentration
5,8,11-Eicosatrynoic acid	DTXSID10159018	Test chemical	250 uM
Actinomycin D	DTXSID9020031	Test chemical	1 uM
Amiodarone hydrochloride	DTXSID7037185	Test chemical	100 uM
Amperozide	DTXSID6048416	Test chemical	100 uM
Aphidicolin	DTXSID5036711	Test chemical	10 uM
Bafilomycin A1	DTXSID201015547	Test chemical	0.5 uM
Berberine chloride	DTXSID8024602	Test chemical	100 uM
Ca-074-Me	DTXSID50881386	Test chemical	3 uM
Cladribine	DTXSID8022828	Test chemical	100 uM
Cucurbitacin I	DTXSID501015546	Test chemical	1 uM
Cycloheximide	DTXSID6024882	Test chemical	100 uM
Cytarabine	DTXSID3022877	Test chemical	10 uM
Docetaxel	DTXSID0040464	Test chemical	0.1 uM
Ethoxyquin	DTXSID9020582	Test chemical	100 uM
Etoposide	DTXSID5023035	Test chemical	10 uM
Exo-1	DTXSID90893483	Test chemical	300 uM
FCCP	DTXSID40190494	Test chemical	100 uM
Fluazinam	DTXSID7032551	Test chemical	100 uM
Lys05	DTXSID9015548	Test chemical	100 uM
Rapamycin	DTXSID5023582	Test chemical	10 uM
Saccharin	DTXSID5021251	Negative chemical	100 uM
Sorbitol	DTXSID5023588	Negative chemical	100 uM
Staurosporine	DTXSID6041131	CV positive control	10 uM

B. Perform a developmental neurotoxicity (DNT) chemical screen. This DNT chemical screen includes approximately 200 compounds that are established DNT chemicals. All chemicals are EFSA_OECD.

C. Assess human recombinant laminin versus mouse laminin for appropriate hNP1 cell attachment and growth. hNP1 cells are typically grown on mouse laminin. In order to eliminate the use of animal products, our laboratory wants to determine whether hNP1 cells will grow on human recombinant laminin (LN111, LN211, LN511, or LN521). hNP1 cells were seeded at 10000 cells/well on respective laminin subtypes and concentrations. Percent (%) confluency (A) and number of analyzed cells/well (B) were quantified as in section III. Data represent one biological replicate and sixteen technical replicates. Note the y-axis scale in (B). % confluency and number of analyzed cells/well were above average for mouse laminin in this experiment. These data demonstrated human recombinant laminin have the potential to support appropriate hNP1 cell attachment and growth in the HTPP assay. **THIS IS PRELIMINARY DATA.**



VIII. Acknowledgments

The authors would like to thank Dr. Timothy Shafer for generously providing the hNP1 human neural progenitor cells, as well as Theresa Freudenrich and Kathleen Wallace for their technical advice and expertise. **This work does not reflect U.S. EPA policy.**