CCTE_Mundy_HCI_Cortical_Synap_Neur_Matur_NeuriteLength

1. General Information

1.1 Assay Title: CCTE's Synaptogenesis and Neurite Maturation Assay in Rat Cortical Cells for Neurite Length, Mundy Lab

1.2 Assay Summary: CCTE_Mundy_HCI_Cortical_Synap&Neurite_Matur is a multiplexed, cell-based-readout assay that uses rat primary cortical cells with measurements taken at 288.33 hours after chemical dosing in a microplate: 96-well plate. CCTE_Mundy_HCI_Cortical_Synap_Neur_Matur_NeuriteLength is one of eight components of the CCTE_Mundy_HCI_Cortical_Synap&Neurite_Matur assay. It measures synaptogenesis and neurite maturation related to neurite length using High Content Imaging of fluorescently labelled markers. Data from the assay component CCTE_Mundy_HCI_Cortical_Synap_Neur_Matur_NeuriteLength was analyzed at the endpoint CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_NeuriteLength in the positive analysis fitting direction relative to DMSO as the negative control and baseline of activity. Using a type of morphology reporter, loss -of-signal activity can be used to understand developmental effects.

1.3 Date of Document Creation: September 05 2024

1.4 Authors and Contact Information:

US Environmental Protection Agency (EPA) Center for Computational Toxicology and Exposure (CCTE)
109 T.W. Alexander Drive (Mail Code D143-02)
Research Triangle Park, NC 27711

1.5 Assay Source: The Mundy Lab at the EPA Center for Computational Toxicology and Exposure utilizes high content imaging to characterize chemical effects in neurodevelopment.

1.6 Date of Assay Development: For date of assay development, see *Section 6: Bibliography*.

1.7 References: For complete list of references, see *Section 6: Bibliography*.

1.8 Proprietary Elements: This assay is considered part of the developmental neurotoxicity in vitro battery. See the OECD Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity (DNT) In Vitro Testing Battery document: https://one.oecd.org/document/ENV/CBC/MONO(2023)13/en/pdf.

1.9 Assay Throughput: 96-well plate. The assay is medium-to-high throughput. It uses primary cortical rat neurons, seeded on a 96-well plate. Each plate may contain 6 test compounds at up to 9 concentrations, in addition to vehicle controls, assay positive controls, and blanks. Cell cultures are exposed to chemicals for 5 days prior to fixation and analysis. Each experiment should be replicated on three separate plates from the same culture preparation.

1.10 Status: The assay is fully developed, and data are publicly available in ToxCast's invitroDB.

1.11 Abbreviations:

AIC: Akaike Information Criterion
AOP: Adverse Outcome Pathway
CV: Coefficient of Variation
DMSO: Dimethyl Sulfoxide
ToxCast: US EPA's Toxicity Forecaster Program
tcpl: ToxCast Data Analysis Pipeline R Package
SSMD: Strictly Standardized Mean Difference

2. Test Method Description

2.1 Purpose: Changes in the neurite length are indicative of neurodevelopment.

The CCTE_Mundy_HCI_Cortical_Synap&Neur assay is designed to investigate changes in synaptogenesis and neurite maturation in response to chemical exposure in developing rat cortical neurons using a high-content imaging (HCI) technology. Synaptogenesis is one of several key processes of neurodevelopment. This endpoint measures the neurite length as a measure of neurite maturation.

2.2 Scientific Principles: During the development of the nervous system, many processes occur to give rise to a functional and healthy neural network and hence nervous system. These important neurodevelopmental processes may be disrupted by potential toxicants, resulting in developmental neurotoxicity. Among these processes is synaptogenesis, where individual cells form close connections that allow for communication by

chemical neurotransmitter. These interconnections between groups of neurons give rise to networks of cells that connect the nervous system together. This assay utilizes a high-content imaging solution to describe synaptogenesis in a rat primary cell culture, via the immunocytochemical labelling of cell bodies, neurites and presynaptic structures.

2.3 Experimental System: adherent NA primary cell used. Primary cortical cultures consist of a mixture of glutamatergic and GABAergic neurons, as well as glial cells (oligodendrocytes and a few microglia) as characterized by immunocytochemistry and functional responses to pharmacological agents (Freudenrich and Mundy, 2000; McConnell et al., 2011; Frank et al., 2017). Primary rat cortical cultures are prepared from the neocortex dissected from the central nervous system (CNS) of newborn (PND0) Long-Evans rat pups using a standard protocol (Section 3.7). In a typical culture, cells are isolated from the combined cortices of 3-5 pups, seeded onto a Poly-L-Lysine coated 96-well plate at a density of 10,000 cells/well and are allowed 2 hours to attach. Sex of pups is not determined, and cultures are presumed to consist of a mixture of male and female pups since multiple pups are used for each culture.

2.4 Metabolic Competence: Metabolic competence has not been characterized extensively to date in this assay.

2.5 Exposure Regime: Cells are plated onto 96 well plates and allowed 2 hr for attachment to the substrate. At days in vitro (DIV) 5, Cytosine Arabinoside (AraC) is added to arrest glial cell growth. Chemicals are dosed and a media change is performed at DIV 7 of differentiation and is continued through DIV 12 when the experiment is terminated. Cells are fixed with 20% paraformaldehyde and Hoechst Dye. Cells are stained using Millipore Mouse anti-MAP2MAB3418 (1:800) and Millipore Rabbit anti-Synaptophysinsc-1750 (1:250) primary antibodies and Alexa Fluor-488 goat anti-mouse and AlexaFluor-546 goat anti-rabbit secondary antibodies to label neuronal cell nuclei, synapses and neurites. A Cellomics ArrayScan VTi HCS Reader is used for automated image acquisition and analysis of neurite outgrowth.

ASSAY DESIGN SUMMARY	
Nominal number of tested concentrations: 9	Target (nominal) number of replicates: 4
Standard minimum concentration tested: 3 µM	Standard maximum concentration tested: 3000 µM
Key positive control: sodium orthovanadate (10uM)	Neutral vehicle control: DMSO or water
Baseline median absolute deviation for the assay (bmad): 6.284	
Response cutoff threshold used to determine hit calls: 30	
Detection technology used: HCS Fluorescent Imaging (Fluorescence)	

2.6 Response: This assay utilizes a high-content imaging solution to describe synaptogenesis and neurite length in a rat primary cell culture, via the immunocytochemical labelling of cell bodies and neurites. An automated image analysis protocol is employed to systematically identify targeted structures based on preassigned criteria. Ultimately, changes in the neurite length, neurite count, number of branch points, the number of pre-synaptic puncta, and number of synapses is quantified.

2.7 Quality and Acceptance Criteria: Each assay may utilize different acceptance criteria and quality assurance methods as it pertains to the individual assay platform and implementation. Pre-processing transformations may indicate issues in plates or wells by setting well quality (wllq) values to 0. Analytical QC calls per sample and substance can also be considered to understand the applicability domain of the chemicals for screening.

2.8 Technical Limitations: ToxCast data can provide initial (screening) information about the capacity for a chemical to illicit a biological response; caution is advised with extrapolation of these results to organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in

complex biological systems but can aid in the prioritization of compound selection for more resource intensive toxicity studies. See *Section 4.4*. for more information on the chemical applicability of the assay.

2.9 Related Assays: For related assays, consult the following assay lists or intended target families. This assay is present in the following assay lists:

DNT-IVB: Assays included in the developmental neurotoxicity in vitro battery, see https://www.regulations.gov/document/EPA-HQ-OPP-2020-0263-0006

Additionally, this assay was annotated to the intended target family of neurodevelopment.

3. Data Interpretation

The ToxCast Data Analysis Pipeline (tcpl) R package includes processing functionality for two screening paradigms: (1) single-concentration ("SC") and (2) multiple-concentration ("MC") screening. SC screening consists of testing chemicals at one concentration, often for the purpose of identifying potentially active chemicals to test in the multiple-concentration format. MC screening consists of testing chemicals across a concentration range, such that the modeled activity can give an estimate of potency, efficacy, etc. MC data is the focus of this documentation, with SC data processing metrics to be incorporated in the future.

3.1 Responses captured in prediction model: See *Section 2.6* for additional information on responses measured.

3.2 Data Analysis: All primary and secondary endpoints are assessed based on an immunocytochemical staining (ICC) of images for each well. Five days after chemical treatment cells were fixed with warm (37C) 4% paraformaldehyde containing 1.5 µg/ml Hoechst 33342 for 20 min followed by permeabilization and blocking steps. Cell bodies and dendrites were labeled using a rabbit primary antibody for microtubule associated protein 2 (MAP2) (Millipore Catalog AB5622, 1:800) and mouse antibody for synaptophysin (Santa Cruz catalog number SC-17750, 1:200) followed by AlexaFluor 488 goat anti-rabbit secondary antibody (Molecular Probes catalog number A11034, 1:500) and AlexaFluor 546 goat antimouse secondary antibody (Molecular Probes catalog number A11029, 1:500). Images are analyzed using the Cellomics Neuronal Profiling BioApplication (version 4) to measure neurite morphology. Optimization of nuclear masking and selection, cell body masking and selection, and neurite tracing parameters is performed on untreated cultures at DIV12 after initial plating. In each well, multiple unique fields-of-view are acquired until at least 200 neurons are counted. Eight morphological features are quantified (see table 8.4.1) Neurites are defined as processes > 10 µm in length.

Prior to the data processing, all the data must go through pre-processing to transform the heterogeneous data into a uniform format before it can be loaded into a database. Level 0 pre-processing is done in R by vendor/dataset-specific scripts with all manual transformations to the data documented with justification. Common examples of manual transformations include fixing a sample ID typo or changing well quality value(wllq) to 0 after identifying problems such a plate row/column missing an assay reagent.

Once data is loaded into the database, tcpl utilizes generalized processing functions provided to process, normalize, model, qualify, and visualize the data. To promote reproducibility, all method assignments must occur through the database and should come from the available list of methods for each processing level. Assigned multiple concentration processing methods include:

Level 2: Component-specific corrections include:

1: none (Use corrected response value (cval) as is; cval = cval. No additional mc2 methods needed for component-specific corrections.)

Level 3: Endpoint-specific normalization include:

5: resp.pc (Calculate the normalized response (resp) as a percent of control, i.e. the ratio of the difference between the corrected (cval) and baseline (bval) values divided the difference between the positive control (pval) and baseline (bval) values multiplied by 100; resp = (cval-bval)/(pval-bval)*100.), 6: resp.multneg1 (Multiply the normalized response value (resp) by -1; -1*resp.), 11: bval.apid.nwlls.med (Calculate the baseline value (bval) as the plate-wise median, by assay plate ID (apid), of the corrected values (cval) for neutral control wells (wllt = n).), 32: pval.zero (Set the positive control value (pval) to 0; pval = 0.)

Level 4: Baseline and required tcplFit2 parameters defined by:

2: bmad.aeid.lowconc.nwells (Calculate the baseline median absolute value (bmad) as the median absolute deviation of normalized response values (resp) for neutral control wells (wllt = n). Calculate one standard deviation of the normalized response for neutral control wells (wllt = n); onesd = sqrt(sum((resp - mean resp)^2)/sample size - 1). Onesd is used to establish BMR and therefore required for tcplfit2 processing.)

Level 5: Possible cutoff thresholds, where higher value for endpoint is selected, include:

1: bmad3 (Add a cutoff value of 3 multiplied by the baseline median absolute deviation (bmad) as defined at Level 4.), 19: pc30 (Add a cutoff value of 30. Typically for percent of control data.)

Level 6: Cautionary flagging include:

5: modl.directionality.fail (Flag series if model directionality is questionable, i.e. if the winning model direction was opposite, more responses (resp) would have exceeded the cutoff (coff). If loss was winning directionality (top < 0), flag if count(resp < -1*coff) < 2*count(resp > coff). If gain was winning directionality (top > 0), flag if count(resp > coff) < 2*count(resp < -1*coff).), 6: singlept.hit.high (Flag single-point hit that's only at the highest conc tested, where series is an active hit call (hitc >= 0.9) with the median response observed above baseline occurring only at the highest tested concentration tested.), 7: singlept.hit.mid (Flag single-point hit that's not at the highest conc tested, where series is an active hit call (hitc >= 0.9) with the median response observed above baseline occurring only at one concentration and not the highest concentration tested.), 8: multipoint.neg (Flag multi-point miss, where series is an inactive hit call (hitc < 0.9) with multiple median responses observed above baseline.), 9: bmd.high (Flag series if modeled benchmark dose (BMD) is greater than AC50 (concentration at 50 percent maximal response). This is indicates high variability in baseline response in excess of more than half of the maximal response.), 10: noise (Flag series as noisy if the quality of fit as calculated by the root mean square error (rmse) for the series is greater than the cutoff (coff); rmse > coff.), 11: border (Flag series if borderline activity is suspected based on modeled top parameter (top) relative to cutoff (coff); |top| <= 1.2(coff) or |top| >= 0.8(coff).), 13: low.nrep (Flag series if the average number of replicates per concentration is less than 2; nrep < 2.), 14: low.nconc (Flag series if 4 concentrations or less were tested; nconc <= 4.), 15: gnls.lowconc (Flag series where winning model is gain-loss (gnls) and the gain AC50 is less than the minimum tested concentration, and the loss AC50 is less than the mean tested concentration.), 17: efficacy.50 (Flag low efficacy hits if series has an active hit call (hitc >= 0.9) and efficacy values (e.g. top and maximum median response) less than 50 percent; intended for biochemical assays. If hitc >= 0.9 and coff >= 5, then flag when top < 50 or max_med < 50. If hitc >= 0.9 and coff < 5, then flag when top < log2(1.5) or max_med < log2(1.5).), 18: ac50.lowconc (Flag series with an active hit call (hitc >= 0.9) if AC50 (concentration at 50 percent maximal response) is less than the lowest concentration tested;if hitc >= 0.9 and ac50 < 10^logc_min, then flag.), 20: no.med.gt.3bmad (Flag series where no median response values are greater than baseline as defined by 3 times the baseline median absolute deviation (bmad); nmed_gtbl_pos and nmed_gtbl_neg both = 0, where nmed_gtbl_pos/_neg is the number of medians greater than 3*bmad/less than -3*bmad.)

The following is an aggregate endpoint summary of the number of samples and chemicals tested, as well as active or inactive hit calls (hitc) and predicted winning models for all samples tested in this endpoint.

SAMPLE AND CHEMICAL COVERAGE		
Number of samples tested: 295		Number of chemicals tested: 260
ACTIVITY HIT CALLS		
Active hit count: hitc≥0.9	Inactive hit count: 0≤hitc<0.9	NA hit count: *hitc<0*
114	181	*0*
WINING MODEL SELECTION		
Number of sample-assay endpoints with winning *hill* model:	10	
gain-loss (gnls) model:	11	

power(pow) model:	31
linear-polynomial (poly1) model:	79
quadratic-polynomial(poly2) model:	30
exponential-2 (exp2) model:	7
exponential-3 (exp3) model:	5
exponential-4 (exp4) model:	73
exponential-5 (exp5) model:	48

For each concentration series, several point-of-departure (POD) estimates are calculated for the winning model. The major estimates include: (1) the activity concentration at the specified benchmark response (BMR) (bmd), (2) the activity concentration at 50% of the maximal response (ac50), (3) the activity concentration at the efficacy cutoff (acc), (4) the activity concentration at 10% of the maximal response, and (5) the concentration at 5% of the maximal response.

3.3 Prediction Model: All statistical analyses were conducted using R programming language, employing the tcpl package to generate model parameters and confidence intervals. Each chemical concentration response series is fit to ten predictive models, encoded by the dependency package tcplfit2. The models include the constant, Hill, gain-loss, two polynomials (i.e. linear and quadratic), power, and four exponential variants. The polynomials, power, and exponential models are all based on BMDExpress2. The winning model (modl) is selected based on the lowest AIC value and is used to determine the activity (or hit call) for the concentration series. If two models have equal AIC values, then the simpler model (i.e. model with fewer parameters) wins. In invitrodb, levels 4 and 5 capture model fit information. mc4 captures summary values calculated for each concentration series, whereas mc4_param stores the estimated model parameters for all models fit to data in long format. mc5 captures the winning model selected and the activity hit call, whereas mc5_param stores the estimated model parameters for the selected winning model in long format. Activity for each concentration-response series is determined by calculating a continuous hit-call for the winning model, which is the product of three proportional weights. The first weight reflects whether there is at least one median response outside the efficacy cutoff band. Second, the top (or maximal change in the predicted response) is larger than the cutoff. The last weight reflects whether the AIC of the winning model is less than the constant model, i.e. the winning model is better fit than a flat line.

The continuous hit call value (hitc), fit category (fitc), and cautionary flags (mc6) can be used to understand the goodness-of-fit, enabling the user to decide the stringency with which to filter and interpret results. Hitc may be further binarized into active or inactive, depending on the level of stringency required by the user; herein, hitc greater than or equal to 0.90 is active, hitc between 0 and 0.90 is inactive, and hitc less than 0 is not applicable, but different thresholds may be used. Fitc was summarize curve behavior relative activity, efficacy, and potency comparisons between the AC50 and the concentration range screened. Cautionary flags on fitting were developed in previous versions of tcpl and have been stored at level 6. These flags are programmatically generated and indicate characteristics of a curve that need extra attention or potential anomalies in the curve or data. Users may review these filtered groupings to understand high-confidence curves.

3.4 Software: The ToxCast Data Analysis Pipeline (tcpl) is an R package that manages, curve-fits, plots, and stores ToxCast data to populate its linked MySQL database, invitrodb. Data for invitrodb v4.2 was processed using the tcpl v3.2. See *Section 7: Supporting Information* on the ToxCast program and tcpl R package.

4. Test Method Performance

4.1 Robustness: The following assay performance metrics surmise the robustness of the method i.e. the reliability of the experimental results and the prediction capability of the model used.

NEUTRAL CONTROL (well type = “n”)
Neutral control well median response value, by plate: *nmed*

	346.092
Neutral control median absolute deviation, by plate: *nmad*	21.741
Coefficient of variation (CV%) in neutral control wells: *(nmad/nmed)*100*	6.75%
POSITIVE CONTROL (well type = "p")	
Positive control well median response value, by plate: *pmed*	258.3
Positive control well median absolute deviation, by plate: *pmad*	20.475
Z Prime Factor for median positive and neutral control across all plates: *(1 - ((3 * (pmad + nmad)) / abs(pmed - nmed))*	NA
Strictly standardized mean difference (SSMD) for positive compared to neutral control wells: *((pmed - nmed) / sqrt($pmad^2$ + $nmad^2$)*	-3.263
Positive control signal-to-noise: *((pmed-nmed)/nmad)*	NA
Positive control signal-to-background: *(pmed/nmed)*	NA
NEGATIVE CONTROL (well type = "m")	
Negative control well median, by plate: *mmed*	271.03
Negative control well median absolute deviation value, by plate: *mmad*	31.229
Z Prime Factor for median negative and neutral control across all plates: *(1 - ((3 * (mmad + nmad)) / abs(mmed - nmed))*	NA
Strictly standardized mean difference (SSMD) for negative compared to neutral control wells: *((mmed - nmed) / sqrt($mmad^2$ + $nmad^2$)*	-1.151
Signal-to-noise (median across all plates, using negative control wells): *((mmed-nmed)/nmad)*	NA
Signal-to-background (median across all plates, using negative control wells): *(mmed/nmed)*	NA

4.2 Reference Chemical Information: Reference chemical curation is ongoing, and this section will be updated as more information becomes available.

4.3 Performance Measures and Predictive Capacity: The performance and predictivity for a given assay may be evaluated with a variety of performance statistics but is dependent upon available data. Predictive capacity (i.e. false negative, false positive rates) will be assessed when reference chemical information is available. Ideally, assays will have sufficient data on reference chemicals (i.e. positive and negative controls) to enable estimation of accuracy statistics, such as sensitivity and specificity.

ToxCast targets may align to a range of event types in the Adverse Outcome Pathway (AOP) framework, however each assay technology may have specific limitations, which may require user discretion for more complex interpretations of the data.

The median root mean squared error (RMSE) across all winning models for active hits was calculated as: 48.

4.4 Chemical Library Scope and Limitations: The ToxCast Chemical Library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. This chemical inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also consider practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Under these constraints, there were three major, interrelated drivers for chemical

selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary *in vivo* and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. Analytical QC calls per sample and substance should be considered to understand the applicability domain of the chemicals for screening.

5. Potential Regulatory Applications

5.1 Context of Use: Examples of end use scenarios could include, but are not limited to:

- *Support Category Formation and Read-Across:* The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across,
- *Priority Setting:* The assay might help prioritize substances within an inventory for more detailed evaluation,
- *Screening Level Assessment of a Biomarker or Mechanistic Activity or Response:* The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency; or
- *Integrated approaches to testing and assessment (IATA):* The assay may form one component of an IATA.

6. **Bibliography:** Harrill JA, Freudenrich T, Wallace K, Ball K, Shafer TJ, Mundy WR. Testing for developmental neurotoxicity using a battery of in vitro assays for key cellular events in neurodevelopment. Toxicol Appl Pharmacol. 2018 Sep 1;354:24-39. doi: 10.1016/j.taap.2018.04.001. Epub 2018 Apr 5. PMID: 29626487., Carstens, K. E., Carpenter, A. F., Martin, M. M., Harrill, J. A., Shafer, T. J., & Paul Friedman, K. (2022). Integrating Data From In Vitro New Approach Methodologies for Developmental Neurotoxicity. Toxicological sciences : an official journal of the Society of Toxicology, 187(1), 62–79. https://doi.org/10.1093/toxsci/kfac018

7. **Supporting Information:**

More information on the ToxCast program can be found at: https://www.epa.gov/chemical-research/toxicity-forecasting. The most recent version of downloadable data can be found at: https://www.epa.gov/chemical-research/exploring-toxcast-data-downloadable-data. The ToxCast Data Analysis Pipeline (tcpl) R package is available on CRAN or GitHub. Check out tcpl's vignette for comprehensive documentation describing ToxCast data processing, retrieval, and interpretation.