ArunA_NOG_BranchPointsPerNeurite

1. General Information

1.1 Assay Title: ArunA Biomedical's Neurite Outgrowth (NOG) Assay for Branch Points Per Neurite

1.2 Assay Summary: ArunA_NOG (Neurite Outgrowth) is a cell-based, image-based assay that uses human H9-derived embryonic differentiated neurons (hNN). Measurements were taken 48 hours after chemical dosing in a 96-well plate. ArunA_NOG_BranchPointsPerNeurite is an assay component measured from the ArunA_NOG assay. It is designed to make measurements of neurite outgrowth related to branch points per neurite, using a form of morphology reporter, as detected with fluorescence intensity signals by HCS Fluorescent Imaging technology. Data from the assay component ArunA_NOG_BranchPointsPerNeurite was analyzed at the endpoint, ArunA_NOG_BranchPointsPerNeurite, with bidirectional fitting relative to DMSO as the negative control and baseline of activity. Using a type of morphology reporter, gain-of-signal activity can be used to understand developmental effects. To generalize the intended target to other relatable targets, this assay endpoint is annotated to the neurodevelopment intended target family, where the subfamily is neurite outgrowth.

1.3 Date of Document Creation: September 05 2024

1.4 Authors and Contact Information:

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1.5 Assay Source: ArunA Biomedical is a privately owned biotechnology company and Contract Research Organization (CRO) formerly providing toxicology screening using neural stem cell-based assays.

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: hN2 cells and growth media were provided through Material Transfer Agreement #466-08 between the U.S. EPA and ArunA Biomedical, Inc.

1.9 Assay Throughput: 96-well plate. ArunA systems offer high throughput chemical screening in a 96-well format for the human neuroprogenitor (hNP) and human neural crest (hNC) migration and cell titer endpoints.

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 fluorescence intensity related to bIII-tubulin/DyLight1 488 antibody labelling is indicative of the neurite outgrowth.

Chemical-induced perturbations to cellular key events across neurogenic outcomes, including migration (neuroprogenitor and neural crest cells) and neural network formation (neurite length, neurite length, and branch points for neurites), in vitro can inform on cell-based prioritization of neurodevelopmental hazard potential.

2.2 Scientific Principles: During the development of the nervous systems, 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 migration of neuroprogenitors and neural crest cells (NCCs). Impaired neuroprogenitor and NCC migration can lead to cerebral malformations and neurodevelopmental disorders, such as diencephalic-mesencephalic dysplasia syndrome, cerebral palsy, cerebellar ataxia, and microcephaly.

2.3 Experimental System: adherent hNN cell line used. The hN2 cell line is derived from neuroepithelial cells of WA09 hESC (Thomson et al., 1998) origin according to a previously described protocol (Shin et al., 2005, Shin et al., 2006). Importantly, as opposed to other methods of deriving neural progenitors through three-dimensional neurosphere and embryoid body formations (Reubinoff et al., 2001, Zhang et al., 2001), these adherent monolayer cultures are uniformly exposed to growth factors and/or morphogens throughout their propagation. Neurogenic lineages from human embryonic stem cell line WA09 were locked into three neural differentiation states: neuroprogenitor (hNP1 – Cat no. 7009), neural crest (hNC – Cat no. 7029), and neural network (hNN – Cat no. hNJL7014). Prior to differentiation into hN2 cells the population was confirmed karyotypically normal, >95% nestin positive and <3% OCT-4 positive (Shin et al., 2006). The cells were produced in bulk by propagation for an additional 2 weeks beyond the neuroepithelial stage by removal of bFGF from the media and cryopreserved (ArunA Biomedical, Athens, GA) for end user applications. The hNP and hNC cell endpoints consisted of cell titer and migratory measurements whereas hNN cell endpoints consisted of neuron count and three neurite-specific metrics to assess network formation: neurite length, neurites per neuron, and branch points for neurites. For this study, ArunA Bio extended the differentiation period of the hNN cells by approximately two weeks more than in the original hN2 protocol. This allowed for increased neural network cell axonation leading to better quantitation of network-specific endpoints. The utility of dissociated hN2 cultures as an in vitro model for neurite outgrowth was assessed using automated high-content image analysis (HCA). In addition, the molecular phenotype of these cells was examined using immunocytochemical staining.

2.4 Metabolic Competence: H9-derived cells are locked at different neuronal developmental states of interest to DNT investigations of chemical exposures. Xenobiotic biotransformation potential has not been characterized.

2.5 Exposure Regime: Chemical treatment: Differentiated hNN cells were seeded and immediately exposed to test medium for 48 h. Following chemical exposure, cell bodies were stained with Hoechst 33,258 to quantitate viable neuron count and neurites were labeled with bIII-tubulin/DyLight1 488. High content imaging assessed the neurite outgrowth endpoints: neurite total length per neuron (μm), neurite count per neuron, and branch points per neurite using the methods described in (Harrill et al 2010). Measurements of hN2 morphology: (Beta)III-Tubulin stained cell cultures were allowed to warm to room temperature. Plates were then loaded into a Cellomics ArrayScan VTI HCS reader high-content imaging system (ThermoFisher Scientific, Waltham, MA) for automated image acquisition and morphometric analyses. This system consists of an epifluorescent microscope with an EXFO X-cite 120 metal-halide arc lamp, motorized imaging objectives, stage and excitation/emission filter wheel and a 12-bit high-resolution CCD camera connected to a Dell Intel Xenon computer terminal with 2 GHz processor. Image acquisition and storage was performed using the vHCS Scan software package, version 6.6.1.4. Matched fluorescent images of Hoechst-stained nuclei and (beta)III-tubulin/DyLight 488 immunolabeled cells were acquired using 365/515 (channel 1) and 475/515 (channel 2) nm excitation/emission filter couplings, respectively, with a 20× objective (Zeiss, Inc., Thornwood, NY). Fixed integration times for image acquisition in each channel were determined by manual sampling of control-treated wells across multiple plates. A matching pseudocolored composite image of Hoechst-stained nuclei (blue) and (beta)III-tubulin/DyLight 488 labeled cell bodies and neurites (green). The Neural Profiling BioApplication performs automated image analysis in a sequential manner as follows. Briefly, nuclei were identified in channel 1 as bright objects on a dark background. Nuclei with size and intensity values outside of the ranges determined a priori for viable cells were identified in the channel 1 image and rejected from further analyses. Spatial coordinates from the channel 1 image were then superimposed on the matching channel 2 image. Cell body masks in channel 2 were then cast based on positional data from channel 1 nuclei and a set of user-defined geometric and signal intensity-based parameters. Cell bodies corresponding to valid neurons were then selected and invalid cell bodies rejected. Parameters for valid cell body selection include the presence of exactly one nucleus within the cell body mask, a requirement that the nucleus met the gating criteria imposed in channel 1, a requirement that at least 25% of the nucleus perimeter is bounded by DyLight 488 labeled cytoplasm and a requirement that the total cell body area not exceed 4000 um2. Neurites emerging from the selected cell bodies were then individually traced and measured. For this study, neurites were defined as processes >10 um in length. Neurites were separated from cell bodies at points when the half-width of the labeled cytoplasm was less 3.6 um across. In the case of neurites with an ambiguous origin (i.e. appearing to emerge from or contact multiple cell bodies) the Neural Profiling

BioApplication traced the neurite from all potential origin points and retained the longest neurite for measurements of length and number of neurites per neuron. This effectively prevented repeated sampling of the same neurite segment within each image. Morphometric data from high-content image analysis (HCA) included measurements of the average number of neurites per neuron and total neurite length per neuron. Data for both endpoints were collected on cell-by-cell basis. The number of neurites and the cumulative length of all neurites associated with each cell body (i.e. total neurite length) were calculated for each cell meeting the selection criteria outlined above. Cell-level measurements were then averaged to obtain a mean measurement for the average number of neurites per neuron and total neurite length per neuron for the cell populations sampled within each well.

ASSAY DESIGN SUMMARY	
Nominal number of tested concentrations: 5	Target (nominal) number of replicates: 4
Standard minimum concentration tested: 1.2 µM	Standard maximum concentration tested: 100 µM
Key positive control: NA	Neutral vehicle control: DMSO
Baseline median absolute deviation for the assay (bmad): 0.228	
Response cutoff threshold used to determine hit calls: 0.683	
Detection technology used: HCS Fluorescent Imaging (Fluorescence)	

2.6 Response: The ArunA neurite outgrowth assay monitors changes in neurite length and number of branch points (both total number of branch points and number formed per neuron) in human neural network cells (hNN) derived from human embryonic stem cells. These different measurements are assessed following 48 hour incubations with test chemical to help predict the potential to disrupt neural network formation in developing human embryos.

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:

NA

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: High content imaging assessed the neurite outgrowth endpoints: neurite total length per neuron (µm), neurite count per neuron, and branch points per neurite. Plate-level raw data, provided by each assay source, were received by EPA from each contractor and analyzed using the ToxCast Pipeline (tcpl). Normalized response values for each assay endpoint were calculated as resp = log2(rval/bval) where rval, bval, and pval correspond to the raw value, the plate level DMSO control median, and the plate level positive/negative control median, respectively.

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:

7: resp.log2 (Transform the response values to log-scale (base 2).), 9: resp.fc (Calculate the normalized response (resp) as the fold change, i.e. the ratio of the corrected (cval) and baseline (bval) values; resp = cval/bal.), 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).)

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.), 28: ow_bidirectional_gain (Multiply winning model hitcall (hitc) by -1 for models fit in the negative analysis direction. Typically used for endpoints where only positive responses are biologically relevant.)

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: 60	Number of chemicals tested: 58

ACTIVITY HIT CALLS		
Active hit count: hitc≥0.9	Inactive hit count: 0≤hitc<0.9	NA hit count: *hitc<0*
10	25	*25*

WINING MODEL SELECTION	
Number of sample-assay endpoints with winning *hill* model:	1
gain-loss (gnls) model:	1
power(pow) model:	5
linear-polynomial (poly1) model:	30
quadratic-polynomial(poly2) model:	4
exponential-2 (exp2) model:	1
exponential-3 (exp3) model:	0
exponential-4 (exp4) model:	16
exponential-5 (exp5) model:	2

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*	0.16
Neutral control median absolute deviation, by plate: *nmad*	0.03
Coefficient of variation (CV%) in neutral control wells: *(nmad/nmed)*100*	18.15%
POSITIVE CONTROL (well type = "p")	
Positive control well median response value, by plate: *pmed*	NA
Positive control well median absolute deviation, by plate: *pmad*	NA
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($pmad^2$ + $nmad^2$)*	NA
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*	NA
Negative control well median absolute deviation value, by plate: *mmad*	NA
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:	NA

((mmed - nmed) / sqrt(mmad^2 + nmad^2)	
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: 2.

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:** Zurlinden TJ, Saili KS, Baker NC, Toimela T, Heinonen T, Knudsen TB. A cross-platform approach to characterize and screen potential neurovascular unit toxicants. Reprod Toxicol. 2020 Jun 24;96:300-315. doi: 10.1016/j.reprotox.2020.06.010. Epub ahead of print. PMID: 32590145.

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.