CCTE_Shafer_MEA_acute_spike_number

1. General Information

1.1 Assay Title: CCTE's Neural Acute Assay for Total Number of Spikes, Shafer Lab

1.2 Assay Summary: CCTE_Shafer_MEA_acute assay is conducted using Axion Biosystems 48 well microelectrode array (MEA) plates and Maestro recording system. Each well of the MEA plate contains a grid of 16 microelectrodes embedded in the culture surface. Electrically active cells, such as neurons, can be cultured over the electrodes, and electrical activity in these cells can be recorded extracellularly. The spontaneous firing of neurons is captured from each electrode on a microsecond timescale providing both temporally and spatially precise data. Because the recordings do not impact the health of the cells, multiple recordings can be made from the same neural network over time in culture. The CCTE_Shafer_MEA_acute assay uses primary cultures of rat cortical neurons. As these cultures mature over time, neural networks develop functional activity and form cohesive networks in which electrical activity can be highly coordinated. Baseline 40-minute recordings are made on days post-plating 13 or 15, followed by a secondary 40-minute recording after chemical exposure. CCTE_Shafer_MEA_acute_spike_number is a component of the CCTE_Shafer_MEA_acute assay. It measures the total number of spikes (action potential firings) in a 40-minute recording in a microelectrode array (MEA) using Axion Biosystems Maestro 768 channel amplifier with the AxIS adaptive spike detector. The recordings before and after chemical treatment are used to calculate a percent change in the spike number in each well. Data from the assay component CCTE_Shafer_MEA_acute_spike_number was analyzed into 1 assay endpoint. This assay endpoint, CCTE_Shafer_MEA_acute_spike_number, was analyzed in the negative analysis fitting direction relative to DMSO as the negative control and baseline of activity. Using a type of functional reporter, gain or loss-of-signal activity can be used to understand electrical activity.

1.3 Date of Document Creation: September 05 2024

1.4 Authors and Contact Information:

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1.5 Assay Source: The Shafer lab at the EPA Center for Computational Toxicology and Exposure focus on developmental neurotoxicity (DNT) hazard identificaton using microelectrode array (MEA) 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: Multi-well microelectrode array plates and assay readout detection technology and AxIS software are commercially available from Axion Biosystems Inc. (Atlanta Ga). MEA systems are also available from other manufacturers. Cell viability assays utilized are commercially available from Promega (Madison, WI). Cell viability assays are also available from other manufacturers.

1.9 Assay Throughput: 48-well plate. The assay uses primary cultures of rat cortical neurons, the Axion Biosystems 48 well microelectrode array (MEA) plates, and Maestro recording system.

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: The number of spikes is a measure of general activity in the network. Changes in electrical activity are indicative of effects on the spontaneous neural activity.

The CCTE_Shafer_MEA_acute assay is designed to investigate changes in neural network activity in response to chemical exposure in rat cortical neurons using a microelectrode array (MEA) technology. This endpoint measures the total number of spikes observed across the well over the recording period.

2.2 Scientific Principles: Nervous system function is sensitive to chemical perturbation. The microelectrode arrays (MEAs) offer an efficient approach to screen compounds for neuroactivity and distinguish between compound effects on firing, bursting, and connectivity patterns.

2.3 Experimental System: adherent primary cortical cell culture primary cell used. Primary rodent cortical cell cultures are prepared on site from the neocortex dissected from the central nervous system of newborn rats using a standardized protocol. In a typical culture, cells are isolated from the combined cortices of 3-5 pups, plated onto multiwell microelectrode array plates and allowed 2 hrs to attach. The cells are maintained in a humidified incubator at 37C and 5% CO2. 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. The primary culture model consists of glutamatergic (excitatory) neurons, GABAergic (inhibitory) neuron, astrocytes and sparse microglia (Harrill et al., 2011; Frank et al., 2017).

2.4 Metabolic Competence: The metabolic capacity of the primary cortical cultures has not been extensively studied. mRNA expression of various Cyp enzymes is low on DIV 1, however, by DIV 14, mRNA for Cyp 211c >> 4x1 > 2d4 > 1s1 > 1a1. Functional expression of these proteins has not been confirmed (Shafer et al., 2015).

2.5 Exposure Regime: Spontaneous activity in the cortical cultures was recorded using an Axion Biosystems Maestro 768 channel amplifier and Axion Integrated Studios (AxIS) v1.8 (or later) software. The amplifier recorded from all channels simultaneously (gain = 1200 ×; sampling rate = 12.5 kHz/channel): raw signals were filtered with a Butterworth band-pass filter (300–5000 Hz), which filters out slower local field potentials leaving only fast potentials, i.e., “spikes”, arising from extracellular currents associated with action potentials (Pine 2006; Wheeler and Nam 2011). On-line spike detection of filtered signals was conducted with the AxIS adaptive spike detector, using a threshold of 8 × the root mean squared (rms) noise on each channel. Any electrodes with rms noise levels greater than 5 µV were grounded (e.g., no data were recorded). Once grounded, an electrode was grounded for the duration of the experiment. All recordings were conducted at 37 C. Wells were deemed usable if on the day of the exposure ≥ 10 electrodes were active (defined as ≥ 5 spikes/min). On DIV 13 or 15, a minimum of three wells from one cortical culture preparation were treated with each compound (0.03–40 µM). Prior to recording baseline activity, each mwMEA plate was placed in the Maestro at 37 C and allowed to sit for 20 min while firing rates stabilized. Baseline activity (40 min) was recorded before the addition of each compound. An additional 40 min of spontaneous activity was recorded in the presence of each compound. Changes in network parameters relative to baseline were assessed following compound treatment.

ASSAY DESIGN SUMMARY	
Nominal number of tested concentrations: 7	Target (nominal) number of replicates: 3
Standard minimum concentration tested: 3 µM	Standard maximum concentration tested: 100 µM
Key positive control: tetrodotoxin	Neutral vehicle control: DMSO or water
Baseline median absolute deviation for the assay (bmad): 23.128	
Response cutoff threshold used to determine hit calls: 69.384	
Detection technology used: Axion Biosystems Maestro 768 channel amplifier with the AxIS adaptive spike detector (microelectrode array (MEA))	

2.6 Response: This endpoint measures the total number of spikes over the duration of the analysis.

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 neuroactivity.

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: Raw recordings were re-played and analyzed with the AxIS 2.3 Neural Statistics Compiler. (Operating Procedure for Axion Maestro Pro Microelectrode Array (MEA) system: I-BCTD-RADB-SOP-5037-0)

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.), 17: bval.apid.nwllslowconc.med (Calculate the baseline value (bval) as the plate-wise median, by assay plate ID (apid), of the corrected values (cval) of test compound wells (wllt = t) with a concentration index (cndx) of 1 or 2 or neutral control wells (wllt = n).), 49: pval.neg.100 (Calculate positive control value (pval) as -100 for endpoints in the down direction; pval = -100.)

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.)

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.)

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: 525		Number of chemicals tested: 508
ACTIVITY HIT CALLS		
Active hit count: hitc≥0.9	Inactive hit count: 0≤hitc<0.9	NA hit count: *hitc<0*
195	330	*0*
WINING MODEL SELECTION		
Number of sample-assay endpoints with winning *hill* model:	35	
gain-loss (gnls) model:	38	
power(pow) model:	56	
linear-polynomial (poly1) model:	122	
quadratic-polynomial(poly2) model:	75	
exponential-2 (exp2) model:	10	

exponential-3 (exp3) model:	3
exponential-4 (exp4) model:	130
exponential-5 (exp5) model:	49

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*	-35.927
Neutral control median absolute deviation, by plate: *nmad*	13.882
Coefficient of variation (CV%) in neutral control wells: *(nmad/nmed)*100*	-39.97%
POSITIVE CONTROL (well type = "p")	

Positive control well median response value, by plate: *pmed*	32.6
Positive control well median absolute deviation, by plate: *pmad*	25.575
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($pmad^2$ + $nmad^2$)*	2.357
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: *((mmed - nmed) / sqrt($mmad^2$ + $nmad^2$)*	NA
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: 49.

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:** NA

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.