Evaluation of *in vitro* New Approach Methodologies for Developmental Neurotoxicity

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Introduction

Background:

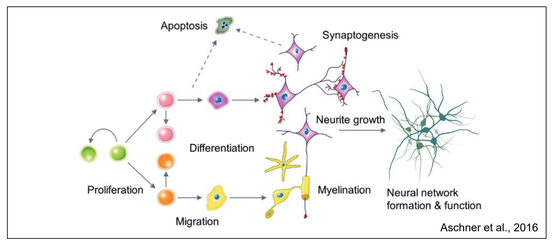
- A 15-year international-coordinated research effort has been under way to develop a battery of *in vitro* developmental neurotoxicity new approach methodologies (DNT-NAMs) to inform the understanding of DNT-related bioactivity.
- Here, we describe the collective performance of a subset of DNT-NAMs developed at the US-EPA for describing putative DNT-relevant bioactivity for 92 chemicals, including 48 reference DNT positives and 11 putative DNT negatives.

DNT-NAMs development → **DNT-NAMs evaluation** → DNT-NAMs implementation

Aims:

- Evaluate the performance of DNT-NAMs assay controls:
  - Activity of assay performance controls
  - Replicability of chemical repeats
  - Reproducibility/ variability: Coefficient of variation (CV) of DMSO controls and Z'-factor of assay positive controls.
- Reveal patterns of potency and selectivity for the 92 substances using hierarchical clustering to inform the understanding of DNT-related bioactivity, with the goal of elucidating adverse outcome pathways for DNT outcomes.
- Determine the accuracy of the DNT-NAM battery in classifying 49 DNT reference positives and 11 putative negatives, with the goal of identifying the most influential assay endpoints.

DNT-NAM battery for key neurodevelopmental processes



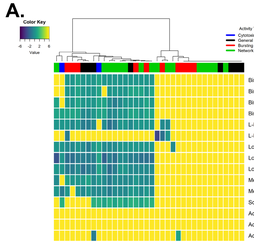
Aschner et al., 2016

Assay technology	Chemicals screened	Cell culture model	Assay/ key neurodevelopmental events	Endpoints measured
Microelectrode array (MEA)	92 (+28 repeats)	Primary rat cortical neurons (DIV 5, 7, 9, 12)	Network Formation assay (NFA); Decreasing neuronal activity	17
			Increasing neuronal activity	17
			Cytotoxicity	2
High-content imaging assays (HCI)	92	Primary rat cortical neurons	Neurite outgrowth (NOG)	4
			Synaptogenesis and Neurite maturation	8
		Human hN2 neural cells	NOG	4
		Human hNP1 neuroprogenitors	Proliferation	3
			Apoptosis	2

DNT-NAM battery with performance controls

The activity of DNT-NAM assay performance controls performed as expected with positive controls demonstrating increased activity and the negative controls demonstrating little to no activity in the battery.

A.



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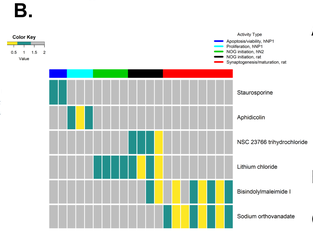


A. Heatmap of NFA performance controls
Color key: activity concentration at 50% maximum activity (AC_{50} values) for each assay endpoint
Positive controls: bisindolylmaleimide I, mevastatin, loperamide HCl, L-domoic acid, and sodium orthovanadate)
Negative control: acetaminophen

B. Heatmap of HCI assay positive controls
Color key: AC_{50} values

C. The CV of DMSO-treated control wells indicates assay reproducibility, the Z'-factor of known assay positive controls indicates variability in efficacy and reproducibility.

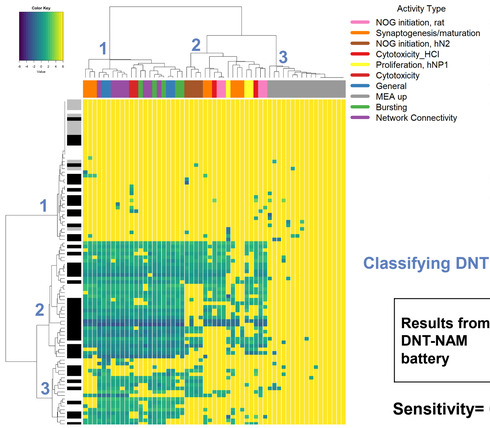
C.

NFA	Endpoint	DMSO CV	Chemical	Median Z'
General activity	MEA_dev_firing_rate_mean	21.26	Loperamide HCl	0.55 (0.29-0.89)
	MEA_dev_burst_rate	20.52		
	MEA_dev_active_electrodes_number	8		
	MEA_dev_bursting_electrodes_number	10.21		
Bursting activity	MEA_dev_per_burst_interspike_interval	24.23	Bisindolylmaleimide I	0.8 (0.33-0.94)
	MEA_dev_per_burst_spike_percent	10.4		
	MEA_dev_burst_duration_mean	25.27		
	MEA_dev_interburst_ interval_mean	21.88		
Network Connectivity	MEA_dev_network_spike_ number	23.04	L-Domoic acid	0.78 (0.26-0.94)
	MEA_dev_network_spike_peak	8.08		
	MEA_dev_spike_duration_mean	13.92		
	MEA_dev_network_spike_ duration_std	23.91	Mevastatin	0.61 (-0.17-0.86)
	MEA_dev_per_network_ spike_interspike_interval_mean	26.4		
	MEA_dev_per_network_ spike_spike_ number_mean	16.99		
	MEA_dev_per_network_ spike_spike_ percent	18.76	Sodium orthovanadate	0.74 (0.08-0.9)
	MEA_dev_correlation_ coefficient_mean	15.67		
	MEA_dev_mutual_ information_norm	21.38		
Cell viability	MEA_dev_LDH	8.06		
	MEA_dev_AB	6.93		

HCI	Endpoint	DMSO CV	Chemical	Z'
Apoptosis/ viability	HCI_hNP1_Casp3_7_gain	3.8	Staurosporine	0.8
	HCI_hNP1_CellTiter_loss	1.96	Staurosporine	0.75
Proliferation	HCI_hNP1_Pro_MeanAvgInten_loss	12.72	Aphidicolin	0
	HCI_hNP1_Pro_ObjectCount_loss	8.51	Aphidicolin	0
	HCI_hNP1_Pro_ResponderAvgInten_ loss	11.9	Aphidicolin	0.1
NOG, hN2	HCI_hN2_NOG_BPCount_loss	13.52	Lithium chloride	0
	HCI_hN2_NOG_NeuriteCount_loss	3.63	Lithium chloride	0.38
	HCI_hN2_NOG_NeuriteLength_loss	7.15	Lithium chloride	0.58
	HCI_hN2_NOG_NeuronCount_loss	13.47	Lithium chloride	0
NOG, rat cortical	HCI_Cortical_NOG_NeuriteLength_loss	8.42	Bisindolylmaleimide I	0.31
	HCI_Cortical_NOG_NeuronCount_loss	7.6	Bisindolylmaleimide I	0
	HCI_Cortical_NOG_BPCount_loss	8.36	Lithium chloride	0.06
	HCI_Cortical_NOG_NeuriteCount_loss	7.6	Lithium chloride	0
	HCI_Cortical_NOG_NeuriteLength_loss	8.42	Lithium chloride	0.3

Evaluating potency collectively informs DNT-relevant bioactivity.

Heatmap of the DNT-NAM battery activity (NFA and HCI assays)



Color key: Potency (AC_{50} values): Yellow = screened negative; Teal = 1-100 µM; Dark blue = 0.0001-0.01 µM

Row label bar (left): DNT reference positives (black) and negatives (gray)

Activity-type (columns):

- **Cluster 1:** *Decreased* network formation activity, synaptogenesis/ neurite maturation. **Cluster 2:** *Decreased* NOG (rat or hN2 cells), proliferation, synaptogenesis/ neurite maturation, and increased apoptosis. **Cluster 3:** *Increased* network formation activity.

Chemicals (rows):

- **Cluster 1:** Little to no activity chemicals. **Cluster 2:** High activity and potency chemicals. **Cluster 3:** Moderate activity and lower potency chemicals

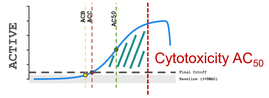
Classifying DNT Reference Chemicals

		Negatives (11)	Positives (49)
Results from DNT-NAM battery	**Potent and high activity** (Clusters 2,3)	False positive: 0	True positive: 32
	Inactive/ equivocal (Cluster 1)	True Negative: 11	False negative:17

Sensitivity= 65%, Specificity= 100%, Accuracy= 72%

Selective bioactivity reveals possibly relevant patterns of activity.

A. Selectivity AUC



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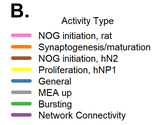

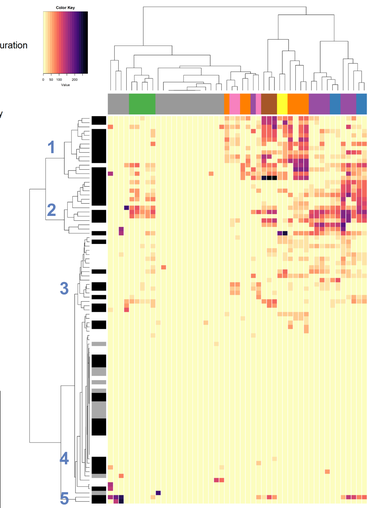

A. Selectivity: area under the curve (AUC) at concentrations lower than the cytotoxicity AC50.

B. Heatmap of DNT-NAM battery selectivity.

Color key: selectivity AUC (purple = high, pink = low to medium, yellow = negative or no selective activity
Row label bar (left): DNT reference positives (black) and negatives (gray)
Chemicals (rows): Hierarchical clustering reveals approximately five main chemical clusters.

Classifying DNT Reference Chemicals by Cluster

	Strong selectivity	Moderate selectivity	DNT Reference: Negative	DNT Reference: Positive
1	Proliferation, synaptogenesis, NOG (hN2 cells)	NOG (rat cortical), firing rate, burst rate, and spike number	0	14
2	Decreased network formation activity	Synaptogenesis, and NOG (hN2 cells), decreased bursting activity	0	10
3		Moderate to low activity across endpoints	0	7
4		Inactive/ equivocal	11	16
5	Increased mean inter-spike interval for network spikes		0	2

		Negatives	Positives
Results from DNT-NAM battery	**Selective activity** (Clusters 1,2,3,5)	False positive:0	True positive: 33
	Inactive/ equivocal (Cluster 4)	True Negative: 11	False negative: 16

Sensitivity= 67%, Specificity= 100%, Accuracy= 73%

Summary and Future Directions

- The performance controls indicates that this battery successfully functions as a broad phenotypic screen of neurodevelopmental processes *in vitro*.
- Potency in the DNT-NAM battery alone does well to capture any effect on DNT-relevant processes, but does little to distinguish patterns of effect in terms of network formation and function.
- Hierarchical clustering of DNT-NAM battery selective activity classifies DNT reference chemicals with 67% sensitivity, with 16 false negatives that may be due to screening or biological limitations. The limited number of true negative reference chemicals may bias specificity, estimated at 100%.
- **Conclusion**: This preliminary evaluation of the DNT-NAM battery reveals differential patterns of DNT-relevant bioactivity that are informative for elucidating substrate-specific biological effects, contributing to a larger effort to use NAMs for identification and prioritization of putative DNT chemicals.
- **Future Direction**: A larger screened chemical dataset, the addition of assays that cover more neurobiological space, and a more balanced DNT reference chemical set will continue to improve data interpretation and model building of the DNT-NAM battery.

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