Developmental Neurotoxicity (DNT) in vitro Battery as an Alternative to DNT in vivo Guideline Studies Used by OPP

Tim Shafer

Board of Scientific Counselors Subcommittee

Chemical Safety for Sustainability and

Health and Environmental Risk Assessment National Research Programs

Virtual Meeting

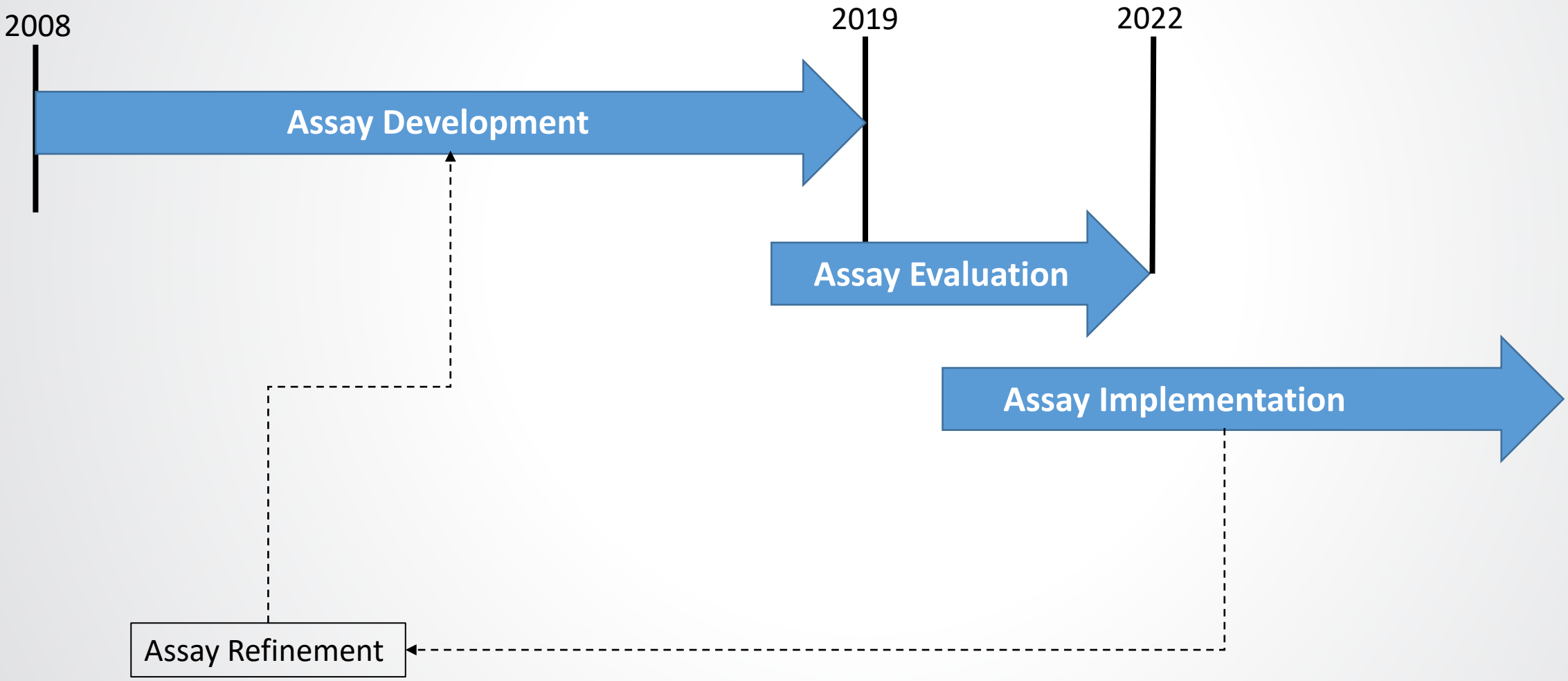
February 3, 2021

The subsequent presentation has been cleared by the Office of Research and Development but is not Agency Policy. This presentation contains unpublished data.

Outline

I. (Re)-Introduction to CSS Research on alternative approaches for developmental neurotoxicity (DNT) hazard assessment
II. International Efforts on use of NAMs for DNT hazard assessment
III. Application of NAMs to OPP and OPPTS issues.
IV. Future Directions

Status of DNT NAMs Research in CSS



Issues with in vivo DNT studies

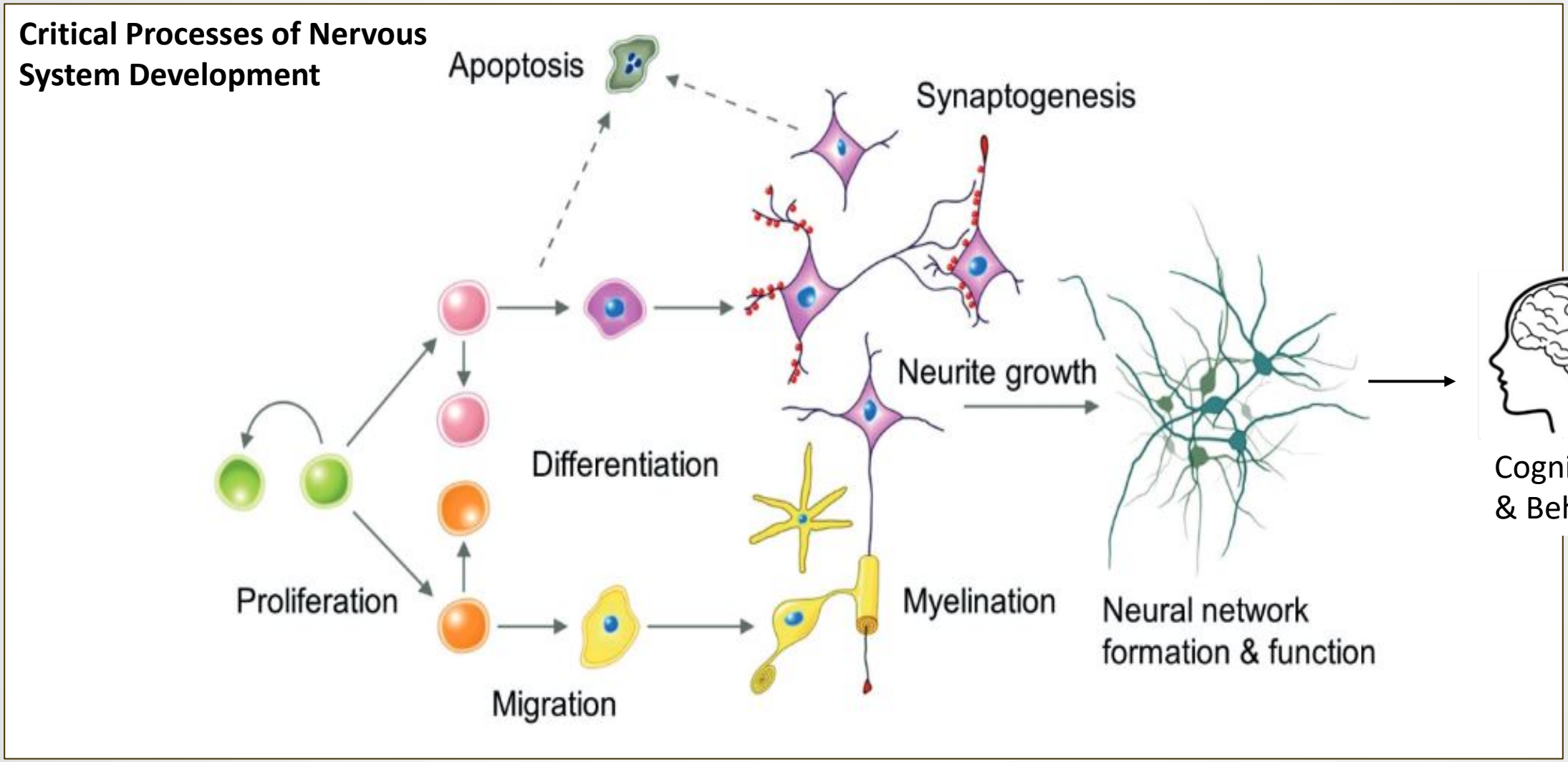
- "Triggered" test- Only requested if concern for neurotoxicity
- Expensive- ~$1,000,000/chemical
- Time-consuming- takes 1-2 years to complete
- Ethically questionable- Estimated ~1000 animals/test
- Value of Information
  - High variability; low precision
  - Not often used (~25%) for point of departure values for risk assessment*

Only ~150 compounds have DNT Guideline Studies

Problem for OPPTS and OPP

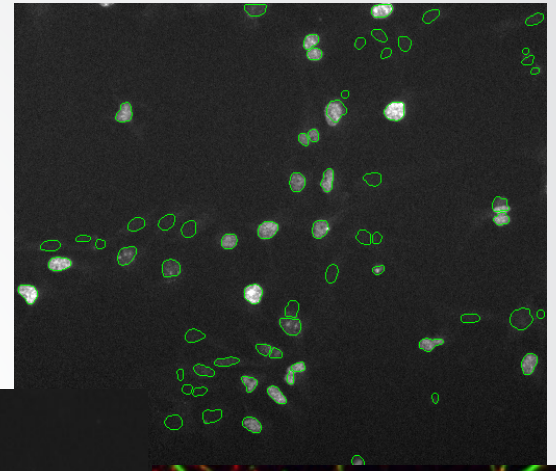
*Raffaele et al. The use of **developmental neurotoxicity** data in pesticide risk assessments. Neurotoxicol Teratol. 2010 Sep-Oct;32(5):563-72.

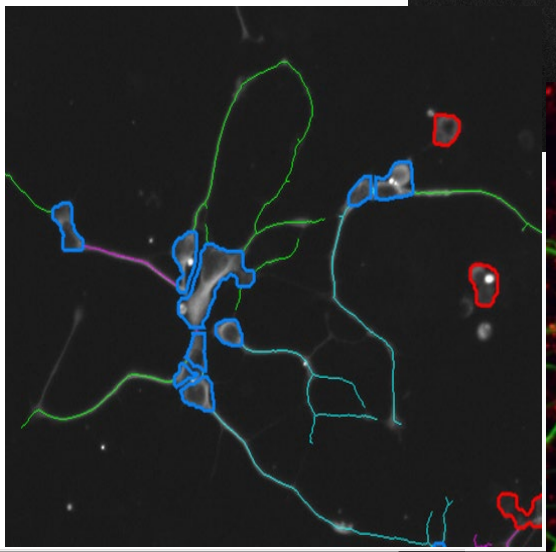
Addressing the limitations of the DNT Guideline Study by using Phenotypic Screens

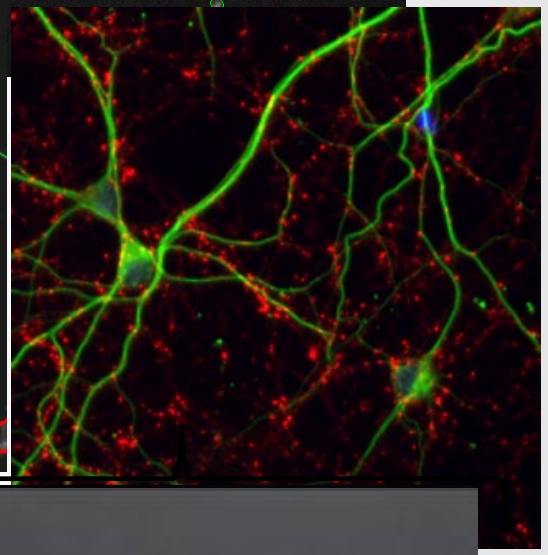


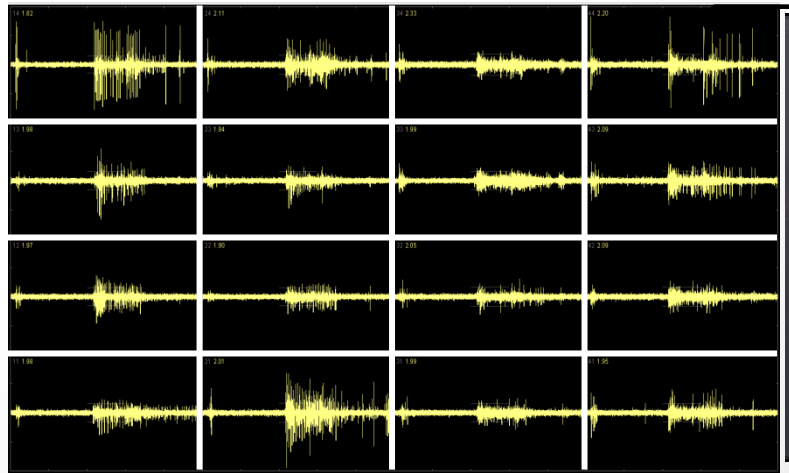
The EPA Assay Battery

Proliferation	-human neuroprogenitors (hNP1)
Apoptosis	-human neuroprogenitors (hNP1)
Neurite initiation	-human neurons (hN2, $iCell_{gluta}$)
Neurite initiation	-rat primary neural culture
Neurite maturation	-rat primary neural culture
Synaptogenesis	-rat primary neural culture
Network formation (MEA)	-rat primary neural culture
Behavior/Anatomy	-zebrafish









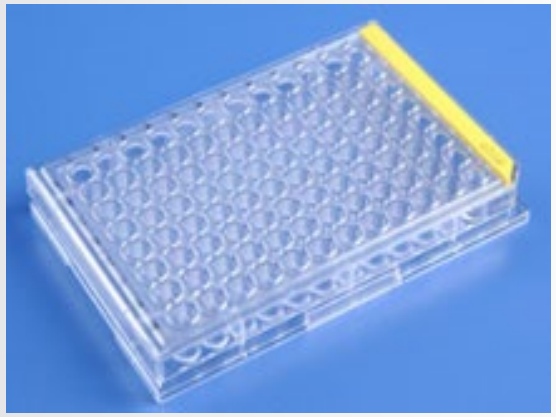
Each assay has concurrent assessments of cell health/viability and has been vetted with assay positive controls as well as by testing DNT reference compounds.

High Content Imaging: Overview

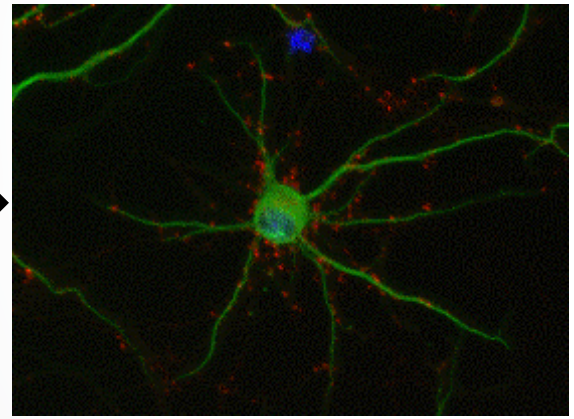
Automated microscopy providing data at the level of the individual cell

High throughput : automated data acquisition and analysis in multi-well plates

High content : large amounts of data from a single image.



Multiwell Culture



Immunocytochemistry

Image Acquisition

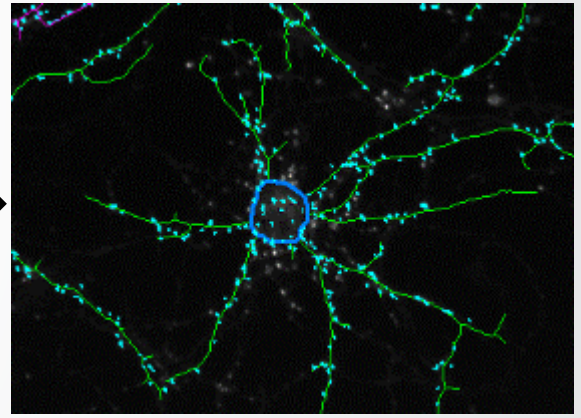
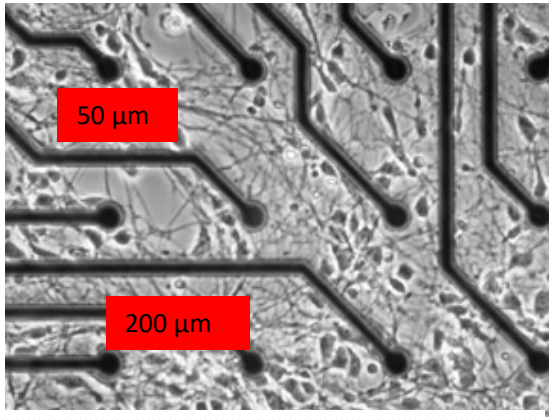


Image Analysis
Feature Extraction

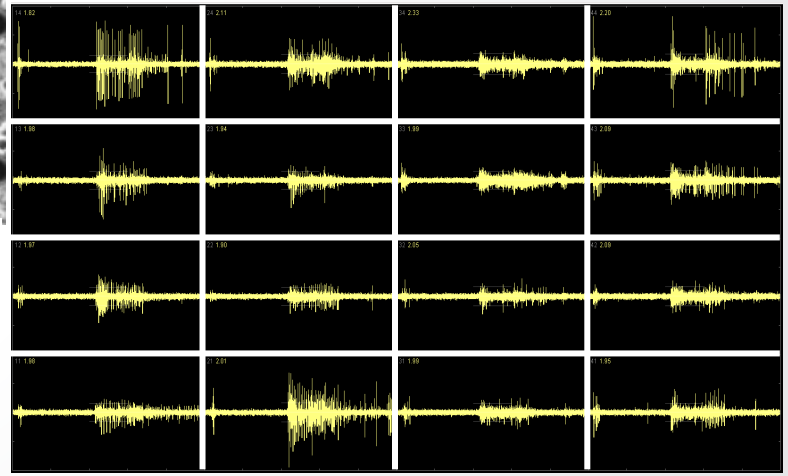
- Epifluorescence microscope and digital camera *in a box*
- Automated stage movement, exposure, and focusing capabilities
- Computer algorithms analyze the images to provide cell-based data (*e.g.* size, shape, location, fluorescence intensity)

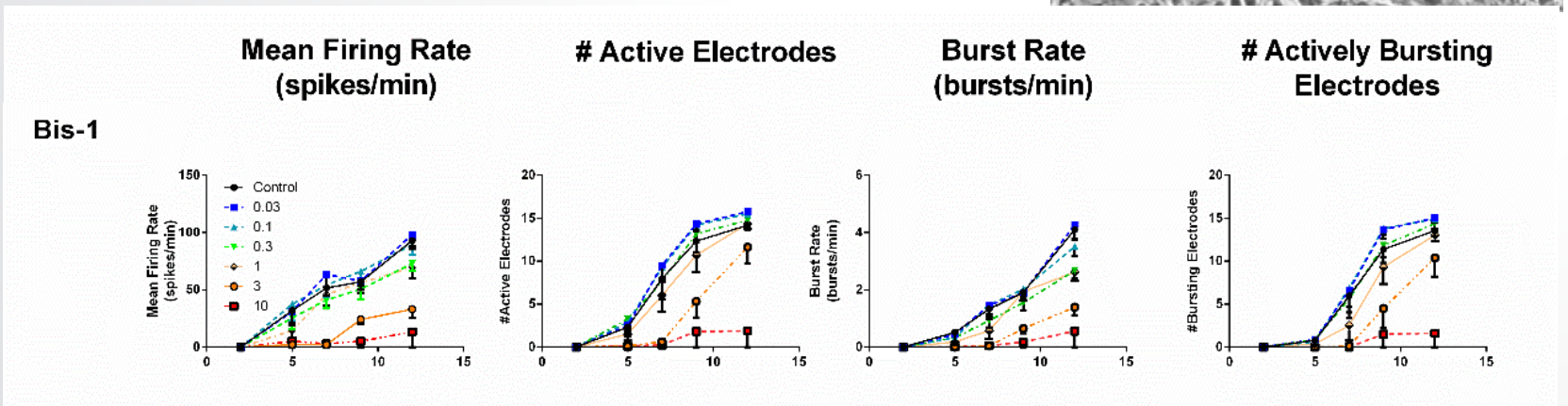
Measurement of Network Formation in vitro using Microelectrode Array (MEA) Recording



"Brain-on-a-Chip": Complex 2D model

- Rat cortical neural networks
- Contains neurons & glia cells
- Spontaneous activity
- Develops rapidly in vitro
- Follow network development over time
- Integrates activity of multiple processes





A snapshot in time of neural network activity in one well. Each box represents the electrical activity of neurons on 1 electrode in the array.

International Efforts on DNT NAMs

OXFORD
SOT | Society of Toxicology
www.toxsci.oxfordjournals.org

TOXICOLOGICAL SCIENCES, 167(1), 2019, 45–57

doi: 10.1093/toxsci/kfy211
Advance Access Publication Date: November 23, 2018
Forum

FORUM

International Regulatory and Scientific Effort for Improved Developmental Neurotoxicity Testing

Magdalini Sachana,*,[1] Anna Bal-Price,† Kevin M. Crofton,‡ Susanne H. Bennekou,§ Timothy J. Shafer,¶ Mamta Behl,‖ and Andrea Terron‖

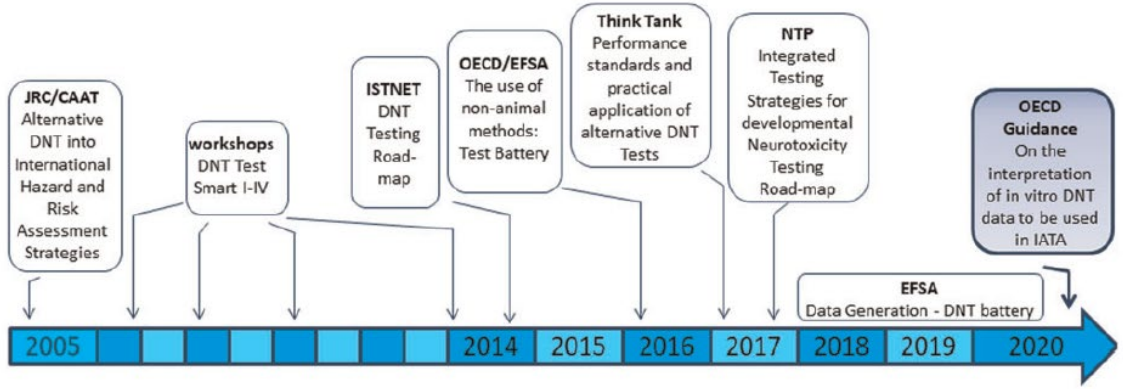
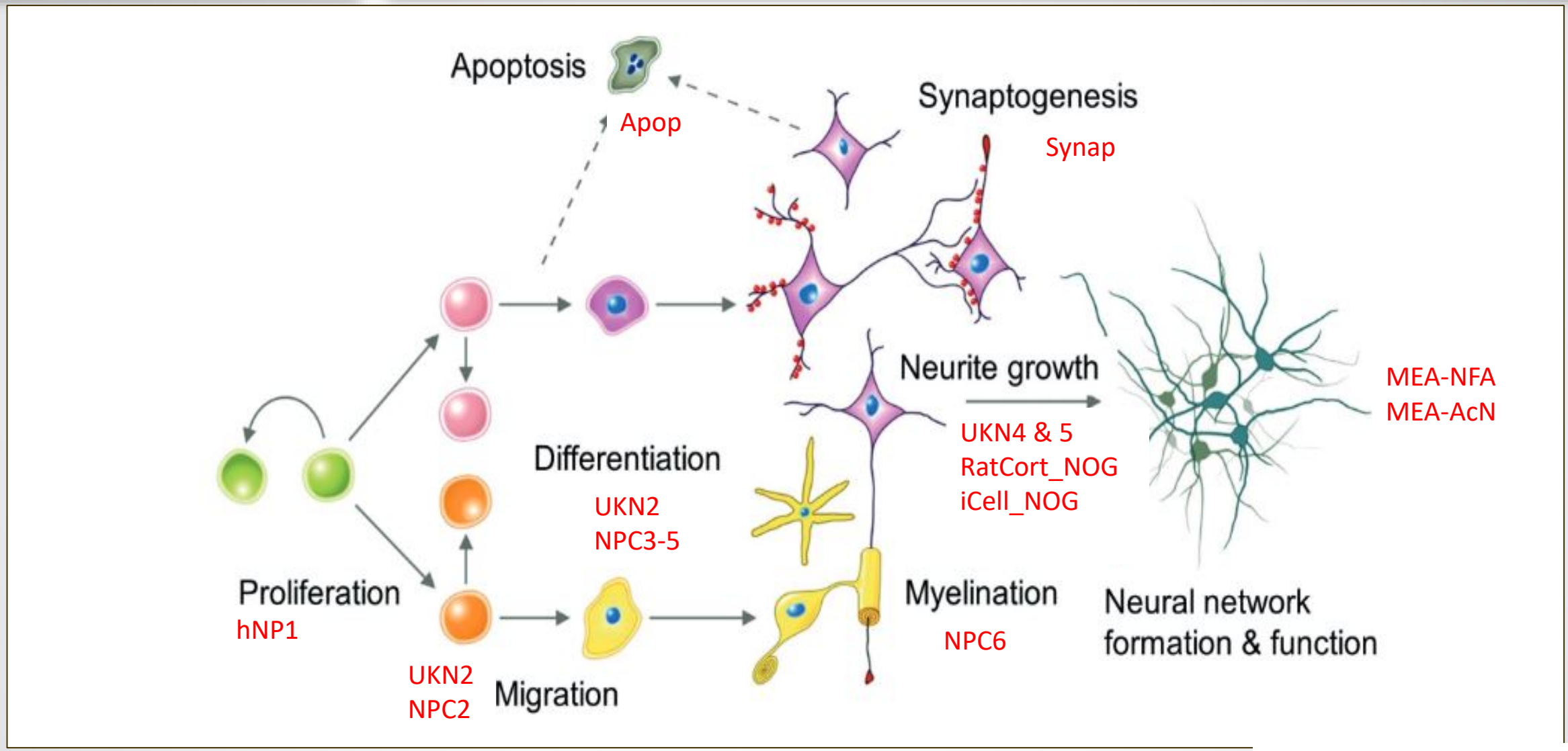


Figure 1. Timeline of efforts to develop and implement new alternative methods for developmental neurotoxicity.

Table 2. Proposed Assays for Evaluation As an *In Vitro* DNT Battery

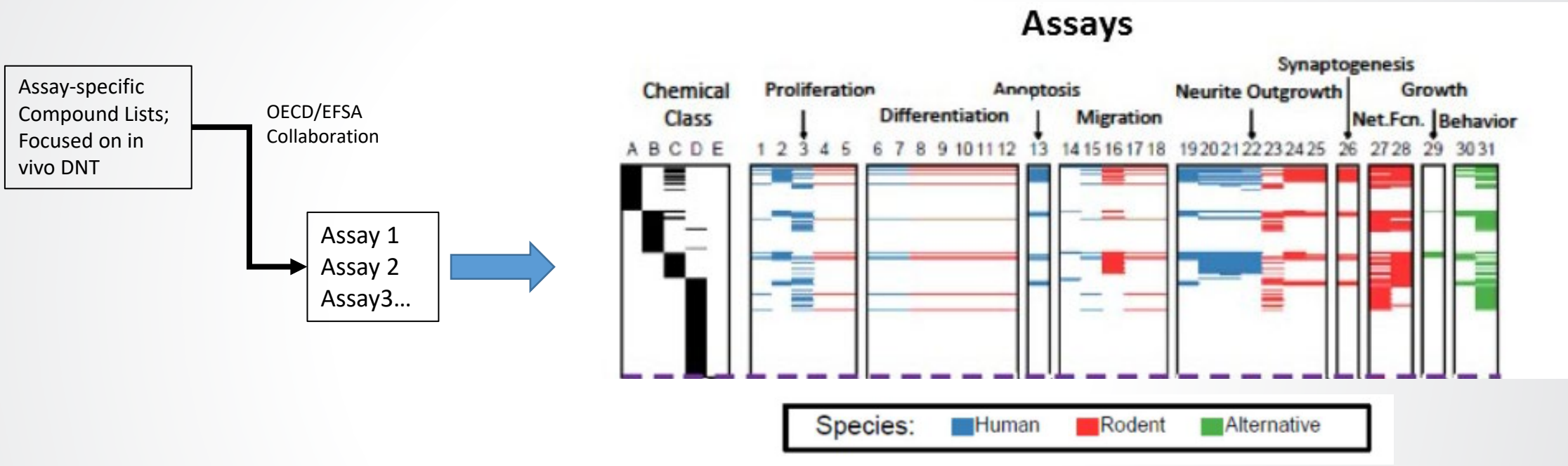
Process	Assays	References
Proliferation	hNP1	Harrill *et al.* (2018)
	NPC1	Baumann *et al.* (2016) and Barenys *et al.* (2017)
	UKN1	Balmer *et al.* (2012)
Apoptosis	hNP1	Harrill *et al.* (2018)
Migration	NPC2	Baumann *et al.* (2016) and Barenys *et al.* (2017)
	UKN2	Nyffeler *et al.* (2017)
Neuron differentiation	NPC3	Baumann *et al.* (2016) and Barenys *et al.* (2017)
Oligodendrocyte differentiation & maturation	NPC5/6	Baumann *et al.* (2016) and Barenys *et al.* (2017)
Neurite outgrowth	iCell gluta hN2	Harrill *et al.* (2018)
	UKN 4 & 5	Krug *et al.* (2013)
	NPC4	Baumann *et al.* (2016) and Barenys *et al.* (2017)
Synaptogenesis	Rat primary synaptogenesis	Harrill *et al.* (2018)
Network formation	MEA-NFA	Brown *et al.* (2016) and Frank *et al.* (2018)

DNT NAMs Provide Good Coverage of Neurodevelopmental Processes



Aschner et al., 2016

OECD/EFSA-EPA Collaboration



Development of a Guidance Document for the use of DNT alternative assays in Integrated Approaches for Testing and Assessment (IATAs)

- Guidance for incorporation of in vitro assays into IATAs
- Case Studies
- Draft Guidance document expected mid 2021

Use of DNT NAMs at EPA

I. **Screening Level information**
   - APCRA, TSCA, PFAS

II. **Understanding species differences**
   - Data from DNT NAMs provided to OPP to help understand rodent-human differences in response to chemicals since the battery has both rodent and human assays

III. **Structure-activity relationships**
   - OPP requested data from selected assays on a set of structurally similar compounds
     - A DNT Guideline study existed for one compound (“compound X”)
     - Assays were selected based on the of activity of compound X in Guideline Study.
     - Structurally similar compounds were tested in vitro
     - OPP will use the data from the in vitro screens in WOE approach to deciding whether or not to request DNT guideline studies on the other compounds (Decisions are in progress).

IV. **Weight of Evidence approaches**
   - Organophosphates

Organophosphates and DNT

Primary Questions: Organophosphate insecticides are currently regulated based on inhibition of acetylcholinesterase (AChE):

Does the DNT battery indicate that this may not be health protective?
Can data from the DNT battery contribute to a WOE approach for OPs?

Organophosphates and DNT

Study Design:

Test 27 Organophosphate insecticides in the EPA DNT assays

8 Parent/oxon pairs

Concentration-response up to 100 µM

Pipeline results through TCPL to generate AC_{50} values

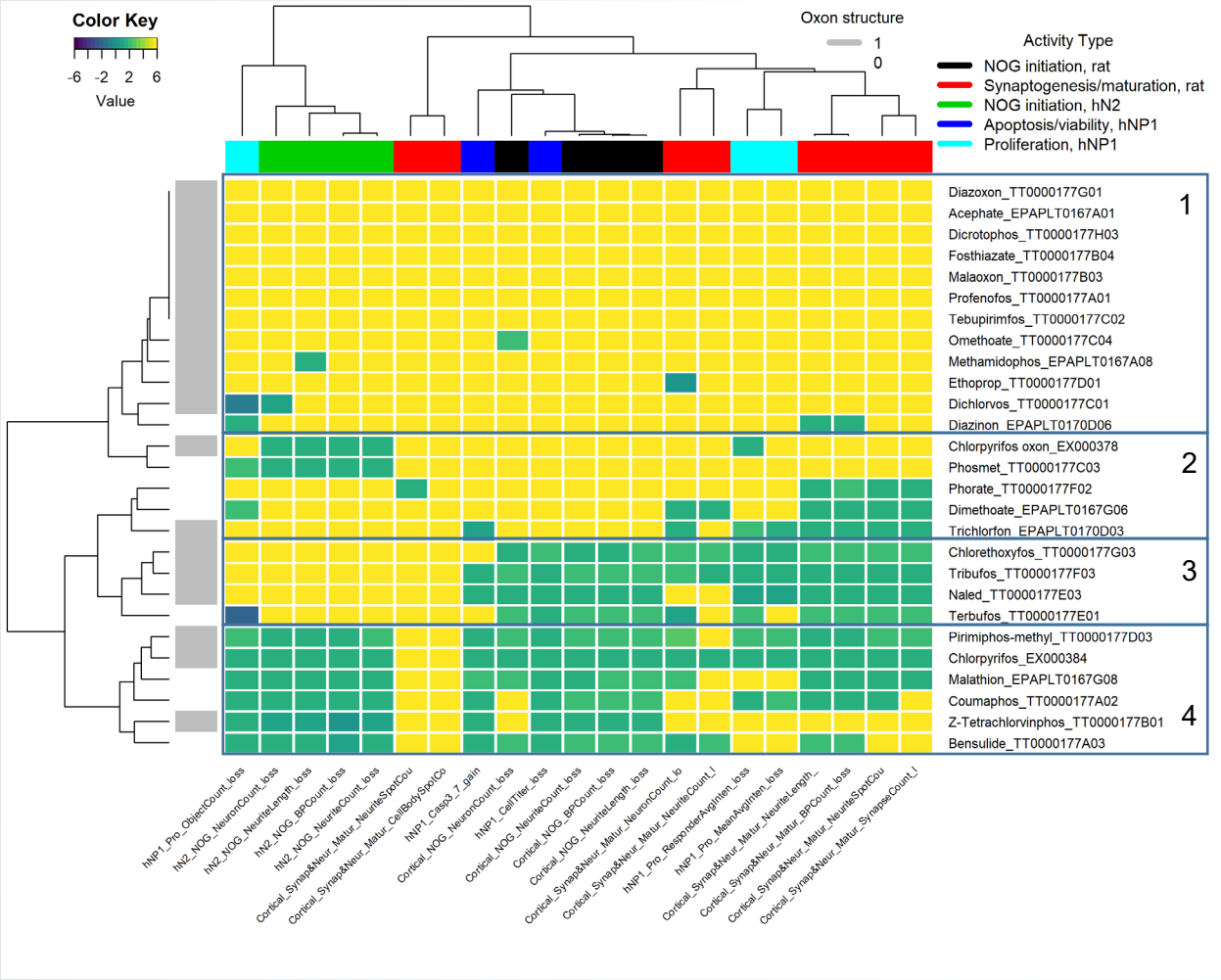
Use HTTK to convert AC_{50} values to AED_{50} values

Compare to BMD/BMDL10 values based on AChE inhibition

Assays:

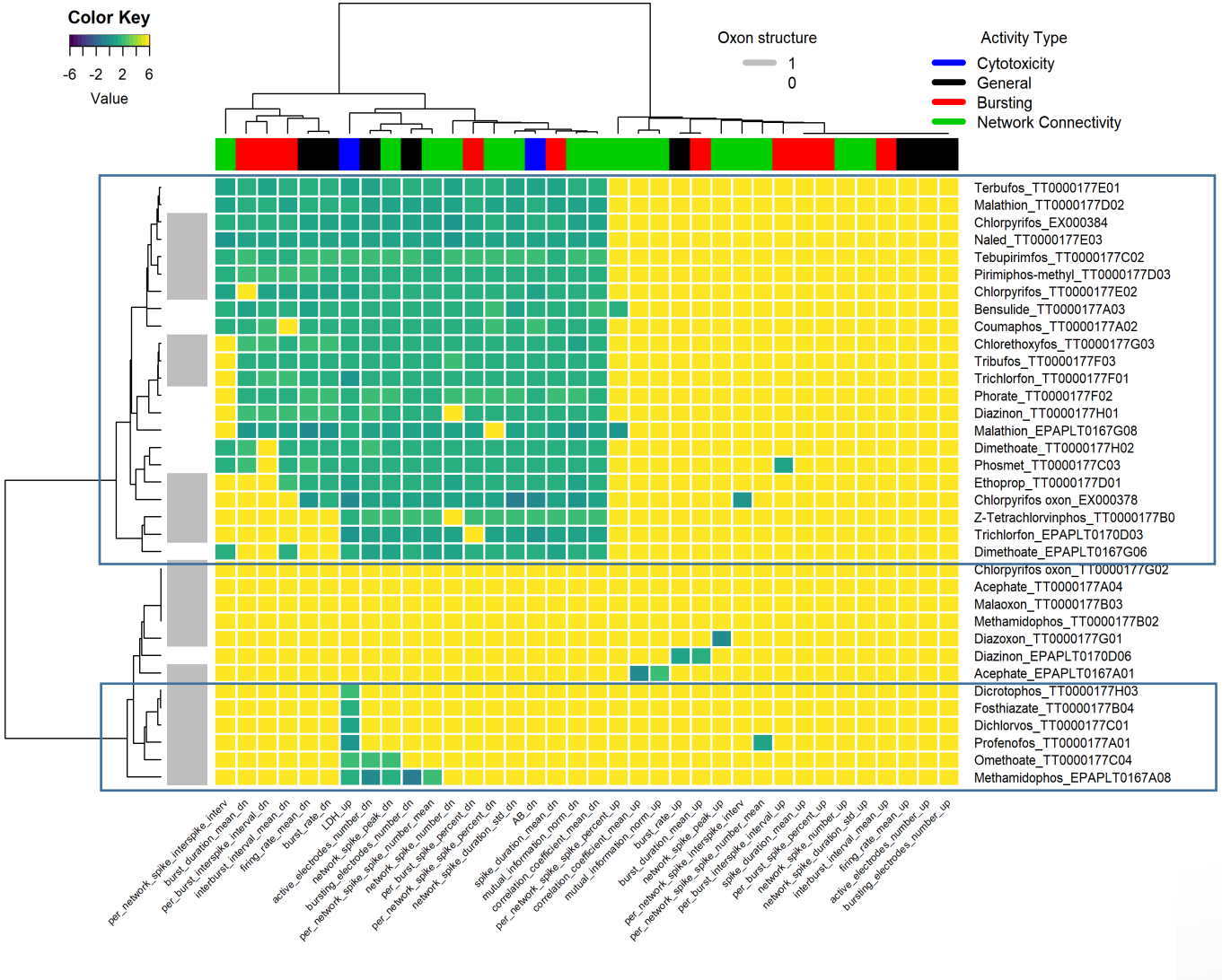
Proliferation	-	human neuroprogenitors (hNP1)
Apoptosis	-	human neuroprogenitors (hNP1)
Neurite initiation	-	human neurons (hN2)
Neurite initiation	-	rat primary neural culture
Neurite maturation	-	rat primary neural culture
Synaptogenesis	-	rat primary neural culture
Network formation (MEA)	-	rat primary neural culture
Behavior/Anatomy	-	zebrafish (data analysis pending)

OPs demonstrate differential responses in the HCI assays.



- Cluster 1: negative or with effects in 1-3 endpoints.
- Cluster 2: effects on 5 or more assay endpoints
- Cluster 3: OP samples with effects on all HCI assay activity types except for NOG initiation in hN2 cells and synaptogenesis in cortical cells
- Cluster 4: widespread effects across activity types

Most OPs decreased MEA NFA activity



- Top active cluster of OPs contains oxon and non-oxon structures.
- These OPs, like the assay performance controls and many other compounds, appear to generally decrease all activity types and most assay endpoints.
- Bottom cluster with minimal actives appears somewhat driven by cytotoxicity in the LDH assay.
- Negative- 0 assay endpoints altered
- Equivocal- 1-3 assay endpoints altered
- Positive- >3 assay endpoints altered

Overall, there was agreement between the HCI and MEA_NFA assays

DTXSID	Chemical	MEA NFA			HCI			
		Neg	Equiv	Pos	1	2	3	4
DTXSID8023846	Acephate	X	X		X			
DTXSID9032329	Bensulide			X				X
DTXSID2032344	Chlorethoxyfos			X			X	
DTXSID4020458	Chlorpyrifos			X,X				X
DTXSID1038666	Chlorpyrifos oxon	X		X		X		
DTXSID2020347	Coumaphos			X				X
DTXSID9020407	Diazinon		X	X		X		
DTXSID5037523	Diazoxon		X		X			
DTXSID5020449	Dichlorvos		X		X			
DTXSID9023914	Dicrotophos		X		X			
DTXSID7020479	Dimethoate			X		X		
DTXSID4032611	Ethoprop			X	X			
DTXSID0034930	Fosthiazate		X		X			
DTXSID9020790	Malaoxon	X			X			
DTXSID4020791	Malathion			X				X
DTXSID6024177	Methamidophos	X	X			X		
DTXSID1024209	Naled			X			X	
DTXSID4037580	Omethoate		X		X			
DTXSID4032459	Phorate			X		X		
DTXSID5024261	Phosmet			X		X		
DTXSID0024266	Pirimiphos-methyl			X				X
DTXSID3032464	Profenofos		X		X			
DTXSID1032482	Tebupirimfos			X	X			
DTXSID2022254	Terbufos			X			X	
DTXSID1024174	Tribufos			X			X	
DTXSID0021389	Trichlorfon			X		X		
DTXSID1032648	Z-Tetrachlorvinphos			X				X

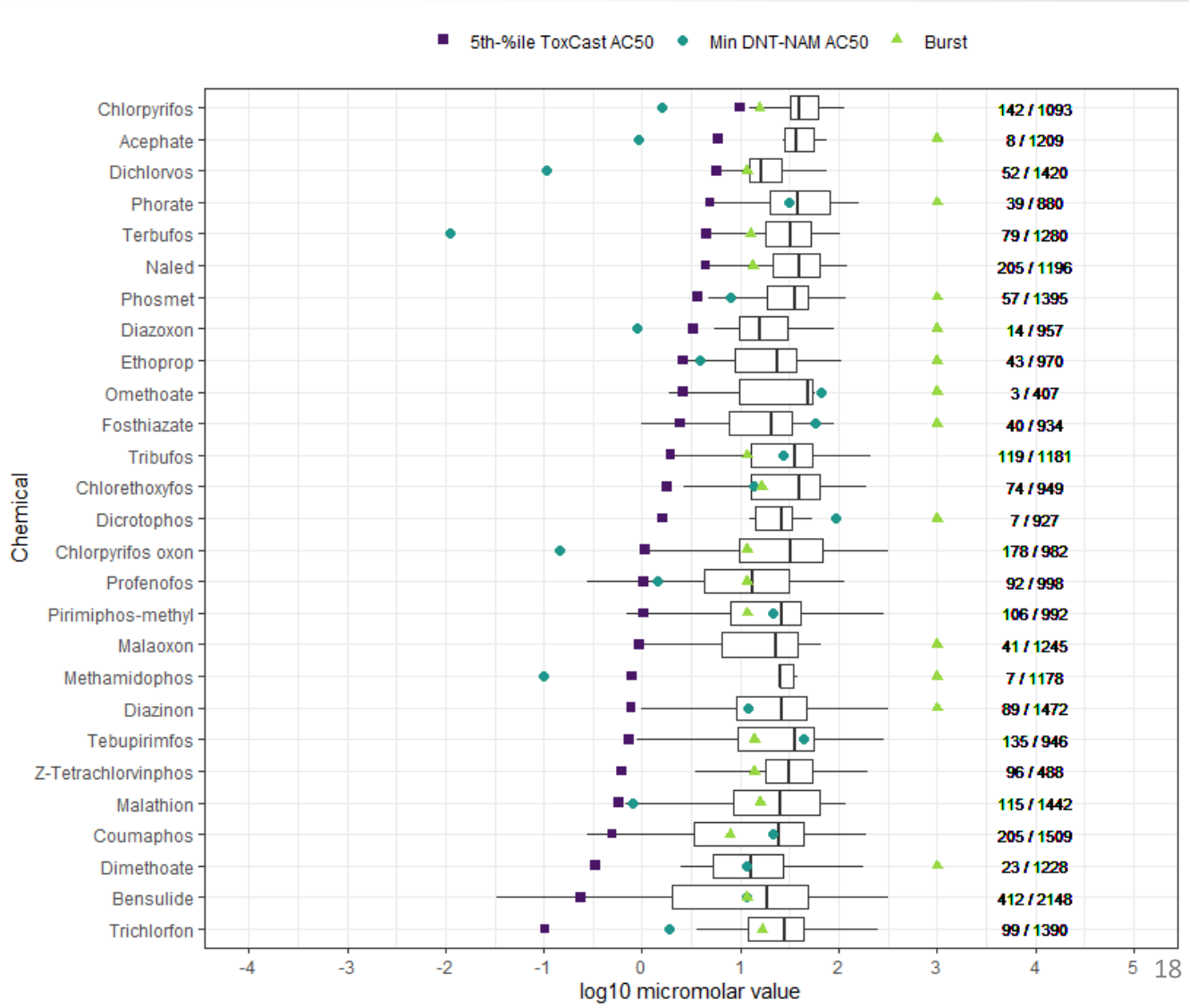
- *Equiv or Pos in MEA NFA and negative in HCI*: Acephate, diazoxon, dichlorvos, dicrotophos, fosthiazate, malaoxon, omethoate, profenofos
- *Positive in MEA NFA and negative in HCI*: Ethoprop
- *Positive in HCI and negative in MEA NFA:* OP chemical (methamidophos) was neg/equiv in the MEA NFA
- **If activity is observed in the HCI assays, it is likely that the OP chemical will also be active in the MEA NFA.**

For some OPs, DNT-NAM AC_{50} < bioactivity estimate from the rest of ToxCast.

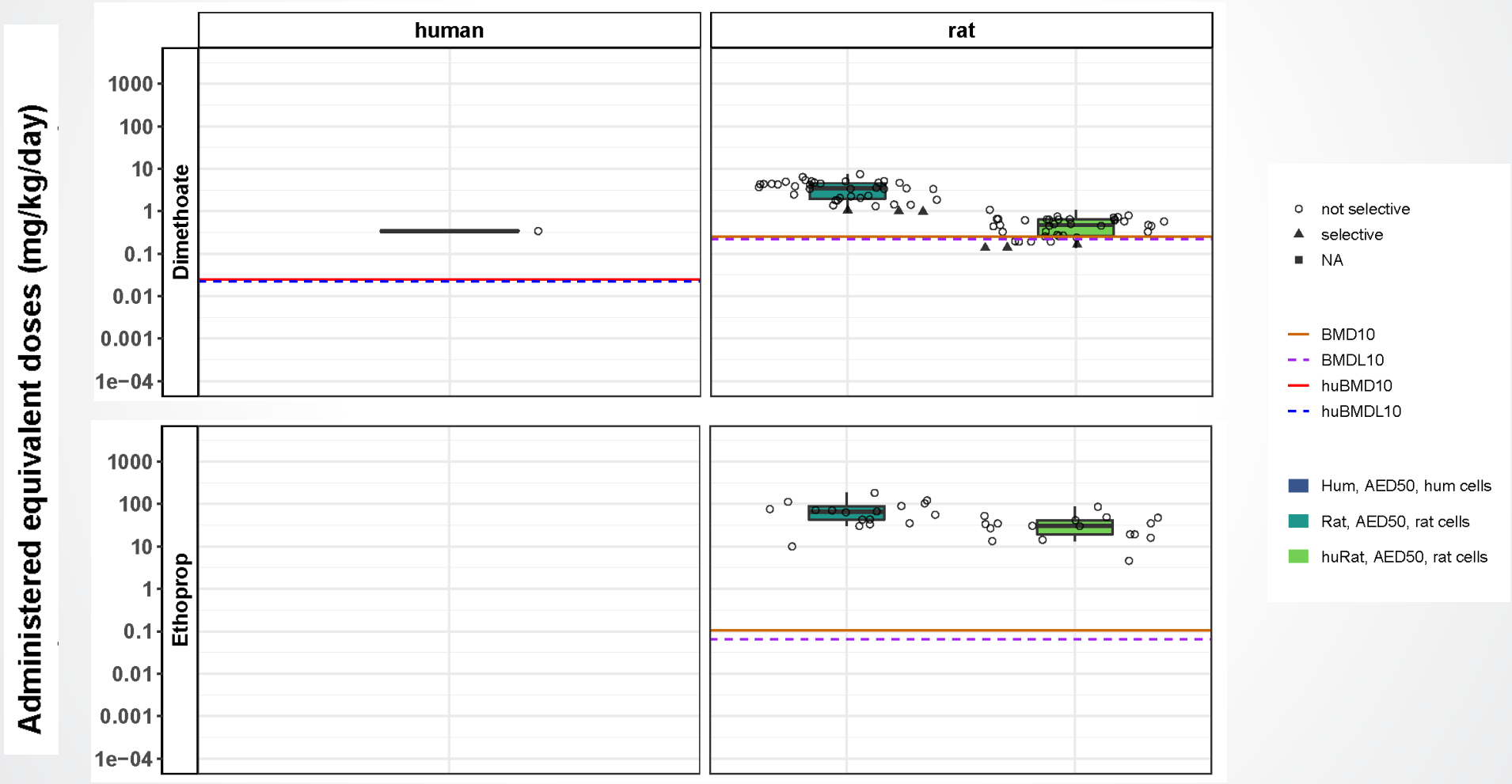
DNT-NAM battery may provide a more potent estimate of bioactivity for substances with minimum DNT-NAM AC50 < 5th percentile of filtered ToxCast AC50 values:

- Chlorpyrifos and chlorpyrifos oxon
- Acephate
- Dichlorvos
- Terbufos
- Diazoxon
- Methamidophos

Suggests that the DNT-NAM battery, in covering some new biology not previously in ToxCast, may yield bioactivity threshold concentrations lower than what is already available for some neuroactive substances in ToxCast.



AED50 to BMD/BMDL10 comparisons



Summary of the AED50 to BMD/BMDL comparison

	Chemicals with AED50 values >>> BMD/BMDL comparator	Chemicals with lowest AED50 within 1 log10 order of magnitude of BMD/BMDL comparator	Chemicals with lowest AED50 approaching BMD/BMDL comparator	Missing in vitro data for comparison
Rat/HuRat	Coumaphos, diazoxon, dicrotophos, ethoprop, fosthiazate, omethoate	acephate, bensulide, chlorpyrifos, chlorpyrifos oxon, diazinon, dimethoate, malathion, methamidophos, and phorate	dimethoate and methamidophos (lower quartile of huRat AED_{50} values dichlorvos (huRat AED_{50}; only one positive rat assay endpoint) overlaps with the BMDL10 value, and it was not based on selective bioactivity in the DNT-NAM battery. malathion (huRat AED_{50} (selective) for also approach the BMD/BMDL10 values.	Malaoxon (negative in all assays)
Human	bensulide, chlorpyrifos, chlorpyrifos oxon, coumaphos, diazinon, dimethoate, malathion, methamidophos, phosmet, pirimiphos-methyl, tribufos, and trichlorfon		dichlorvos, only two AED_{50} values are available for comparison, and these values are centered around the BMD10/10 and BMDL10/10 values. terbufos, only 3 human AED_{50} values are available for comparison, and the lowest one of these values approaches the BMD10/10 value.	**Negative in all assays with human cells:** Acephate, diazoxon, dicrotophos, ethoprop, fosthiazate, omethoate, phorate, profenofos, and tebupirimfos Malaoxon was negative in all assays.

AEDs from DNT NAMS are more sensitive than LOAELs for other compounds

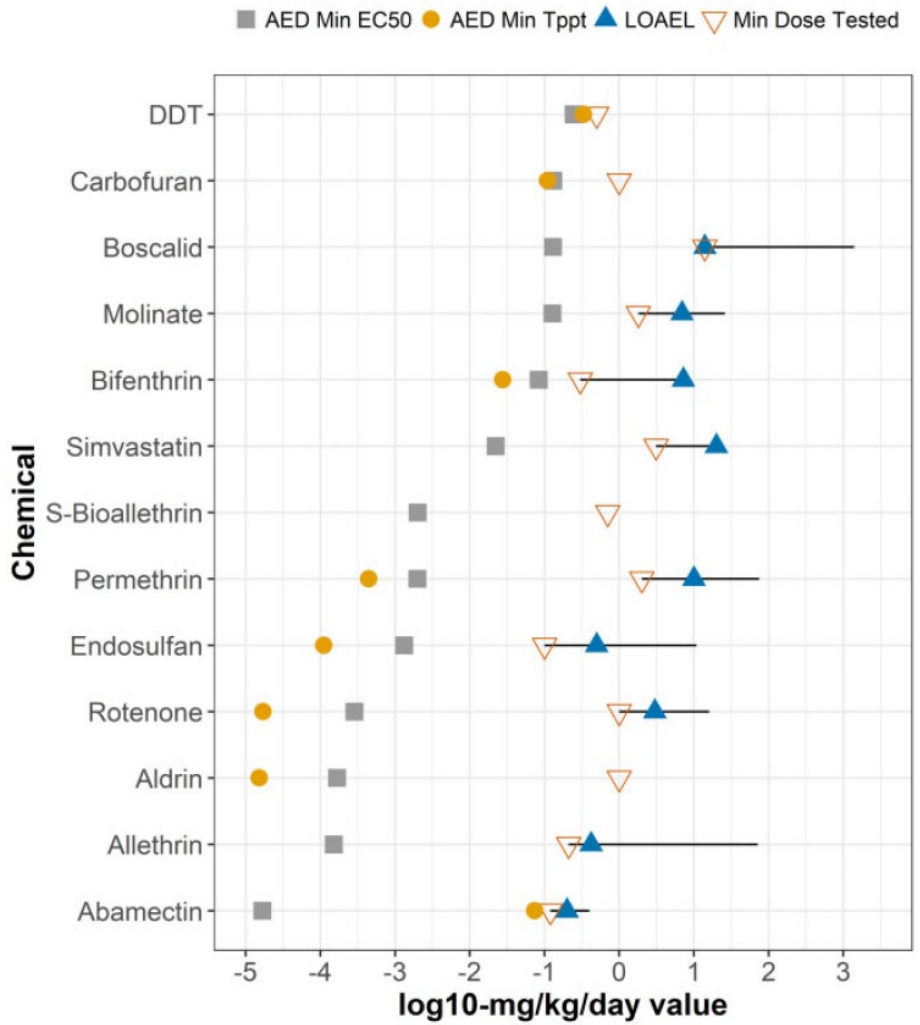
OXFORD SOT Society of Toxicology
www.toxsci.oxfordjournals.org

TOXICOLOGICAL SCIENCES, 169(2), 2019, 436–455

doi: 10.1093/toxsci/kfz052
Advance Access Publication Date: February 28, 2019
Research Article

Evaluation of Chemical Effects on Network Formation in Cortical Neurons Grown on Microelectrode Arrays

Timothy J. Shafer,*,[1] Jasmine P. Brown,*,[2] Brittany Lynch,†
Sylmarie Davila-Montero,‡ Kathleen Wallace,* and Katie Paul Friedman§



Even though AEDs were not more sensitive than BMDLs for OPs, DNT NAMs can still be sensitive indicators of potential disruption of nervous system development

Overall conclusion

The development of a DNT-NAM battery for assessing potential DNT-related effects provides:

- an opportunity to overcome some of the challenges with the *in vivo* DNT guideline study
- Evaluates critical processes underlying neurodevelopment
- Incorporating human relevant information.
- Represents a significant advancement toward developing a DNT-NAM battery for DNT evaluation.
- Is currently being utilized for a variety of regulatory decision-making processes at EPA

Future Directions

I. Continue to Improve Current Assays
    I. Scale up to higher throughput
    II. Increase # compounds tested
II. Contribute to Development of AOPs (CSS 4.2.4)
III. Incorporate Next Generation Technologies
IV. Incorporate 3D Models

Collaborators

EPA

- Theresa Freudenrich
- Kathleen Wallace
- Jasmine Brown
- Chris Frank
- Stephanie Padilla
- Josh Harrill
- Megan Culbreth
- Bill Mundy (retired)
- Kevin Crofton (retired)
- Katie Paul-Friedman
- Richard Judson
- Anna Lowit (OPP)
- Monique Perron (OPP)
- Liz Mendez (OPP)

University of Konstanz

- Marcel Leist
- Johanna Nyffeler

Düsseldorf

- Ellen Fritsche

Support:

- EPA CSS Research Program
- EPA Pathway Innovation Projects