EPA Innovative Approaches for DART-related Toxicities

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October 21, 2020

Disclosure Statement

Portions of this work have been funded by the US. Environmental Protection Agency. I have no conflicts to declare.

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DART-Related research at EPA

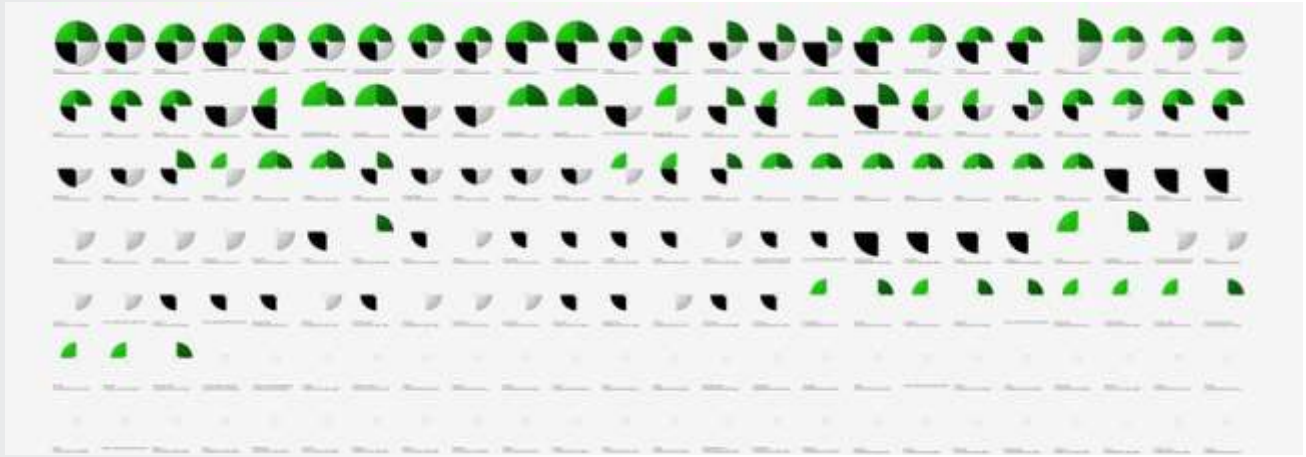
EPA Chemical Safety for Sustainability (CSS) Research Program has over 30 Projects Related to DART

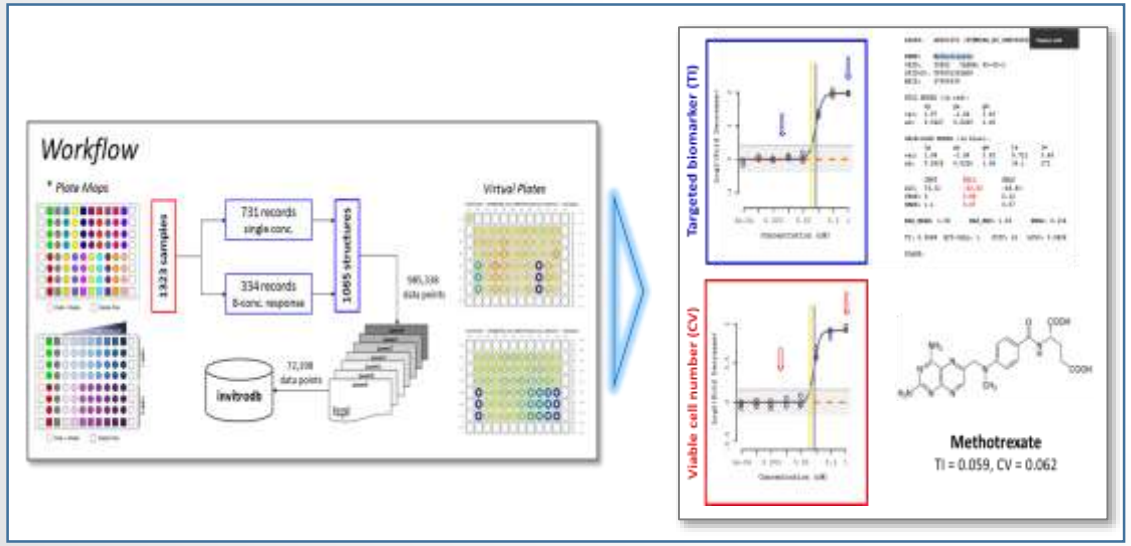
Overarching Areas

- Application of Stem Cells for Screening
- Complex/Virtual Tissue Models
- Thyroid
- Developmental Neurotoxicity (DNT)

DART-Related research at EPA

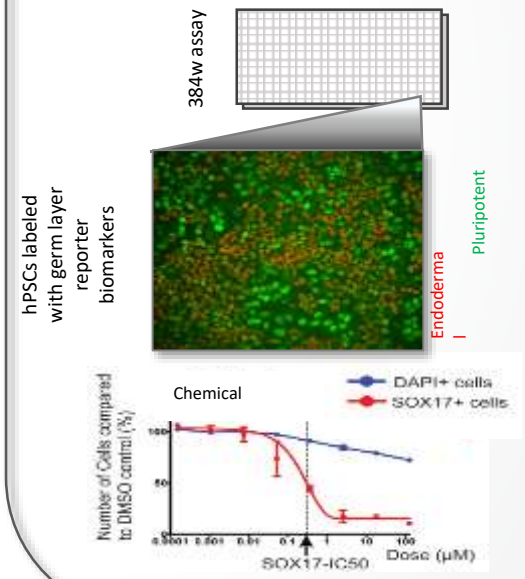
Application of Stem Cells for Screening





Biomarker-based Assay in a hESC Germ Layer Reporter +/- Metabolic Screen

2D HTS screening platform for developing endoderm +/- metabolism screening that utilizes a transgenic hESC line with fluorescent protein reporters for representative germ layer genes.



hPSCs seeded in 2D 384-well plate.

Definitive endoderm media is added to induce differentiation.

Chemical exposure of hPSCs +/-additional hepatic metabolism method.

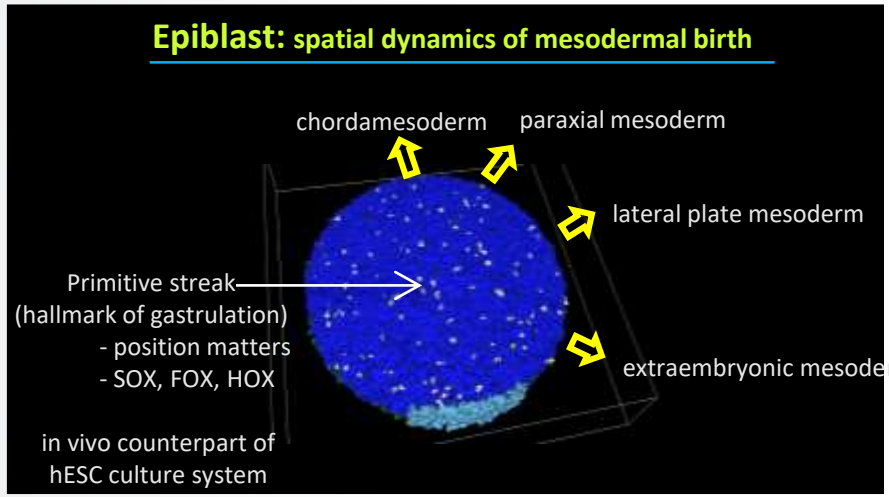
High content imaging to identify percentage of differentiated (SOX17+) and pluripotent cells.

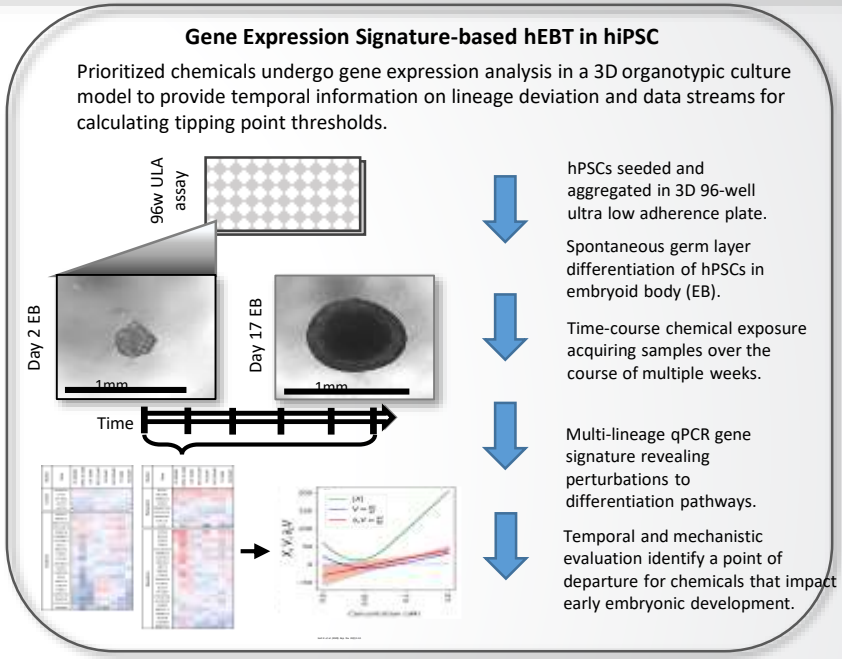
Chemicals that increase or decrease hPSCs endoderm differentiation are prioritized for further evaluation.

Kameoka S. et al. (2014). Toxicol Sci. 137(1):76–90.

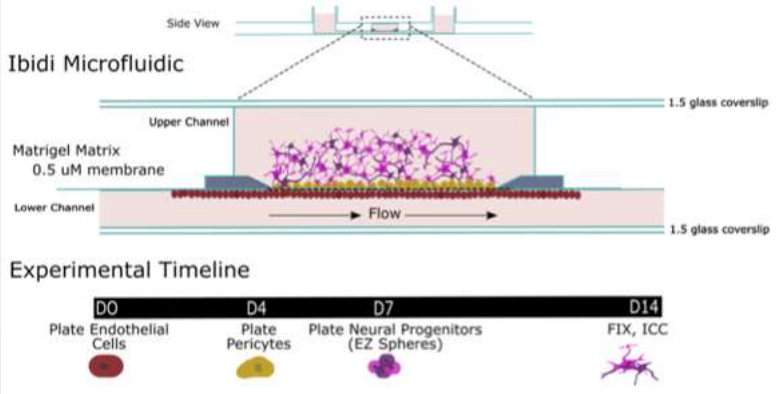
DART-Related research at EPA

Virtual Tissue Models

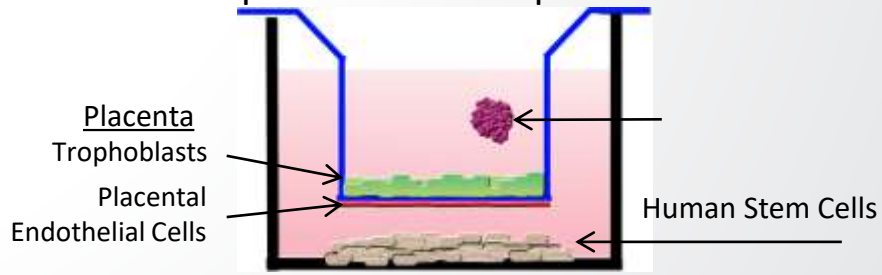




Microfluidic Models of Development



Three Compartment Developmental NAM

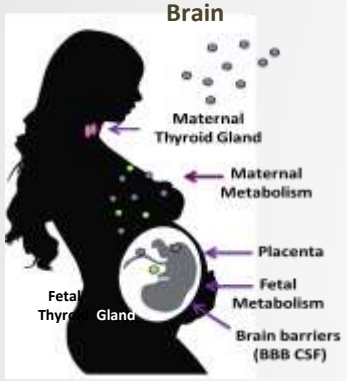


Assess chemical effects on liver, placenta, stem cell differentiation

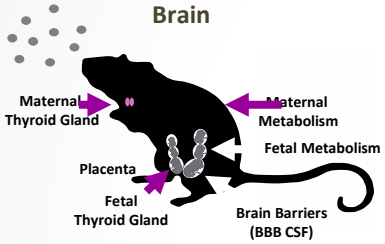
DART-Related research at EPA

Thyroid

Human Pregnancy
Thyroid
Brain



***In vivo* Rodent Models**
Thyroid
Brain



?

Gestational timing
Multiple births
Metabolism differences
Equivalent outcomes?

***In vitro* Models**
Thyroid
Brain

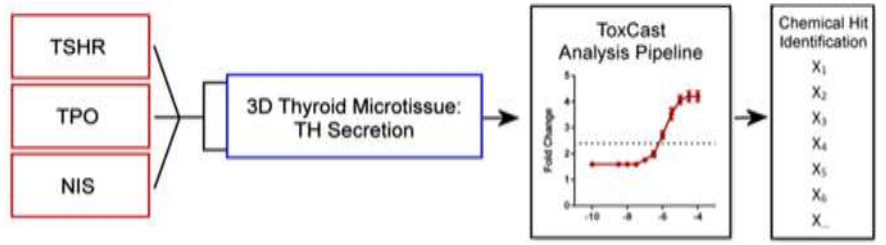
? ? ? ?

Are these the cells affected *in vivo*?
Is the dose relevant?
Metabolism - maternal, placental, fetal
Placenta, Brain Barriers
Assay for Chemical vs Hormone?

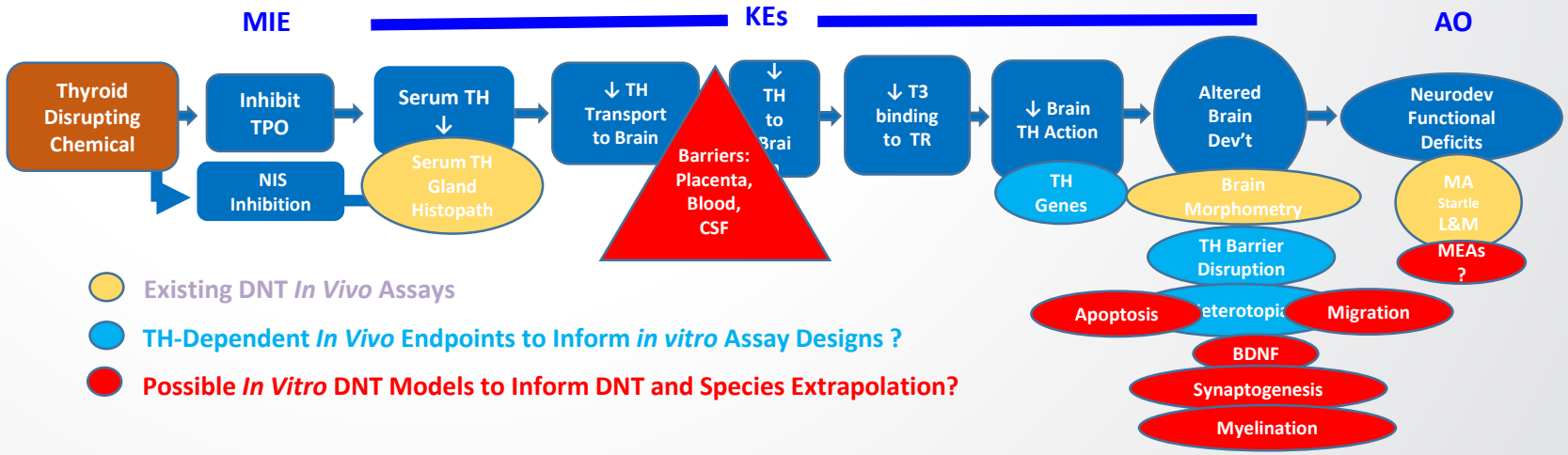
AOP & HTT ***VTM***

High-Throughput Chemical Hit Prioritization — Tier 2

Medium-Throughput Orthogonal Bioactivity Profiling and Human Relevance — Tier 3



Status of DART Testing for TH-Dependent DNT?

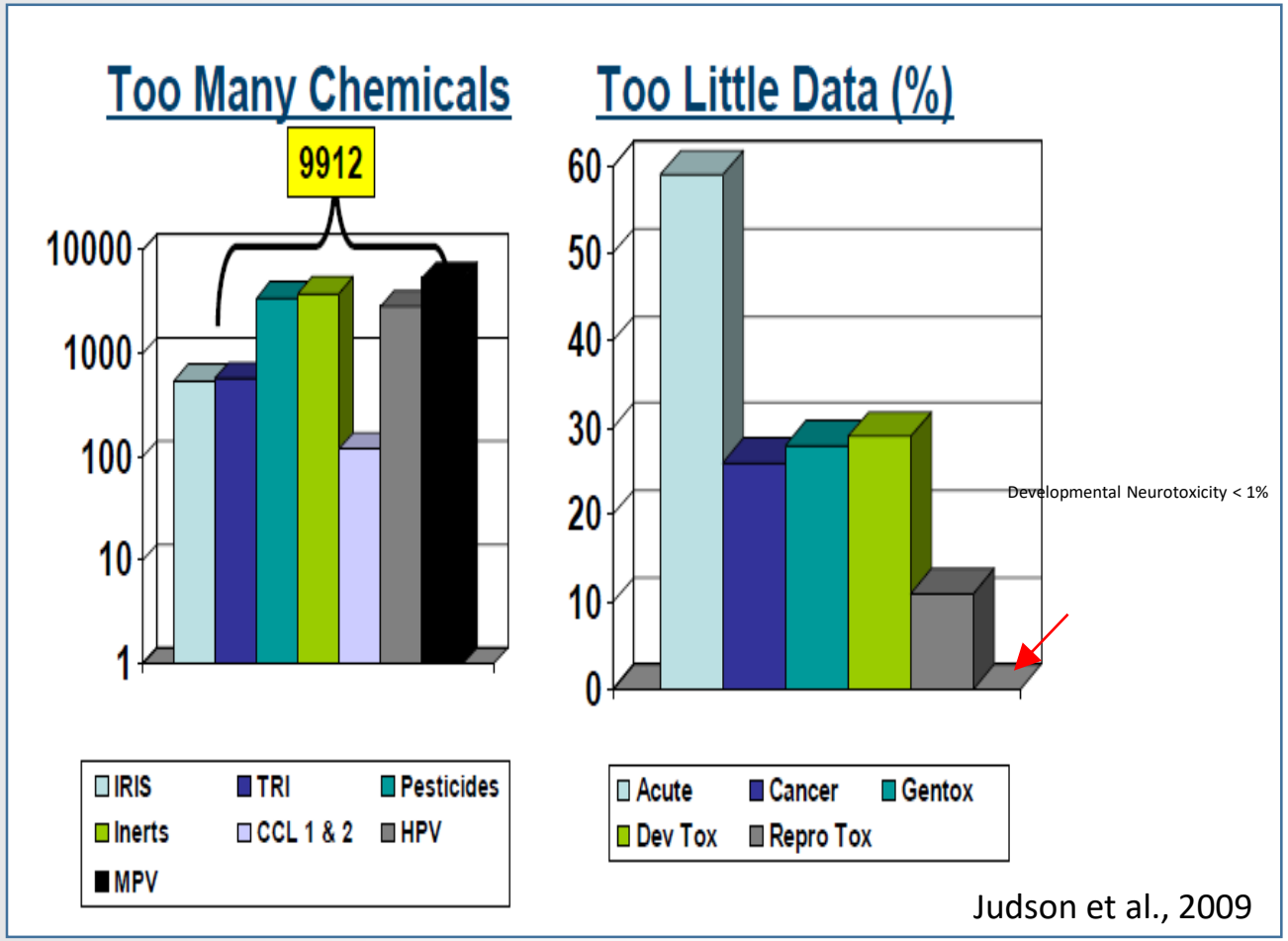


Existing DNT *In Vivo* Assays

TH-Dependent *In Vivo* Endpoints to Inform *in vitro* Assay Designs ?

Possible *In Vitro* DNT Models to Inform DNT and Species Extrapolation?

Many Chemicals Lack Developmental Neurotoxicity (DNT) Data



Current testing too slow

- Not Required under FIFRA
- Animal "Guideline" DNT; 1 chemical, $1M cost; 2 yr
- At current pace, ~150 chemicals in 20+ yrs
- Not often used (~25%) for point of departure values for risk assessment*

The absence of DNT hazard data on chemicals impedes consideration of this adverse outcome in environmental decision-making.

Reports of the potential involvement of environmental chemicals in increased rates of neurodevelopmental disease contributed to increasing public concern about DNT hazard of chemicals

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

Solution

Faster, inexpensive and predictive methods are needed to detect and characterize compounds with developmental neurotoxicity hazard

- Develop high throughput, *in vitro* assays,
- Characterize chemicals for developmental neurotoxicity hazard
- Data from these assays can provide information for decision-making

- Use human models whenever possible

International Efforts to Develop Alternatives for DNT Guideline Studies

- European Food Safety Organization
  - Funding research to develop and evaluate a battery of in vitro DNT assays
- Danish EPA
  - Supporting evaluation of DNT alternatives
  - Combination of structural and functional endpoints
  - Qualification of primary hits by secondary testing (same assay; and hit confirmation testing using an alternative assay)
  - Integration of dosimetry to improve hit prediction from screening results
- US EPA
  - Internal research on development of alternatives to DNT Guideline
    - Focus on Screening and Prioritization
- National Toxicology Program (NTP, National Institutes of Environmental Health Sciences (NIEHS))
  - Evaluating alternatives as a decision tool to best utilize limited resources for in vivo testing of nominated chemicals
  - Provided compounds for testing to a number of laboratories;
  - Built an interactive database (DNT DIVER) to house data and facilitate utilization of data for decision-making
- Organization for Economic Cooperation and Development (OECD)
  - DNT Expert Group

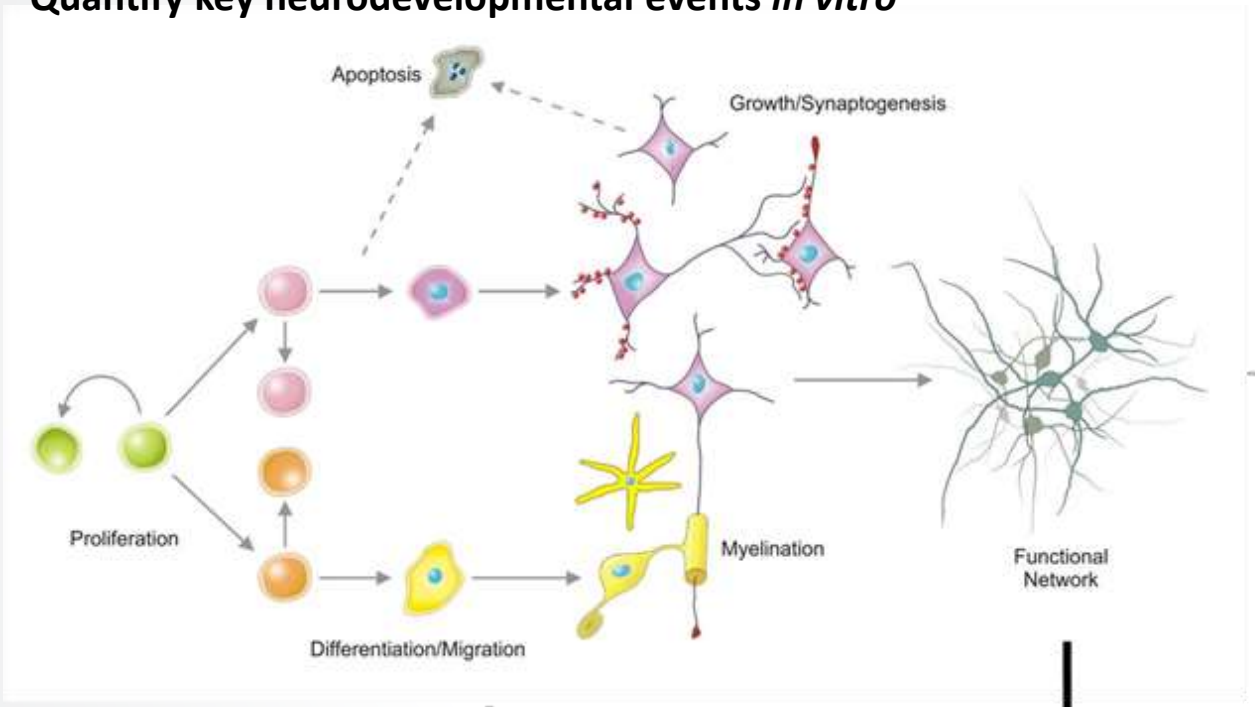
Challenges to Development of DNT Screens

- Central nervous system development is complex
  - Multiple potential targets
  - Time-dependent processes
  - Spatially dependent processes
- Which target? Where? When?

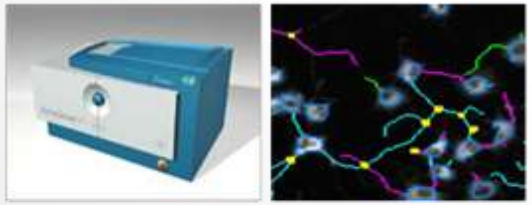
Research focus on *key neurodevelopmental processes*

Phenotypic Screening for DNT Hazard

Quantify key neurodevelopmental events *in vitro*



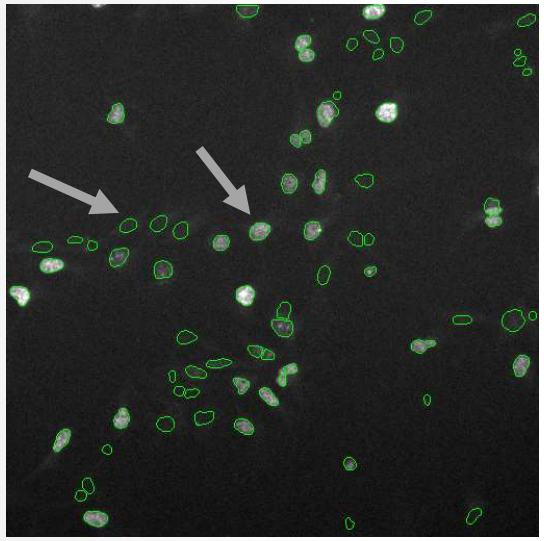
Key Neurodevelopmental Events (High Content Imaging)



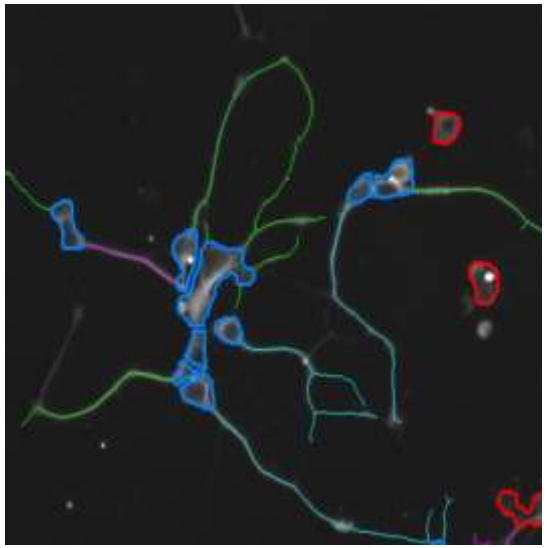
Network Formation (Multi-Electrode Array)

EPA Assays for DNT

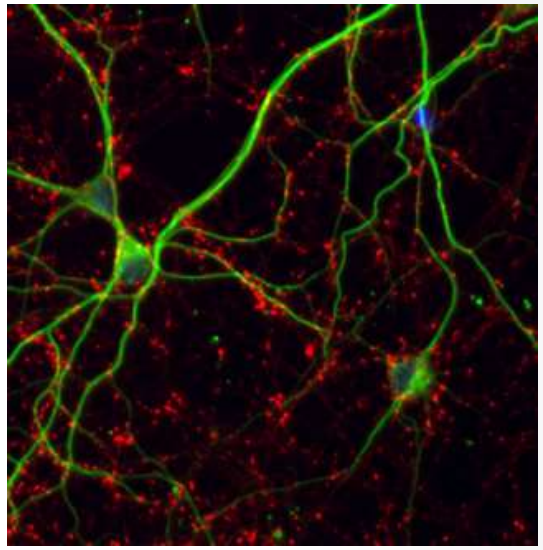
Proliferation



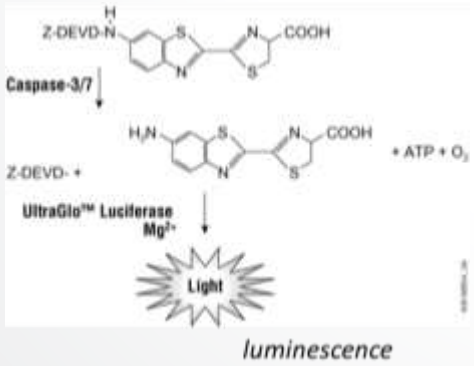
Neurite Outgrowth



Synaptogenesis

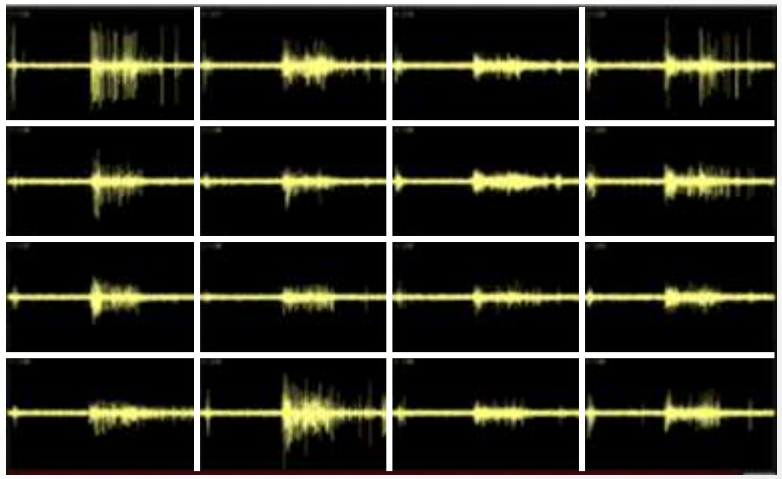


Apoptosis



luminescence

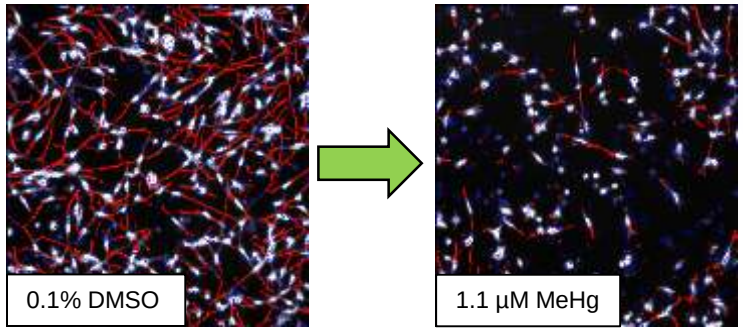
Network Function and Formation



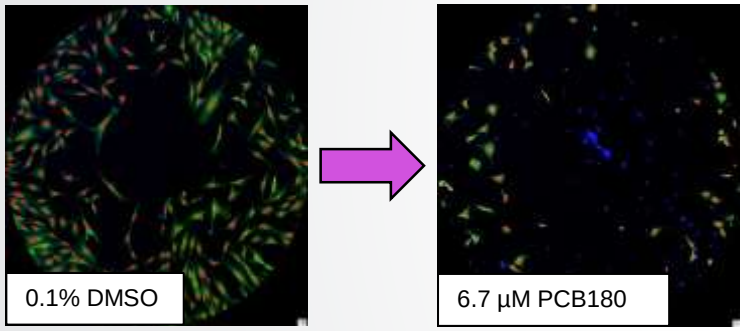
EFSA-Sponsored Assays

DNT compounds

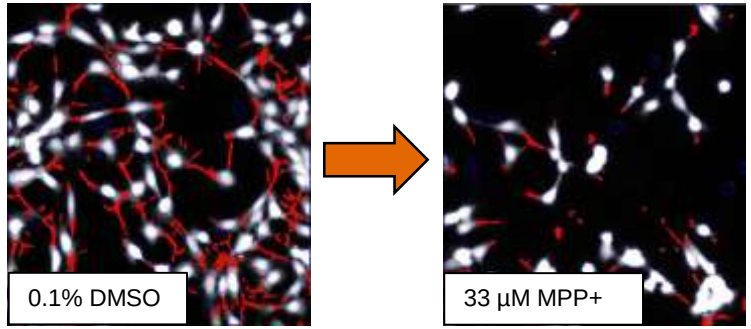
Effect on neurite outgrowth of PNS neurons

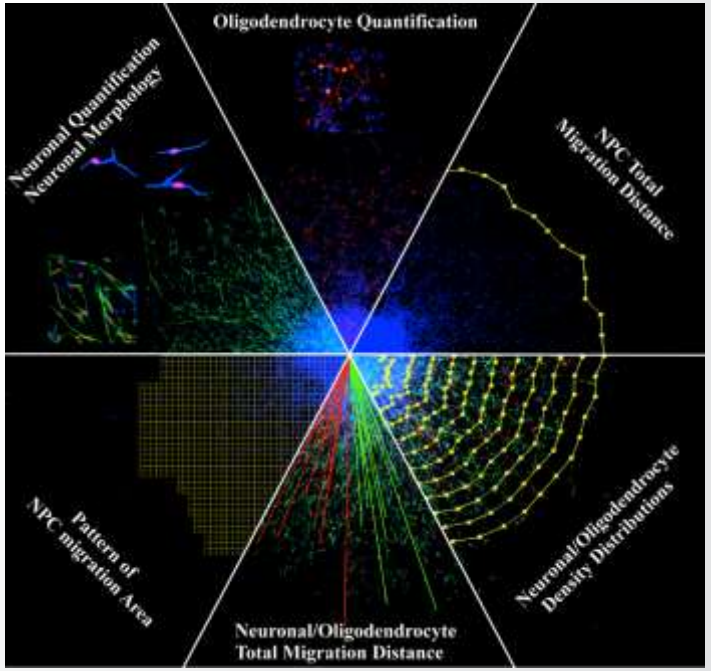


Effect on migration of neural crest cells

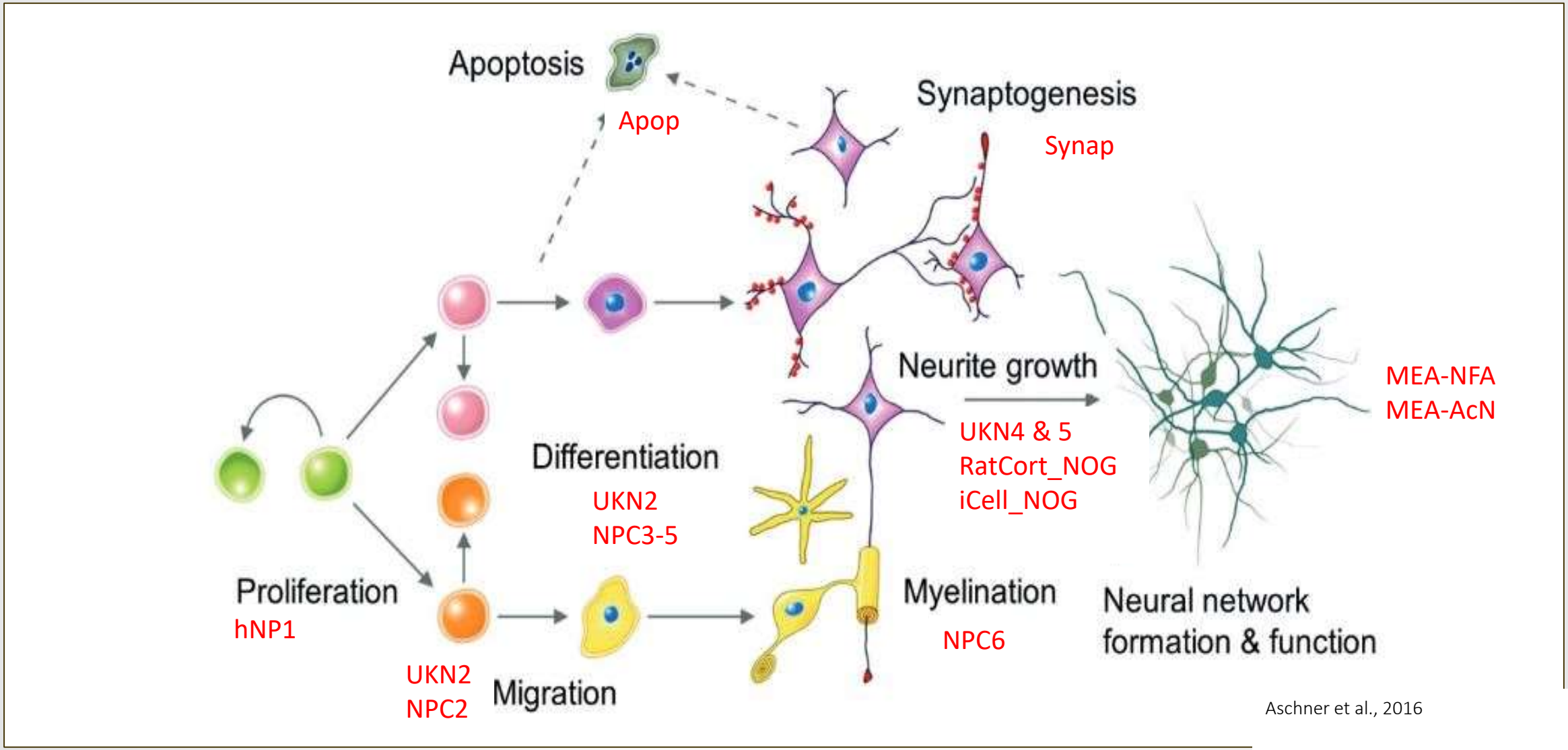


Effect on neurite outgrowth of CNS neurons





This Combination of Assays Provides Good Coverage of Neurodevelopmental Processes



Aschner et al., 2016

Encouraging Regulatory Use of Alternative Methods and for Guidance Document- What is Needed?

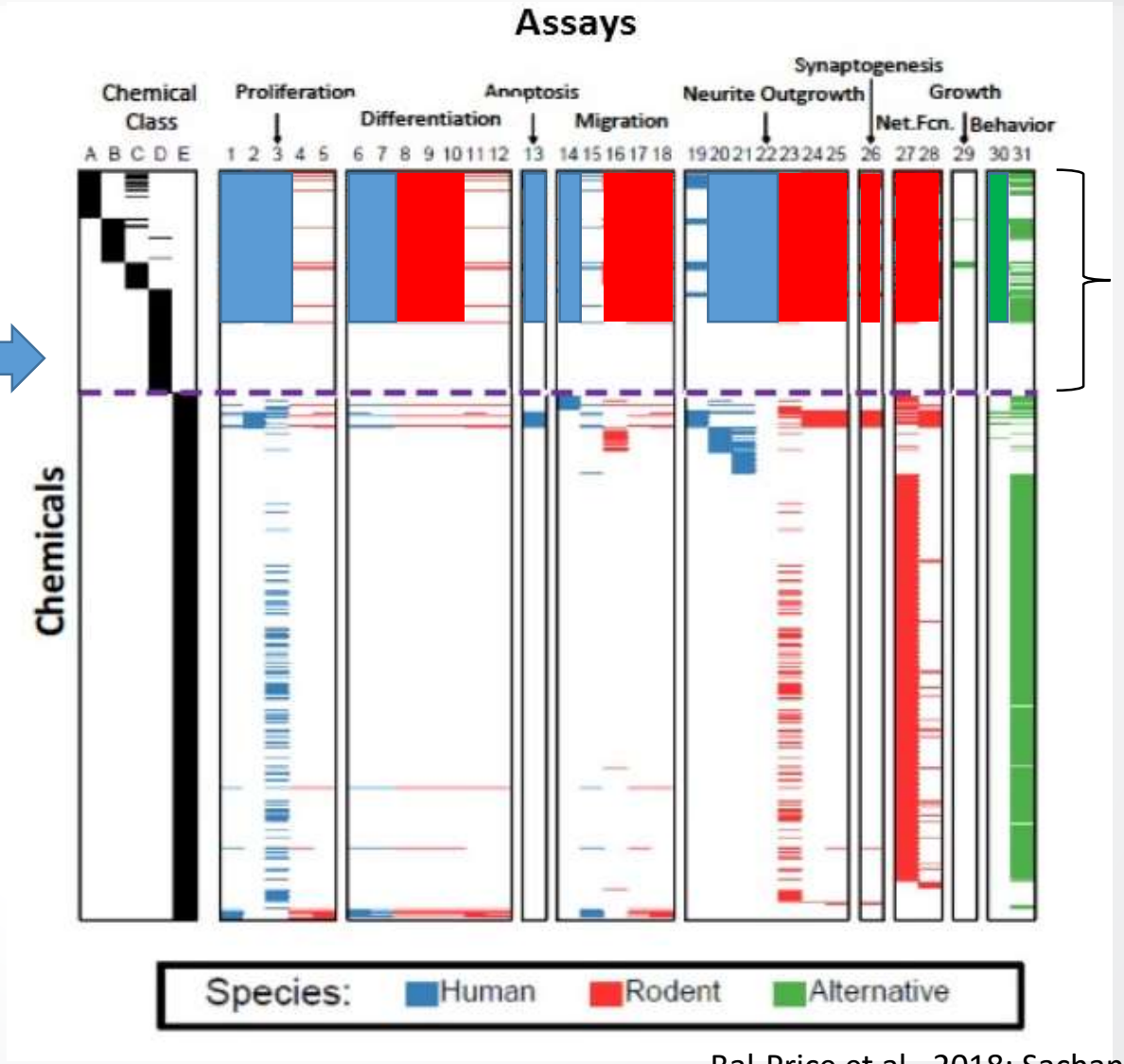
- Data from alternative assays
- Understanding of how the assays work and what they measure
- Evaluation of individual assays and the battery of assays
- Understanding of what can be done with the data
- Accessibility to the data

Regulatory decision-makers must have confidence in the assays and data in order to incorporate them into the decision-making process

The Need for More Data

Assay-specific Compound Lists; Focused on in vivo DNT

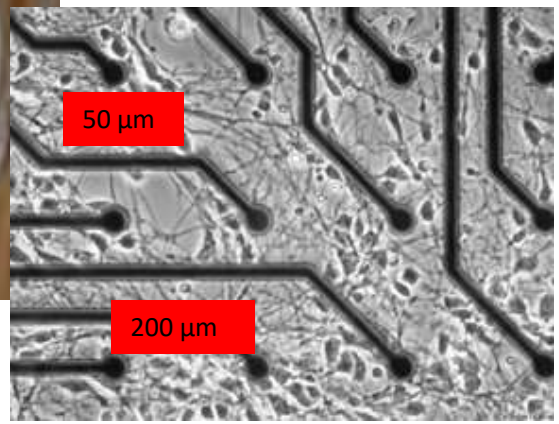
Assay 1
Assay 2
Assay3…



Assay Evaluation

Bal-Price et al., 2018; Sachana et al., 2019

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



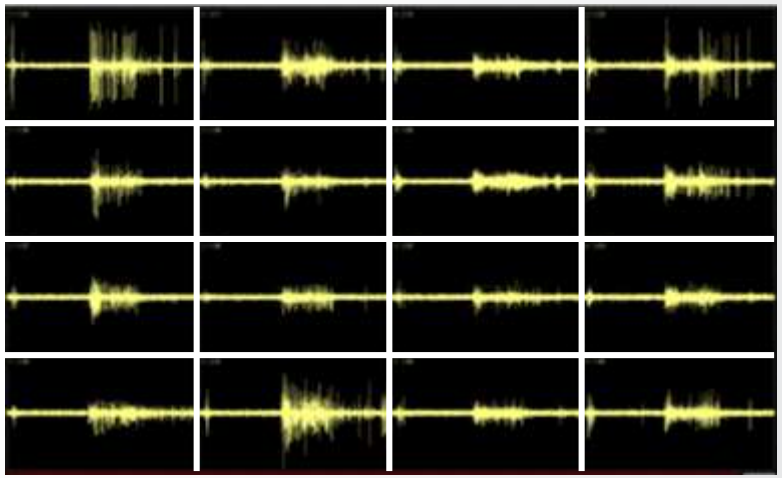
"Brain-on-a-Chip": Complex 2D model

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

Microelectrode Array Recording

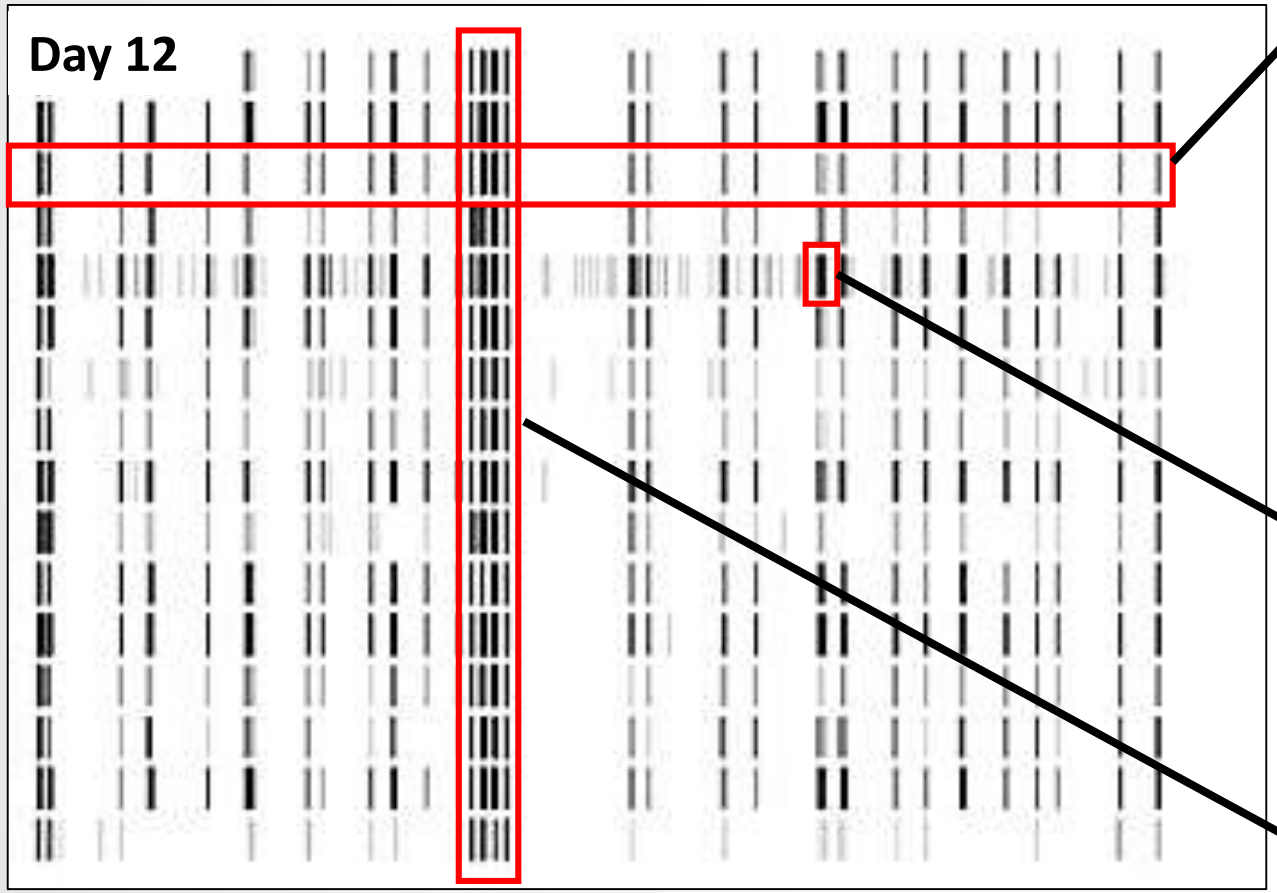
- Planar microelectrodes are non-invasive
- Records electrical activity of any tissue type
- Repeated recordings from same sample

The electrical activity recorded by MEAs are the biological underpinnings of EEG recordings.



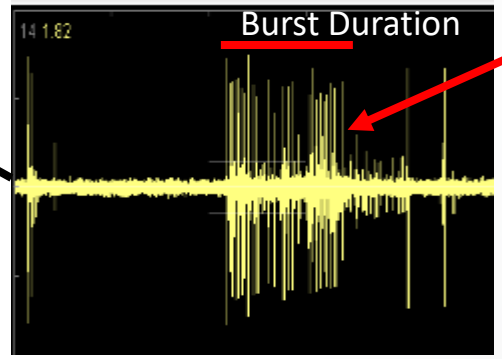
A snapshot in time of neural network activity in one well. Each box represents the electrical activity of neurons on 1 electrode in the array.

MEAs Measure Multiple Characteristics of Network Function



General Activity- overall rate of firing or bursting; measured on each electrode and averaged across the well.

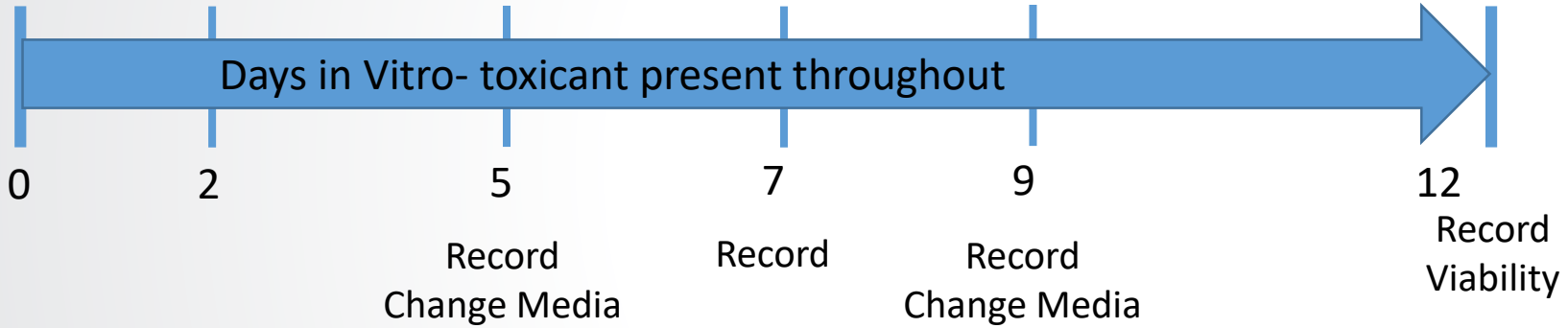
Bursting Structure- the length and number of events in a burst; measured on each electrode and averaged across the well.



Connectivity- Communication of information across electrodes (Correlation coefficients, Network Spikes, Mutual Information); averaged for the well.

Chemical Effects on Network Formation

Network Formation Assay (NFA)

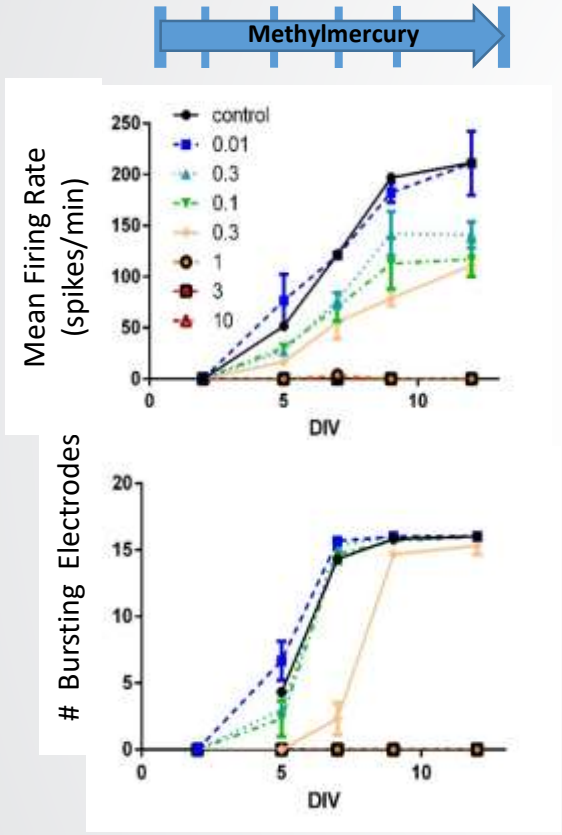


Tested to Date:

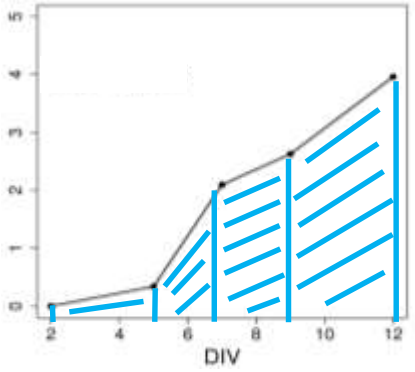
- **Brown et al., ToxSci. 2016.**
  - **Proof of Concept-6 Chemicals**
- **Frank et al., ToxSci. 2017.**
  - **DNT Reference Set-60 Chemicals**
  - **ToxCast/Uncharacterized- 20 Compounds**
- **Shafer et al., ToxSci. 2019**
  - **96 ToxCast Compounds**
  - **~40 NTP Compounds**
- **Unpublished**
  - **27 Organophosphates**
  - **75 PFAS Compounds**
  - **61 EFSA/EPA Compounds**

~300 compounds

Testing Chemicals for Effects on Neural Network Formation: Data Analysis in Brief

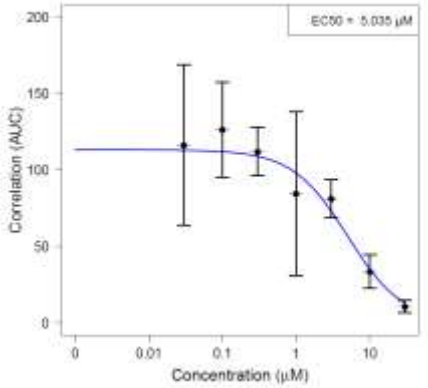


Determine Area Under the Curve for each dose (8) and parameter (17)

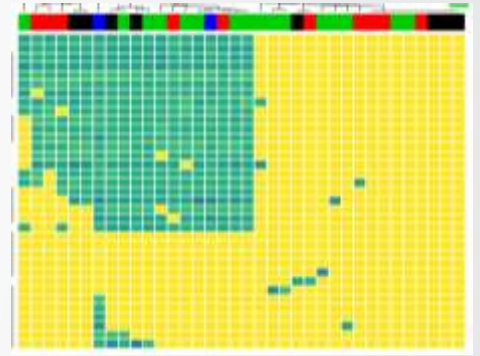


tcpl

Determine concentration-response

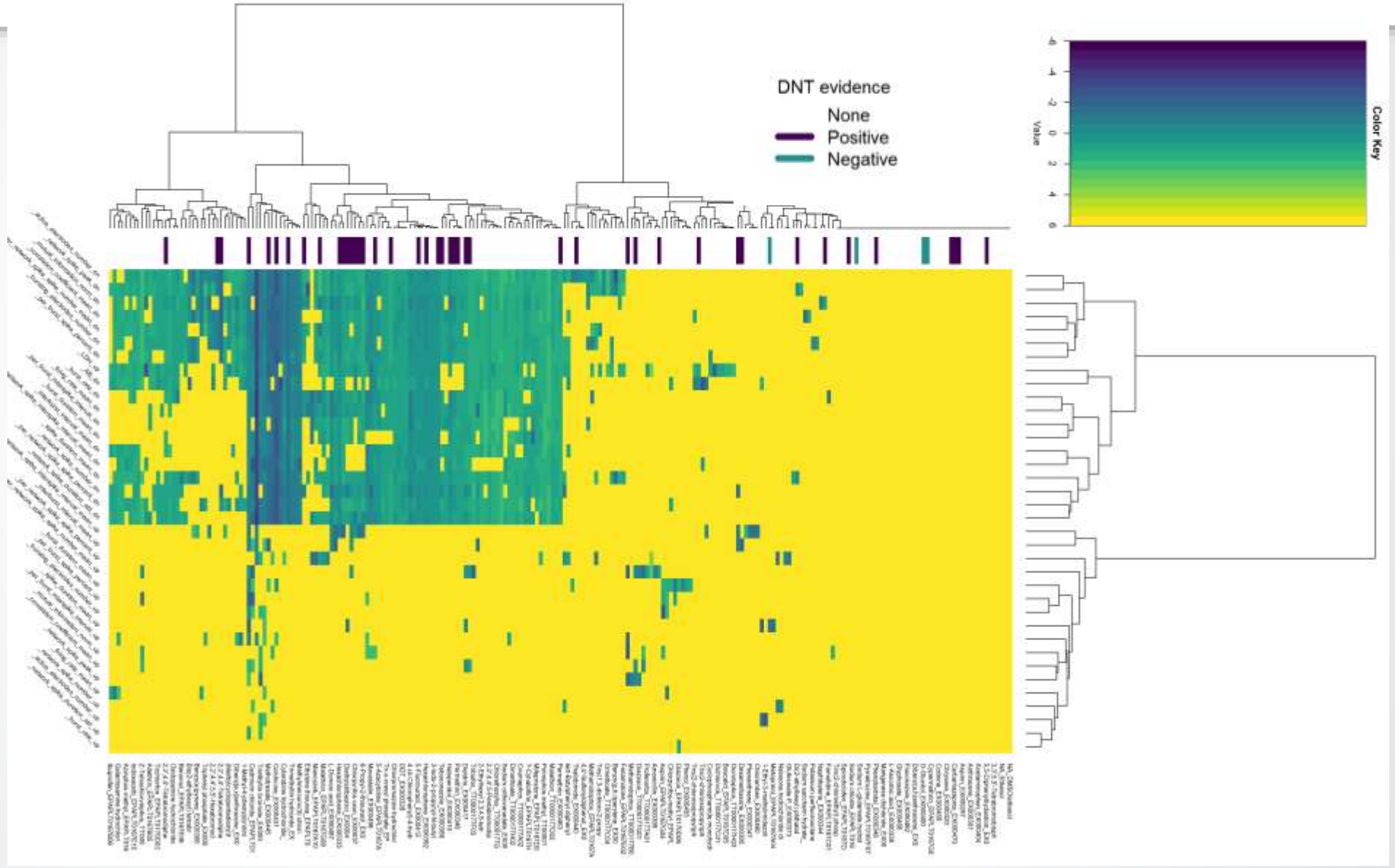


Generate comparisons of potency for many chemicals and endpoints

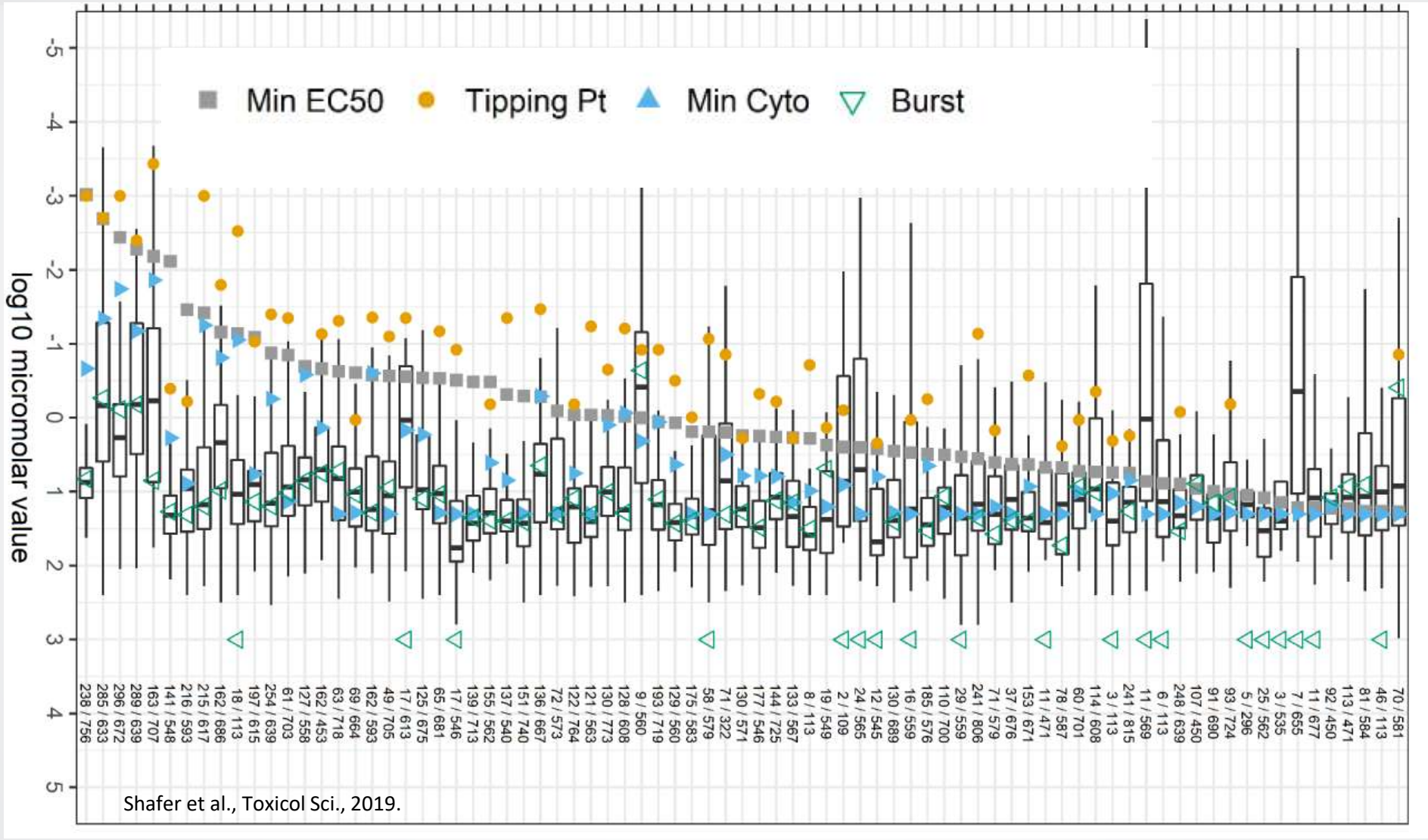


A snapshot of compound effects on network development

- A few main clusters; “on” and “off” behavior?
- Ability to distinguish negatives
- Cannot expect that one assay domain would identify all DNT positive chemicals.



The MEA_NFA covers different biology than other ToxCast Assays



Shafer et al., Toxicol Sci., 2019.

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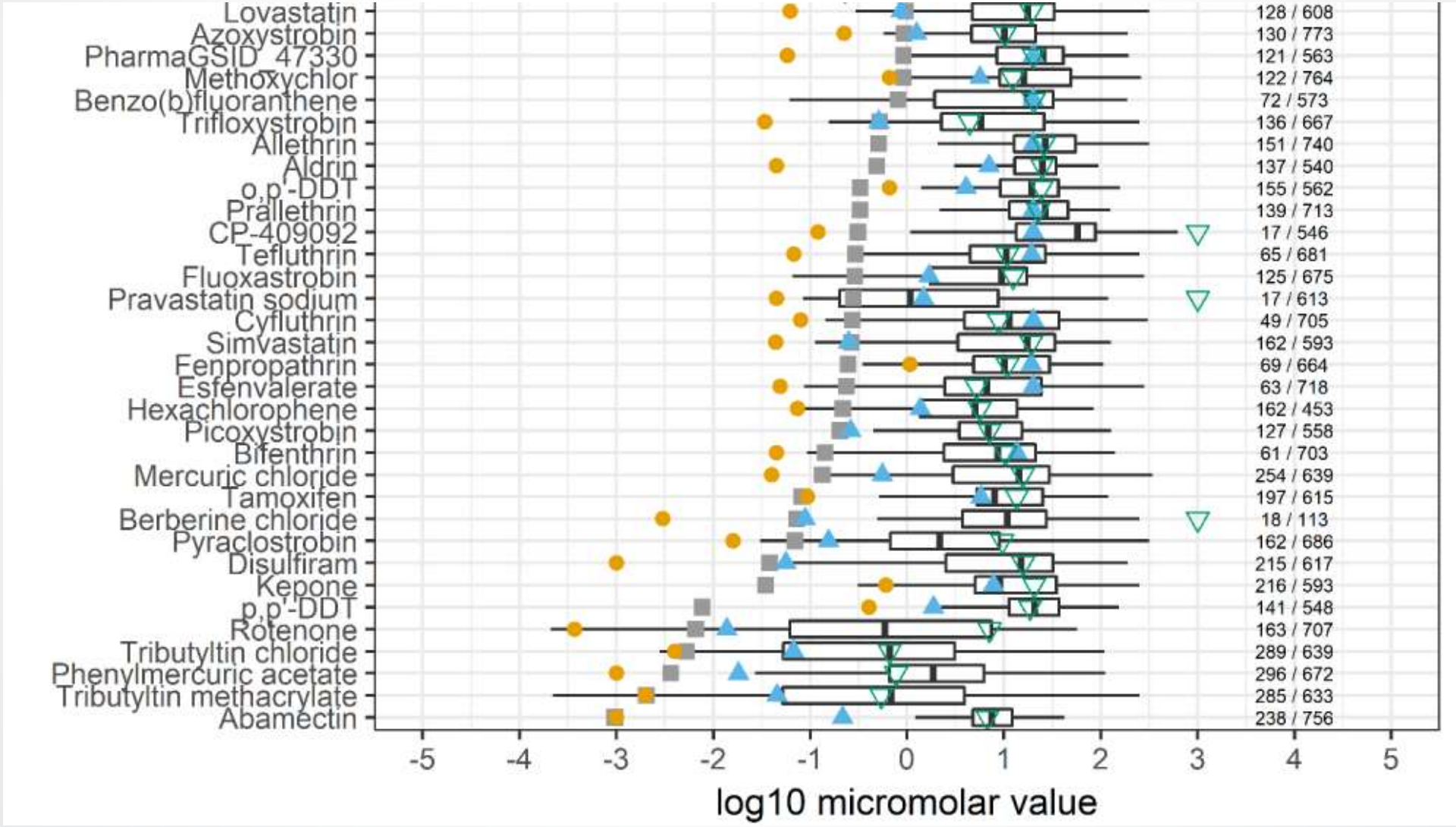
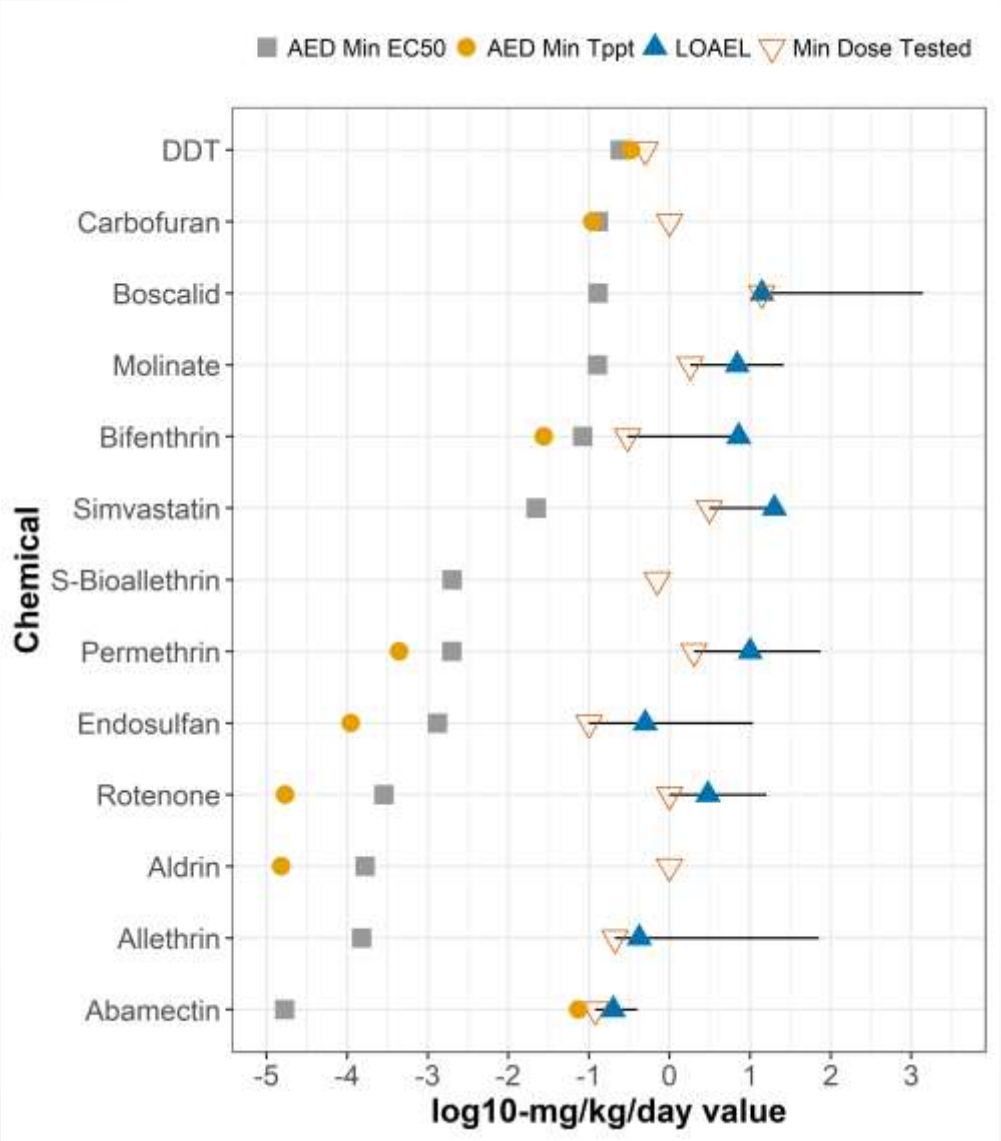


In vitro to *in vivo* Extrapolation indicates that MEA_NFA values are relevant



Shafer et al., Toxicol Sci., 2019

Adverse Outcome Pathway Development

Well established AOPs for developmental neurotoxicity may reduce the uncertainty in using data from DNT NAMs

While several of the few DNT-relevant AOPs in the AOPWiki include alterations in network function as a key event, overall there are few established AOPs linked to Acute Neurotoxicity or DNT

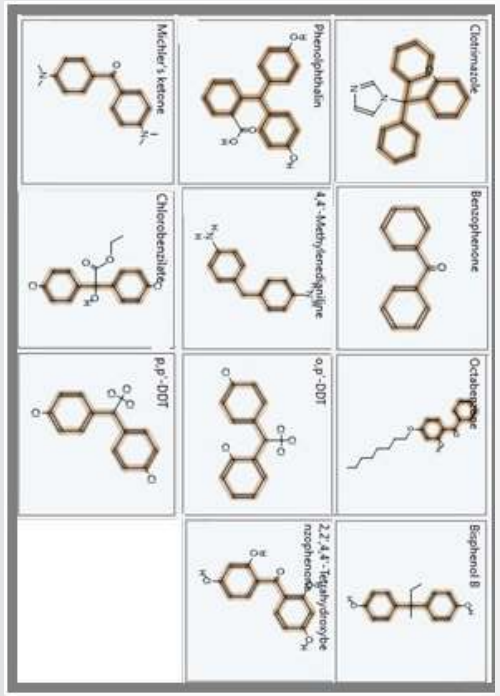
https://aopwiki.org/aops/15

High-throughput assays for DNT provide information for Adverse Outcome Pathway Development

Toxicant → **Molecular Initiating Event** → **Cellular Responses** → **Organ Responses** → **Individual Responses**

Chemical Property Profile → **?** **(many postulated, few confirmed)** → **Key Events** proliferation, differentiation, migration, neurite growth, synaptogenesis → **Nervous System** Δ connectivity (morphology, neurochemistry, physiology) → behavior, cognition

"Chemotyping"

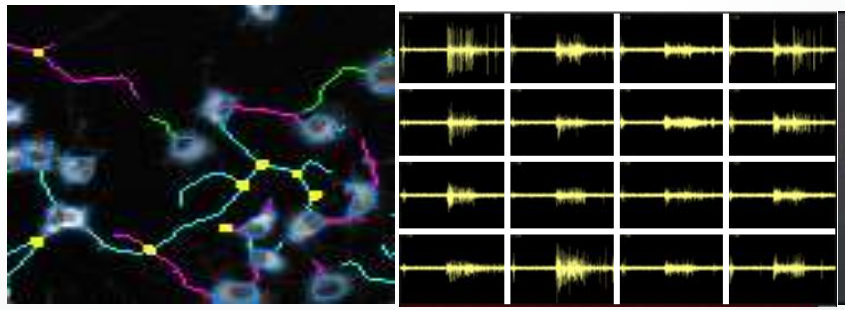


Target-based Screening (known MIEs)

HTP Molecular assays (ToxCast)
ion channels
AChE
neurotransmitter receptors
growth factor receptors
transcription factors
kinases
…

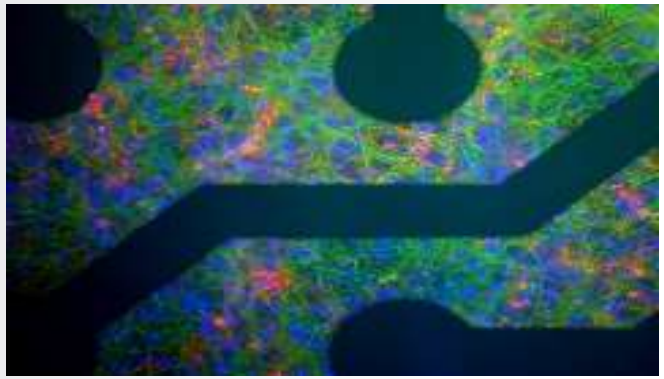
Phenotypic Screening in physiologically-relevant models (MIE not assumed)

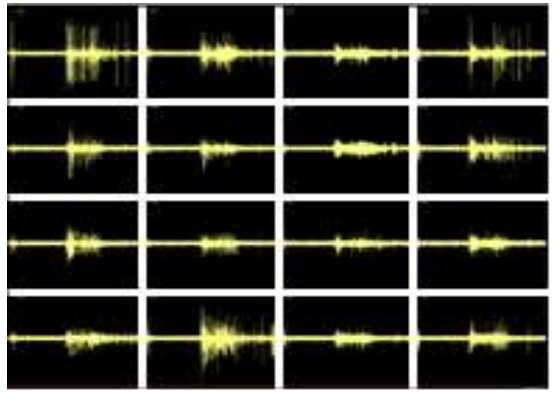
In Vitro Approaches

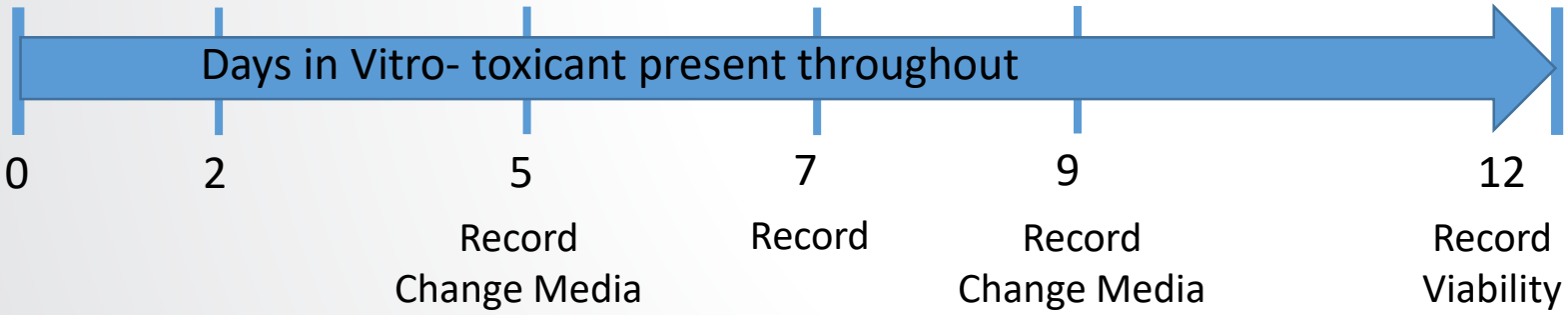


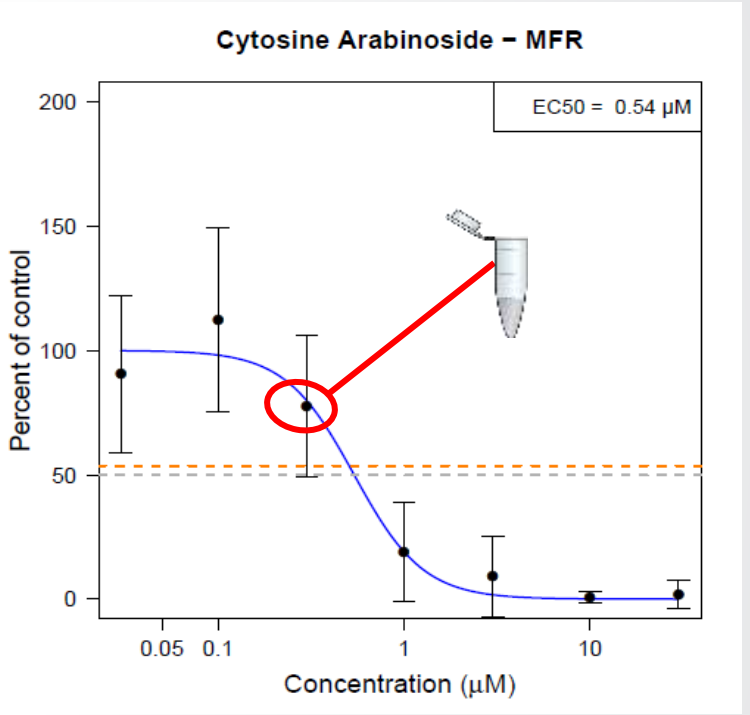
Behavior

Application of Transcriptomics and Metabolomics to in vitro DNT assays for AOP development





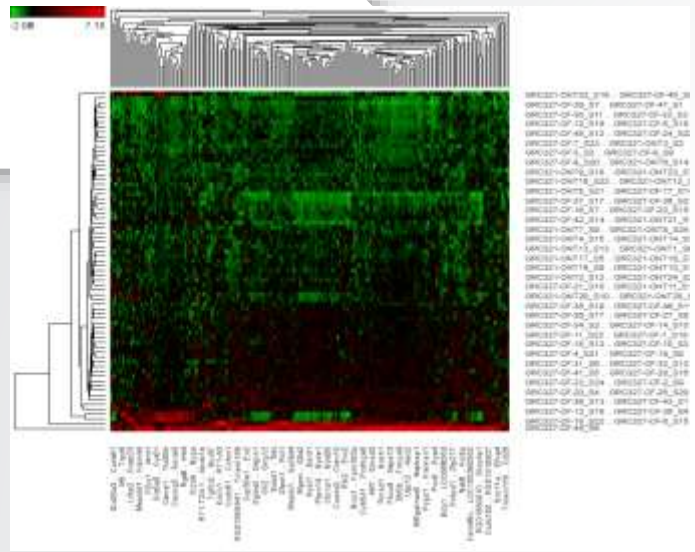




Critical concentration ("tipping point") determined

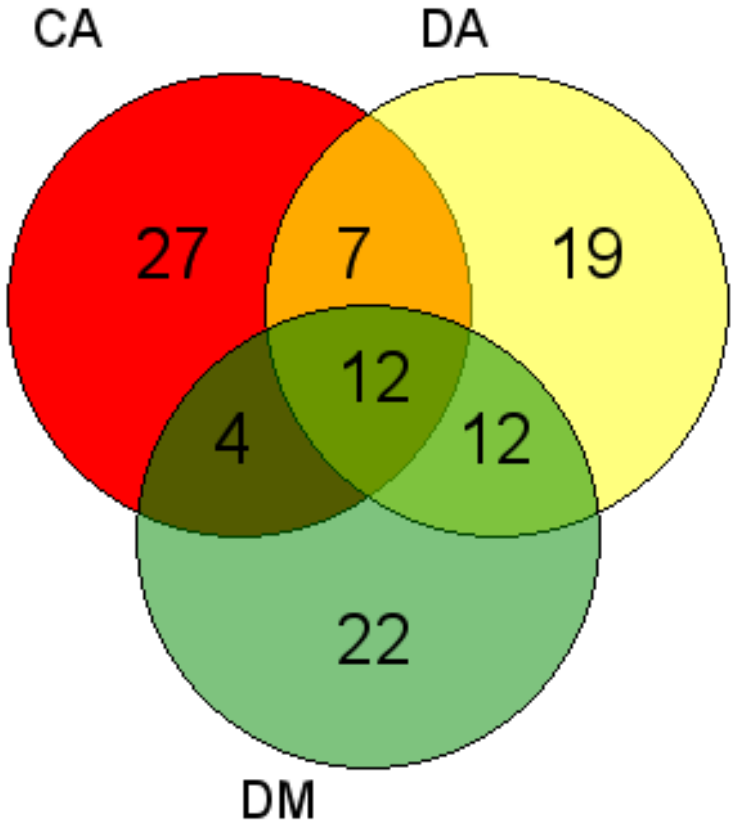
Six Chemical Proof of Concept

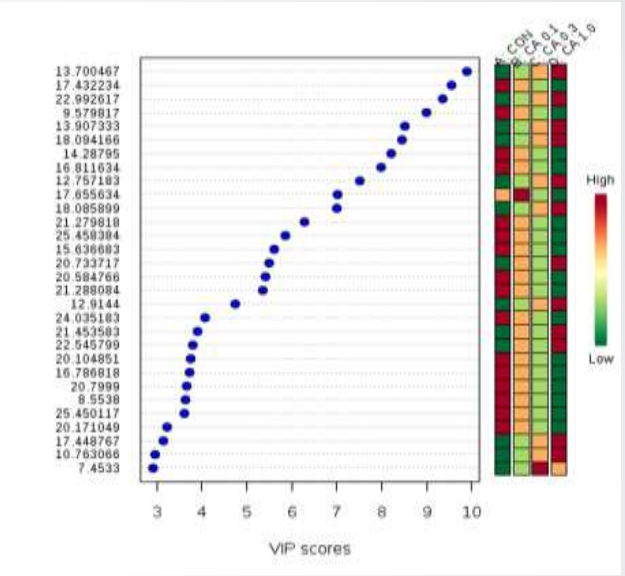
Domoic acid	Haloperidol
Cypermethrin	Deltamethrin
Cytosine Arabinoside	5-Fluorouracil



Transcriptomics

Canonical Pathway: Axonal Guidance





Metabolomics

Found in all three gene lists

ACTR3	EFNA5
ADAM15	EPHA7
ADAMTS5	FZD2
BMP7	FZD5
BRCC3	FZD7
EFNA4	GLI2

Summary

Promises:

- Data on DNT hazard for many more chemicals
- Characterization of DNT hazard on biologically-relevant processes
- Data from human models
- Substantially lower cost and faster results than *in vivo* studies
- Provide information on Key Events for AOP development

Challenges:

- Demonstration that the *in vitro* assays provide results that are equivalent to or better than animal models for DNT
- Development of additional case-studies using *in vitro* DNT assays
- Development of additional AOPs related to DNT that will increase confidence in using these assays
- Development of assays that cover areas of neurodevelopmental processes not well covered in the current battery

Thanks for your attention!

Questions?

Acknowledgements:

EPA:

- Theresa Freudenrich
- Kathleen Wallace
- Katie Paul-Friedman
- Bill Mundy
- Josh Harrill
- Imran Shah
- Chris Frank (EPA post-doc)

Students/Student contractors:

- Jasmine Brown
- Amy Carpenter
- Seline Choo

Support:

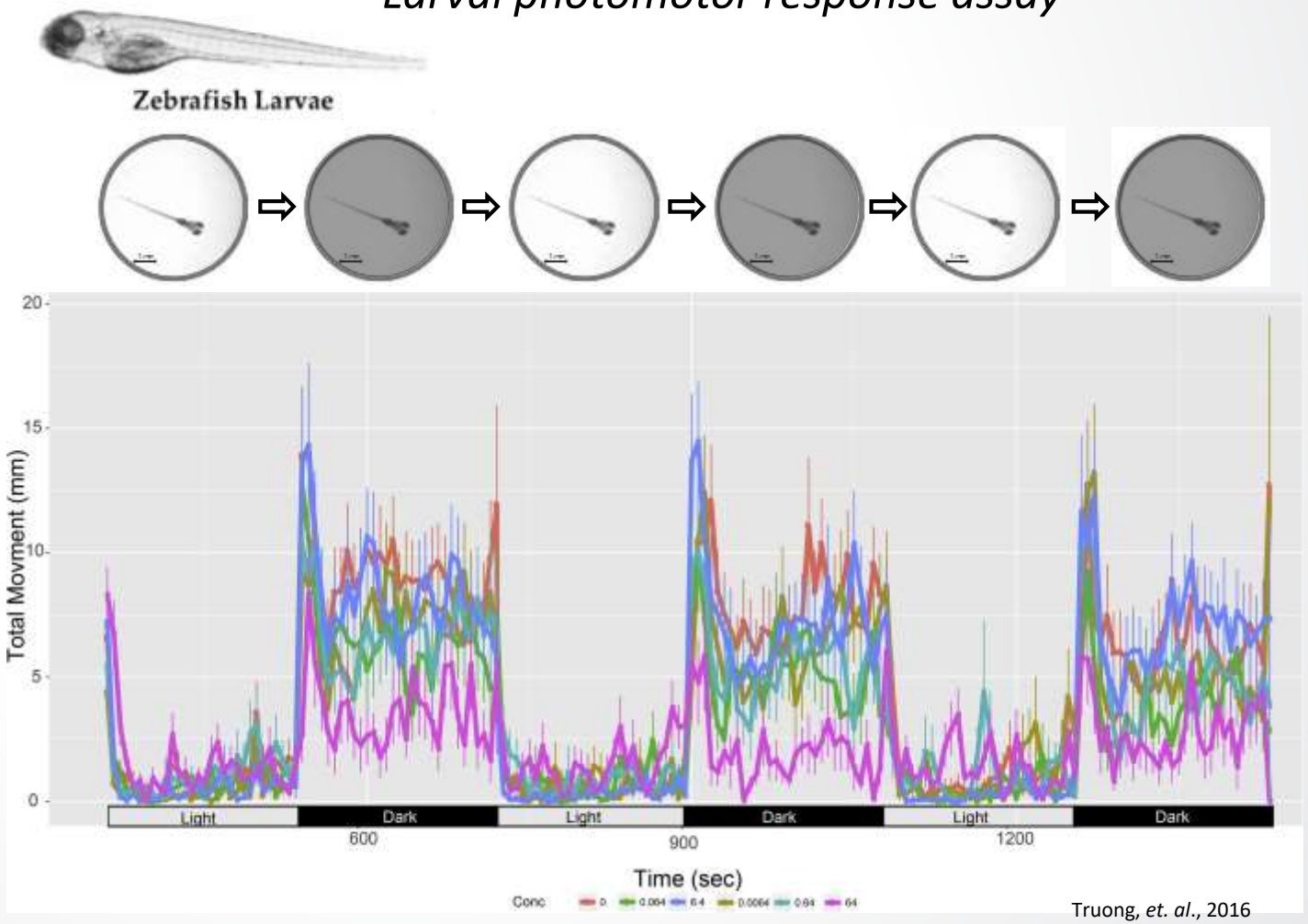
- Chemical Safety for Sustainability Research Program
- EPA Pathfinder Innovation Project Awards

“Fish on a Chip”: Linking network formation and behavior

- Their ease of accessibility, genetic engineering, and behavioral screens make zebrafish useful models for many neurological diseases
- Compared to *in vitro* assays, *in vivo* behavioral assays more closely recapitulate human neurodevelopmental disorders
- Zebrafish have been established for the *in vivo* assessment of DNT
  - Larval photomotor response: an assay typically used to assess the zebrafish startle response
- Lack throughput and mechanistic information

Zebrafish Behavior:

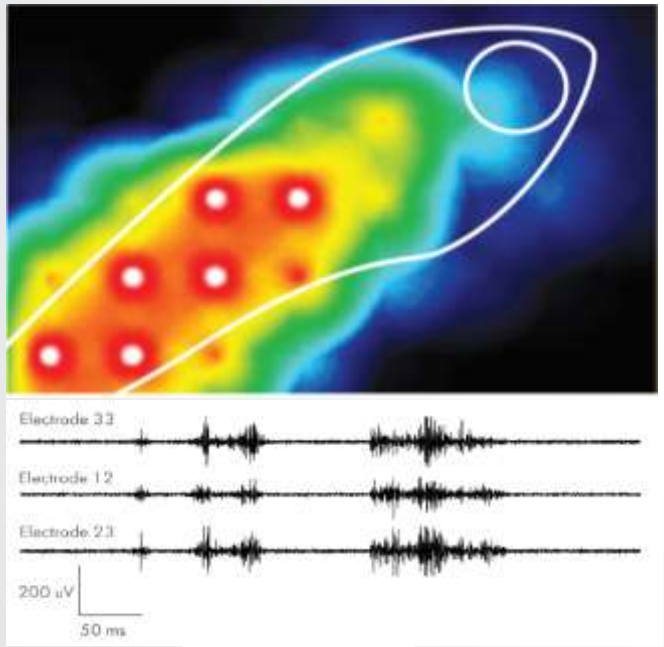
Larval photomotor response assay



Truong, *et. al.*, 2016

Specific Aim 2: Develop a novel zebrafish larval photomotor response assay using MEA technology

Spontaneous electrical activity in brain of zebrafish larvae



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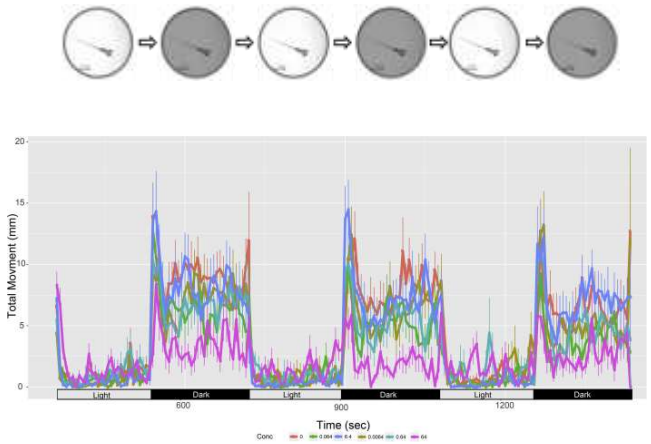
Zebrafish larval photomotor response assay



Truong, *et. al.*, 2016

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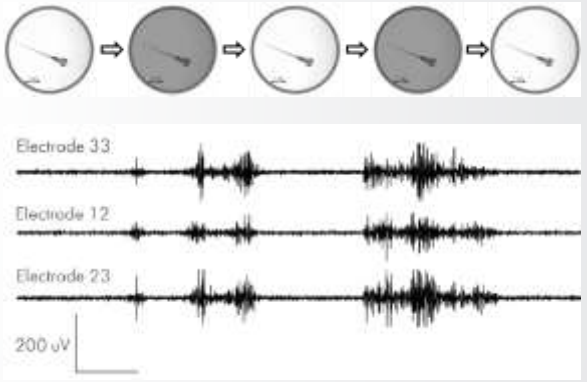
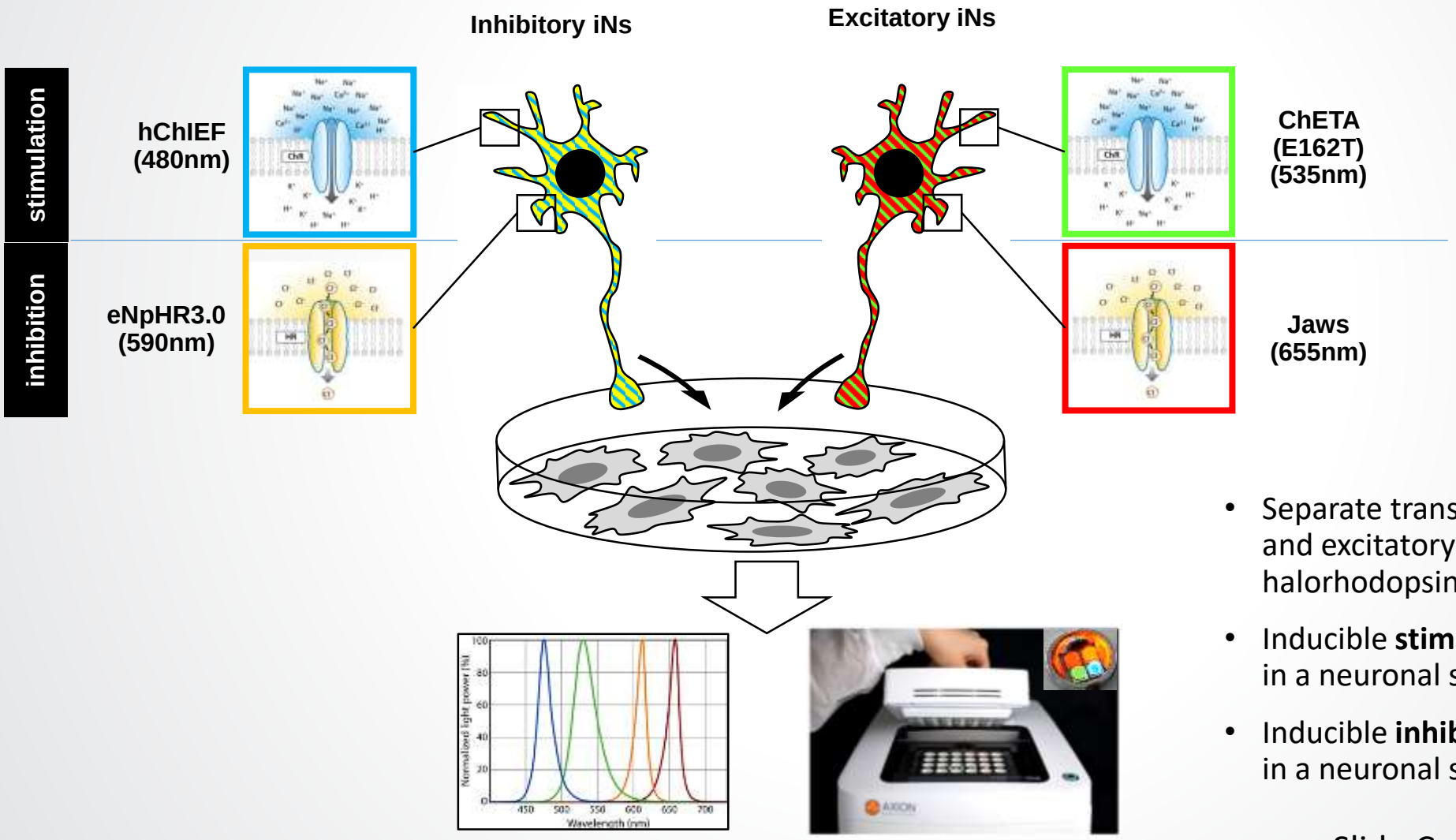
Modified larval photomotor response assay



Record electrical brain activity at baseline and during dark to light transition period

Specific Aim 1. Using optogenetic approaches, develop human neural network models in which we can specifically increase or decrease activity in excitatory or inhibitory neurons.



Lumos multi-well optical stimulator uses different wavelengths of light to activate the opsins

- Separate transduction of inhibitory iNs and excitatory iNs with rhodopsin and halorhodopsin variants
- Inducible **stimulation** of neuronal activity in a neuronal subpopulation
- Inducible **inhibition** of neuronal activity in a neuronal subpopulation

Slide Courtesy of NeuCyte

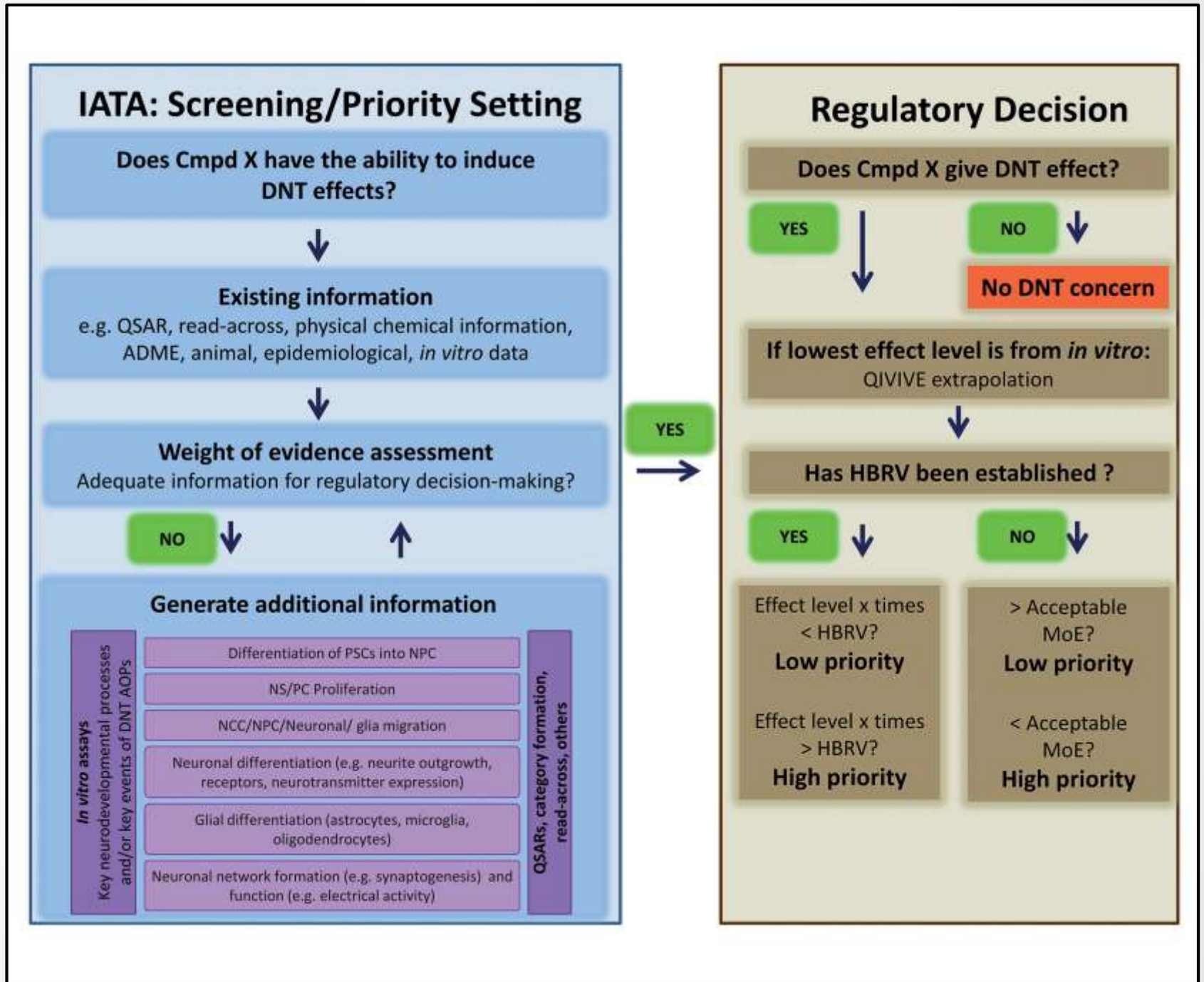
Development of a Chemical Library

- Identified ~136 compounds:
  - Compounds for which DNT Guideline studies are available
  - Compounds of interest for Integrated Approaches to Testing and Assessment (IATAs)
  - Compounds where the Danish EPA has in vivo data
  - Negative compounds
  - Modulators of developmental pathways
- These compounds will be tested in the 12 different DNT assays
- ToxCast has supplied most of these compounds

- Compounds will be tested by EPA, University of Konstanz and University of Dusseldorf in a variety of in vitro assays

Status and Timelines

- Partners have received ToxCast compounds.
  - Testing is Completed at Konstanz and Duesseldorf
    - Report is expected to be released to public in October 2020.
  - EPA testing is nearing completion
    - Data expected in late 2020
  - Zebrafish behavioral testing
    - Focus on ~30 IATA compounds
    - Data collection has started and will be completed later in 2020.



HBRV = health-based reference value