# *International Progress on New Approach Methods for Developmental Neurotoxicity Hazard Identification*

Timothy J Shafer, PhD

November 11, 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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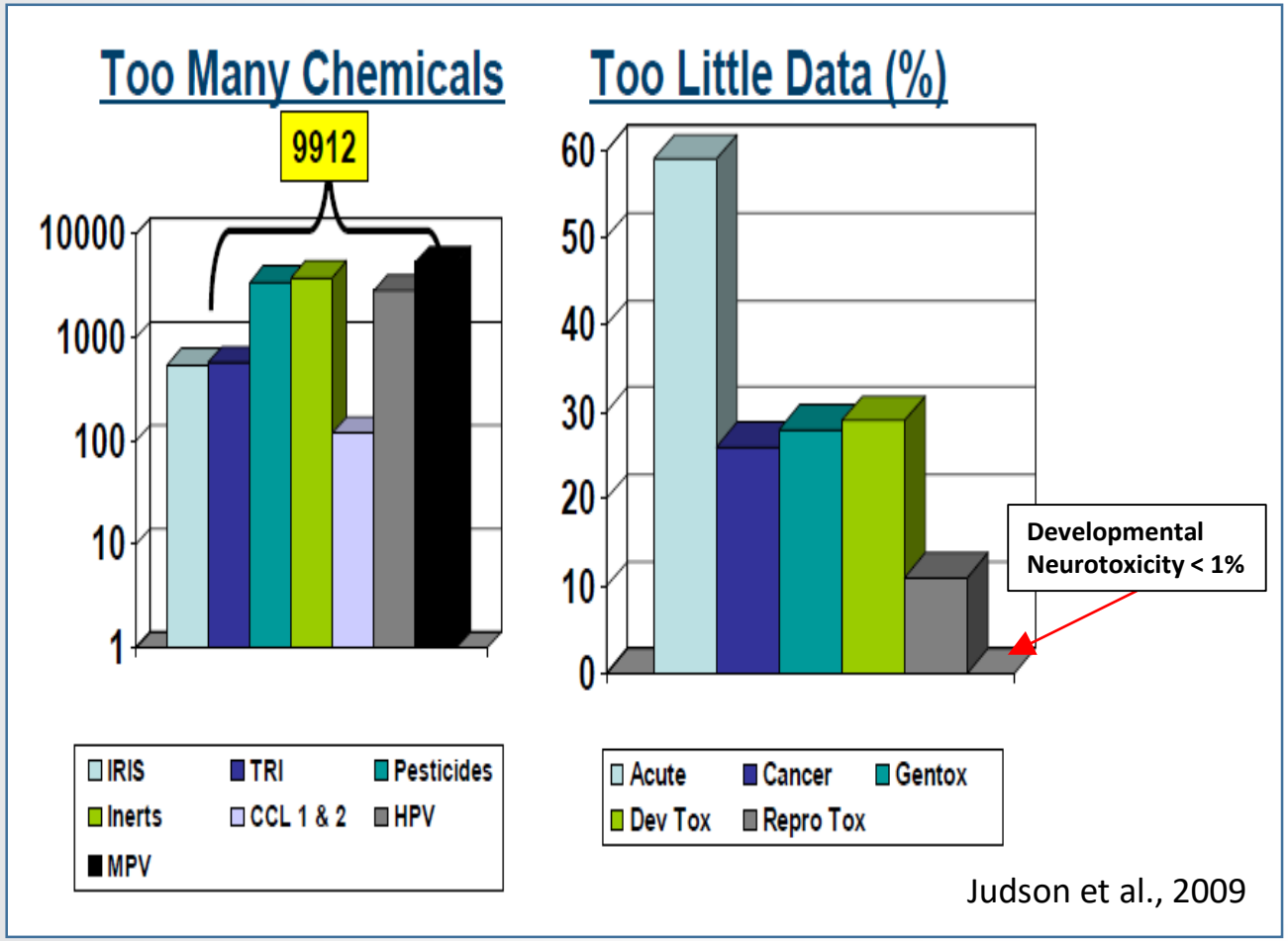
# Many Chemicals Lack Developmental Neurotoxicity (DNT) Data




Judson et al., 2009

**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.

# Requirements of EPA 870.6300 (OECD TG 426/443)

- 6 Pregnant females/dose (20 litters/dose recommended)
- 10 pups/litter (5 male/5 female)
- Minimum 3 doses + control
- Dosing period GD6-PND10
- Assessments on PND 4, 11, 21, 35, 45, 60

- Signs of Maternal Toxicity
- Developmental landmarks
- Brain/body weights (4, 11, 17, 21 PND)
- Motor activity (13, 17, 21, 60 PND)
- Auditory Startle (weaning, PND 60)
- Learning and memory (weaning, PND 60)
- Neuropathology (PND 11 and termination)
  - Major brain regions

https://beta.regulations.gov/document/EPA-HQ-OPPT-2009-0156-0042
https://www.oecd-ilibrary.org/environment/test-no-426-developmental-neurotoxicity-study_9789264067394-en
https://www.oecd.org/chemicalsafety/test-no-443-extended-one-generation-reproductive-toxicity-study-9789264185371-en.htm

# 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
  - Quality of data varies considerably
  - Not often used for point of departure values for risk assessment*

*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

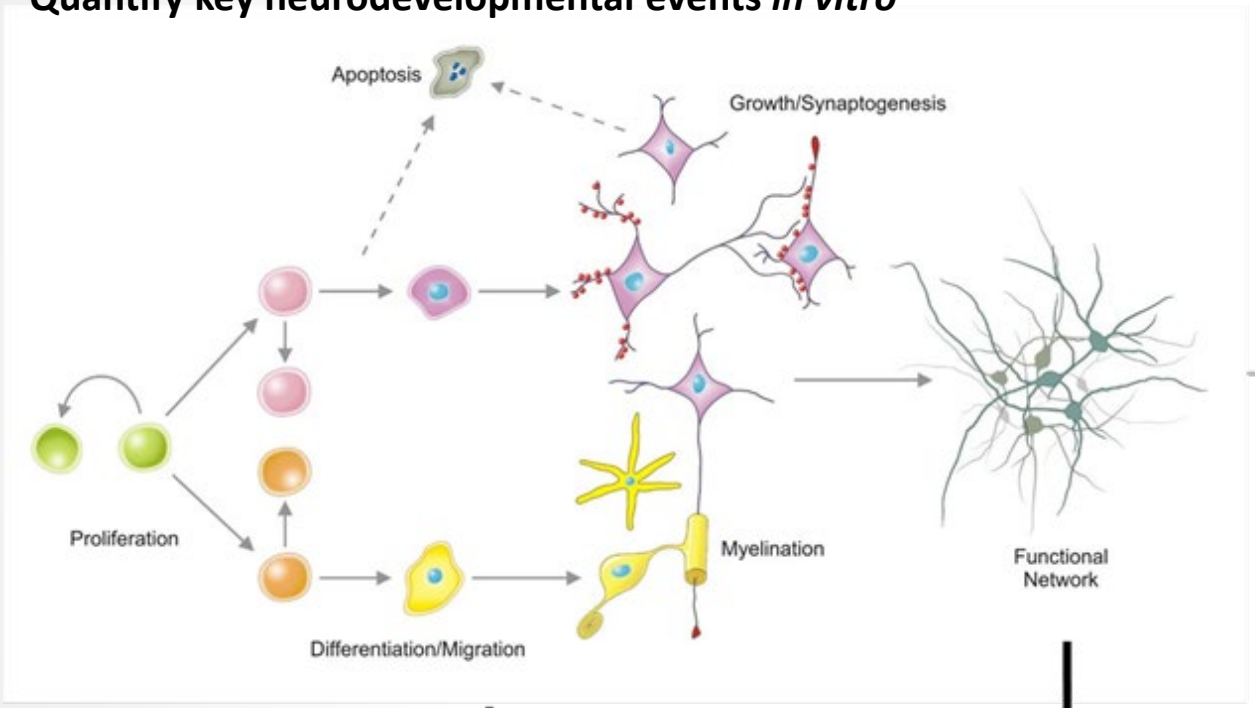
# Challenges to Development of DNT Screens

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

Therefore, focus research on *key neurodevelopmental processes*

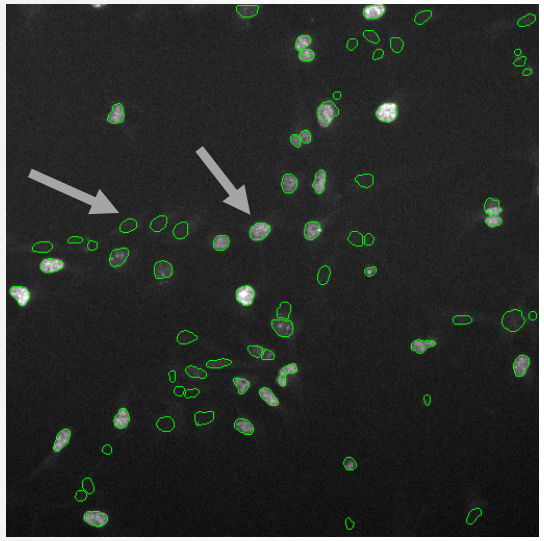
# Phenotypic Screening for DNT Hazard

**Quantify key neurodevelopmental events *in vitro***

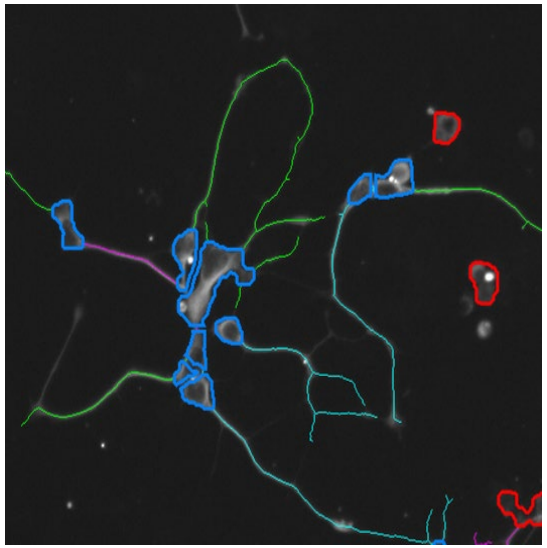


# EPA Assays

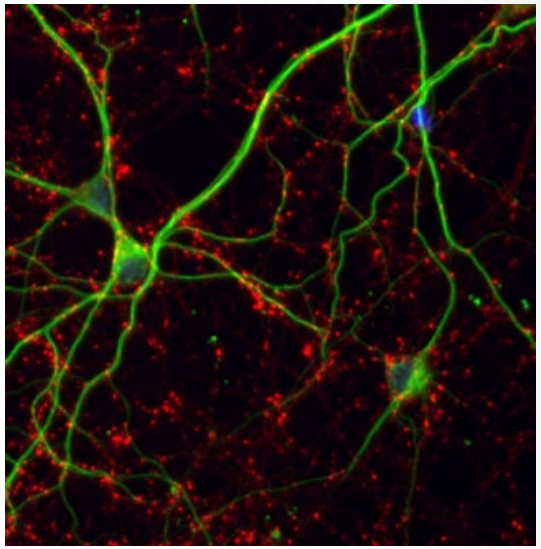
Proliferation



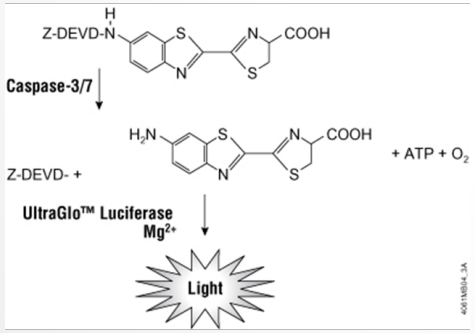
Neurite Outgrowth



Synaptogenesis

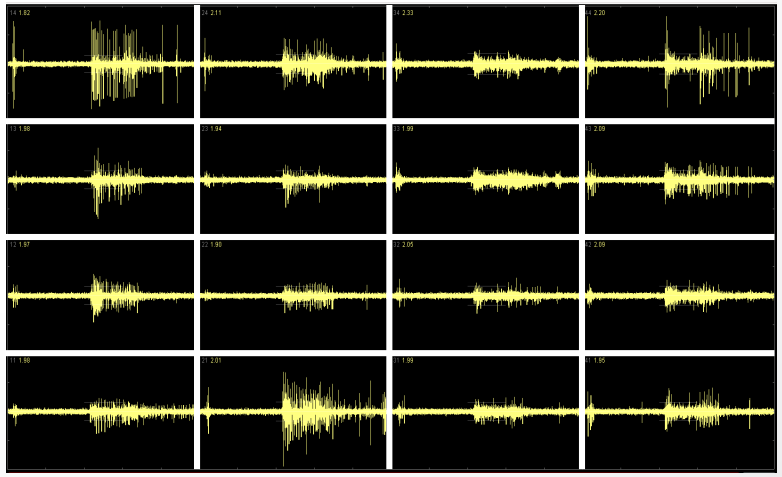


Apoptosis




*luminescence*

Network Function and Formation



# 2004 – Today
# National and International Science and Stakeholder Engagement

## Workshops to promote the development and use of *in vitro* DNT assays for regulatory use.

- 2005 - *In Vitro* Alternative Methods for DNT, Ispra, Italy (Coecke at al. EHP, 2007)
- 2006 - DNT TestSmart I (Lein et al. EHP, 2007)
- 2008 - DNT TestSmart DNT II (Crofton et al. ALTEX 2011)
- 2011 - DNT TestSmart III (Bal-Price et al. ALTEX 2012)
- 2014 - DNT TestSmart IV
- 2014 - ISTNET DNT (Bal-Price et al., Arch Toxicol 2015)

- 2016 – Brussels OECD/EFSA Workshop
  - Consensus that several *in vitro* assays are ready to use for screening chemicals
  - These could comprise an "*in vitro* DNT Battery" of tests
  - OECD Developmental Neurotoxicity Expert Panel working on Guidance in the use of DNT NAMs for Integrated Approaches to Testing and Assessment (IATA)

# International Efforts to Develop Alternatives for DNT Guideline Studies

- European Food Safety Authority (EFSA)
  - 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

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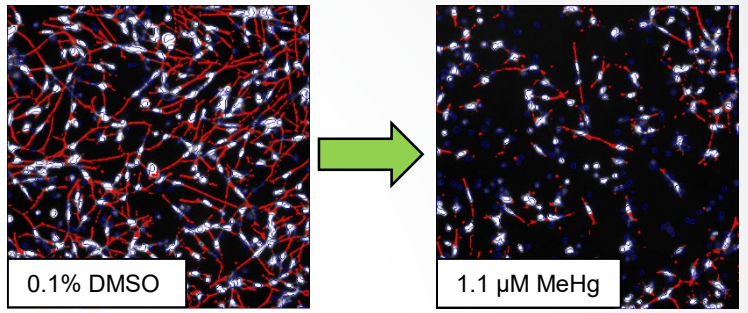
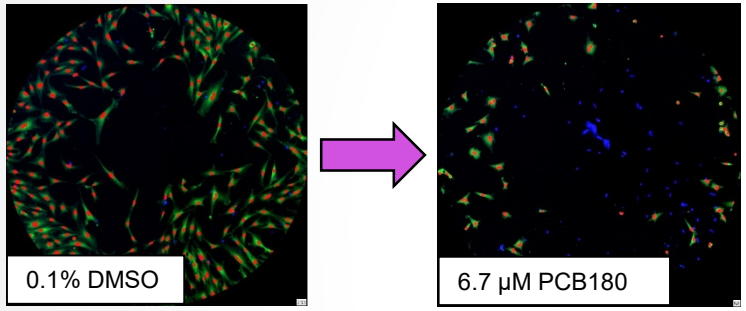
# University of Konstanz Assays

DNT compounds

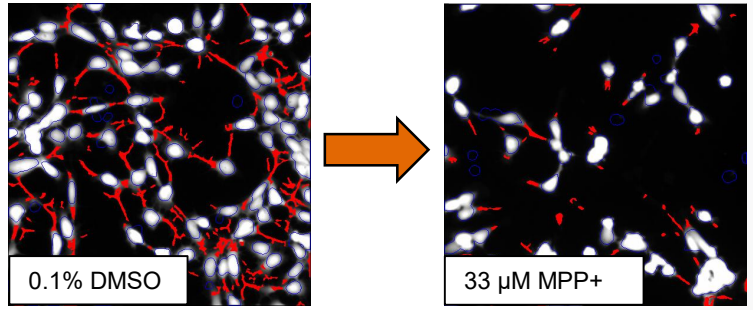
Effect on neurite outgrowth of PNS neurons

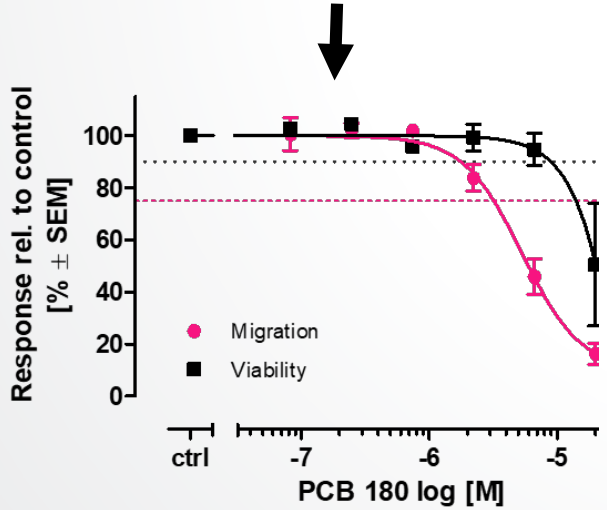



Effect on migration of neural crest cells




Effect on neurite outgrowth of CNS neurons

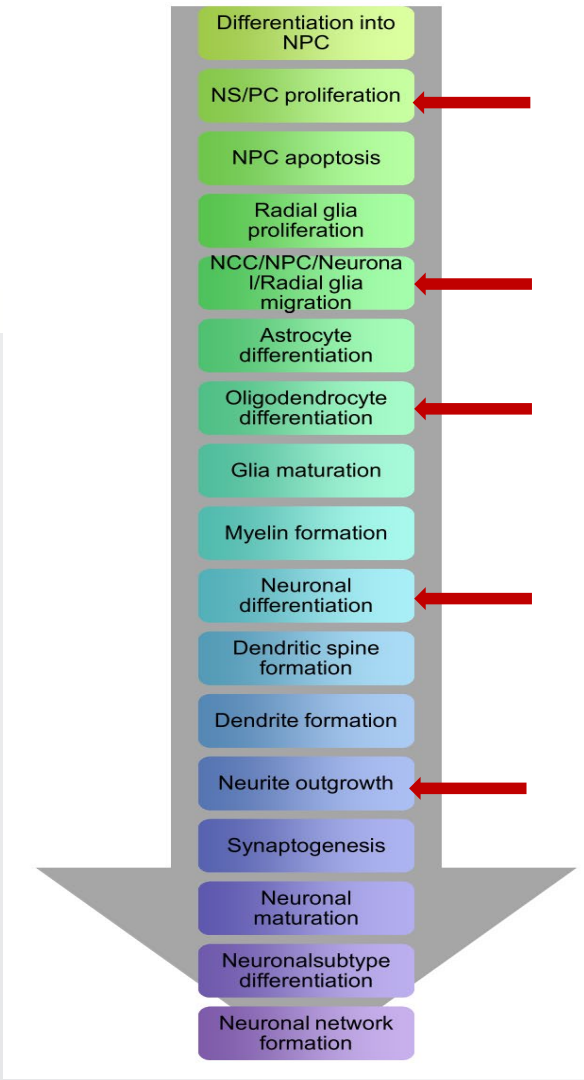


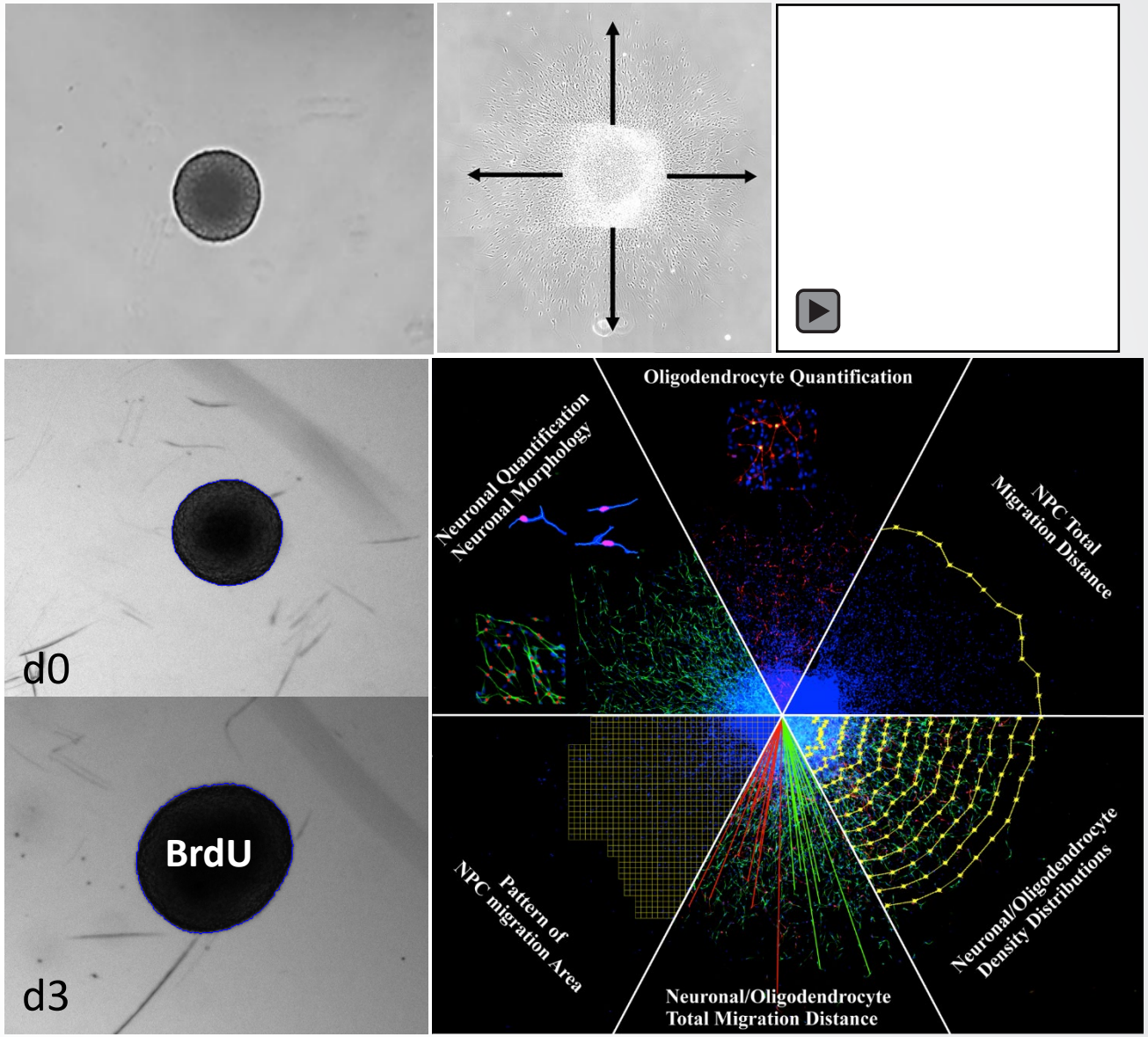




Prediction model & hit definition

Slide courtesy of M. Leist

# The ‘Neurosphere Assay’ (Düsseldorf)



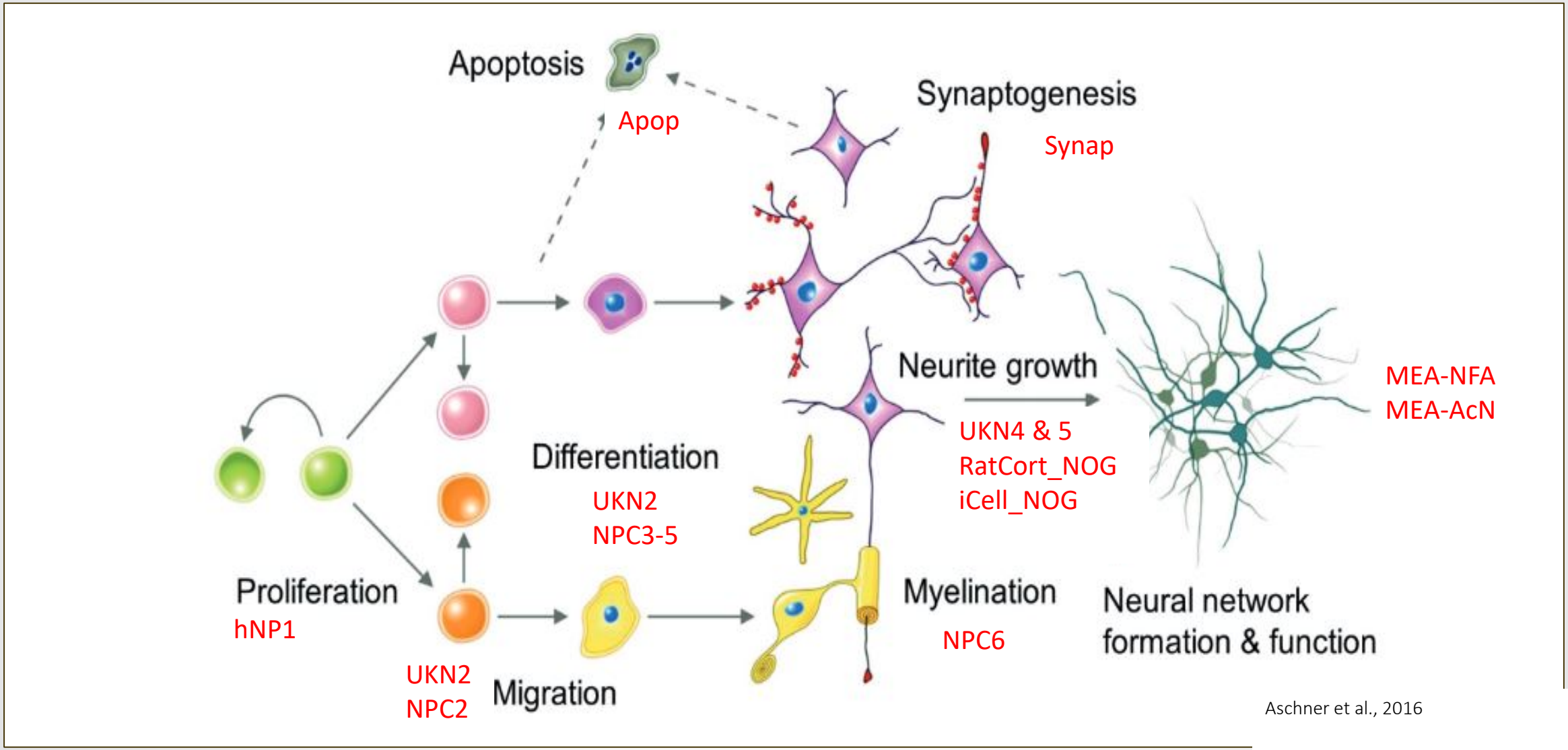


Baumann *et al.*, 2015 Methods in Pharmacology and Toxicology
Schmuck *et al.*, 2016 Archives of Toxicology
Masjosthusmann et al 2018 Toxicology and Applied Pharmacology


Slide courtesy of E. Fritsche

BrdU= bromdesoxyuridine

# This Combination of Assays Provides Good Coverage of Neurodevelopmental Processes




Aschner et al., 2016

# What is Needed to Encourage Regulatory Use of Alternative Methods?

- Understanding of how the assays work and what they measure
- Evaluation of individual assays and the battery of assays
- Data from alternative assays
  - Particularly for compounds that will be used for IATA case studies
- 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**

# Evaluating the Performance of DNT NAMs

Several different approaches can be taken to evaluate the performance of the DNT-NAMS

- Demonstrate that they recapitulate the *in vivo* neurobiology
- Evaluation of the Sensitivity/Specificity
- Evaluate Performance of Positive Controls
- Evaluate the Reproducibility when retesting compounds

# Evaluation of Specificity and Sensitivity

**True Positive Rate (sensitivity)** = True positives/Known Positives

**True Negative Rate (specificity)** = True negatives/Known Negatives

**Precision** = True positives/(True Positives + False Positives)

**Accuracy** = (True Positives + True Negatives )/(Known Positives + Known Negatives)

# Evaluating Sensitivity and Specificity of DNT NAMs Represents a Challenge

NTP Report on Human Carcinogens (2016)

- 62 recognized, human carcinogens
- >170 “Anticipated” human Carcinogens
- >1000 compounds evaluated

By Contrast, for DNT:

- 12 recognized human developmental neurotoxicants (Grandjean and Landrigan, Lancet Neurol. 2014).
- ~150 compounds evaluated in Guideline DNT studies (rodents).

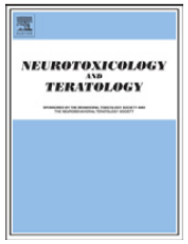
As a result, benchmarking the performance of *in vitro* DNT assays against *in vivo* data is confounded

Neurotoxicology and Teratology 52 (2015) 25–35

Contents lists available at ScienceDirect

Neurotoxicology and Teratology

journal homepage: www.elsevier.com/locate/neutera




Review article

# Expanding the test set: Chemicals with potential to disrupt mammalian brain development

William R. Mundy [a,*], Stephanie Padilla [a], Joseph M. Breier [a,1], Kevin M. Crofton [b], Mary E. Gilbert [a], David W. Herr [a], Karl F. Jensen [a], Nicholas M. Radio [a,2], Kathleen C. Raffaele [c], Kelly Schumacher [d], Timothy J. Shafer [a], John Cowden [b]

[a] National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC, USA
[b] National Center for Environmental Assessment, U.S. Environmental Protection Agency, Research Triangle Park, NC, USA
[c] Office of Solid Waste and Emergency Response, U.S. Environmental Protection Agency, Washington, DC, USA
[d] Region 7, U.S. Environmental Protection Agency, Lenexa, KS, USA

Goal: Assess the level of information in the literature that a chemical has *DNT hazard*

**This is a *scientific* summary of evidence, not a *regulatory* decision. Does not necessarily reflect dose.**

**A vetted list of "negative" compounds is in progress and expected by Jan 2021.**

# Sensitivity and Specificity of the Network Formation Assay

| | Actual Positive | Actual Negative | Total |
|---|---|---|---|
| Predicted Positive | 49 | 14 | 63 |
| Predicted Negative | 2 | 11 | 13 |
| Total | 51 | 25 | 76 |

**True Positive Rate (sensitivity)** = True positives (49)/Known Positives (63) = **0.78**

**True Negative Rate (specificity)** = True negatives (11)/Known Negatives (14) = **0.84**

**Precision** = True positives (49)/(True Positives (49) + False Positives (2)) = **0.96**

**Accuracy** = (True Positives (49) + True Negatives (11))/(Known Positives (63) + Known Negatives (14)) = **0.78**

Shafer et al., Toxicol Sci., 2019.

# What is Needed to Encourage Regulatory Use of Alternative Methods?

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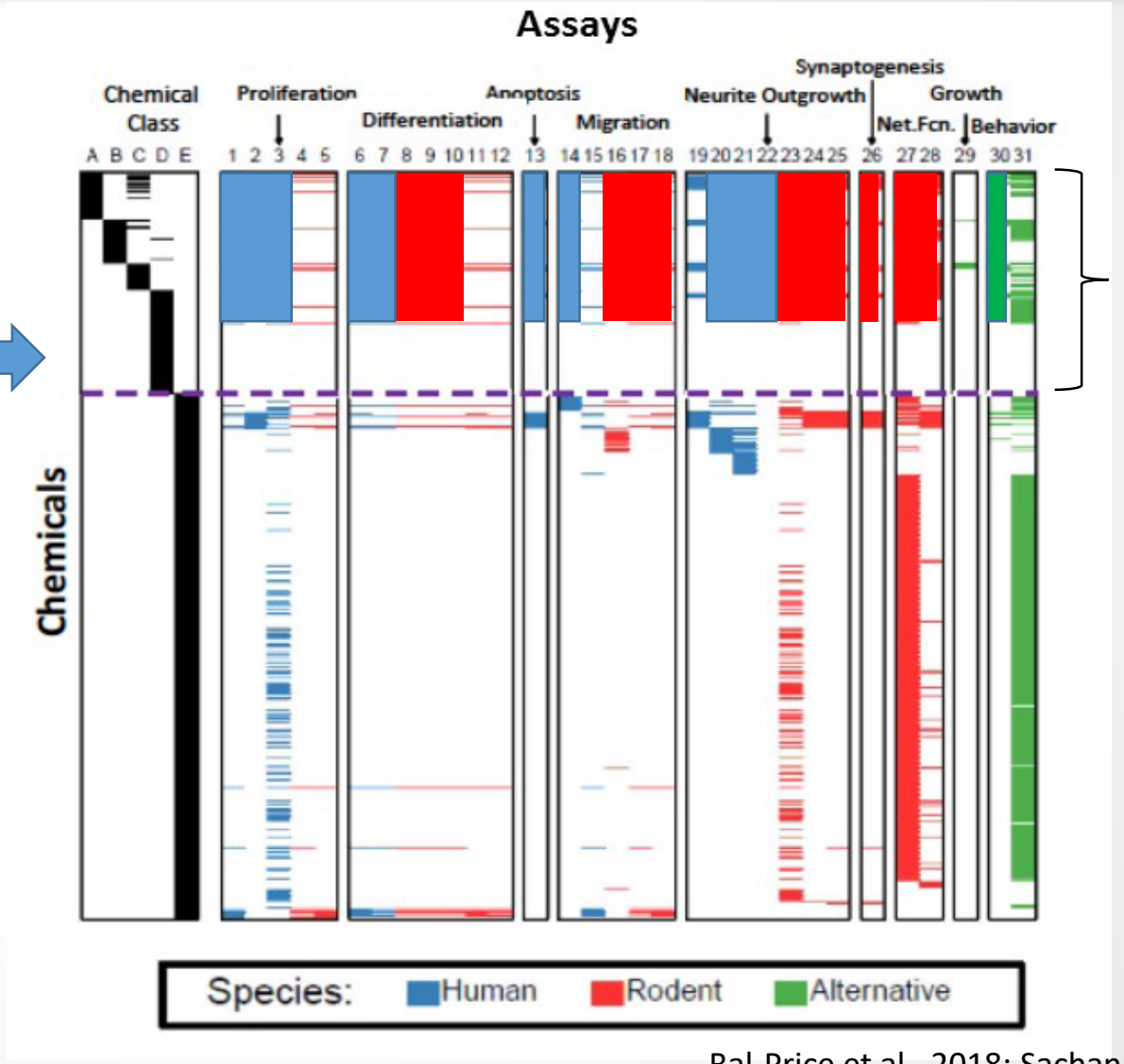
**Regulatory decision-makers must have confidence in the assays and data in order to incorporate them into the decision-making process**

# The Need for More Data:
# Priority on compounds with *in vivo* DNT information

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

# Development of a Chemical Library

- Identified ~120 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 are being 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
- A subset (~30 IATA) of these compounds are being tested by 5 labs in zebrafish behavioral assays

# Status and Timelines

- Partners have received ToxCast compounds.
  - Testing is Completed at Konstanz and Duesseldorf
    - Report has been released to the public.
      - https://www.efsa.europa.eu/en/supporting/pub/en-1938
  - 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.

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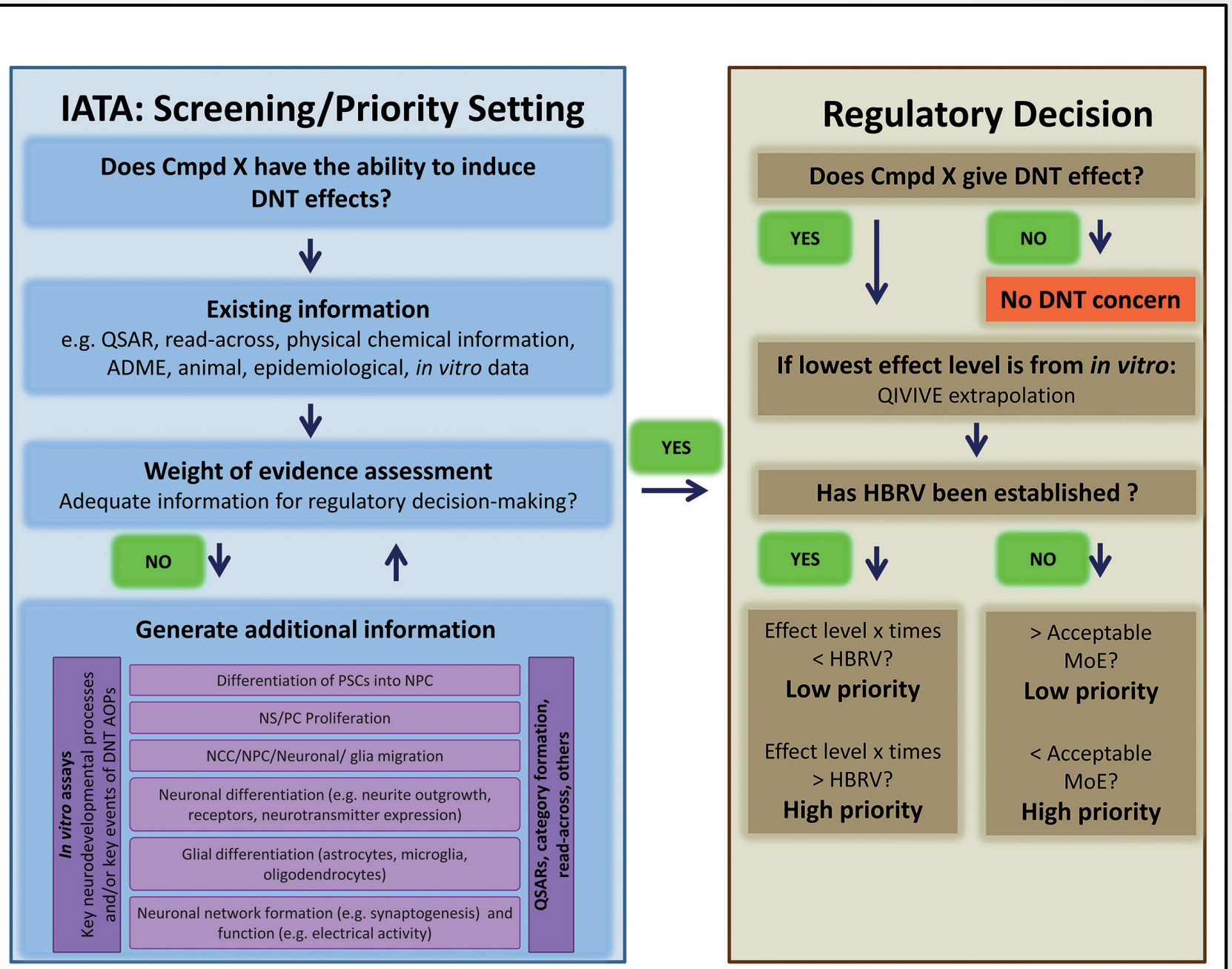


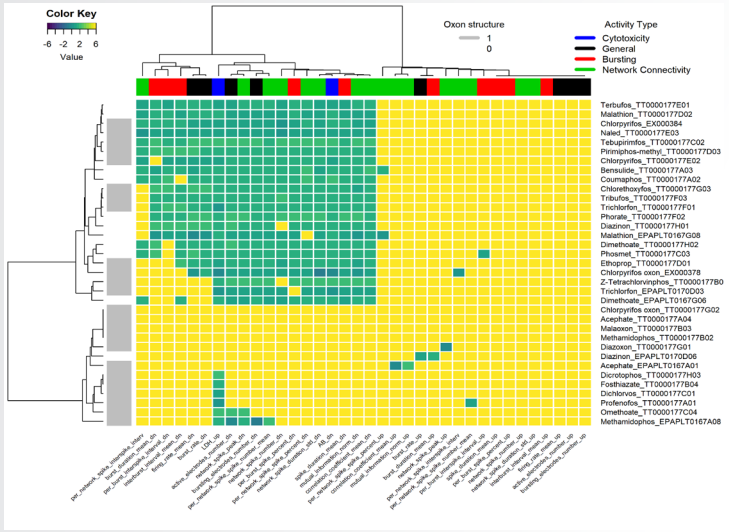

HBRV = health-based reference value

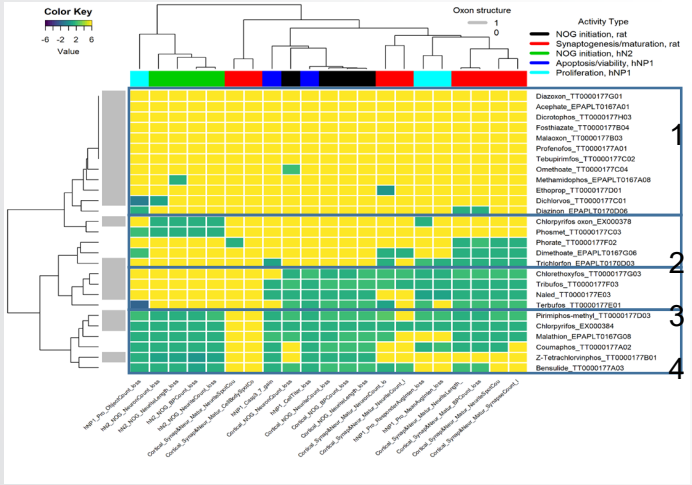
# Current Focus of the OECD DNT Expert Group

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

- Introduction and Rationale
- Issues with the Current Guideline testing approaches
- Guidance for incorporation of *in vitro* assays into IATAs
- **Case Studies**

# Examples of what has been done with DNT NAMs Data




FIFRA Scientific Advisory Panel on Organophosphates

- Consensus that DNT-NAMs can be used for WOE approaches

Informing decisions on compounds for *in vivo* DNT Studies:

- NTP had nominations for DNT studies of several organophosphate flame retardants
  - Used DNT NAMs to inform which compounds to test *in vivo*

Should a DNT Guideline study be required?

- Chemical proposed for registration that was structurally similar to a compound for which a Guideline DNT study already existed.
- Data from DNT NAMs is being considered as part of deciding whether or not to require a Guideline DNT study on the new compound.

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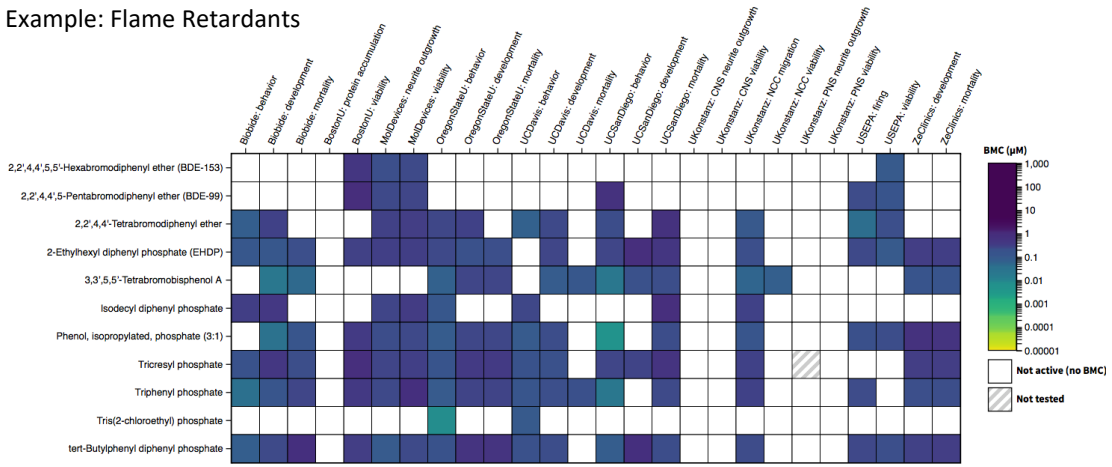
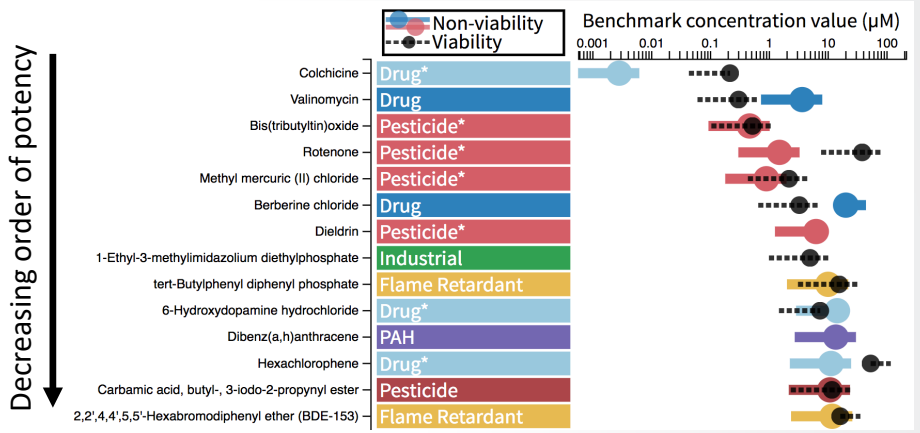
- Understanding of what can be done with the data
- Accessibility to the data
  - Pipelining data into ToxCast
  - EFSA-Funded researchers are building a database
  - NTP has developed a visualization tool

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

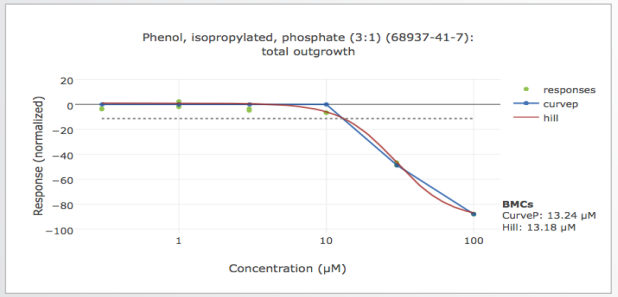
# NTP's DNT-DIVER: Free Data Integration & Visualization Tool




Compare activity of compounds/ classes across multiple assays




Compare activity of compounds within an assay




Individual dose-response curves

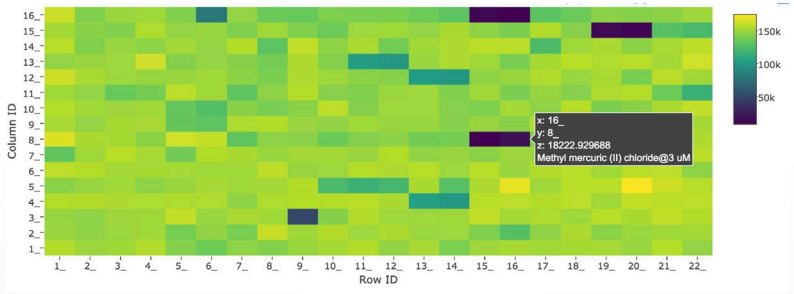
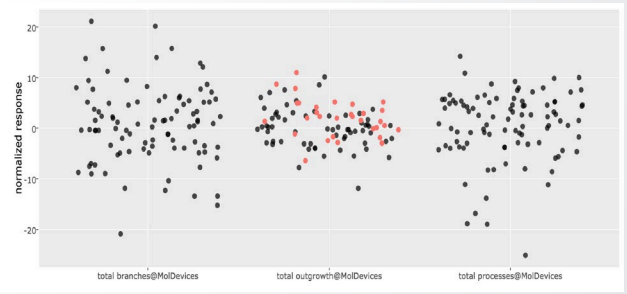



Plate and well level information

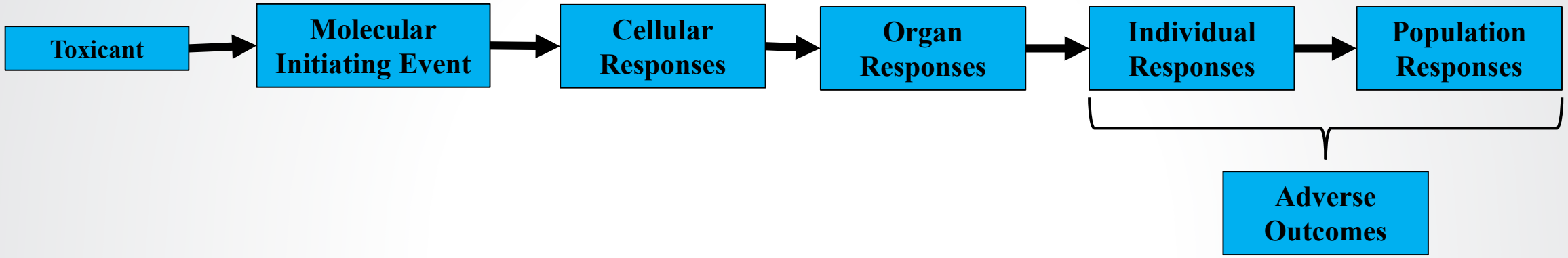



Control variability in assay

For further information contact Mamta Behl: mamta.behl@nih.gov

- https://sandbox.ntp.niehs.nih.gov/neurotox/

# Adverse Outcome Pathways (AOPs) provide biological context for *in vitro* results




AOPs are a construct to describe the biological relationships that contribute to toxicological effects (Adverse Outcomes)

- AOPs are lacking for DNT related outcomes that are not the result of disruption of Thyroid Hormone Pathways

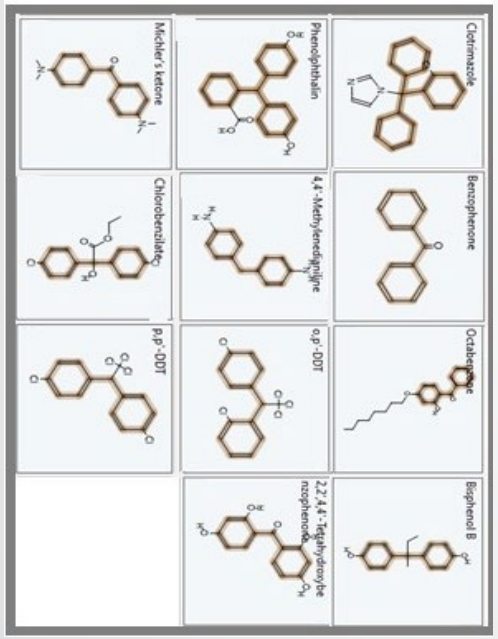
https://aopwiki.org/aops/15

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

**Toxicant**

Chemical Property Profile

→ "Chemotyping"




**Molecular Initiating Event**

? (many postulated, few confirmed)

→ **Target-based Screening** (known MIEs)

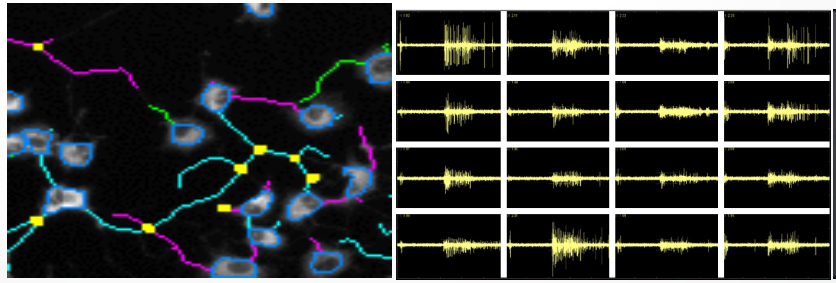
**HTP Molecular assays (ToxCast)**
ion channels
AChE
neurotransmitter receptors
growth factor receptors
transcription factors
kinases
…

**Cellular Responses**

<u>Key Events</u>
proliferation
differentiation
migration
neurite growth
synaptogenesis

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

***In Vitro* Approaches**



**Organ Responses**

<u>Nervous System</u>
Δ connectivity
(morphology, neurochemistry, physiology)

**Individual Responses**

behavior
cognition

**Behavior**

# *In vitro* assays to identify developmental neurotoxicity hazard:
## Promises and challenges

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

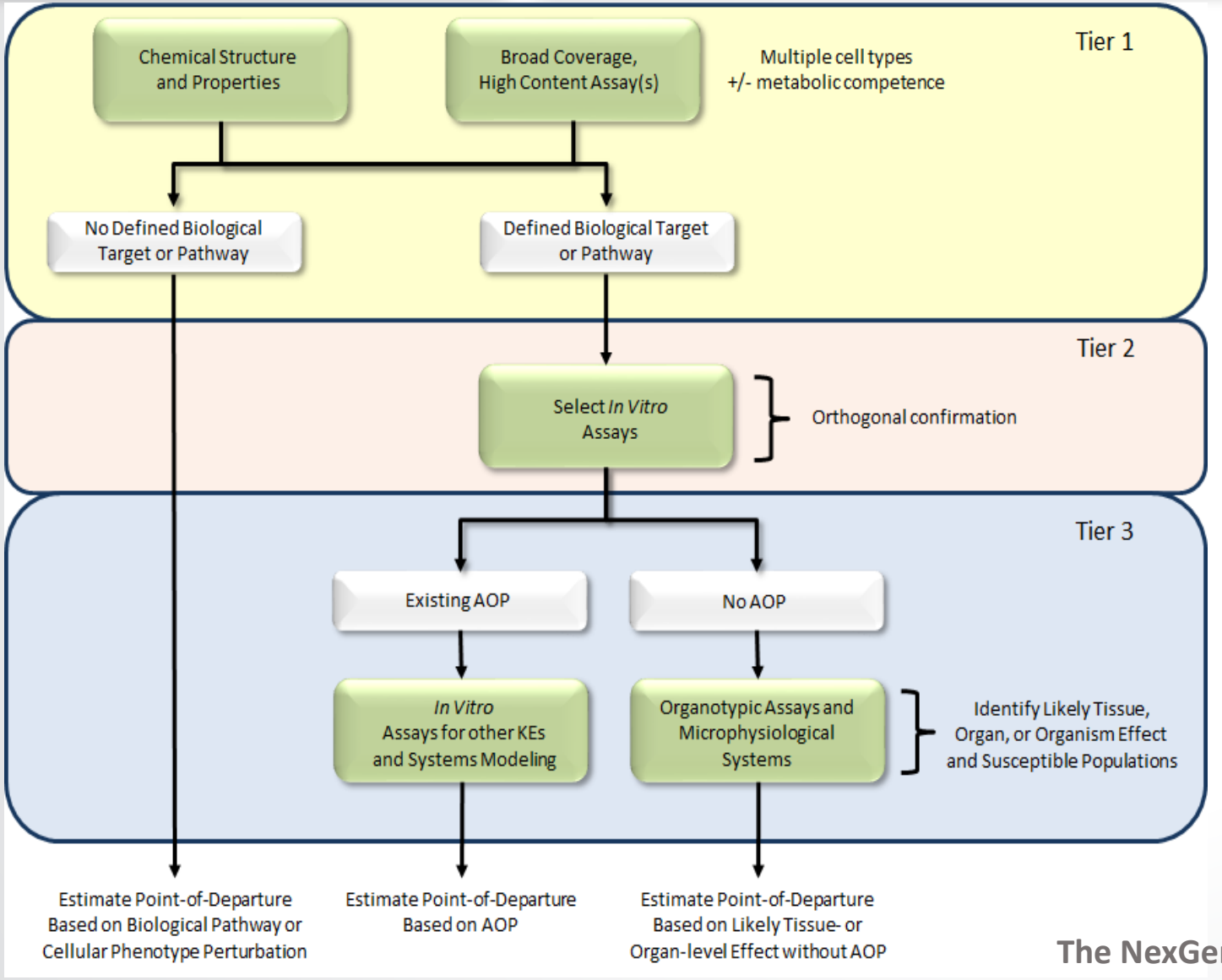
Challenges:

- Further evaluation of the battery
- 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

# Future Directions

- NTP is assembling a second set of ~96 compounds to test in DNT NAMs
  - Solicited input from a wide array of stakeholders, including EPA
  - List will include additional compounds where there are *in vivo* Guideline DNT Studies
  - Test sites TBD, but EPA will participate via an interagency agreement
  - Results expected mid-2022

# Tiered Hazard Evaluation Approach

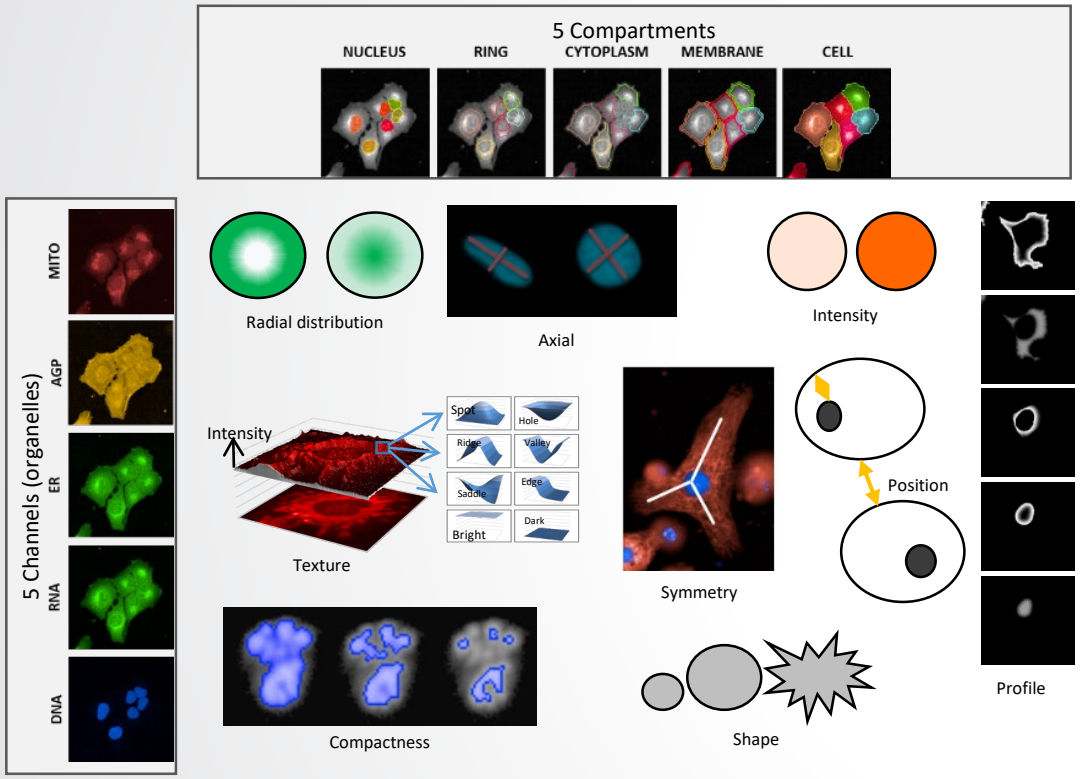



- Increasing efficiency and declining cost of generating whole transcriptome profiles has made **high-throughput transcriptomics (HTTr)** a practical option for *in vitro* chemical screening.
  - **Whole Transcriptome TempO-Seq**
- Imaging-based **high-throughput phenotypic profiling (HTPP)** provides a cost-effective means for characterizing the effects of chemicals on apical cellular morphology (i.e. cellular pathology).
  - **Cell Painting**
- Both methods are **complementary** to each other and can be used in **human-derived** *in vitro* models.
- The resulting bioactivity profiles can potentially be used for **potency estimation**, **mechanistic prediction** and evaluation of **chemical similarity**.



The NexGen Blueprint of CompTox as USEPA Tox. Sci. 2019; 169(2):317-322

# Cell Painting

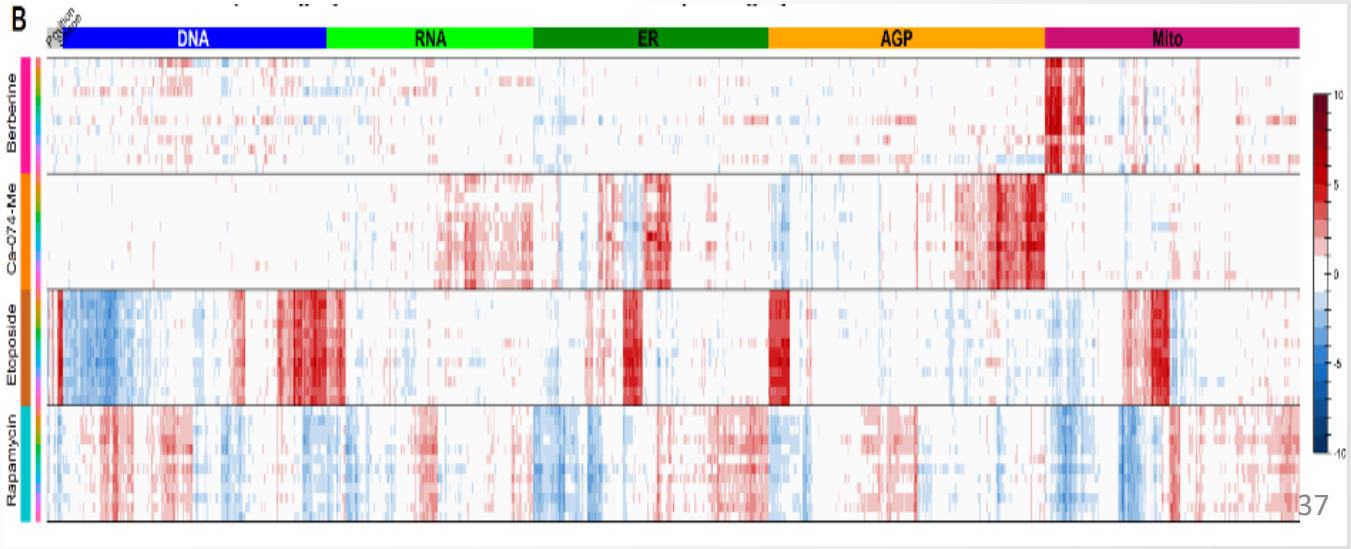



**PerkinElmer Opera Phenix**

| | |
|---|---|
| **Modality:** | Confocal (single *z*) |
| **Objective:** | 20X Water |
| **Plate:** | CellCarrier-384 Ultra |
| **Fields:** | 5 or 9 |

*With illustrations from Perkin Elmer*

1300 features / cell



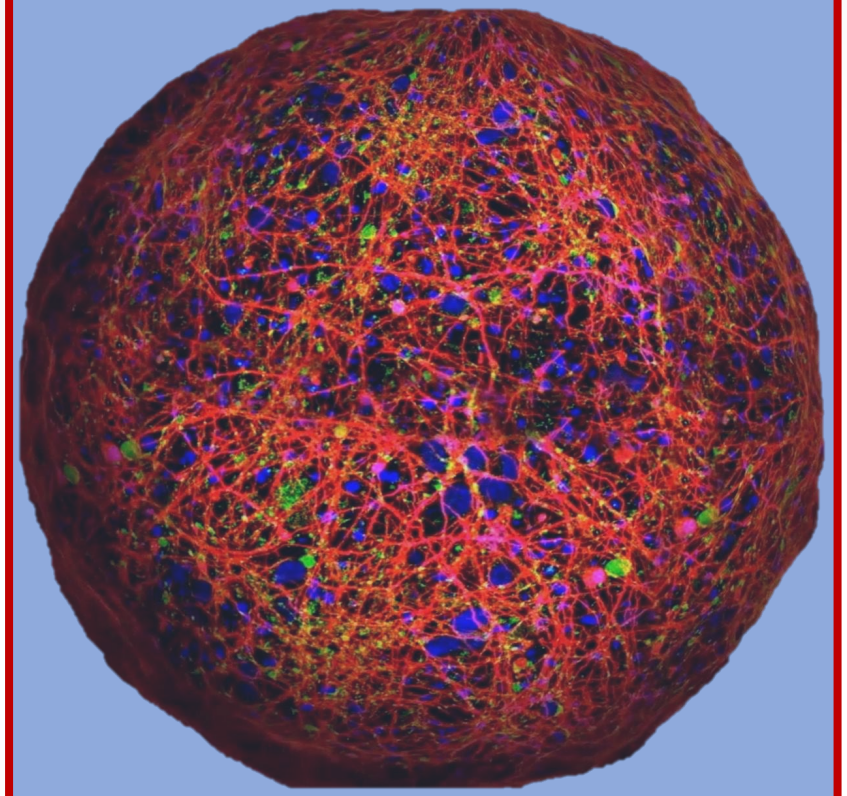

Currently- non-neural cell models only

**Pilot Project**: mouse and human neuroprogenitors

**ADVANCING ACTIONABLE ALTERNATIVES TO VERTEBRATE ANIMAL TESTING FOR CHEMICAL SAFETY ASSESSMENT (EPA-G2018-STAR-C1)**

**Smirnova, Hartung, Berlinicke, Gracias**

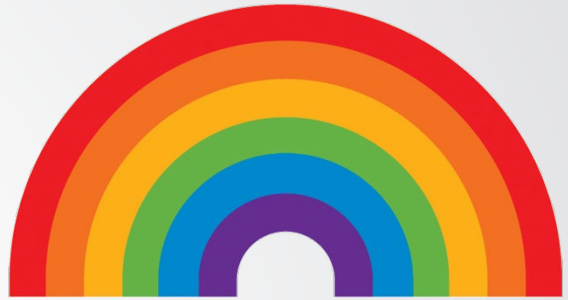
6-in-1 BrainSphere assay anchors key neurodevelopmental processes



**Neuronal differentiation**
**Myelination**
**Neurite outgrowth**
**Synaptogenesis**
**Glia migration & Gliosis**
**Neural network (Electrophysiology)**

**CRISPR/CAS9**

**Reporter/ Fusion proteins**



**Mini- Brainbow**

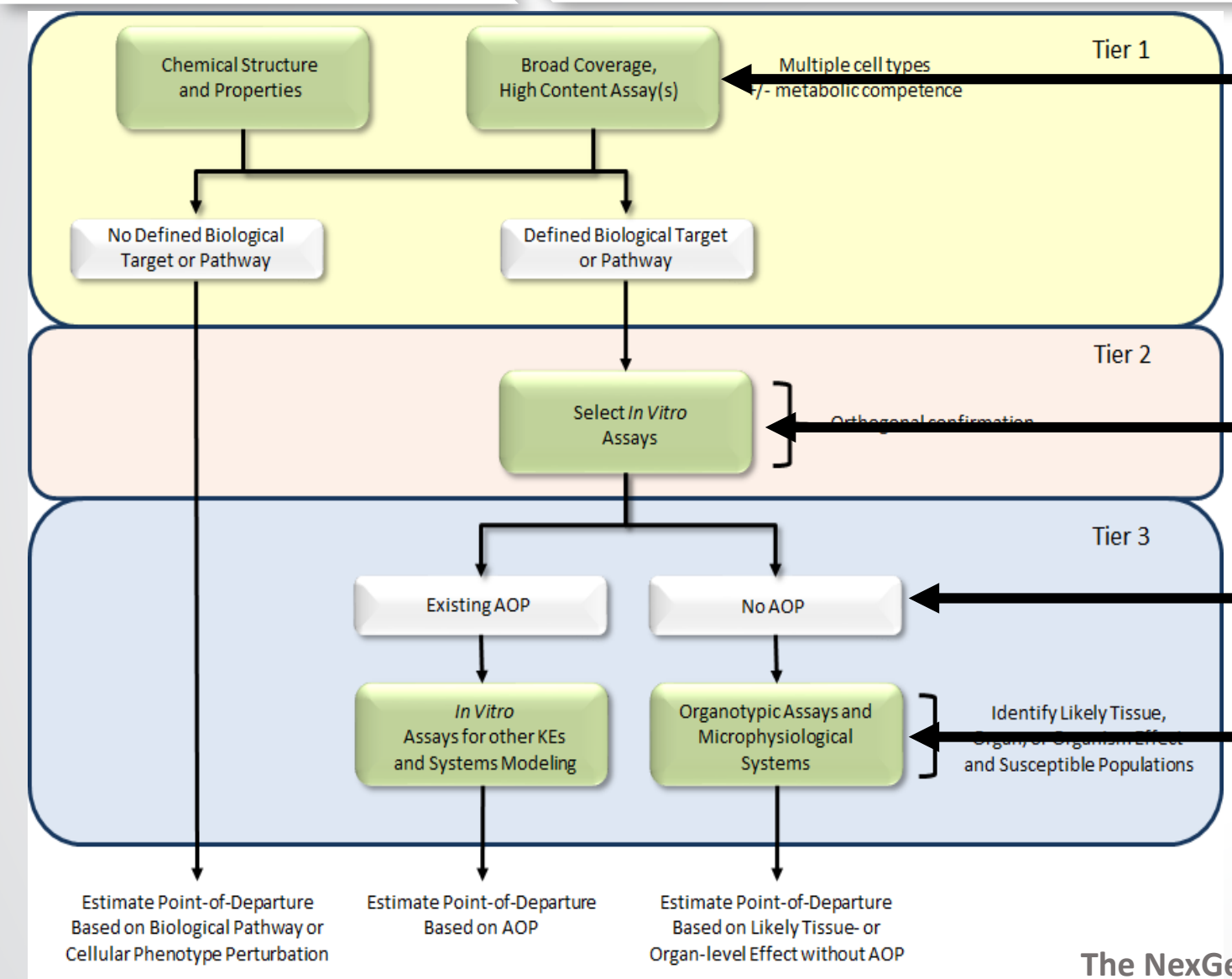
**Neurons**
**Astrocytes**
**Oligodendrocytes**
**Synapses**
**3D electrophys**

**High content imaging**

**Toxicant screening**

Slide courtesy of Lena Smirnova, JHU

# Tiered Hazard Evaluation Approach




Cell painting in neuroprogenitor cells

Current DNT Assay Battery

AOPs for DNT

Neurospheres & Zebrafish



The NexGen Blueprint of CompTox as USEPA Tox. Sci. 2019; 169(2):317-322

# Thank you!
# Questions?

**EPA Colleagues:**
- Kathleen Wallace
- Theresa Freudenrich
- Bill Mundy (retired)
- Josh Harrill
- Jasmine Brown
- Katie Paul-Friedman

**EFSA Collaborators**
- Ellen Fritsche
- Marcel Leist

**OECD Expert Group on DNT**
- Magda Sachana
- Andrea Terron