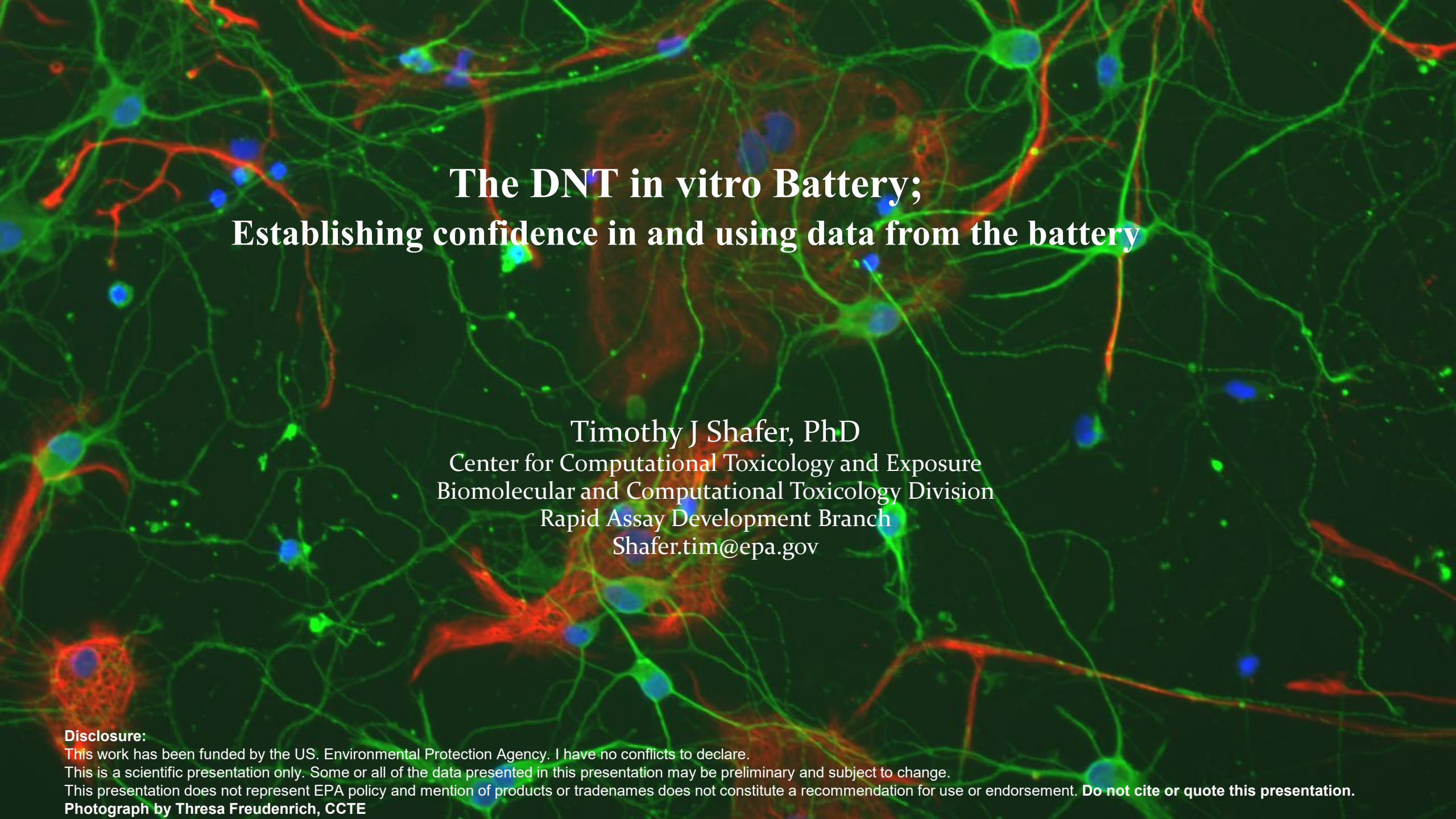

The DNT in vitro Battery;
Establishing confidence in and using data from the battery

Timothy J Shafer, PhD
Center for Computational Toxicology and Exposure
Biomolecular and Computational Toxicology Division
Rapid Assay Development Branch
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Disclosure:
This work has been funded by the US. Environmental Protection Agency. I have no conflicts to declare.
This is a scientific presentation only. Some or all of the data presented in this presentation may be preliminary and subject to change.
This presentation does not represent EPA policy and mention of products or tradenames does not constitute a recommendation for use or endorsement. **Do not cite or quote this presentation.**
Photograph by Thresa Freudenrich, CCTE

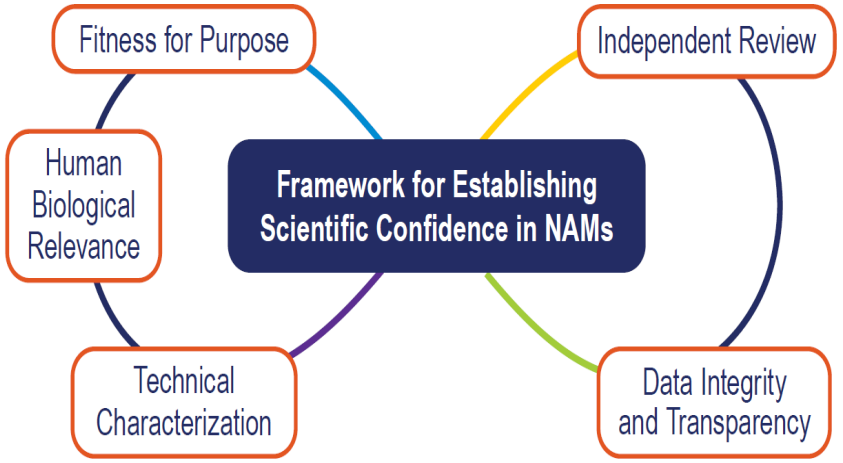
Outline

I. Introduction to Developmental Neurotoxicology in vitro battery (DNT-IVB)

II. Comparing the DNT-IVB to criterion for confidence in NAMs
   a) Review
   b) Fit for purpose
   c) Data transparency
   d) Technical characterization
   e) Human relevance

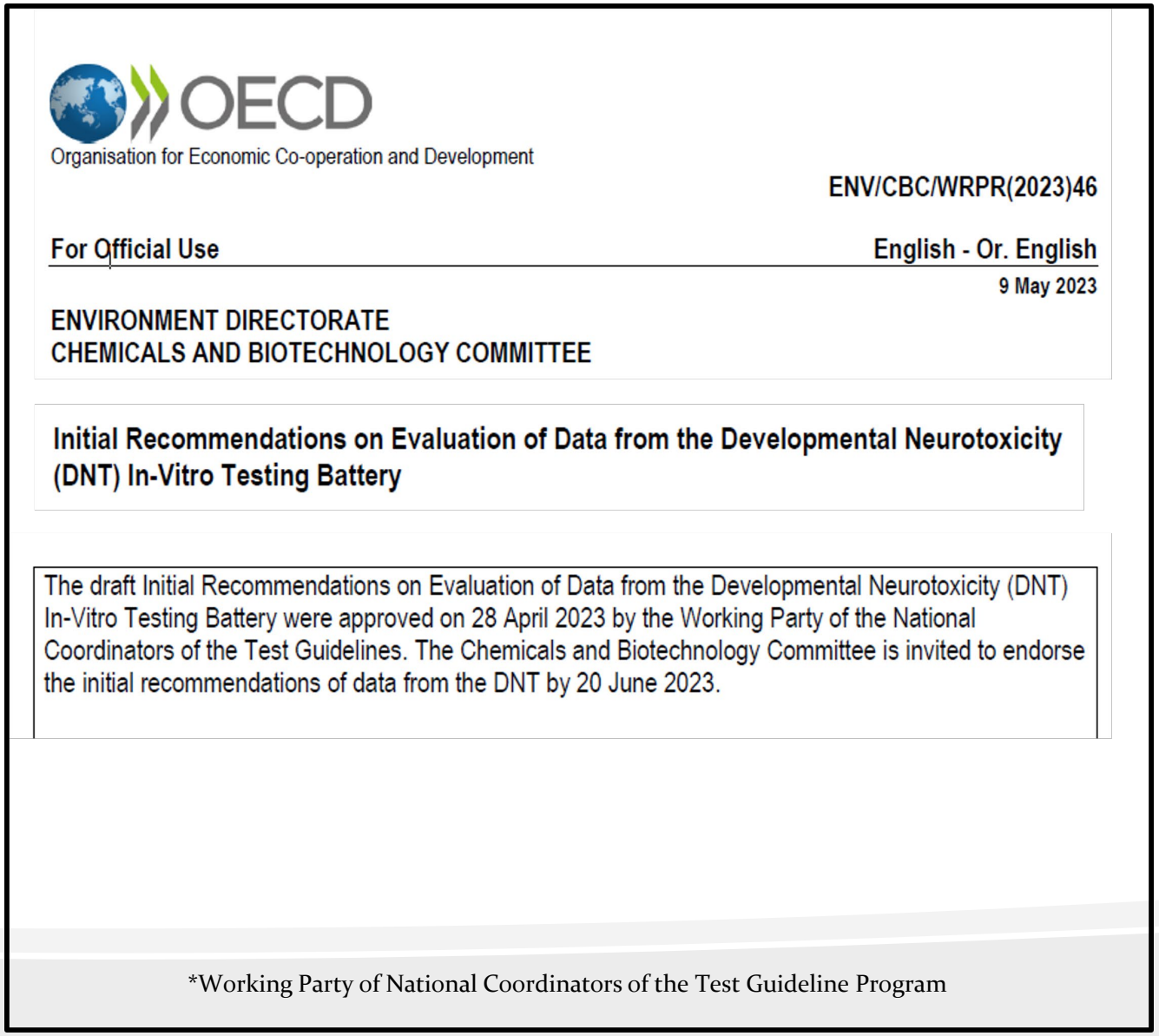
III. Case Studies

IV. Conclusion



from Van der Zalm, et al., Arch Toxicol. 2022 Nov;96(11):2865-2879. doi: 10.1007/s00204-022-03365-4.

On April 26, 2023, the OECD WNT approved the following document:



OECD

Organisation for Economic Co-operation and Development

ENV/CBC/WRPR(2023)46

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9 May 2023

ENVIRONMENT DIRECTORATE
CHEMICALS AND BIOTECHNOLOGY COMMITTEE

Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity (DNT) In-Vitro Testing Battery

The draft Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity (DNT) In-Vitro Testing Battery were approved on 28 April 2023 by the Working Party of the National Coordinators of the Test Guidelines. The Chemicals and Biotechnology Committee is invited to endorse the initial recommendations of data from the DNT by 20 June 2023.

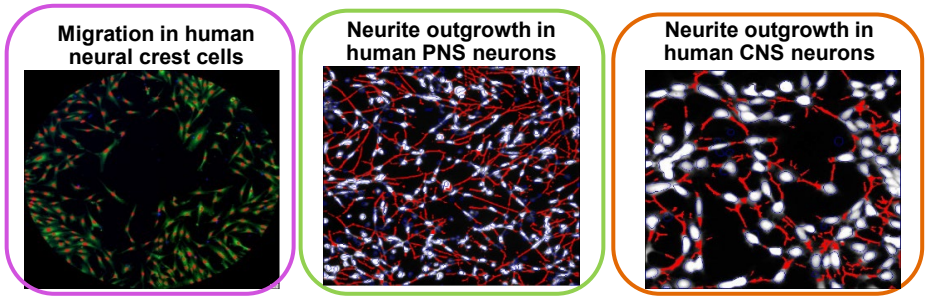
*Working Party of National Coordinators of the Test Guideline Program

- Recognized a battery of in vitro assays for DNT
- Provides international recognition and credibility to the DNT in vitro assays.
- 38 member countries
  - Americas, Europe, Asia, Australia, Africa

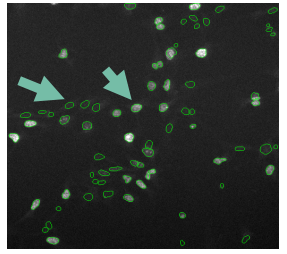
Implementing the DNT IVB

Now that we have the DNT-IVB, how do we facilitate its use for decision making?

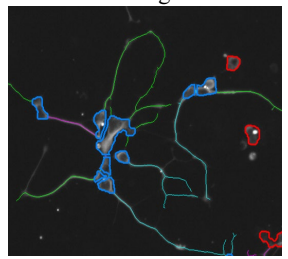
- Establishing confidence in the battery
  - Need a “roadmap” to establish confidence
- Demonstrating Utility
  - Need Case Studies to demonstrate utility



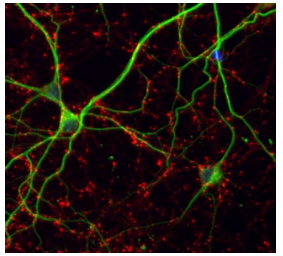
Human Neuroprogenitor Proliferation



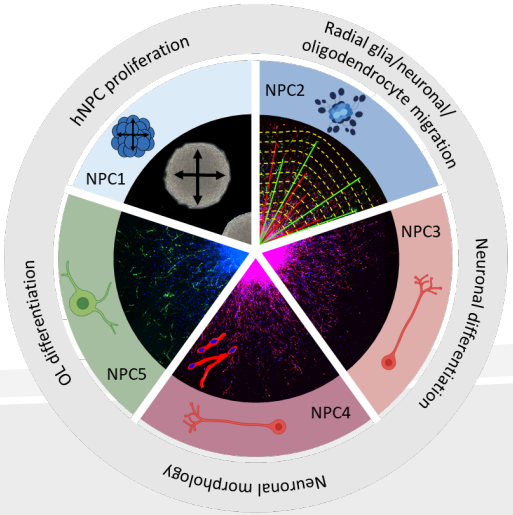
Human and Rat Neuron Neurite Outgrowth



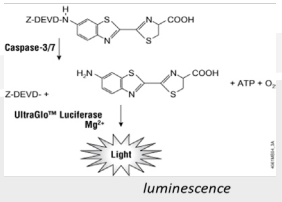
Synaptogenesis in Rat Cortex Neurons



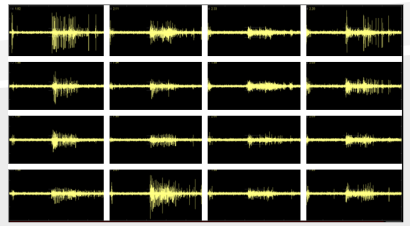
Human Neurospheres



Human Neuroprogenitor Apoptosis

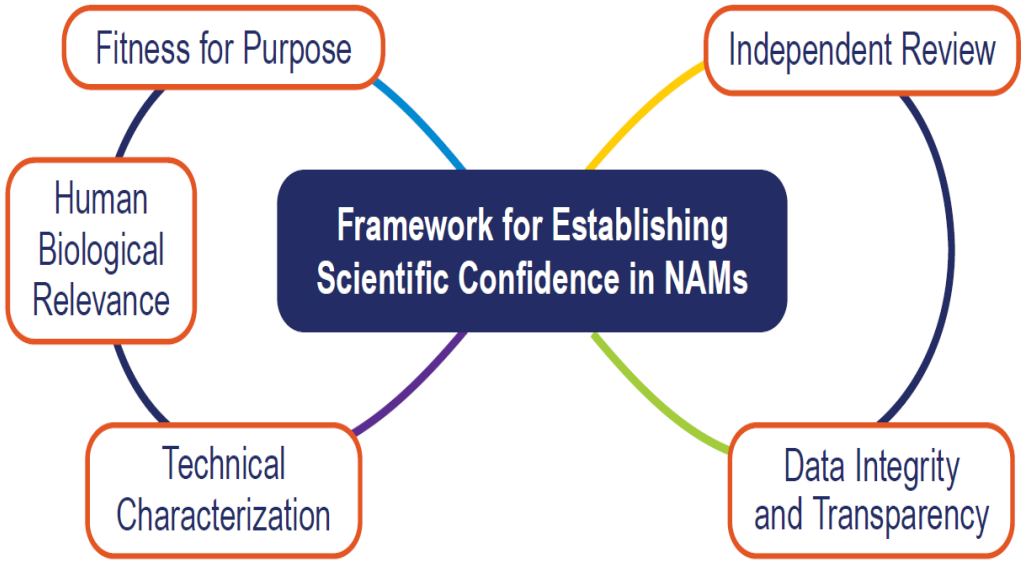


Rat Network Formation Assay



Figures courtesy of Drs Marcel Leist, and Ellen Fritsche

Establishing Confidence in the Assays



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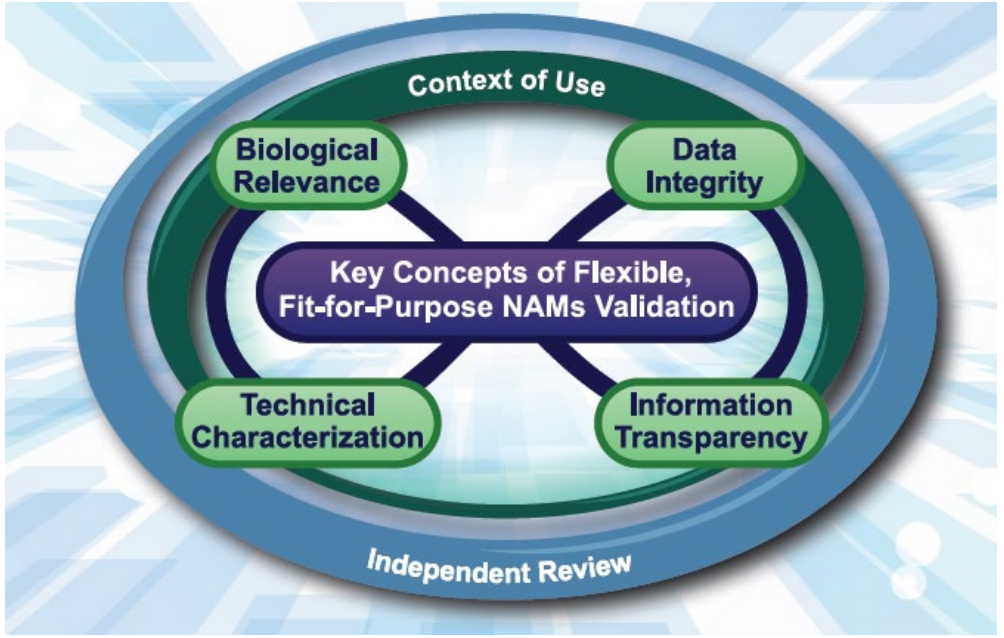


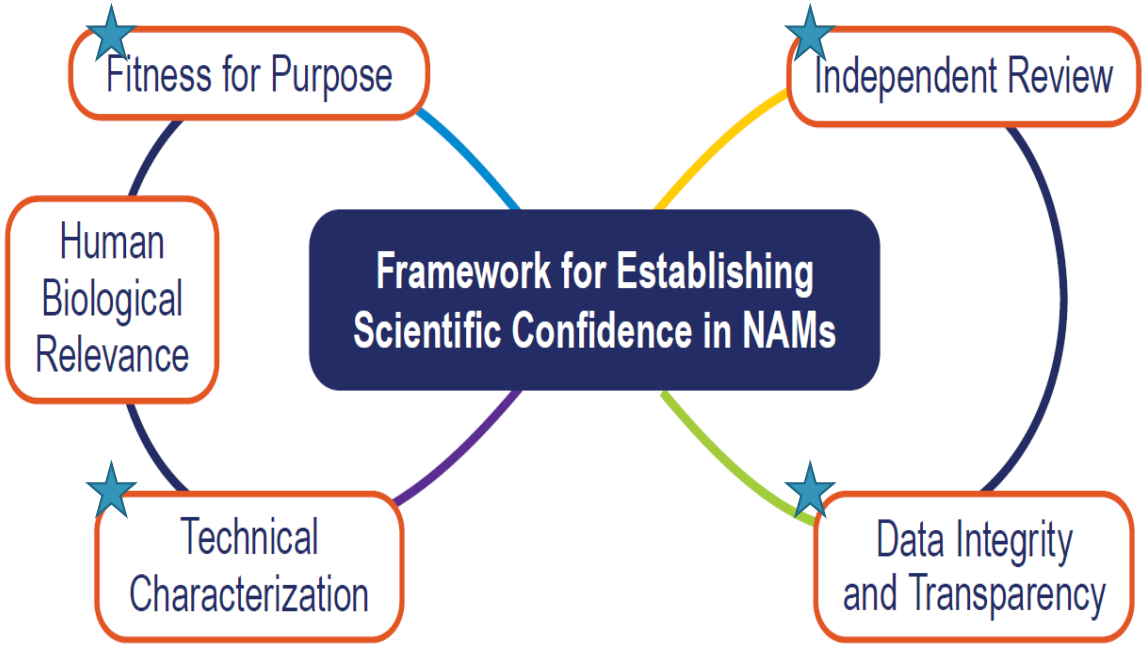
Validation, Qualification, and Regulatory Acceptance of New Approach Methodologies

Interagency Coordinating Committee on the Validation of Animal Methods (ICCVAM). March 2024

Establishing Confidence in the DNT-IVB

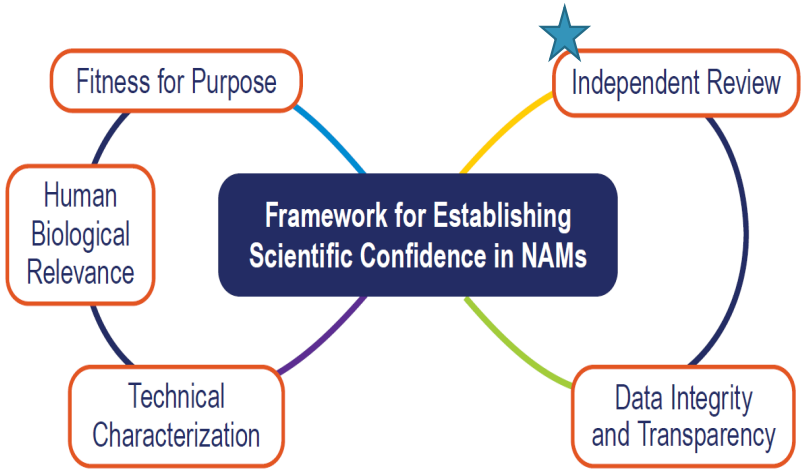
Assay Inclusion in the Battery:

- Deemed ready for use in screening and prioritization (Fritsche et al. 2017; Bal-Price et al. 2018; Sachana et al. 2019)
- Tested a common set of chemicals
- Analyzed using the USEPA's ToxCast Pipeline (TCPL)
- Detailed methodological descriptions in the ToxTemp (Krebs et al. 2019)



from Van der Zalm, et al., Arch Toxicol. 2022 Nov;96(11):2865-2879. doi: 10.1007/s00204-022-03365-4. .

Establishing Confidence in the Assays: Independent Review



All assays in the battery have been described in the peer-reviewed literature.

The Developmental Neurotoxicity Battery- DNT-IVB

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

Process	Assays	References
Proliferation	hNP1	Harrill *et al.* (2018)
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	UKN1	Balmer *et al.* (2012)
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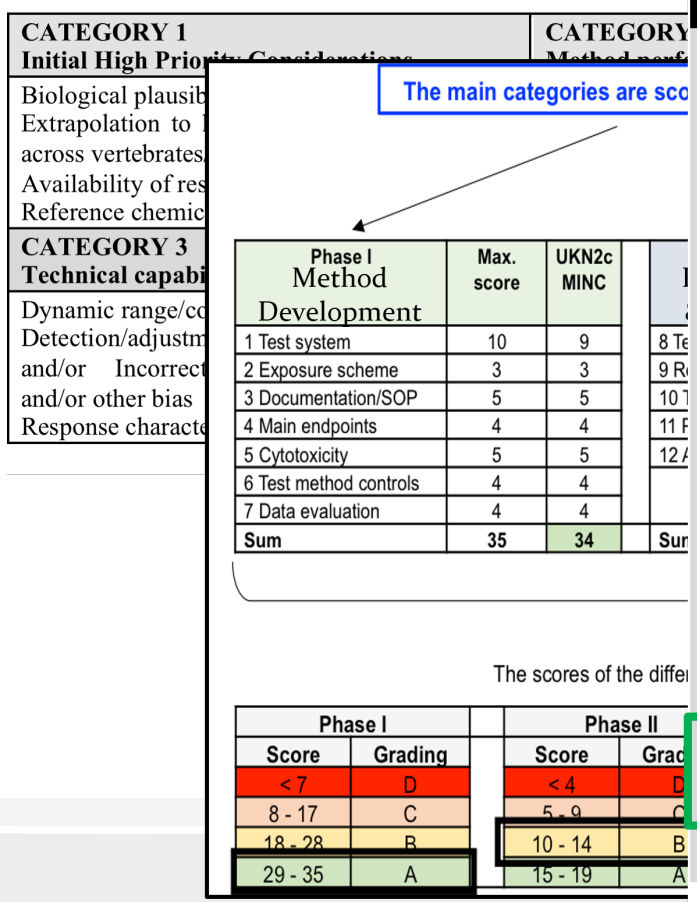
	UKN2	Nyffeler *et al.* (2017)
Neuron differentiation	NPC3	Baumann *et al.* (2016) and Barenys *et al.* (2017)
Oligodendrocyte differentiation & maturation	NPC5/6	Baumann *et al.* (2016) and Barenys *et al.* (2017)
Neurite outgrowth	iCell gluta hN2	Harrill *et al.* (2018)
	UKN 4 & 5	Krug *et al.* (2013)
	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)

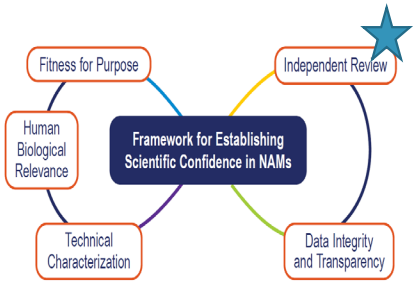
From Sachana et al., Toxicol Sci. 2019 Jan 1;167(1):45-57. doi: 10.1093/toxsci/kfy211

Establishing Confidence in the Assays: Independent Review

Ranking Parameters



Readiness/ Test method	Phase I	Phase II	Phase III	Overall readiness
UKN1	A	B	B	B+
NPC1	A	A	A	A
NPC2	A	A	A	A
NPC3	A	A	B	A-
NPC4	A	B	C	B
NPC5	A	A	B	A-
NPC6	A	B	B	B+
UKN2 (cMINC)	A	B	A	A-
MESn	C	D	D	D+
UKN4 (NeuriTox)	A	A	A	A
NSR	C	D	D	D+
SYN	B	B	B	B
Nnff	B	A	B	B+
3Dh	B	C	C	C+



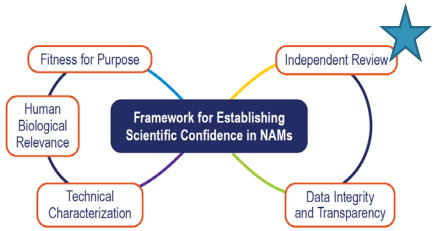
Phase I: Method Development

Phase II: Performance & Replicability

Phase III: Screening

From Bal-Price et al. 2018 ALTEX. 2018;35(3):306-352. doi: 10.14573/altex.1712081

Review of DNT *in vitro* battery



OECD WNT* accepts the inclusion of the DNT Project in its workplan

Sept- EPA SAP† review of DNT-IVB

Expert Group comments

WNT Comments & Revisions 3 rounds

April 2023-WNT approval

1st Draft of Guidance Document July 2021

2nd Draft

2017 | 2018 | 2019 | 2020 | 2021 | 2022 | 2023

Release of DNT-IVB data in ToxCast

FIFRA Scientific Advisory Panel Meeting Minutes and Final Report No. 2020-02

Peer Review of the Use of New Approach Methodologies (NAMs) to Derive Extrapolati Evaluate Developmental Neuroto Health Risk Assessr

OECD
Organisation for Economic Co-operation and Development

ENV/CBC/WRPR(2023)46

For Official Use — English - Or. English

9 May 2023

ENVIRONMENT DIRECTORATE
CHEMICALS AND BIOTECHNOLOGY COMMITTEE

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Regulatory Toxicology and Pharmacology 143 (2023) 105444

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

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

ELSEVIER

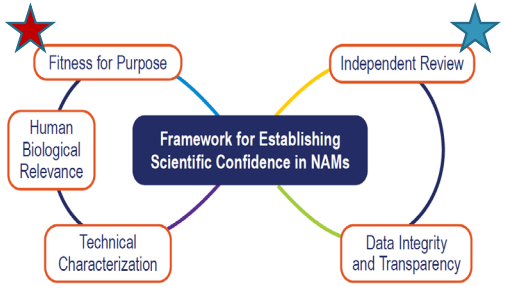
A perspective on *In vitro* developmental neurotoxicity test assay results: An expert panel review

D.R. Juberg [a], D.A. Fox [b,1], P.A. Forcelli [c,1], S. Kacew [d,1], J.C. Lipscomb [e,1], S.A. Saghir [f,1], C.M. Sherwin [g,1], C.M. Koenig [h], S.M. Hays [i], C.R. Kirman [i,*]

*Working Party of National Coordinators of the Test Guideline Program

†SAP=Scientific Advisory Panel (an external review board to provide input to US EPA's Office of Pesticides)

Establishing Confidence in the Assays:
Fit for Purpose



Juberg et al 2023.

"...the in vitro DNT test battery could be used as a screening tool..."

"One might employ as much robust in vitro data as possible to inform on DNT potential, but ultimately there will be the need to employ some (e.g., limited or more in-depth) in vivo data to aid in the interpretation of generated in vitro data."

"In evaluating DNT, there are various approaches including experimental animal models, human epidemiological and clinical studies, and increasingly in vitro methodologies, each with utility in providing insight on DNT. As all model systems and approaches have limitations, integration of data across these methodologies becomes critical to the accuracy and sensitivity of detecting DNT."

OECD Initial Guidance "Target Uses" of the DNT-IVB

- Screening for Prioritization
- Weight of Evidence evaluations

"The structure of these initial recommendations should be expanded in the future to encompass improvements to the current assays in the DNT IVB, updated validation information, and/or new and novel assays that complement or expand the DNT IVB as it currently exists."

EPA 2020 SAP

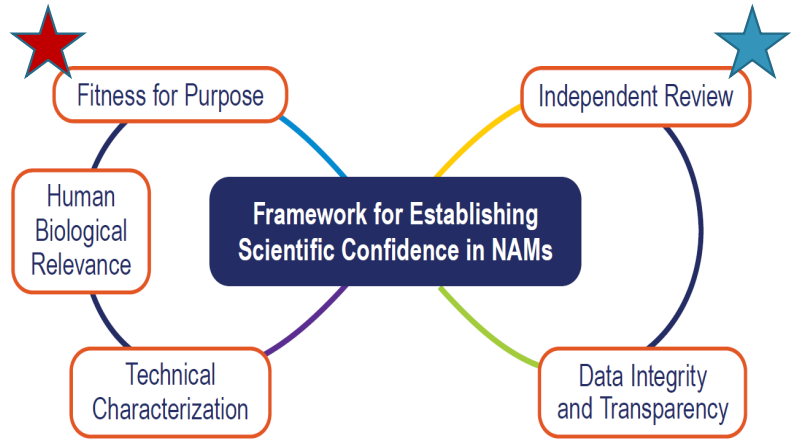
"In general, the Panel agreed that if the Agency uses published data in their evaluation, then there is no reason to exclude peer-reviewed published in vitro assay data - whether screening or mechanistic - in that final "weight of evidence". "

Establishing Confidence in the Assays:
Fit for Purpose (2)

Consensus

All three reviews agreed that data from the DNT IVB could be used for:

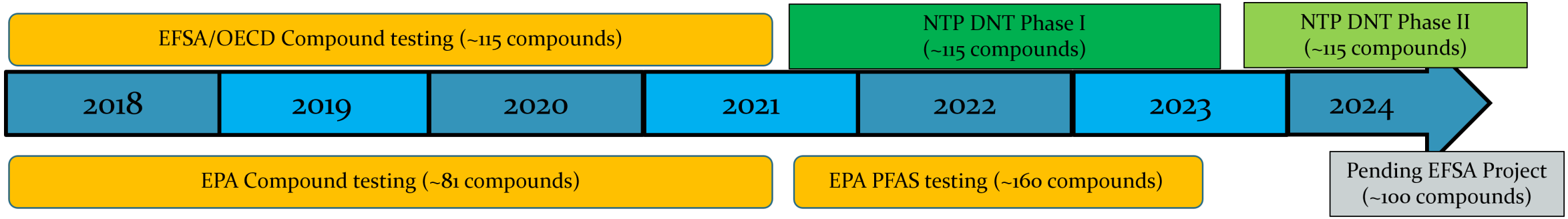
- Screening and Prioritization
- Weight of Evidence Decision-Making



and

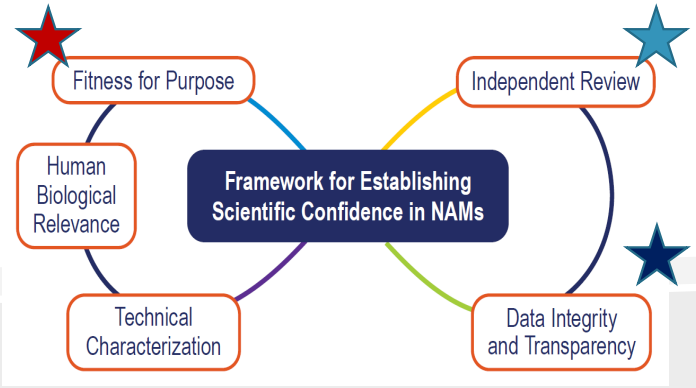
- The battery should be a “living process” that should evolve

Establishing Confidence in the Assays:
Data Integrity & Transparency



Testing has focused on:

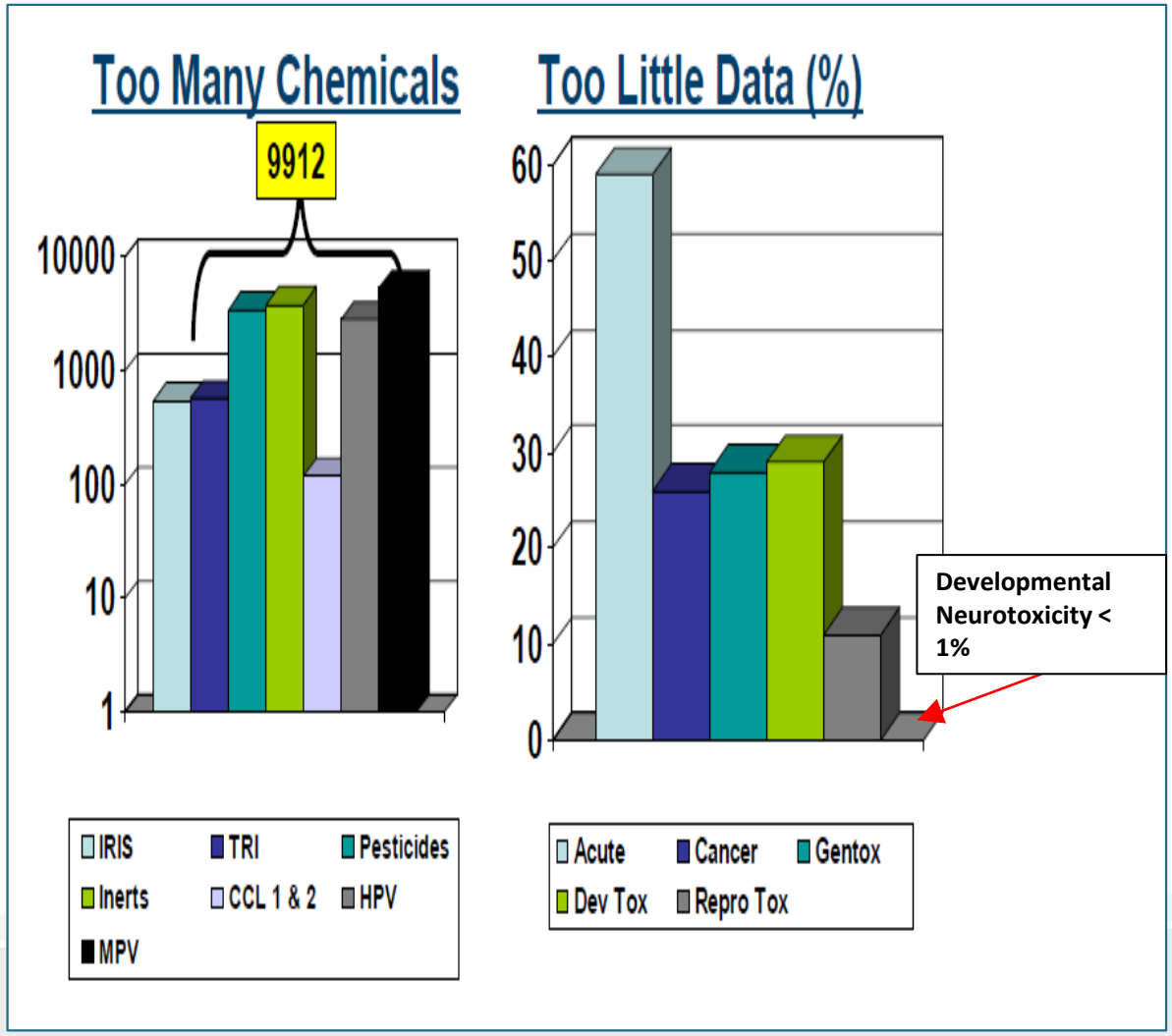
- DNT Reference positive and negative chemicals
- Chemicals with *in vivo* DNT Guideline studies
- Chemicals with specific programmatic interest (PFAS; OPs; botanicals, cannabinoids)

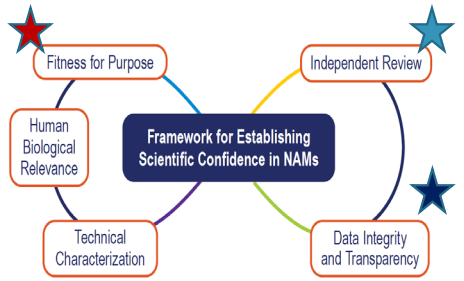


- Tested, data available on "Dashboard*"
- Tested, data pending on Dashboard
- Testing initiated
- Testing in planning process

* Dashboard= https://comptox.epa.gov/dashboard/

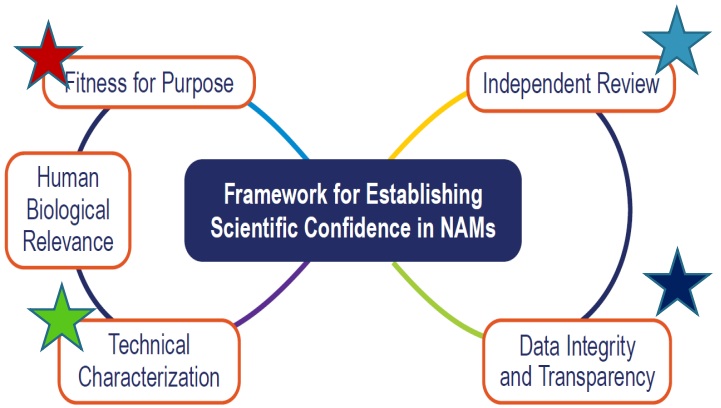
Establishing Confidence in the Assays:
Data Integrity & Transparency





Note: Much of the raw data underlying conclusions in DNT Guideline studies is not available for public review

Establishing Confidence in the Assays:
Technical Characterization



ToxTemp forms are included in the "Initial Guidance" as Appendices B.1-B.10

Summary of Topics in ToxTemp forms;
Aspects of technical characterization

1.Overview
- 1.1.Descriptive full-text title
- 1.2.Abstract

2.General information
- 2.1. Name of test method
- 2.2. Version number and date of deposition
- 2.3. Summary of introduced changes in comparison to previous version(s)
- 2.4. Assigned data base name
- 2.5. Name and acronym of the test depositor
- 2.6. Name and email of contact person
- 2.7. Name of further persons involved
- 2.8. Reference to additional files of relevance

3. Description of general features of the test system source
- 3.1. Supply of source cells
- 3.2. Overview of cell source component(s)
- 3.3. Characterization and definition of source cells
- 3.4. Acceptance criteria for source cell population
- 3.5. Variability and troubleshooting of source cells
- 3.6. Differentiation towards the final test system
- 3.7. Reference/link to maintenance culture protocol

4. Definition of the test system as used in the method
- 4.1. Principles of the culture protocol
- 4.2. Acceptance criteria for assessing the test system at its start
- 4.3. Acceptance criteria for the test system at the end of compound exposure
- 4.4. Variability of the test system and troubleshooting
- 4.5. Metabolic capacity of the test system
- 4.6. Omics characterization of the test system
- 4.7. Features of the test system that reflect the in vivo tissue
- 4.8. Commercial and intellectual property rights aspects of cells
- 4.9. Reference/link to the culture protocol
- 4.10. Exposure scheme for toxicity testing
- 4.11. Endpoint(s) of the test method
- 4.12. Overview of analytical method(s) to assess test endpoint(s)
- 4.13. Technical details (of e.g. endpoint measurements)
- 4.14. Endpoint-specific controls/mechanistic control compounds (MCC)
- 4.15 Positive controls
- 4.16 Negative and unspecific controls
- 4.17 Features relevant for cytotoxicity testing
- 4.18 Acceptance criteria for the test method
- 4.19 Throughput estimate

5. Handling details of the test method
- 5.1. Preparation/addition of test compounds
- 5.2. Day-to-day documentation of test execution
- 5.3. Practical phase of test compound exposure
- 5.4 Concentration settings
- 5.5 Uncertainties and troubleshooting
- 5.6 Detailed protocol (SOP)
- 5.7 Special instrumentation
- 5.8 Possible Variations
- 5.9 Cross-reference to related test methods

6. Data management

7. Prediction model and toxicological application

8. Publication/validation status

9. Test method transferability

10.Safety, ethics and specific requirements

CVs, sd, etc of control wells
Z' scores

Establishing Confidence in the Assays:
Human Biological Relevance

The Developmental Neurotoxicity Battery- DNT-IVB

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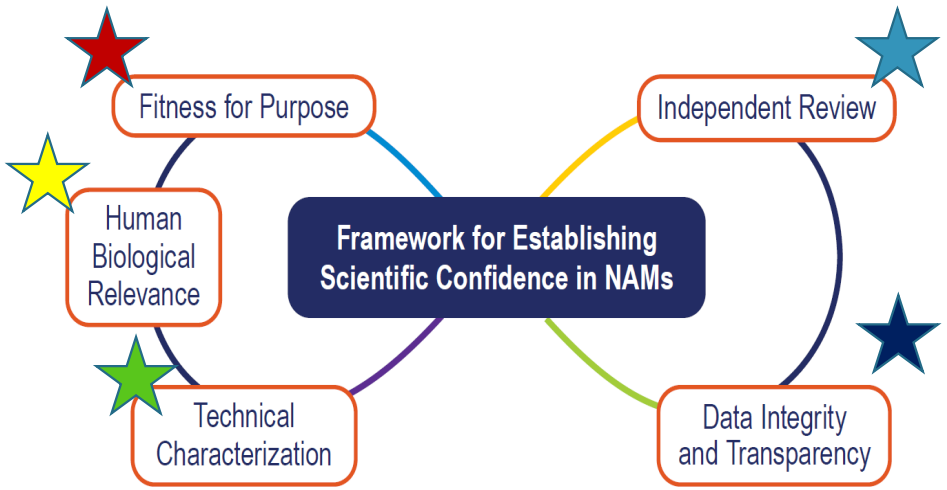

Demonstrate the similarities between the physiology of the test system or the biology measured by the test system, and human biology. Confidence in a NAM is bolstered when it adequately reflects human biological understanding (or, for example, key events in a relevant adverse outcome pathway, AOP).
(From van der Zalm et al., 2022)

Neurodevelopmental Processes, Outcomes and Environmental Chemicals.

Neurodevelopmental Process	Environmental Agents Related to each Process	Clinical Conditions Related to each Process
Proliferation	Ionizing radiation, MAM, MeHg, Chlorpyrifos	Autism
Migration	Ethanol, MeHg	Cerebral Palsy
Apoptosis	Ethanol, MeHg, Chlorpyrifos	Autism
Differentiation (Neurite Outgrowth)	Nicotine, Pb, MeHg	Schizophrenia (reduced axons and dendrites)
Synaptogenesis	Triethyltin, Pb, permethrin, PCBs	IQ/learning decrements
Gliogenesis/Myelination	Ethanol, Pb	

Rice and Barone, Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ Health Perspect. 2000 Jun;108 Suppl 3(Suppl 3):511-33. doi: 10.1289/ehp.00108s3511.

Relationship between Neurodevelopmental Processes and *in vivo* metrics of altered neurodevelopment.

Methods *in vivo*	Outcome	Cell Biological Causes
Gross Morphology	Brain measures ↑↓	→ Proliferation, Apoptosis
	Brain parts missing	→ Proliferation, Differentiation
	Malformation	→ Proliferation, Migration, Differentiation
Histopathology	Necrosis	→ Cytotoxicity
	Pyknosis	→ Apoptosis, Necrosis
	Neuronal Degeneration	→ Neurotoxicity
	Astrocytosis	→ Glia proliferation, GFAP content
	Layer thickness ↑↓	→ Proliferation, Migration, Myelination, Cell death
Morphometry	Layer thickness ↑↓	→ Proliferation, Migration, Myelination
	Morphology	→ Proliferation, Migration, Differentiation
Learning/Memory/Motor Activity	↑↓	→ Synaptogenesis
		→ Network formation
		→ Specific death of neuronal subpopulations
		→ Myelination

Fritsche (2014) Anna Bal-Price and Paul Jennings (eds.), *In Vitro Toxicology Systems*, Methods in Pharmacology and Toxicology, DOI 10.1007/978-1-4939-0521-8_16

Establishing Confidence in the Assays:
Human Biological Relevance

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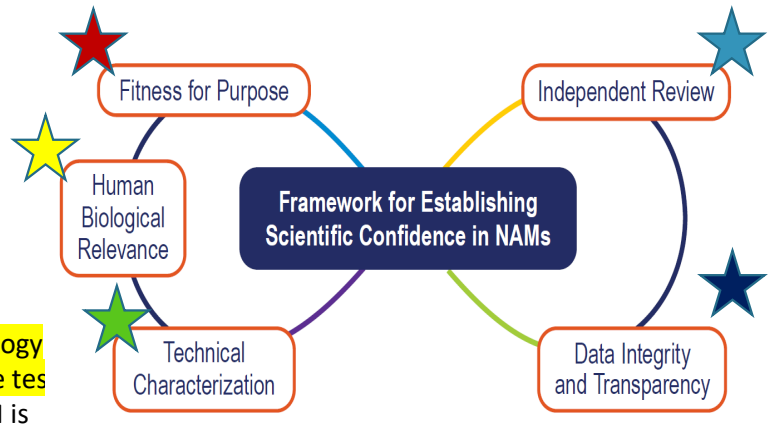
	UKN2	Nyffeler *et al.* (2017)
Neuron differentiation	NPC3	Baumann *et al.* (2016) and Barenys *et al.* (2017)
Oligodendrocyte differentiation & maturation	NPC5/6	Baumann *et al.* (2016) and Barenys *et al.* (2017)
Neurite outgrowth	iCell gluta hN2	Harrill *et al.* (2018)
	UKN 4 & 5	Krug *et al.* (2013)
	NPC4	Baumann *et al.* (2016) and Barenys *et al.* (2017)
	Rat primary	
Synaptogenesis	Rat primary synaptogenesis	Harrill *et al.* (2018)
Network formation	MEA-NFA	Brown *et al.* (2016) and Frank *et al.* (2018)



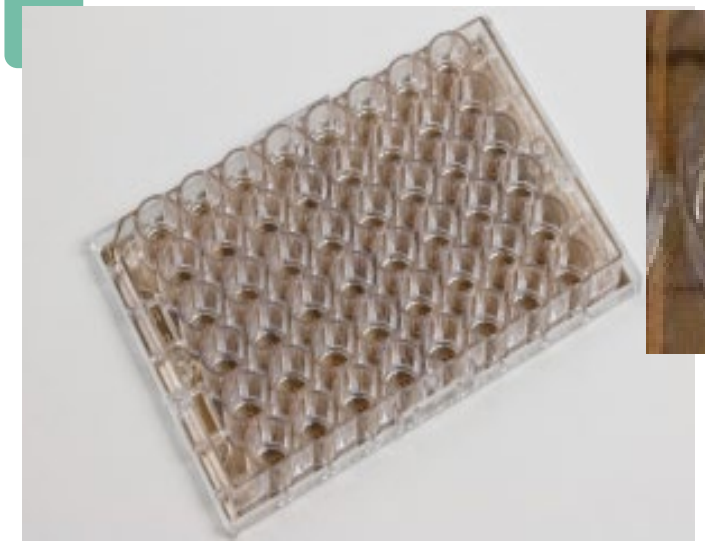
Demonstrate the similarities between the physiology of the test system or the biology measured by the test system, and human biology. Confidence in a NAM is bolstered when it adequately reflects human biological understanding (or, for example, key events in a relevant adverse outcome pathway, AOP). (From van der Zalm et al., 2022)

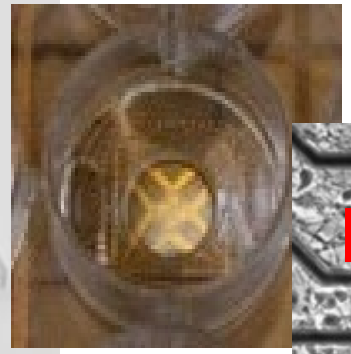
DNT-IVB: 12 Assays use human cell models.

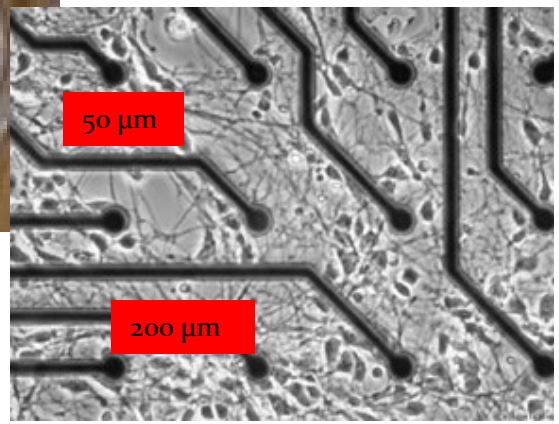
3 Assays use rat primary cortical cell models

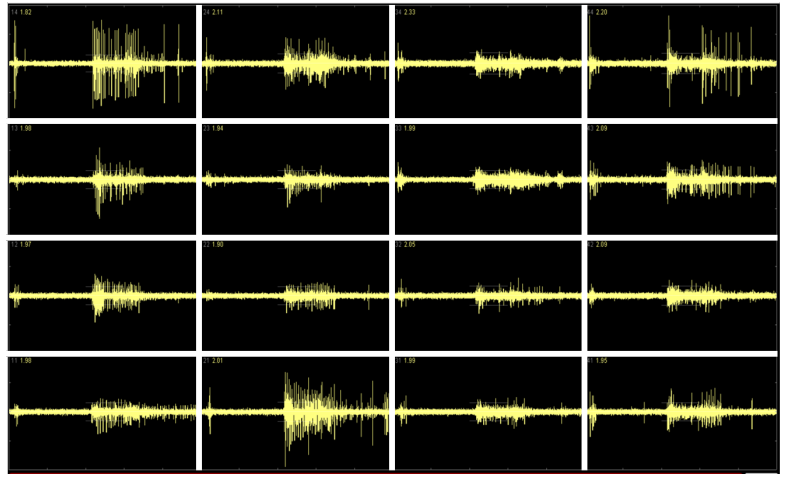
- Neurite initiation
- Neurite Maturation & Synaptogenesis
- Network Formation on microelectrode arrays

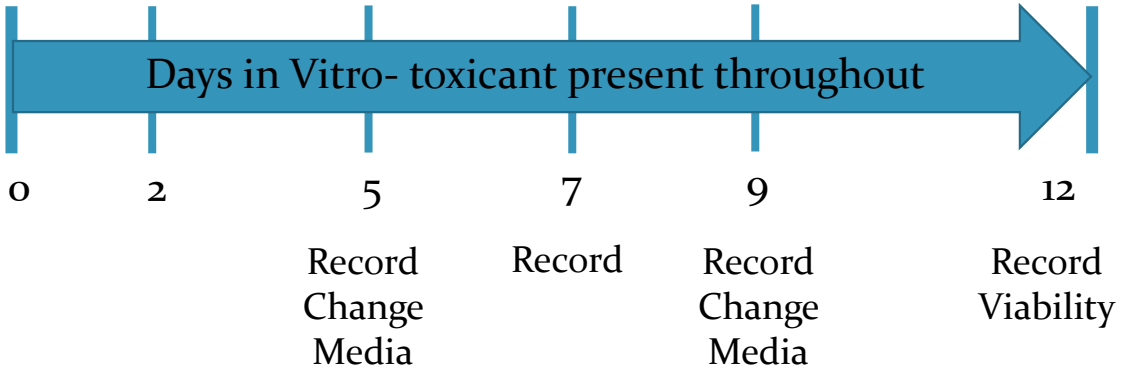
Human Relevance of the Network Formation Assay









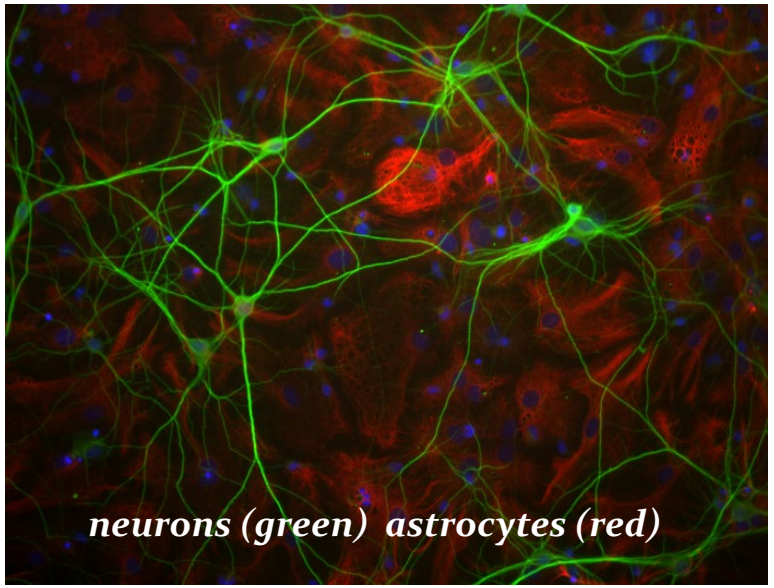


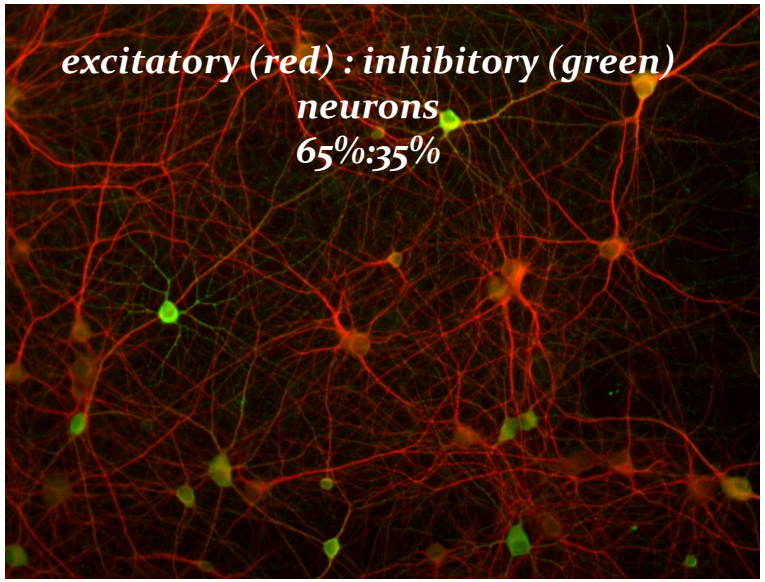
A snapshot in time of neural network activity in one well. Each box represents the electrical activity of neurons on 1 electrode in the array.

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

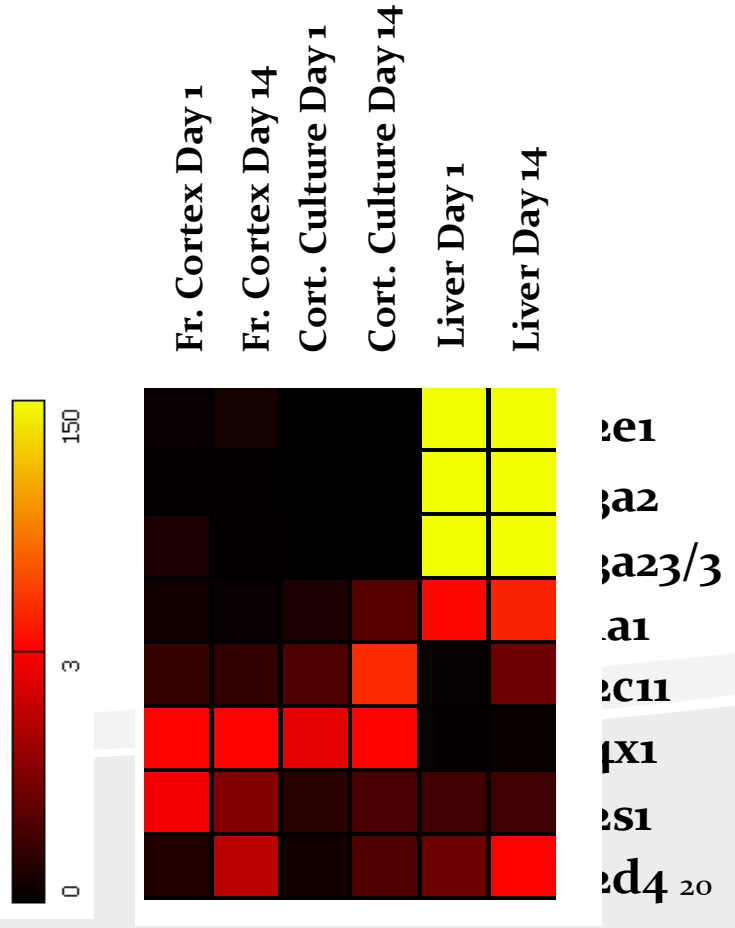
Transcriptomic data have recently been collected for all days in vitro covered by the NFA assay.

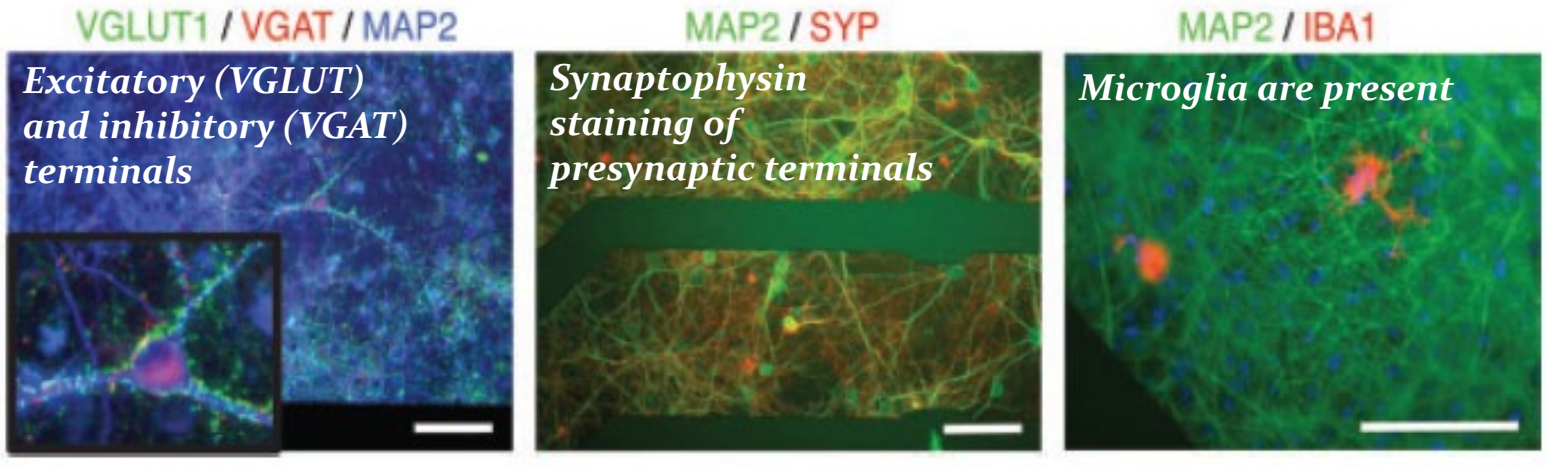
Primary Cultures of Cortical Neurons are Complex and Representative of in vivo Cortex





P450 expression is similar to Frontal Cortex, but lower than and different from the liver





Frank et al., ToxSci. 160,121-135. 2017

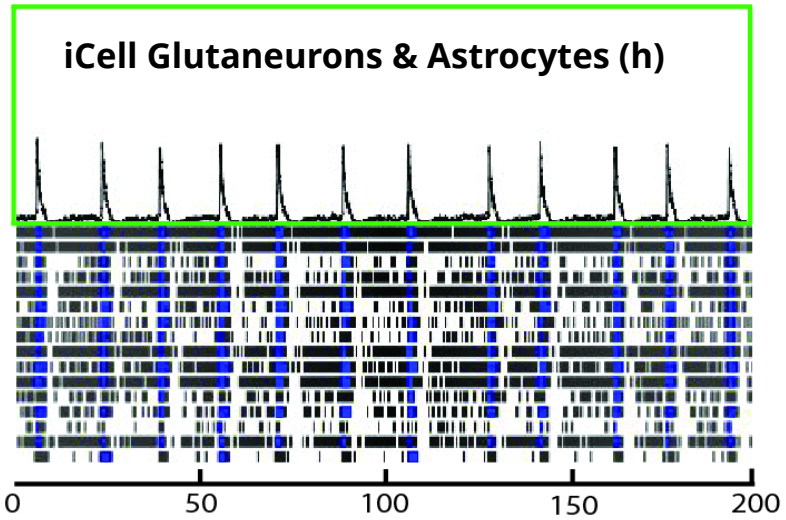
Functional Responses in Cortical Networks include Major Neurotransmitters and Channels

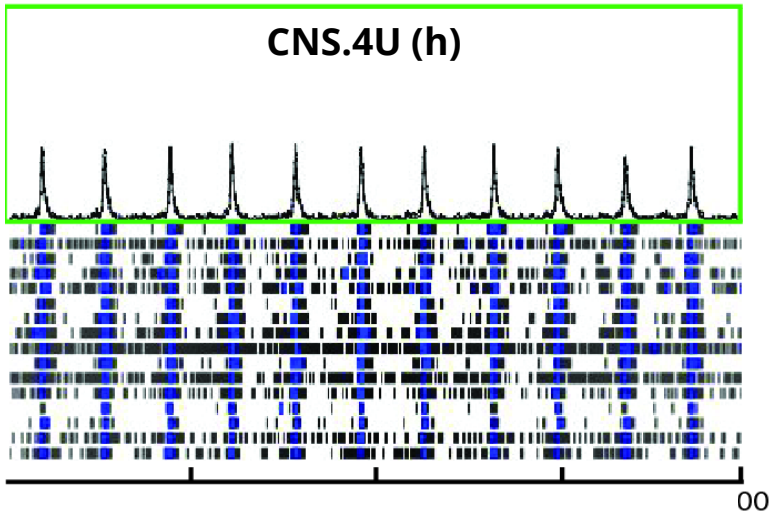
Receptor Type	Functional Response
AMPA-R	+
Kainate-R	+
NMDA-R	+
$GABA_A$-R	+
nACh-R	-/+
Dopamine R	+
VGSC	+

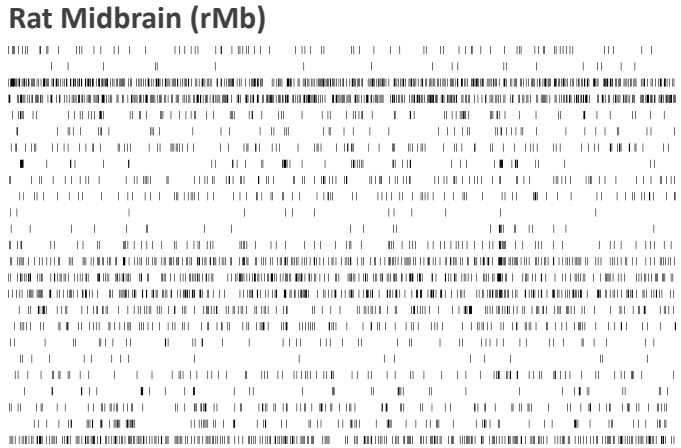
Development of Network Function is Crucial for Neurodevelopment across species

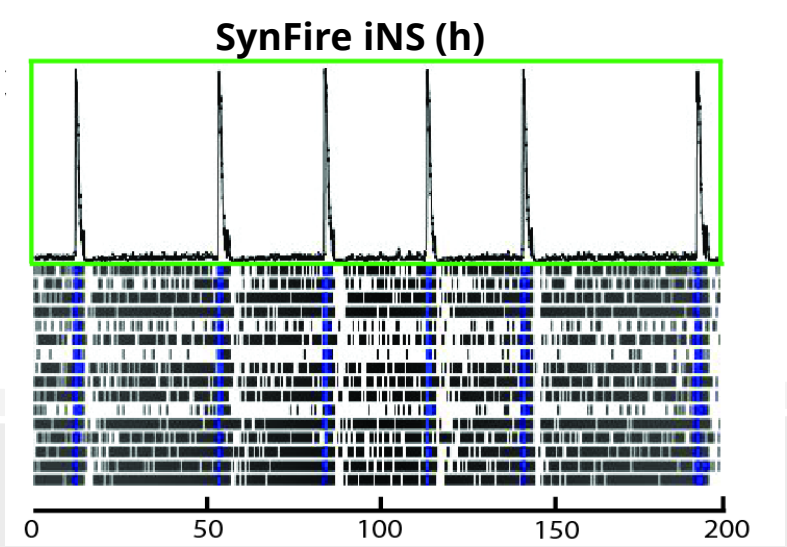
- Spiking, bursting, and synchronous activity are intrinsic network functions.
  - These properties of networks develop spontaneously in vivo and in vitro
- Neurodevelopmental processes are influenced by electrical activity.
- Synchronous activity in networks is integral to sensory awareness, attention, memory and other cognitive processes.
- Patterns of network activity are **highly conserved**.
  - There is greater similarity across the same brain regions of different species than between different brain regions within the same species

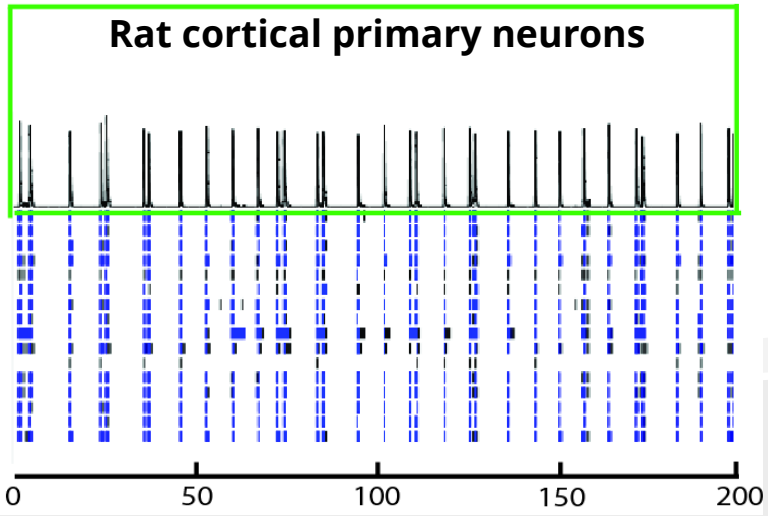
Human and Rodent Tissues have Similar Phenotypic Patterns of Spontaneous Activity

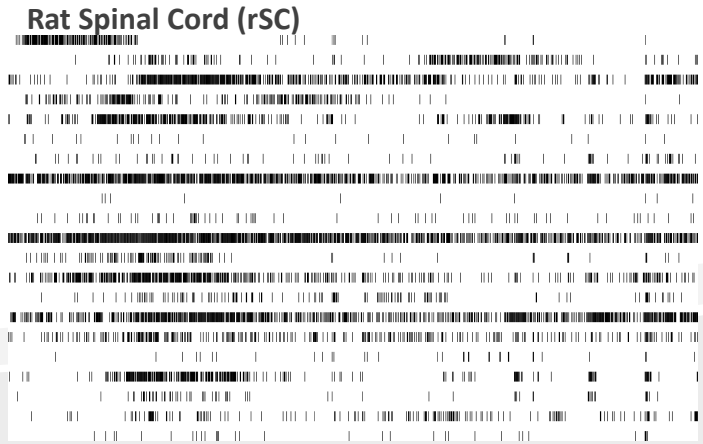












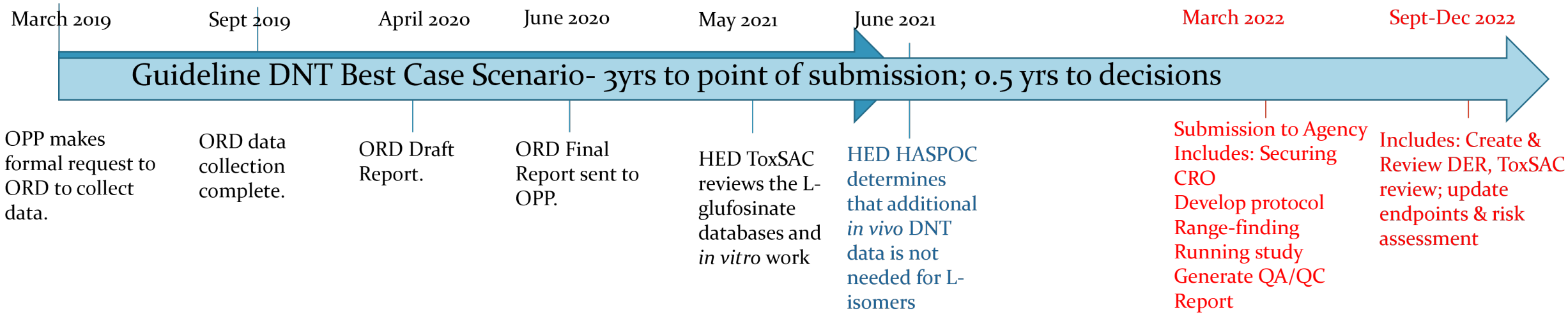
Tukker et al., 2020 ALTEX; Slide Courtesy of Anke Tukker

Case studies using DNT NAMs

- Screening and Prioritization
  - 150 PFAS compounds (Carstens et al., 2023)
  - Organophosphate flame retardants (DTT; OECD case-study)

- Weight of Evidence
  - Glufosinate DNT Guideline Waiver (Dobreniecki et al., 2022)
  - Deltamethrin and flufenacet (OECD Case-studies)
  - DCNA (Dichloran)
    - Required the DNT guideline study; based on WoE and positive effects in acute MEA study
  - Organophosphates
    - Evaluate DNT potential and relative sensitivity to AChE inhibition to inform FQPA determinations
    - Individual OP WoE assessments
      - Acephate, methamidophos, others pending

Impacts of DNT NAMs: Glufosinate example



Animals Used:

- *In vitro* study- 3 Pregnant Dams (~12-15pups)
- Guideline study- 160 Pregnant Dams (2 compounds X 3 doses + control @20/dose (recommended))
  - ~1600 pups

Cost:

- *In vitro* study- $1000 for Assays + $96,000 labor = $97,000
- Guideline study- $2,000,000 (2 compounds x $1M each)

Summary and Conclusions

- The DNT-IVB meets criteria for establishing confidence

- There is consensus that this DNT-IVB is ready for use in decisions regarding:
  - Screening and prioritization
  - Weight of Evidence
  - Case-studies document the use of the DNT-IVB in these contexts

- These Case-Studies demonstrate that data from the DNT NAMs can:
  - Speed decision making
  - Reduce costs
  - Contribute to health protective decisions.

There is consensus that the science behind DNT NAMs will continue to evolve and improve.

Implementation of the battery does not need to wait for future improvements

Thank you!
Questions?

EPA ORD Colleagues:
- Kathleen Wallace
- Theresa Freudenrich
- Bill Mundy (retired)
- Kevin Crofton (retired)
- Josh Harrill
- Jasmine Brown
- Katie Paul Friedman
- Melissa Martin
- Kelly Carstens
- Megan Culbreth
- Gabby Byrd
- Amy Carpenter (ORISE)
- Seline Choo (ORISE)
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- Chris McPherson
- Jui-Hua Hsieh
- Mamta Behl (formerly DTT)

International Collaborators
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- Marcel Leist (U. Konstantz)
- Andrea Terron (EFSA)
- Iris Mangas (EFSA)

OECD
- Magda Sachana