

A fluorescence microscopy image of neurons. The neurons have cell bodies (soma) stained blue with DAPI. Their processes, including dendrites and axons, are stained with two different fluorescent markers: one in green and one in red. The green staining appears more extensive and forms a dense network, while the red staining is more localized and highlights specific cellular structures. The background is black.

The DNT in vitro Battery

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Disclosure:

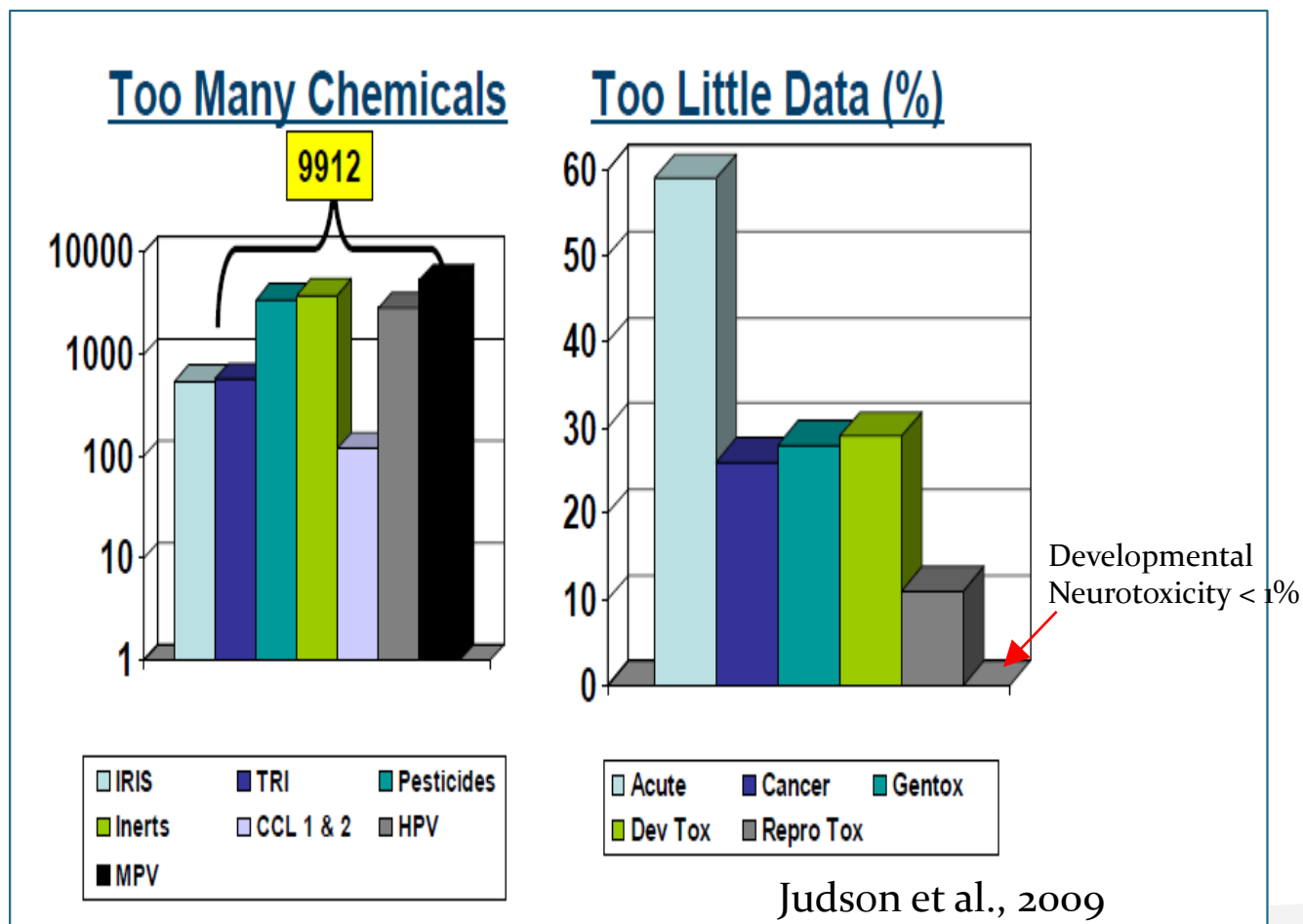
This work has been funded by the US. Environmental Protection Agency. I have no conflicts to declare.

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Photograph by Thresa Freudenrich, CCTE

The Problem: Developmental Neurotoxicity (DNT) has been examined for too few chemicals



Current testing too slow

- Not Required under FIFRA
- Animal “Guideline” DNT; 1 chemical, \$1M cost; 2+ yr
- At current pace, ~220 chemicals in 20+ yrs

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

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



Solution to obtaining more data about potential DNT hazard



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

- Develop high throughput, in vitro assays,
- Rapid, cost-effective characterization of developmental neurotoxicity hazard
- Data from these assays can provide information for decision-making

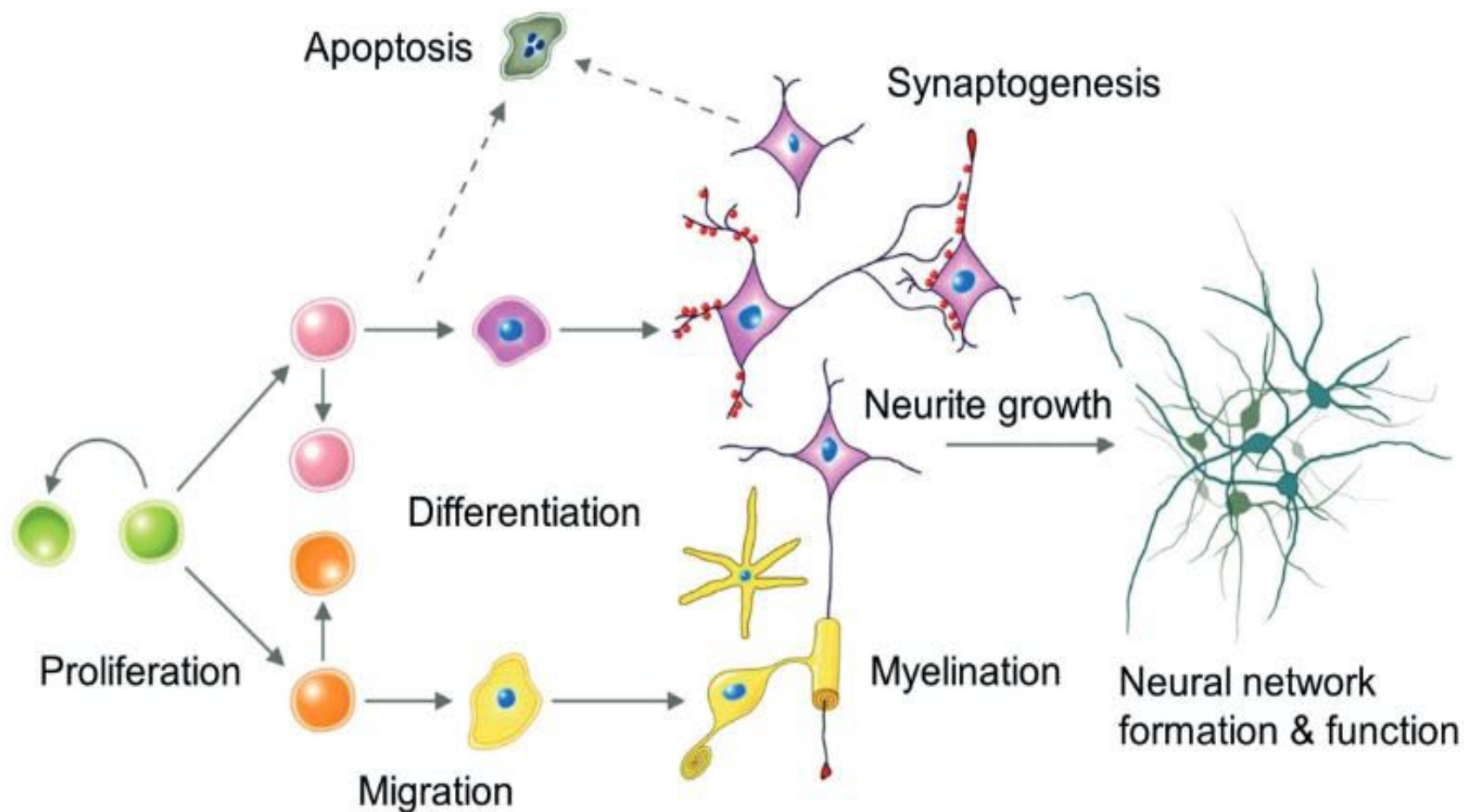


Requirements for in vitro DNT assays

Develop assays using cell-based endpoints that are amenable to medium-high throughput/content testing. Useful methods must be:

- Phenotypic/functional assays rather than target-based (MIE) screening
 - To ensure coverage of a wide range of potential targets
- Scalable to medium to high-throughput
- Cost effective (or at least not cost prohibitive)
- Reproducible
- Transferrable
- Translatable (decision-makers need to understand what is being measured and how it relates to neurodevelopment in vivo).

Key Neurodevelopmental Processes



Aschner et al., ALTEX. 2017;34(1):49-74. doi: 10.14573/altex.1604201

Key Neurodevelopmental Processes, Outcomes and Environmental Chemicals.



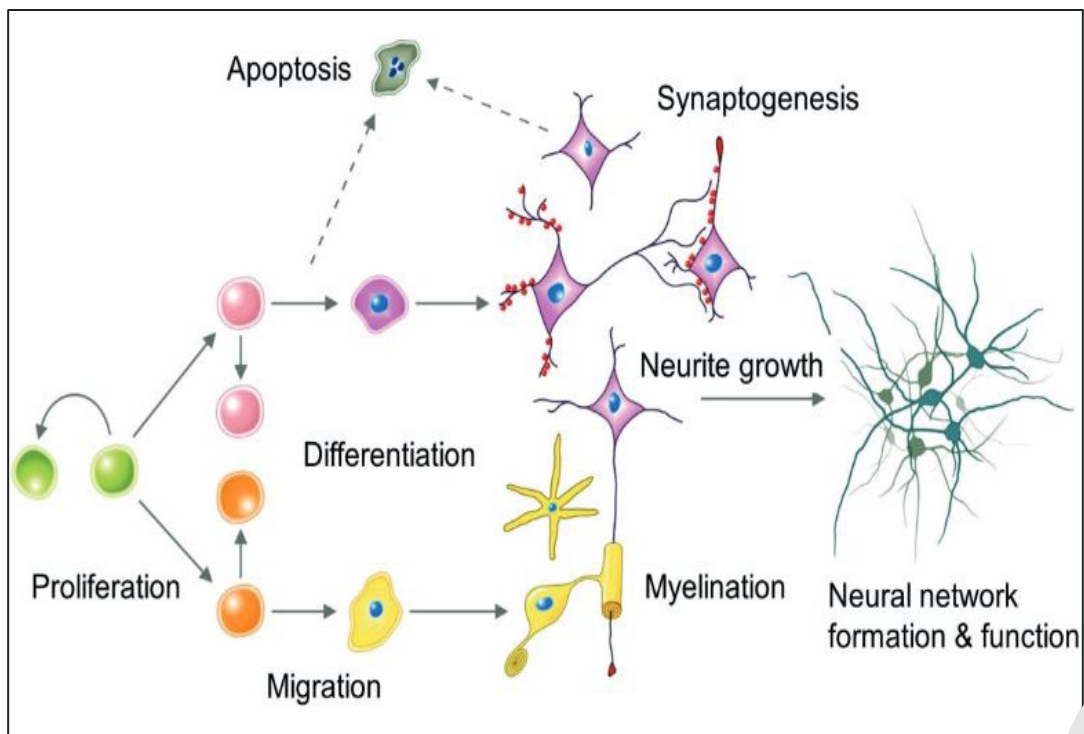
Neurodevelopmental Process	Environmental Agents Related to each Process	Clinical Conditions Related to each Process
Proliferation	Ionizing radiation, MAM, MeHg, Chlorpyrifos	Autism
Migration	Ethanol, MeHg	Cerebral Palsy
Apoptosis	Ethanol, MeHg, Chlorpyrifos	Autism
Differentiation (Neurite Outgrowth)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

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

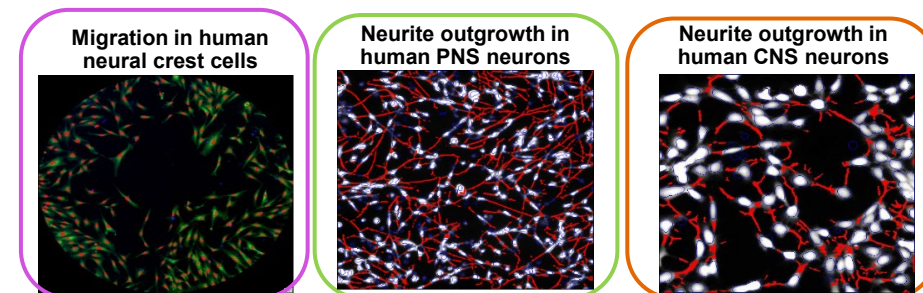
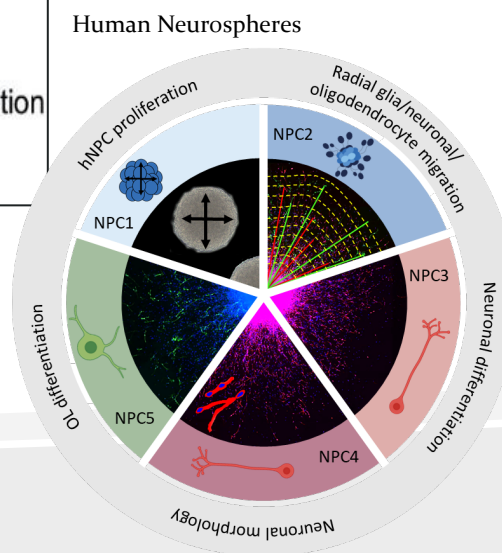


Methods <i>in vivo</i>	Outcome	Cell Biological Causes
Gross Morphology	Brain measures ↑↓	→ Proliferation, Apoptosis
	Brain parts missing	→ Proliferation, Differentiation
	Malformation	→ Proliferation, Migration, Differentiation
Histopathology	Necrosis	→ Cytotoxicity
	Pyknosis	→ Apoptosis, Necrosis
	Neuronal 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

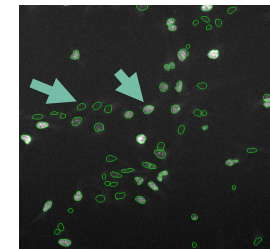
DNT-IVB Coverage of Neurodevelopmental Processes



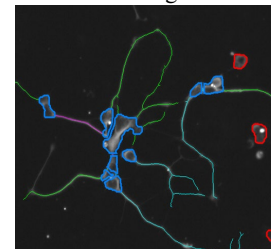
Aschner et al., ALTEX. 2017;34(1):49-74. doi: 10.14573/altex.1604201



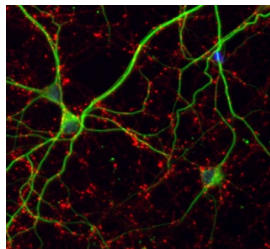
Human Neuroprogenitor Proliferation



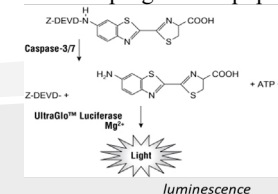
Human and Rat Neuron Neurite Outgrowth



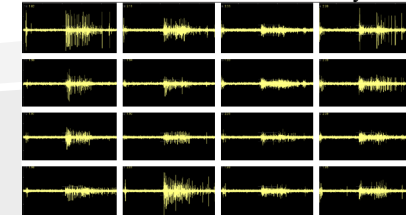
Synaptogenesis in Rat Cortex Neurons



Human Neuroprogenitor Apoptosis



Rat Network Formation Assay



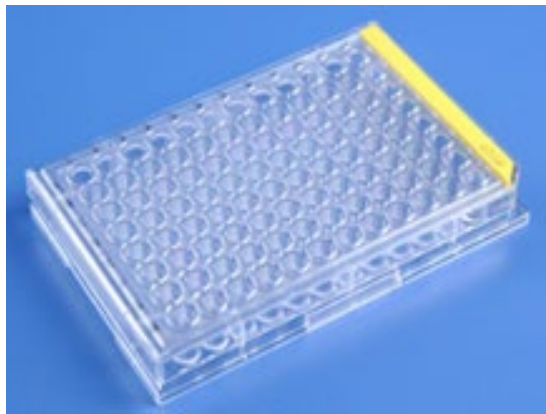
Figures courtesy of Drs Marcel Leist, and Ellen Fritsche

High Content Imaging: Overview

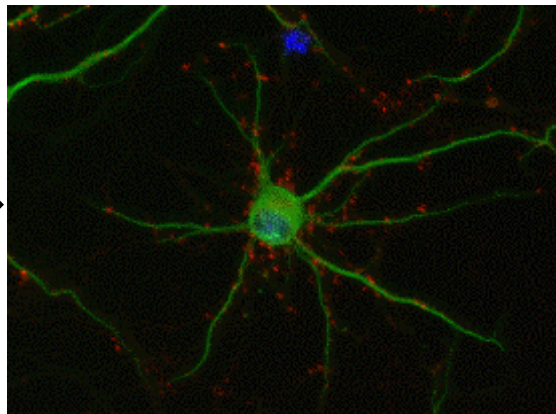
Automated microscopy providing data at the level of the individual cell

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

High content : large quantity of cell morphometric measurements simultaneously for multiple cells in one field.



Multiwell Culture



Immunocytochemistry



Image Acquisition

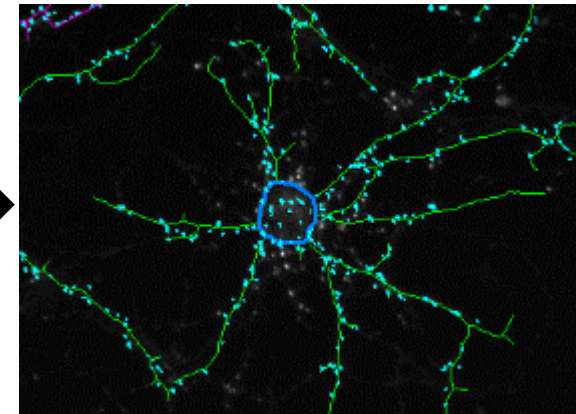


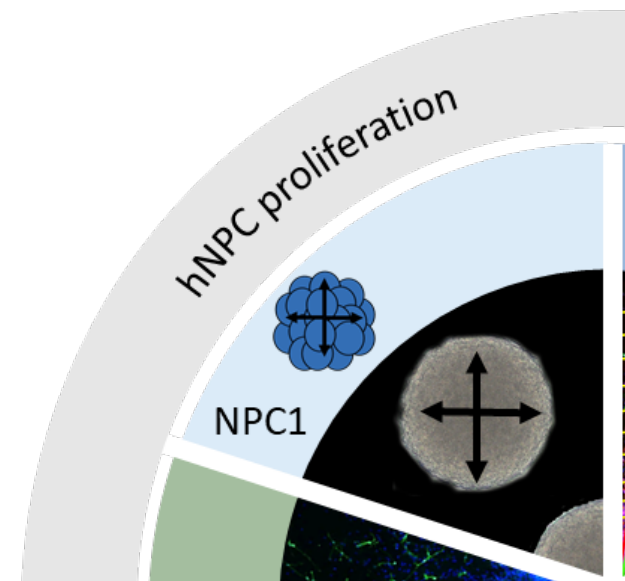
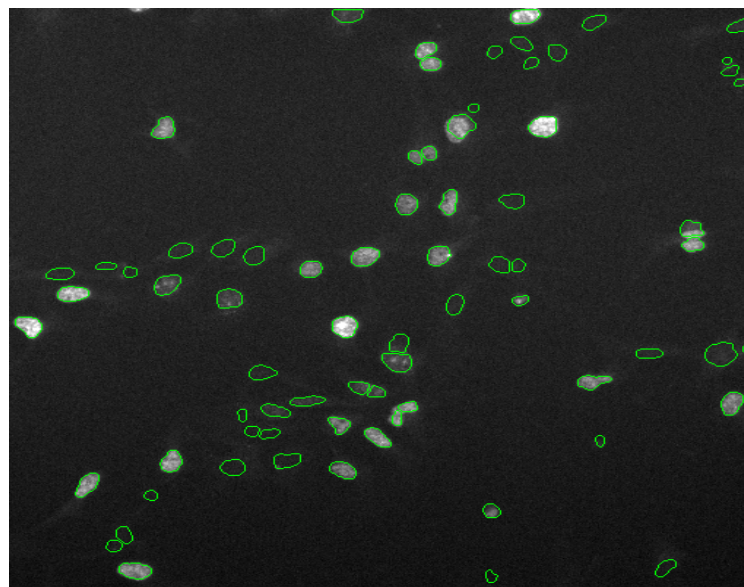
Image Analysis
Feature Extraction

- 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)

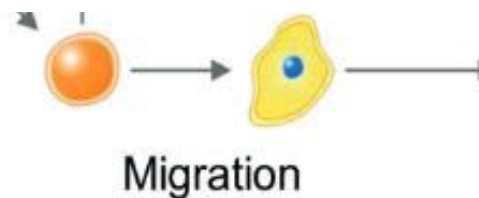
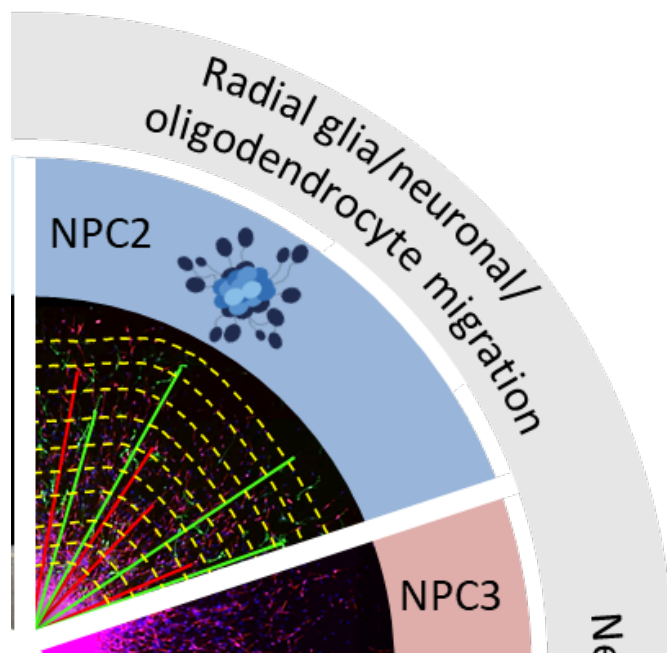
DNT-IVB Coverage of Neurodevelopmental Processes



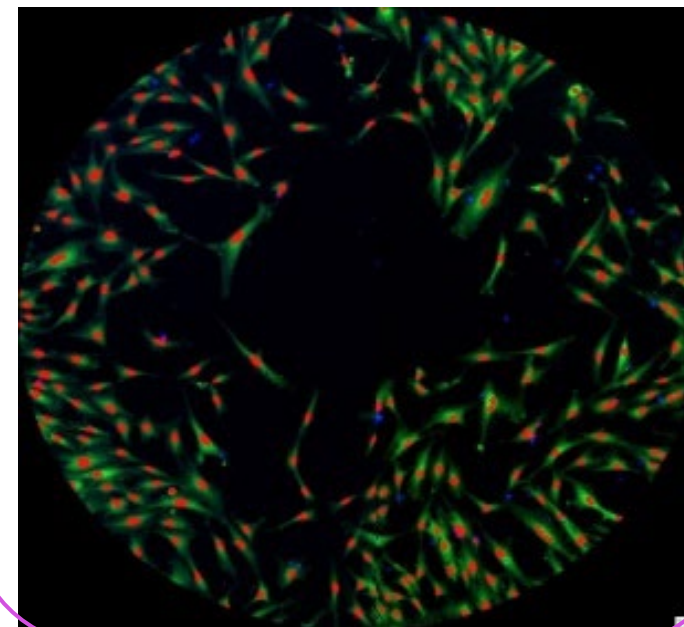
Human Neuroprogenitor Proliferation



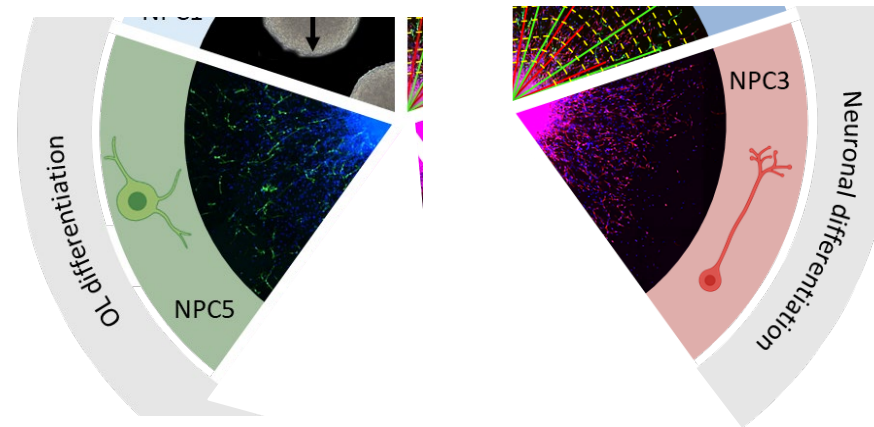
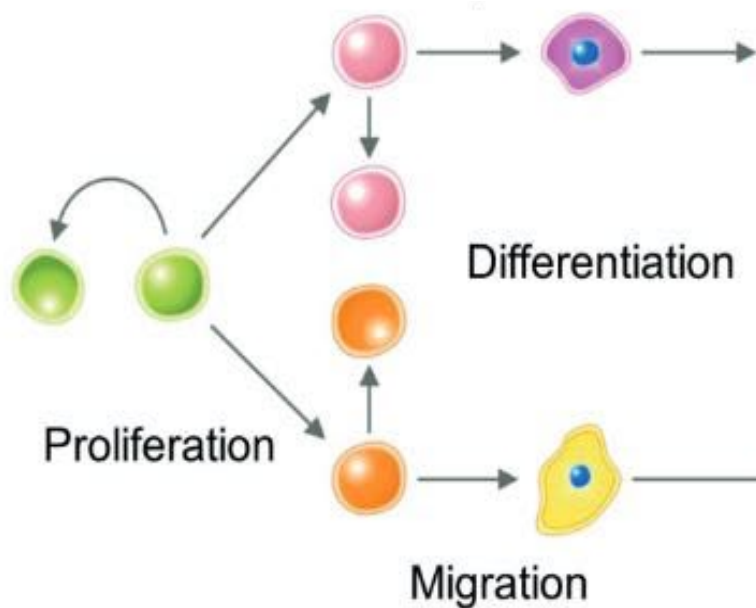
DNT-IVB Coverage of Neurodevelopmental Processes



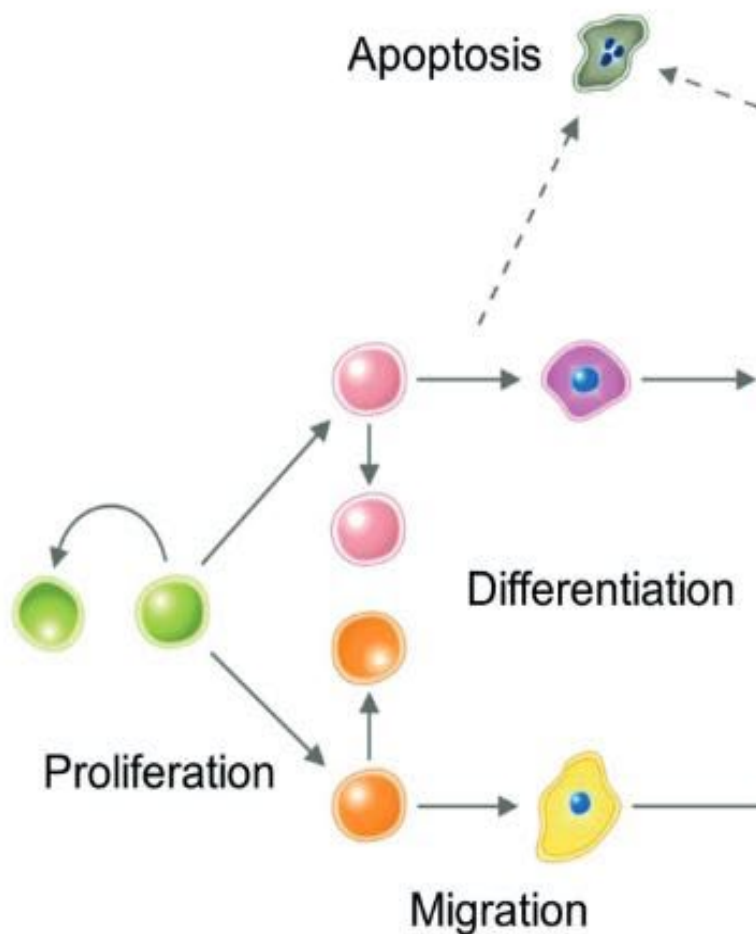
Migration in human neural crest cells



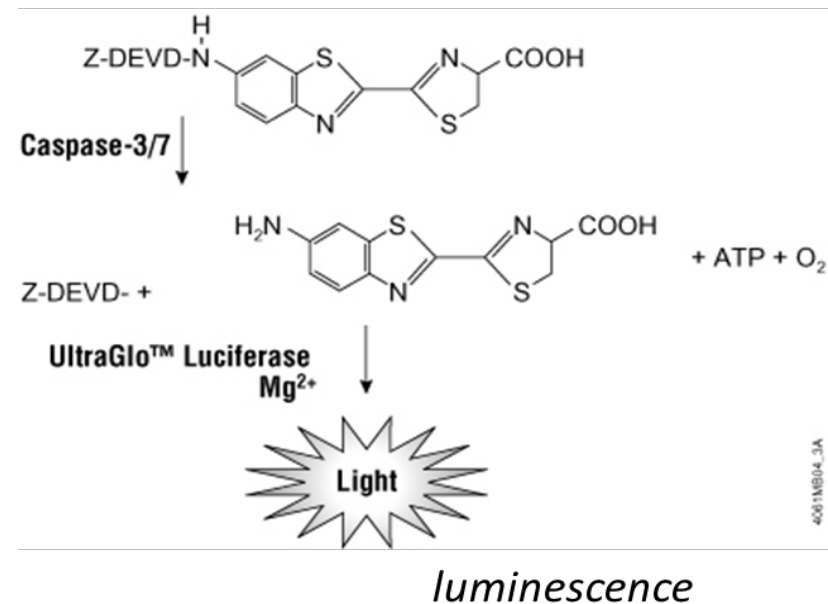
DNT-IVB Coverage of Neurodevelopmental Processes



DNT-IVB Coverage of Neurodevelopmental Processes



Human Neuroprogenitor Apoptosis



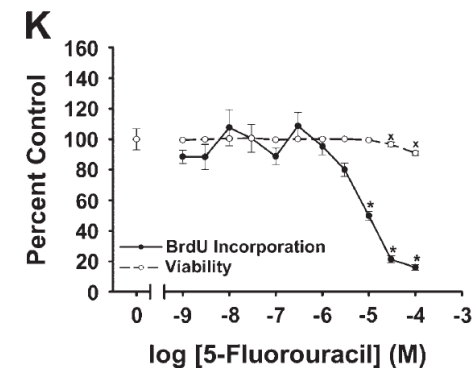
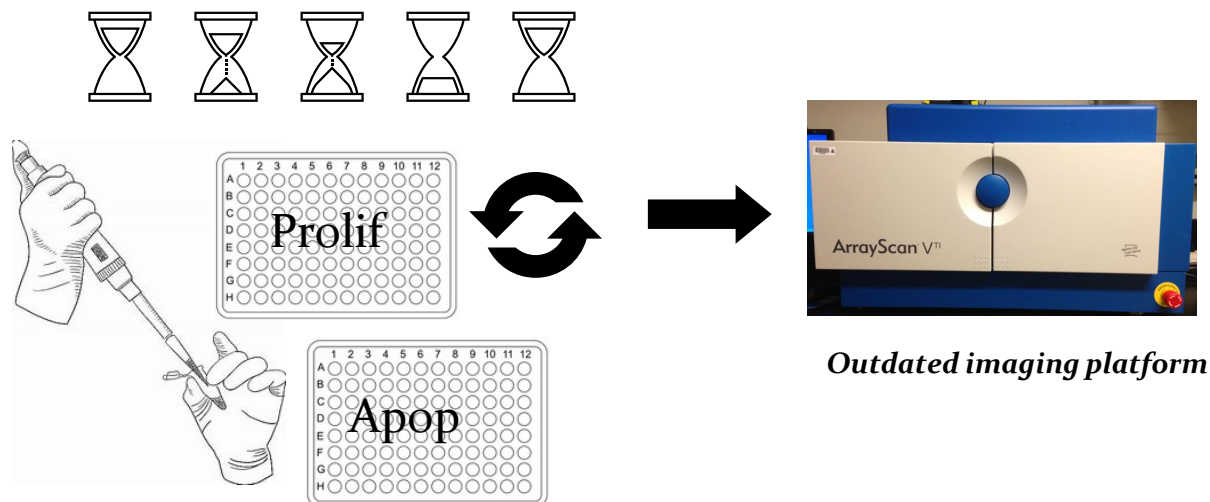
Multiplexing Proliferation and Apoptosis

Development of Increased Throughput DNT NAMs to facilitate chemical screening

96-well → 384-well

Manual pipetting → Laboratory automation

Updated imaging equipment



Scale up of proliferation/apoptosis in hNP1 Cells to 384 wells

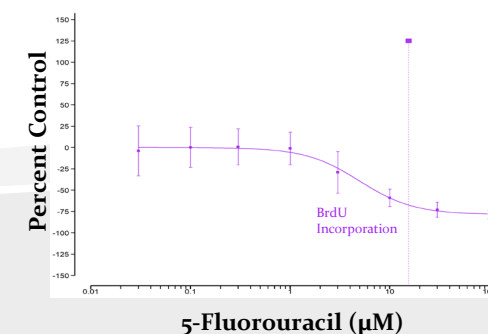
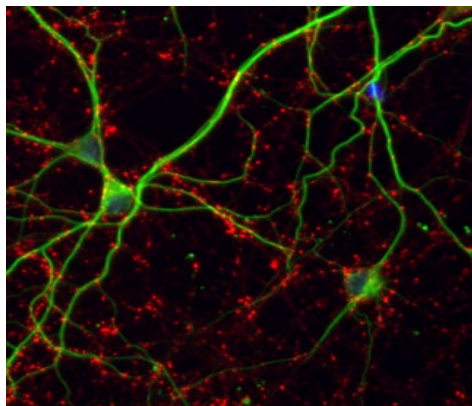


Figure courtesy of Gabby Byrd

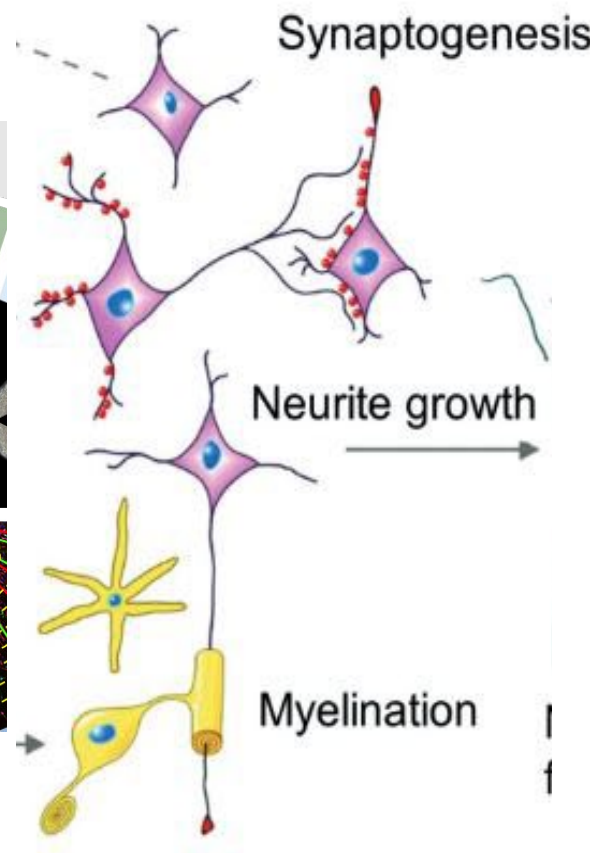
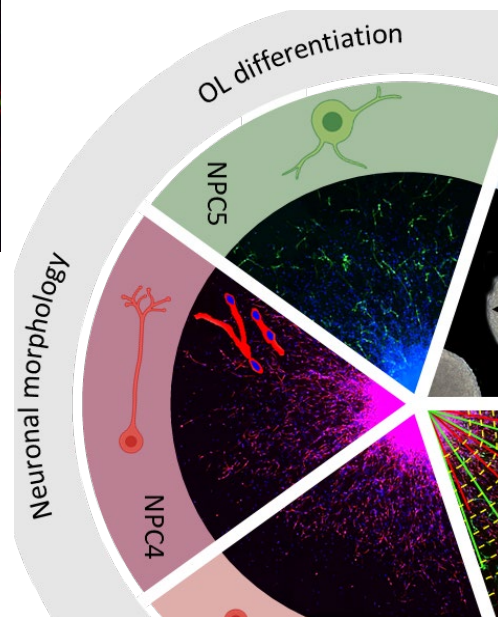
DNT-IVB Coverage of Neurodevelopmental Processes



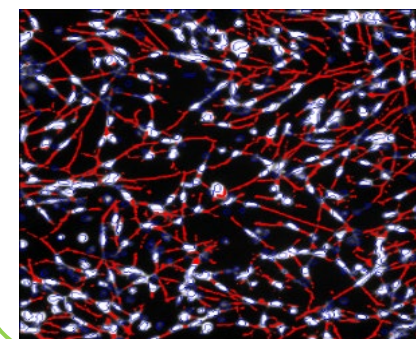
Synaptogenesis in Rat
Cortex Neurons



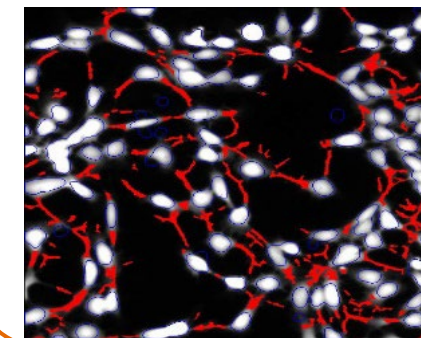
Human and Rat Neuron
Neurite Outgrowth



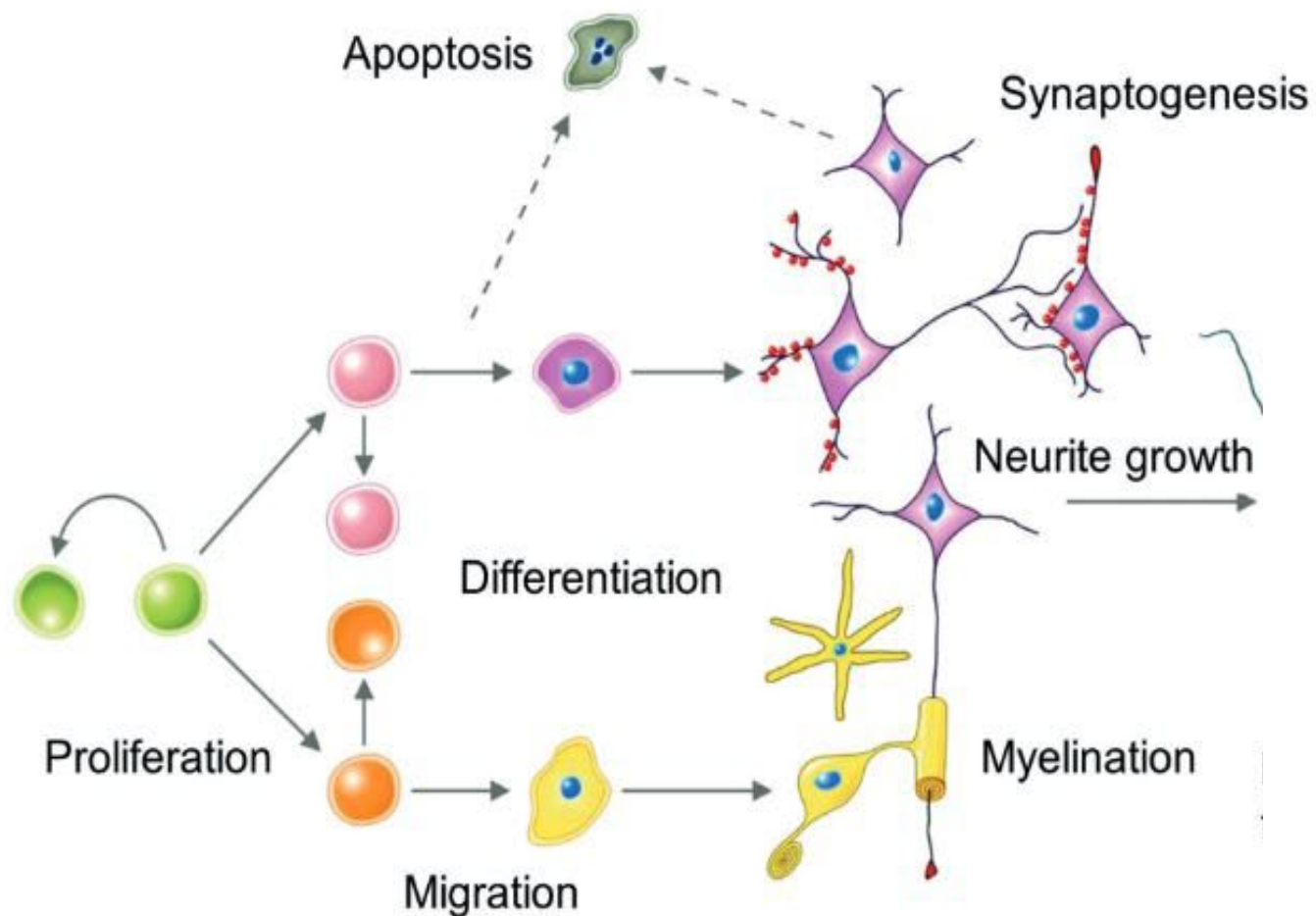
Neurite outgrowth in human
PNS neurons



Neurite outgrowth in human
CNS neurons



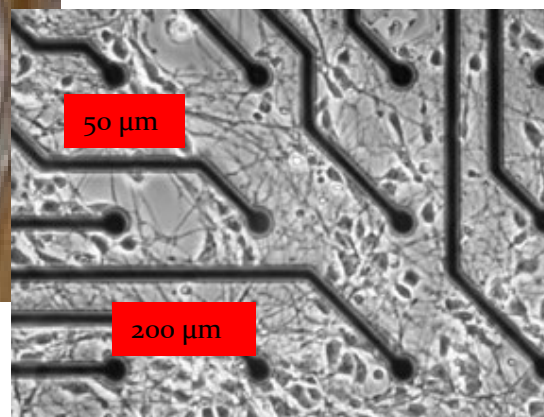
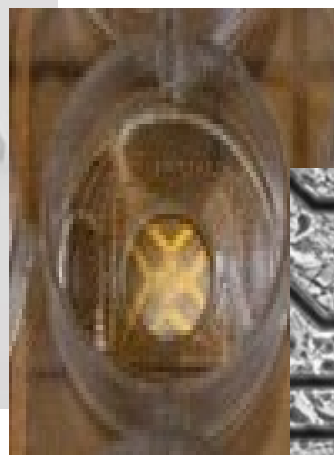
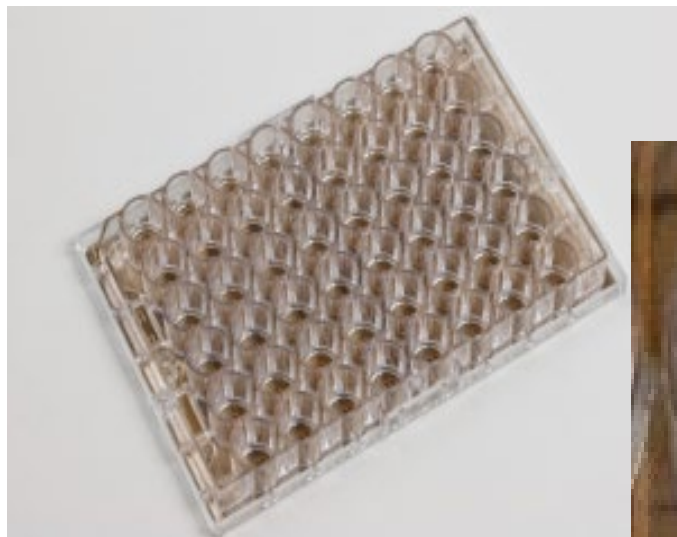
DNT-IVB Coverage of Neurodevelopmental Processes



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



“Brain-on-a-Chip”: Complex 2D model



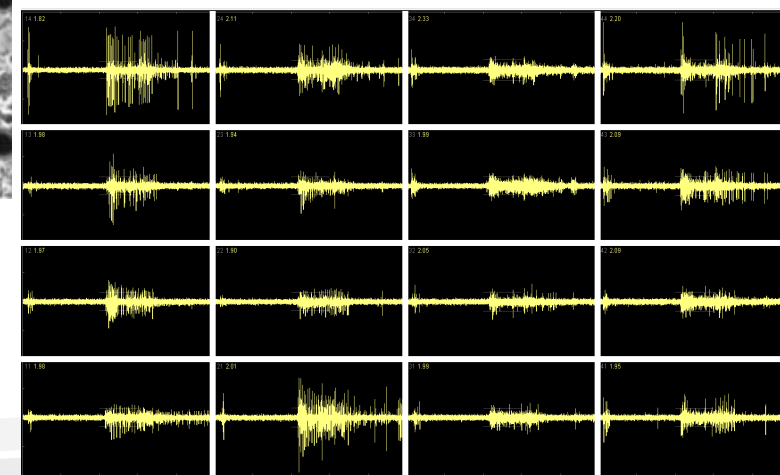
- Rat cortical neural networks
- Contains neurons & glia cells
- Spontaneous activity
- Develops rapidly in vitro
- Follow network development over time
- Integrates activity of multiple processes
- **Replicates human biology and pharmacology**

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.

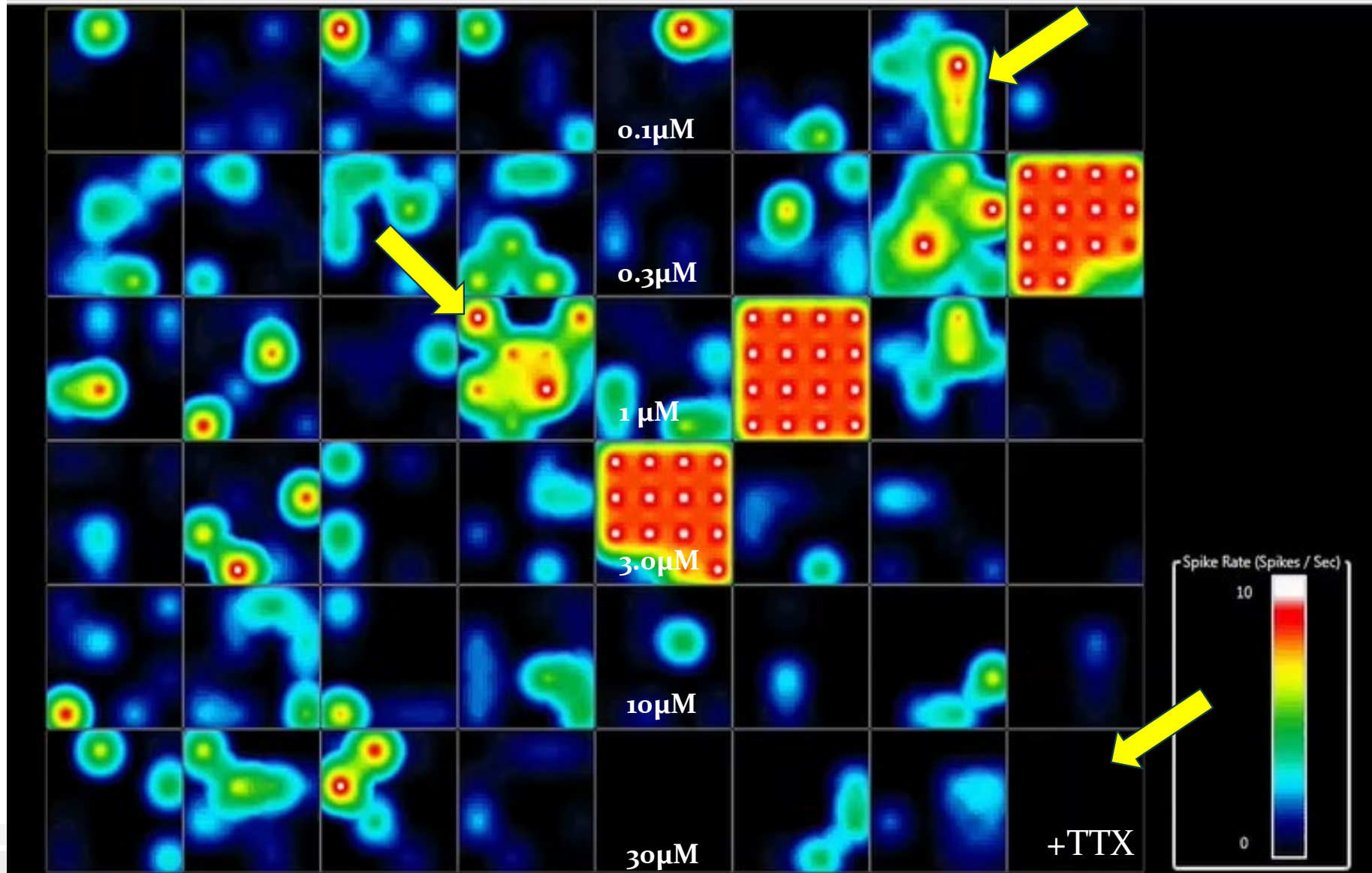


A snapshot in time of neural network activity in one well. Each box represents the electrical activity of neurons on 1 electrode in the array.

DMSO

Picrotoxin

BIC (25
 μ M)



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



ENV/CBC/MONO(2023)13 | 1

Unclassified

English - Or. English

3 November 2023

ENVIRONMENT DIRECTORATE
CHEMICALS AND BIOTECHNOLOGY COMMITTEE

Cancels & replaces the same document of 9 October 2023

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

Series on Testing and Assessment
No. 377

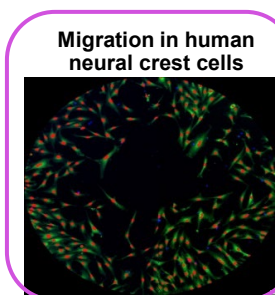
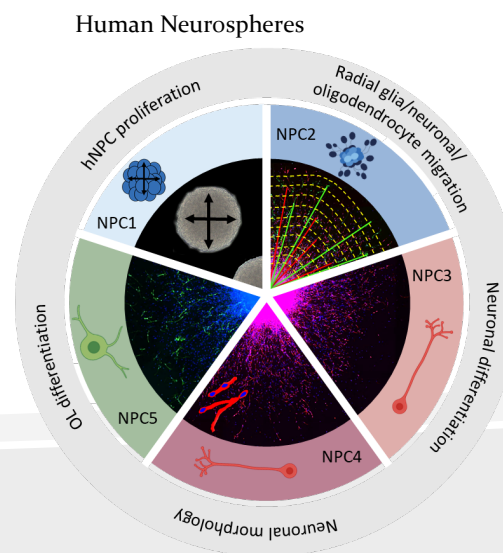
- Recognized a battery of in vitro assays for DNT
- Provides international recognition and credibility to the DNT in vitro assays.

Application of the DNT IVB

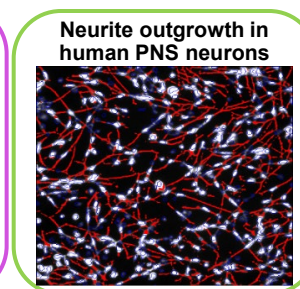


Now that we have the DNT-IVB, how do we facilitate its use for decision making?

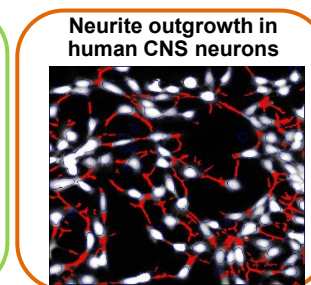
- Establishing confidence in the battery
 - Need a “roadmap” to establish confidence
- Demonstrating Utility
 - Case studies of regulatory use



Migration in human neural crest cells

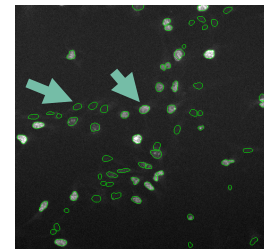


Neurite outgrowth in human PNS neurons



Neurite outgrowth in human CNS neurons

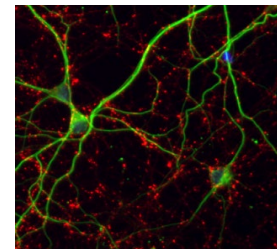
Human Neuroprogenitor Proliferation



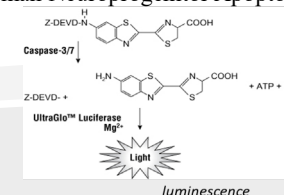
Human and Rat Neuron Neurite Outgrowth



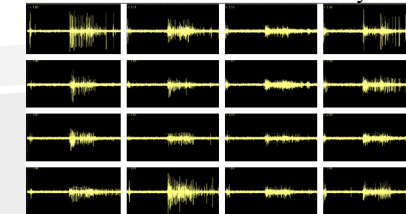
Synaptogenesis in Rat Cortex Neurons



Human Neuroprogenitor Apoptosis



Rat Network Formation Assay

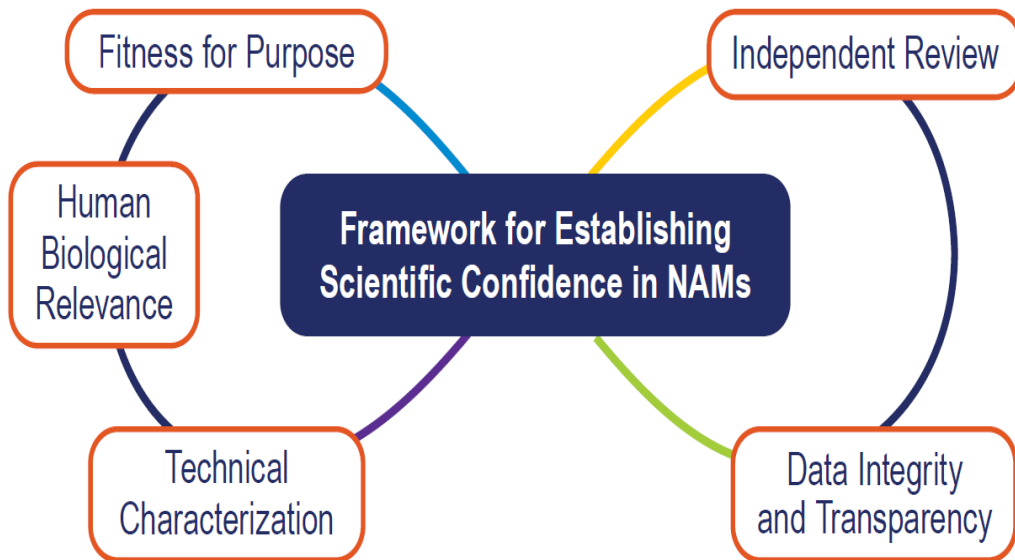


Figures courtesy of Drs Marcel Leist, and Ellen Fritsche

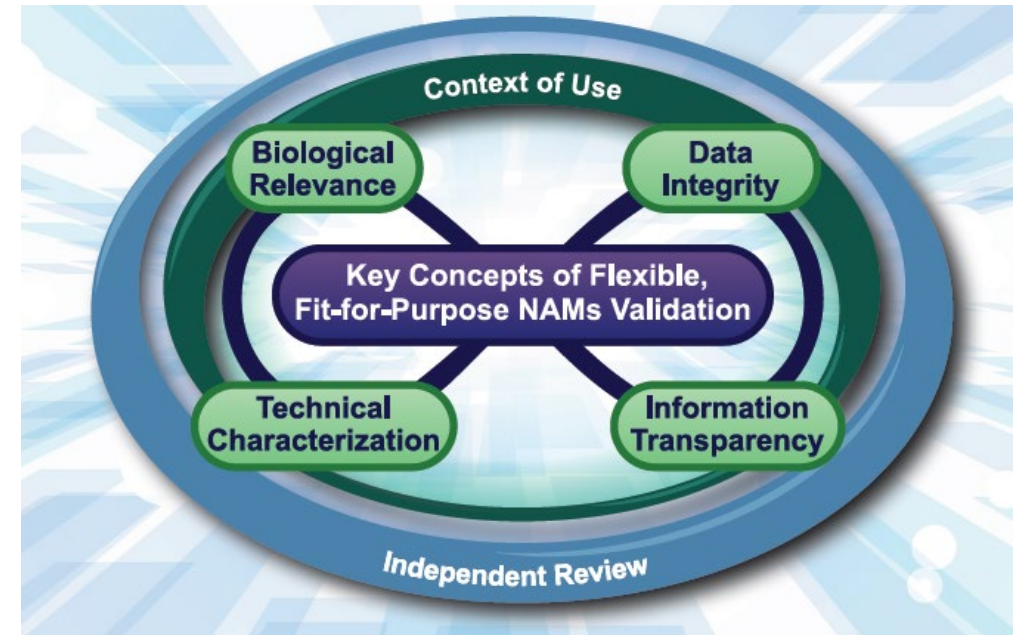
Establishing Confidence in the Assays



OECD 34 = Under revision



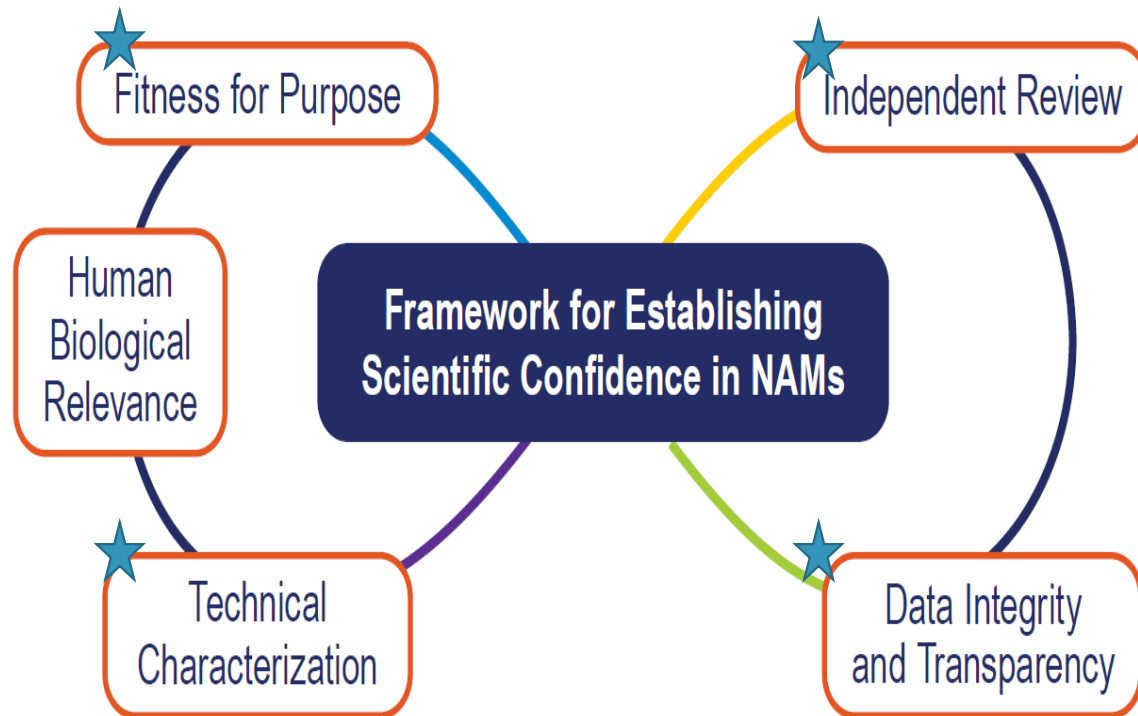
from Van der Zalm, et al., Arch Toxicol. 2022 Nov;96(11):2865-2879. doi: 10.1007/s00204-022-03365-4.



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 format (Krebs et al. 2019)

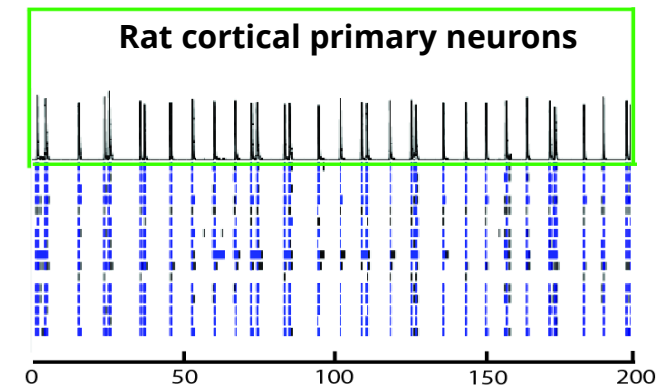
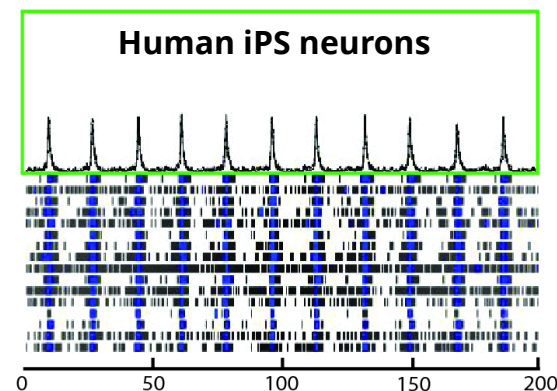
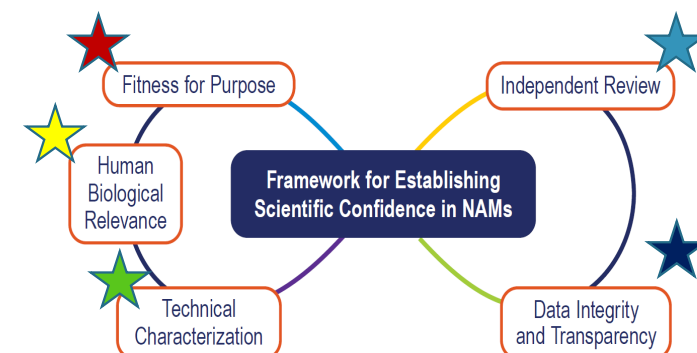
Establishing Confidence in the Assays: Human Biological Relevance

The Developmental Neurotoxicity Battery- DNT-IVB

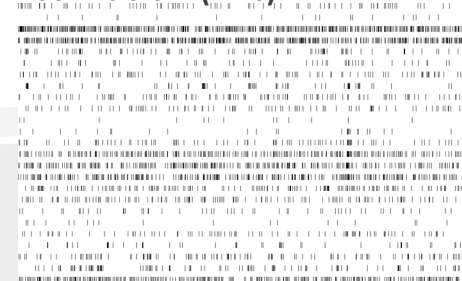
Table 2. Proposed Assays for Evaluation As an *In Vitro* DNT Battery

Process	Assays	References
Proliferation	hNP1	Harrill et al. (2018)
	NPC1	Baumann et al. (2016) and Barenys et al. (2017)
Apoptosis Migration	UKN1	Balmer et al. (2012)
	hNP1	Harrill et al. (2018)
	NPC2	Baumann et al. (2016) and Barenys et al. (2017)
Neuron differentiation	UKN2	Nyffeler et al. (2017)
	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	Harrill et al. (2018)
Synaptogenesis	Rat primary synaptogenesis	Harrill et al. (2018)
Network formation	MEA-NFA	Brown et al. (2016) and Frank et al. (2018)

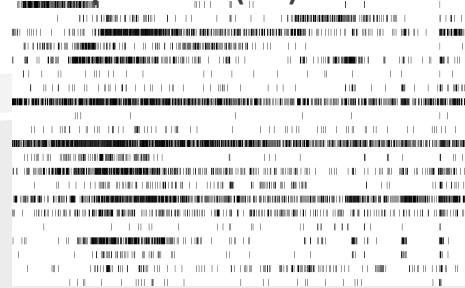
Demonstrate the similarities between the physiology of the test system or the biology measured by the test system, and human biology. Confidence in a NAM is bolstered when it adequately reflects human biological understanding (or, for example, key events in a relevant adverse outcome pathway, AOP). (From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



Rat Midbrain (rMb)



Rat Spinal Cord (rSC)





Summary



- Data on the potential developmental neurotoxicity is not available for thousands of chemicals
- Testing all of these chemicals in Guideline DNT studies is not feasible
- A battery of in vitro assays has been developed to test chemicals for potential DNT hazard
- This battery consists of several assays that:
 - Cover a wide variety of key processes critical for neurodevelopment
 - Provide information about the biological activity of chemicals towards the developing nervous system
- Disruption of these processes can contribute to:
 - Clinical neurodevelopmental diseases
 - Are relevant to metrics evaluated in guideline studies
- An initial guidance document on the use of data from these assays has been endorsed by the OECD WNT



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)
- Richard Judson
- Grace Patlewicz

EPA Program Office Colleagues

- Anna Lowit
- Liz Mendez
- Monique Perron
- Sarah Dobreniecki
- Mike Metzger (Retired)

NIEHS DTT Colleagues

- Helena Hogberg
- Laura Hall
- Chris McPherson
- Jui-Hua Hsieh
- Mamta Behl (formerly DTT)

International Collaborators

- Ellen Fritsche (IUF)
- Marcel Leist (U. Konstanz)
- Andrea Terron (EFSA)
- Iris Mangas (EFSA)

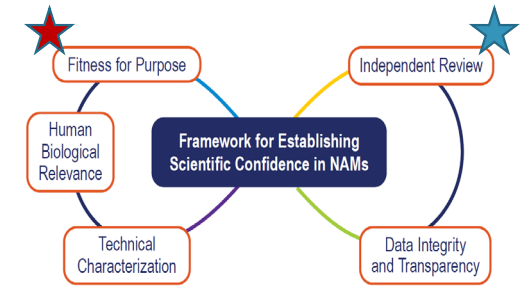
OECD

- Magda Sachana

 Extra slides



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.”

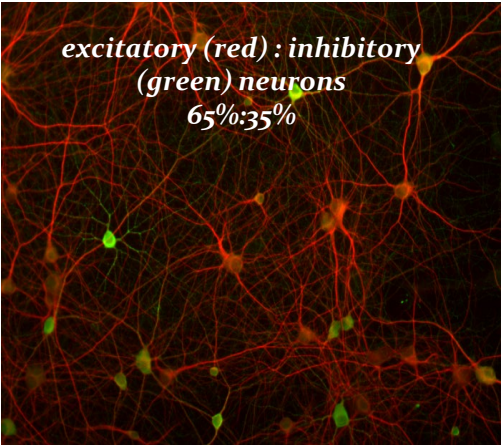
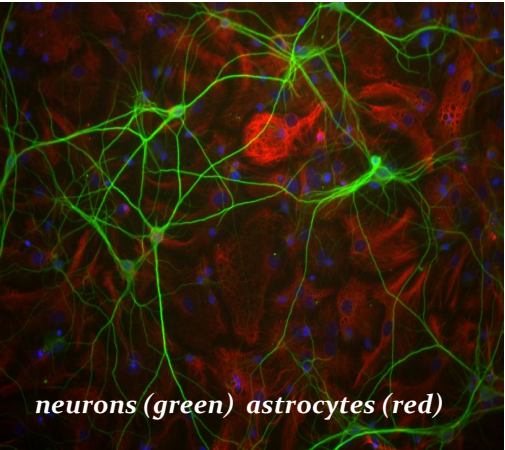
EPA 2020 SAP

“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**”. ”

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

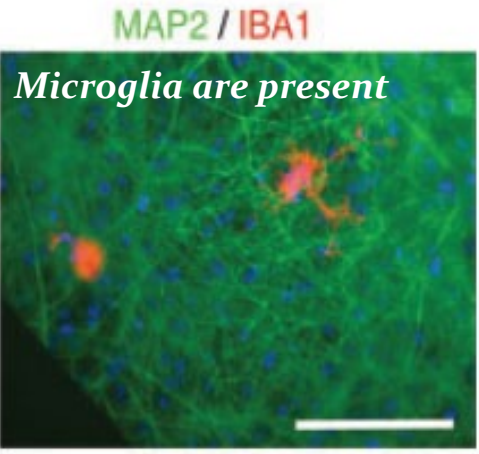
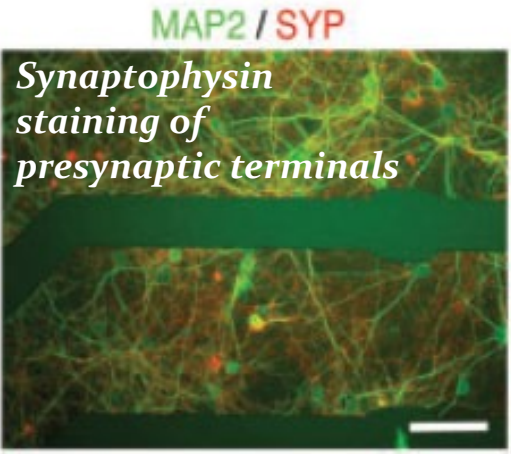
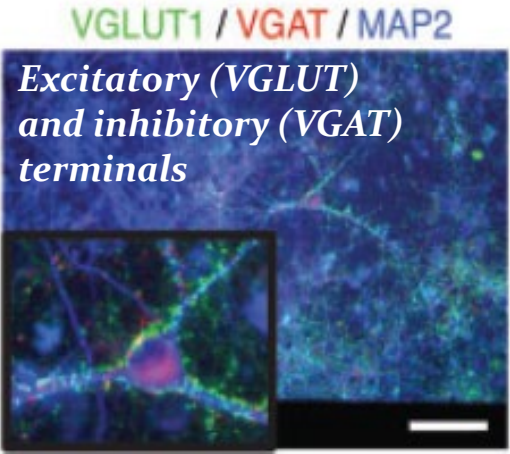


P450 expression is similar to Frontal Cortex, but lower than and different from the liver



Receptor Type	Functional Response
AMPA-R	+
Kainate-R	+
NMDA-R	+
GABA _A -R	+
nACh-R	-/+
Dopamine R	+
VGSC	+

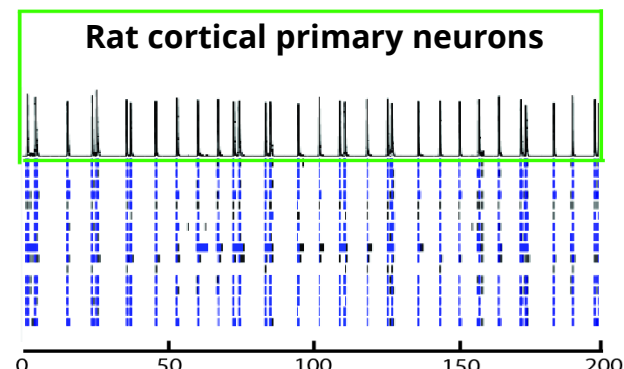
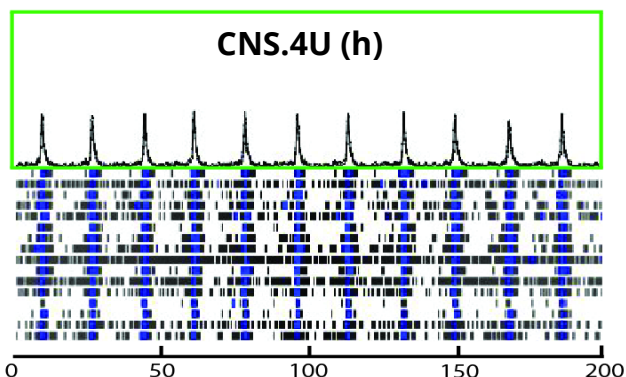
Fr. Cortex Day 1
Fr. Cortex Day 14
Cort. Culture Day 1
Cort. Culture Day 14
Liver Day 1
Liver Day 14



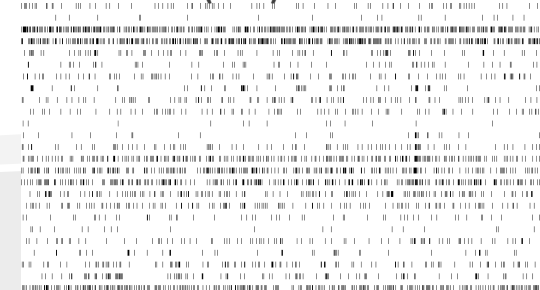
Development of Network Function is Crucial for Neurodevelopment across species



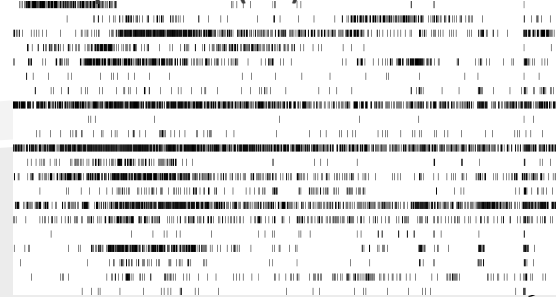
- Spiking, bursting, and synchronous activity are intrinsic network functions.
 - These properties of networks develop spontaneously in vivo and in vitro
- Neurodevelopmental processes are influenced by electrical activity.
- Synchronous activity in networks is integral to sensory awareness, attention, 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



Rat Midbrain (rMb)



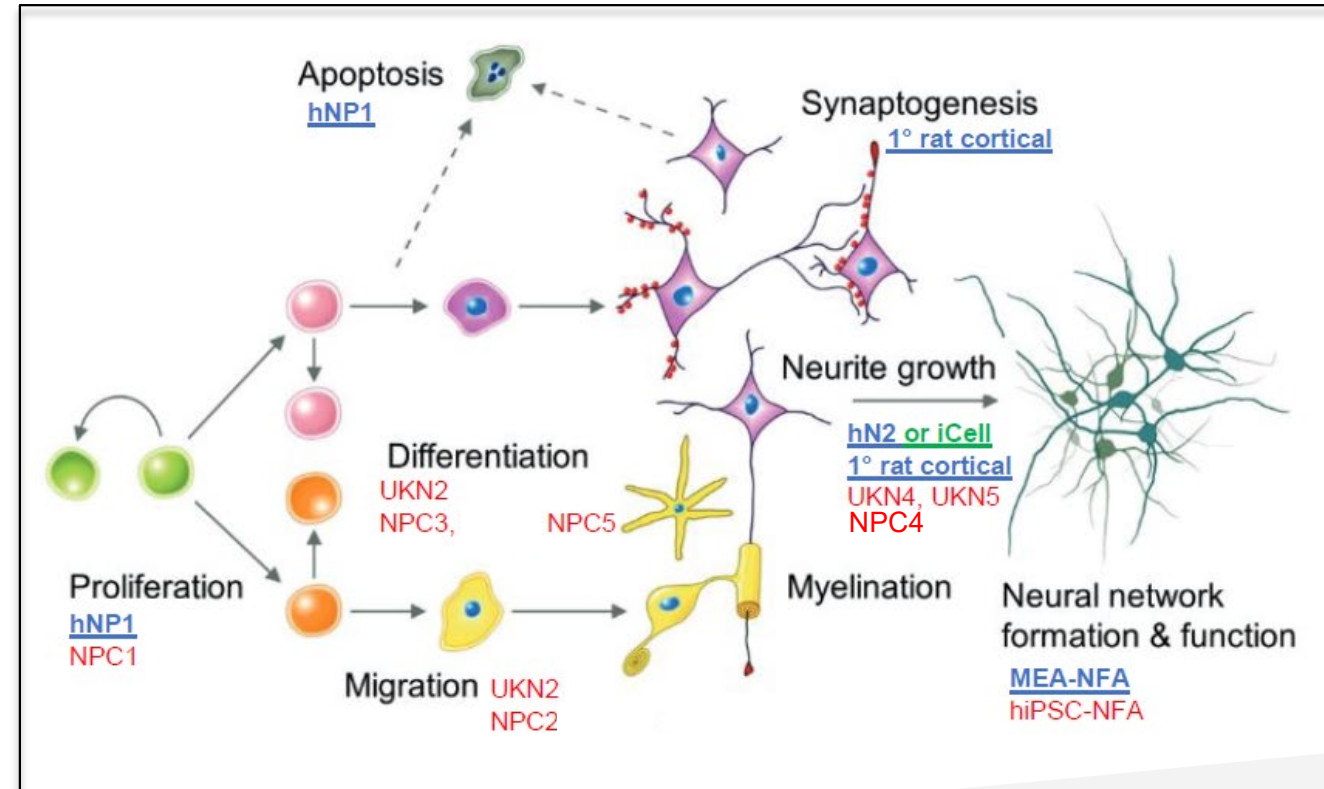
Rat Spinal Cord (rSc)



List of DNT assays currently in the ToxCast database



Neurodevelopmental Process	Species	Assay
Apoptosis	Human	Apoptosis, hNP1
Differentiation	Human	Neuronal Differentiation, NPC3
Differentiation	Human	Oligo Differentiation, NPC5
Migration	Human	Migration, UKN2
Migration	Human	Migration, neuronal, NPC2
Migration	Human	Migration, oligo, NPC2
Migration	Human	Radial glial migration, NCP2
Network formation & function	Rat	MEA NFA
Neurite Outgrowth	Rat	NOG, rat
Neurite Outgrowth	Human	NOG, hN2
Neurite Outgrowth	Human	NOG, CDI
Neurite Outgrowth	Human	NOG, SBAD2
Neurite Outgrowth	Human	NOG, LUHMES
Neurite Outgrowth	Human	NOG, NPC4
Proliferation	Human	Proliferation, hNP1
Proliferation	Human	Proliferation, NPC1
Synaptogenesis	Rat	Synaptogenesis, rat



<https://www.regulations.gov/document/EPA-HQ-OPP-2020-0263-0054>