Screening for Chemical Effects on Neuronal Proliferation and Neurite Outgrowth Using High-Content/High-Throughput Microscopy

Joseph M. Breier, Ph.D.

Curriculum in Toxicology
University of North Carolina at Chapel Hill
Chapel Hill, NC

Neurotoxicology Division
National Health and Environmental Effects Research Laboratory
United States Environmental Protection Agency
Research Triangle Park, NC

Chemical Prioritization Community of Practice
Research Triangle Park, NC
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In Vitro Screening for Developmental Neurotoxicity

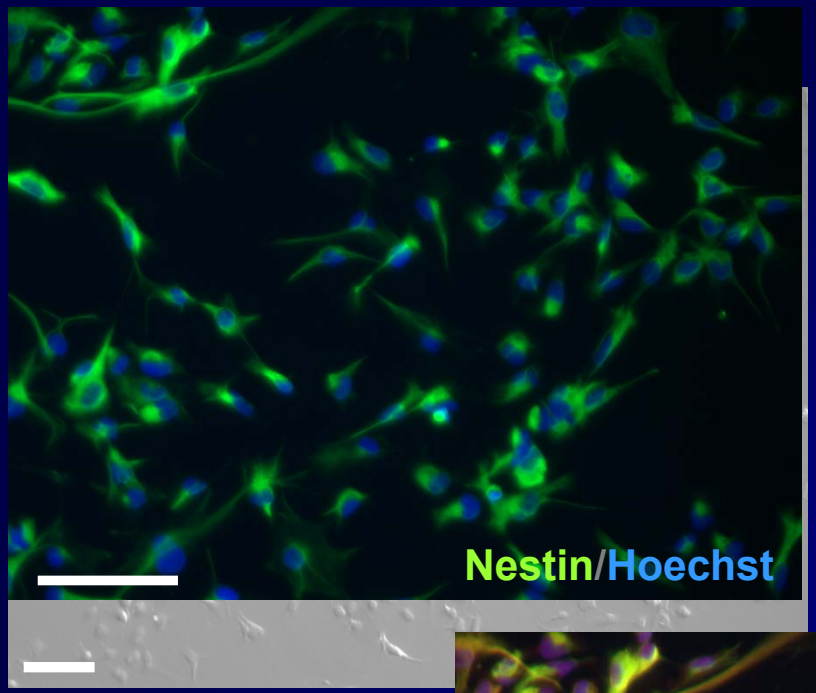
- Central nervous system development is complex
- Research focus on *processes* of development rather than specific targets (*e.g. proliferation, migration, neurite growth, synaptogenesis)*
- Possible cell-based models:
  - Rodent models (primary cell culture, PC12 cells)
  - Human-derived models (primary neural cells, SH-SY5Y)
  - Embryonic stem cells
- Limitations
  - Need for fresh tissue
  - System of interest
  - Phenotypic/genotypic stability over multiple passages
- Goal is to develop *in vitro* models of human origin
  - **Human neural progenitor cells**

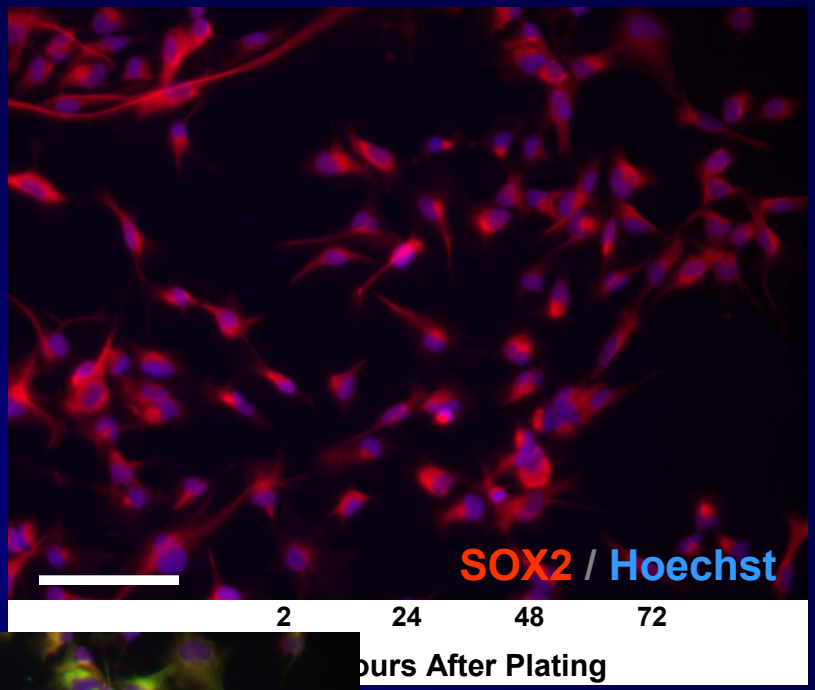
ReNcell CX Cells

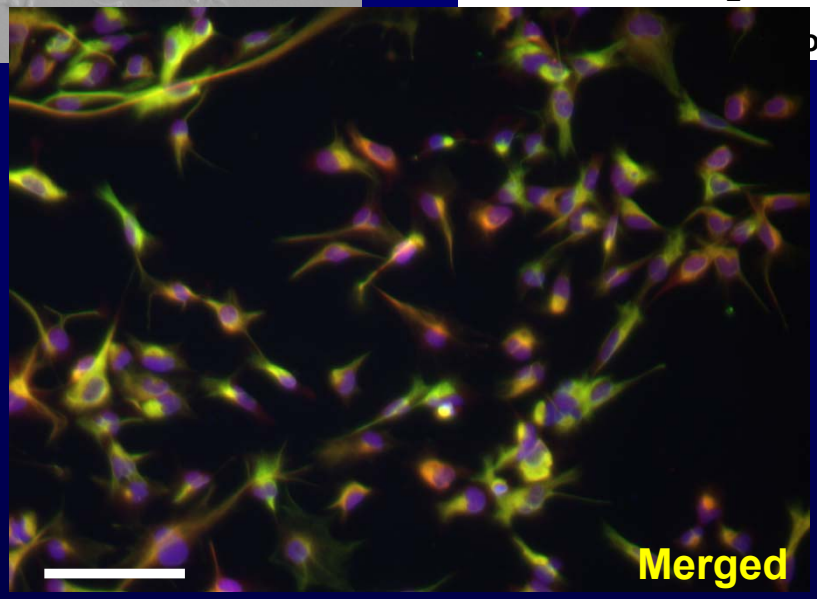
- Immortalized neural progenitor cells derived from a 14-week sample of human cortex
- Express intermediate filament protein nestin
- Proliferate in the presence of growth factors EGF and FGF-2
- Differentiate into neuronal, astrocytic, and oligodendrocytic cell populations with growth factor removal

Donato et al., 2007

ReNcell CX Cells Are Neural Progenitor Cells





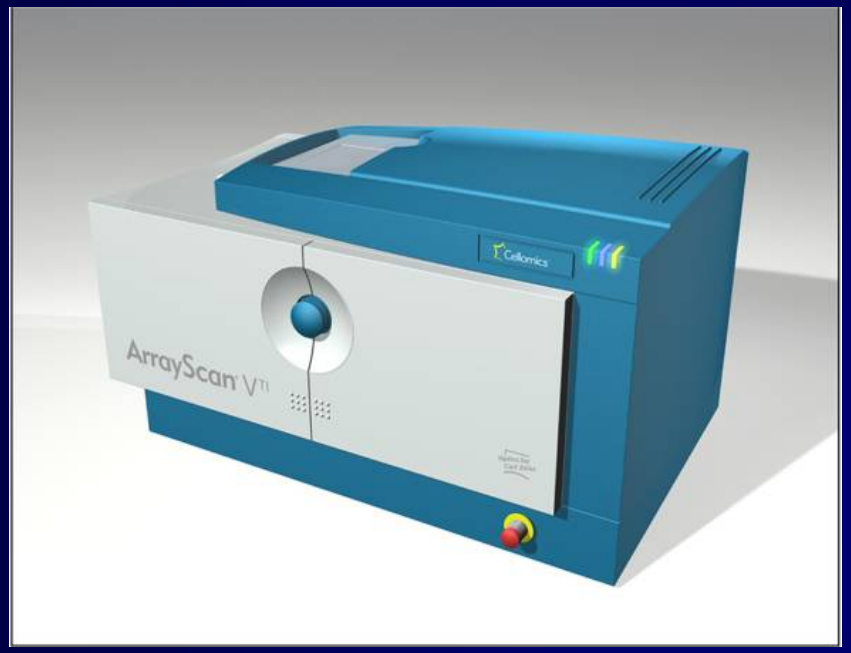


Breier et al., 2008

Cell Proliferation as a Screening Endpoint for Developmental Neurotoxicity

- Cell proliferation is a critical developmental process
- Proliferation is inhibited by chemicals for which evidence of developmental neurotoxicity exists
  - MeHg, Pb, EtOH
- Proliferation has been used as a screening endpoint
- Screening for effects on proliferation
  - BrdU incorporation is one of the most well-established methods
  - Amenable to high-throughput screening
- Cell viability was assessed to evaluate any overt toxicity associated with the chemicals of interest
  - Propidium iodide exclusion

High-Content Microscopy to Assess Cell Proliferation and Viability



Cellomics ArrayScan V^{TI}:

- Fully automated image acquisition and analysis that is time-efficient
- High-content and high-throughput capacity
- Accompanying software (bioapplications) allows automated image analysis and provide data for individual cells

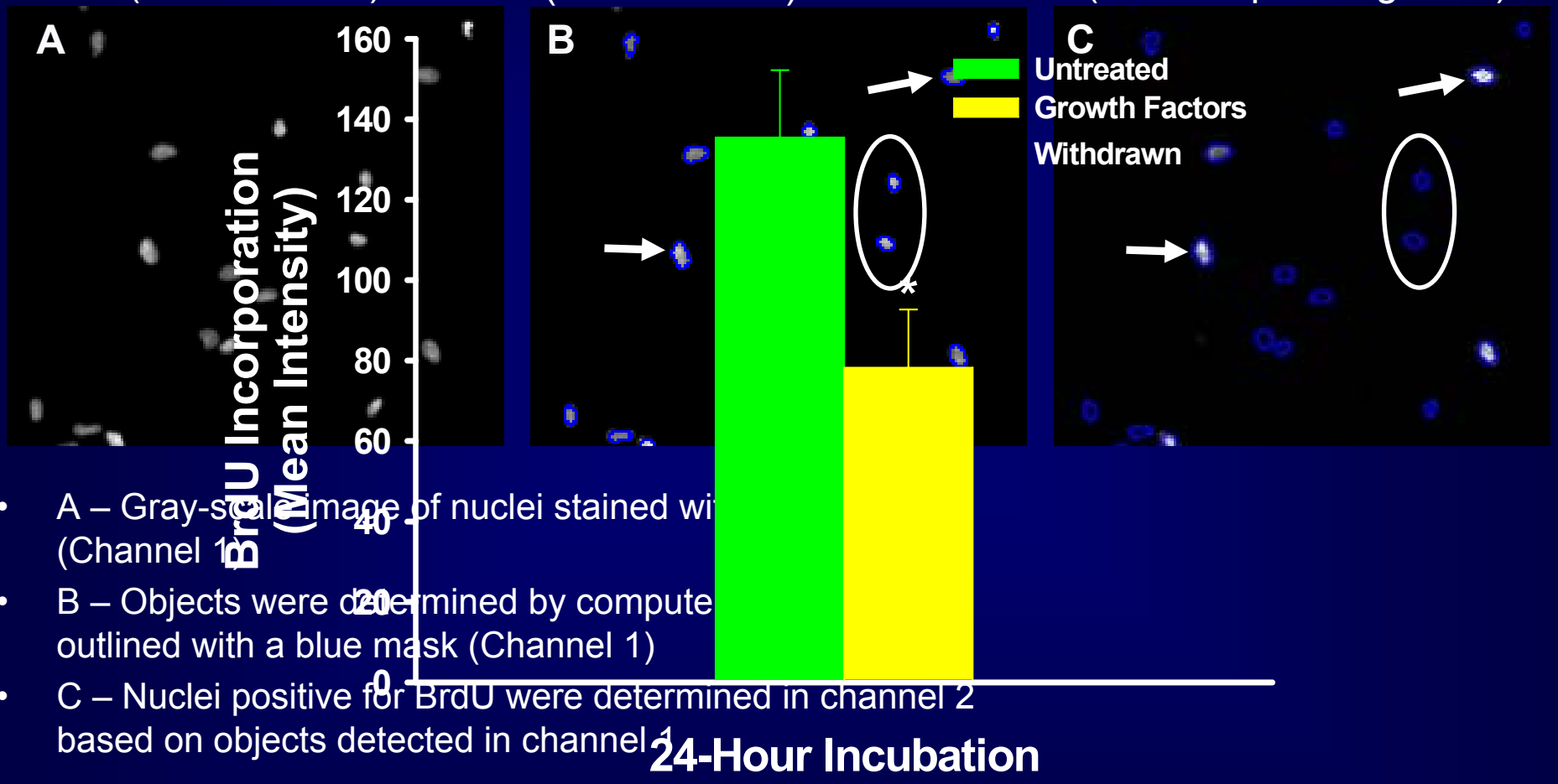
Potential to examine chemical effects on cell proliferation and viability using a 96-well format

Detection of BrdU Incorporation Using a High-Content Screening System

DAPI Dye
(nuclear stain)

DAPI Dye
(nuclear stain) with masks

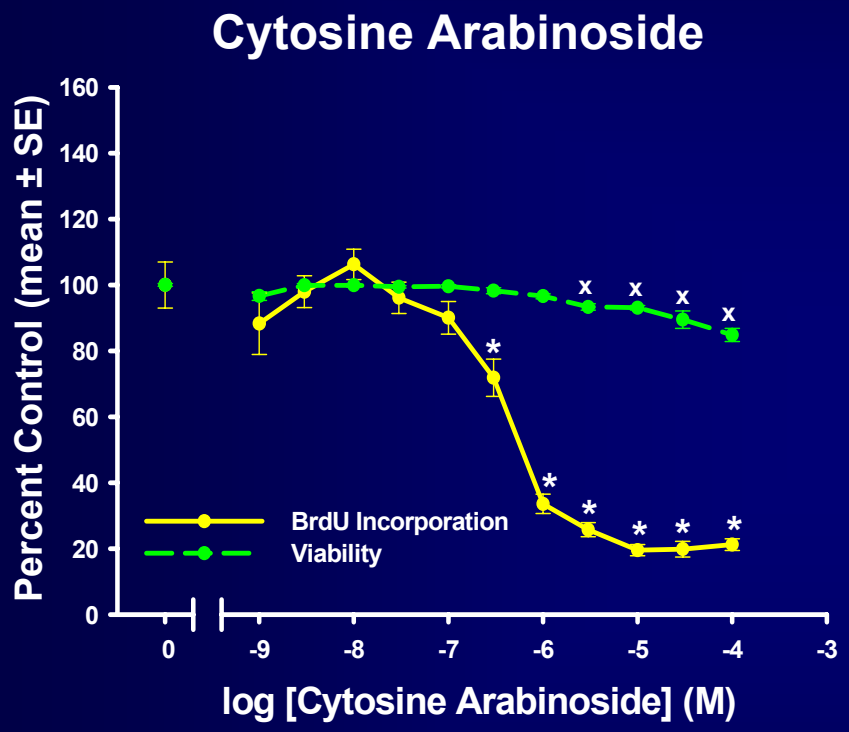
BrdU Incorporation
(stains replicating DNA)

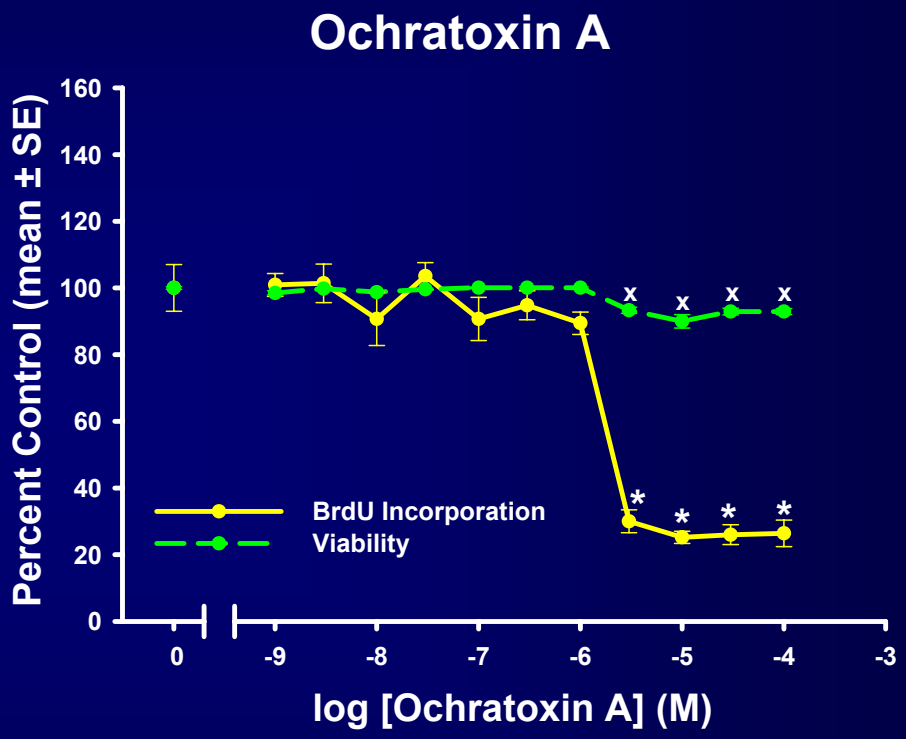


- A – Gray-scale image of nuclei stained wi
  (Channel 1)
- B – Objects were determined by compute
  outlined with a blue mask (Channel 1)
- C – Nuclei positive for BrdU were determined in channel 2
  based on objects detected in channel 1

Propidium iodide staining was evaluated using a similar approach

Known Anti-Proliferative Compounds Inhibit ReNcell CX Cell Proliferation



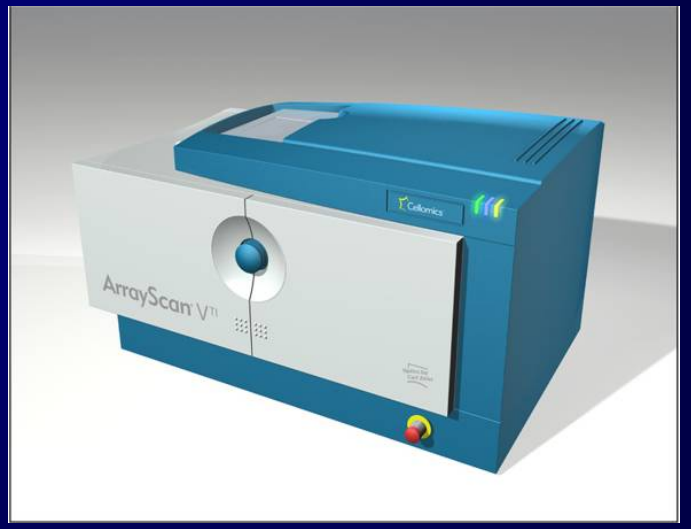


Others tested: Aphidicolin, 5-fluorouracil, hydroxyurea

Protocol for Chemical Screening Using ReNcell CX cells

Cell expansion with EGF and FGF-2

Subcultured at 10,000 cells per well

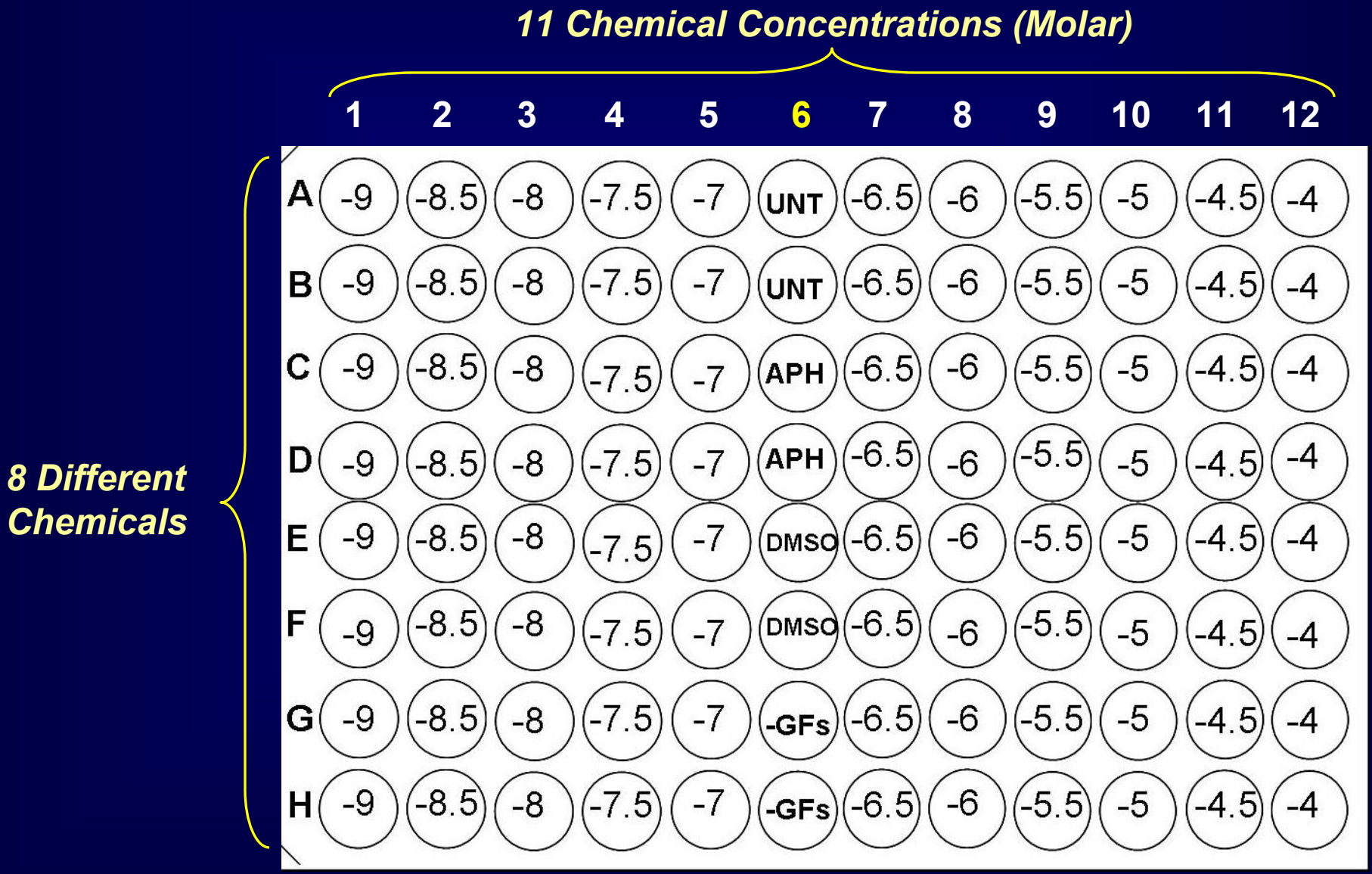


Proliferation (BrdU incorporation) or cell viability (propidium iodide exclusion) were determined 24 hours later

Cells were exposed 16 hours later to chemicals from stock plate in the final concentration range of 1 nM – 100 µM

Chemicals dissolved in DMSO vehicle diluted in growth media

Plate Layout for Chemical Screening Using ReNcell CX Cells



Known Anti-Proliferative Compounds *In Vitro*

Chemical	Proliferation		Viability	
Concentration Range: (*1 nM – 100 µM*)	**Lowest Effective Concentration**	**Percent Inhibition**	**Lowest Effective Concentration**	**Percent Inhibition**
D-Amphetamine Sulfate	.01 µM	20	---	---
Methylmercury (II) chloride	3 µM	75	3 µM	20
Cadmium chloride, hydrate	3 µM	30	30 µM	40
Lead (II) chloride	10 µM	20	---	---
Trans-Retinoic Acid	30 µM	80	30 µM	50
Dexamethasone	100 µM	50	---	---
5,5-Diphenylhydantoin	---	---	---	---
Valproic Acid	---	---	---	---

Non-Neurotoxicants Have Minimal Effect on Cell Proliferation

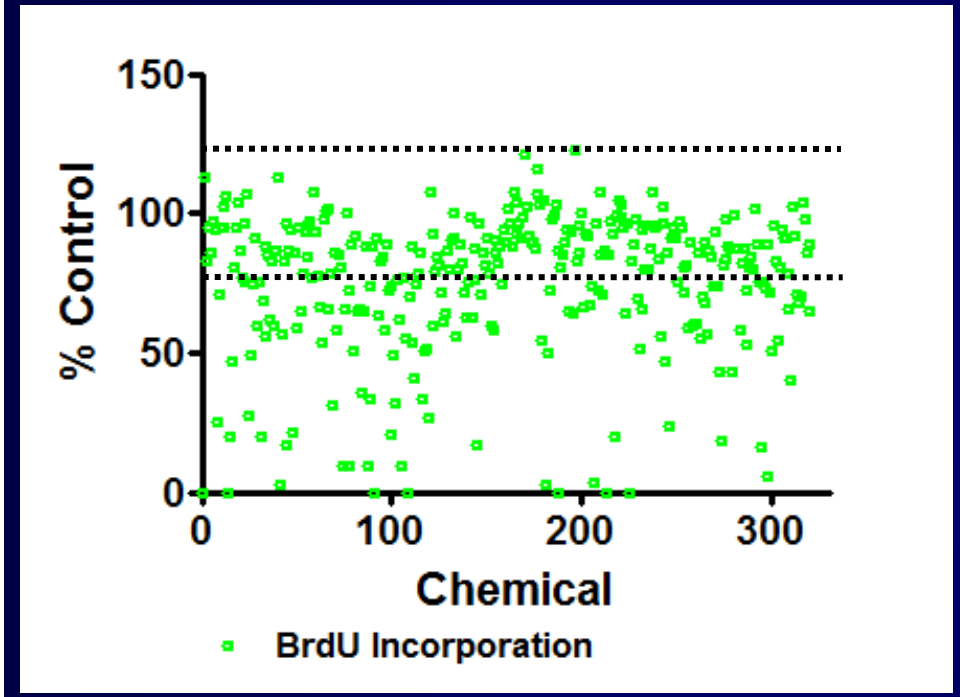
Chemical	Proliferation		Viability	
Concentration Range: (*1 nM – 100 µM*)	Lowest Effective Concentration	Percent Inhibition	Lowest Effective Concentration	Percent Inhibition
Omeprazole	30 µM	30	100 µM	40
Diphenhydramine hydrochloride	---	---	100 µM	7
Amoxicillin	---	---	---	---
Acetaminophen	---	---	---	---
Glyphosate	---	---	---	---
Saccharin sodium salt hydrate	---	---	---	---
D-Sorbitol	---	---	---	---
Dimethyl Phthalate	---	---	---	---

NCCT 320: Screening for Effects on ReNcell CX Cell Proliferation and Viability

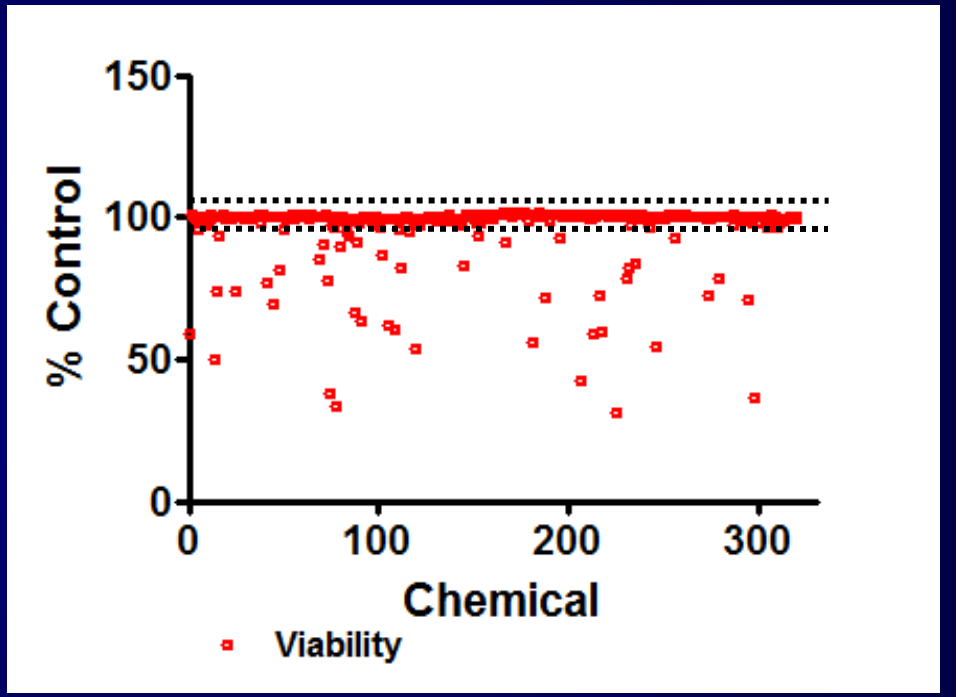
- National Center for Computational Toxicology (NCCT) – launched ToxCast in 2007
- Using methodology described above, 320 chemicals provided by the NCCT were screened for effects on ReNcell CX cell proliferation and viability
- **Initial Screen: ReNcell CX cells exposed to every chemical at highest concentration only (40 µM)**

NCCT 320: Screening for Effects on ReNcell CX Cell Proliferation and Viability

Proliferation



Viability



"Hit" – chemical effects ≥ 3 standard deviations from control

NCCT 320: Hits for Effects on ReNcell CX Cell Proliferation and Viability

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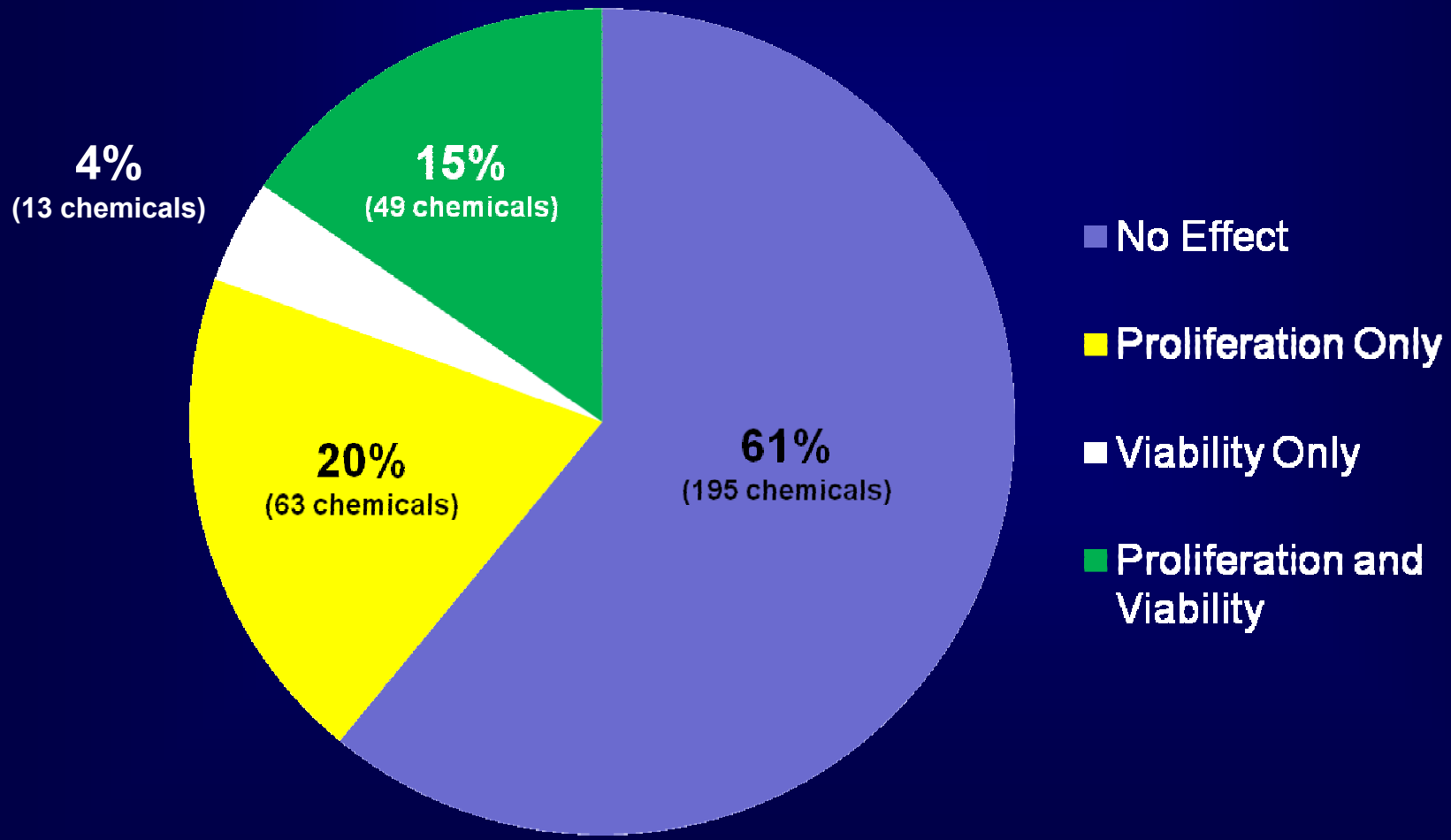
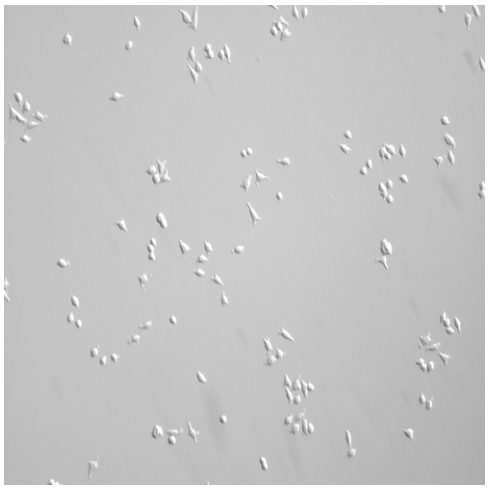
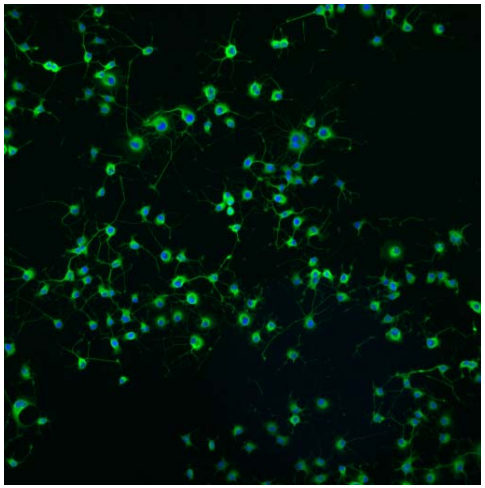


High Content Screening - Neurite Outgrowth

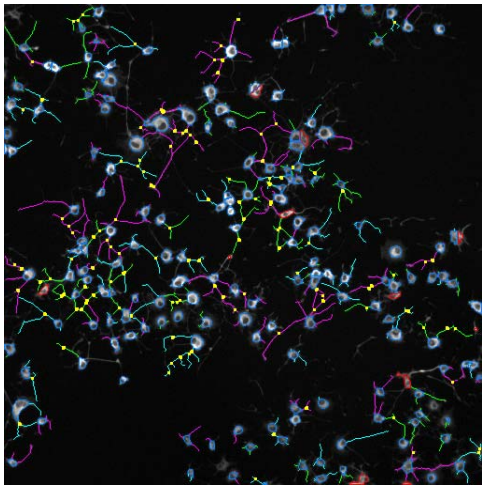
seed, treat, grow **PC12 cells** in 96-well plate (4 days)



stain cells to visualize neurites (4 hrs)



analyze 96-well plate (30 min) using ArrayScan

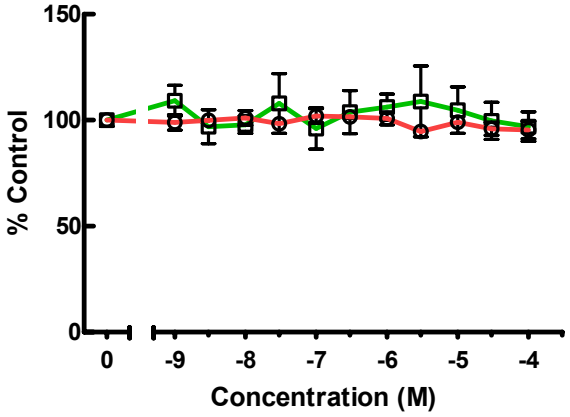


Patterns of Effects - Neurite Growth and Cytotoxicity

96hr exposure

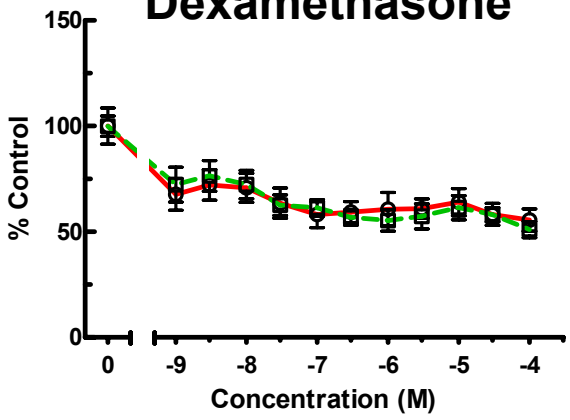
1) No effect

Diphenhydramine



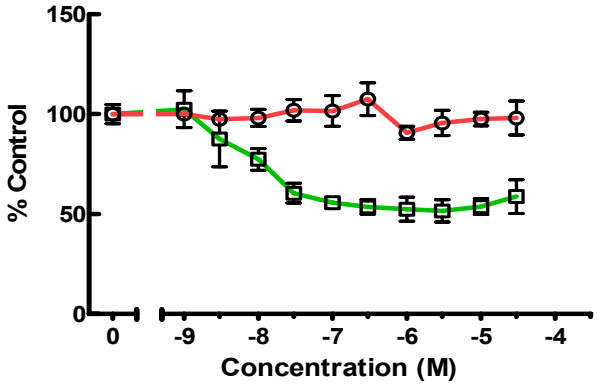
2) Outgrowth inhibition at cytotoxic concentrations

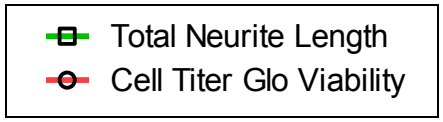
Dexamethasone



3) Outgrowth inhibition at concentrations that are not cytotoxic

trans-Retinoic Acid





Training Set Results

Positive

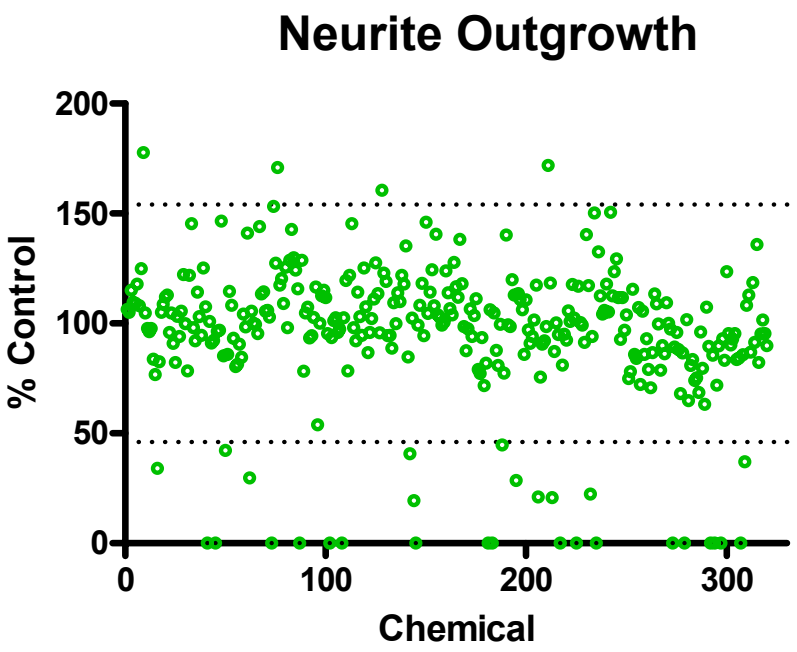
Chemical	Neurite Growth	DNT in vivo
K252a	+	*nd*
U0126	+	*nd*
Okadaic Acid	+	*nd*
Vincristine	+	+
Lead Acetate	+	+
Valproic Acid	+	+
Dexamethasone	+	+
Methylmercury	+	+
Trans-Retinoic Acid	+	+
*Amphetamine	+	+

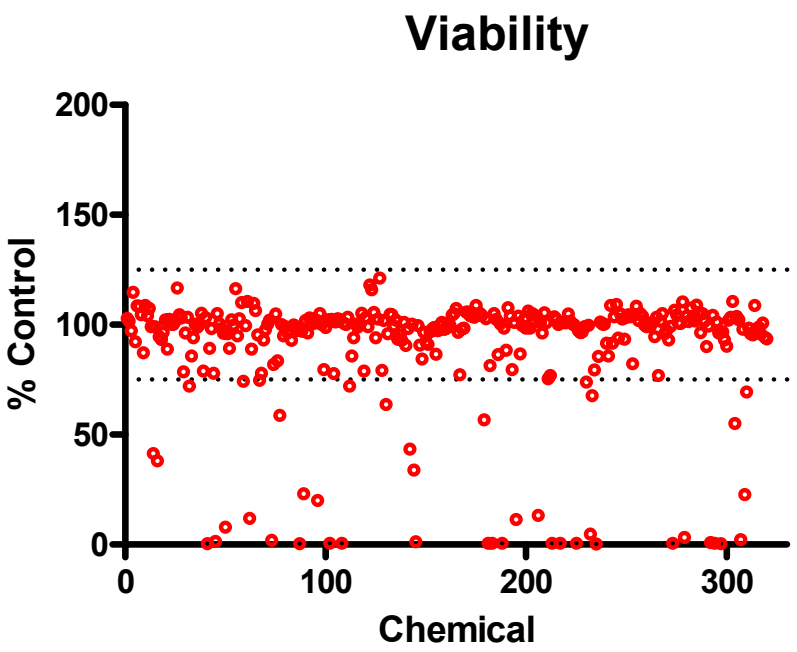
Negative

Chemical	in vitro/in vivo
*Dimethyl phthalate	-
d-Sorbitol	-
Acetaminophen	-
*Omeprazole	-
Amoxicillin	-
Diphenhydramine	-
Saccharin	-
Glyphosate	-

* Increase at highest concentration tested

NCCT 320: Screening for Effects on NS-1 Neurite Outgrowth and Viability





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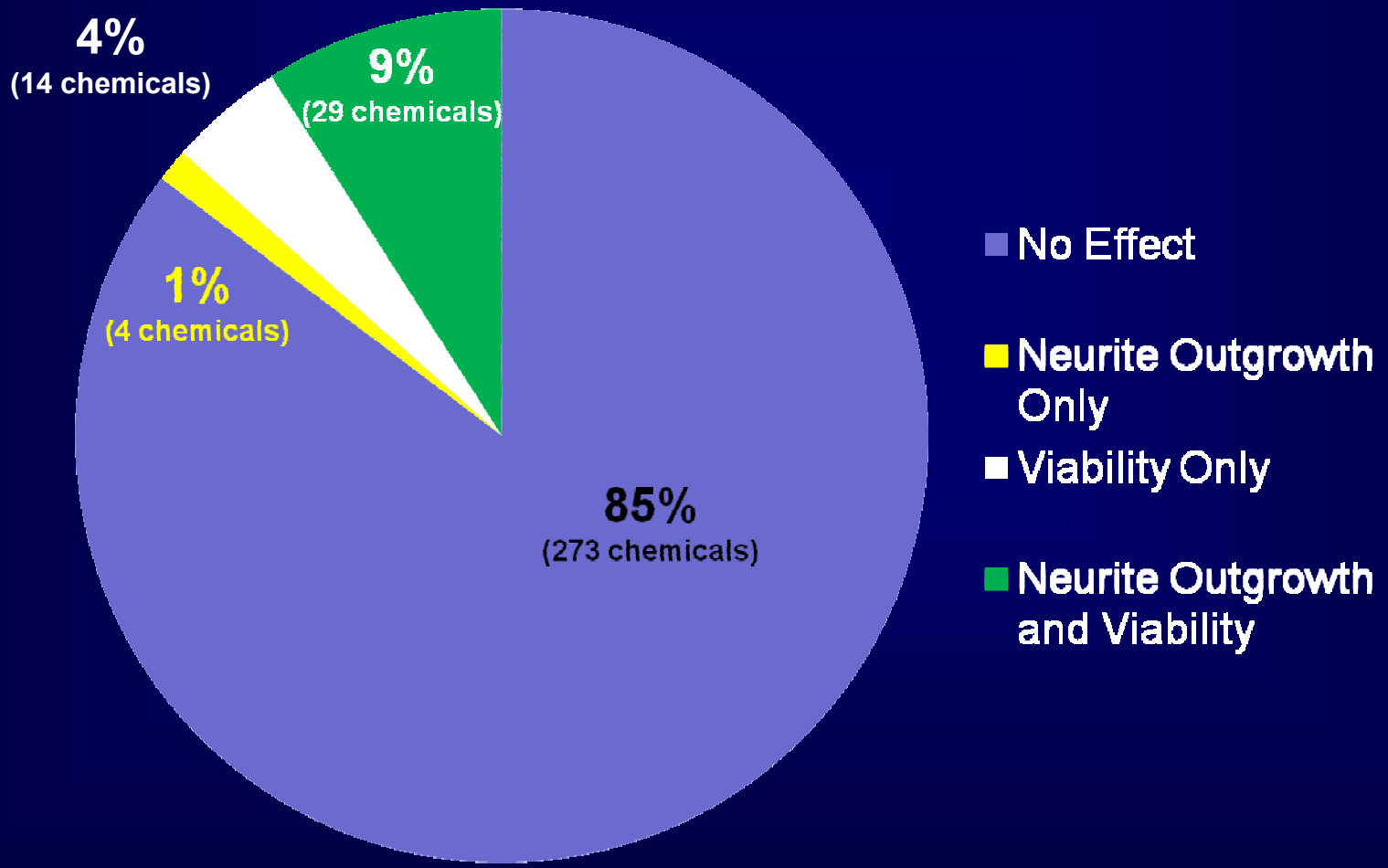
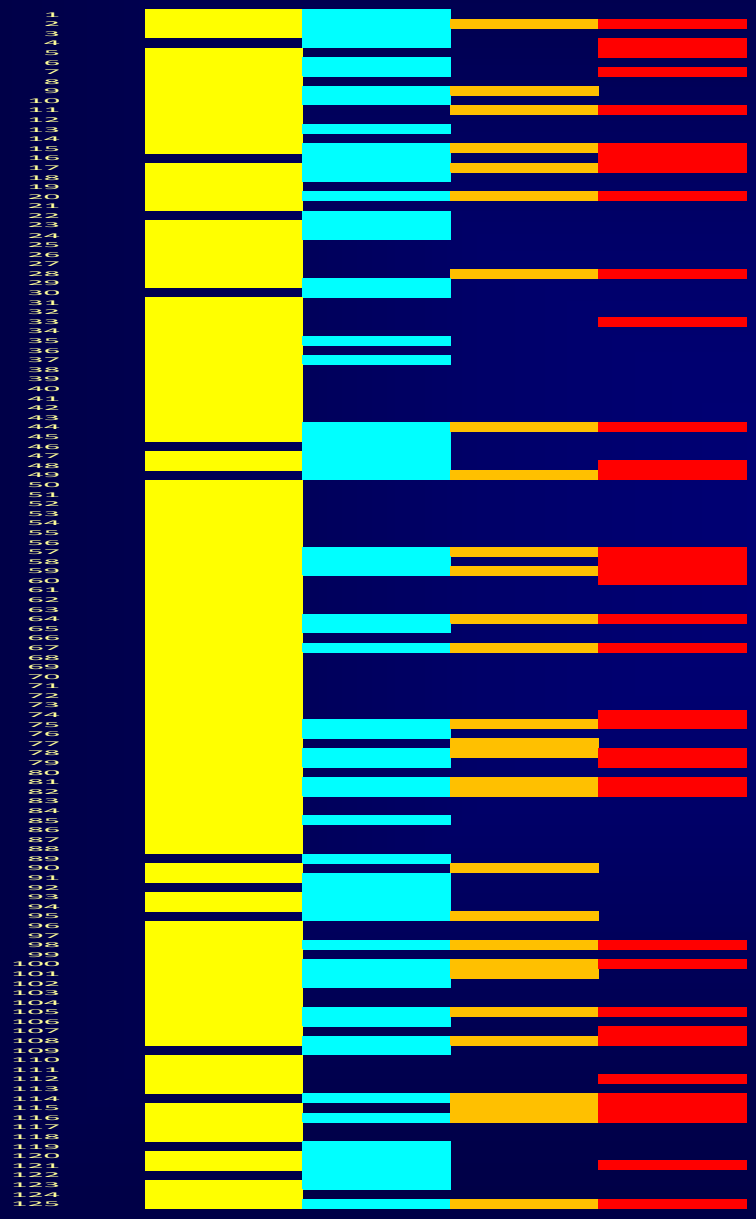


Summary of Screening Effects on Cell Proliferation, Neurite Outgrowth, and Viability



Proliferation Hits (ReNcell CX cells) (112 hits)

Viability Hits (ReNcell CX cells) (63 hits)

Neurite Outgrowth Hits (NS-1 cells) (33 hits)

Viability Hits (NS-1 cells) (43 hits)

- 20 chemicals were hits on all endpoints
- Proliferation most sensitive endpoint
- Neurite outgrowth was not uniquely affected as proliferation was with regard to effects on viability

Summary / Conclusions

- ReNcell CX cells are a useful hNPC model for screening for developmental neurotoxicity
- Screening for chemical effects on cell proliferation, neurite outgrowth and viability can be achieved in a high-throughput format
- Protocols were developed for screening and prioritization of chemicals for further testing that may reduce the demands associated with toxicity testing *in vivo*
- These data will be incorporated into the larger ToxCast dataset and evaluated for their ability to predict *in vivo* toxicities

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