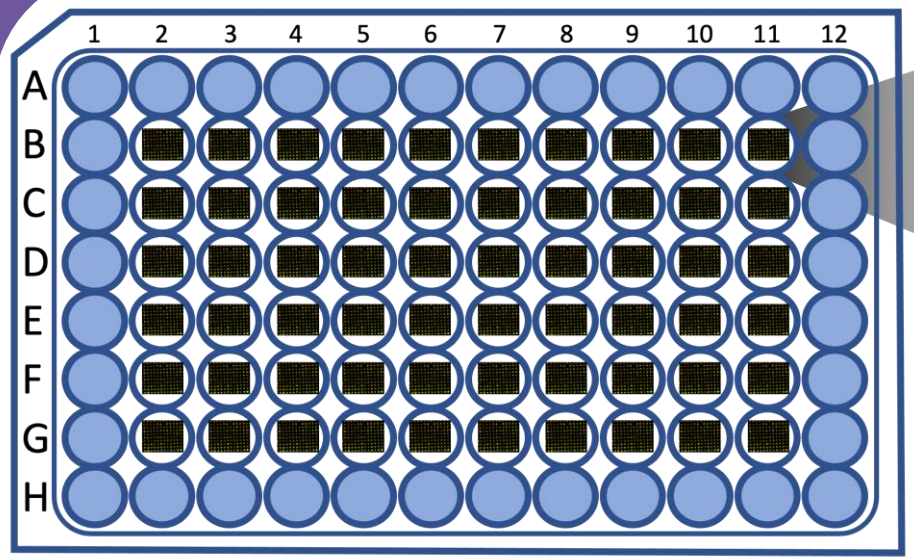
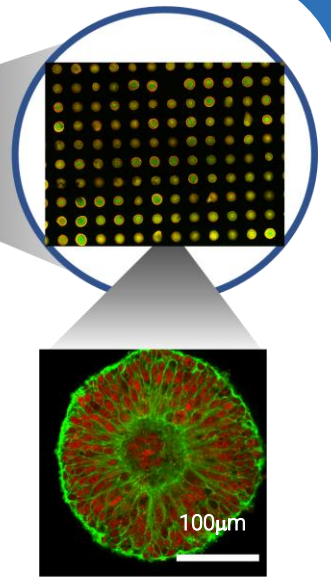
RosetteArray® Platform for Quantitative High-Throughput Screening of Human Neurodevelopmental Risk



Patent #US 11,767,508 B2



> 9,000 per 96-well plate

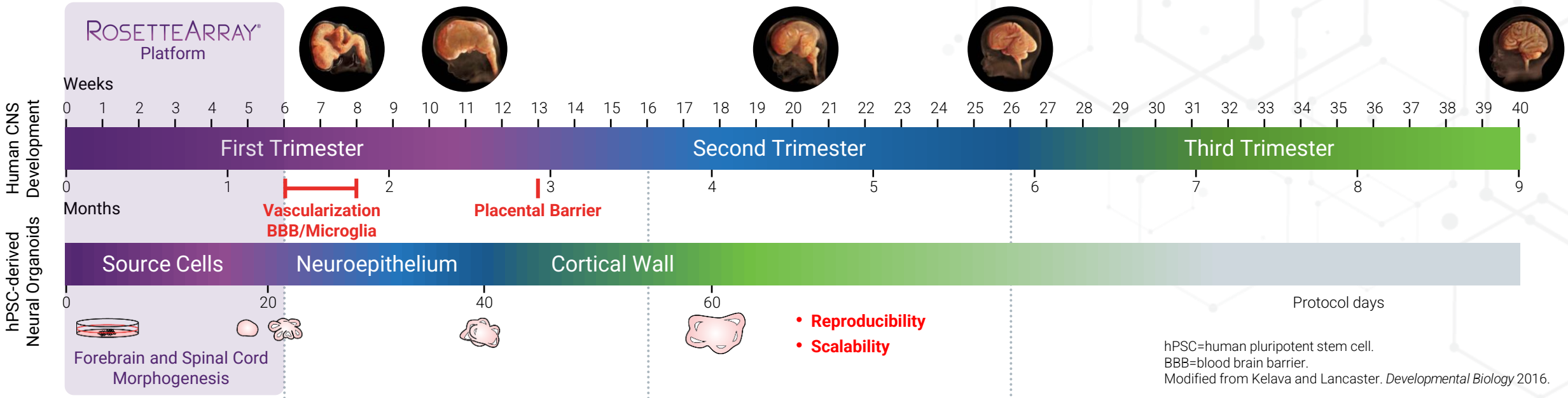
Randolph Ashton, Ph.D.
Co-founder & CEO

Associate Professor
Biomedical Engineering
UW-Madison

NEUROSETTA

330 N. Orchard Street, Rm. 4140A, Madison, WI 53715
(o) 608-316-4312, www.neurosetta.com

RosetteArray Platform: In vitro risks assessment for human brain and spinal cord development



hPSC=human pluripotent stem cell.
BBB=blood brain barrier.
Modified from Kelava and Lancaster. *Developmental Biology* 2016.

Initial pathogenesis of Neural Tube Defects, Autism Spectrum Disorder, Huntington's Disease, . . .

Developmental Neurotoxicity-In Vitro Battery

(OECD Series on Testing and Assessment No. 377)

- UKN2 (neural crest cell migration/viability; forebrain)
- UKN5 (immature peripheral neuron viability and morphology; dorsal root ganglia)
- hN Initiation (human glutamatergic neuron outgrowth; cortex)
- UKN4 (neuronal differentiation and viability of dopaminergic progenitors; midbrain)
- hNP1 (human NPC Proliferation/apoptosis/viability; cortex; **source line no longer available**)
- NPC1 (NPC proliferation; cortex)
- NPC2a,b,c, NPC3,NPC4, NPC5 (RGC, neuron, oligodendrocyte migration; cortex)
- Rat cortical initiation/neuron outgrowth
- Rat cortical neuron maturation/synaptogenesis
- Rat cortical neuron MEA

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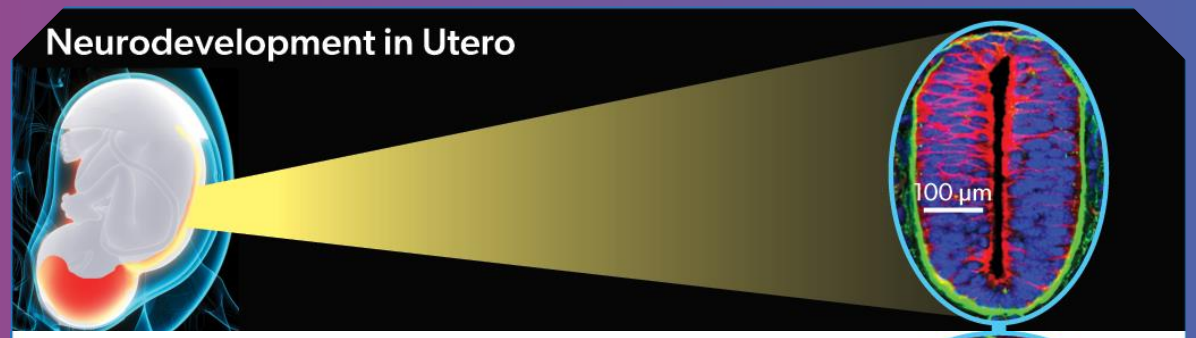
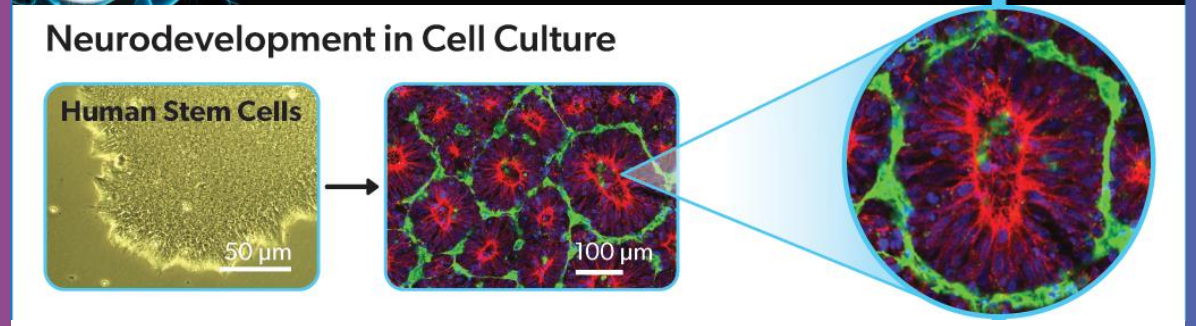
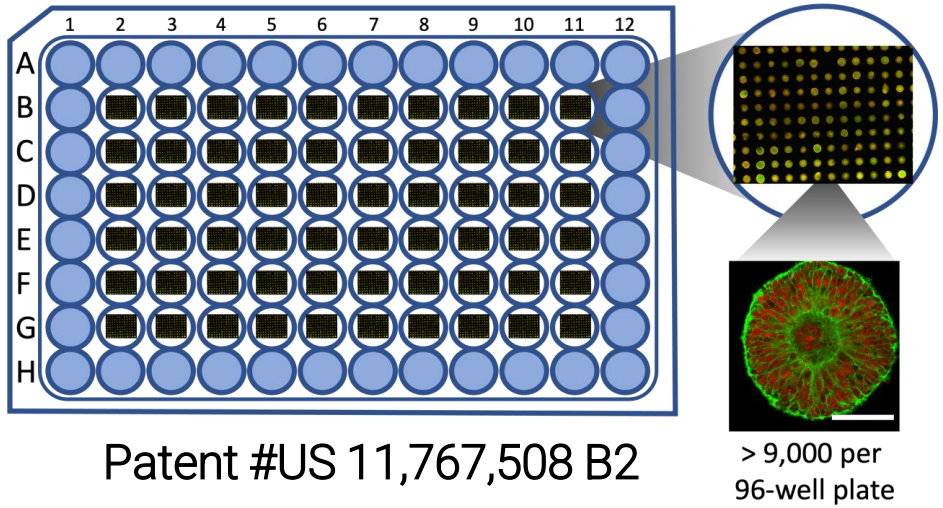
Neurosetta's Solution: Use human pluripotent stem cells (hPSCs) to bioengineer human brain and spinal cord development in-a-dish





- In culture, random neural organoid structure
- Protracted derivation/analysis (> 6 months)
- Prohibitive cost and time at scale

RosetteArray® Technology

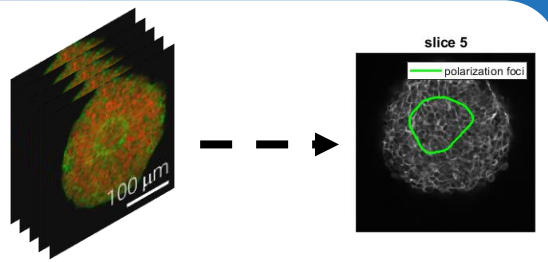


Patent #US 11,767,508 B2

> 9,000 per 96-well plate

- Rapid, reproducible, scalable derivation

RosetteDetect™ (Artificial Intelligence Image Analysis Software)



- Derivation/quantitative analysis (~2.5 weeks)

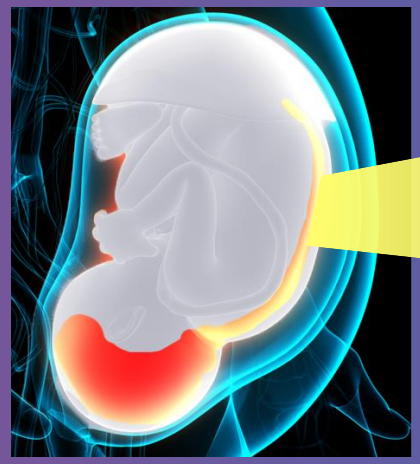
- Knight GT, Lundin BF, Iyer N, Ashton LMT, Sethares WA, Willett RM, Ashton RS. ***eLife*** (2018)
- Lundin B.F. et al. RosetteArray Platform for Quantitative High-Throughput Screening of Human Neurodevelopmental Risk. bioRxiv[Preprint].2024 Apr https://doi.org/10.1101/2024.04.01.587605.

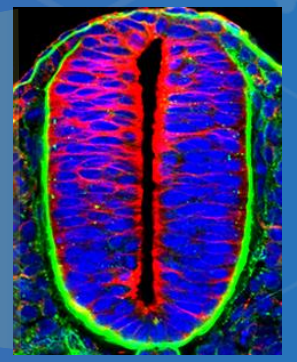
NEUROSETTA

Neurosetta's Solution: Use human pluripotent stem cells (hPSCs) to bioengineer human brain and spinal cord development in-a-dish

In Vivo

Neural Tube (~GW6)

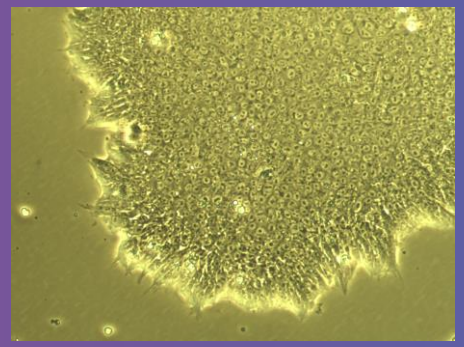




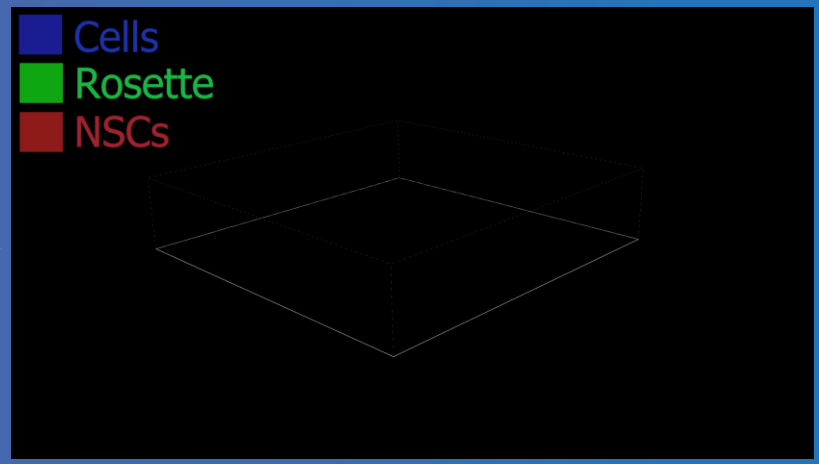
(By Gwenval LeDreau et al.-
Instituto de Biología Molecular de Barcelona-CSIC)

In Vitro

Human Pluripotent Stem Cells (hPSCs)



Micropatterned Substrate



Knight GT, Lundin BF, Iyer N, Ashton LMT, Sethares WA, Willett RM, Ashton RS. ***eLife*** (2018)

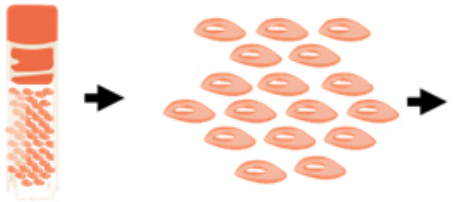
Patent #US 11,767,508 B2

NEUROSETTA

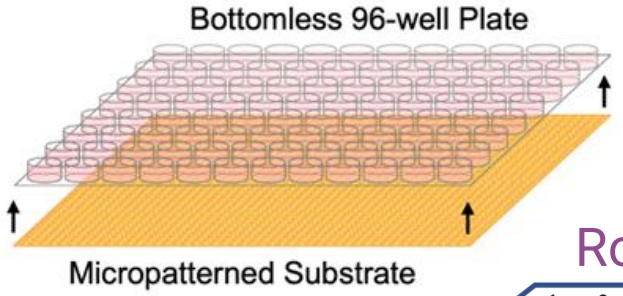
RosetteArray® Platform for qHTS of human DNT

Human w/wo Disorder (Genetic Factors)

Human Pluripotent Stem Cells (hPSCs)



Seed into RosetteArray Plate (96-well)

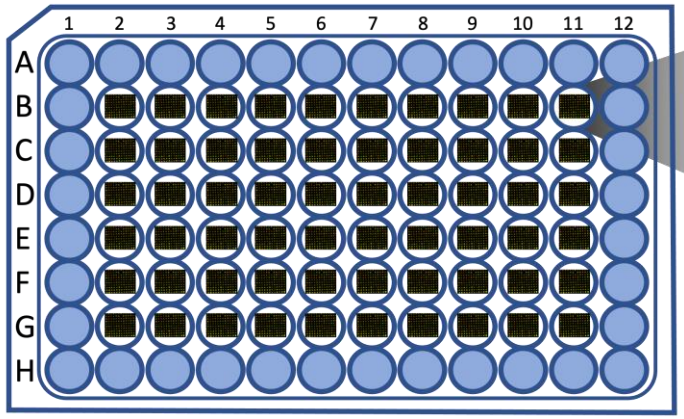


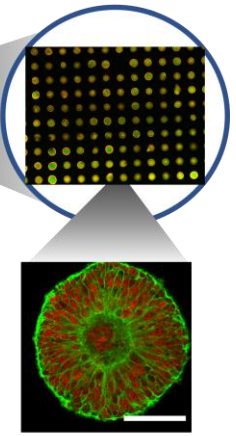
Cell Culture w/wo Chemicals or Drugs (~1 week)

Transformative Tool for :

- **Developmental neurotoxicity (DNT)** testing for chemical and drug safety
- **Precision Medicine/Drug Discovery**
  - Neural Tube Defects/Spina Bifida
  - Hydrocephalus
  - Autism Spectrum Disorder
  - Rett & Fragile-X Syndrome
  - Epilepsy
  - Frontal Temporal Dementia/ALS
  - Huntington's Disease
  - . . .

RosetteArray® Assay





x 3000 per 96-well plate

Automated Microscope Imaging (~6 hrs per plate)

(~2.5 hrs per plate)

RosetteDetect™ Software

✓ **Unprecedented, quantitative, high-throughput screening of neural organoid culture**

Lundin B.F. et al. RosetteArray Platform for Quantitative High-Throughput Screening of Human Neurodevelopmental Risk. bioRxiv[Preprint].2024 Apr https://doi.org/10.1101/2024.04.01.587605.

NEUROSETTA

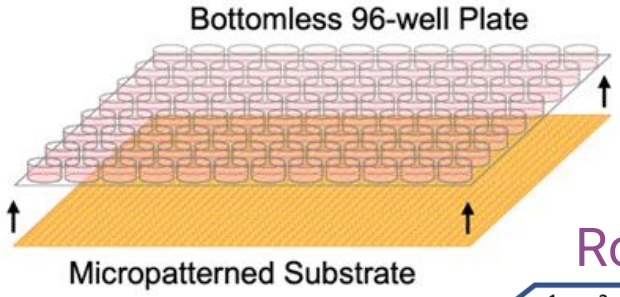
RosetteArray® Platform for qHTS of human DNT

Human w/wo Disorder (Genetic Factors)

-OR-

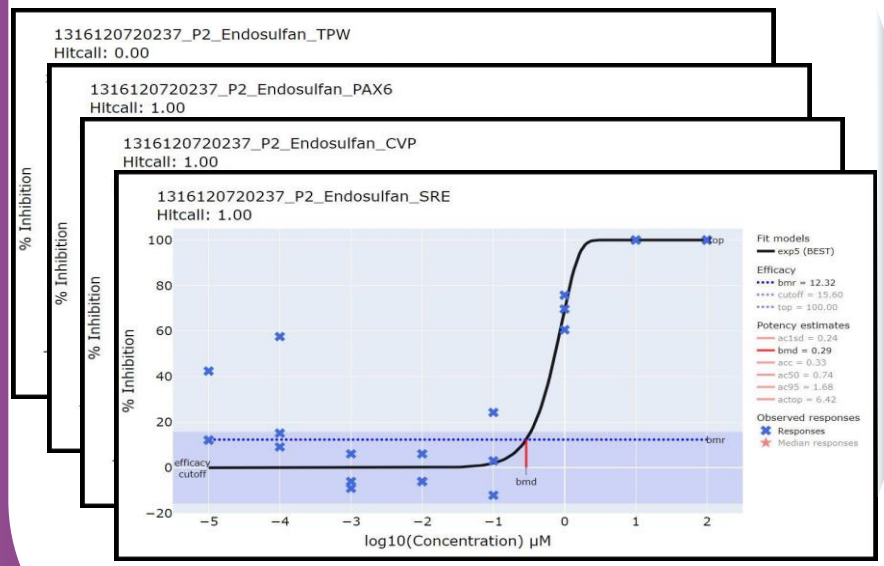
Human Pluripotent Stem Cells (hPSCs)

Seed into RosetteArray Plate (96-well)



Cell Culture w/wo Chemicals or Drugs (~1 week)

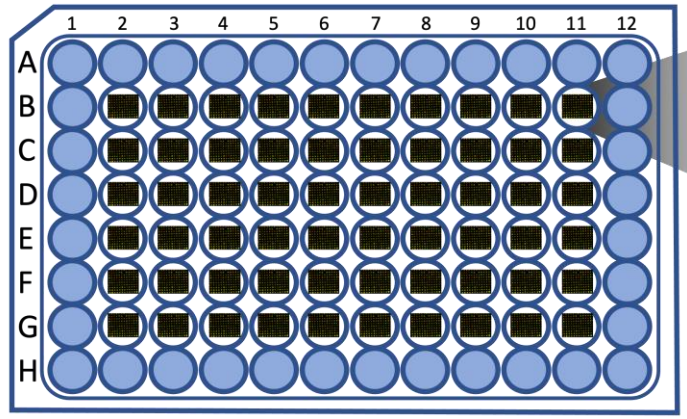
Results provided in tcplfit2 format:



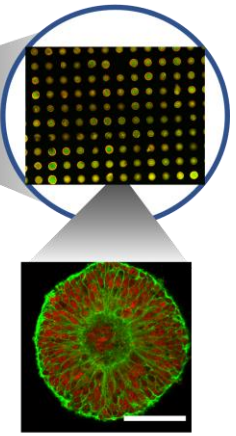
(~2.5 hrs per plate)

RosetteDetect™ Software

RosetteArray® Assay



Automated Microscope Imaging (~6 hrs per plate)



x 3000 per 96-well plate

✓ **Unprecedented, quantitative, high-throughput screening of neural organoid culture**

Lundin B.F. et al. RosetteArray Platform for Quantitative High-Throughput Screening of Human Neurodevelopmental Risk. bioRxiv[Preprint].2024 Apr https://doi.org/10.1101/2024.04.01.587605.

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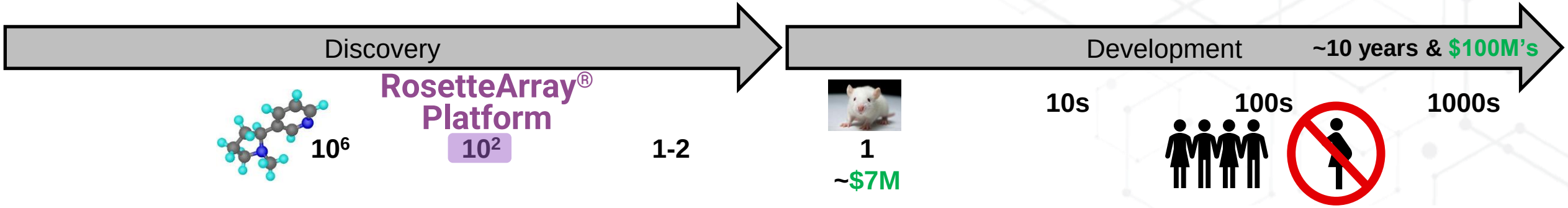
Value Proposition: RosetteArray screens can provide a quantitative rank-ordered predication of HUMAN DNT risk



1-2 month turnaround
(20 or less compounds)

"10% improvement in predicting failure before the initiation of expensive and time-consuming clinical trials could save upwards of $100 million in the cost associated with drug development"

Allen, Jeffrey, Williams & Ratcliffe *Drugs of the Future* 2010

↓ Risk ↓ Time ↓ Cost

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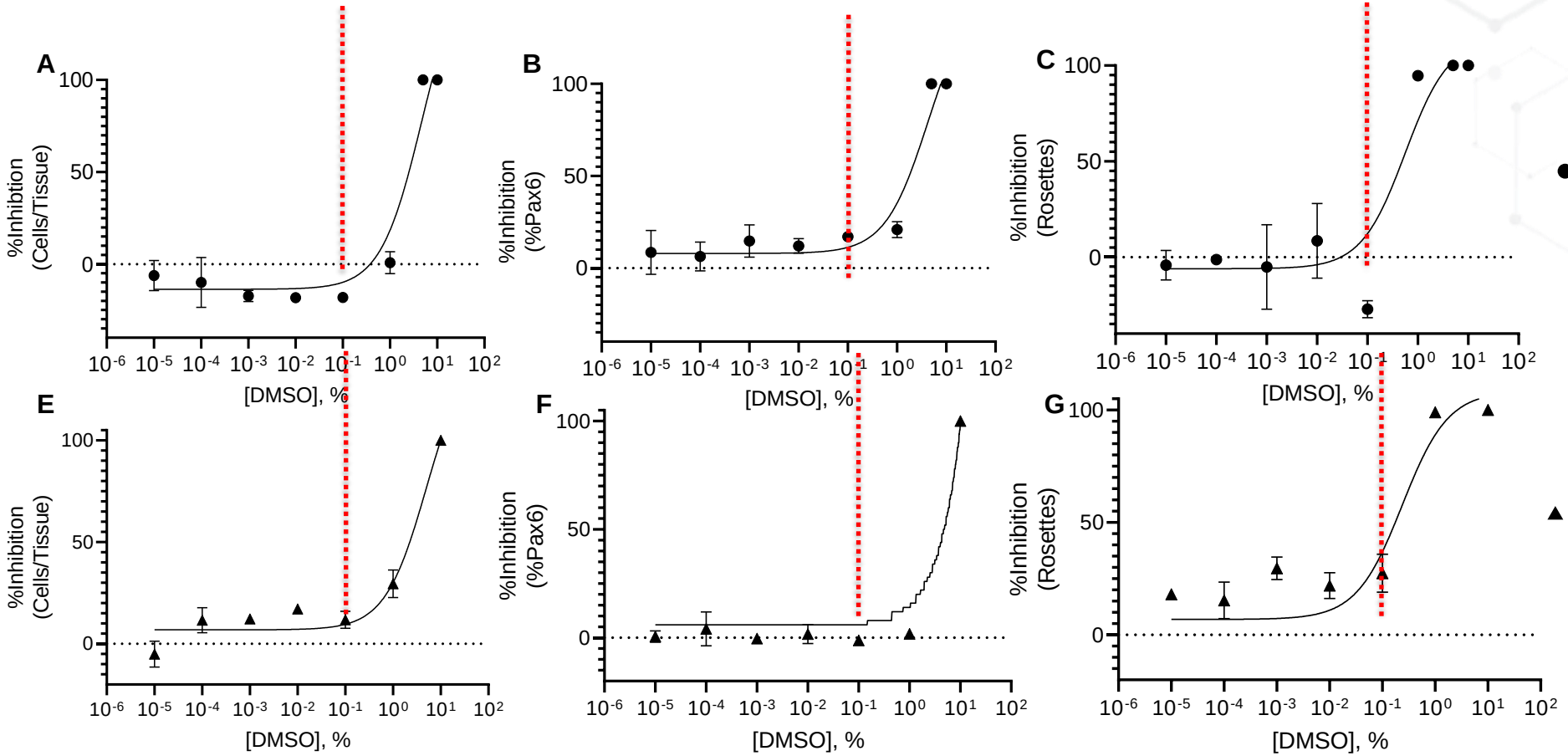
Fundamental Question:

How good is the RosetteArray assay at predicting **HUMAN** DNT hazard?

NEUROSETTA

RosetteArray Platform can screen compounds solvated in DMSO.

- Works with DMSO solvent up to 0.1% (i.e., 1:1000 dilution)



FB

	DMSO		
	IC_{50}	95% Confidence Interval	R^2
Cells/Tissue	4.87%	2.50-10.06%	0.96
%Pax6	4.21%	2.25-8.84%	0.93
Rosettes	0.56%	0.27-1.19%	0.86

SC

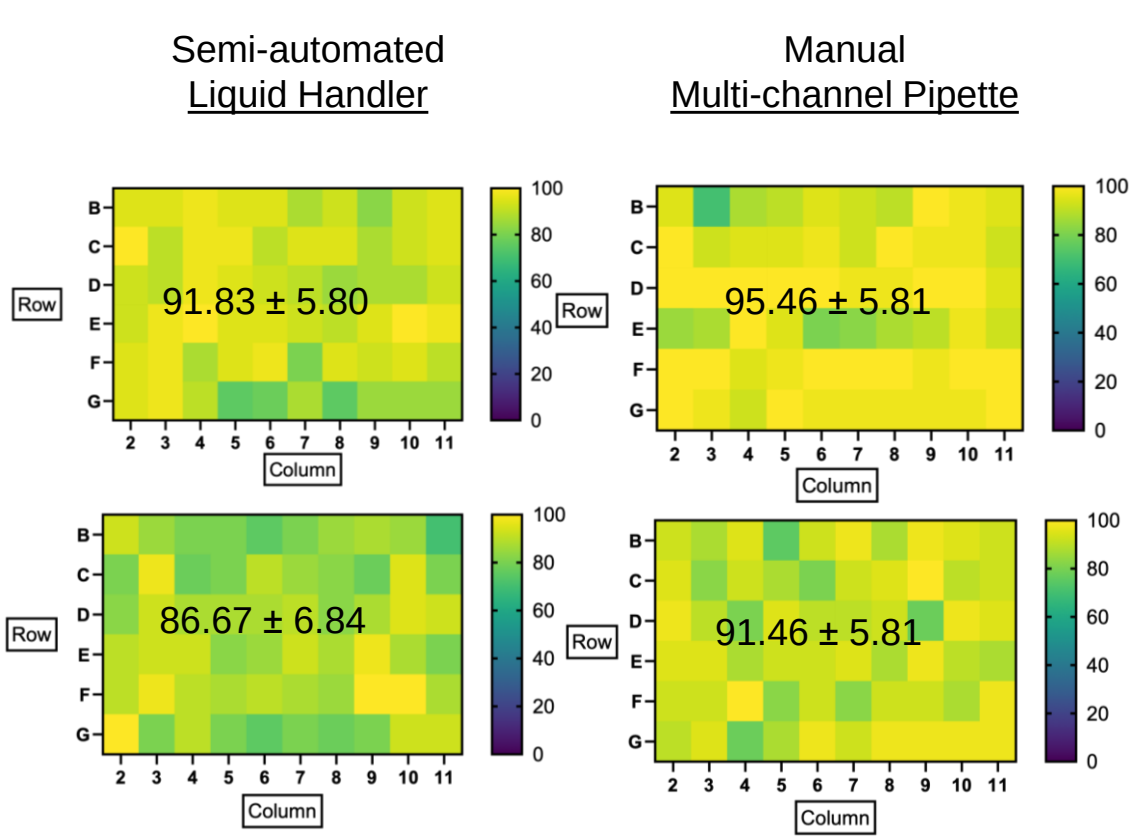
	DMSO		
	IC_{50}	95% Confidence Interval	R^2
Cells/Tissue	5.05%	5.50--%	0.94
%Pax6	--	--	0.95
Rosettes	0.24%	.015-0.35%	0.86

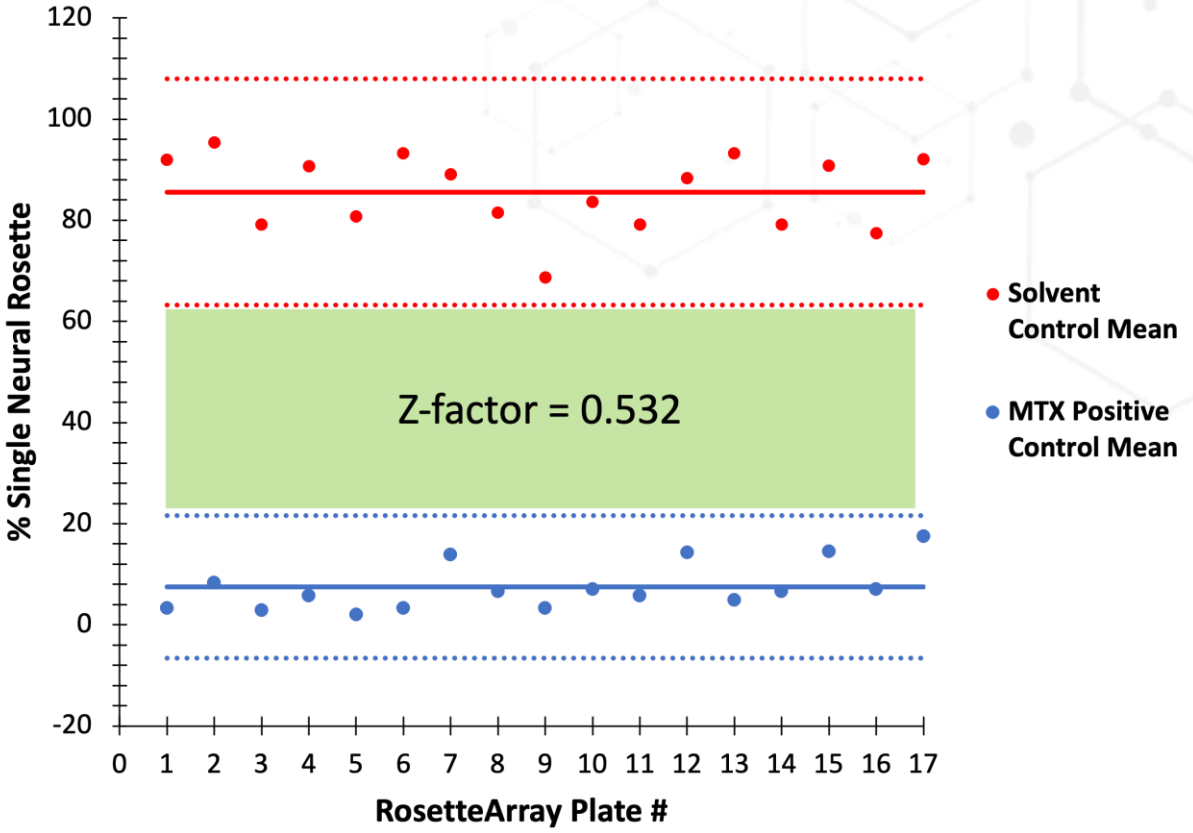
NEUROSETTA

Lundin B.F. et al. bioRxiv[Preprint].2024 Apr https://doi.org/10.1101/2024.04.01.587605

RosetteArray Platform is reproducible.

- Good reproducibility (Forebrain: 86.3 ± 9.06%; Lumbosacral Spinal: 93.8 ± 4.1%)

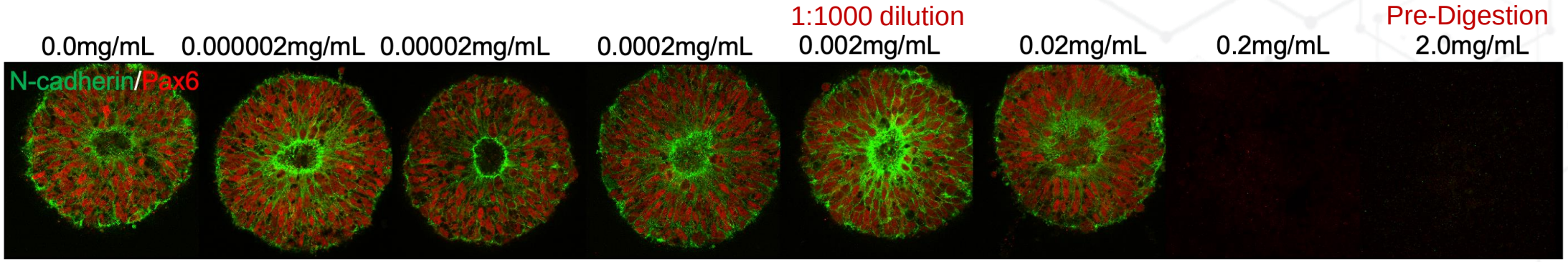


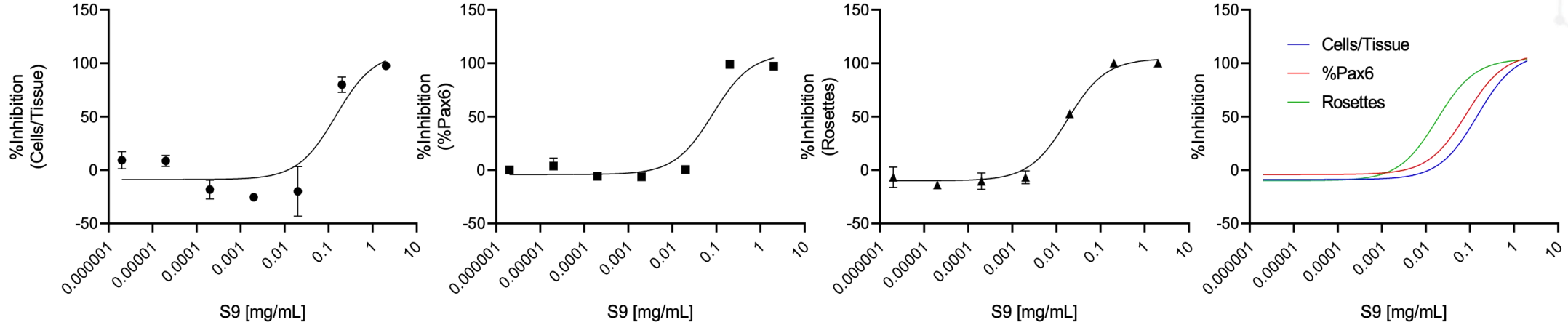


Lundin B.F. et al. bioRxiv[Preprint].2024 Apr https://doi.org/10.1101/2024.04.01.587605

RosetteArray Platform can integrate simulated human metabolism.

- Pre-digestion of compound with S9-pooled liver fraction





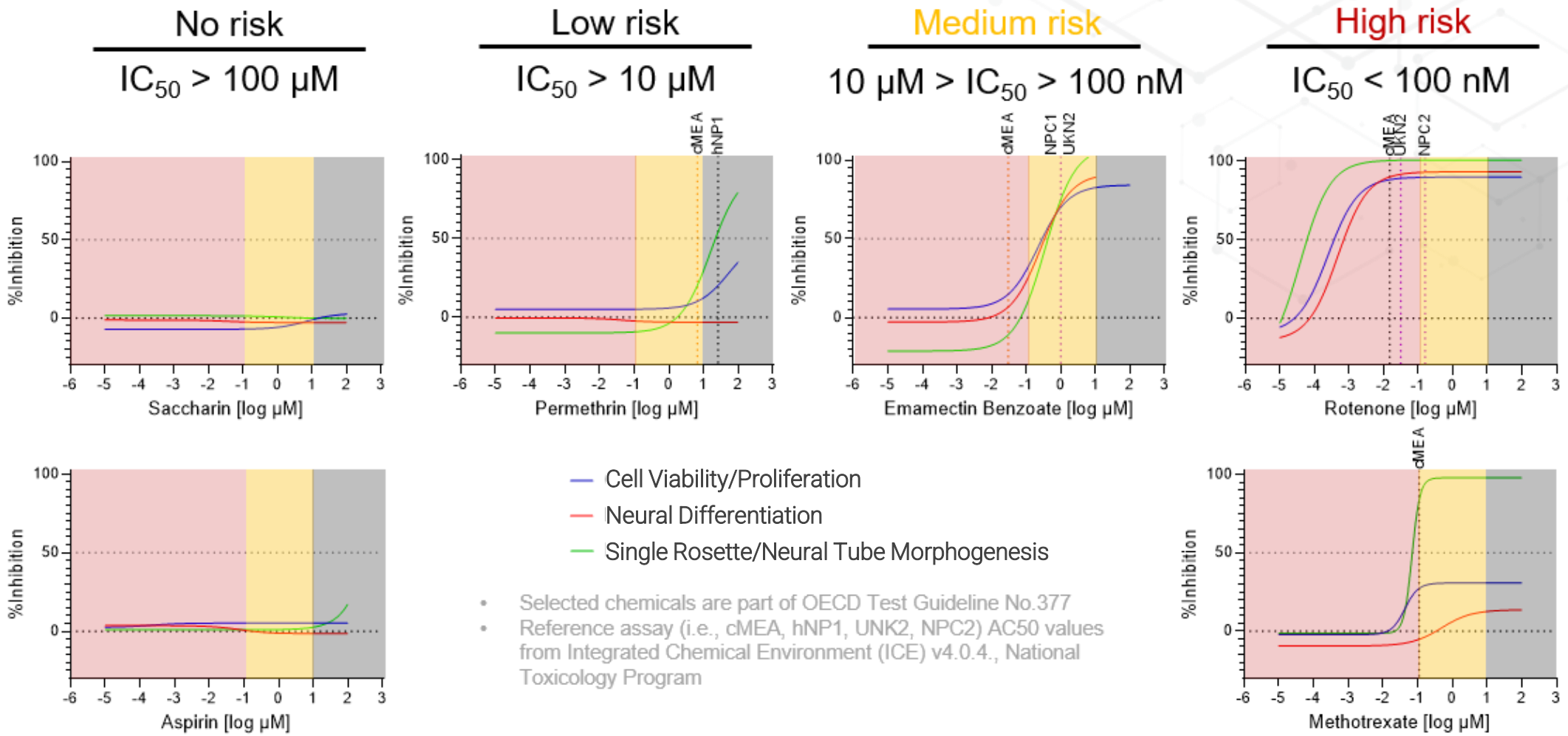
p=0.0002, F=5.244

	S9 Fraction		
	IC_{50}	95% Confidence Interval	R^2
Cells/Tissue	0.146 mg/mL	0.00603-0.391 mg/mL	0.82
%Pax6	0.0828 mg/mL	0.0482-0.144 mg/mL	0.93
Rosettes	0.0178 mg/ml	0.0127-0.0249 mg/mL	0.98

NEUROSETTA

Lundin B.F. et al. bioRxiv[Preprint].2024 Apr https://doi.org/10.1101/2024.04.01.587605

Forebrain RosetteArray: Sample DNT Data



- Selected chemicals are part of OECD Test Guideline No.377
- Reference assay (i.e., cMEA, hNP1, UNK2, NPC2) AC50 values from Integrated Chemical Environment (ICE) v4.0.4., National Toxicology Program

Lundin B.F. et al. RosetteArray Platform for Quantitative High-Throughput Screening of Human Neurodevelopmental Risk. bioRxiv[Preprint].2024 Apr https://doi.org/10.1101/2024.04.01.587605.

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Forebrain RosetteArray Assay: Ongoing ToxCast Library DNT Screen

- 8-point dose response (10pM – 100μM)
- No simulated human metabolism
- Blinded ToxCast ph1/2 screen with the EPA (OECD 377 overlap); *- compounds provided by Neurosetta.

#	Compound	CAS	Reference	RosetteArray
1	Acetylsalicylic acid*	50-78-2	Negative	Negative
2	Caffine*	58-08-2	Positive	Negative
3	Emamectin Benzoate*	155569-91-8	Positive	Positive
4	Permethrin*	52645-53-1	Positive	Positive
5	Rotenone*	83-79-4		Positive
6	Methotrexate*	58197	Positive	Positive
7	Saccharin*	82385-42-0	Negative	Negative
8	Rotenone	83-79-4		Positive
9	Flufenacet	142459-58-3	Positive	Positive
10	Azinphos-methyl	86-50-0		Positive
11	Cyfluthrin	68359-37-5		Positive
12	Tebupirimfos	96182-53-5		Positive
13	Acibenzolar-S-methyl	135158-54-2	Positive	Positive
14	Retinoic acid	302-79-4	Positive	Positive
15	S-Bioallethrin	28434-00-6		Positive
16	Dichlorvos	62-73-7	Positive	Positive
17	Benomyl	17804-35-2	Positive	Positive
18	Acephate	30560-19-1	Positive	Positive
19	Trichlorfon	52-68-6	Positive	Positive
20	Malathion	121-75-5	Positive	Positive
21	3-Iodo-2-propynyl-N-butylcarbamate	55406-53-6		Positive
22	1,1-Dimethylpiperidinium chloride (mepiquat chloride)	24307-26-4		Positive
23	Thiamethoxam	153719-23-4	Positive	Positive
24	Acetaminophen	103-90-2	Negative	Negative

#	Compound	CAS	Reference	RosetteArray
25	Methamidophos	10265-92-6	Positive	Positive
26	Mifepristone	84371-65-3		Positive
27	Thiacloprid	111988-49-9	Positive	Positive
28	Malaoxon	1634-78-2		Positive
29	Permethrin	52645-53-1	Positive	Negative
30	Diazinon	333-41-5	Positive	Positive
31	Fenamidone	161326-34-7		Positive
32	Endosulfan	959-98-8	Positive	Positive
33	Carbaryl	63-25-2	Positive	Positive
34	Clothianidin	210880-92-5	Positive	Positive
35	Bensulide	741-58-2		Positive
36	Dimethoate	60-51-5	Positive	Positive
37	Imidacloprid	138261-41-3	Positive	Negative
38	Di(2-ethylhexyl) phthalate	117-81-7		Negative
39	Cyclophosphamide monohydrate	6055-19-2	Positive	Positive
40	Profenofos	41198-08-07	Positive	Positive
41	Boscalid	188425-85-6	Positive	Positive
42	Tri-allate	2303-17-5	Positive	Positive
43	Acetamiprid	135410-20-7	Positive	Positive
44	Pirimiphos-methyl	29232-93-7		Positive
45	Aldicarb	0116-06-03	Positive	Negative
46	Fipronil	120068-37-3	Positive	Positive
47	Captan	0133-06-02		Positive
48	4,5-Dihydro-2-mercaptoimidazole	96-45-7		Negative
49	Valproic Acid	99-661	Positive	Positive
50	3,3',5,5'–Tetrabromobisphenol A		Positive	Positive

Forebrain RosetteArray (all active hits):

- **Sensitivity (n=31): 87.1%**
- **Specificity (n=3): 100%**

DNT-IVB (all active hits):

- **Sensitivity : 75 - 87%**
- **Specificity : 71 - 86%**

Fundamental Questions:

The RosetteArray assay is predictive of **HUMAN** DNT hazard.

How good is the RosetteArray assay at predicting clinical **HUMAN** neurodevelopmental risk?

NEUROSETTA

POLYGENETIC VARIANTS

?

CHEMICALS / POLLUTANTS

Lundin B.F. et al. bioRxiv[Preprint].2024 Apr https://doi.org/10.1101/2024.04.01.587605

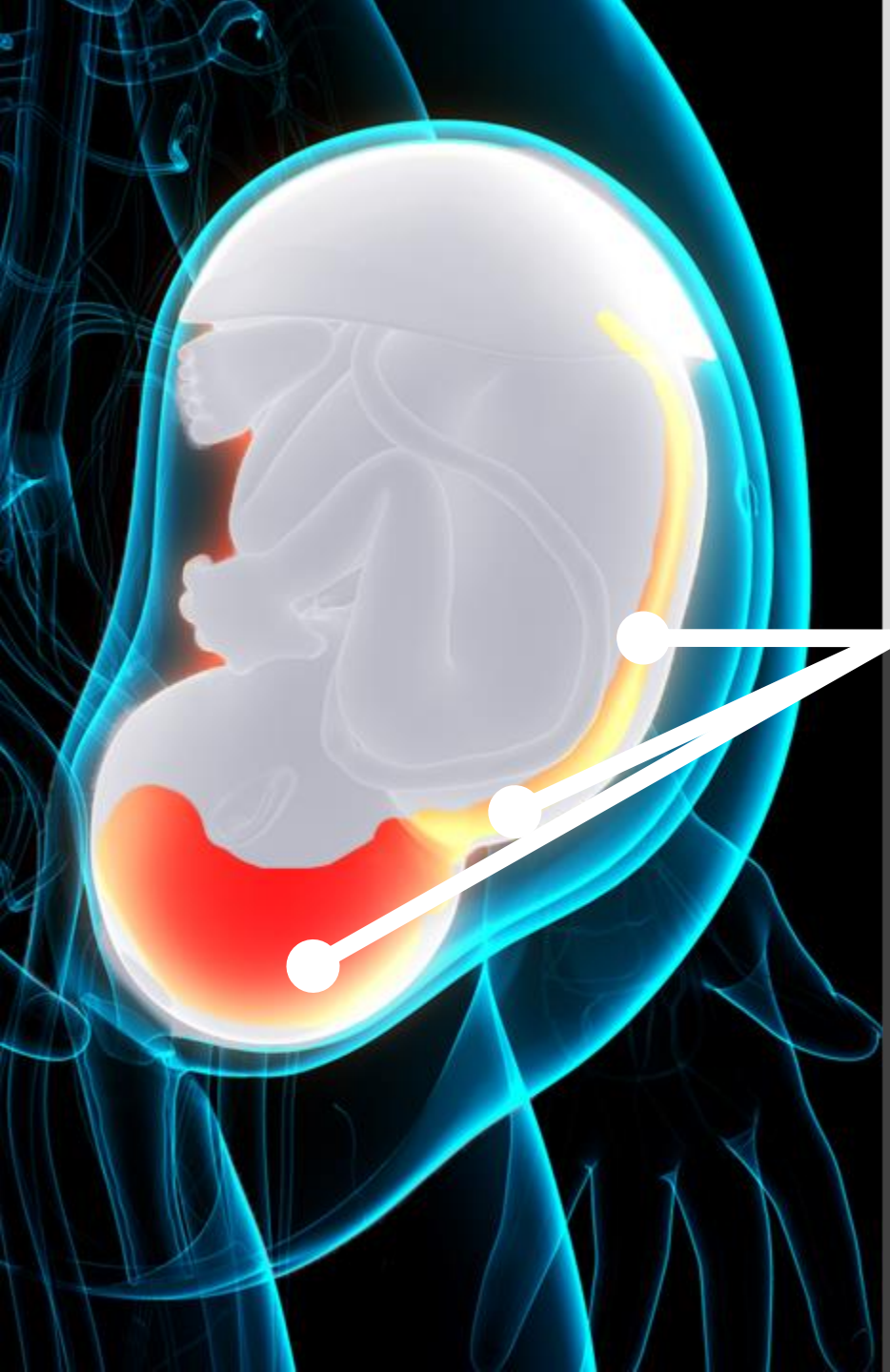
Spina Bifida

Birtele, M. *et al. Nat. Neurosci.* 26, 2090–2103 (2023)

Autism

Haremaki, T. *et al. Nature Biotechnology* 37, 1198–1208 (2019).

Huntington's Disease



POLYGENIC VARIANTS

?

CHEMICALS / POLLUTANTS

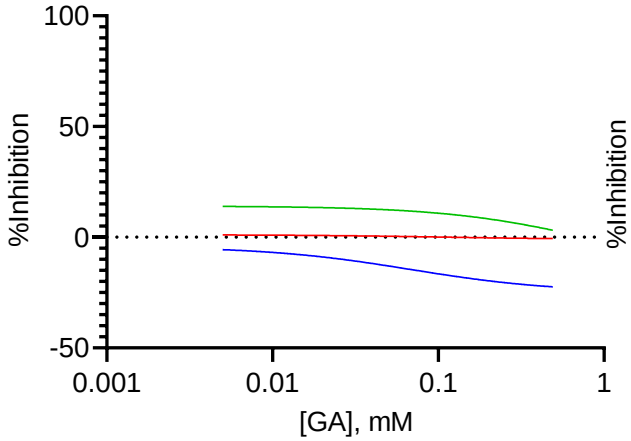
Neural Tube Defects (NTD)

Autism

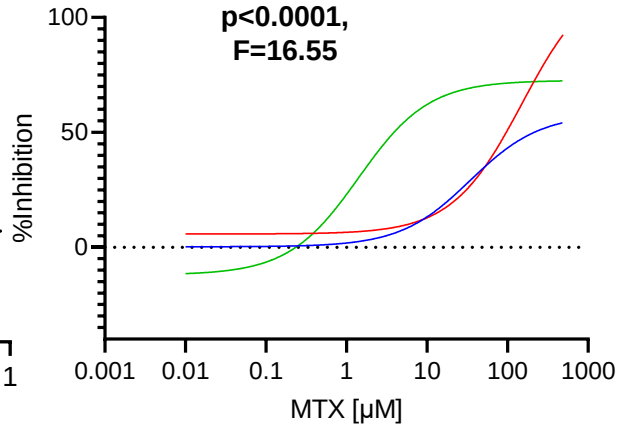
Huntington’s Disease

Forebrain RosetteArray Platform: Spina Bifida Risk Reference Compounds

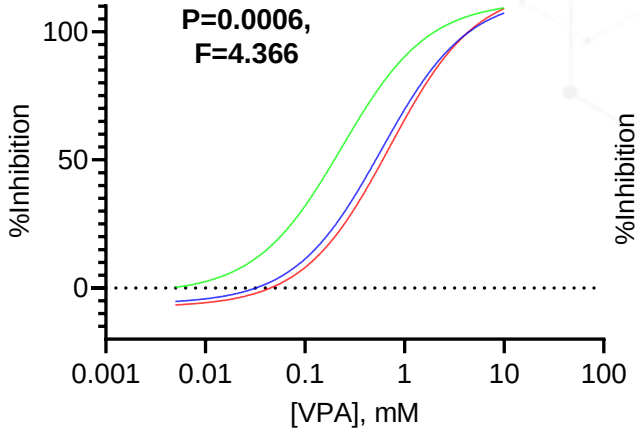
Glycolic Acid (GA)



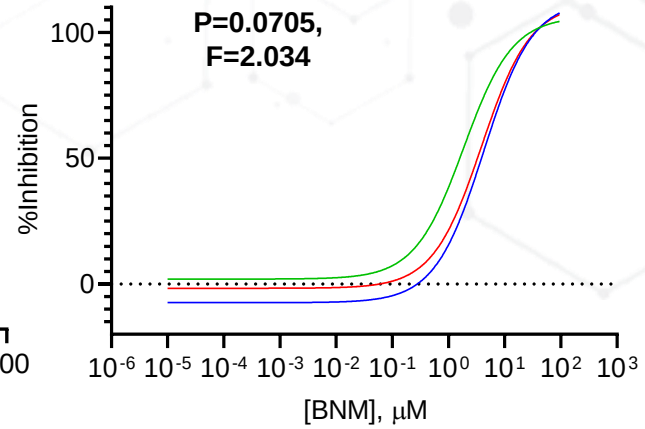
Methotrexate (MTX)



Valproic Acid (VPA)



Benomyl (BNM)



Cells/Tissue, Neural Induction (%Pax6), %Singular Rosette

NEUROSETTA

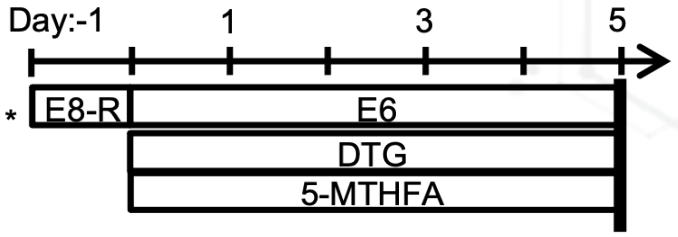
Lundin B.F. et al. bioRxiv[Preprint].2024 Apr https://doi.org/10.1101/2024.04.01.587605

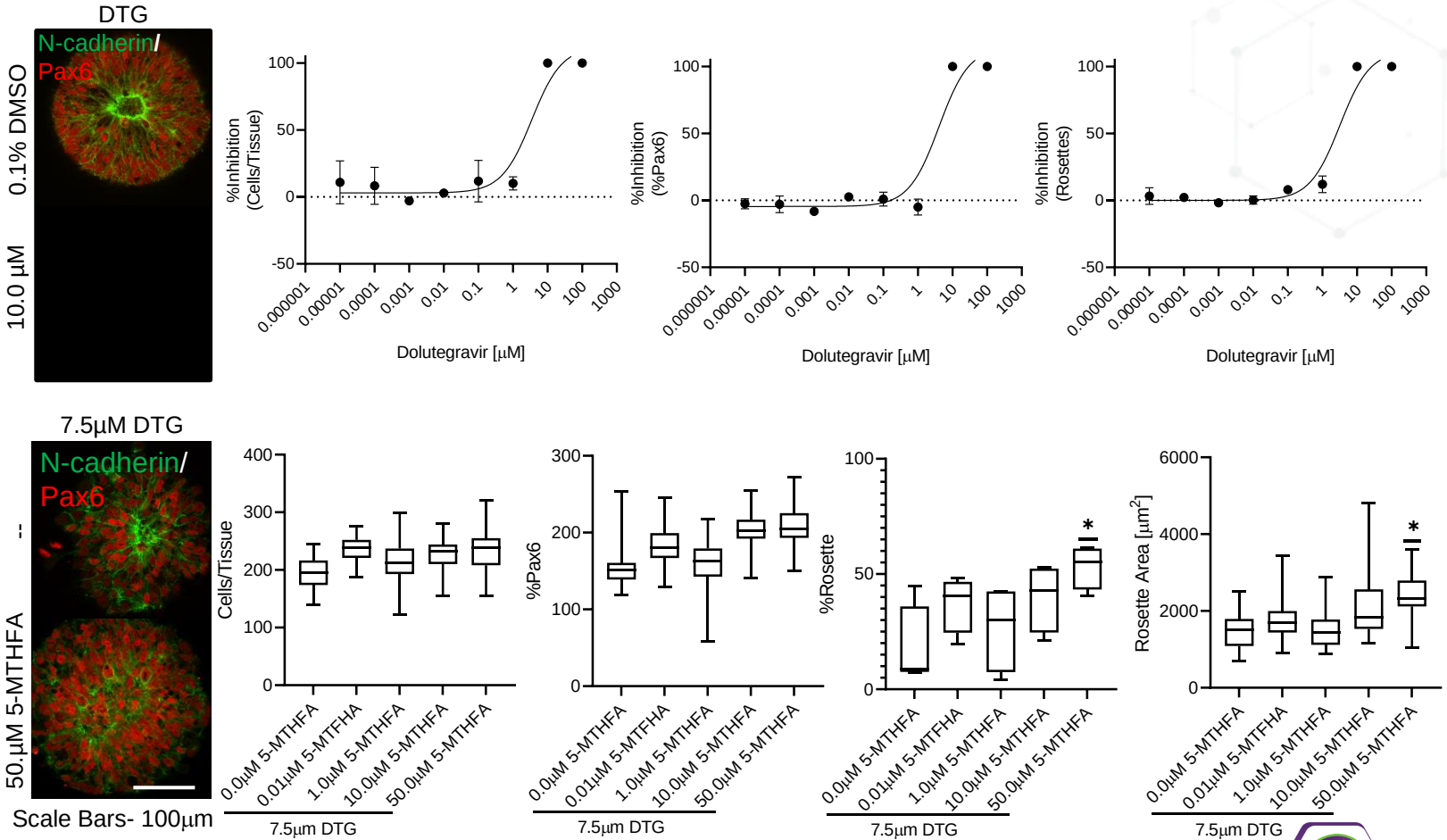
Forebrain RosetteArray Platform: Spina Bifida Risk Reference Compounds

Dolutegravir (DTG):

- 3-10μM therapeutic range
  (Eron JJ *et al. J Infect Dis* 2013 & Castagna A *et al. J Infect Dis* 2014)
- DTG caused 3 vs. 1 NTDs per 1000 deliveries.

(Zash R. *et al. NEJM* 2019 & Zash R. *et al. NEJM* 2018)





POLYGENIC VARIANTS

?

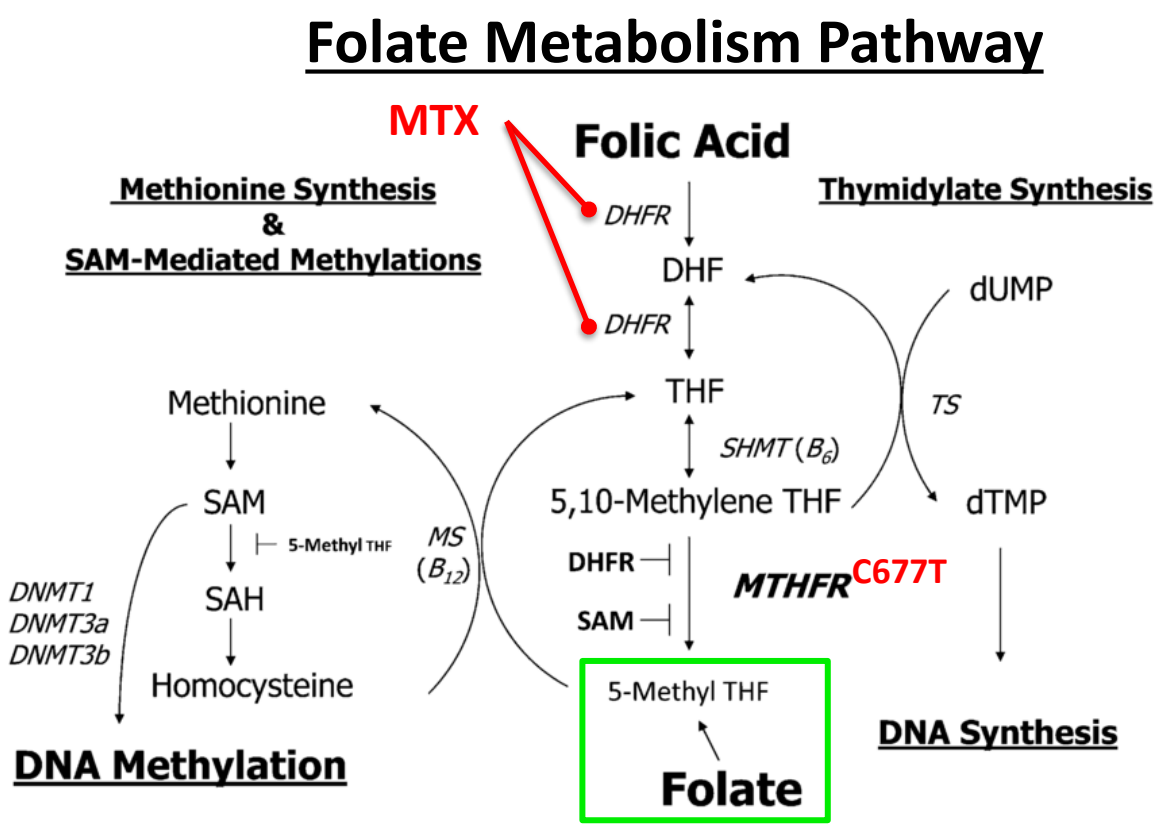
CHEMICALS / POLLUTANTS

Spina Bifida

Autism

Huntington’s Disease

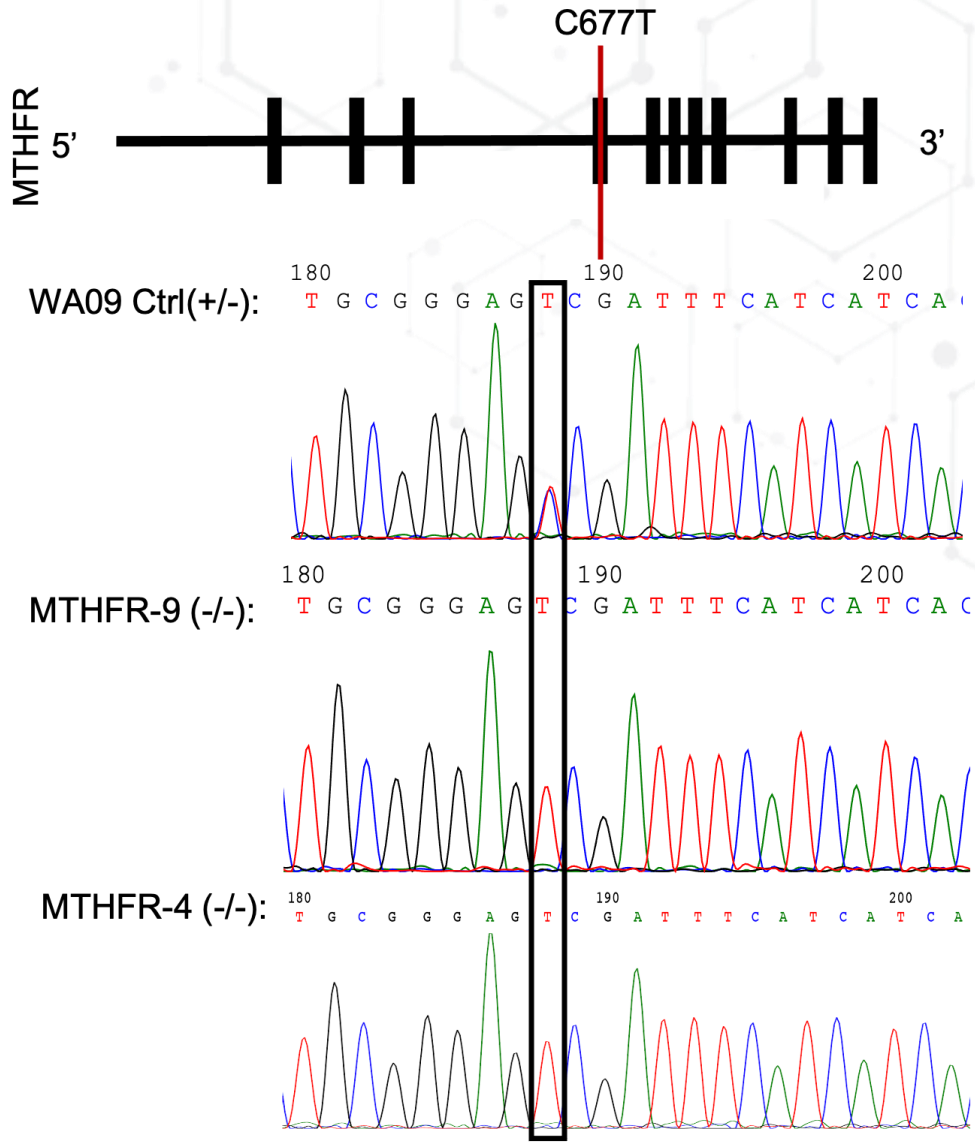
Forebrain RosetteArray Platform: Spina Bifida Genetic + Chemical Risk Detection



Crider, K. S. *et al. Adv. Nutr.* **3**, 21–38 (2012).

- 19 different clinical studies
- ~6438 participants
- TC vs. TT odds ratio of 1.427 (1.247-1.634, 95% CI)

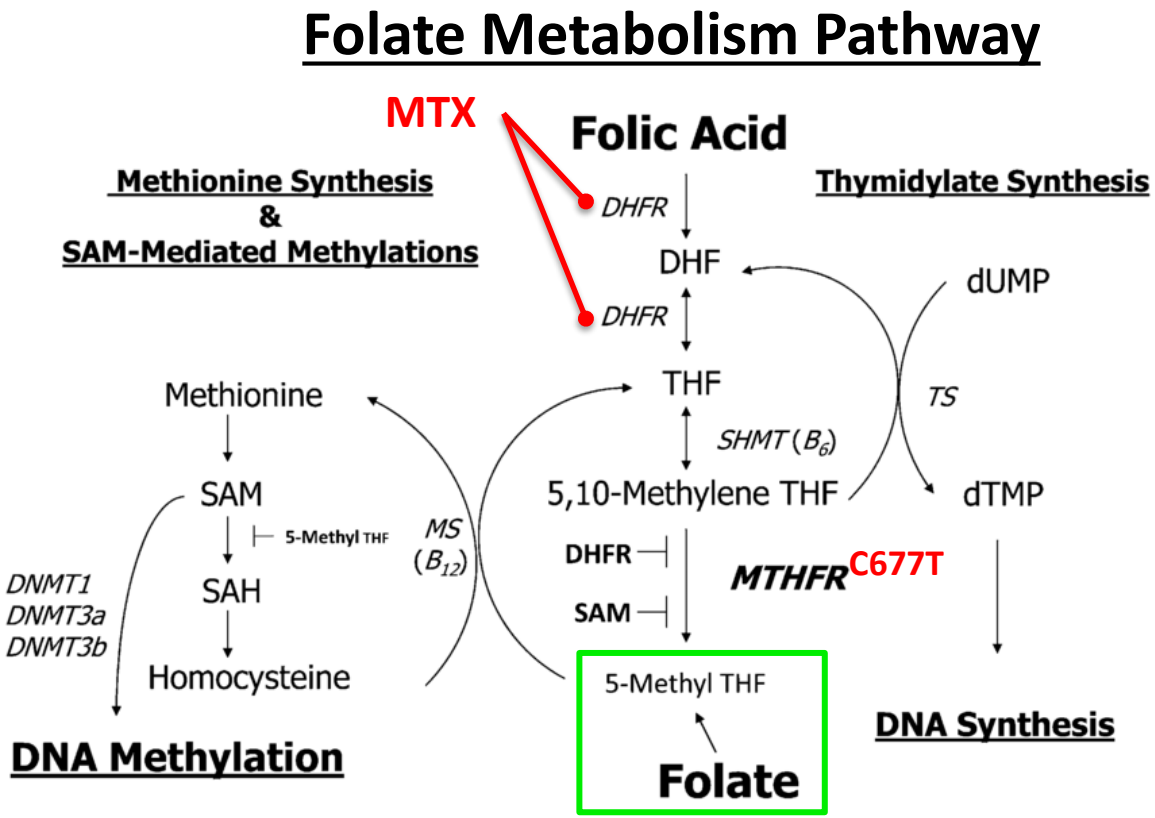
Tabatabaei, R. S. *et al. Fetal and pediatric Pathology* 1–17 (2020)



NEUROSETTA

Lundin B.F. et al. bioRxiv[Preprint].2024 Apr https://doi.org/10.1101/2024.04.01.587605

Forebrain RosetteArray Platform: Spina Bifida Genetic + Chemical Risk Detection



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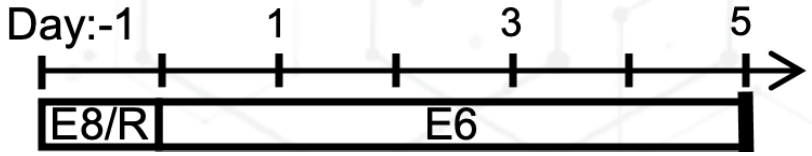

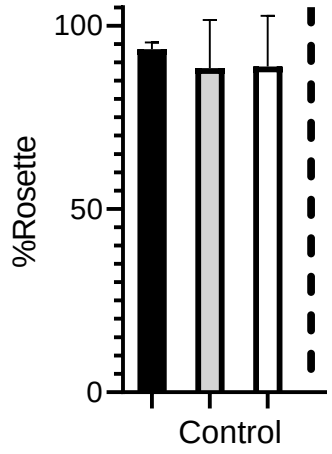

WA09 Ctrl (+/-)
MTHFR Clone 1 Ctrl (-/-)
MTHFR Clone 2 Ctrl (-/-)

**p<0.01
***p<0.001
****p<0.0001

- 19 different clinical studies
- ~6438 participants
- TC vs. TT odds ratio of 1.427 (1.247-1.634, 95% CI)

Tabatabaei, R. S. *et al. Fetal and pediatric Pathology* 1–17 (2020)

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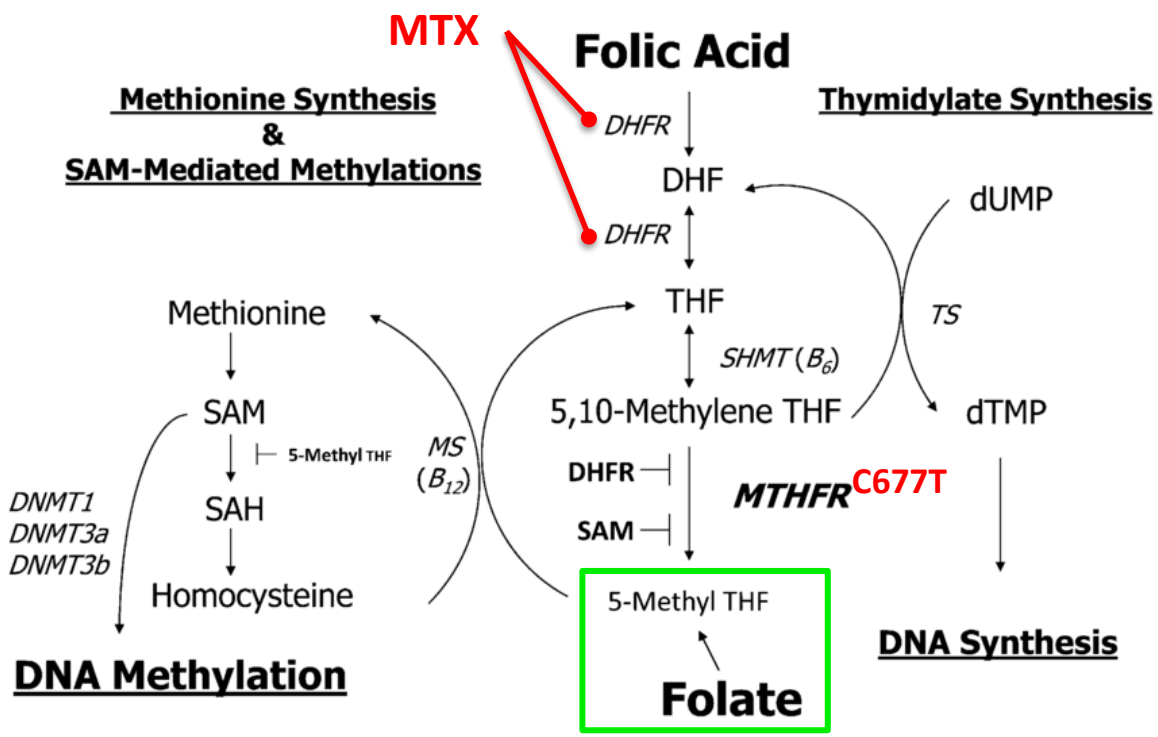
Forebrain RosetteArray Platform: Spina Bifida Genetic + Chemical Risk Detection

Folate Metabolism Pathway



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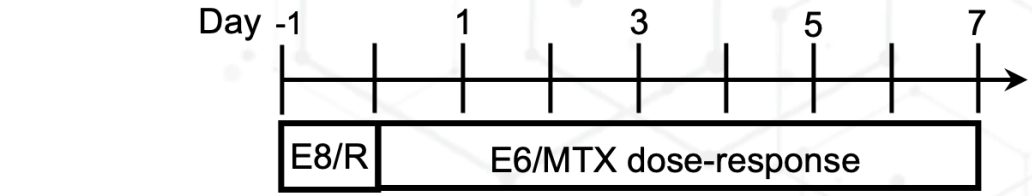

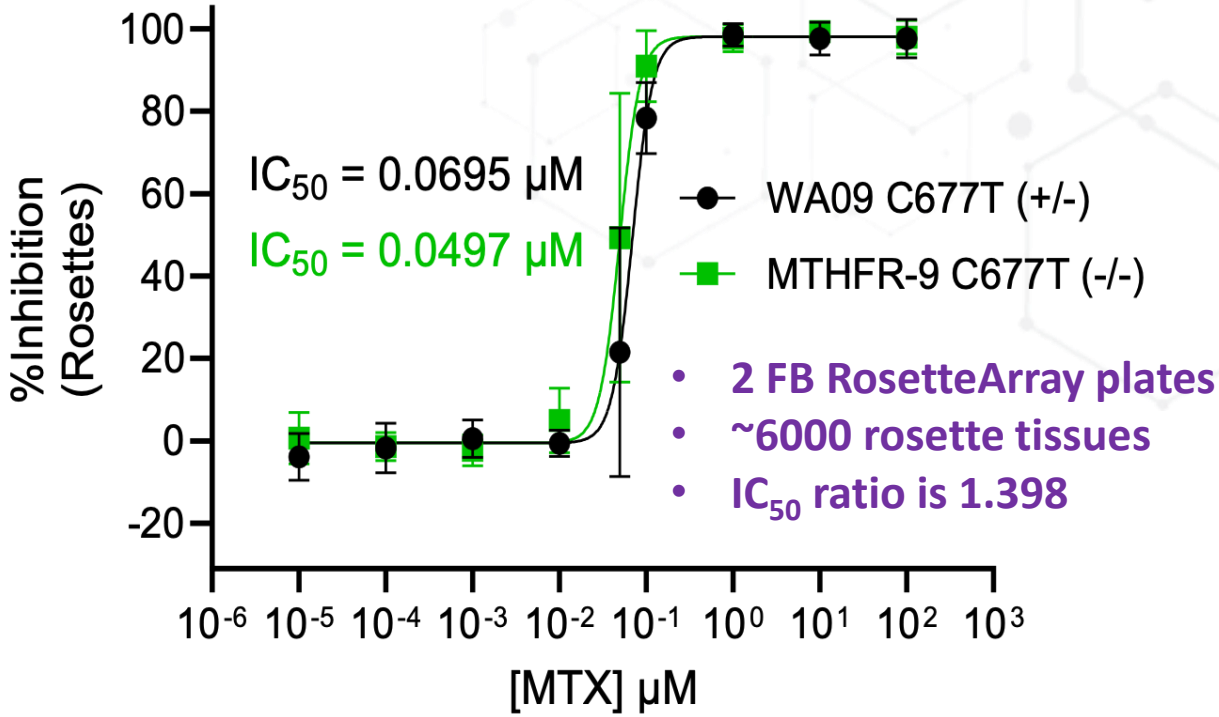

- 2 FB RosetteArray plates
- ~6000 rosette tissues
- IC_{50} ratio is 1.398

p<0.0001, F=27.45

	WA09 Ctrl C677T (+/-)		MTHFR-9 C677T (-/-)	
	IC_{50} (95% CI)	R^2	IC_{50} (95%CI)	R^2
Rosettes	0.0695 (0.0629-0.0768) µM	0.95	0.0497 (0.0452-0.0544) µM	0.93

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POLYGENIC VARIANTS

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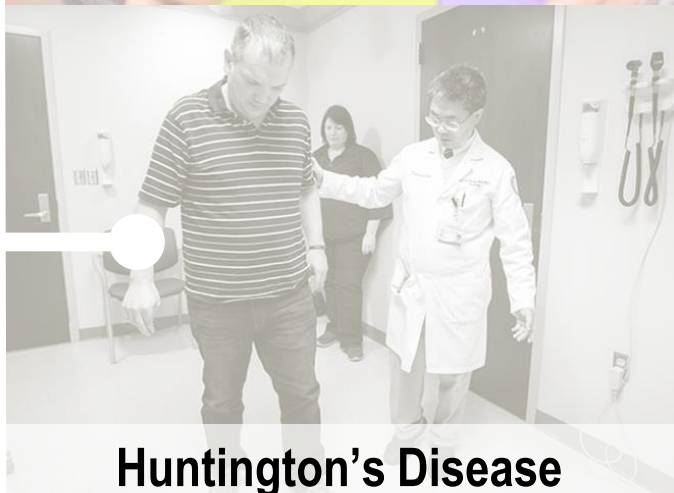
CHEMICALS / POLLUTANTS

Spina Bifida

Autism

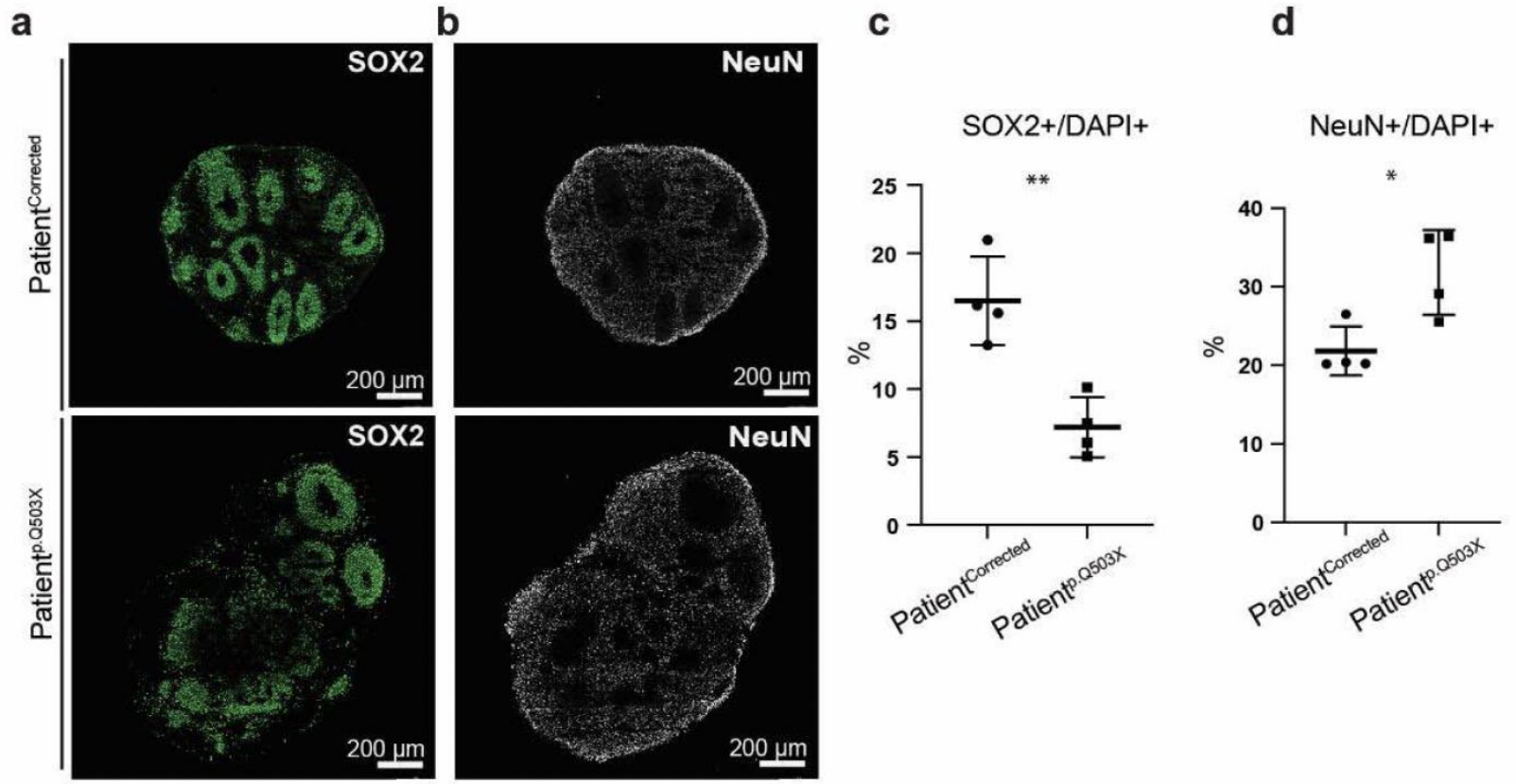


Huntington's Disease

Forebrain RosetteArray Platform: Autism Genetic Risk Detection

Dr. Giorgia Quadrato (USC) collaboration:

- SYNGAP1 mutations are a top genetic risk factor for ASD; represent ~1-2% of all intellectual disability cases.
- iPSC line with SYNGAP1$^{p.Q503X}$ haploinsufficent mutation and corrected isogenic control



**** 2-month Cortical Organoid Derivation ****

NEUROSETTA

Birtele, M. *et al. Nat. Neurosci.* **26**, 2090–2103 (2023)

Forebrain RosetteArray Platform: Autism Genetic Risk Detection

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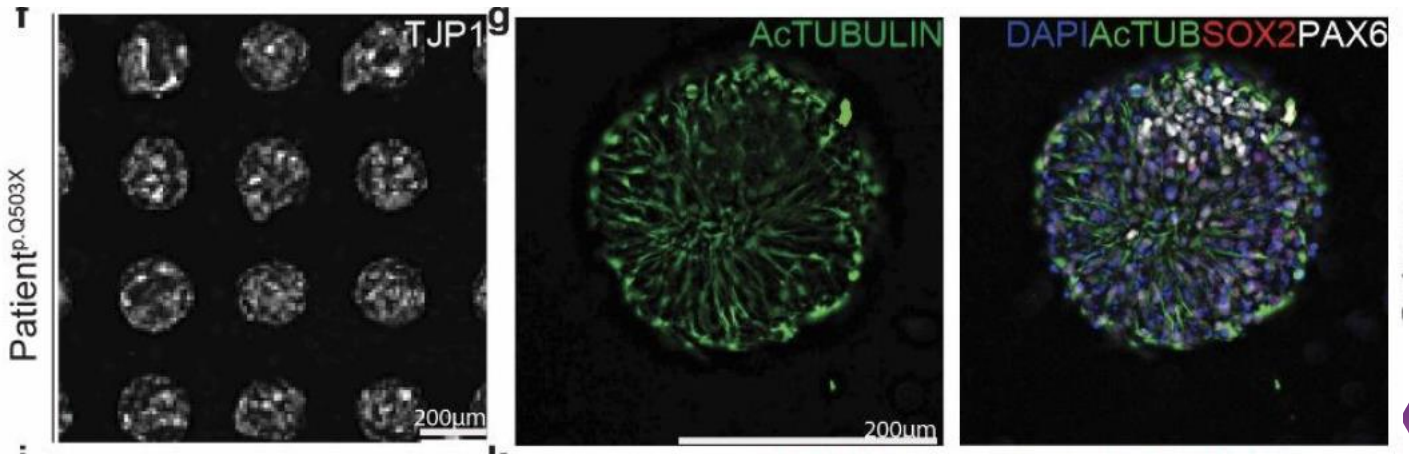
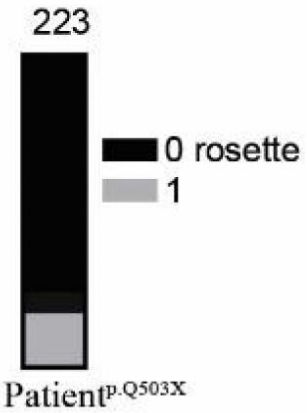
D.I.V 8 Forebrain RosetteArray™

**** 8-day Forebrain RosetteArray ****





Birtele, M. *et al. Nat. Neurosci.* **26**, 2090–2103 (2023)

NEUROSETTA

Forebrain RosetteArray Platform: Autism Genetic Risk Detection

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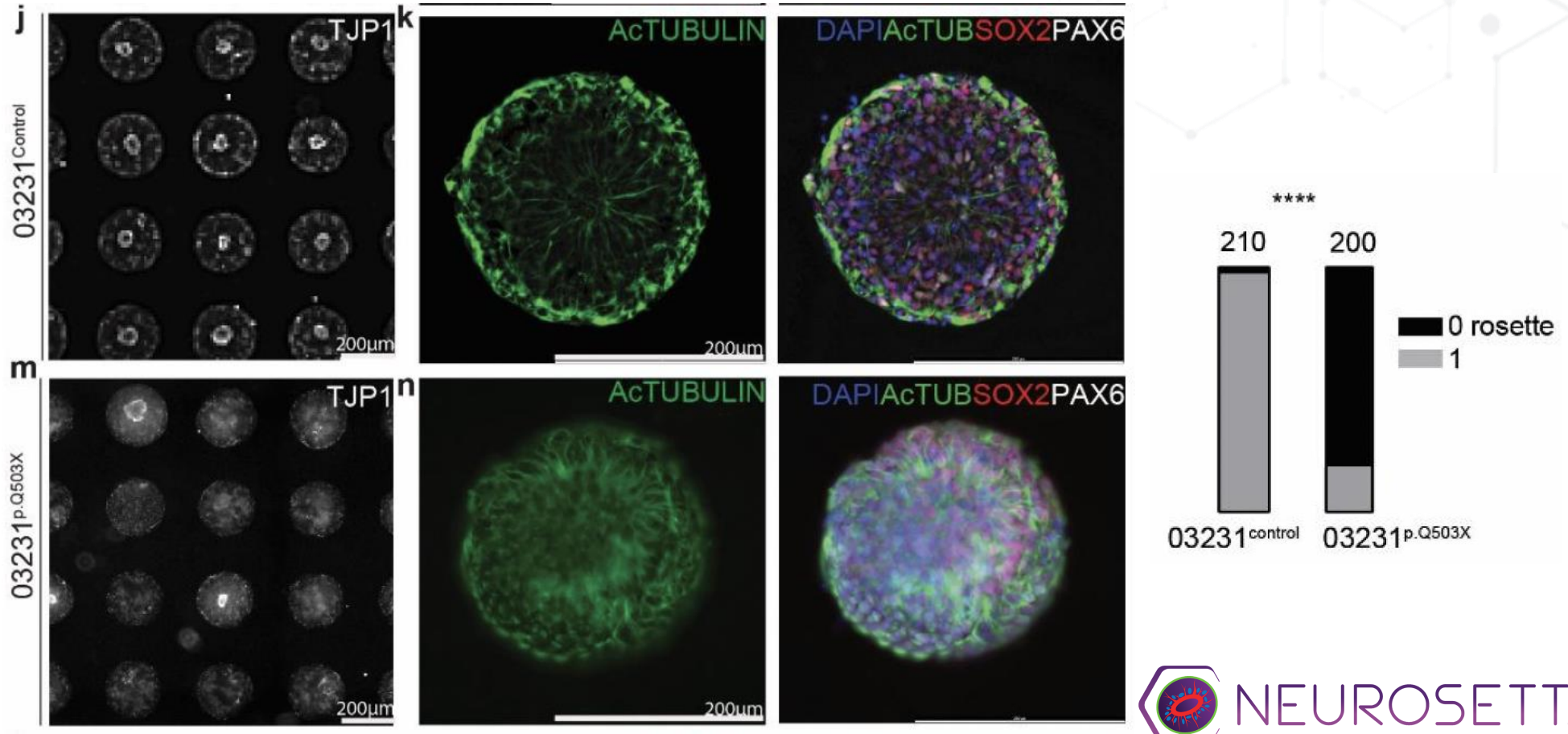
- SYNGAP1 mutations are a top genetic risk factor for ASD; represent ~1-2% of all intellectual disability cases.
- 03231 iPSC control line edited to contain SYNGAP1$^{p.Q503X}$ haploinsufficent mutation

**** 8-day Forebrain RosetteArray ****

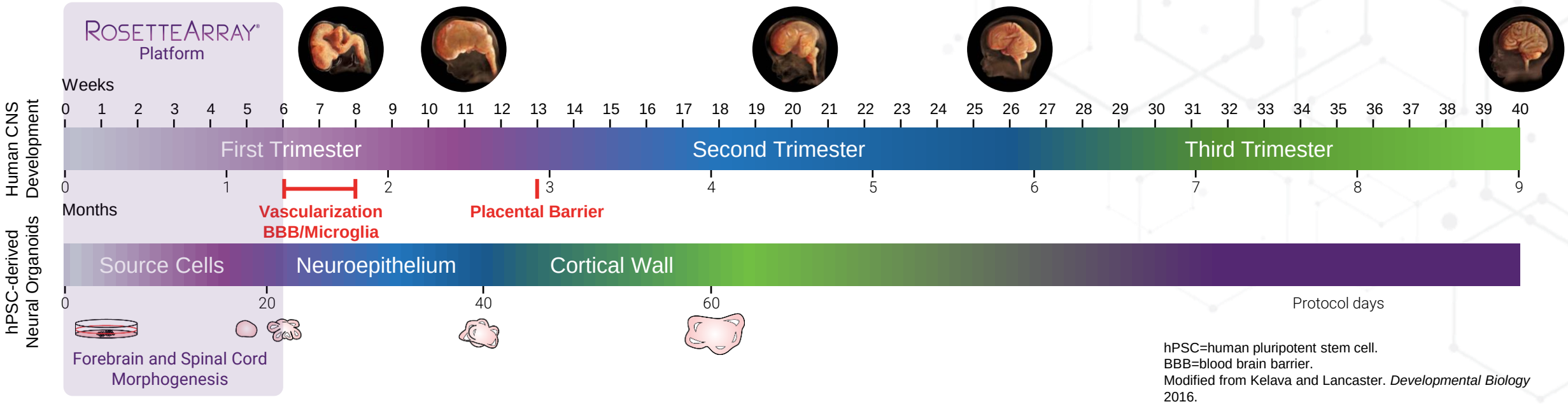
D.I.V 8 Forebrain RosetteArray™



Birtele, M. *et al. Nat. Neurosci.* **26**, 2090–2103 (2023)

NEUROSETTA

RosetteArray® Platform simplifies **HUMAN** DNT hazard/risk screening

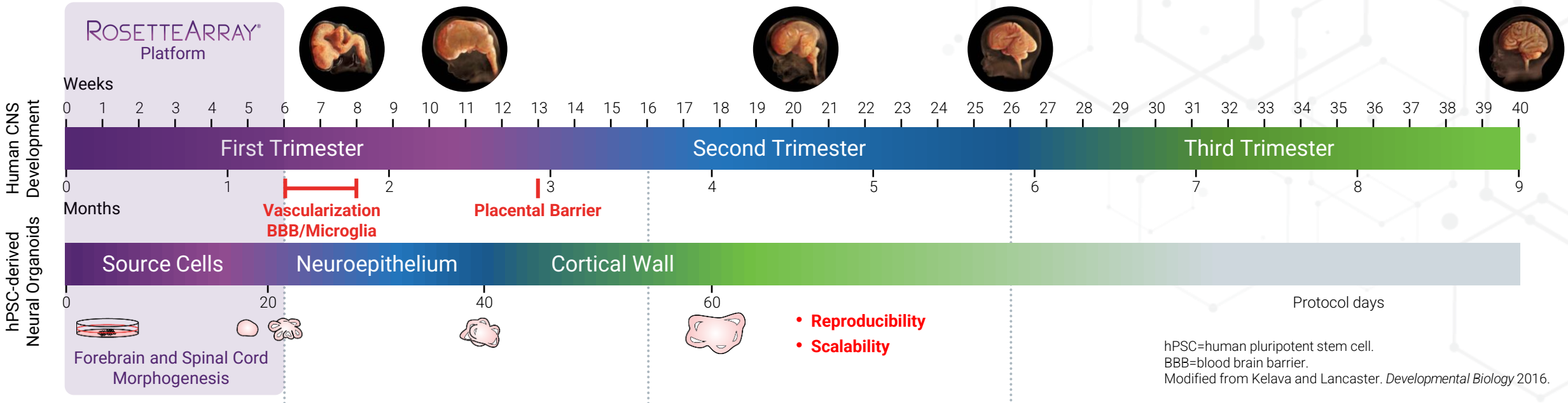


hPSC=human pluripotent stem cell.
BBB=blood brain barrier.
Modified from Kelava and Lancaster. *Developmental Biology* 2016.

- Works with DMSO solvent up to 0.1% (i.e., 1:1000 dilution)
- Excellent reproducibility
  - Forebrain RosetteArray: 86.3% ± 9.06 (stdv) single rosette emergence efficiency
  - Lumbar Spinal RosetteArray: 93.8% ± 4.10 (stdv) single rosette emergence efficiency
  - Z-factor score of 0.532 across >20 assays
- Integration of human metabolism for developmental neurotoxicity (DNT) screening
- DNT screening to date indicates a Sensitivity (n=31) of 87.1% and a Specificity (n=3) of 100%
- Used to model 2 different genetic causes of Spina Bifida (Lundin, B. *et al.* bioRxiv preprint 2024)
- Used to model 1 genetic causes of Autism Spectrum Disorder (Birtele, M. et al. *Nat. Neurosci.* 2023)

NEUROSETTA

RosetteArray assay is predictive of human DNT hazard (chemical) and neurodevelopmental risk (genetic)



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Modified from Kelava and Lancaster. *Developmental Biology* 2016.

Initial pathogenesis of Neural Tube Defects, Autism Spectrum Disorder, Huntington's Disease, . . .

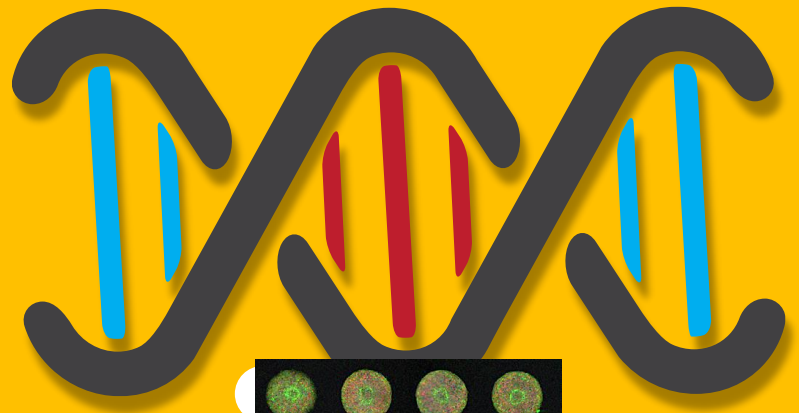
Developmental Neurotoxicity-In Vitro Battery

(OECD Series on Testing and Assessment No. 377)

- UKN2 (neural crest cell migration/viability; forebrain)
- UKN5 (immature peripheral neuron viability and morphology; dorsal root ganglia)
- hN Initiation (human glutamatergic neuron outgrowth; cortex)
- UKN4 (neuronal differentiation and viability of dopaminergic progenitors; midbrain)
- hNP1 (human NPC Proliferation/apoptosis/viability; cortex; **source line no longer available**)
- NPC1 (NPC proliferation; cortex)
- NPC2a,b,c, NPC3,NPC4, NPC5 (RGC, neuron, oligodendrocyte migration; cortex)
- Rat cortical initiation/neuron outgrowth
- Rat cortical neuron maturation/synaptogenesis
- Rat cortical neuron MEA

NEUROSETTA

(POLY)GENETIC VARIANTS



ROSETTEARRAY®

CHEMICALS / POLLUTANTS

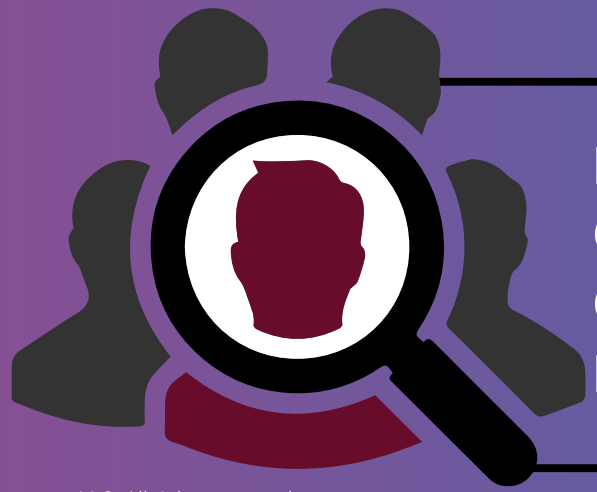
Spina Bifida

Autism

Huntington's Disease

Neurosetta Products/Services

- Contract human DNT screening services
- RosetteArray® plates and RosetteDetect™ Image Analysis
- Personalized disease model development
  - e.g., Spina Bifida and Autism Spectrum Disorder
- Precision Medicine Drug Discovery



Regulatory Agencies
Chemical/Pharmaceutical Companies
Contract Research Organizations
Research Institutes and Labs

Randolph Ashton, Ph.D.
Co-founder & CEO

customerservice@neurosetta.com

NEUROSETTA

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Jack Maher
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Lydia MT Ashton (UW-Madison)
Joshua Robinson (UCSF)
Bermans Iskandar (UW-Madison)
Giorgia Quadrato (USC)

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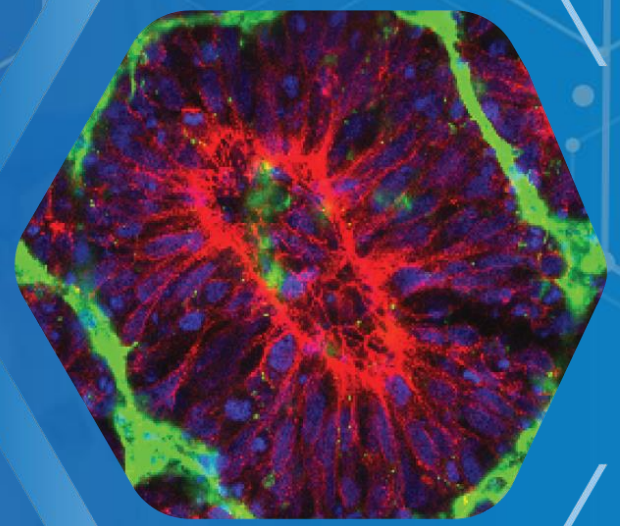
AI-Enabled High-Throughput Screening of Human Neurodevelopment for Toxicology and Precision Medicine



Questions?