American College of Toxicology

Society for Birth Defects Research & Prevention

# Practical Reproductive and Developmental Toxicology

American College of Toxicology and
Society for Birth Defects Research and Prevention

## Future of Reproductive and Developmental Toxicity Testing: Computational and Organoids

*Thomas B. Knudsen, PhD*

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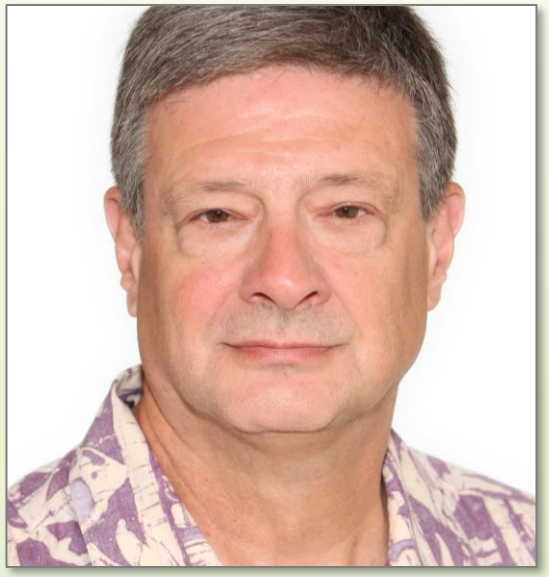
# Thomas B. Knudsen, PhD



Developmental Systems Biologist at the US EPA Center for Computational Toxicology and Exposure, since 2007. Expertise is building and testing computational systems models for predictive developmental toxicity enabling the synthetic reconstruction of morphogenesis and simulation of dysmorphogenesis. He is a Past-President of the Society for Birth Defects Research and Prevention and serves as the Editor-in-Chief of ‘Current Research in Toxicology’.

# Disclosures

*DISCLAIMER: the views expressed here are my own and do not necessarily reflect Agency policy.*


https://stemcells.nih.gov/research/registry.htm

Funding: our research with human pluripotent stem cell lines (hPSCs) was performed under EPA's *Chemical Safety for Sustainability Research Program, Research Area 5 'Virtual Tissue Models' (VTMs).*

Compliance*:* work involving established hPSC lines is compliant with Executive Order 13505 (issued 2009) to ensure that is ethically responsible, scientifically worthy, and conducted in accordance with applicable law.

*The H9 cell line is registered in the NIH Human Embryonic Stem Cell Registry:* WA09 (H9): NIH Approval Number: NIHhESC-10-0062 (EPA contract EP-D-13-055 with Stemina Biomarker Discovery).

*Other pluripotent stem cell lines:* endodermal hPSC line from Allele Biotech #ABPSC-HDFAIPS (EPA contract EP-D-13-054 with Vala Sciences, Inc.).

# Take-home points

- Tiered approaches are now available to help shift developmental hazard detection to virtual animal-free alternatives, based on *in vitro* data and *in silico* models.
- Reducing a complex system to simpler *in vitro* assays to enable high-throughput screening (HTS) disrupts the integrated properties of energy, control, and robustness.
- Complex HTS data and information now in hand, the need arises for synthetic microsystems, computational intelligence, and artificial life to rebuild this complexity.
- Focus of this lecture is on the predictive power of computational models and computer simulation for human-relevant pathways underlying prenatal developmental toxicity.

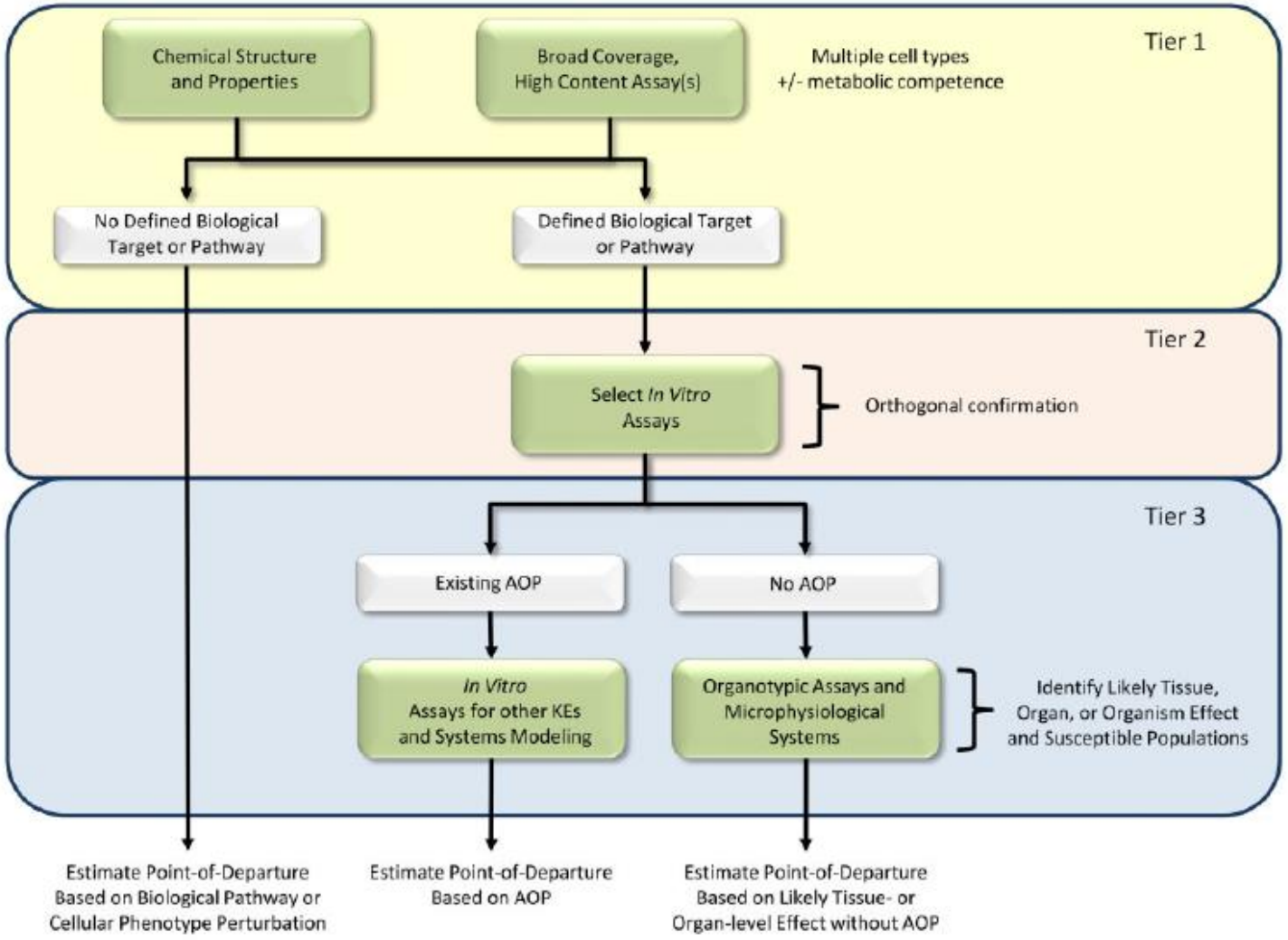
*"Predicting the future isn't magic, it's artificial intelligence"*
- Dave Waters, January 2020

# A.I. and Predictive Toxicology

- Refers to the ability of a computer to learn from complex data and identify meaningful connections; see for example:
  - *Luechtefeld et al. (2018) Machine learning of toxicological big data enables read-across structure activity relationships (RASAR) outperforming animal test reproducibility. Toxicol Sci 165: 198-212.*
  - *Ciallella and Zhu (2019) Advancing computational toxicology in the big data era by artificial intelligence: data-driven and mechanism-driven modeling for chemical toxicity. Chem Res Toxicol 32: 536-547*
- Minimal requirements for predictive toxicology:
  1. availability, type, and quality of high-dimensional data;
  2. ontologies for systematic organization of input/output parameters;
  3. evolutionary algorithms that can handle complex cellular dynamics;
  4. sophisticated computer models to visualize cells in space and time.

# CompTox Blueprint:

*USEPA's tiered testing framework for hazard detection*





- information on 883K chemicals
- HTS data on >1K assays in ToxCast/Tox21
- HTS coverage for up to 8K chemicals
- give it a try at:

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

*Thomas et al. 2019, Toxicol Sci*

# New Approach Methods (NAMs)

- Frank R. Lautenberg Chemical Safety for the 21st Century Act (LCSA) promotes use of non-animal alternatives to identify chemical risks in vulnerable populations/lifestages.
- USEPA established a strategic work plan for 'new approach methods' (NAMs) to address critical information gaps in *in vitro* testing for chemical hazard detection and assessment.
- *In vitro* assays and *in silico* models that reflect key aspects of embryo-fetal development will be indispensable for NAM-based detection of developmental hazard potential.
- *In vitro* profiling of **human pluripotent stem cell (hPSC)** lines is an active area of investigation and one of the most promising alternatives to pregnant animal testing.

# Novel Features of PSC Lines

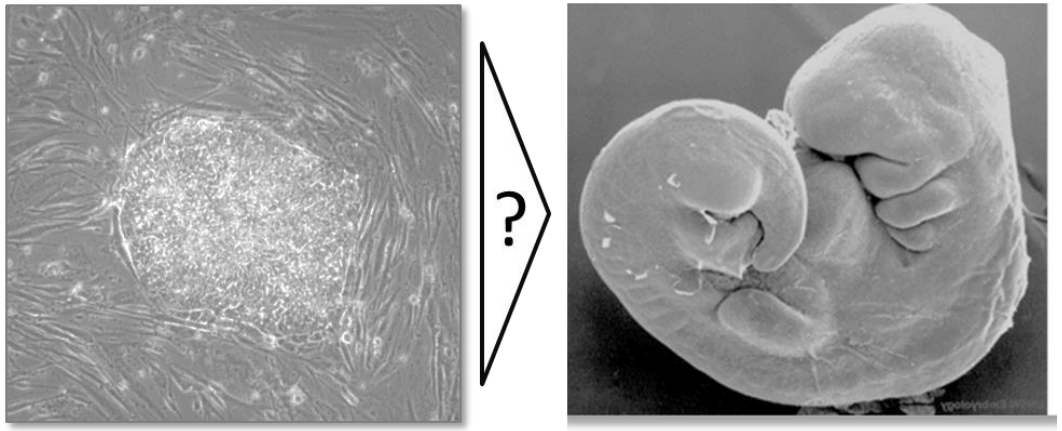
- **Self-renewal:** cells replicate themselves indefinitely when cultured under appropriate growth factor conditions.
- **Pluripotency:** cells have the potential to form most of the different cell types comprising the embryo/fetus.
- **Autopoiesis:** capacity to self-organize into rudimentary tissues and more complex organoid structures.

PSC lines established from the embryoblast (mouse, human) can recapitulate **some** of the biology driving embryogenesis during the period covered by guideline prenatal studies (e.g., OECD TG 414).

# A Few Milestones in hPSC Research …

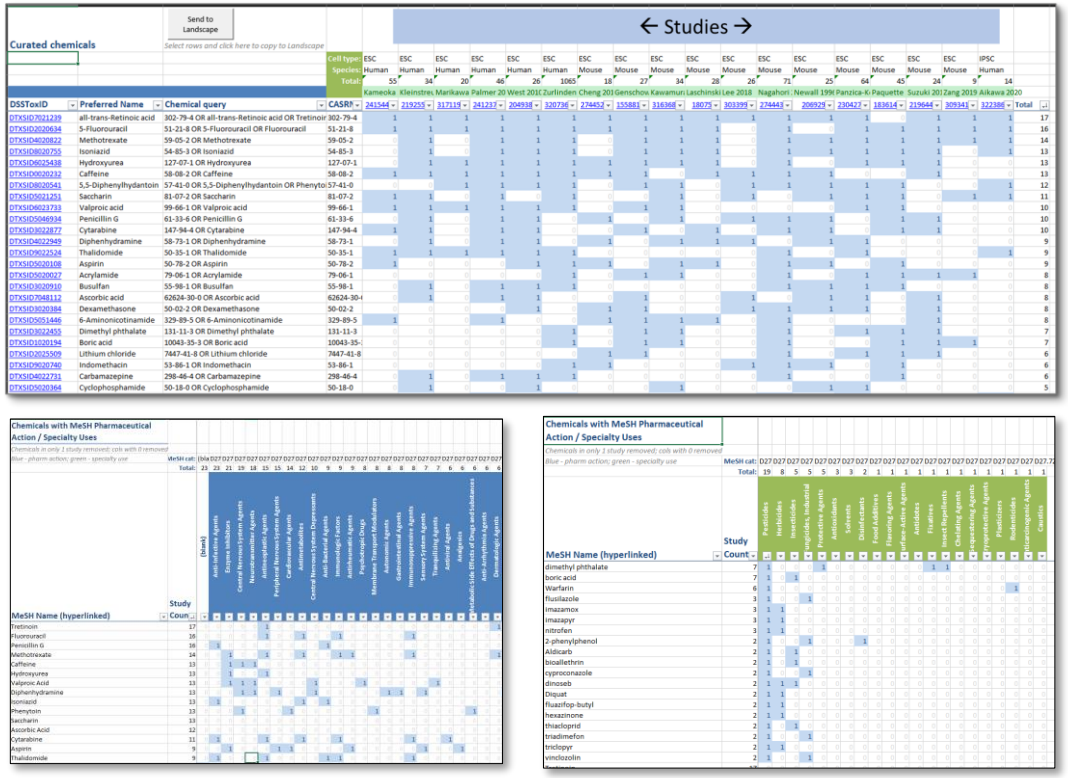
- **1975**: the term ‘ES cell’ was first coined to distinguish pluripotent cells from a pre-implantation mouse embryoblast versus pluripotent embryonal carcinoma cell lines.
- **1998:** PSCs isolated from human blastocysts and cultured under conditions to maintain self-renewal can form derivatives of all 3 embryonic germ layers even after 4-5 months.
- **2001:** ethical debate led POTUS to issue an executive order limiting federally-funded research on ES cells to 21 hPSC lines established before August 2001.
- **2006:** researchers could reprogram dermal fibroblasts to a pluripotent state (iPSCs) simply by altering expression of 4 genes (Oct3/4, Sox2, c-Myc, Klf).
- **https://stemcelldb.nih.gov/** NIH database of genomic profiling data on the 21 hPSC lines approved under the GW Bush administration, and also on registered human iPSCs.

# Can an hPSC Assay Live up to the NAM challenge?




- Does not encompass the full complexity of anatomical development;
- Blind to the precise spatial-temporal control of cell-cell interactions *in vivo* ;
- Misses developmental effects secondary to maternal or placental toxicity;
- Uncertainty of post-organogenesis vulnerability and post-natal manifestations;
- Cross-species extrapolation (mPSC to human, hPSC to animals);
- Limited xenobiotic metabolism and other ADME considerations (toxicokinetics);
- Uncertainties in translatability to the intact embryo (toxicodynamics).

# Conceptual and Practical Considerations




*Abstract Sifter, SWIFT, MeSH terms, Chemicals Dashboard, …*

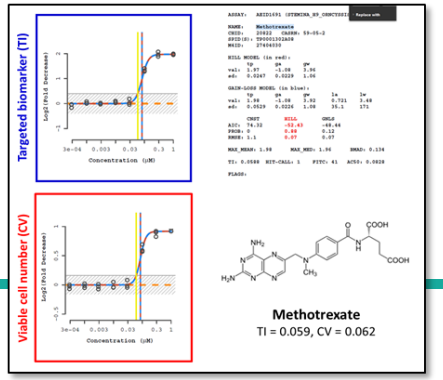
- **Detailed literature review:** survey of extant ES cell assays used to classify developmental toxicants:
  - Chemical domain
  - Biological domain
  - Standardized protocols
  - Reproducibility
  - Biomarker readouts
  - Predictive power.

***1,533 records in PubMed reduced to 333 (AI for relevance) and 192 (manual curation).***

- **1,250 annotated chemicals (through 2020):**
  - 18 publications tested ≥ 10 compounds (primary)
  - 174 publications tested 1-9 (evidentiary support)
  - Most frequently represented: ATRA, 5-FU, MTX.

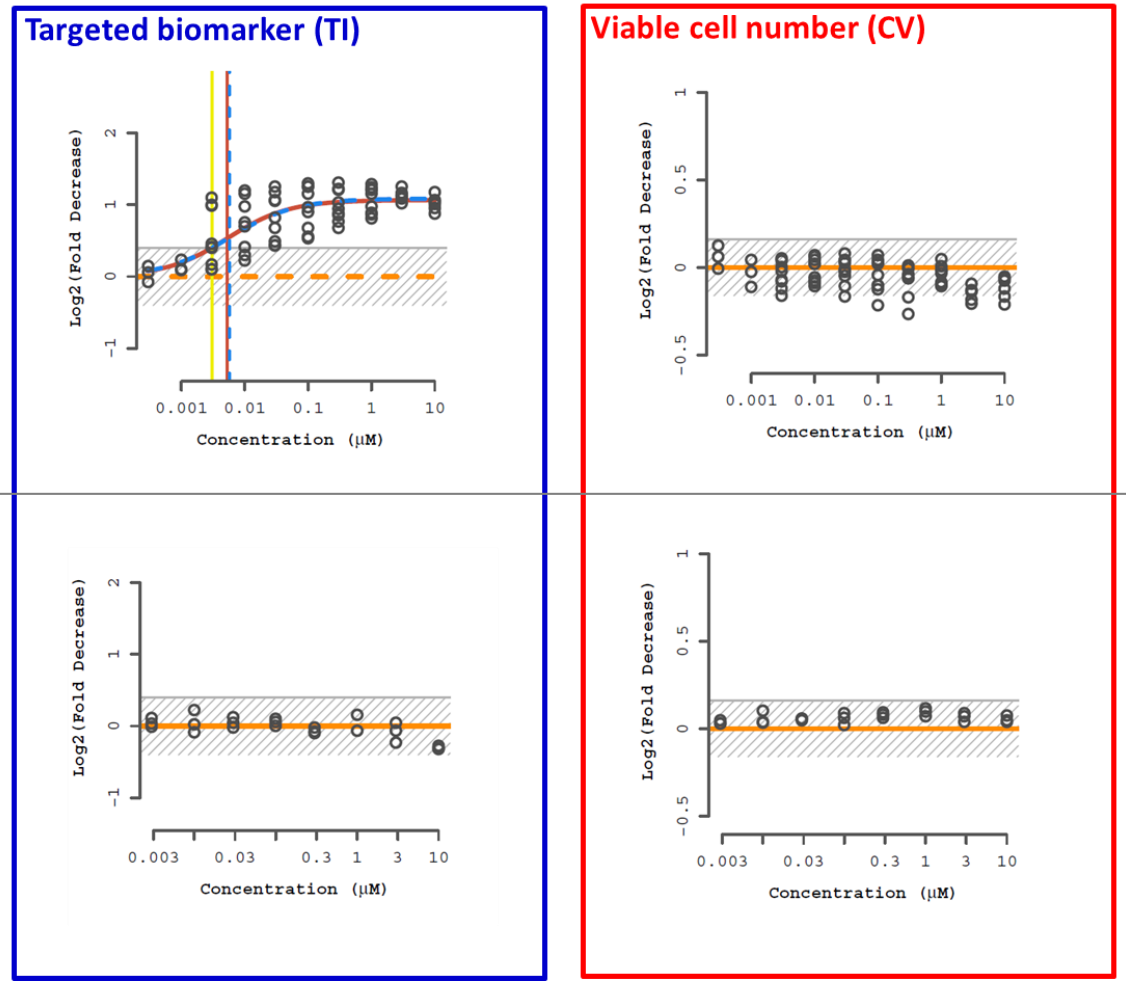
*Piersma et al., manuscript in preparation*

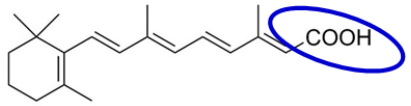
# HTS Profiling with the devTOX$^{qP}$ Assay




- Pluripotent human (H9) stem cell-based biomarker assay for developmental toxicity screening developed by Stemina Biomarker Discovery [*Palmer et al., 2013, BDRB*].
- Developmental toxicity potential defined by the concentration of a test chemical reducing the ratio of ornithine (secreted) to cystine (utilized) to a critical level (77% accuracy).
- We used this assay to test 1065 ToxCast chemicals for teratogenicity index (TI) and pipelined the dataset into EPA's CompTox Chemicals Dashboard [*Zurlinden et al., 2020, Toxicol Sci*].
- Observed a 19.2% positivity rate across the 1065 chemicals tested, with a performance reaching 79%–82% balanced accuracy to well-curated teratogens and non-teratogens.

# **Example:** *Vitamin A and Its Active Metabolite (All-Trans Retinoic Acid)*



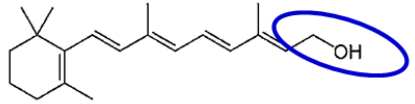




**all trans Retinoic acid**

TI = 0.003 µM, CV = NA

dLEL rat = 2.5 mg/kg/day (> mLEL)

dLEL rabbit = 0.5 mg/kg/day (=mLEL)



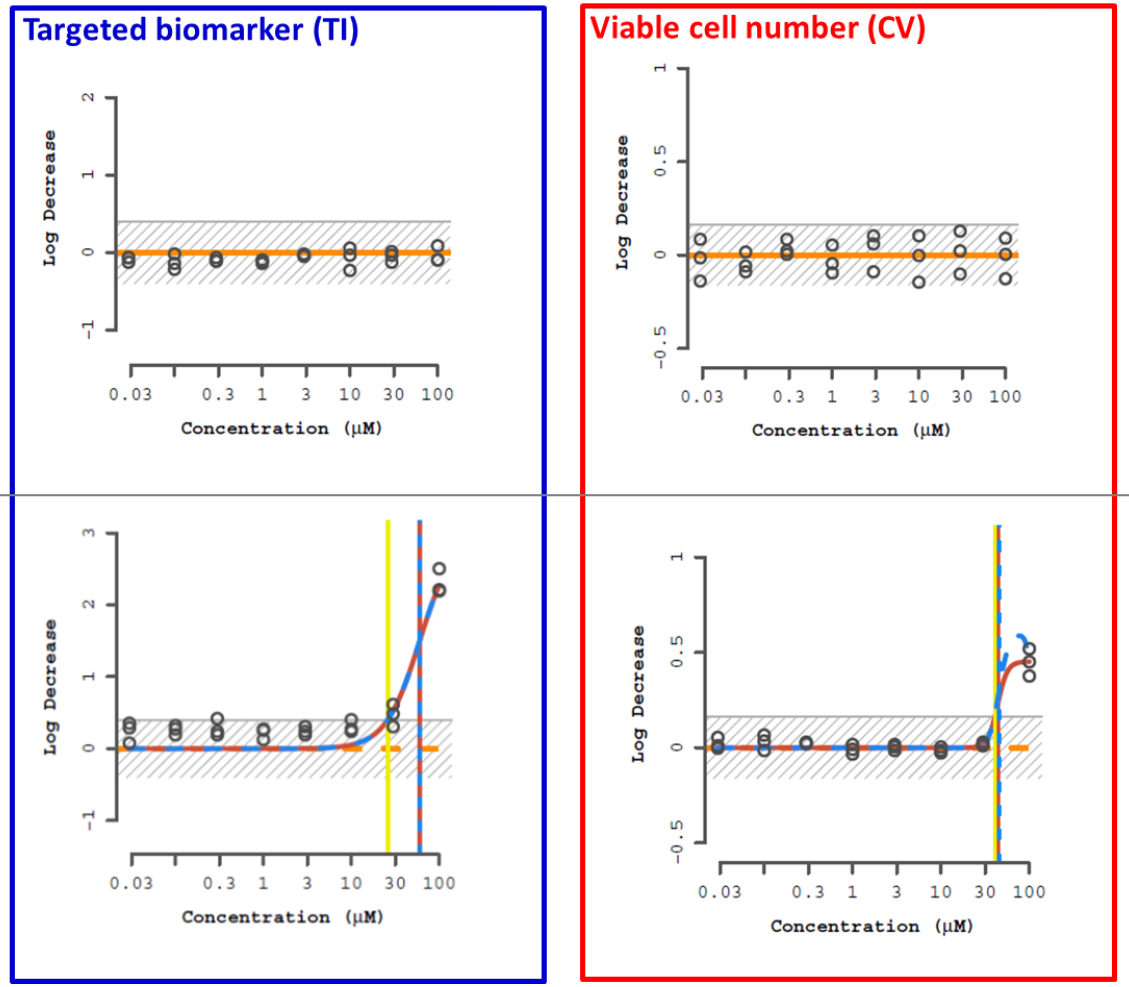

**Retinol (vitamin-A)**

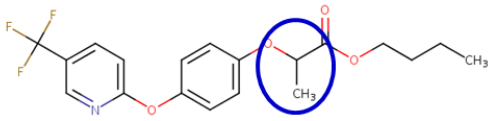
TI = NA, CV = NA

(True Negative)

*Zurlinden et al., 2020, Toxicol Sci*

# **Example:** *R-enantiomer (Fluazifop-P-butyl) Is the Active Herbicide*



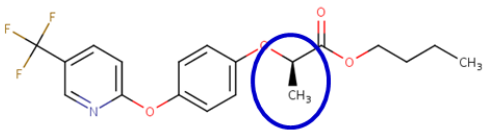



**Fluazifop butyl**

TI = not active, CV = no effect

dLEL rat = 10 mg/kg/day (< mLEL)

dLEL rabbit = 90 mg/kg/day (mLEL)



**Fluazifop-P-butyl**

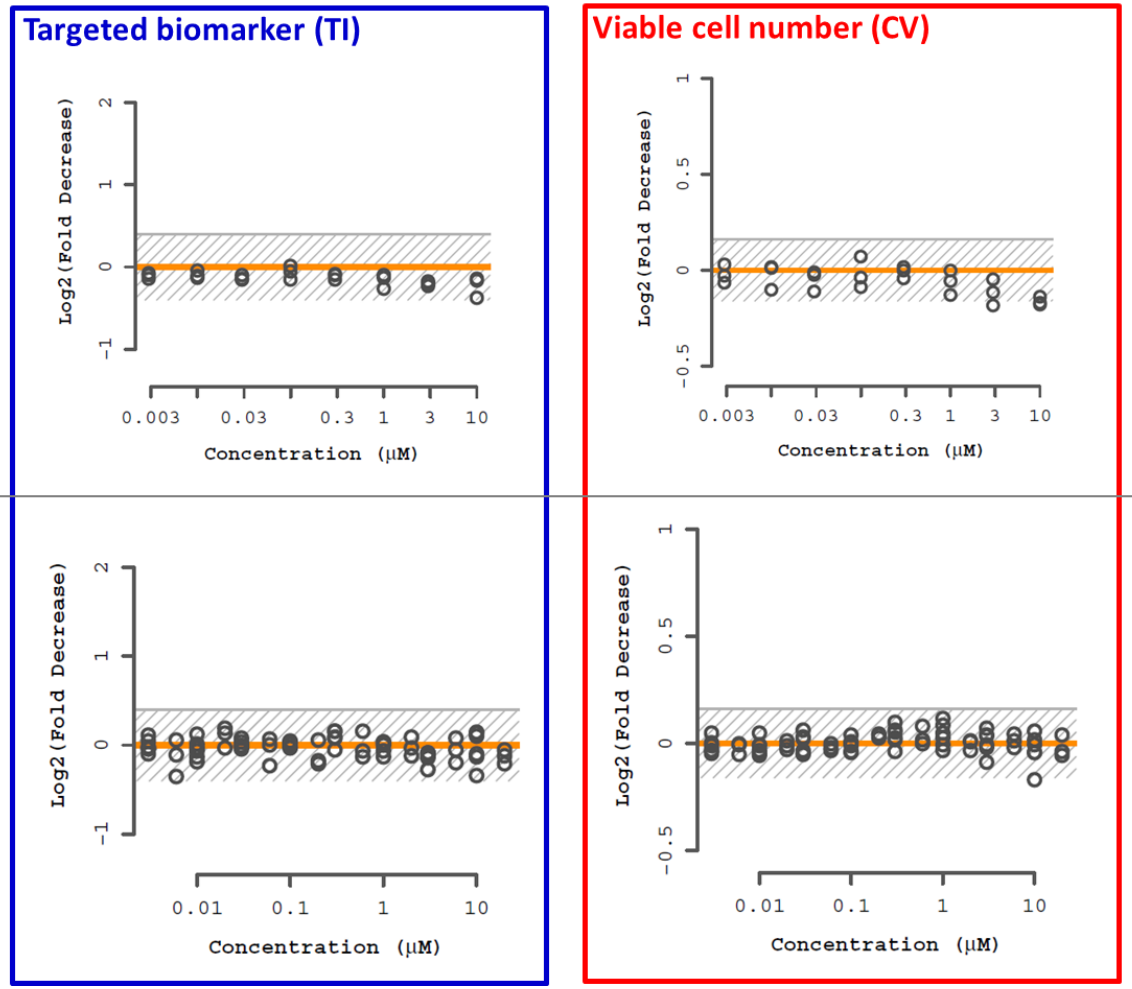
TI = 26 µM, CV = 40.8 µM

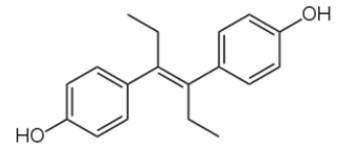
dLEL rat = 5 mg/kg/day (< mLEL)

dLEL rabbit = 50 mg/kg/day (mLEL)

*Zurlinden et al., 2020, Toxicol Sci*

# **Example:** *False Negatives (Not Detected in ToxCast_STM)*



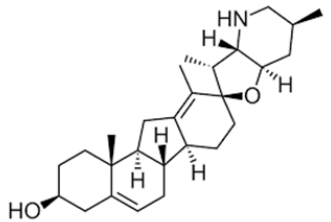




**Diethylstilbestrol (DES)**

TI = NA, CV = NA

dLEL rat = 0.03 mg/kg/day (= mLEL)

(no rabbit data in ToxRefDB)



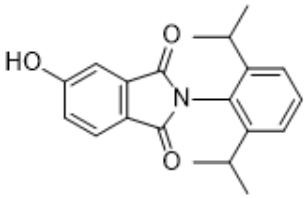

**Cyclopamine**

TI = NA, CV = NA

*Zurlinden et al., 2020, Toxicol Sci*

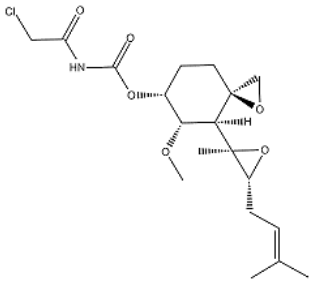
# Case Study to Confirm Forward Predictivity

Colleagues at Dow Chemical, led by Ed Carney, tested T.I. predictions for two structurally diverse potential vascular disrupters (pVDCs) in rat whole embryo culture (WEC):



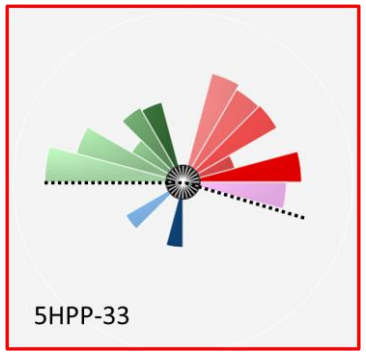

**5HPP-33:** *synthetic thalidomide analog*

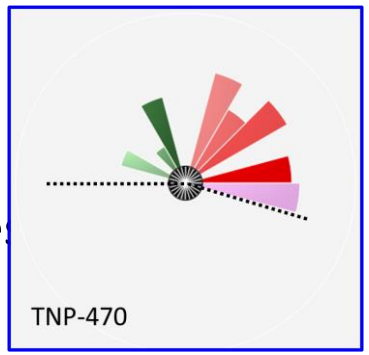
- T.I. predicted by hESC 10.5 µM
- AC50 observed in WEC 21.2 µM (embryo viability)

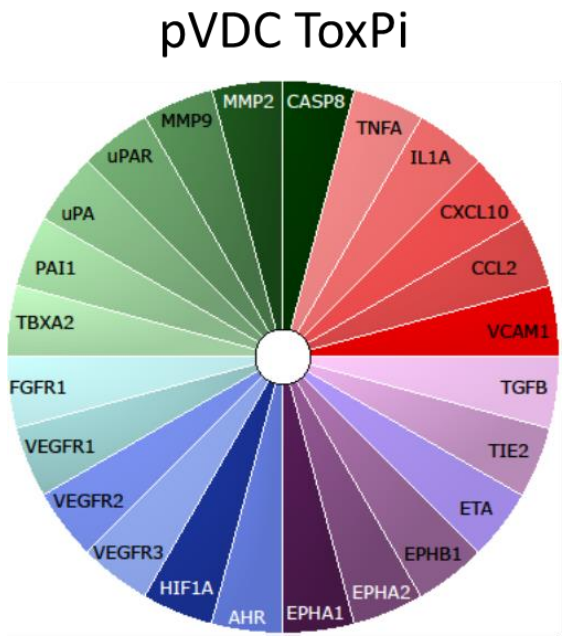



**TNP-470:** *synthetic fumagillin analog*

- T.I. predicted by hESC 0.01 µM
- AC50 observed in WEC 0.04 µM (dysmorphogene



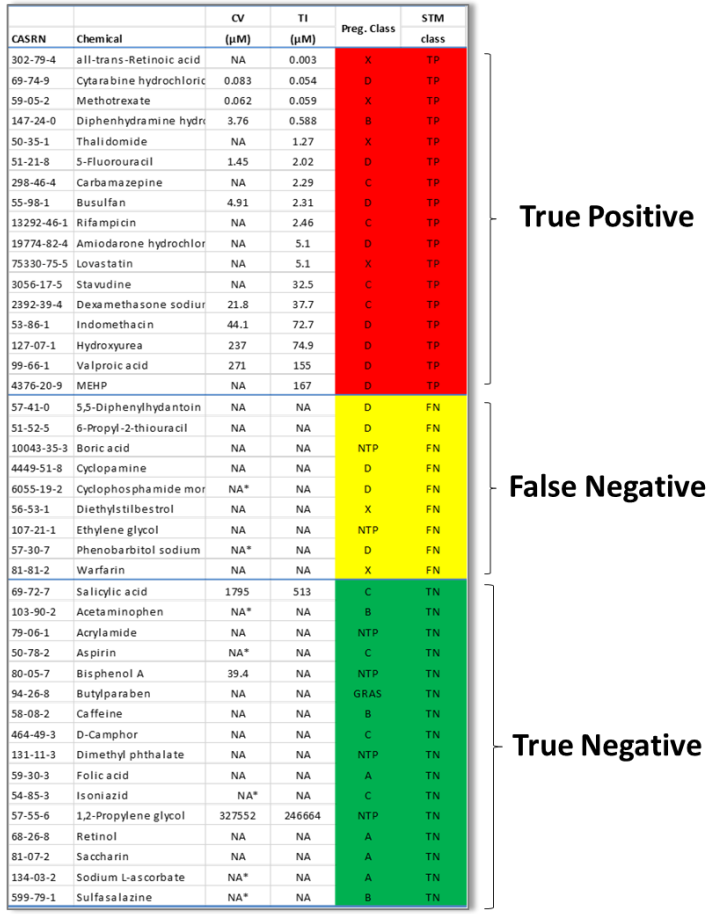




*Ellis-Hutchings et al. (2017) Reprod Toxicol*

# Performance Check for Classification of DevTox

- Qualification on 42 well-curated reference compounds often used to validate alternative DevTox platforms[1].
- Balanced Accuracy (BAC) = 82% (0.65 sensitivity, 1.00 specificity) for these reference chemicals.
- Metrics are consistent with the original pharma-trained model [Palmer et al. 2013].

Many alternative assays have been validated with a limited set of data-rich chemicals, inflating predictive capacity of >80%; this has hampered regulatory acceptance.

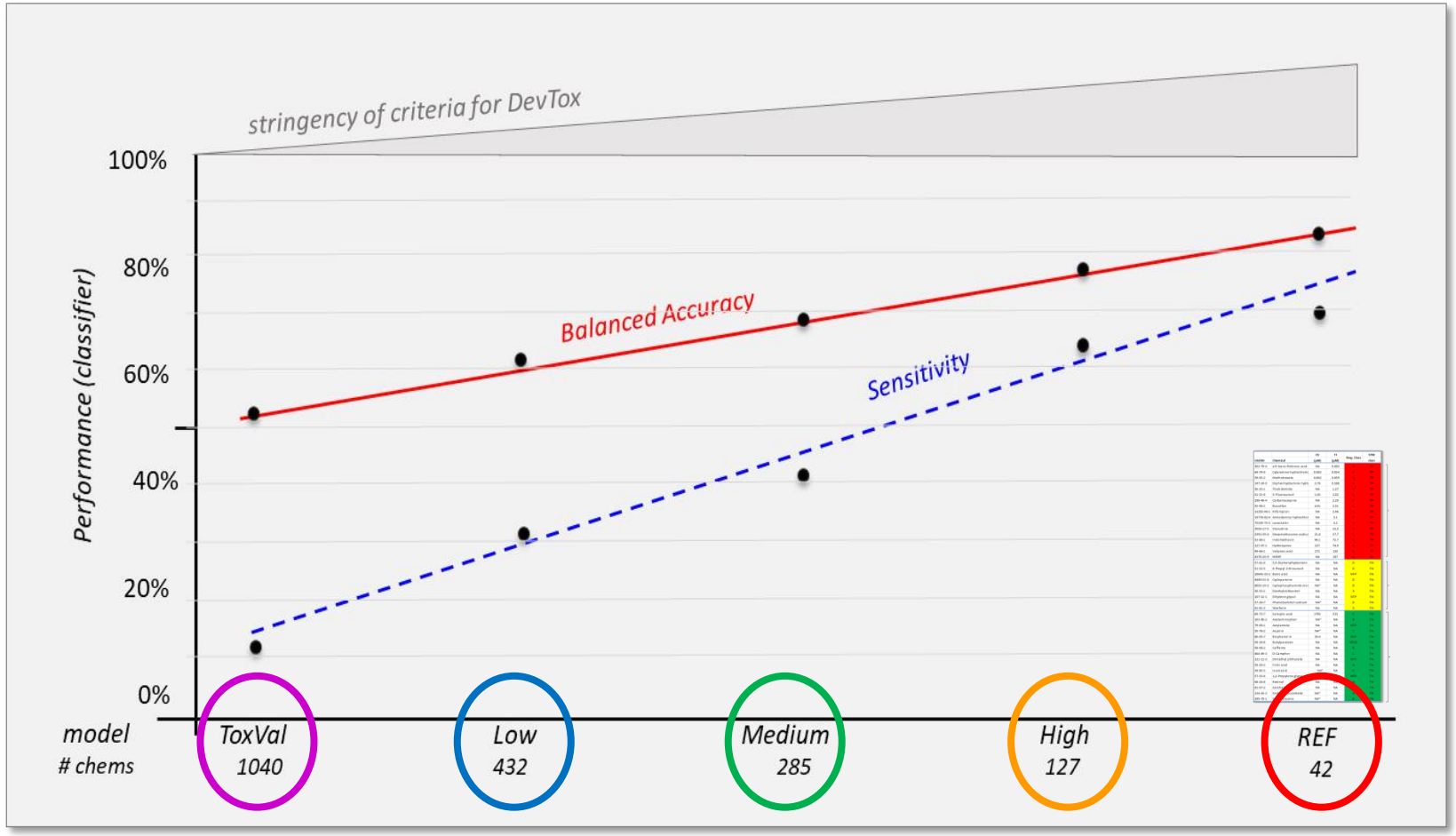


| CASRN | Chemical | CV (µM) | TI (µM) | Preg. Class | STM class | |
|---|---|---|---|---|---|---|
| 302-79-4 | all-trans-Retinoic acid | NA | 0.003 | X | TP | True Positive |
| 69-74-9 | Cytarabine hydrochloric | 0.083 | 0.054 | D | TP | |
| 59-05-2 | Methotrexate | 0.062 | 0.059 | X | TP | |
| 147-24-0 | Diphenhydramine hydr | 3.76 | 0.588 | B | TP | |
| 50-35-1 | Thalidomide | NA | 1.27 | X | TP | |
| 51-21-8 | 5-Fluorouracil | 1.45 | 2.02 | D | TP | |
| 298-46-4 | Carbamazepine | NA | 2.29 | C | TP | |
| 55-98-1 | Busulfan | 4.91 | 2.31 | D | TP | |
| 13292-46-1 | Rifampicin | NA | 2.46 | C | TP | |
| 19774-82-4 | Amiodarone hydrochlor | NA | 5.1 | D | TP | |
| 75330-75-5 | Lovastatin | NA | 5.1 | X | TP | |
| 3056-17-5 | Stavudine | NA | 32.5 | C | TP | |
| 2392-39-4 | Dexamethasone sodiur | 21.8 | 37.7 | C | TP | |
| 53-86-1 | Indomethacin | 44.1 | 72.7 | D | TP | |
| 127-07-1 | Hydroxyurea | 237 | 74.9 | D | TP | |
| 99-66-1 | Valproic acid | 271 | 155 | D | TP | |
| 4376-20-9 | MEHP | NA | 167 | D | TP | |
| 57-41-0 | 5,5-Diphenylhydantoin | NA | NA | D | FN | False Negative |
| 51-52-5 | 6-Propyl-2-thiouracil | NA | NA | D | FN | |
| 10043-35-3 | Boric acid | NA | NA | NTP | FN | |
| 4449-51-8 | Cyclopamine | NA | NA | D | FN | |
| 6055-19-2 | Cyclophosphamide mor | NA* | NA | D | FN | |
| 56-53-1 | Diethylstilbestrol | NA | NA | X | FN | |
| 107-21-1 | Ethylene glycol | NA | NA | NTP | FN | |
| 57-30-7 | Phenobarbitol sodium | NA* | NA | D | FN | |
| 81-81-2 | Warfarin | NA | NA | X | FN | |
| 69-72-7 | Salicylic acid | 1795 | 513 | C | TN | True Negative |
| 103-90-2 | Acetaminophen | NA* | NA | B | TN | |
| 79-06-1 | Acrylamide | NA | NA | NTP | TN | |
| 50-78-2 | Aspirin | NA* | NA | C | TN | |
| 80-05-7 | Bisphenol A | 39.4 | NA | NTP | TN | |
| 94-26-8 | Butylparaben | NA | NA | GRAS | TN | |
| 58-08-2 | Caffeine | NA | NA | B | TN | |
| 464-49-3 | D-Camphor | NA | NA | C | TN | |
| 131-11-3 | Dimethyl phthalate | NA | NA | NTP | TN | |
| 59-30-3 | Folic acid | NA | NA | A | TN | |
| 54-85-3 | Isoniazid | NA* | NA | C | TN | |
| 57-55-6 | 1,2-Propylene glycol | 327552 | 246664 | NTP | TN | |
| 68-26-8 | Retinol | NA | NA | A | TN | |
| 81-07-2 | Saccharin | NA | NA | A | TN | |
| 134-03-2 | Sodium L-ascorbate | NA* | NA | A | TN | |
| 599-79-1 | Sulfasalazine | NA* | NA | B | TN | |

*Zurlinden et al. (2020), Toxicol Sci*

[1] Genschow et al. 2002; West et al. 2010; Daston et al. 2014; Augustine-Rauch et al. 2016; Wise et al. 2016

# Expanding the Chemical Landscape: *up to 432 Chemicals with Prenatal Rat and/or Rabbit Studies in EPA's ToxRefDB Database*




***Scaling Criteria (ToxRefDB)***

- **BM-42 reference**
- **concordant, rat AND rabbit**
- **dLEL < mLEL, rat OR rabbit**
- **dLEL ≤ 200 mg/kg/day**
- **LEL for any study type**

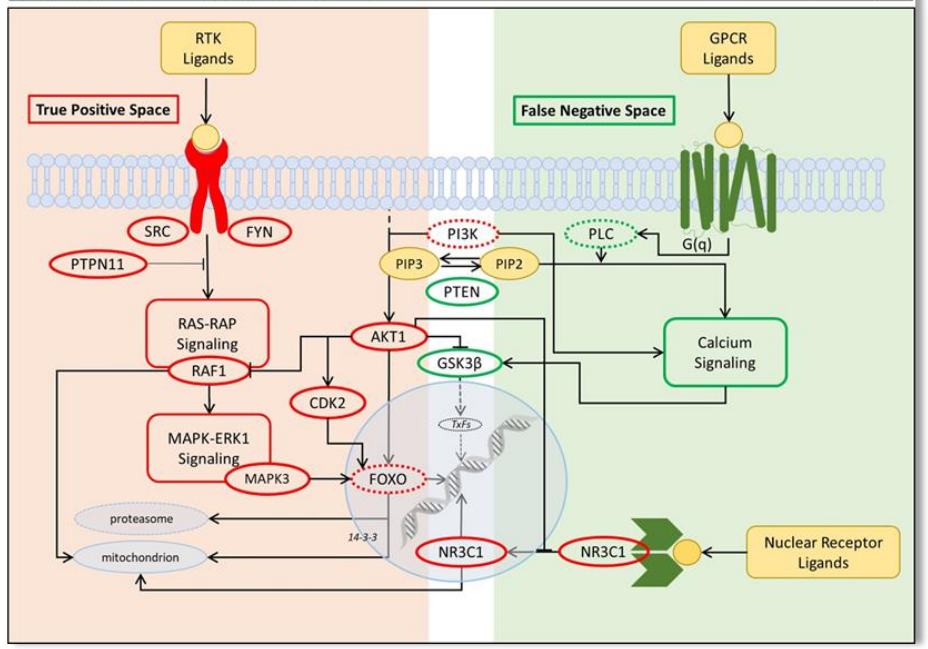
Predictivity of the hPSC biomarker declines as fetal outcomes gain less concordance between rat-rabbit studies, and concurrent maternal toxicity.

*Zurlinden et al. (2020), Toxcol Sci*

# What is the Biological Domain?

**Sensitive Domain** **Insensitive Domain**

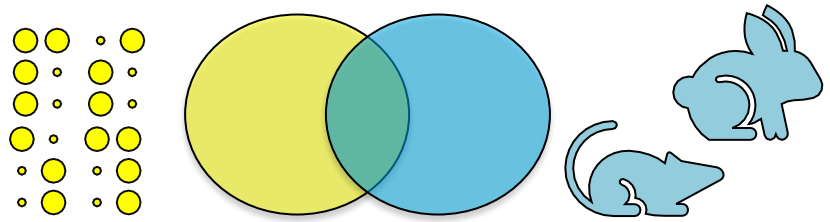
| Annotation System | Keystone Pathway / Process | # MIEs | Class |
|---|---|---|---|
| GOTERM_BP_DIRECT | GO:0014066~regulation of phosphatidylinositol 3-kinase signaling | 6 | TP |
| KEGG_PATHWAY | hsa04068:FoxO signaling pathway | 8 | TP |
| KEGG_PATHWAY | hsa04510:Focal adhesion | 13 | TP |
| GOTERM_BP_DIRECT | GO:0007200~phospholipase C-activating G-protein coupled receptor signaling pathway | 10 | FN |
| INTERPRO | IPR001723:Steroid hormone receptor | 7 | FN |
| GOTERM_MF_DIRECT | GO:0005496~steroid binding | 5 | FN |




- ToxCast_STM correlations for well-curated chemicals to 337 ToxCast_NVS biochemical targets;
- Composite model found the top annotated pathways to which the hPSC assay was sensitive and insensitive;
- Flow of regulatory information to AKT/FOXO signaling in the sensitive domain (true positives);
- G(q) signaling and steroid hormone signaling in the insensitive domain (false negatives).

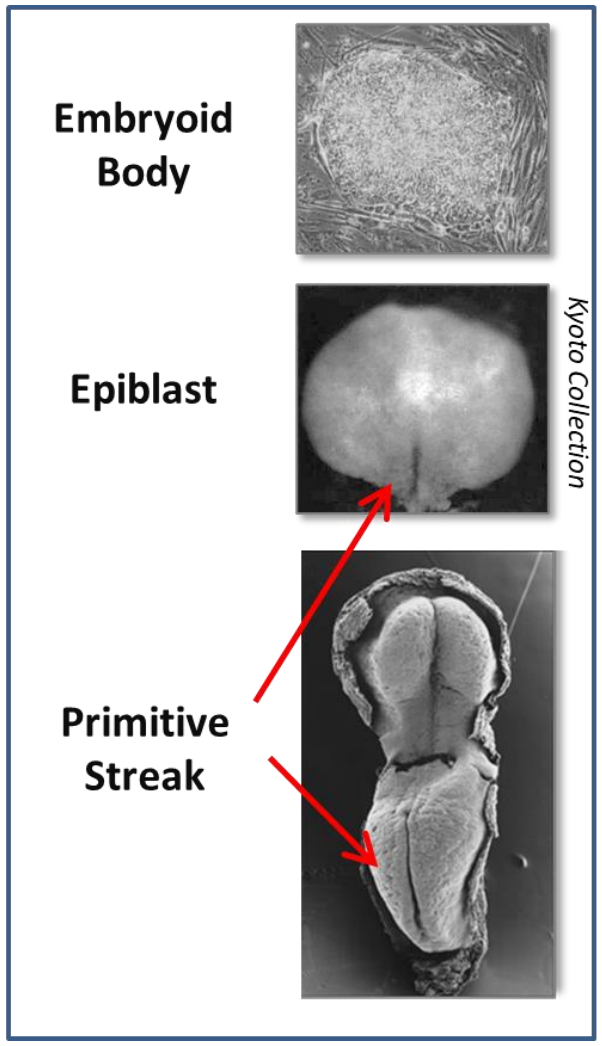
*Zurlinden et al. (2020) Toxicol Sci*

# Bridging Animal-Human Studies



- Query of prenatal developmental studies in EPA's ToxRefDB database found adverse fetal outcome for 53 of 283 (18.7%) chemicals tested in pregnant rats and rabbits.
- Although this incidence closely matches the 19.2% positivity rate from the ToxCast_STM assay, only a subset of the compounds tested positive in both platforms.
- Discordance: (i) biology missed by the hPSC platform; (ii) concurrence of fetal outcomes with maternal toxicity; (iii) mesoscopic properties of complex systems.
- Motivation for building and testing complex 3D **synthetic microsystems** with PSCs to improve mechanistic understanding of developmental processes and toxicities.

# Gastrulating Embryo: *Remarkable Example of a Self-Organizing System*



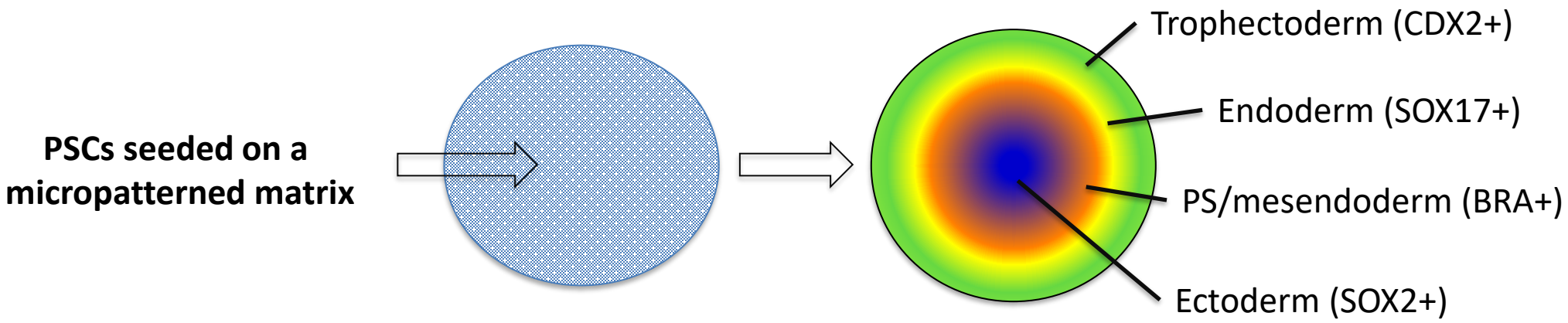

- The molecular biology and behavior of hPSCs in culture most closely resembles the **epiblast** of an early embryo during 'gastrulation'.
- The hallmark of gastrulation in *Vertebrata* is **primitive streak** formation through which the genomic body plan is set up.
- Cell migration through the primitive streak is essential for spatial organization, regional specification, and lineage determination.
- Although cultured hPSCs can form most cell types in the fetus they lack **positional information** of an intact epiblast.

"It is not birth, marriage, or death, but **gastrulation** which is truly the most important time in your life." *- Lewis Wolpert*

# Geometric Confinement

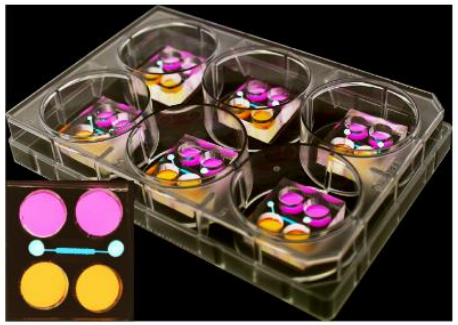
- Randomly seeded PSCs readily generate primary germ layers in culture; however, patterns of differentiation are heterogeneous and spatially disordered.

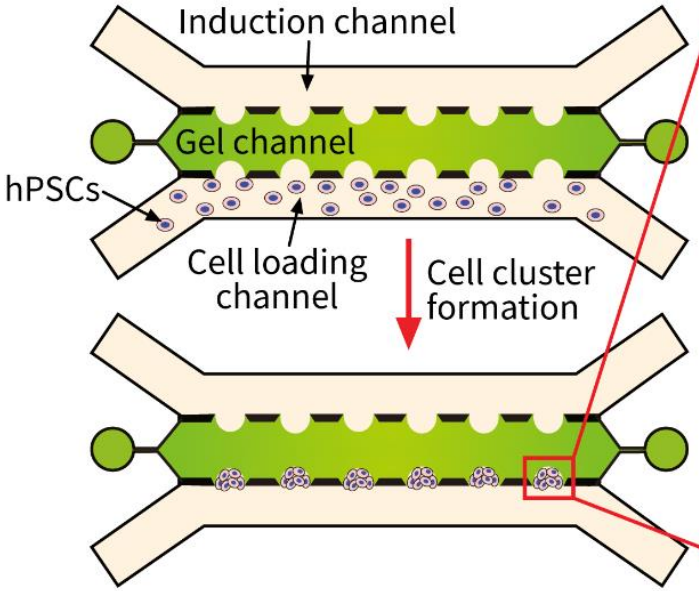
- PSCs differentiated on a micropatterned surface express lineage-specific markers and self-organize in symmetrical domains [Martinez Arias et al. (2014) *Development;* Warmflash et al. (2014) Nat Meth].

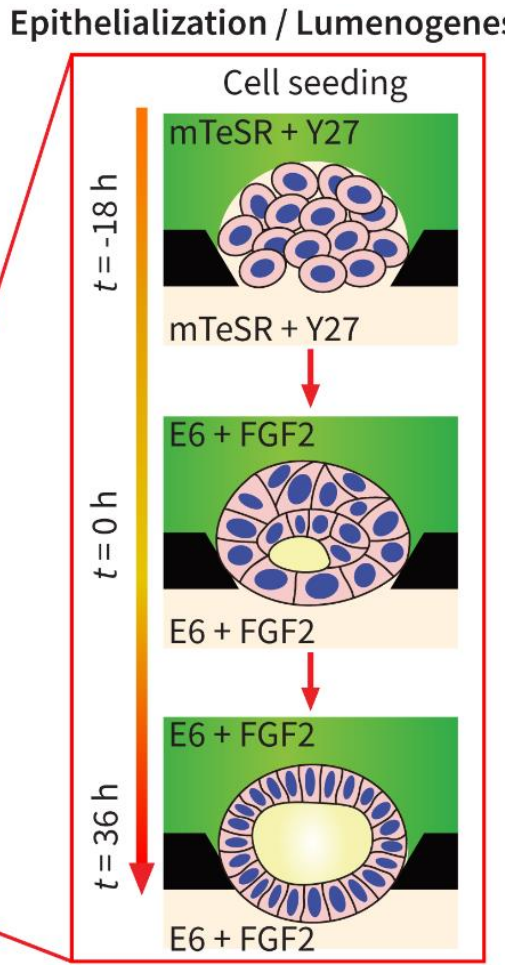


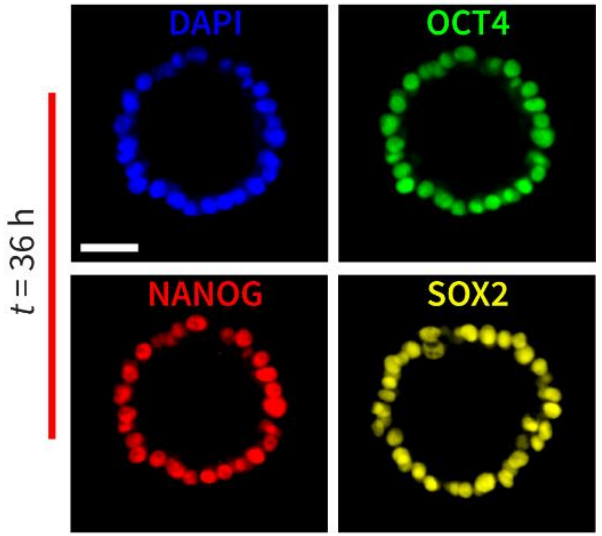

***Micropatterned differentiation***

# Synthetic Epiblast: *Microphysiological System*

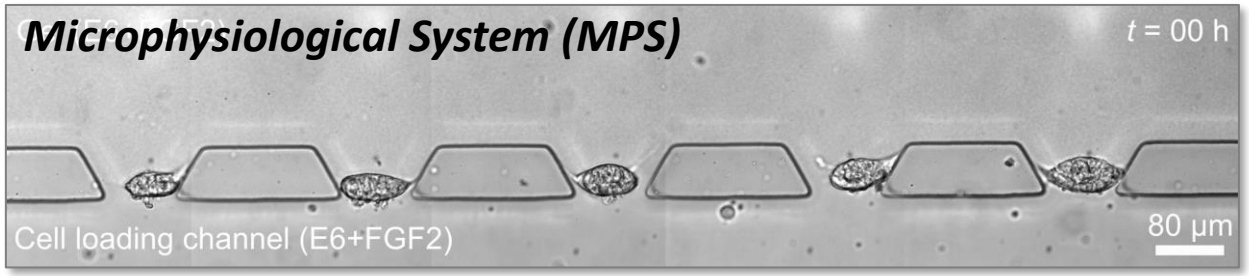




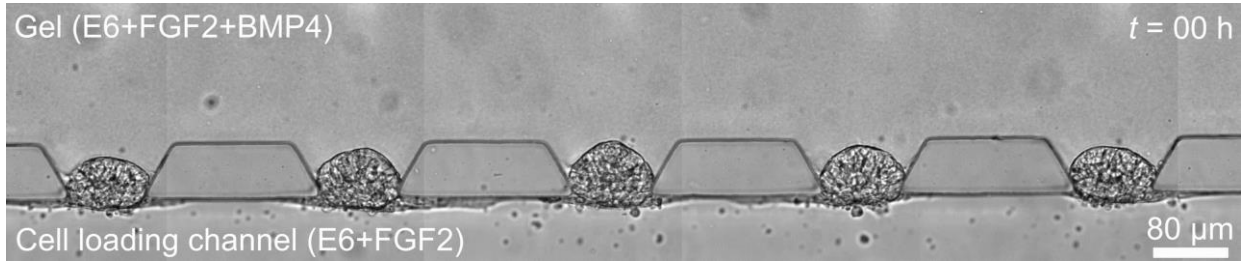



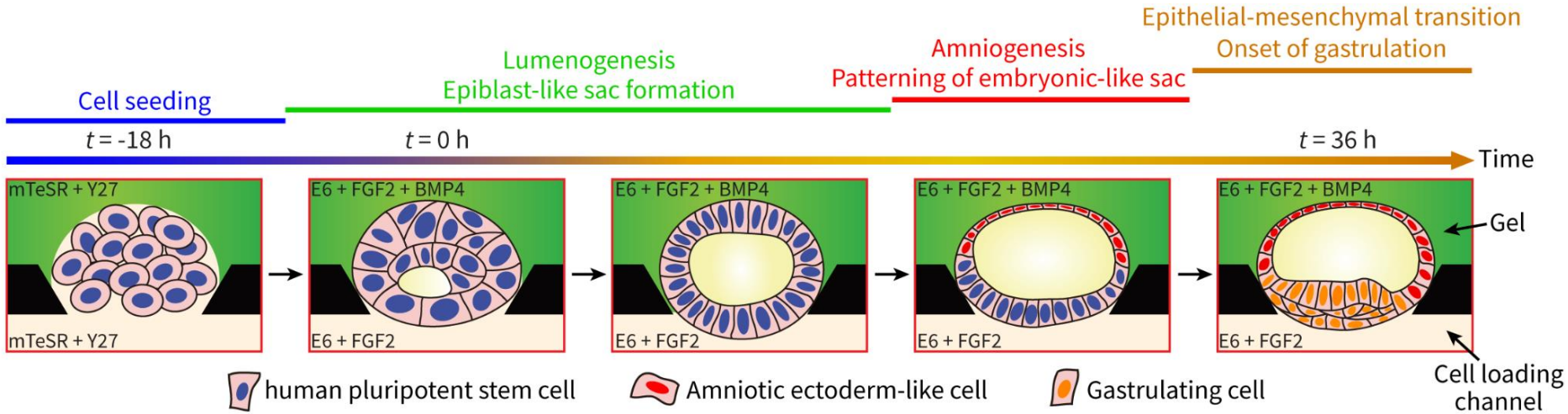

- FGF at 18h induces lumenization (36h):
- Pluripotency markers (OCT4/NANOG/SOX2);
- Intrinsic radial symmetry (spheroid).

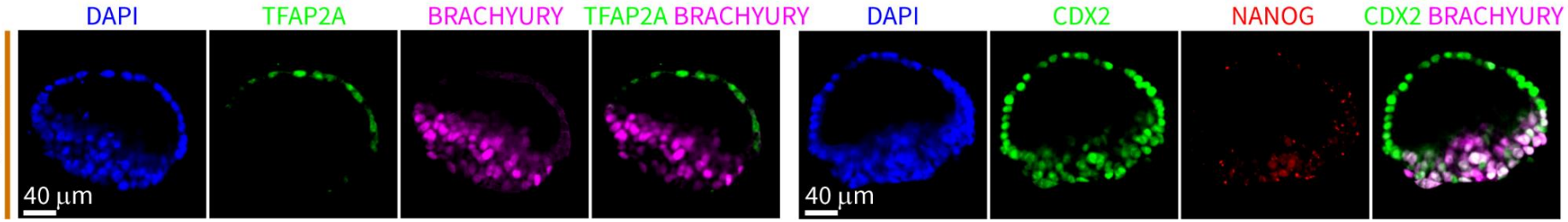



*Shared by Jianping Fu, from Zheng et al. Nature (2019)*

# Breaking the Symmetry



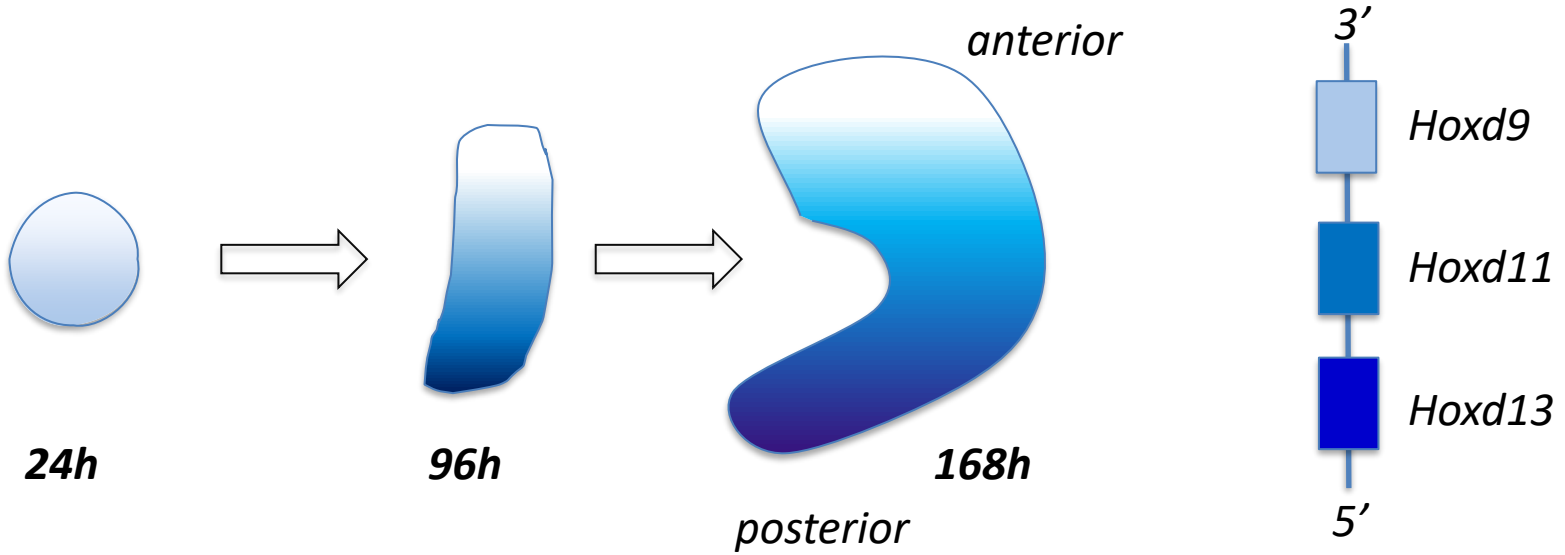

- BMP4 gradient breaks intrinsic symmetry.
- BMP4 primes posterior cell fate.
- Distinct axial domains emerge.
- Pluripotency advances to a determined state.
- But a bona fide primitive streak has not formed.

*Shared by Jianping Fu, from Zheng et al. Nature (2019)*

# Gastruloids

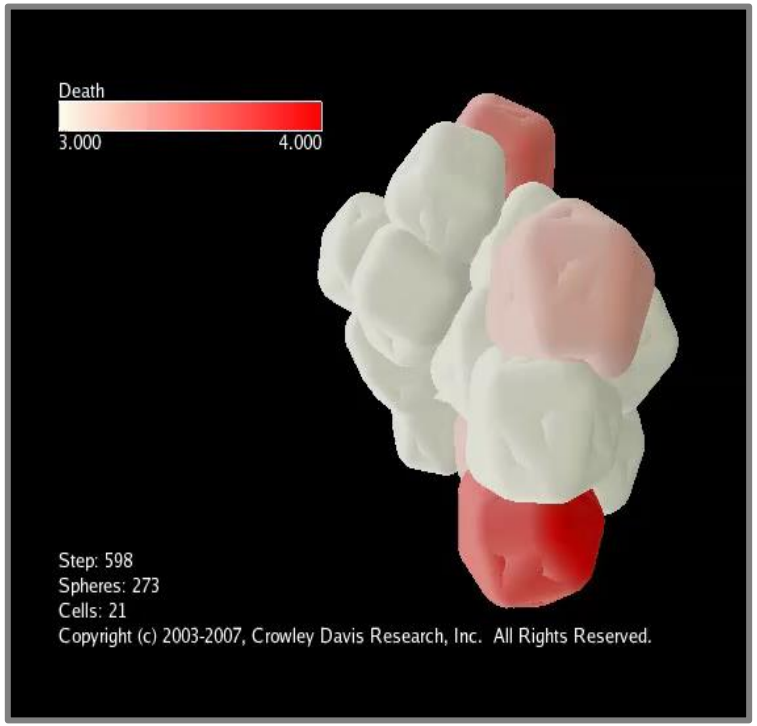
- Mouse ESCs aggregated with defined numbers of cells and grown under certain culture conditions spontaneously organize into axial structures, referred to as ‘gastruloids’.

- These display hallmarks of postcranial axial gene regulatory systems such as colinear Hox expression along an extending antero-posterior axis [*Beccari et al. (2018), Nature*].



# A More Synoptic View …

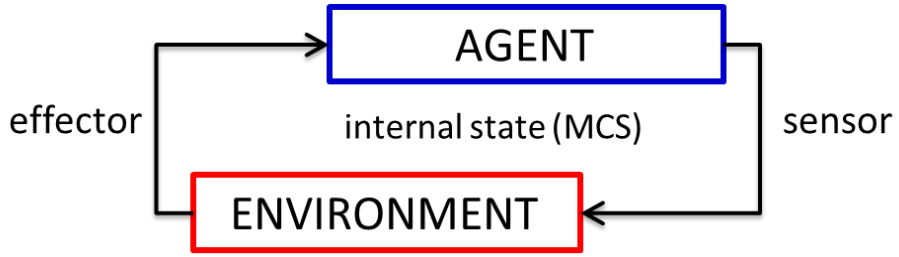
*Anatomical homeostasis in a self-regulating 'Virtual Embryo'*




*Shared by Tim Otter, from Andersen et al. (2006) Am. Assoc. Artif. Intel.*

- **synthetic microsystems:** recapitulate the microphysiology and cellular behaviors of a physical system.
- **computational intelligence:** biological-inspired algorithms use fuzzy logic to fill in missing or incomplete information.
- **artificial life:** computer simulation of biological processes evolved through automation, control networks.

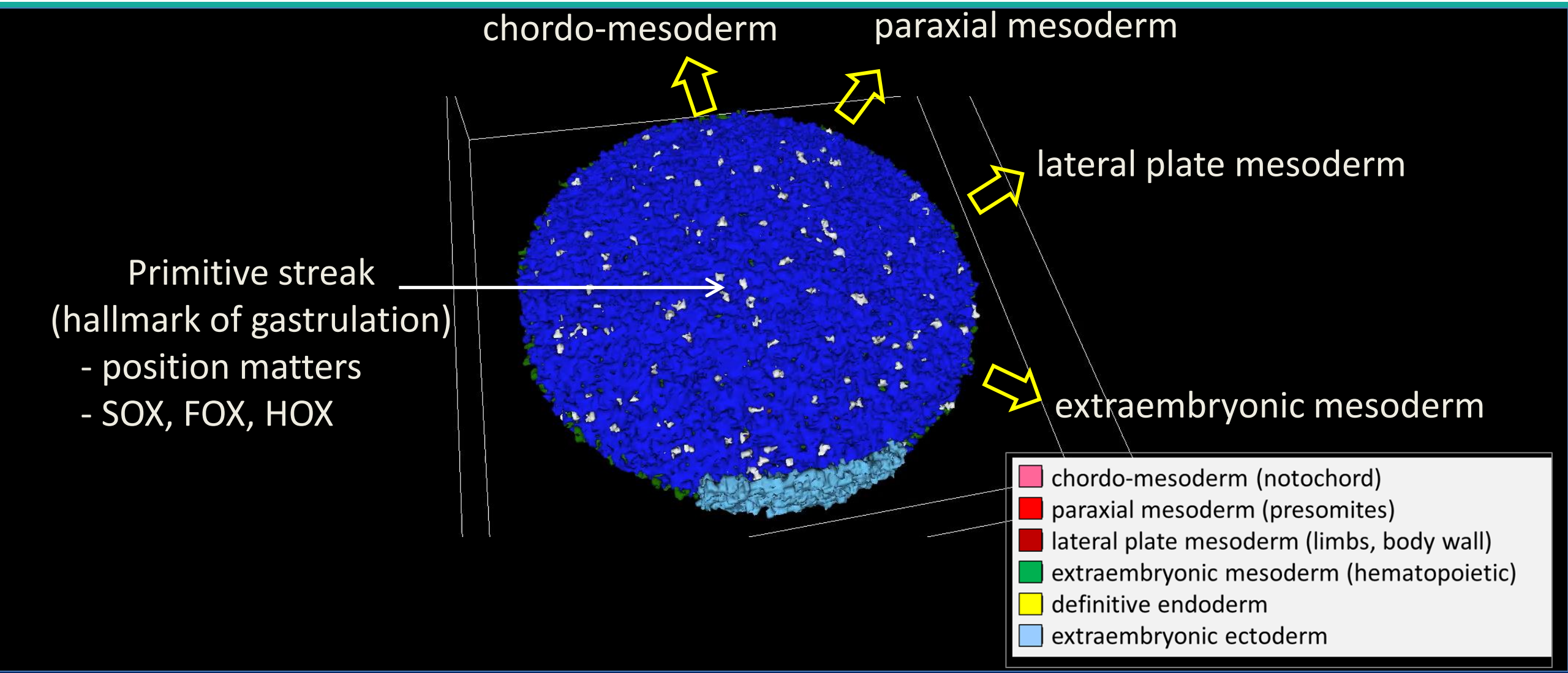
# Agent-Based Models (ABMs)




- Nature-inspired *agents* (cells) and *rules* (behaviors) are set into motion as a self-organizing virtual system, using an open-source modeling environment (**CompuCell3d.org**).
- Soft-computing uses 'fuzzy logic' to simulate forces or properties governing cell fate and behavior where rules are inexact or knowledge incomplete (**computational intelligence**).
- Can change course in response to a particular situation or stimulus, such as genetic errors or biomolecular lesions fed to the model from real world data (**dynamic translation**).
- Probabilistic rendering of where, when and how a particular condition might lead to an adverse developmental outcome (**cybermorphs**).

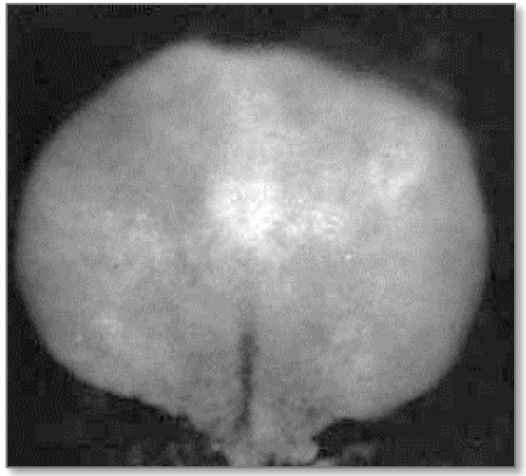
*"Molecular biology took Humpty Dumpty apart … mathematical modeling is required to put him back together again."* – *Schnell et al. (2007) Amer Scientist*

# Quasi-gastrulation: *recoding the genomic blueprint of the fetal body plan?*



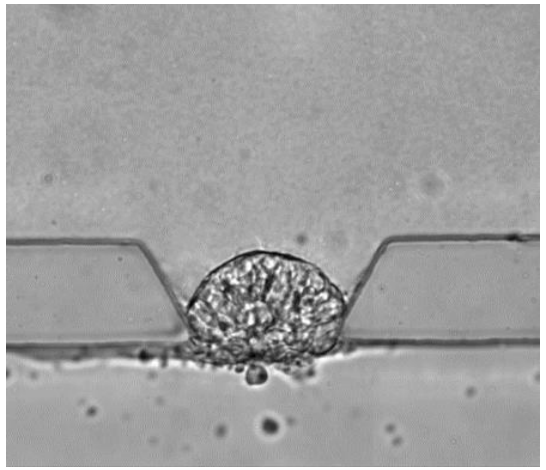
# Practical Use of a Synoptic Manifold

***In vivo***



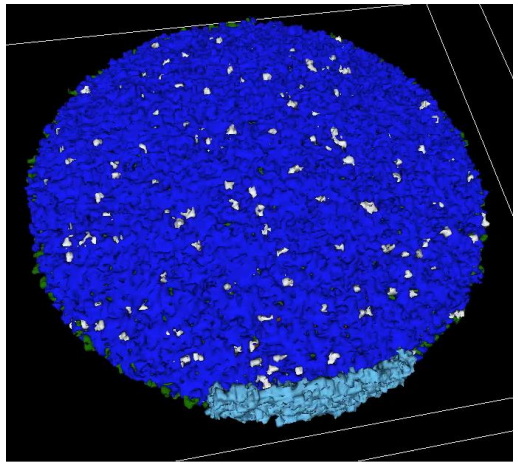
*Kyoto Collection*

***In vitro***



*Jianping Fu, Univ Michigan*

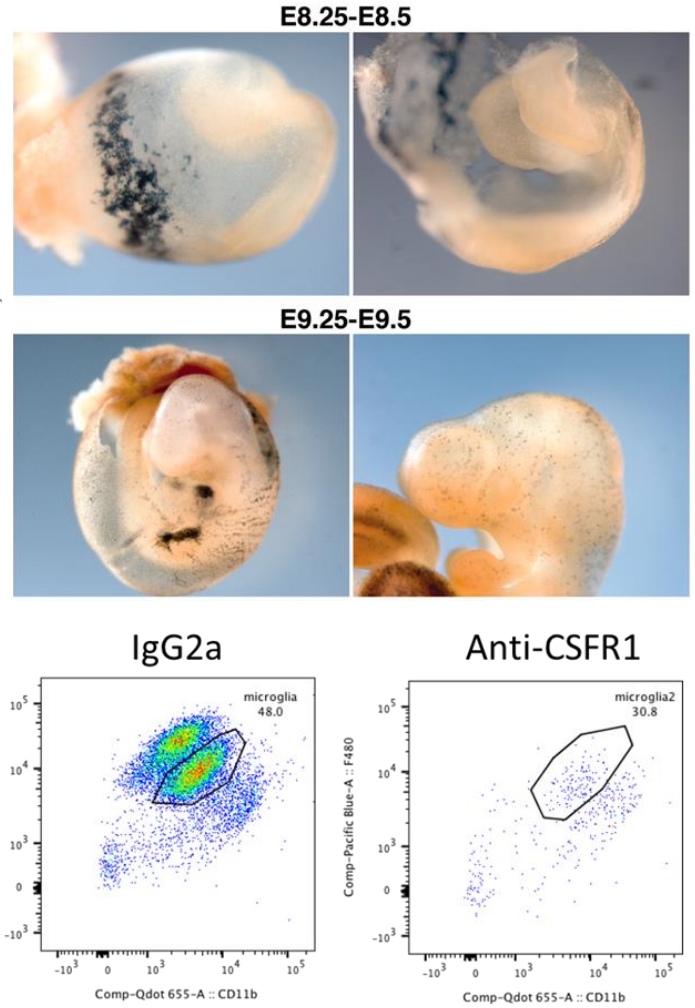
***In silico***



*Richard Spencer, EPA-EMVL*

- MPS models can probe the interaction of physical geometry and cell signaling;
- FGF2 and BMP4 is a start, but still other signals needed to position a primitive streak;
- ABM adds positional information and tracks individual cell behaviors;
- quantitatively simulate what chemical exposures could impose at the cellular level;
- provide inferences on developmental effects in a human-relevant manner.

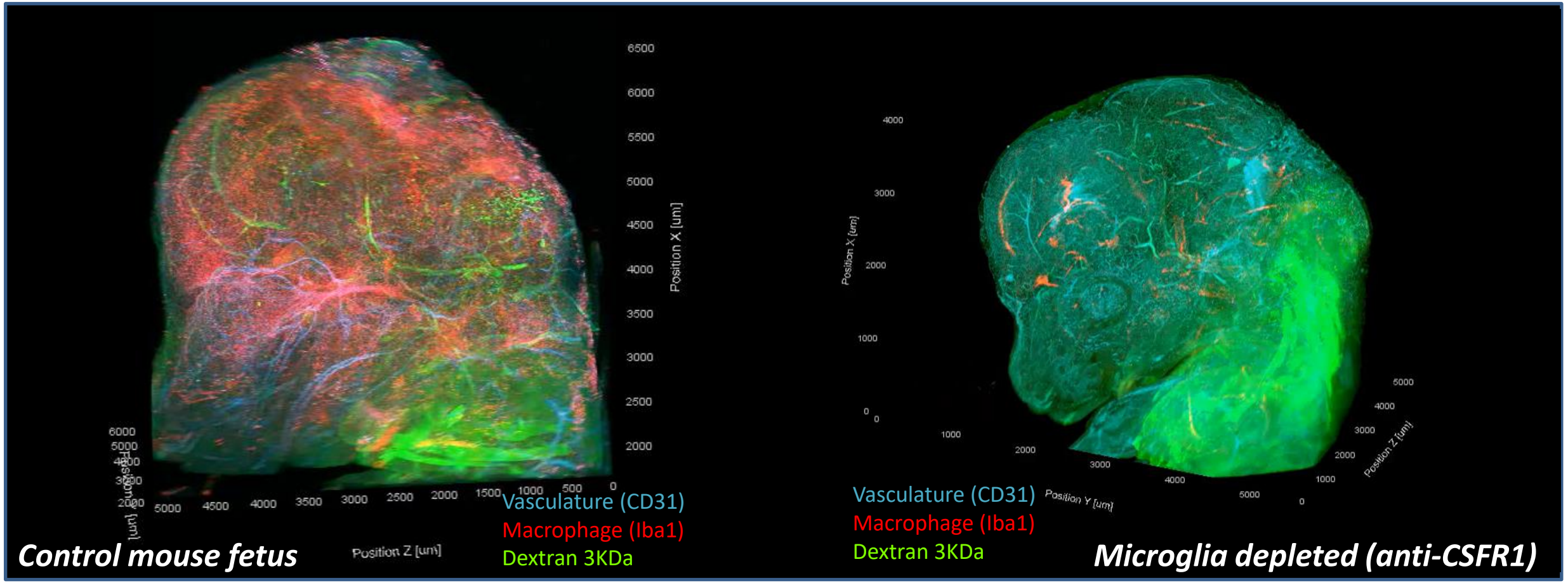
# Microglia and Neurovascular Patterning




Ginhoux et al 2010, *Science*

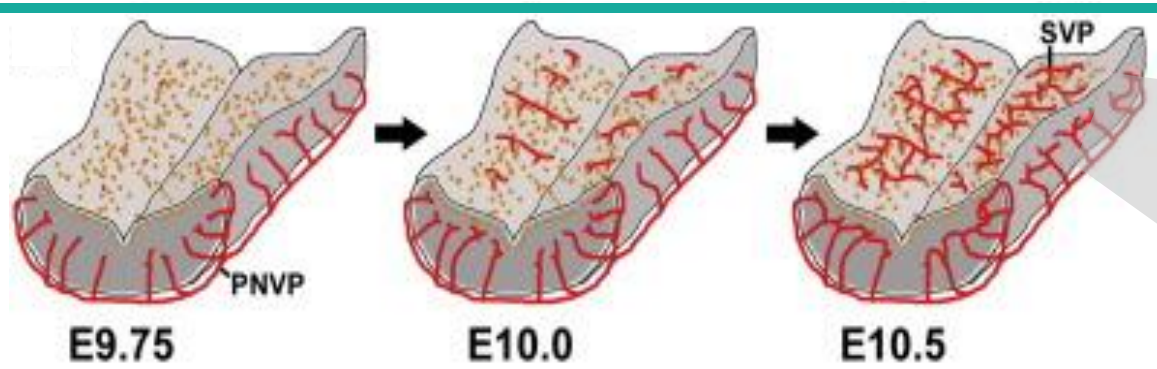
- Vascularization of the neural tube commences on E9-10 (mouse) with formation of blood-brain barrier by E11.
- Microglia from yolk sac blood islands form on E8 and circulate to colonize the neuroepithelium by E9.
- Anti-CSFR1 treatment on E6.5 -7.5 depletes 95-99% of the microglial population in the brain by E14.5.
- Microglia have 3 phenotypic states: M0 (resting), M1 (activated), M2 (protective).
- Microglia orchestrate neurovascular patterning, but when stress-activated → neuroinflammatory response.

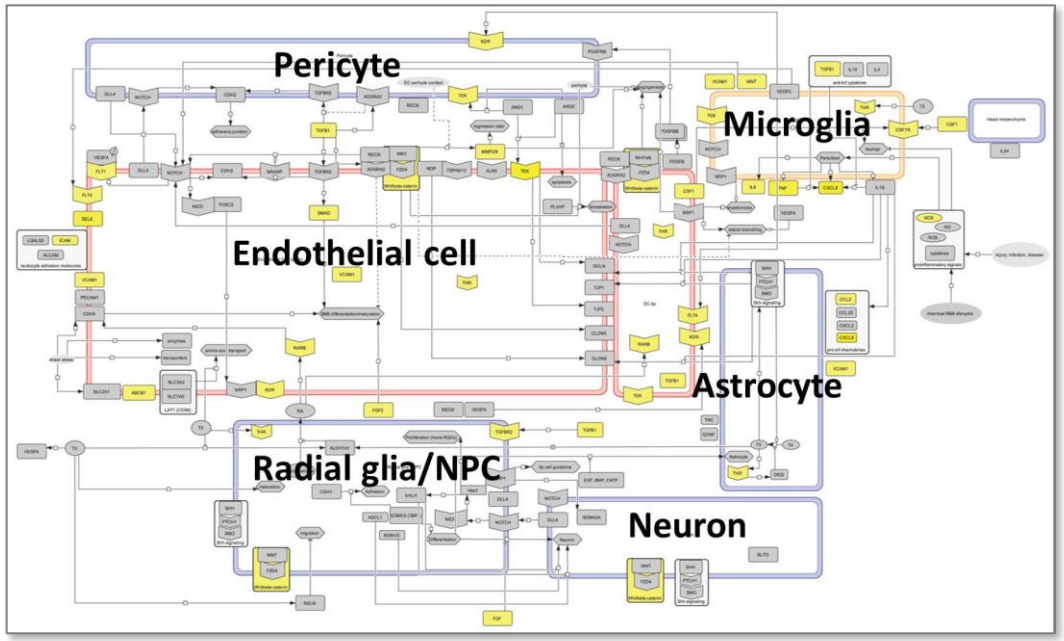
# **Microglia Depletion:** *reduces angiogenesis of the fetal brain and impairs or delays the development of barrier function of the microvasculature.*



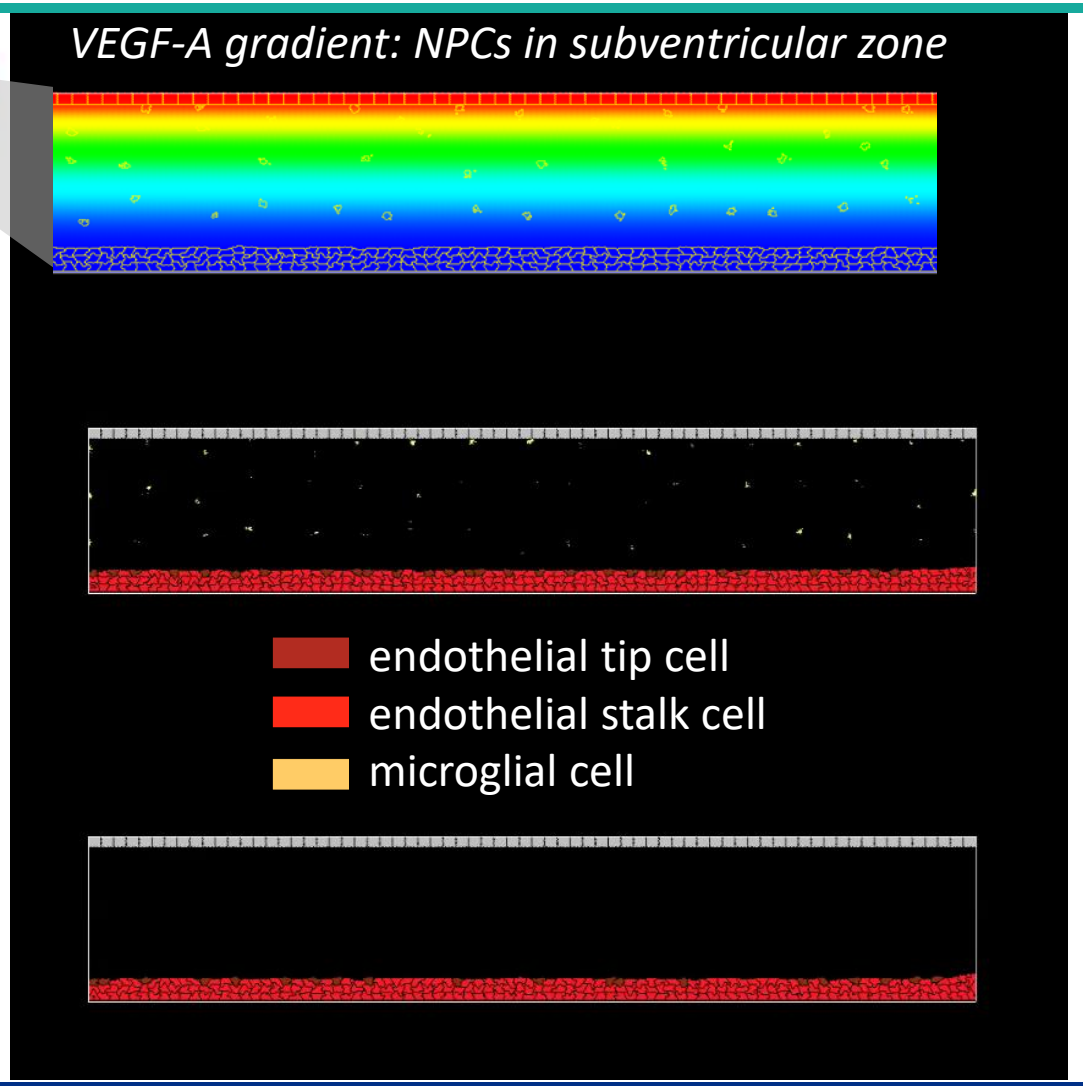

*EPA-A*STAR collaboration with A Silvin, F Ginhoux – A*STAR/SIgN*

# Computational Systems Model

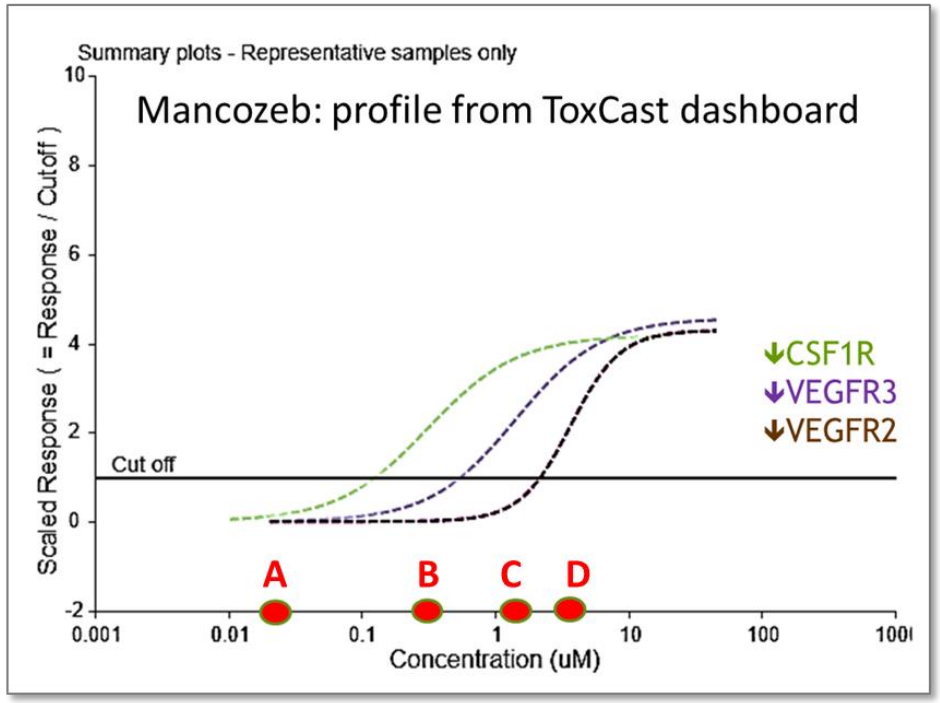


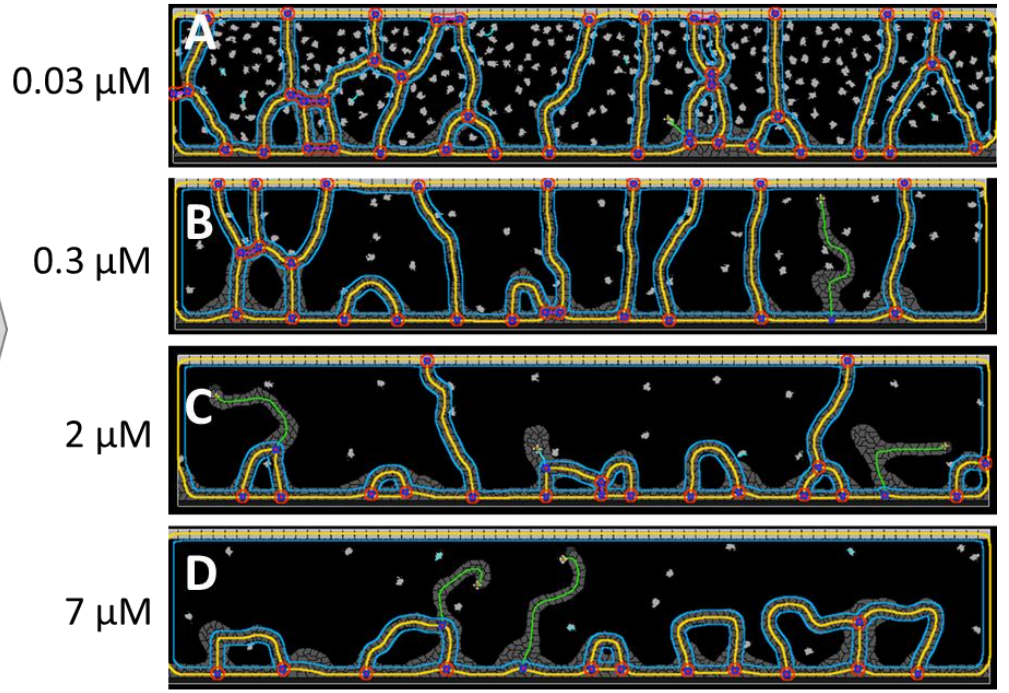



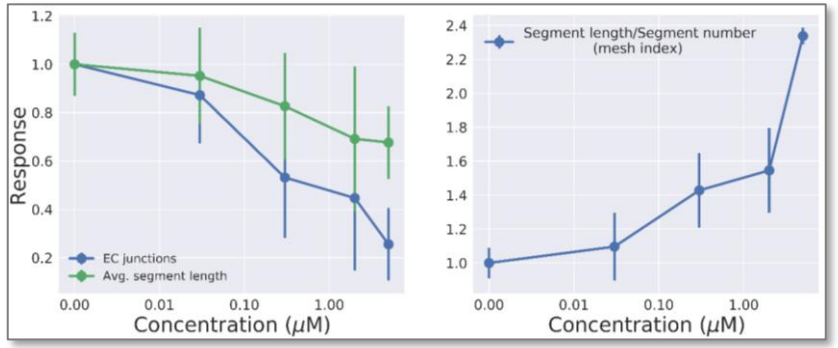

*Produced in CellDesigner v4.4*



# Executing a simulated concentration-response

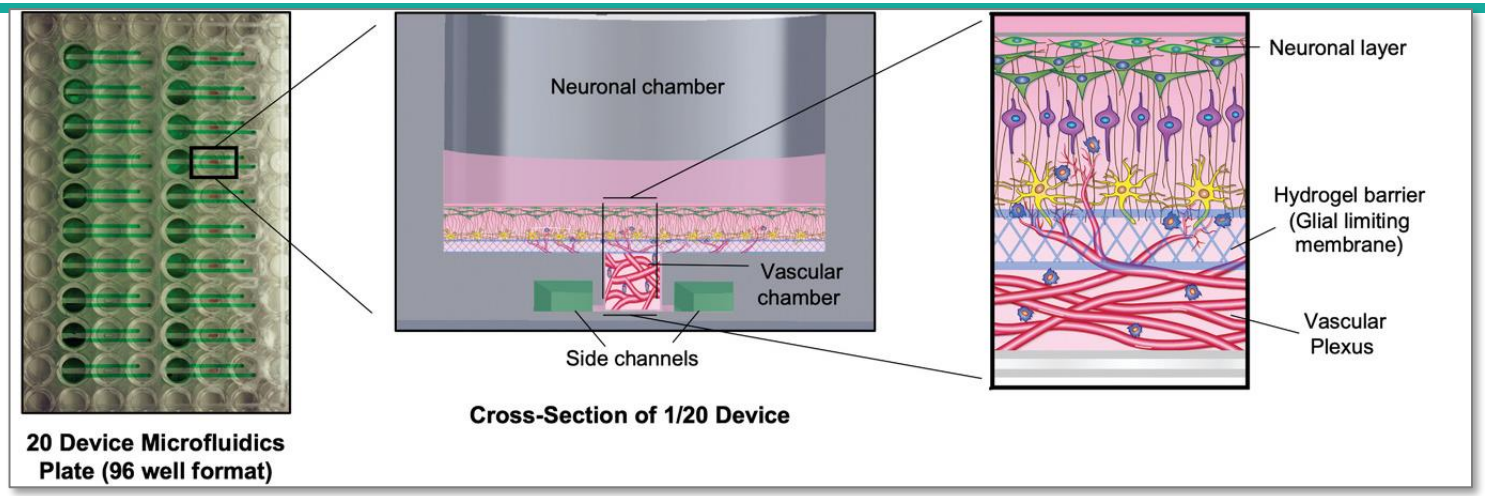



- Prediction: affects microglial-endothelial interaction (reduced tortuosity → deficiency of SVZ).
- Quantitative microvascular 'cybermorphs' predicts an AC50 for Mancozeb disruption at 0.5 µM.

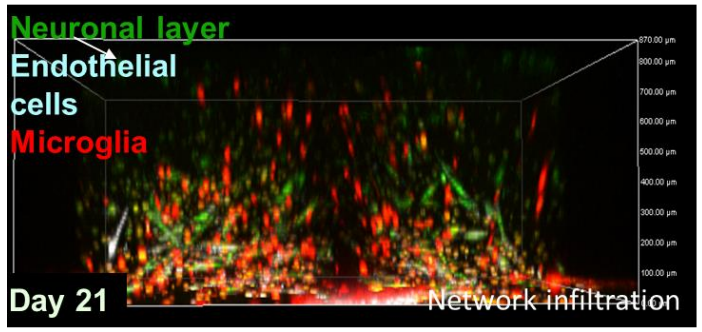
# Checking the prediction: *microglial integration in a synthetic microsystem*



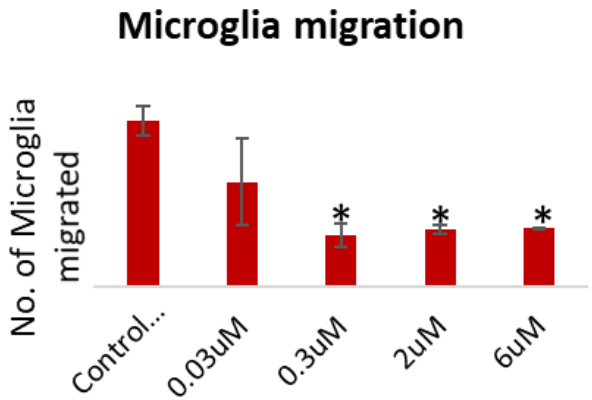

**Engineered Perineural Vascular Plexus for Modeling Developmental Toxicity**

*Gaurav Kaushik, Kartik Gupta, Victoria Harms, Elizabeth Torr, Jonathan Evans, Hunter J. Johnson, Cheryl Soref, Suehelay Acevedo-Acevedo, Jessica Antosiewicz-Bourget, Daniel Mamott, Peyton Uhl, Brian P. Johnson, Sean P. Palecek, David J. Beebe, James A. Thomson, William T. Daly,* and William L. Murphy**

*Kaushik et al. (2020), Adv Hlthc Materials*




Microglia Migration into Neural Layer



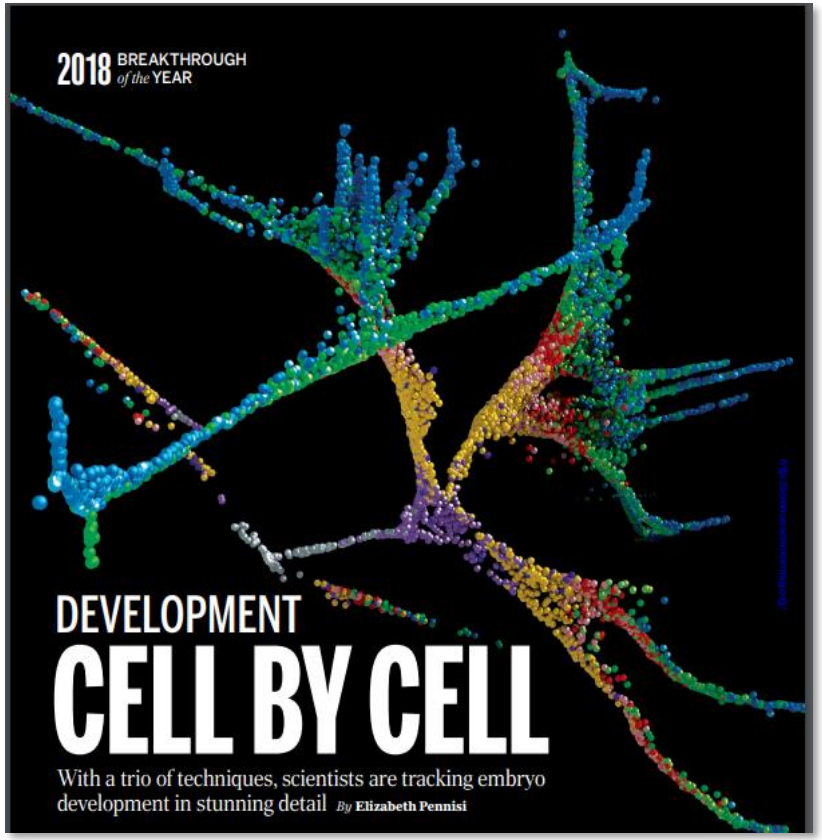

Critical concentration (PoD) for Mancozeb on neural tube vascularization:

- ***predicted*** *by in silico cNVU = 0.5 µM*
- ***observed*** *in organotypic culture = 0.3 µM.*

*Microglial states may be an important sentinel for neurodevelopmental toxicity.*

*EPA STAR Center Co-operative grant #835737 , Univ Wisconsin (W Murphy)*

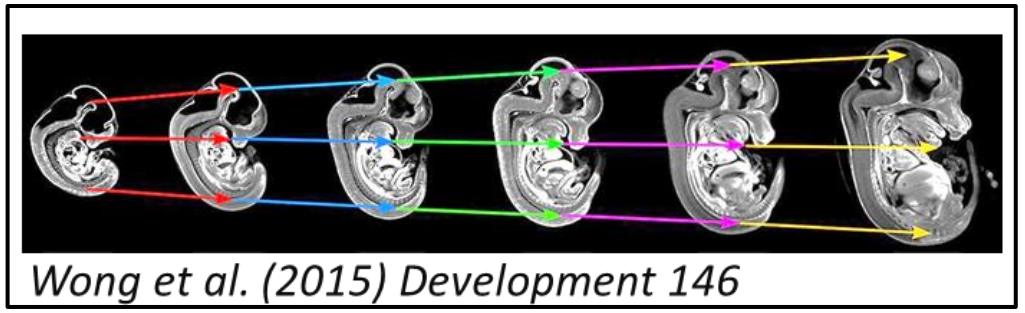
# An Evolving Challenge . . .

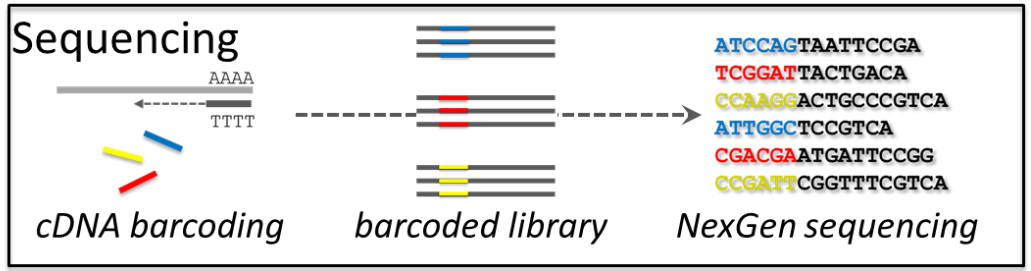


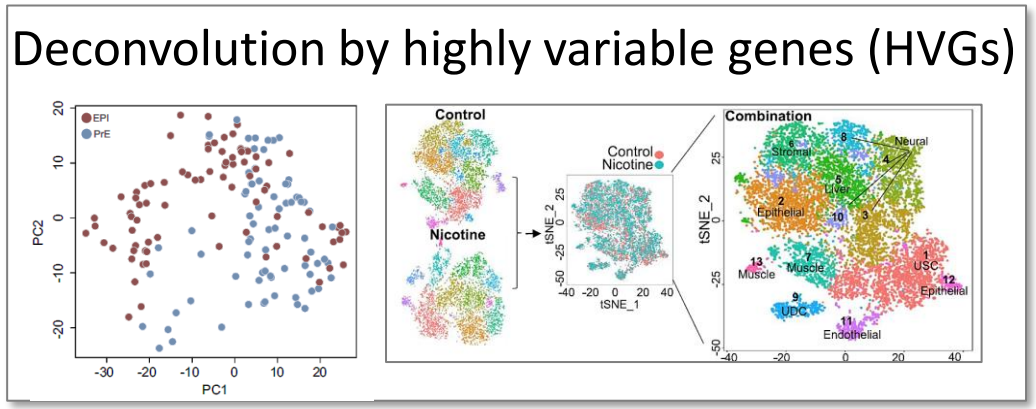

- Define **sentinel cells** that propagate chemical injury to a structural defect.
- Bringing embryology into the fold to improve mechanistic understanding.
- Profiling development at the single cell level voted *Science* magazine's breakthrough of the year 2018.

https://science.sciencemag.org/content/sci/362/6421/1344.full.pdf

# Why Profile Transcriptomes at the Single Cell Level?



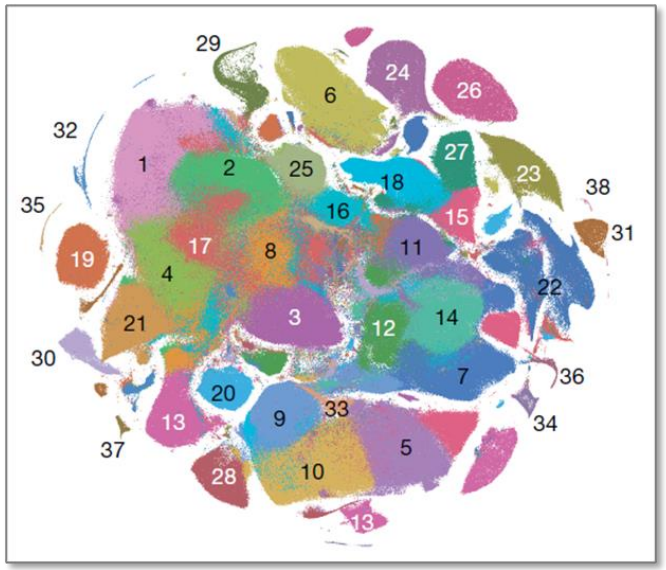

- **scRNAseq fulfills the need for greater detail:**
  - Molecular progression of all cell lineages;
  - Cellular control of progression at every step;
  - Higher resolution of pathogenesis.

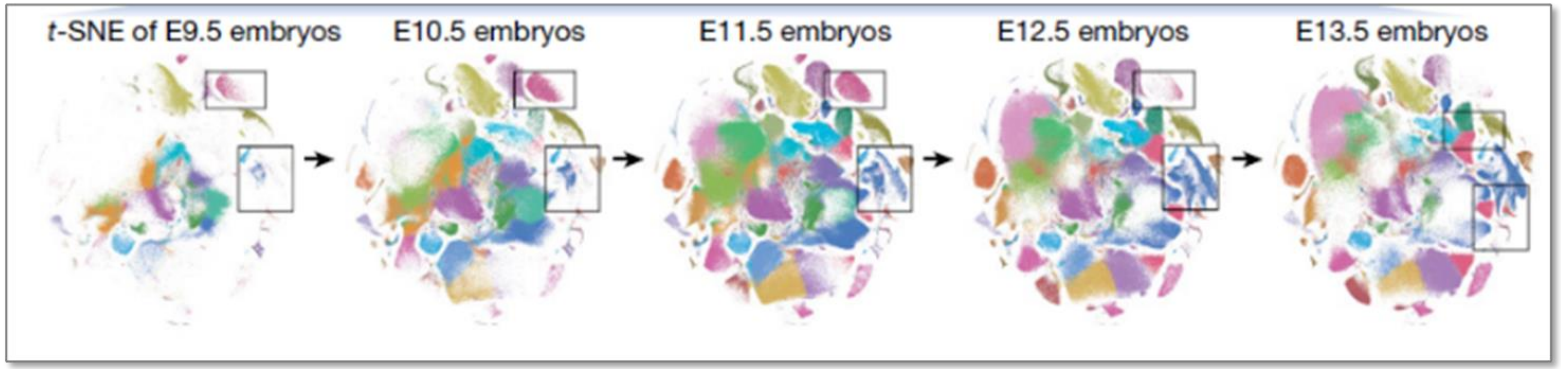
- **Practical applications:**
  - Map pathogenesis with cellular precision;
  - Mechanistic detail for predictive toxicology;
  - New ways to unravel biological complexity.

# Organogenesis

*Cell state landscape has a higher dimensionality (655 cell states) than the lineage map (38 cell types)*

1–Connective tissue progenitors
2–Chondrocytes and osteoblasts
3–Intermediate mesoderm
4–Jaw and tooth progenitors
5–Excitatory neurons
6–Epithelial cells
7–Radial glia
8–Early mesenchyme
9–Neural progenitor cells
10–Postmitotic premature neurons
11–Oligodendrocyte progenitors
12–Isthmic organizer cells
13–Myocytes
14–Dorsal neural tube cells
15–Inhibitory neurons
16–Stromal cells
17–Osteoblasts
18–Inhibitory neuron progenitors
19–Premature oligodendrocytes
20–Endothelial cells
21–Chondroctye progenitors
22–Definitive erythrocyte lineage
23–Schwann cell precursors
24–Sensory neurons
25–Limb mesenchyme
26–Primitive erythroid lineage
27–Inhibitory interneurons
28–Granule neurons
29–Hepatocytes
30–Notochord and floor plate cells
31–White blood cells
32–Ependymal cells
33–Cholinergic neurons
34–Cardiac muscle lineage
35–Megakaryocytes
36–Melanocytes
37–Lens
38–Neutrophils




*Video courtesy of M Spielmann, from Cao et al. (2019), Nature*

# Looking Ahead …

**Translational:** what do synthetic models of human development - both computational and organoids - bring to future of DART testing?

**Investigational:** how smart must these models be (A.I.) to support decision-making in the animal-free (3Rs) zone?

**Operational:** what best practices are needed to implement synthetic models into an integrative decision framework (eg, AOP-based IATAs)?

**Communication:** what are the practical considerations for science, engineering, and stakeholder engagement (academics, government, industry, NGOs, policy, …)?

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