Contemporary Reproductive and Developmental Toxicity Testing: New Approaches and Methodologies

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Dedicated to the Prevention of Birth Defects

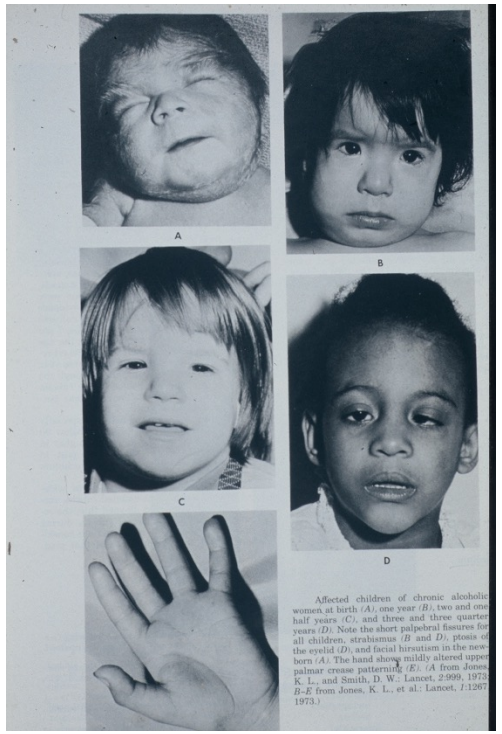
methyl mercury

environmental

thalidomide

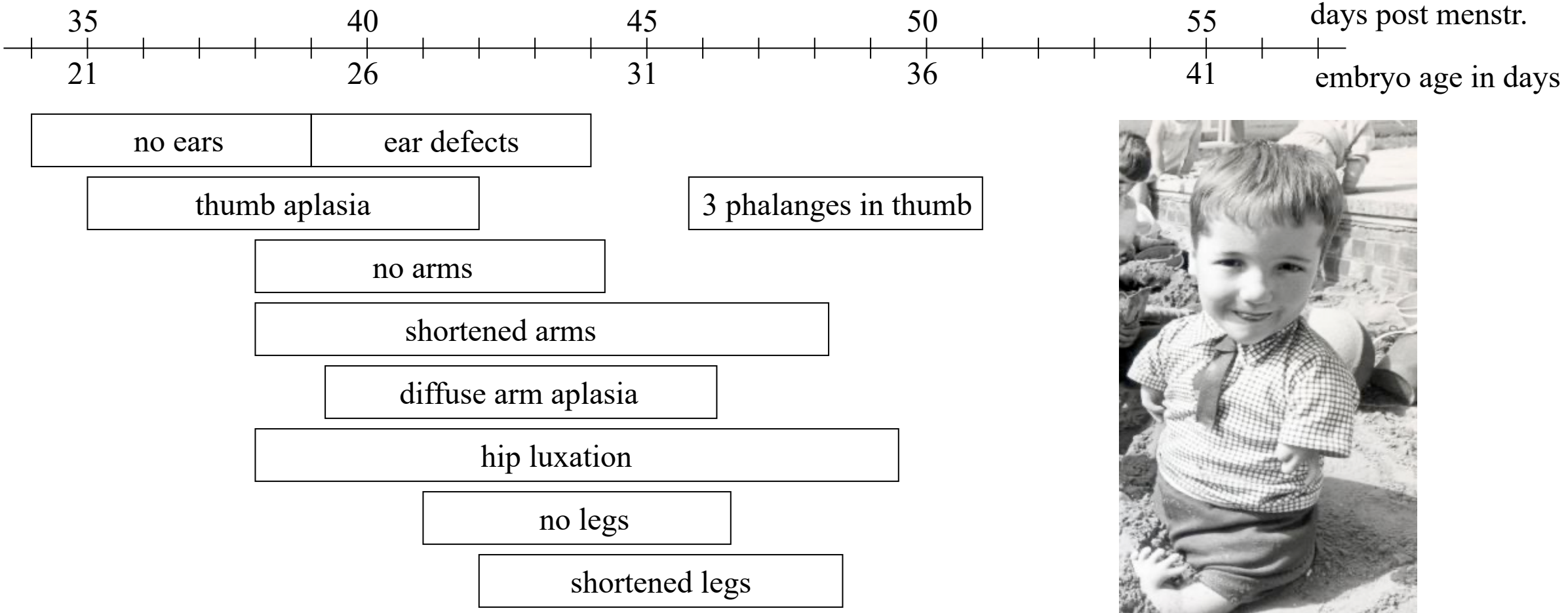
pharmaceutical

alcohol

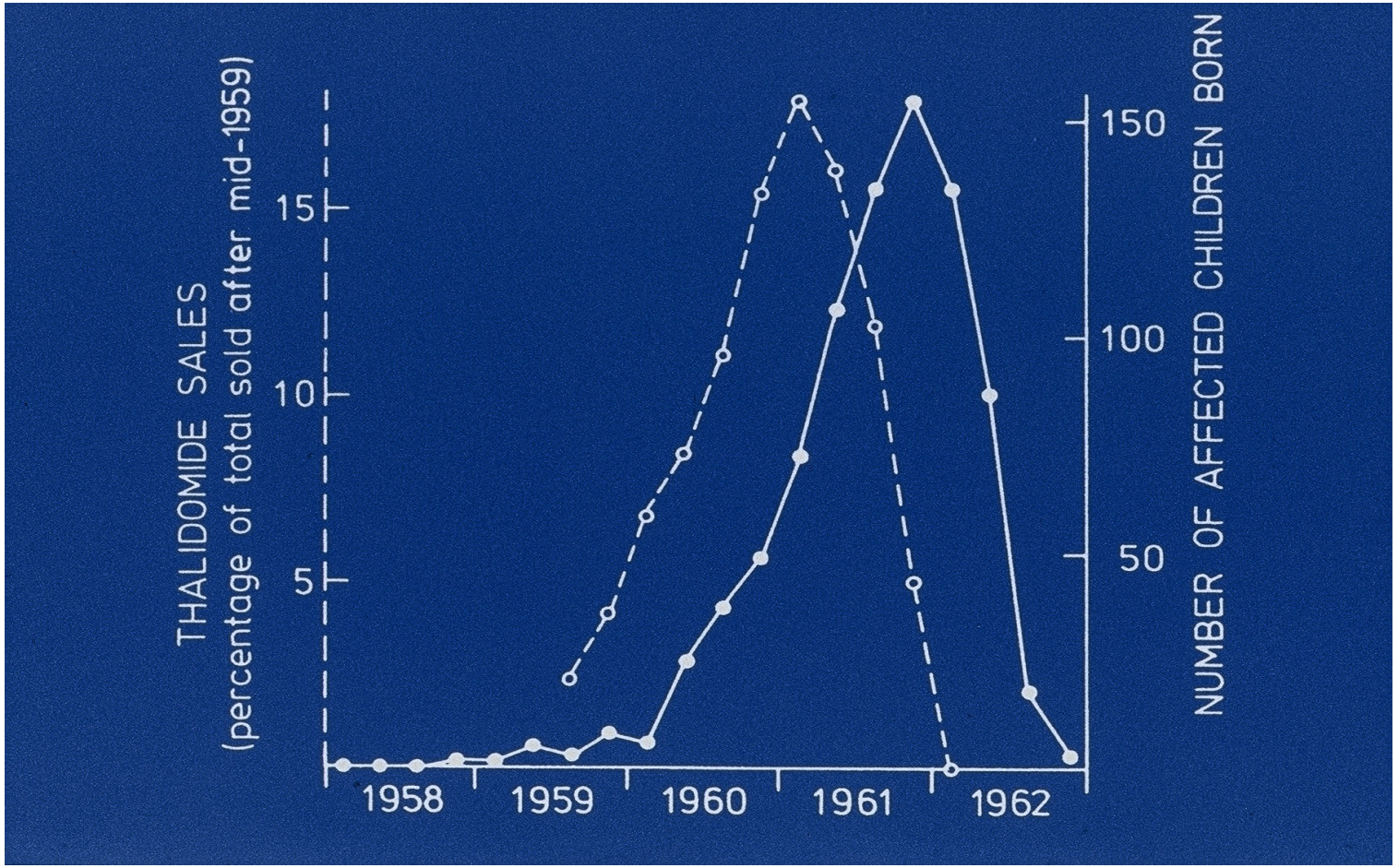


behavioural

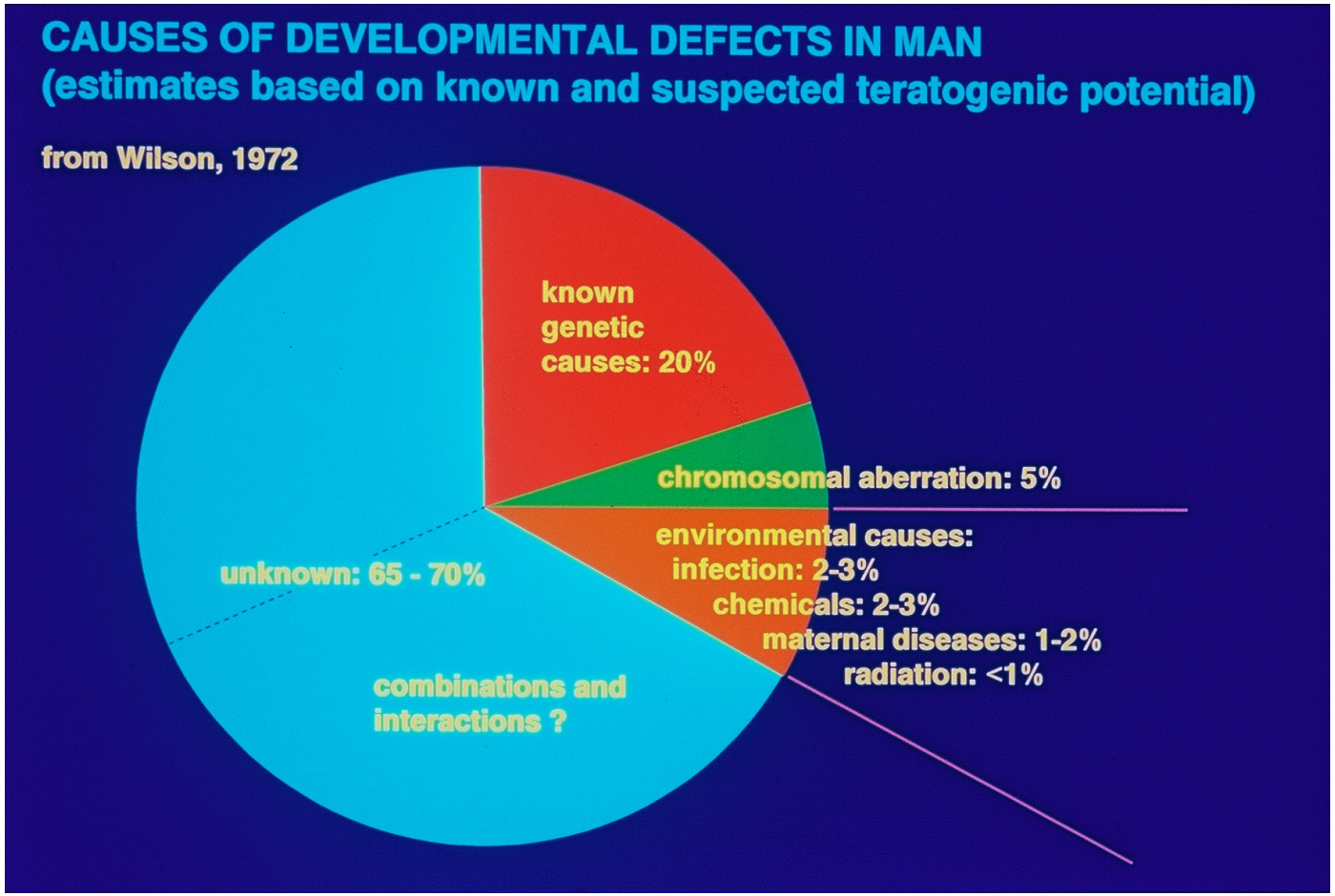
Thalidomide exposure-effect timing



Thalidomide epidemiology



(Un)known causes of birth defects



RDT safety evaluation of chemicals

- Since the 1960ies hazard and risk assessment has been largely based on animal studies.
- Guidelines for standardized animal study protocols have been harmonized globally through OECD.
- The prenatal developmental toxicity study and the generation study became central for RDT assessment.
- Guideline studies have been largely focused on adverse health outcomes.
- Extrapolation factors are used to translate overall No Observed Adverse Effect Level (NOAEL) into Point of Departure for human risk assessment (default 10x10 for intra- and interspecies differences).

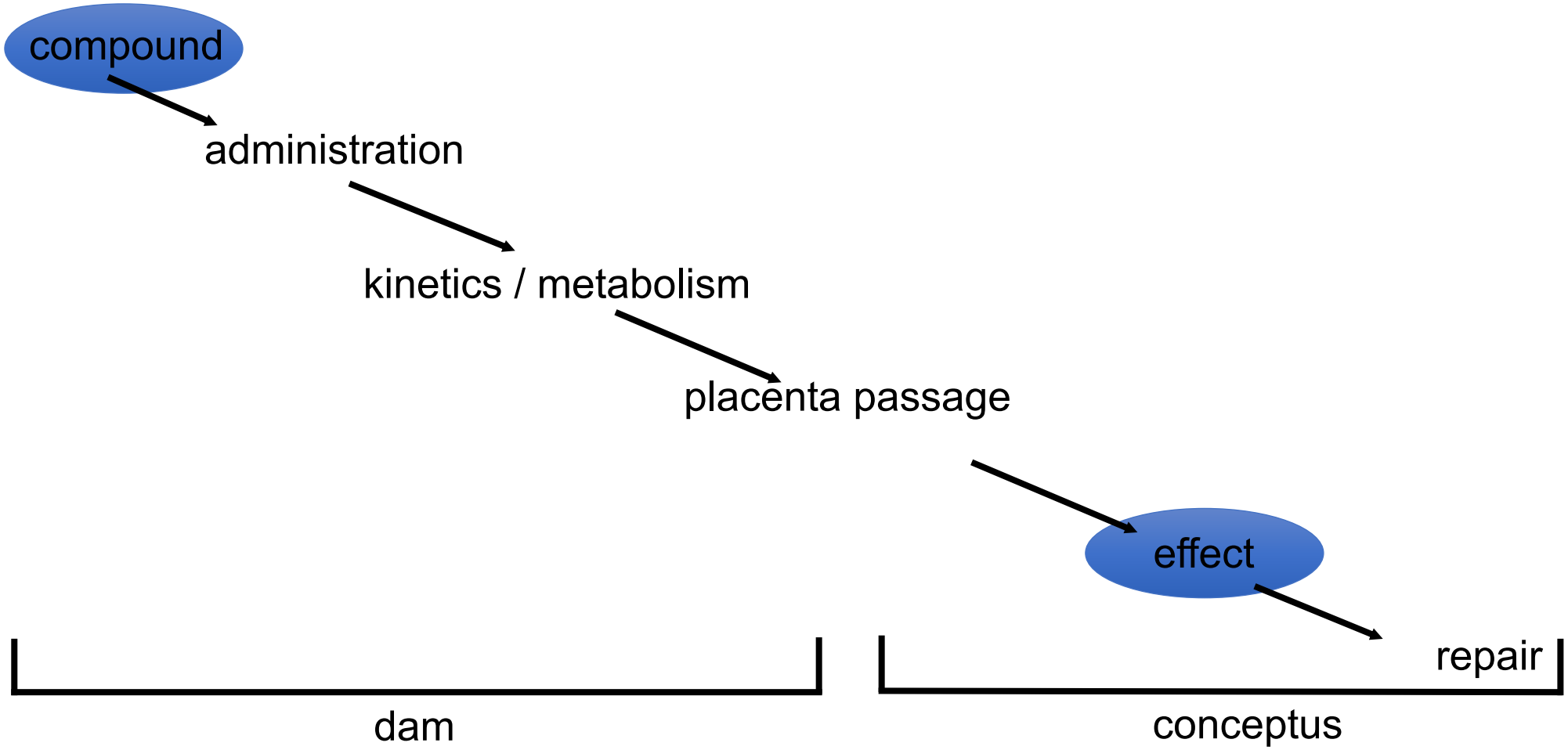
Five decades of regulatory animal studies

- The system based on animal testing appears to perform generally well.
- Reproductive and developmental toxicity studies have low power of detection for low incidence adverse effects.
- Relevance of animal findings for humans is not always clear.
- Meanwhile information on underlying mechanisms of RDT has increased significantly.
- Ethical issues with animal studies for human safety and public awareness has increased.
- Non-animal alternative methods have increasingly been developed and have found applications over time.
- Regulatory acceptance of non-animal methods as alternatives for animal studies still lags behind.

Objectives of in vitro testing: The three R's

- Replace in vivo testing
- Reduce animal use, cost, time
- Refine effect assessment

In vitro = reductionistic approach



Two applications of in vitro tests

screening

compounds

↓

in vitro prescreen

↓

selection of compounds

↓

in vivo test

mechanistic studies

compounds

↓

in vivo effect

↓

selection of in vitro model

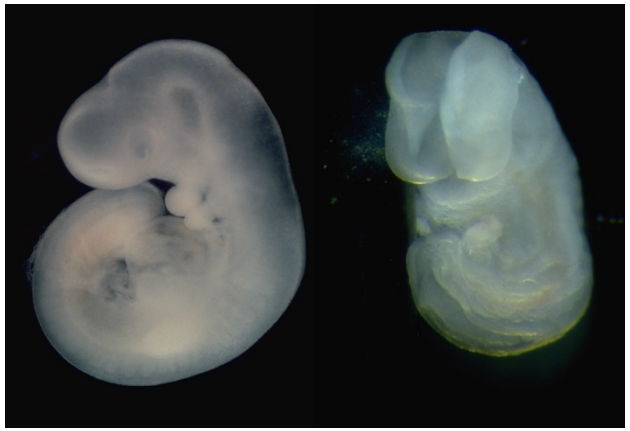
↓

mechanism

In vitro complexity: pro-con

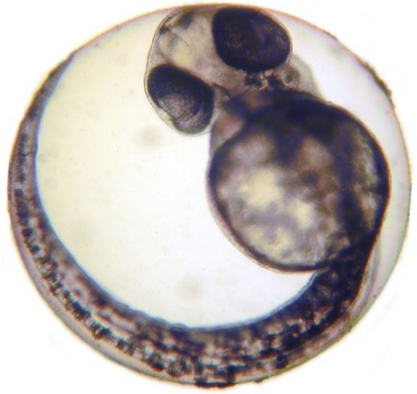
type of test	animal use	complexity
continuous cell lines	-	-
primary cell cultures	+	-
organ cultures	++	+
embryo cultures	+++	++

Selected devtox alternatives



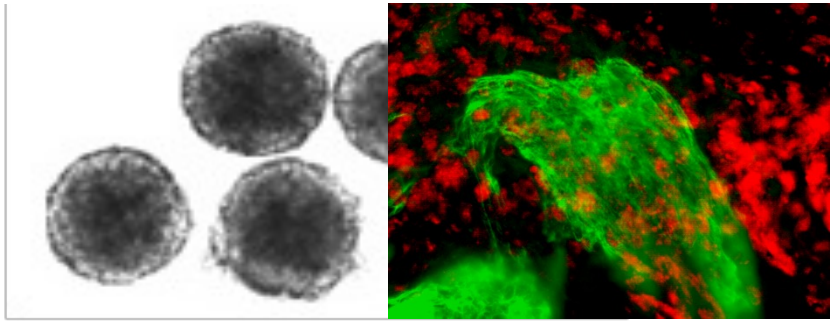
Rodent whole embryo culture

WEC (photo Aart Verhoef, RIVM)



Zebrafish embryo test

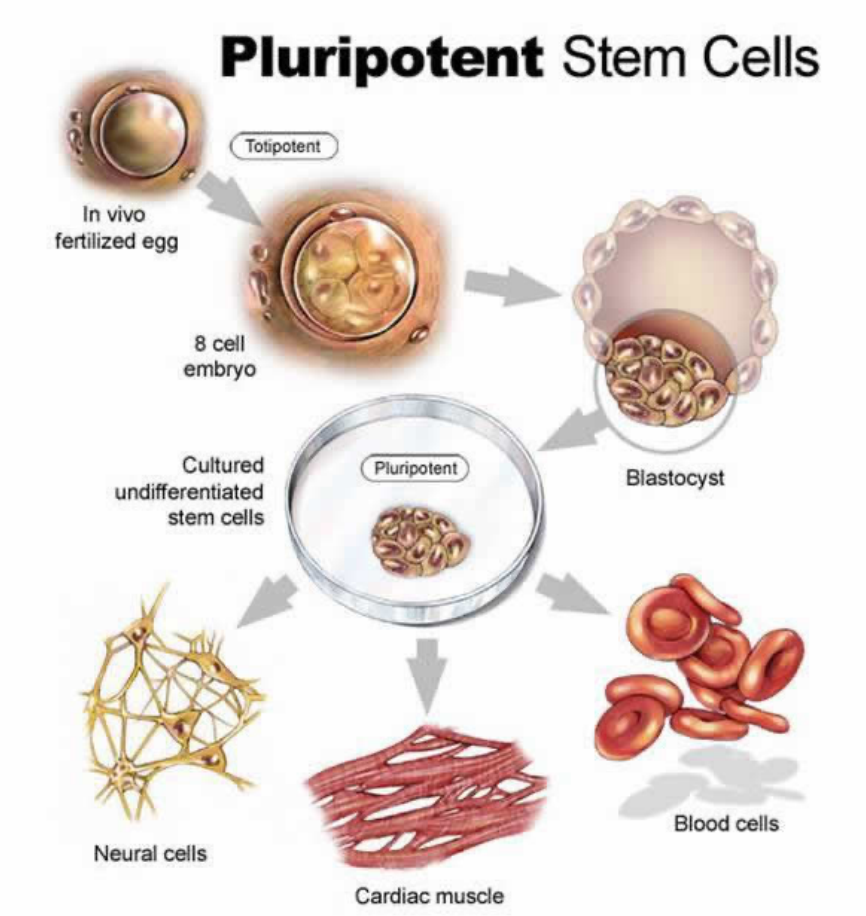
ZFET (photo Sanne Hermsen, RIVM)



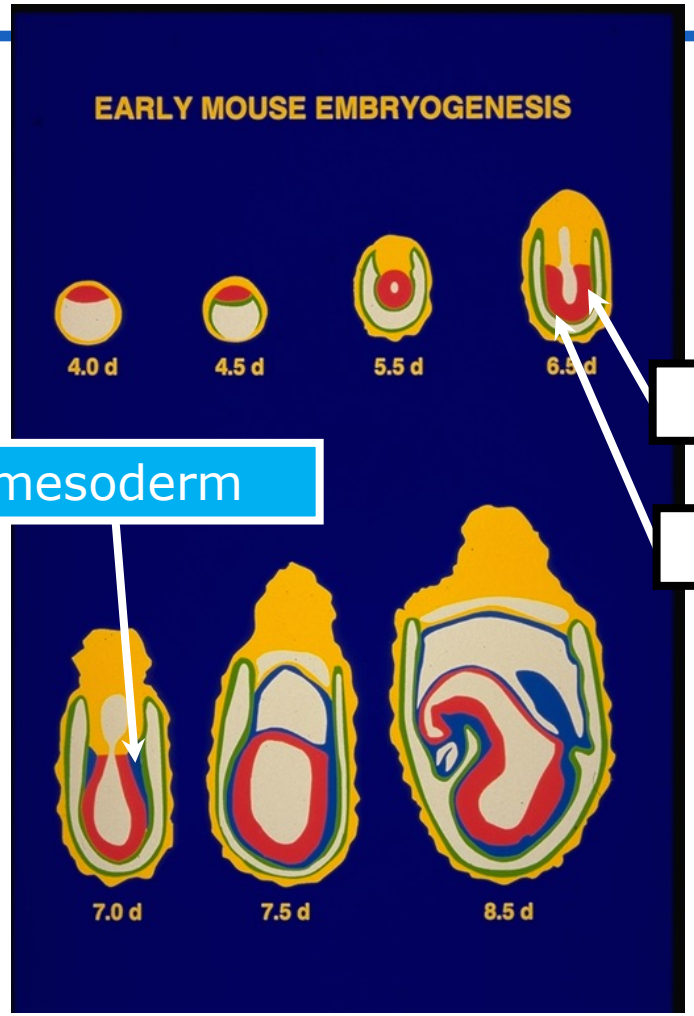
Embryonic stem cell test

EST (photo Dorien van Dartel, RIVM)

Embryonic stem cell differentiation



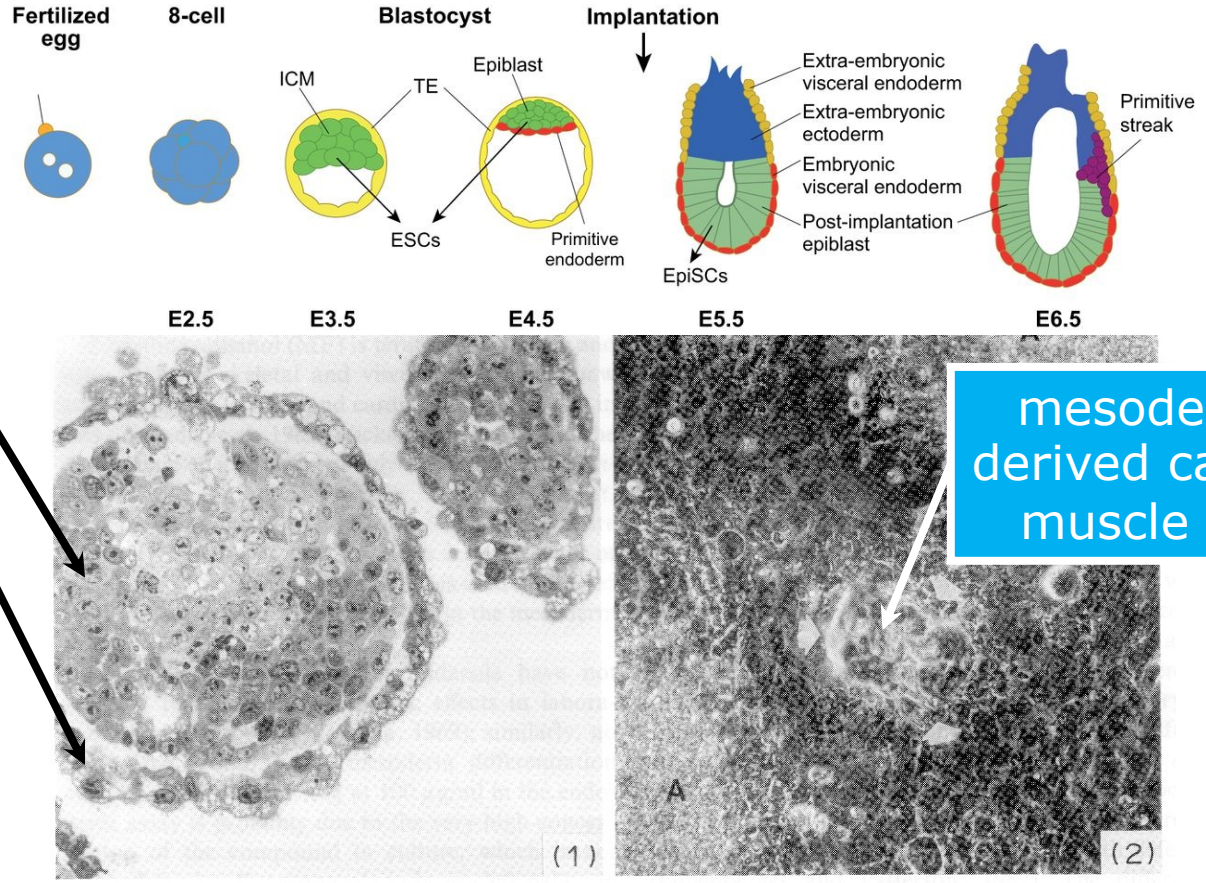
Embryonic stem cell differentiation



mesoderm

epiblast

endoderm

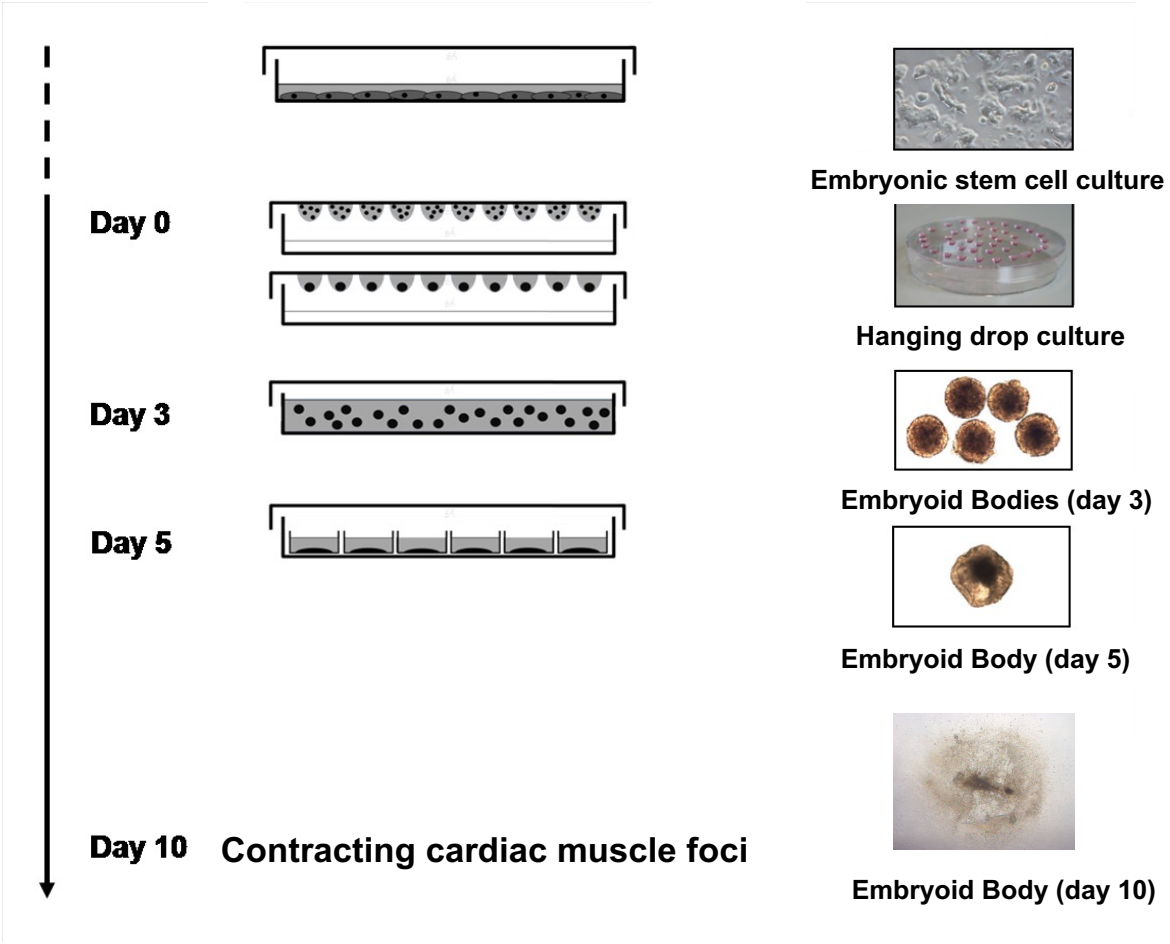


mesoderm-derived cardiac muscle foci

ESC embryoid body formation in vitro

Piersma et al., 1993

Embryonic stem cell test (cardiac)



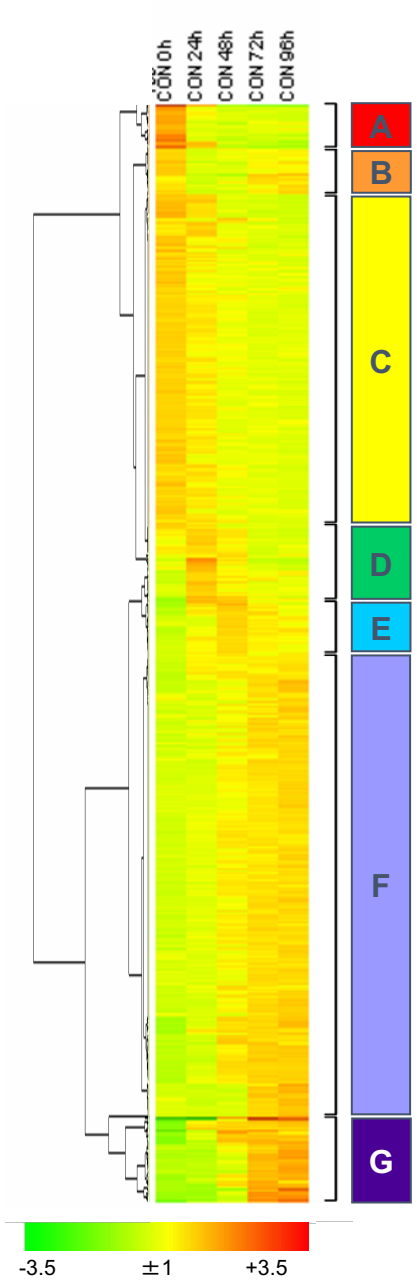
Van Dartel et al., 2009

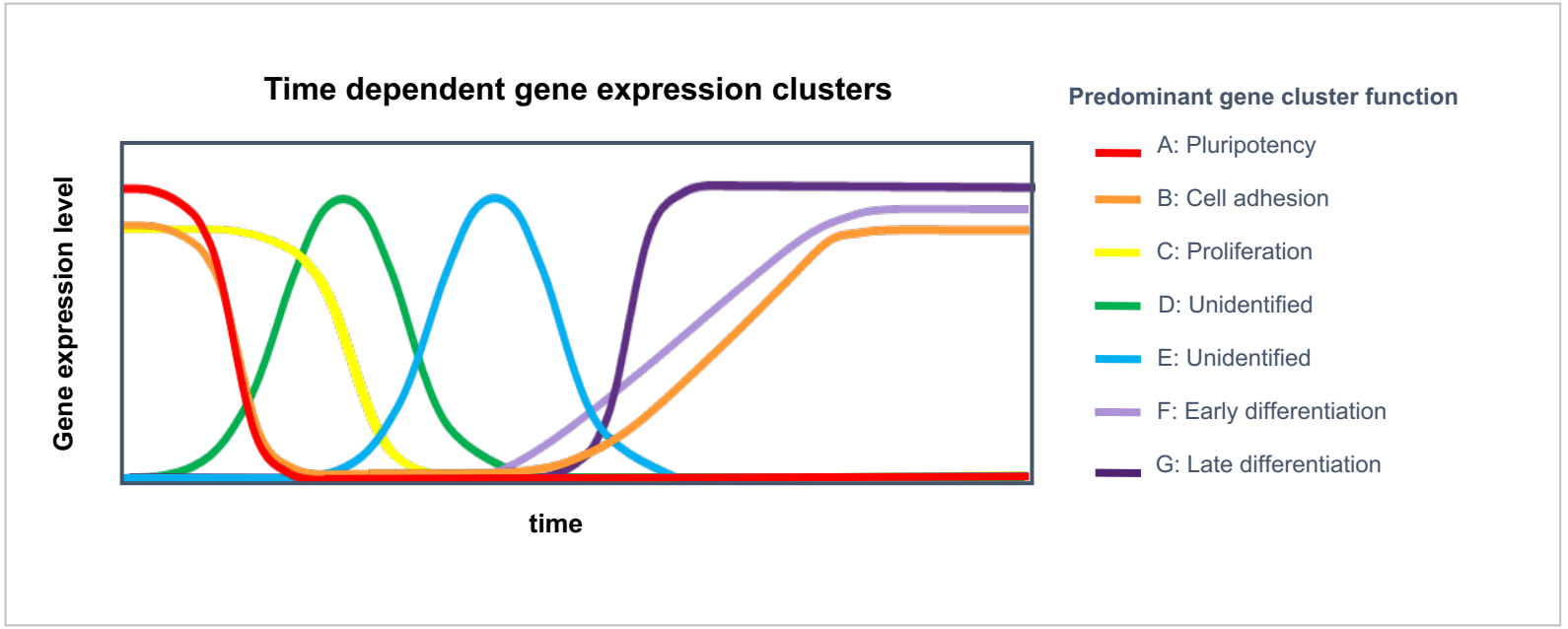
EST validation study

- 20 compounds were tested, discriminating strong/weak/none embryotoxicants
- Scoring beating muscle foci by eye proved laborious and subjective
- Predictivity versus animal study data was around 80% (sensitivity and specificity combined)
- ECVAM listed the validated assay on their website
- Subsequent testing with other compounds appeared less predictive
- Prompted need for better definition of biological domain
- Prompted further research into alternative end points that were less laborious and more objective

Genschow et al., 2002; Marx-Stoelting et al., 2009

EST differentiation – related gene expression

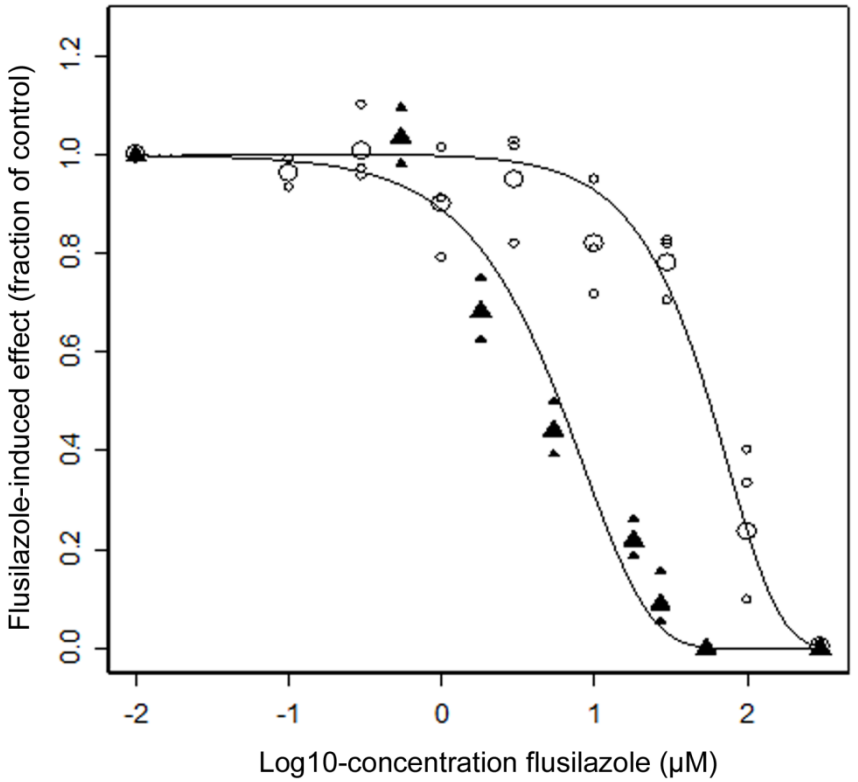




Van Dartel et al., 2010

Threshold of adversity in vitro?

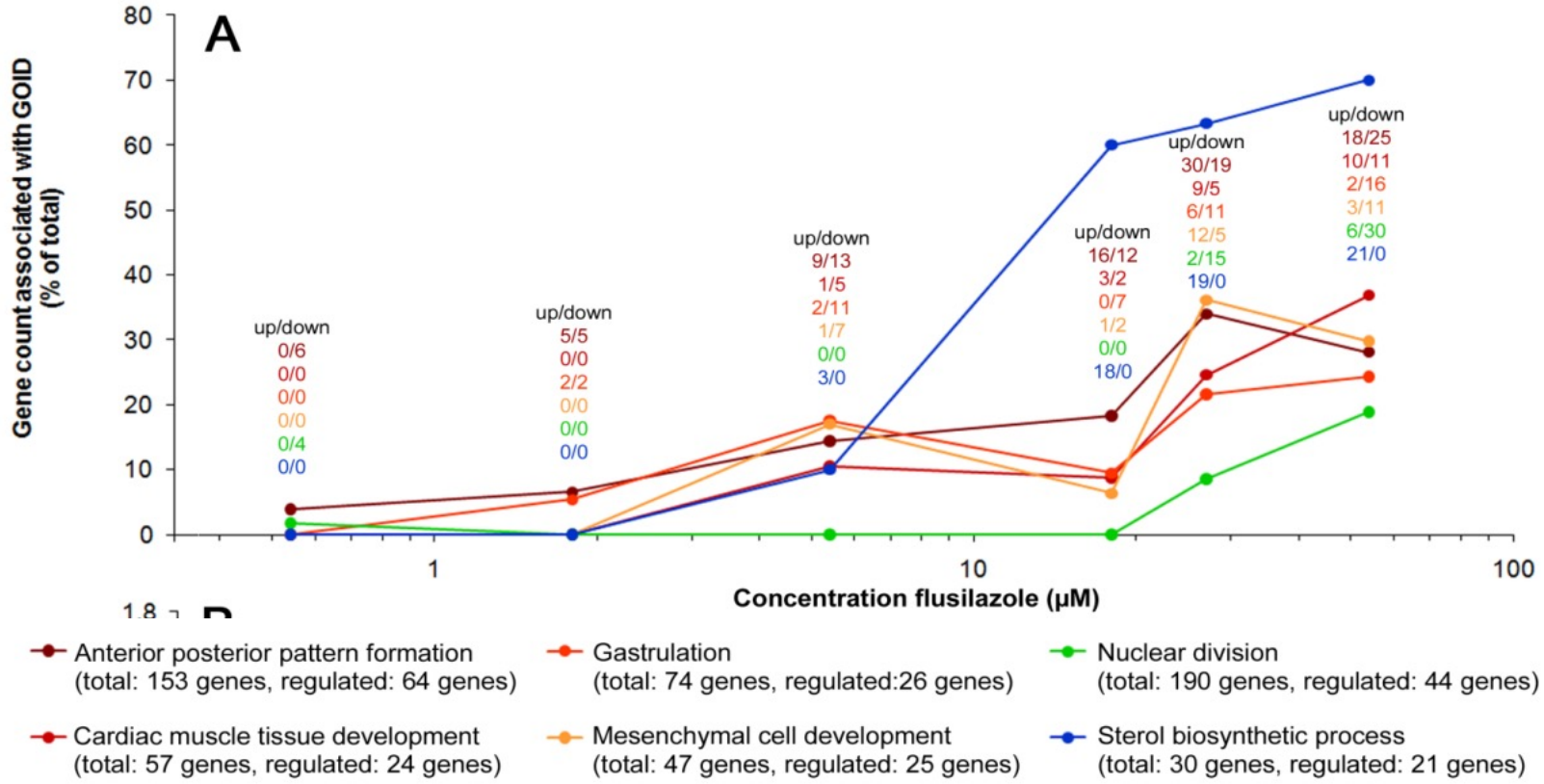
- Flusilazole dose-response
- EST classical dose-response on contracting muscle foci readout
- Differentiation is more sensitive than proliferation
- Interpreted as indicative of a specific developmental toxicant

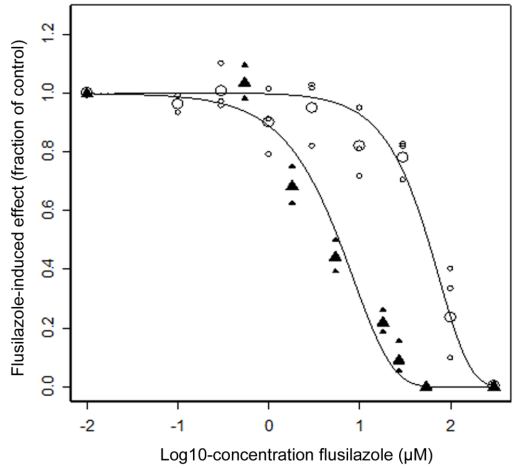


Concentration-response curves of ESC differentiation (▲) (n=2) and cell viability (○) (n=3) after flusilazole exposure.

Van Dartel et al., 2011

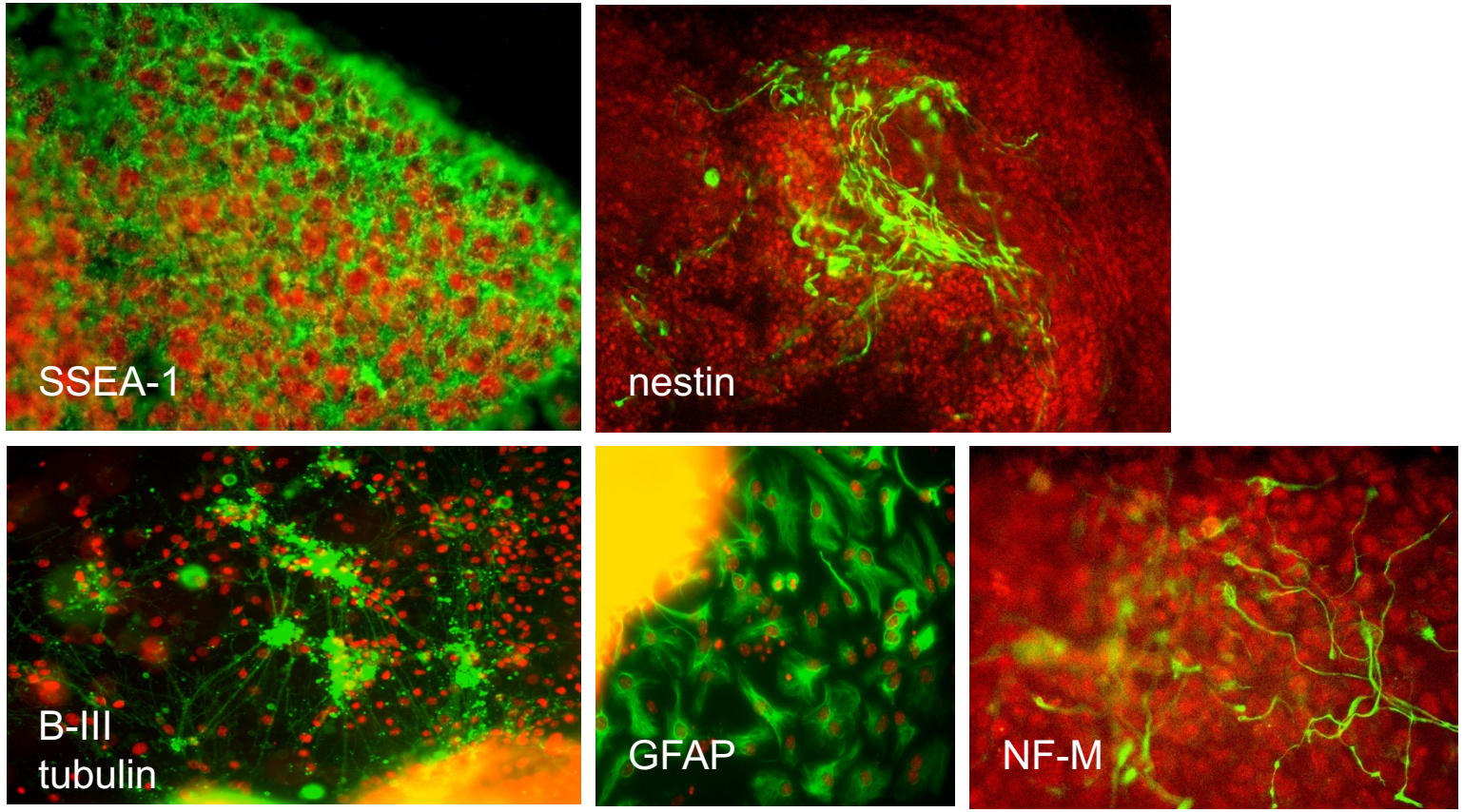
Flusilazole-induced GO-term enrichment





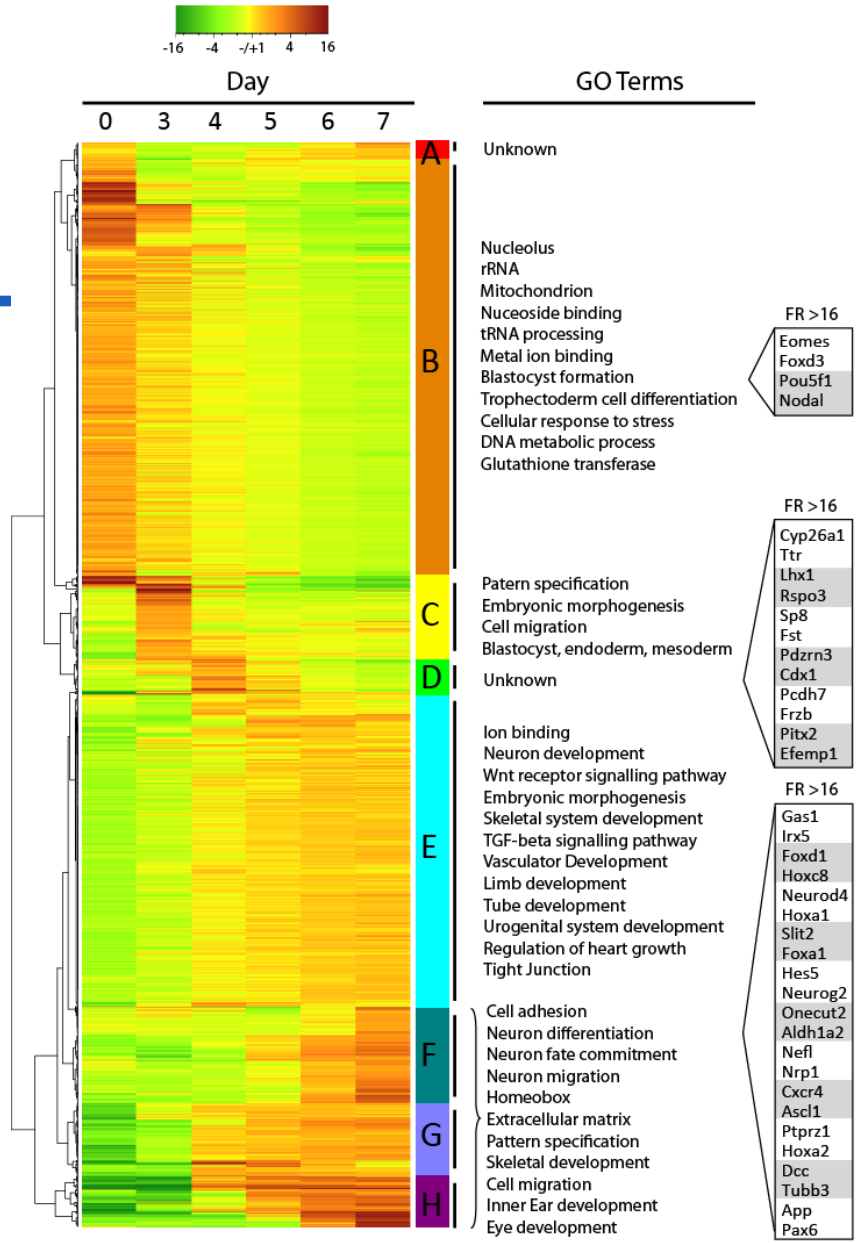
Van Dartel et al., 2011

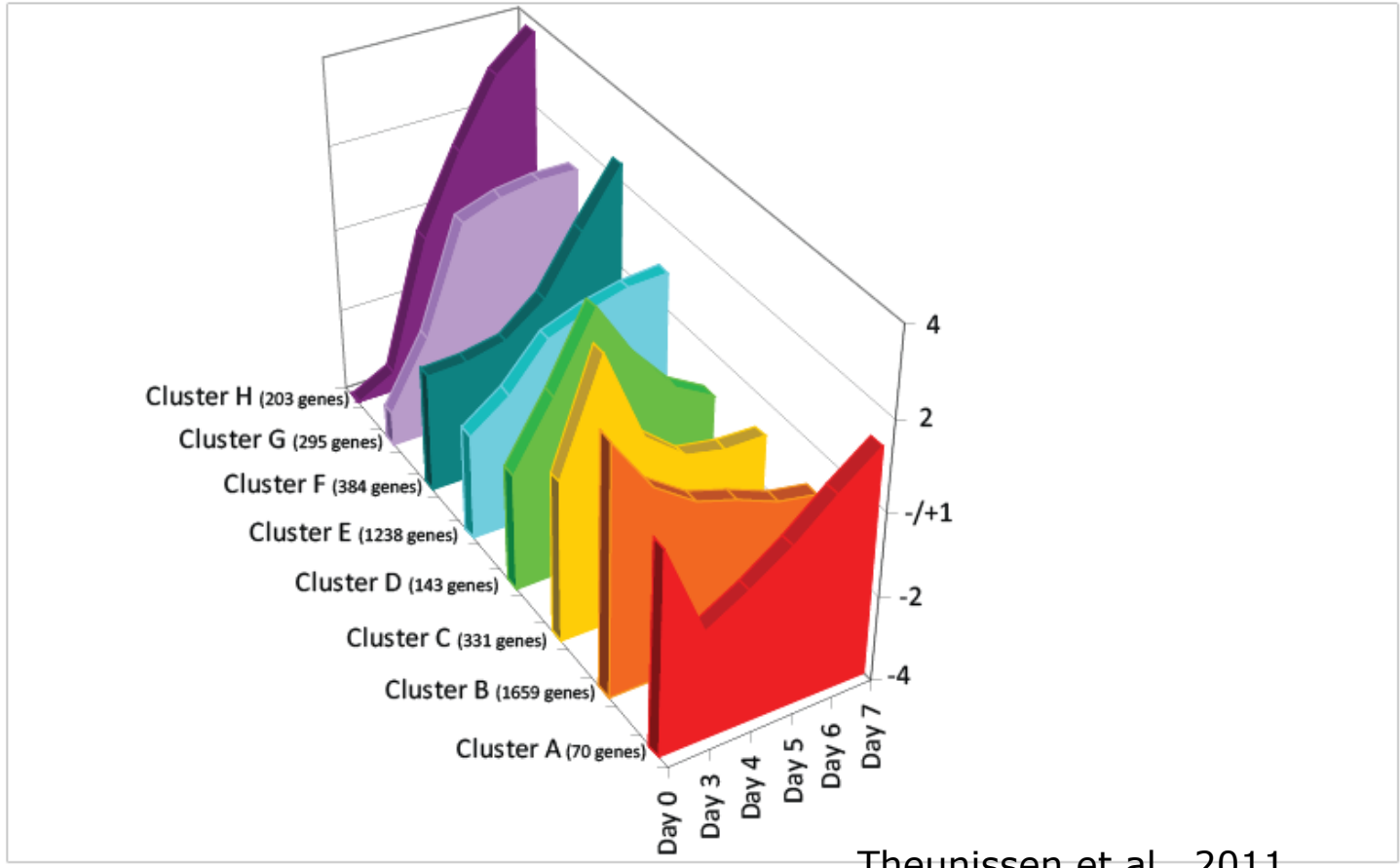
EST neural differentiation assay



Photo's Peter Theunissen, RIVM

EST neural gene expression

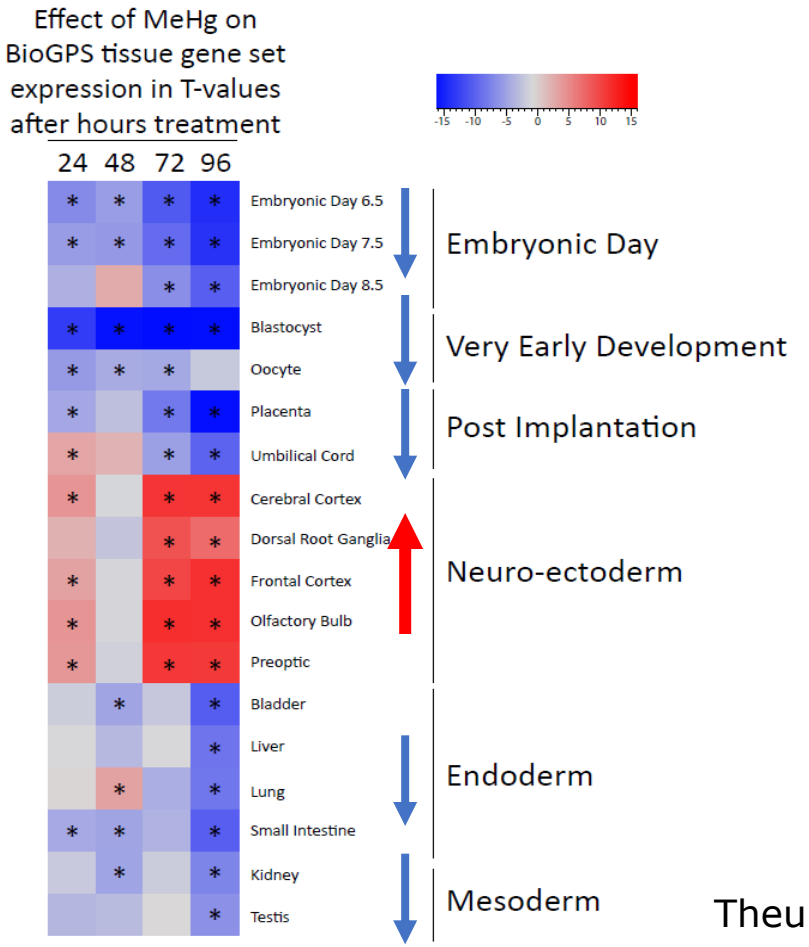




Theunissen et al., 2011

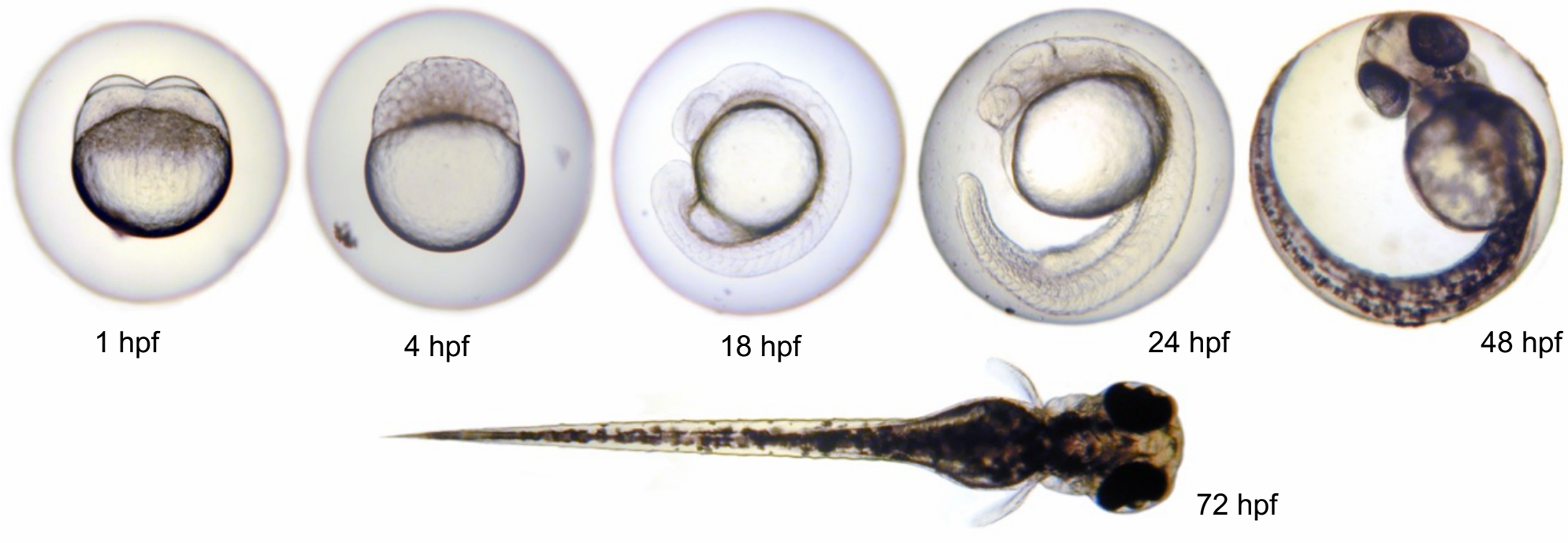
MeHg effects on gene sets over time

- Embryonic time gene sets decrease
- Gene sets for early development decrease
- Neuroectodermal gene sets increase
- Gene expression for non-neuro-ectodermal germ layers decreases
- Agrees with in vivo studies showing enhanced neural differentiation at the expense of proliferation and migration causing brain malformations



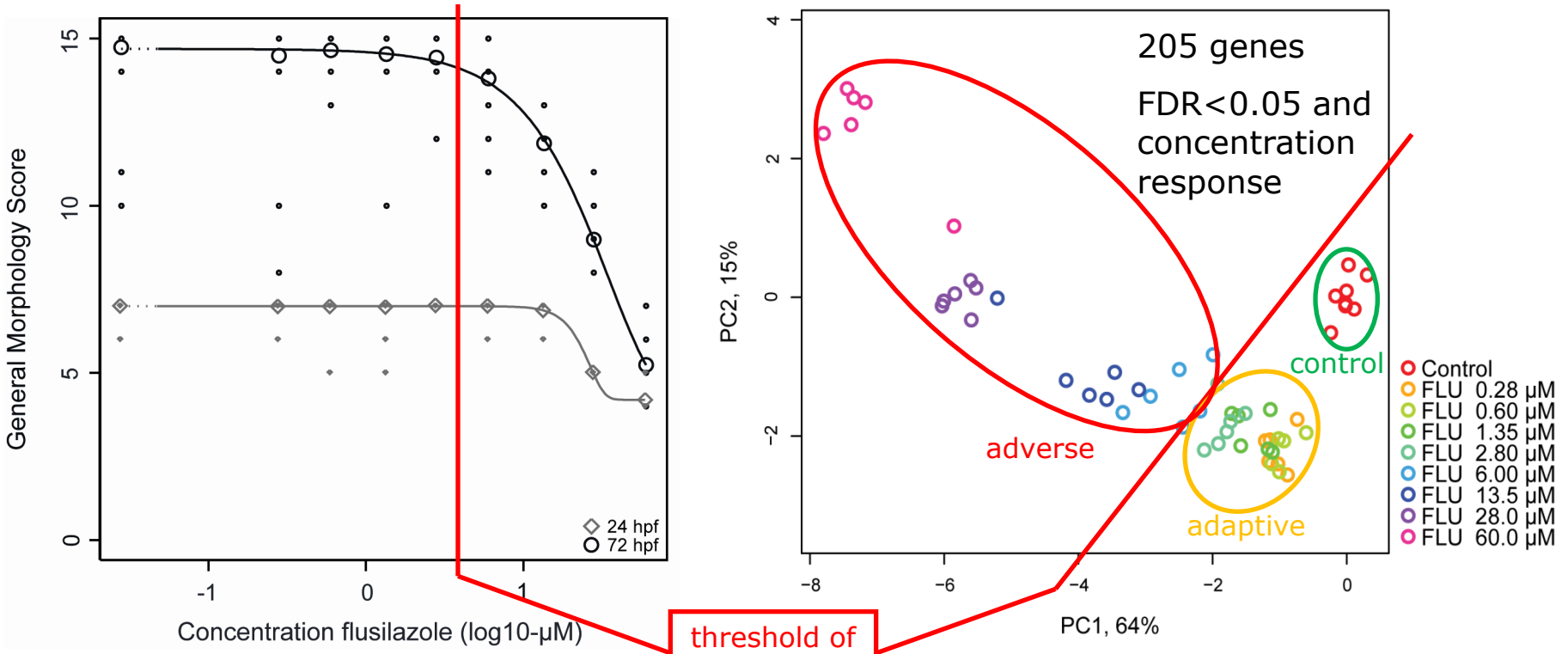
Theunissen et al., 2011

Zebrafish embryotoxicity test



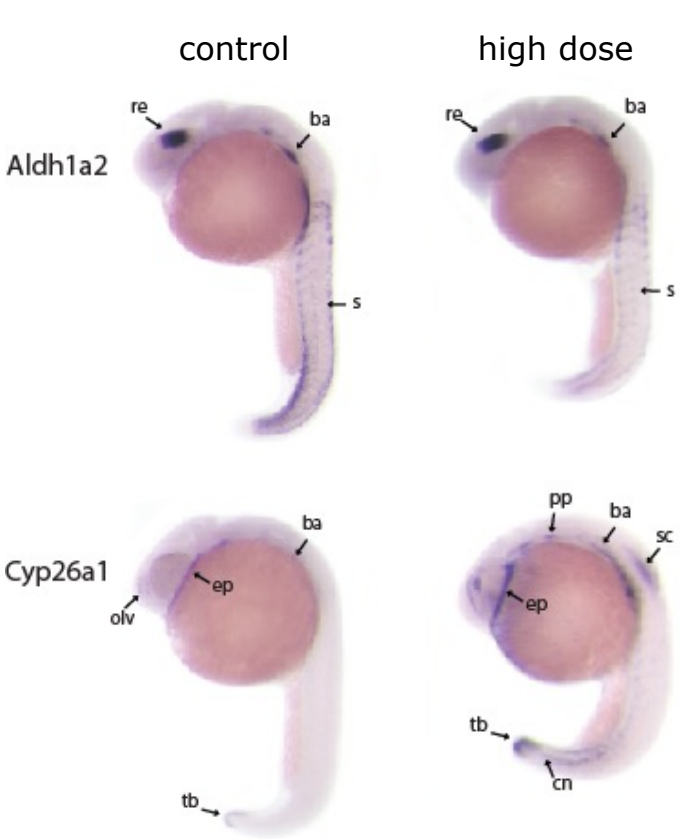
Hermsen et al., 2011

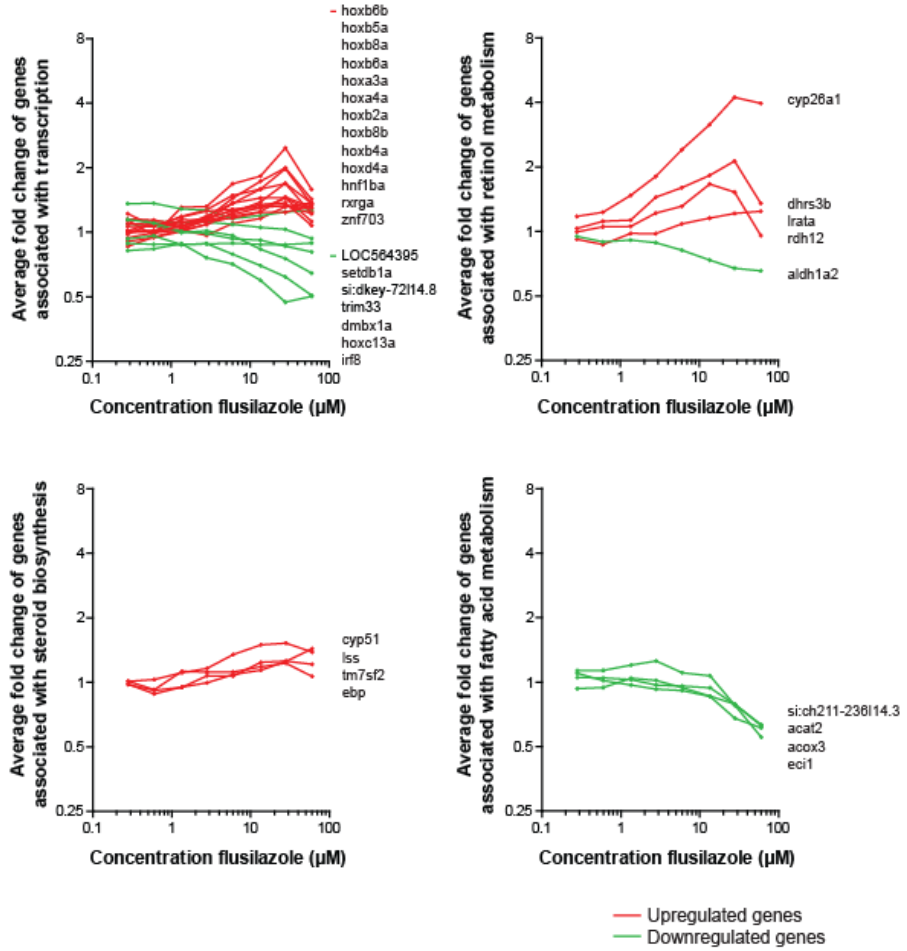
Flusilazole dose-response morphology versus gene expression



Hermsen et al., 2011

Flusilazole-induced gene set regulation

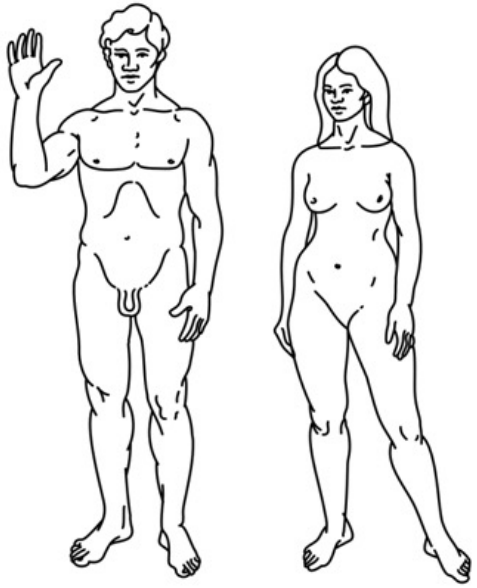




Hermsen et al., 2011

Reductionist models for human risk assessment

the species of interest



the test species

the replacement test

Issues with classical validation

- The animal study as the gold standard
- The 1:1 replacement paradigm
- The relevance of mathematical / statistical prediction models
- The exposure and kinetics of compounds

- Cf. the human is the target for risk assessment
- Cf. the reductionist nature of in vitro assays
- Cf. the biological domain of the in vitro assay
- Cf. external exposure in intact individuals versus direct exposure to cells in vitro

Two extreme options for innovation of chemical risk assessment for humans

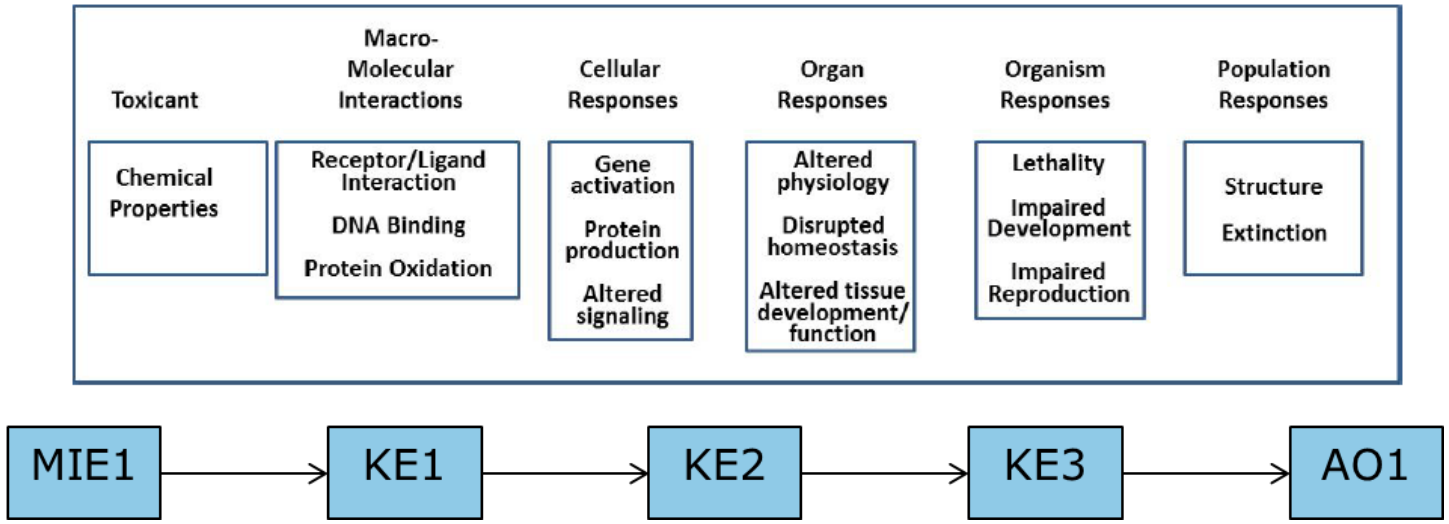
“Evolution”

- Improve current approaches
- Animal study as the gold standard
- Framed within current legislation

“Revolution”

- Start from scratch
- Human biology as the gold standard
- Independent of current legislation

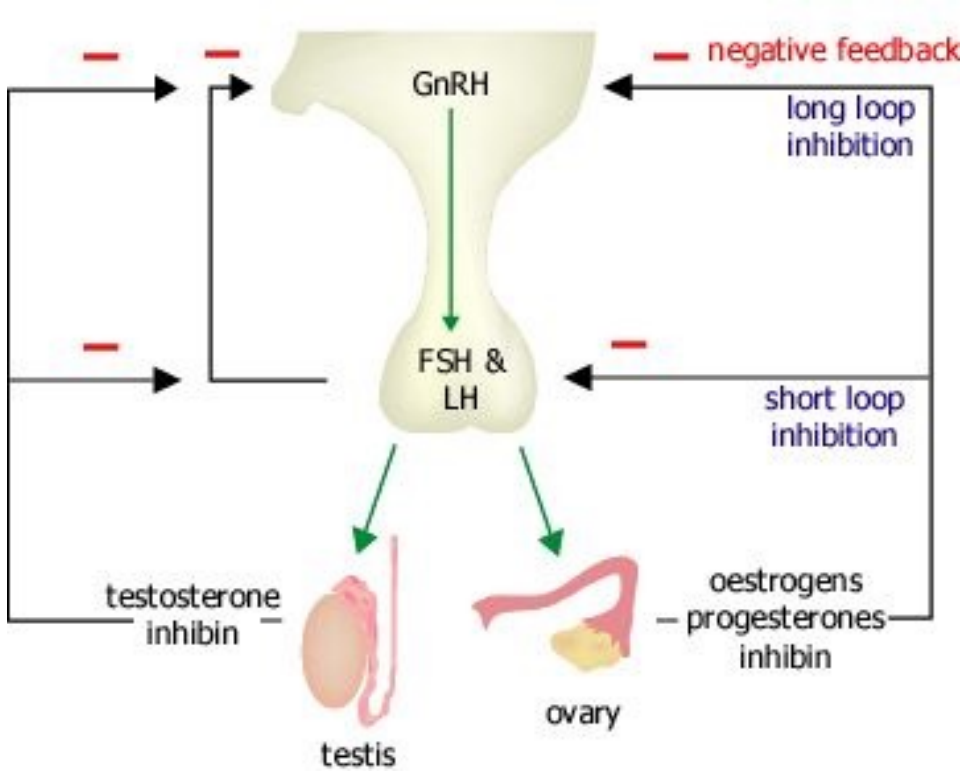
Adverse outcome pathways: Merits and issues



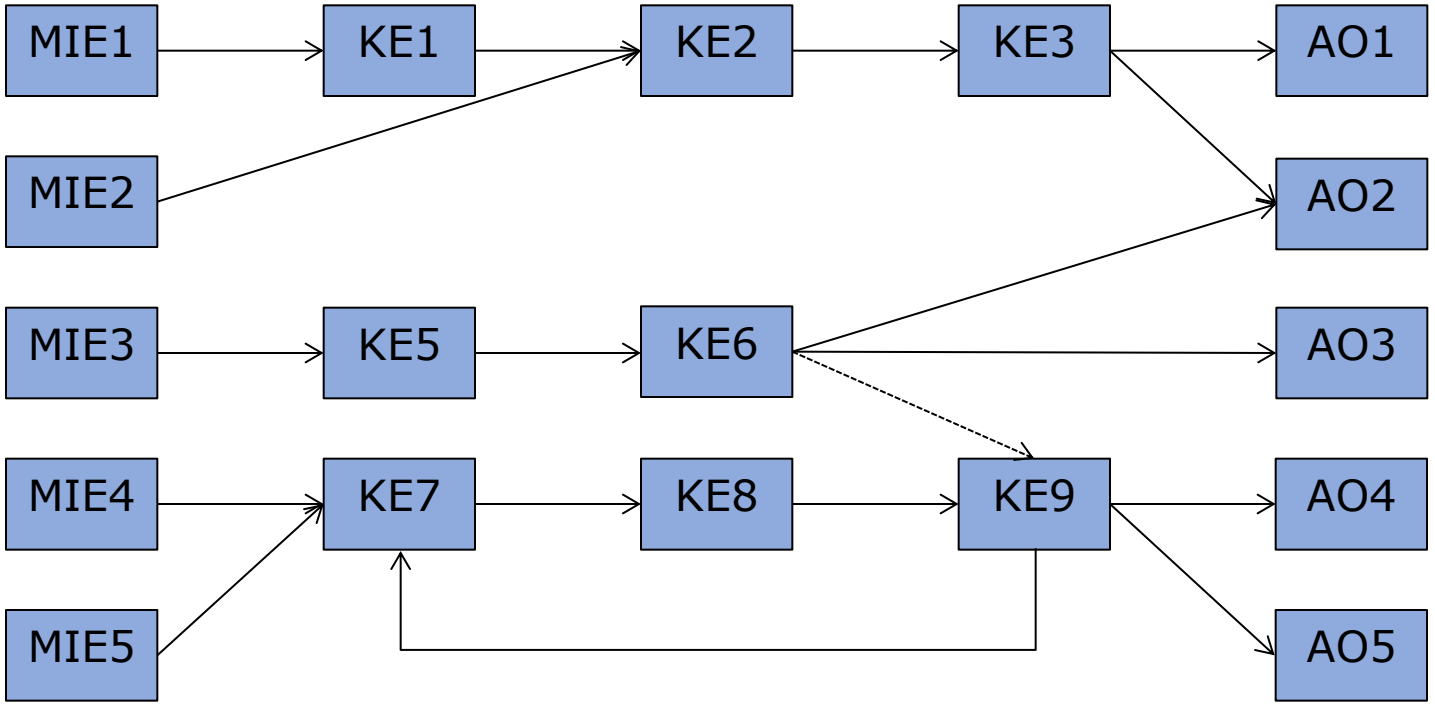
- AOP approach has stimulated the use mechanistic information in human risk assessment
- Physiology is not one-directional; Toxicological mechanisms are not linear; AOPs do not work in isolation
- Complex mechanistic network modelling is required for toxicity prediction

Homeostasis through feedback mechanisms:

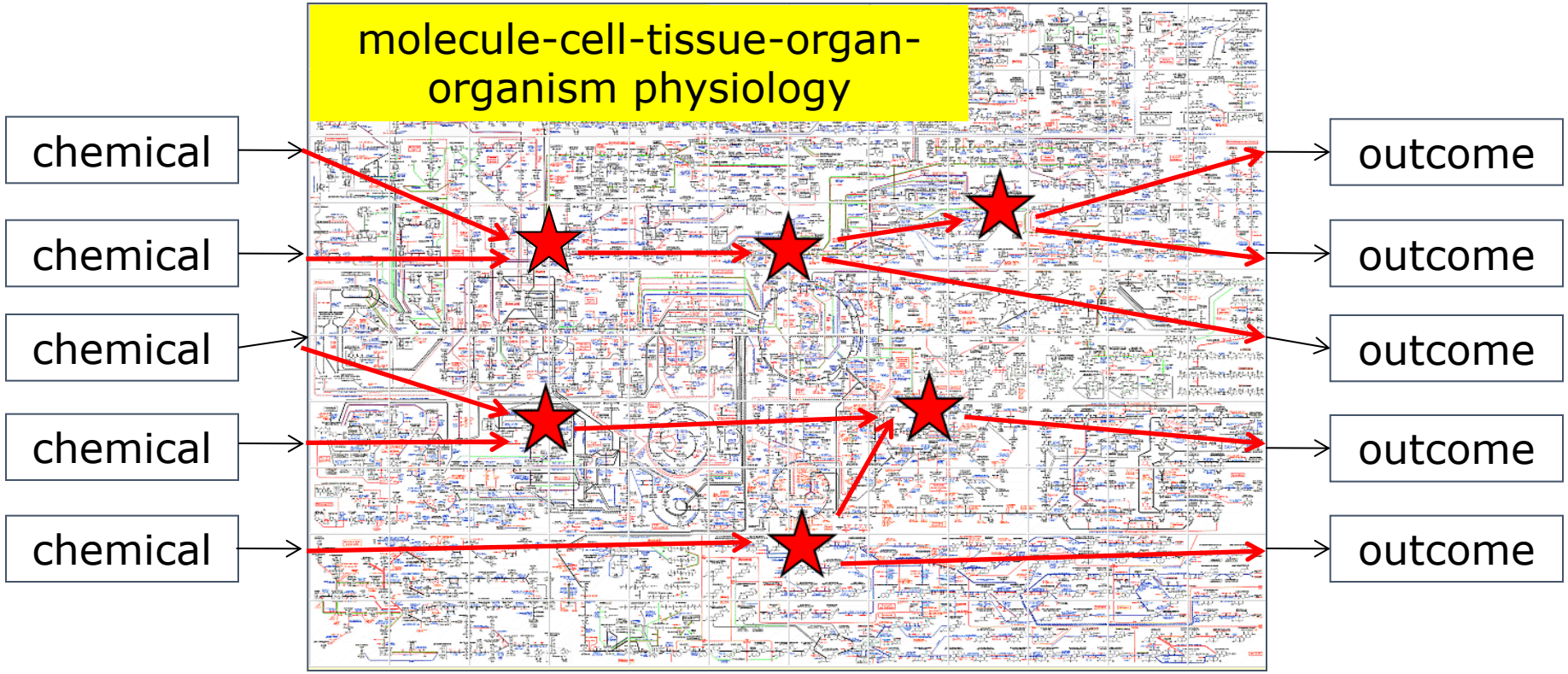
the Hypothalamus-Pituitary-Gonadal axis



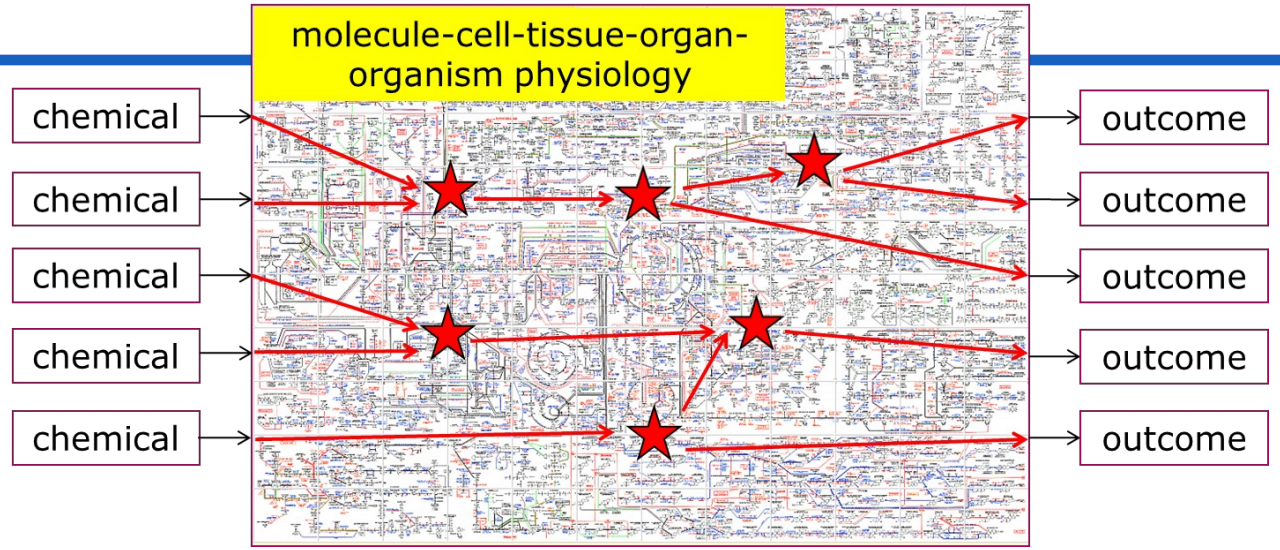
Adverse Outcome Pathway Networks

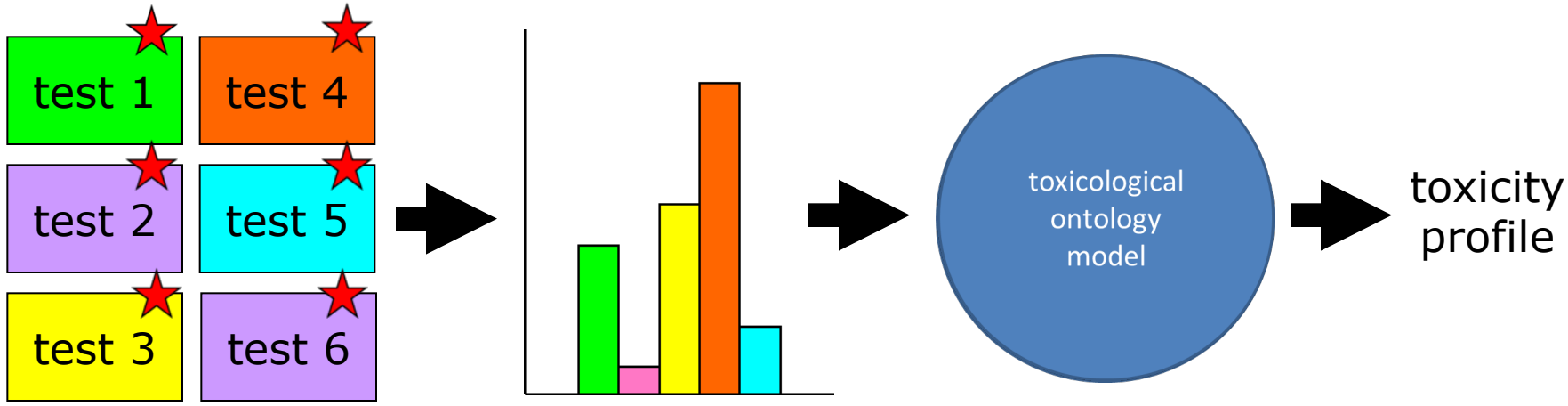


Toxicological Ontology

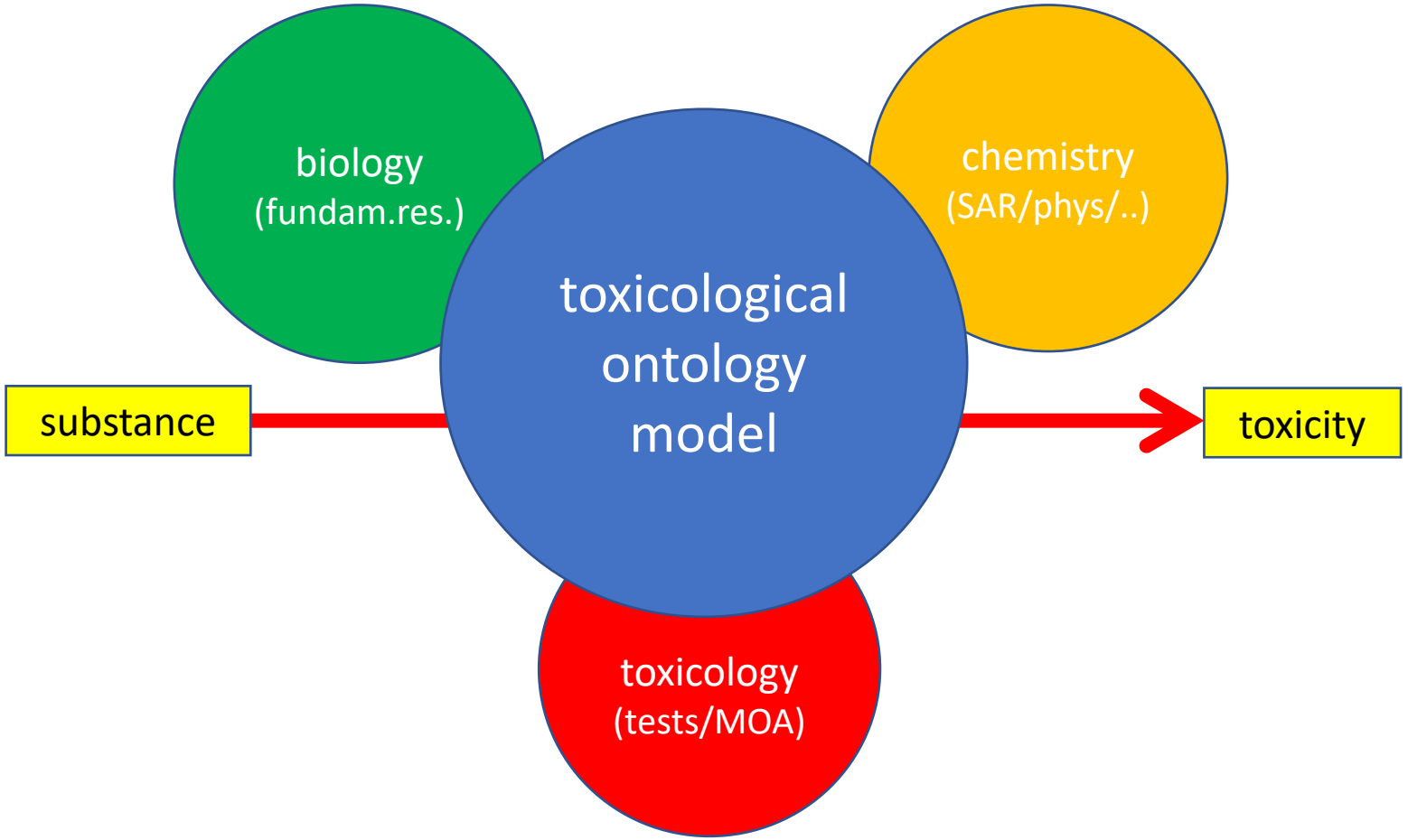


Animal-free risk assessment





Toxicological ontology modelling



Computational ontology models: a proof of principle

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PLOS COMPUTATIONAL BIOLOGY

A Computational Model Predicting Disruption of Blood Vessel Development

Nicole Kleinstreuer[1], David Dix[1], Michael Rountree[1], Nancy Baker[2], Nisha Sipes[1], David Reif[1], Richard Spencer[2], Thomas Knudsen[1]*

1 National Center for Computational Toxicology, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina, United States of America, 2 Lockheed-Martin, Research Triangle Park, North Carolina, United States of America

Kleinstreuer et al., 2013

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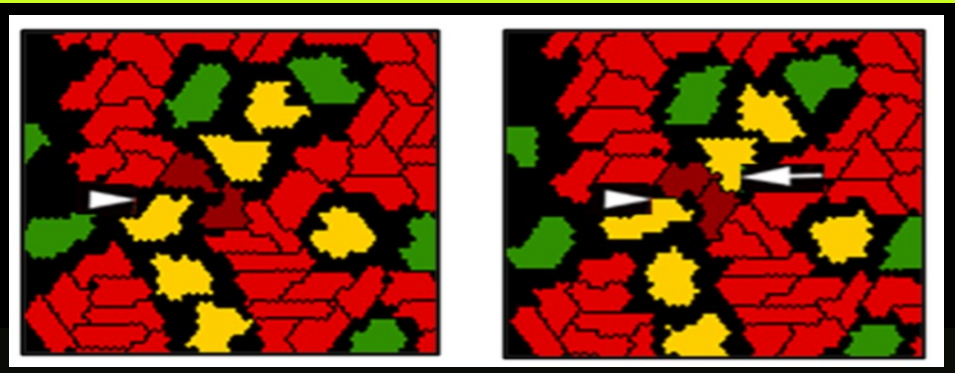
Table 3. Cell types, behaviors, and associated angiogenic signals represented in the computational model of early embryonic vascular plexus formation.

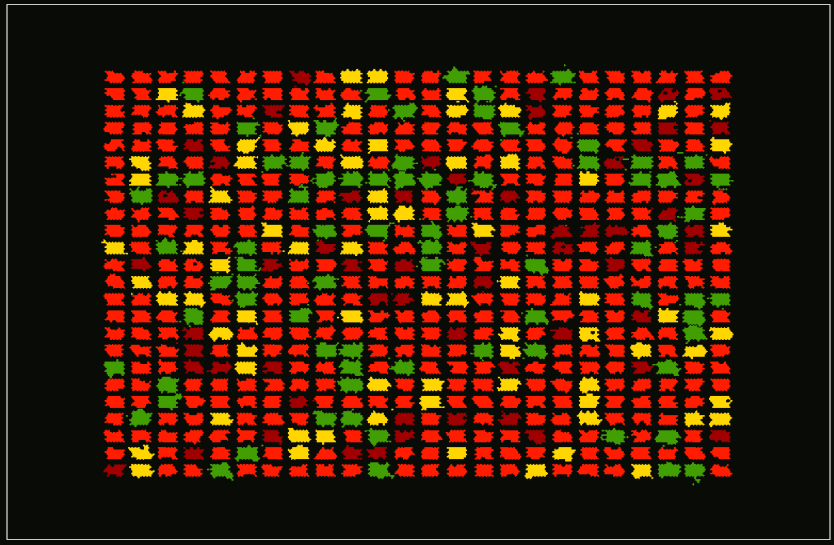
Cell Type	Behavior	Signal
Endothelial Tip Cell (EC_t)	migration up chemotactic gradients	VEGF165, VEGF121, CCL2
	secretion of proteases that break down ECM and release growth factors	PAI1, Proteases, VEGF165
	expression of chemokines	CCL2
	motility along the ECM	uPAR, VCAM1
	Apoptosis	
Endothelial Stalk Cell (EC_s)	proliferation in response to growth factors	VEGF165, VEGF121
	inhibition of proliferation	CXCL10
	secretion of proteases that break down ECM and release growth factors	PAI1, Proteases, VEGF165
	secretion of soluble decoy receptors that bind and sequester growth factor	sVEGFR1
	adhesion to other cell types	Tie2, VCAM1
	assumption of tip cell type based on free surface area and growth factor concentration	VEGF165, VEGF121
	motility along the ECM	uPAR, VCAM1
	quiescence based on shared surface area with other cells	
	Apoptosis	
Inflammatory Cell (IC)	migration up chemotactic gradients	CCL2
	expression of chemokines/growth factors	VEGF121, CCL2, CXCL10
	interaction with the ECM to release bound growth factor	VEGF165
	adhesion to other cell types	VCAM1
	Apoptosis	
Mural Cell (MC)	expression of chemokines/growth factors	VEGF165, VEGF121, CCL2
	adhesion to other cell types	ANG1, VCAM1
	motility along the ECM	PAI1
	promotion of endothelial quiescence based on shared surface area	
	Apoptosis	

Signaling molecules diffuse uniformly and isotropically. Lattice boundary conditions are periodic.

doi:10.1371/journal.pcbi.1002996.t003



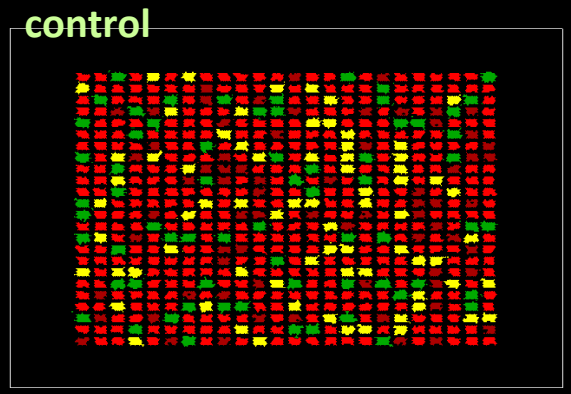
Agent-Based Model (CompuCell3D)

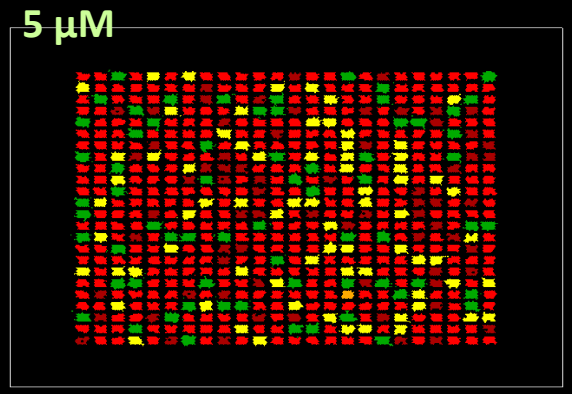


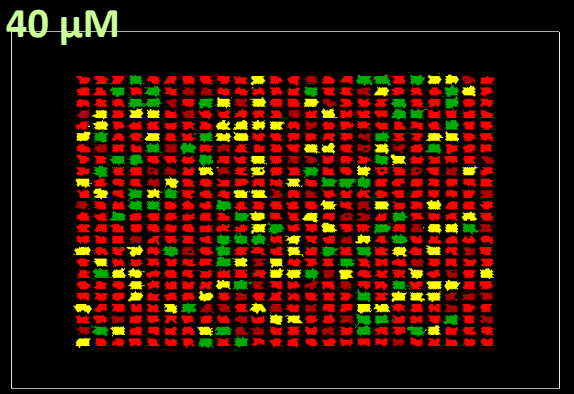
VEGF165
MMPs
VEGF121
sFlit1
TIE2
CXCL10
CCL2

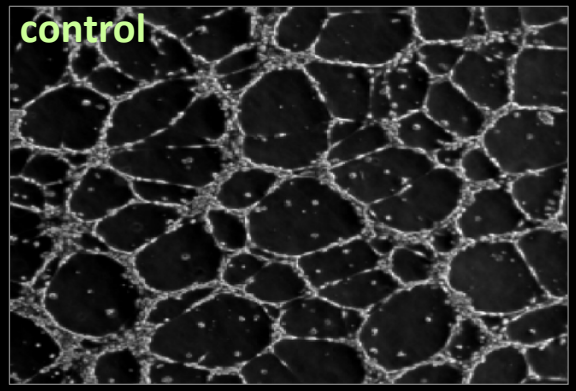
Endothelial Stalk
Endothelial Tip
Mural Cell
Inflammatory Cell

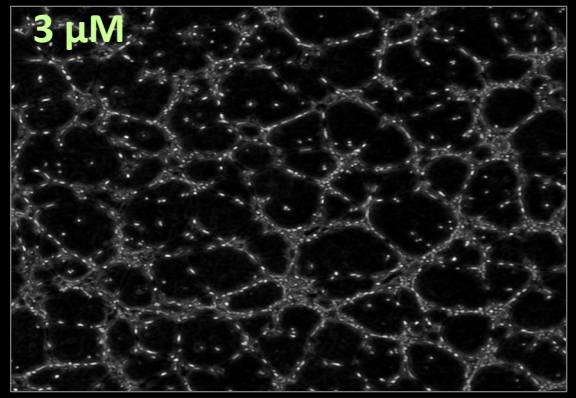
Simulation of 5HPP-33 Concentration Response

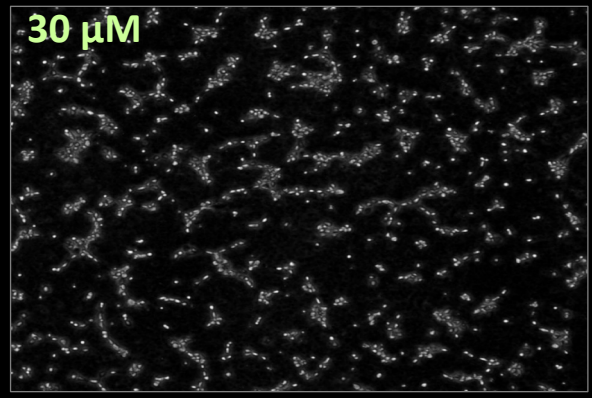






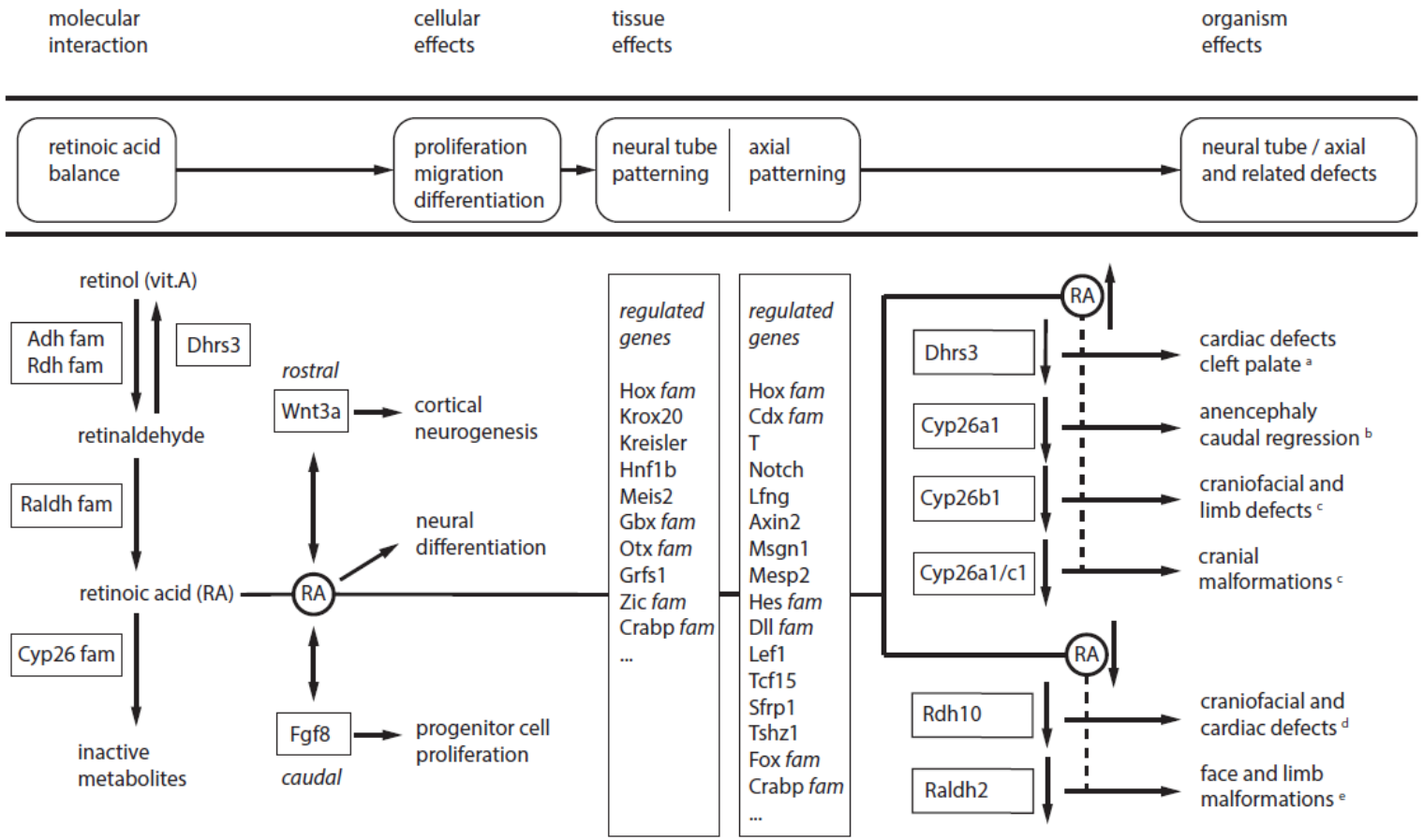






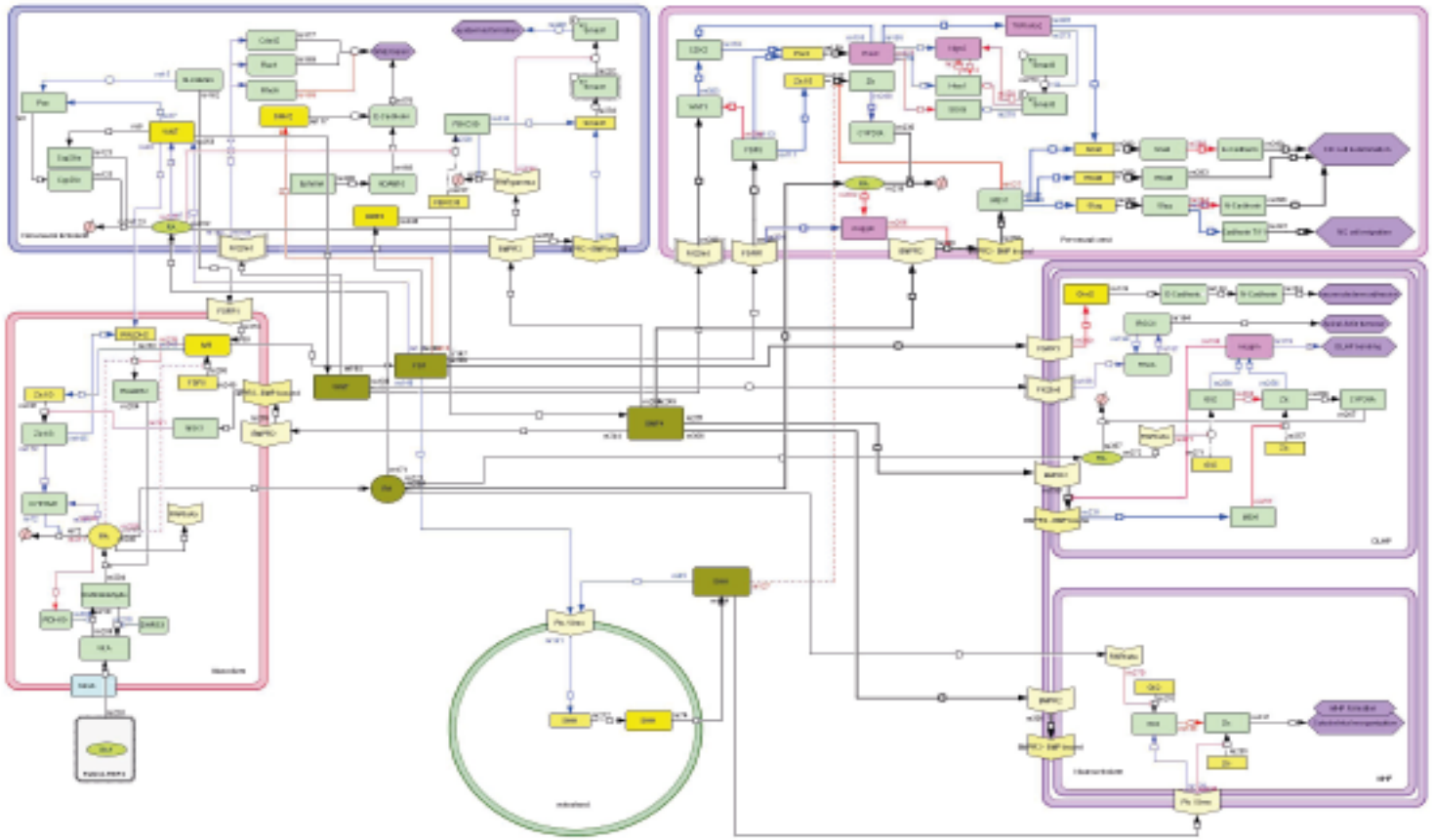
SOURCE: Kleinstreuer et al. (2013) PLoS Comp Biol 9(4): e1002996

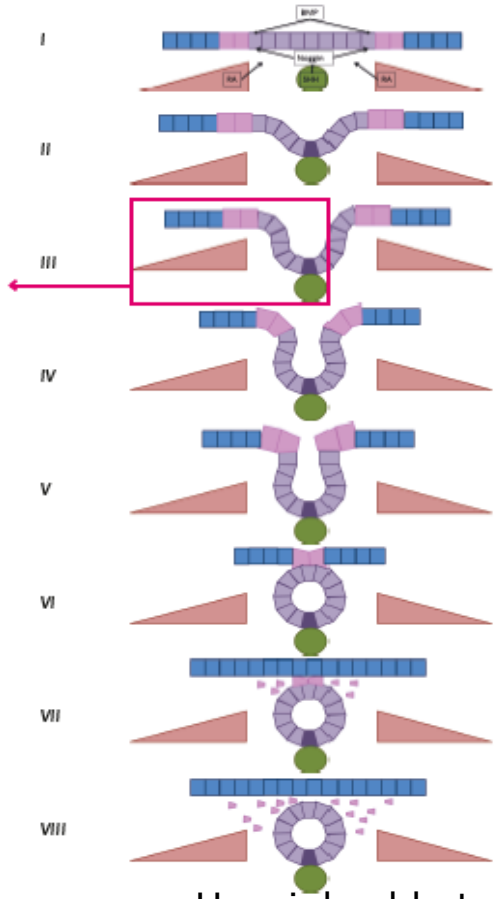
An RA neural tube - axial patterning AOP Framework



Tonk, Pennings & Piersma 2015

Neural tube closure molecular network

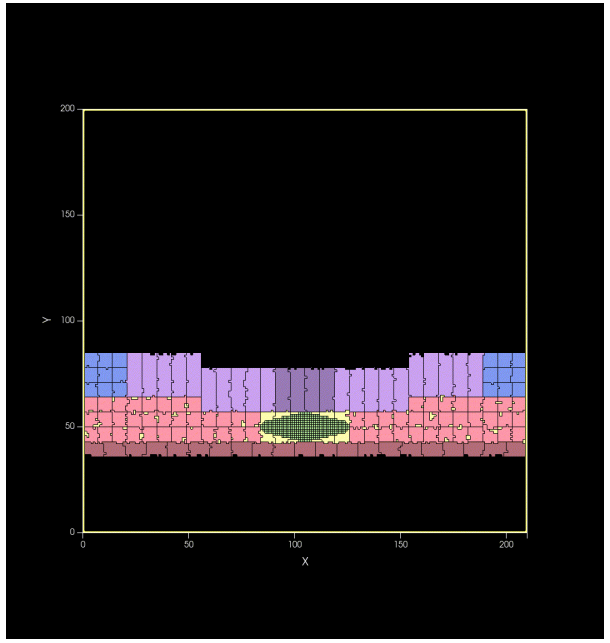




Heusinkveld et al., 2020

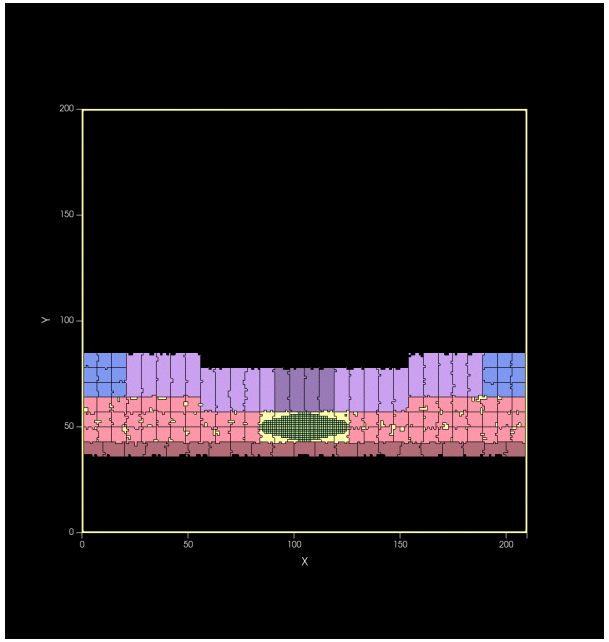
Computed neural tube closure

BMP Hypoactivation



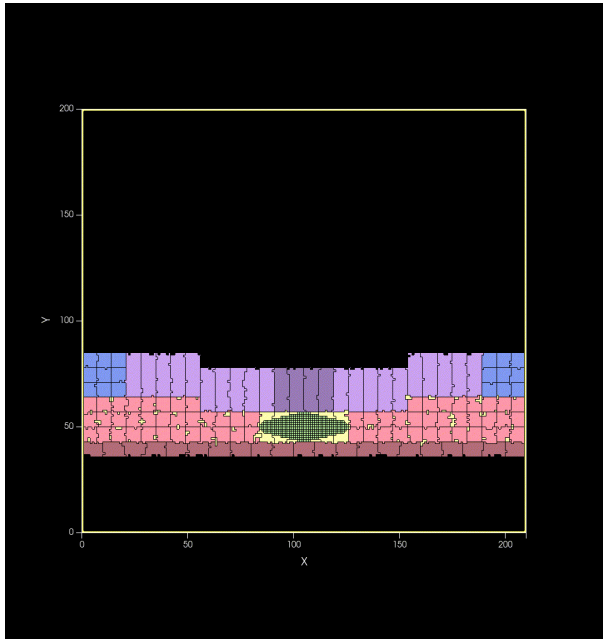
spina bifida occulta

Normal



closed neural tube

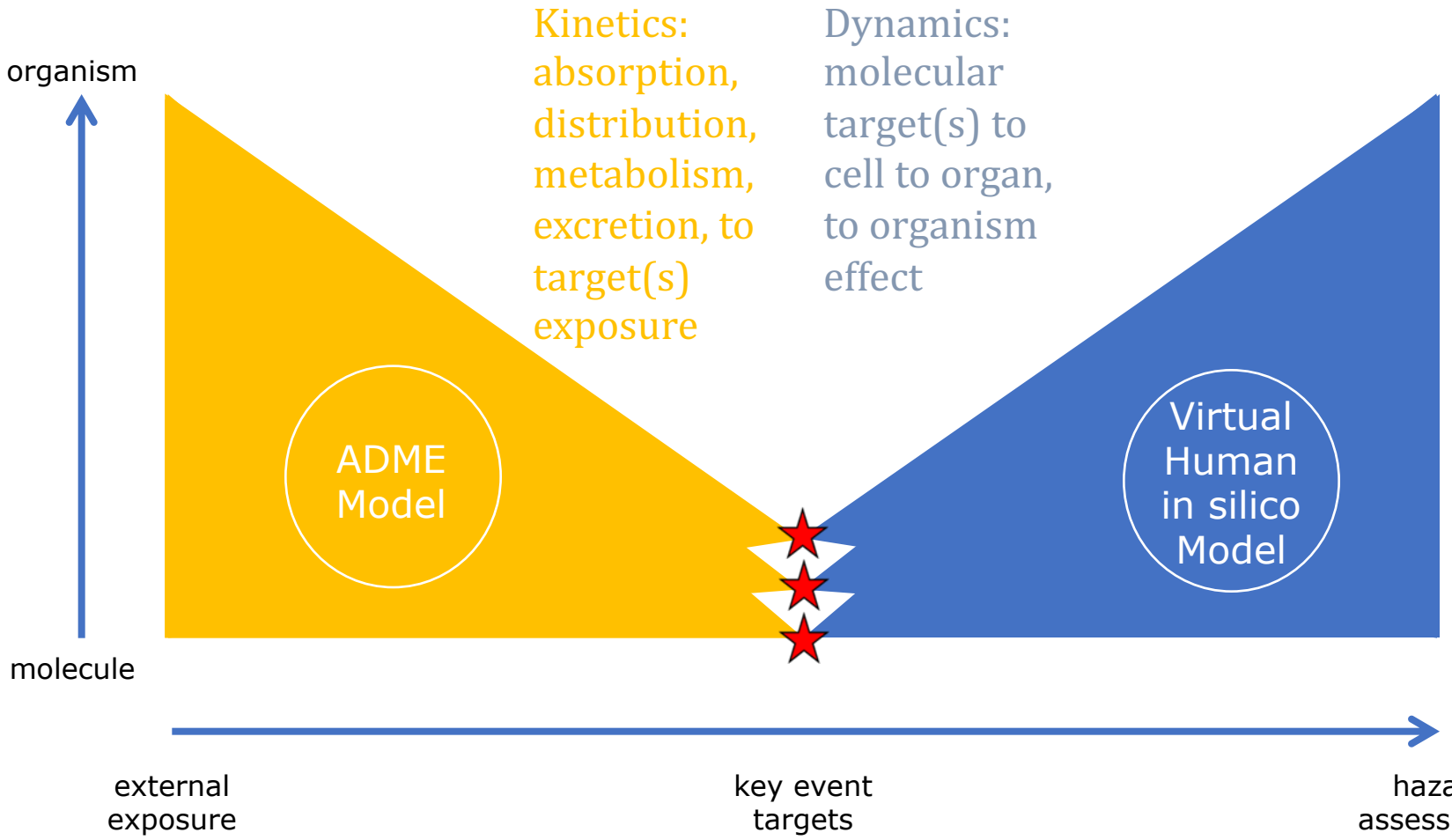
BMP Hyperactivation



open spina bifida

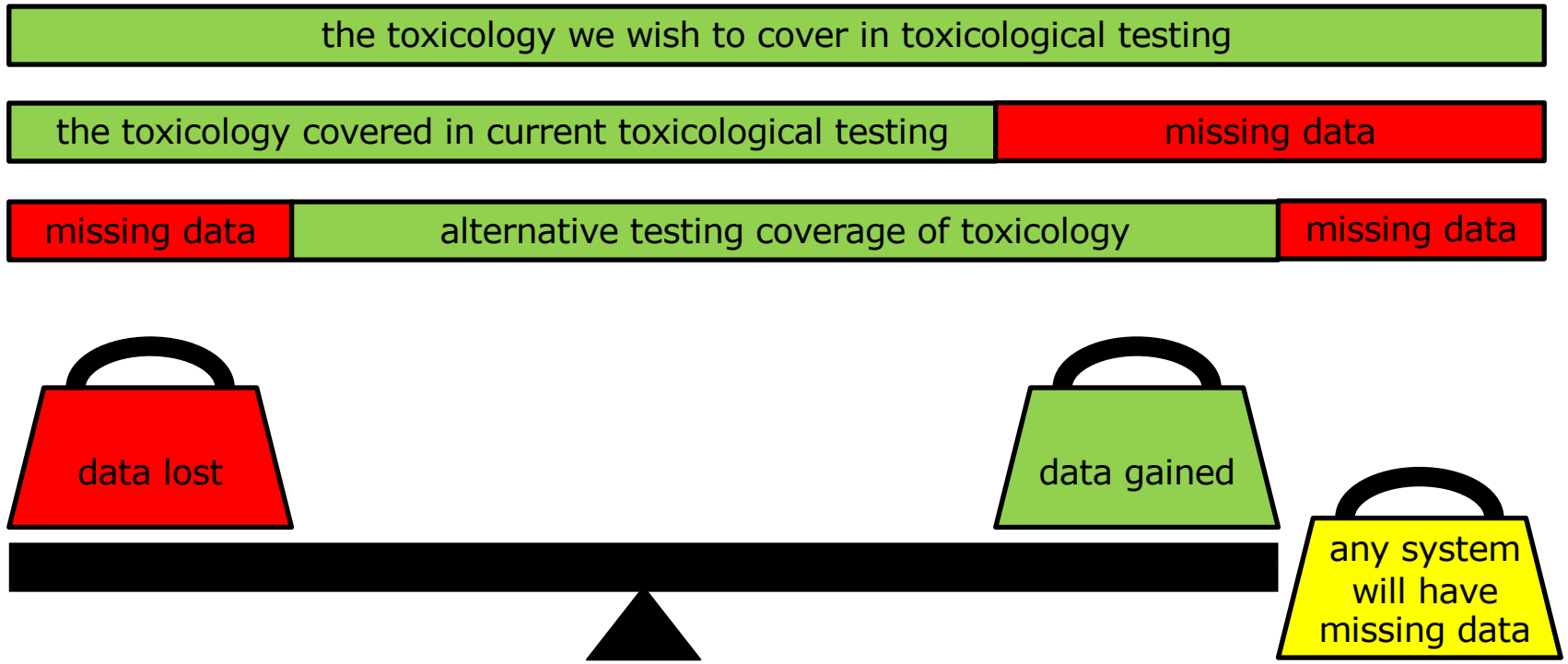
CompuCell3D animation Berkhout et al., in progress

Considering compound kinetics

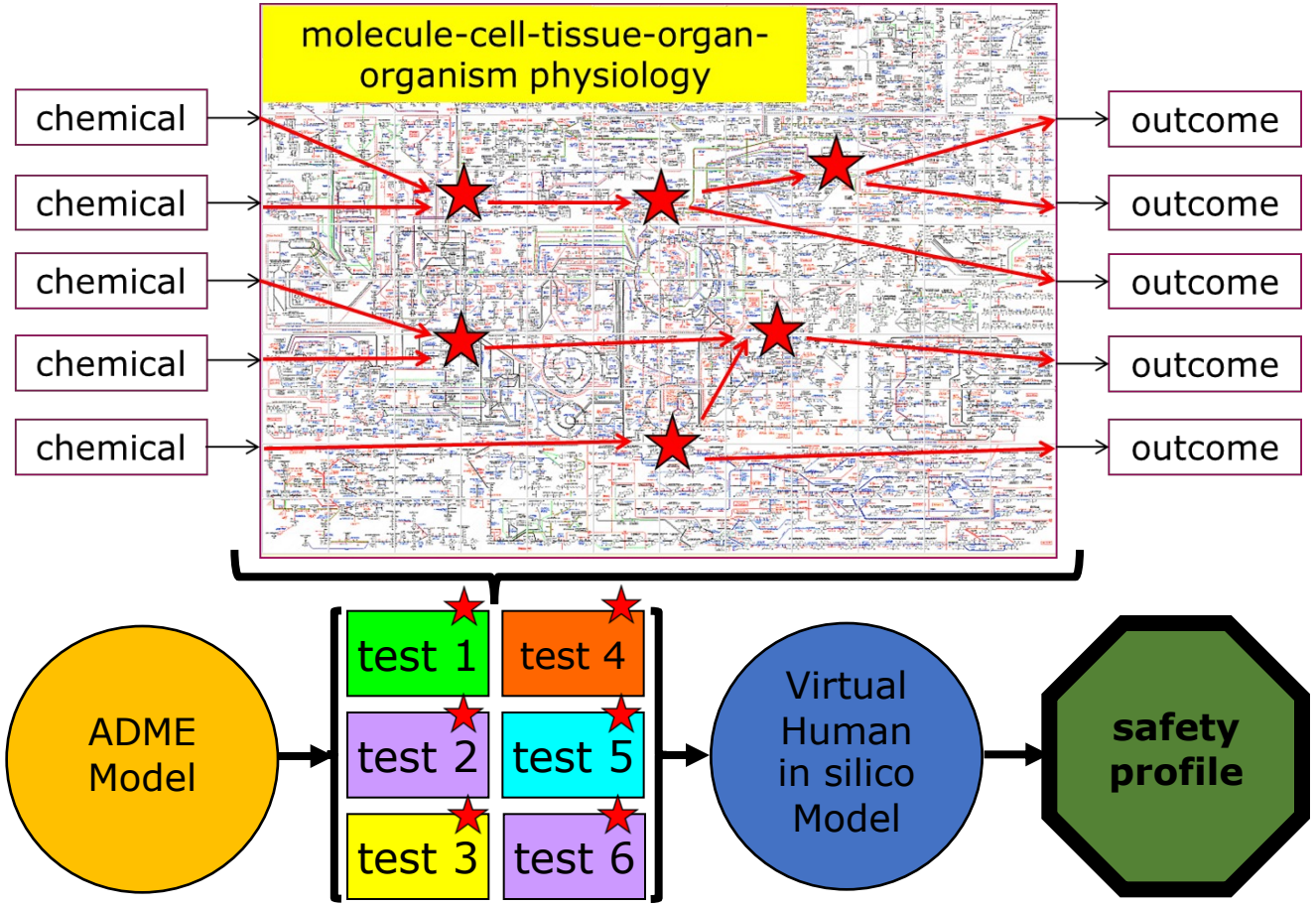


Piersma et al., 2019

The balance of transition in toxicological testing



Animal-free risk assessment



Piersma et al., 2019

“Revolutionizing” chemical risk assessment

- Based on integrated knowledge of the biology of the system
- Fit for purpose - Comprehensive as to toxicity pathways
- Employing all existing knowledge in chemistry and toxicology (incl *man* and *animal* and *in vitro* and *in silico*)
- Targeting the human
- Avoiding the detour of the animal (time, cost, ethics)

A way forward

- Serving both “Revolution” and “Evolution”
- Develop tools that fit both Revolution and Evolution
- Aim at transition to innovative approach on the horizon whilst implementing low hanging fruit now
- Intensify stakeholder interaction
  - (science, government, industry, NGO, politics)
- Intensify multidisciplinary collaboration
  - (biology, toxicology, data scientists, software developers, artificial intelligence experts)

Thank You

"The computer is claiming its intelligence is real, and ours is artificial."