Texas Indiana STAR Center on Developmental Toxicology

Investigational Area 1; Zebrafish as a model to elucidate the morphological and mechanistic effects of environmental pollutants

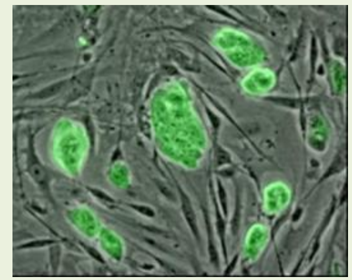
March 22, 2012

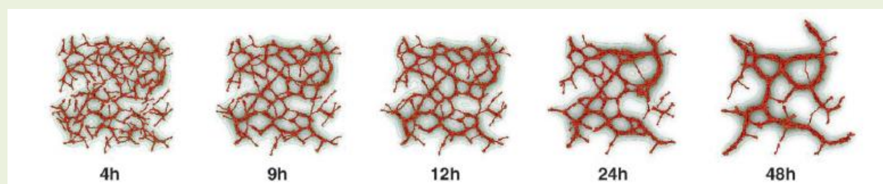
Maria Bondesson

University of Houston

New screening models for developmental toxicity

From Biological Models of Developmental Toxicity to Computer Simulations



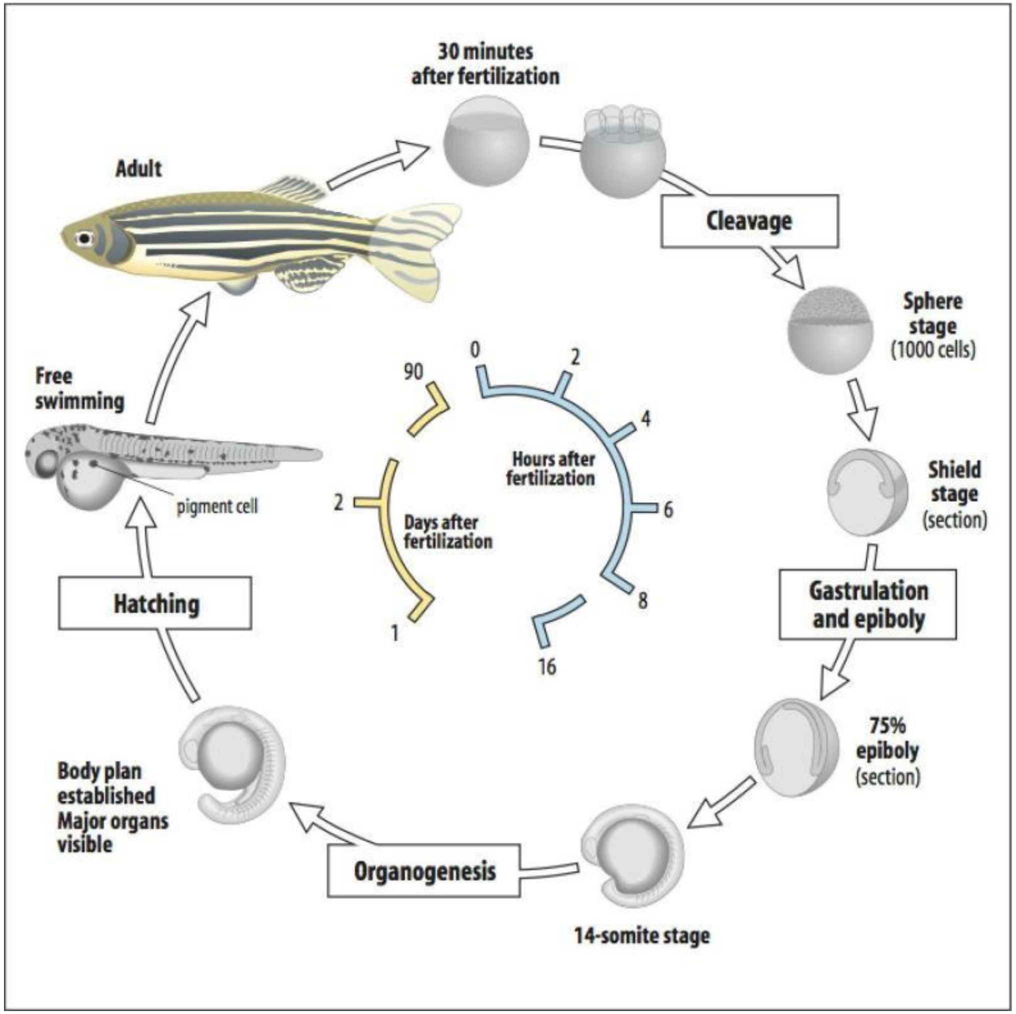


Zebrafish, mouse stem cells and computer models

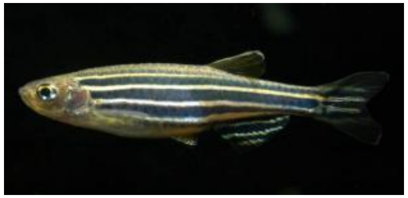
Jan-Ake Gustafsson, UH, Rick Finnell, UT-Austin, James Glazier, IU

Why use zebrafish as a model for developmental toxicity?

- External, ex-utero, rapid embryonic development
- Small size, small test volumes
- Transparent embryos/fish
- Hundreds of embryos weekly/pair
- Genome almost sequenced
- 75% of genes have human homologues
- Conserved developmental processes and signaling pathways
- Many mutants
- Morpholino knockdown
- Cost-efficient
- Adaptable to medium or high throughput screening
- Many transgenic fish available



Adapted from Lewis Wolpert *et al. Principles of Development* (3rd ed.)

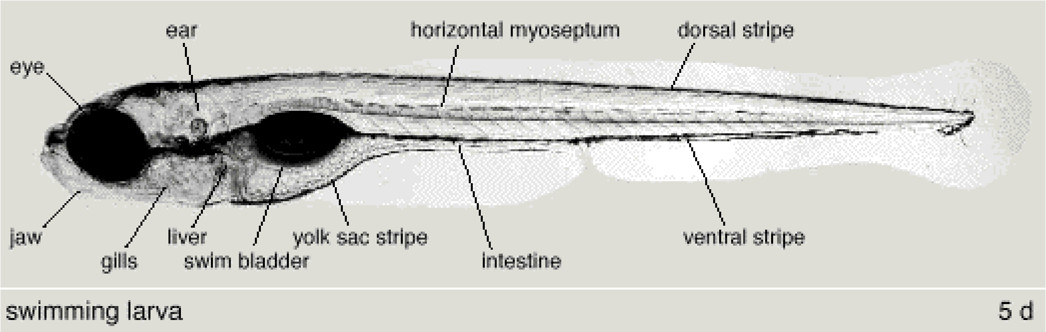


Developmental toxicity in ZF

Lethality | Gastrulation/epiboly
Hatching | Size | Curvature | Yolk sac edema | Swim bladder inflation
Notochord | Somite | Neuronal system | Vascular system

Eye

Ear



Cardiac | Pancreas | Liver | Kidney | Gut
Muscle | Skeletal | Immune system | Lipid metabolism
Behavior

Signaling pathways: HSP, oxidative stress, apoptosis

ZeToxDB

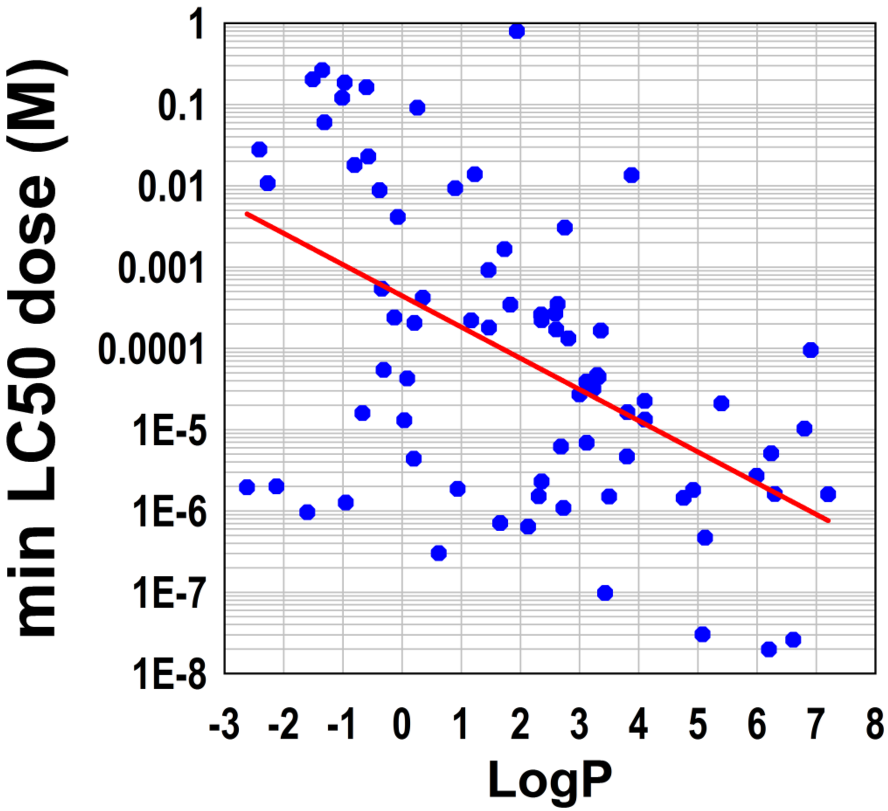
140 Chemicals

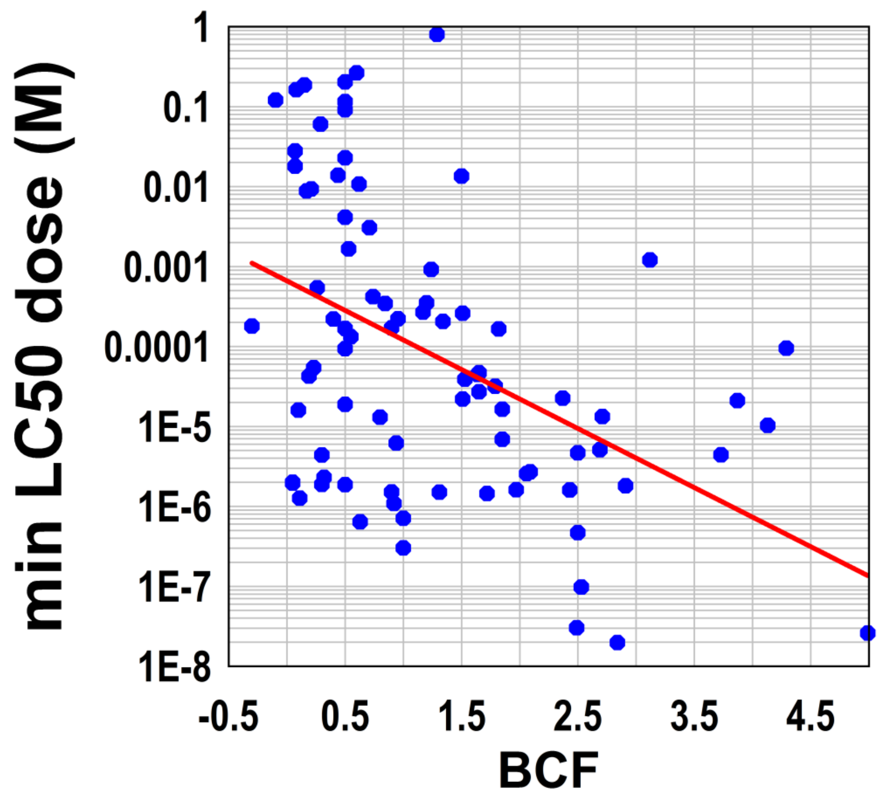
Conclusions from PubMed articles:

1. Many of the phenotypic perturbations are correlated, as the same compound causes many different effects. For example, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), perfluorooctanesulfonate (PFOS), arsenite and ethanol all affect hatching time, body size and curvature, yolk sac edema, and swim bladder inflation .
2. While some endpoints are investigated extensively (i.e., cardiovascular and gross morphological phenotypes) others have been less studied, such as pancreas, immune system, and lipid metabolism.

McCollum CW, Ducharme NA, Bondesson M and Gustafsson J-A. Developmental toxicity screening in zebrafish. Birth Defects Research (Part C) 2011, 93:67–114

Can toxicity in ZF be predicted?





$$\log\ P_{\text{oct/wat}} = \log\left(\frac{[\text{solute}]_{\text{octanol}}}{[\text{solute}]^{\text{un-ionized}}_{\text{water}}}\right)$$

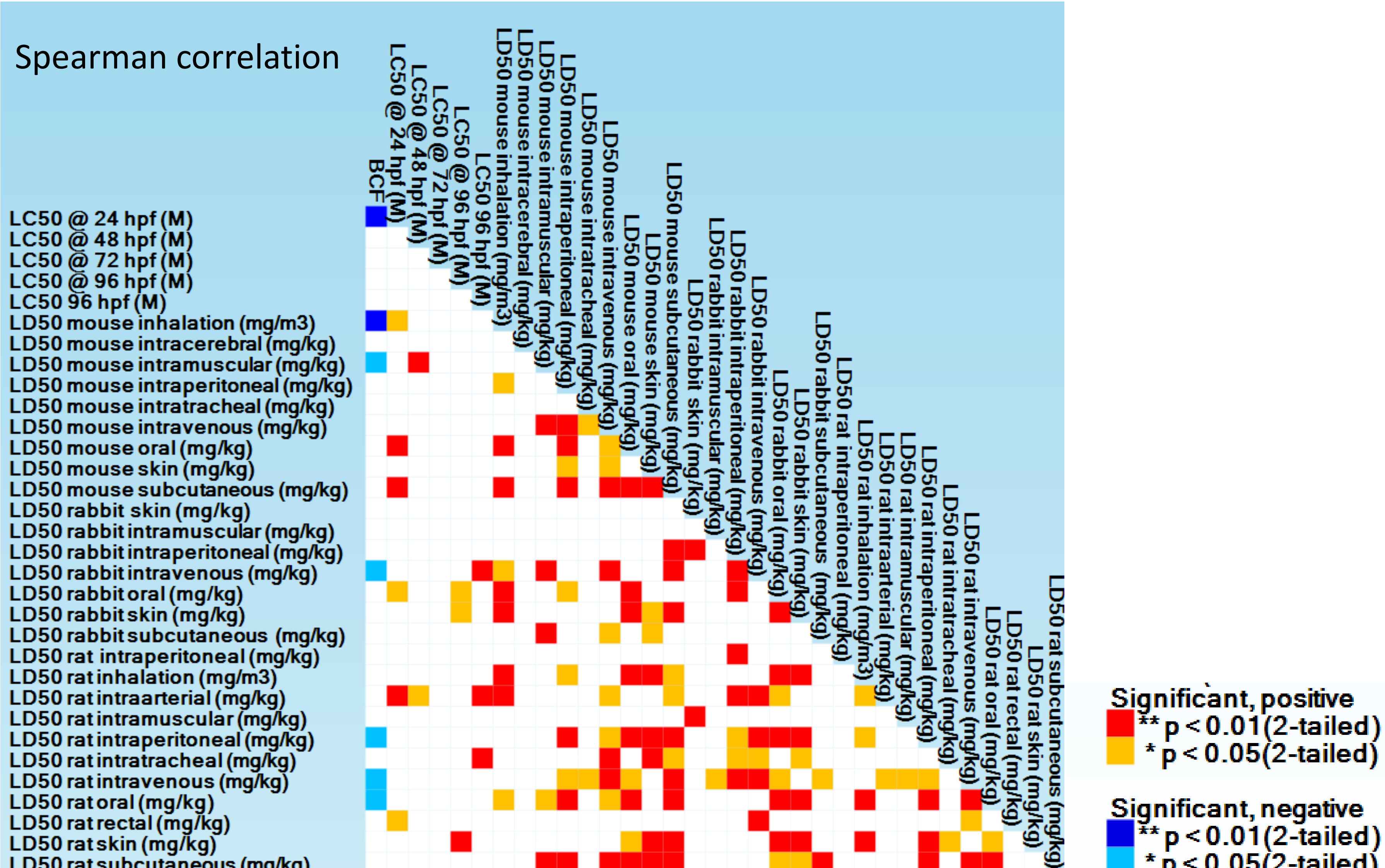
$$BCF = \frac{Concentration_{Organism}}{Concentration_{Environment}}$$

The **partition coefficient** is a ratio of concentrations of un-ionized compound between the two solutions.
The logarithm of the ratio of the concentrations of the un-ionized solute in the solvents is called **log *P***: The log P value is also known as a measure of lipophilicity.

Bioconcentration factor (**BCF**) is the concentration of a particular chemical in a biological tissue per concentration of that chemical in water surrounding that tissue.

Leif Petersen, Nicole Ducharme, Emilio Benfenati

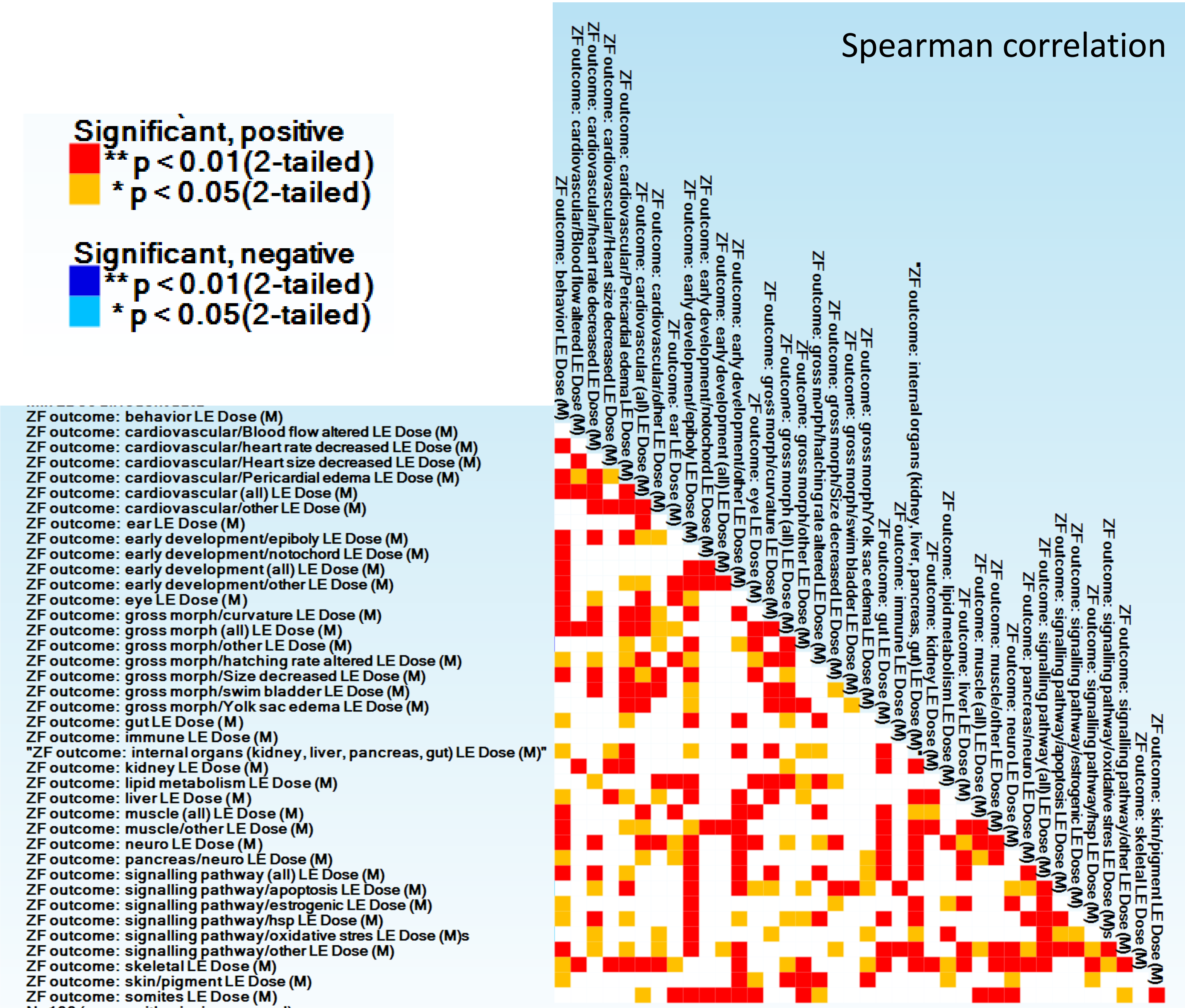
Do the fish LC50s correlate to rodent LD50s?



OECD guidelines 212: Treatment period: as soon as possible after fertilization (early gastrula stage) to 5 days post-hatch (8-10 days)

Leif Petersen, Nicole Ducharme

Do different zebrafish perturbations correlate to each other?



Leif Petersen, Nicole Ducharme

Aims of TIVS project/ZF part

The aims are to:

1. Develop *in vivo* screening models

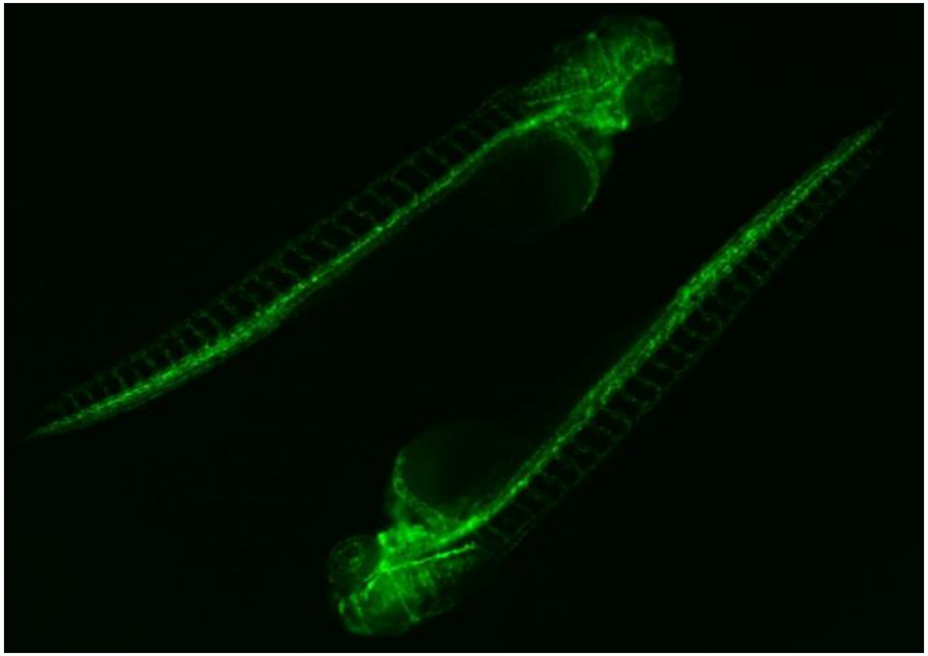
2. Produce high information content models.

The selected endpoints for the screening models include:

•*Gastrulation and early embryonic cell movements*
•*Patterning of CNS and neurogenesis*
•*Hematopoiesis and angiogenesis*
•*Yolk utilization and morphological effects on somitogenesis*

The workflow is to:

1. Use transgenic zebrafish expressing fluorescent markers in a tissue/organ/cell and to record the expression of the marker under normal and perturbed embryonic development.
2. Quantify effects of perturbations
3. Automate screening

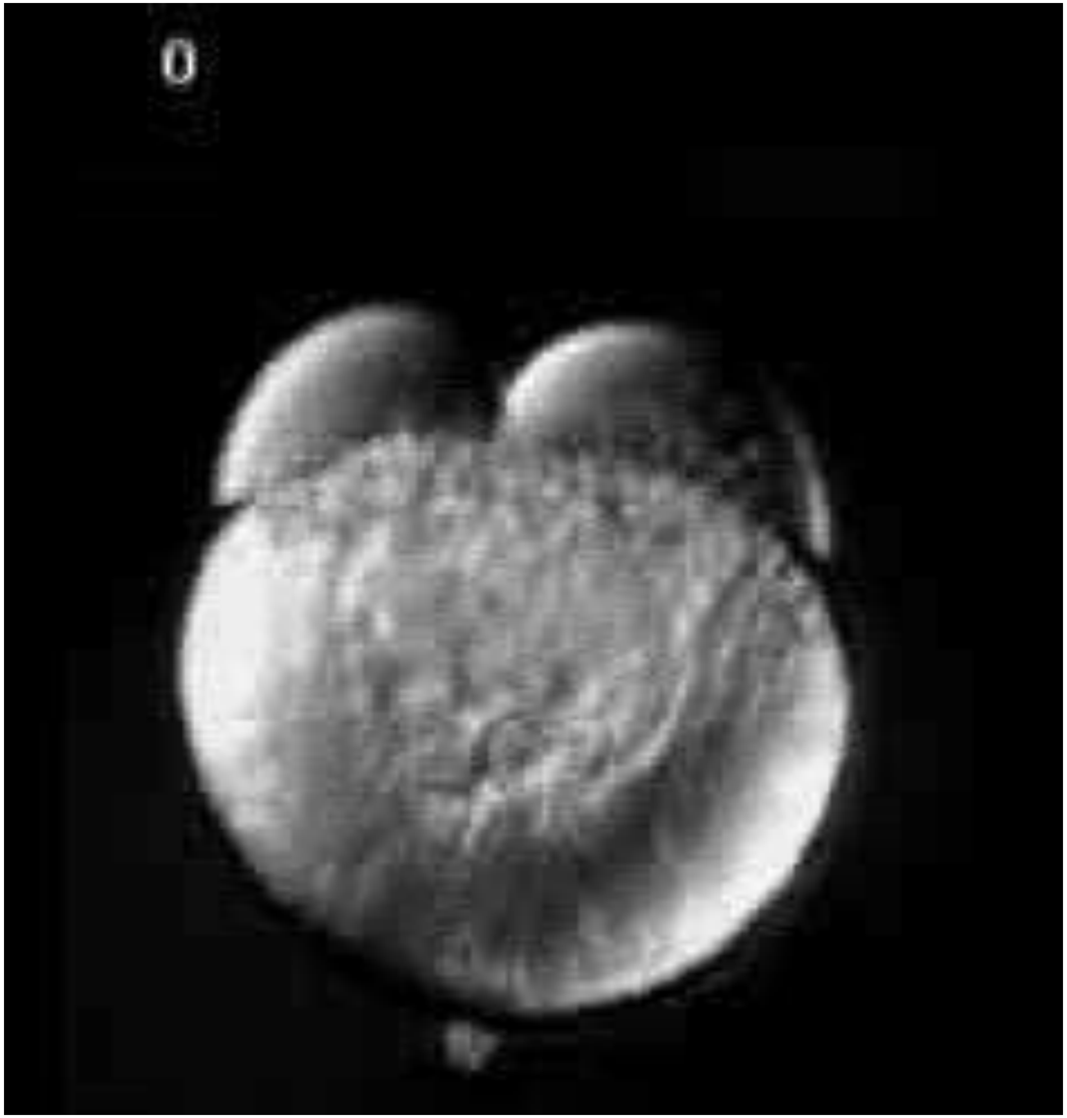


Transgenic fish

#	Gene	Used for	Reporter	Start time of expected expression (hpf)
1	*dharma*	Early patterning, epiboly, early cell movements and developmental delay	EGFP	3.5 hpf
2	*wnt8*	Patterning (anterior-posterior symmetry), early cell movements	GFP-	1 cell stage 0 hpf
3	*ngn1*	Neurogenesis, Axon guidance, early, developmental delay	GFP	10 hpf
4	*fli1*	Angiogenesis and blood vessel remodeling, heart morphology and function	EGFP-	11 hpf
5	*flk1*	Angiogenesis and blood vessel remodeling, heart morphology and function. Expressed in tip cells.	EGFP-	11 hpf
6	*kdr membrane*	Membranes of vascular cells	Cherry	
7	*kdr nuclear*	Nuclei of vascular cells	dsRed	
8	*gata-1*	Red blood cells	dsRed	
9	*unc45b*	A myosin chaperone, Muscle development and somitogenesis	GFP	9hpf
10	*smyhc-1*	Slow myosin heavy chain 1, Muscle development and somitogenesis	GFP	
11	*HGn39B*	Muscle expression, Muscle development and somitogenesis	GFP	
12	*Yolk (HGn50D)*	Yolk expression, Yolk utilization	GFP	
13	*ERE*	Estrogen response element driven reporter	GFP	
14	*hPPARg reporter*	Gal4-human PPARgamma fusion together with an UAS reporter	GPF	
15	*zfPPARg reporter*	Gal4-zebrafish PPARgamma fusion together with an UAS reporter	GFP	

Rapid external development

Time lapse movie by Karlstrom and Kane



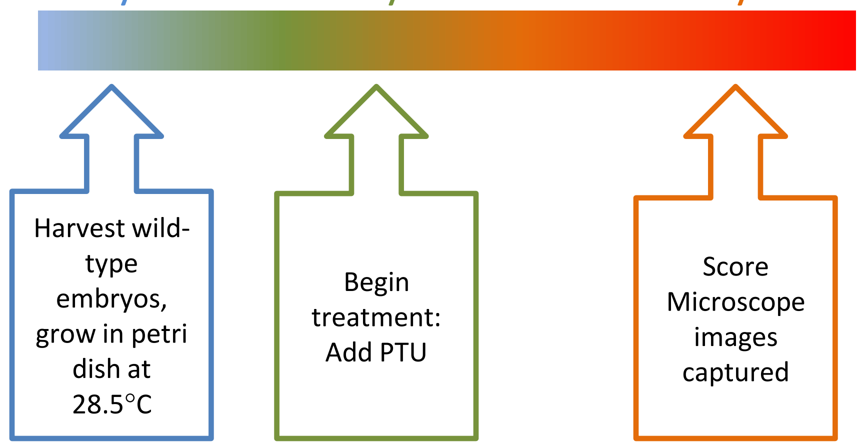
1-24hpf

Screening model for yolk utilization

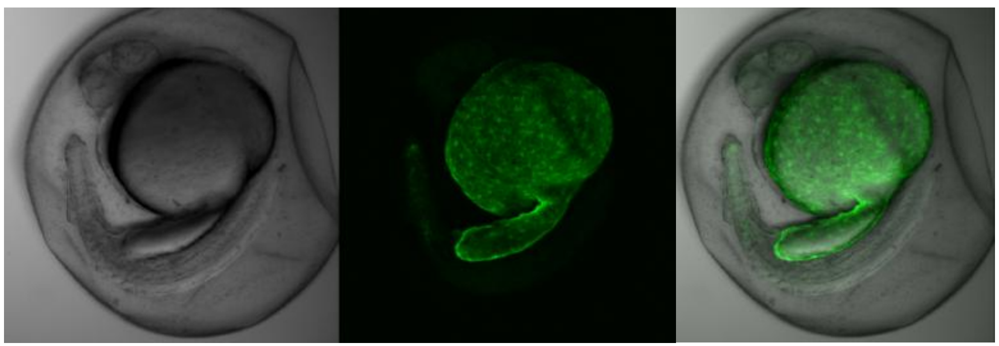
The yolk is the source of nutrients during the first week of development.
The components that make up the yolk are mostly phospholipids and triacylglycerols in the form of yolk globules. Yolk lipids are processed and transferred to the embryo at the yolk-embryo interface, also known as the yolk syncytial layer (YSL). This process requires microsomal triglyceride transfer protein (mtp).

We used the fish Tg(*Yolk;GFP)* (originally called HGn50D). This is one of the so called enhancer trap (CET) zebrafish lines, generated by Tol2 transposon-mediated transgenesis of GFP into random sites.
http://kawakami.lab.nig.ac.jp/ztrap/

Experimental Design

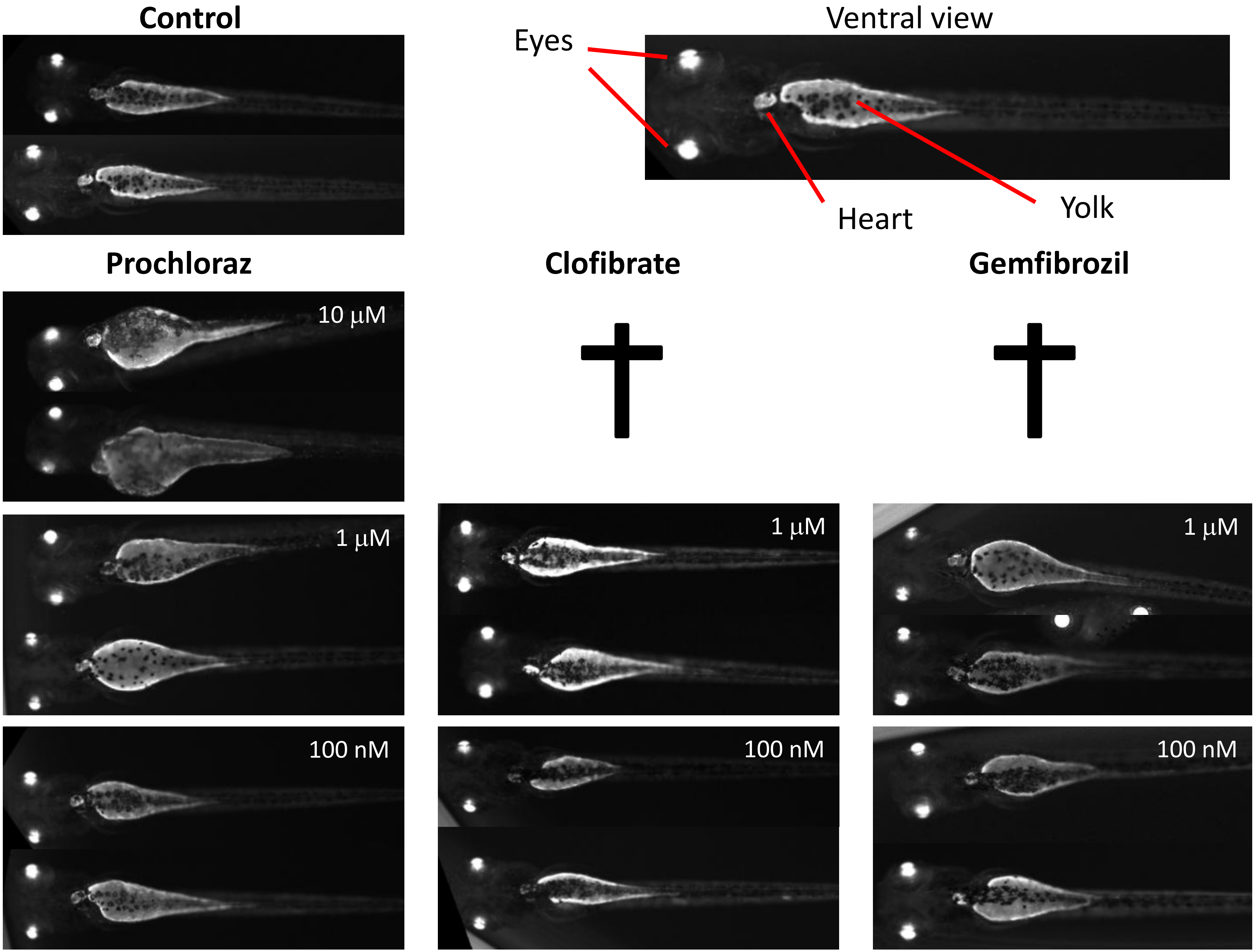


Confocal images 1dpf



DIC GFP Merge

PTU = phenylthiouracil (inhibits pigmentation)



Control
Ventral view
Eyes
Heart
Yolk
Prochloraz
Clofibrate
Gemfibrozil
10 μM
1 μM
100 nM
1 μM
100 nM
1 μM
100 nM

Screening model for yolk utilization

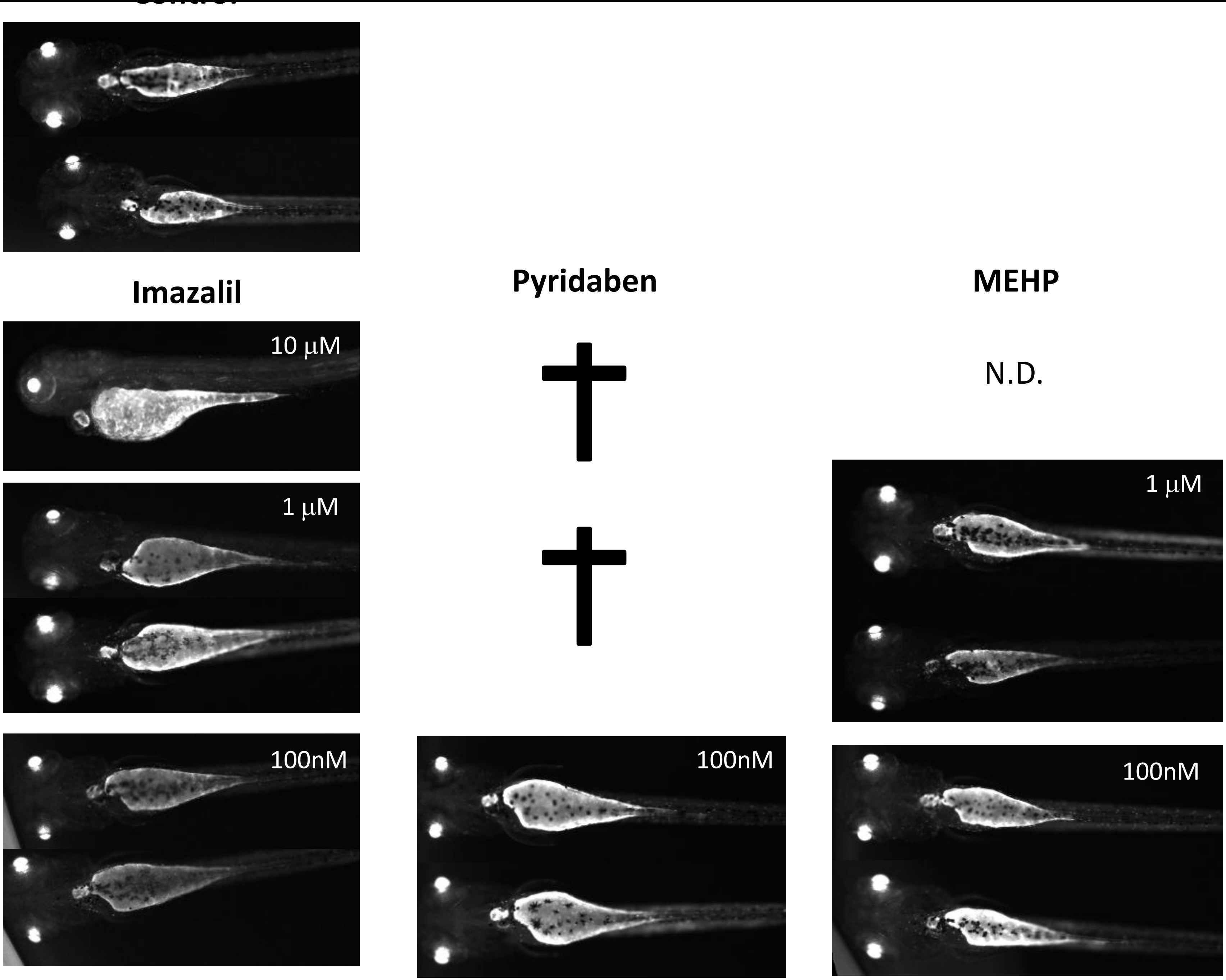
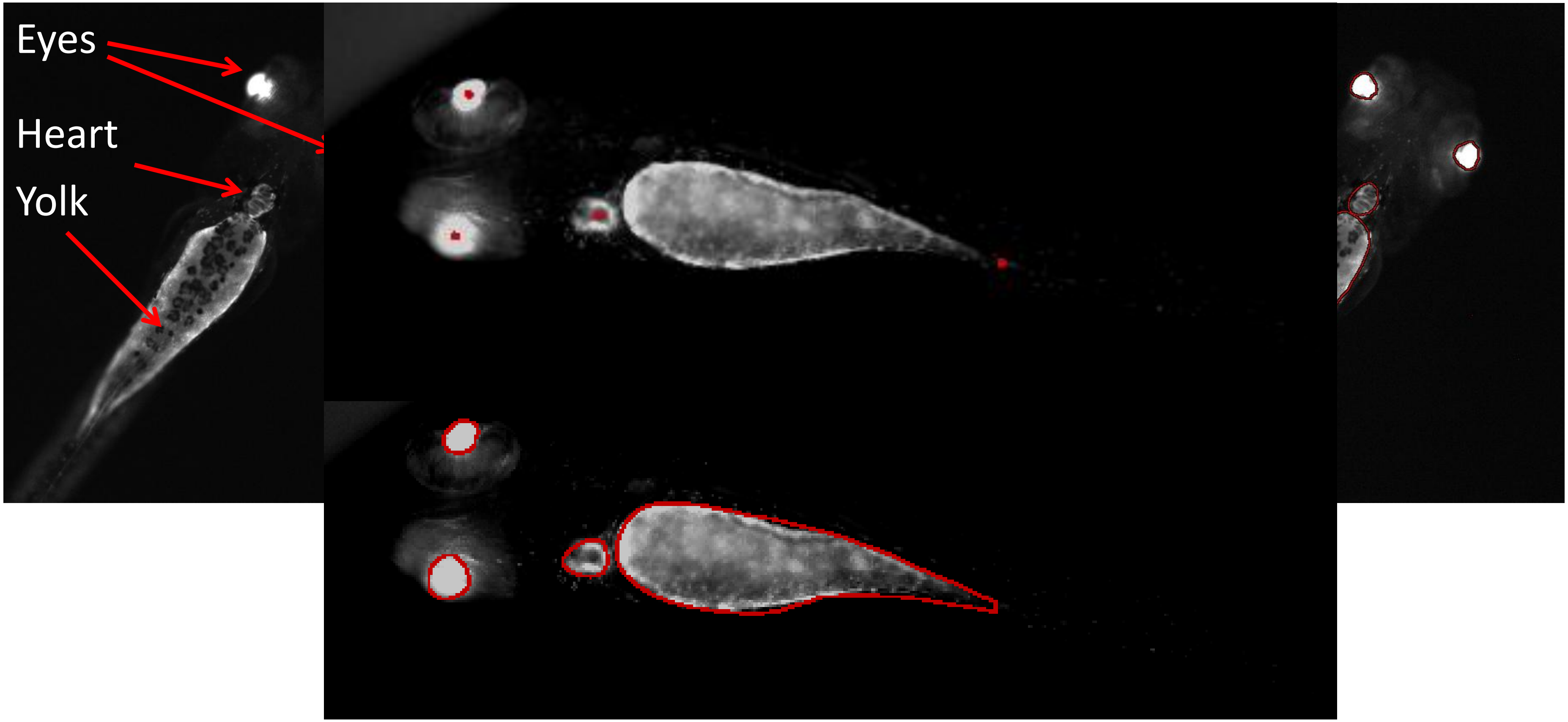


Image Segmentation

In collaboration with Eleni Zacharia and Ioannis Kakadiaris, Dept. of Computer Sciences, UH

- The purpose of image segmentation is to partition an image into the following 4 *meaningful regions: Eyes, Heart and Yolk*

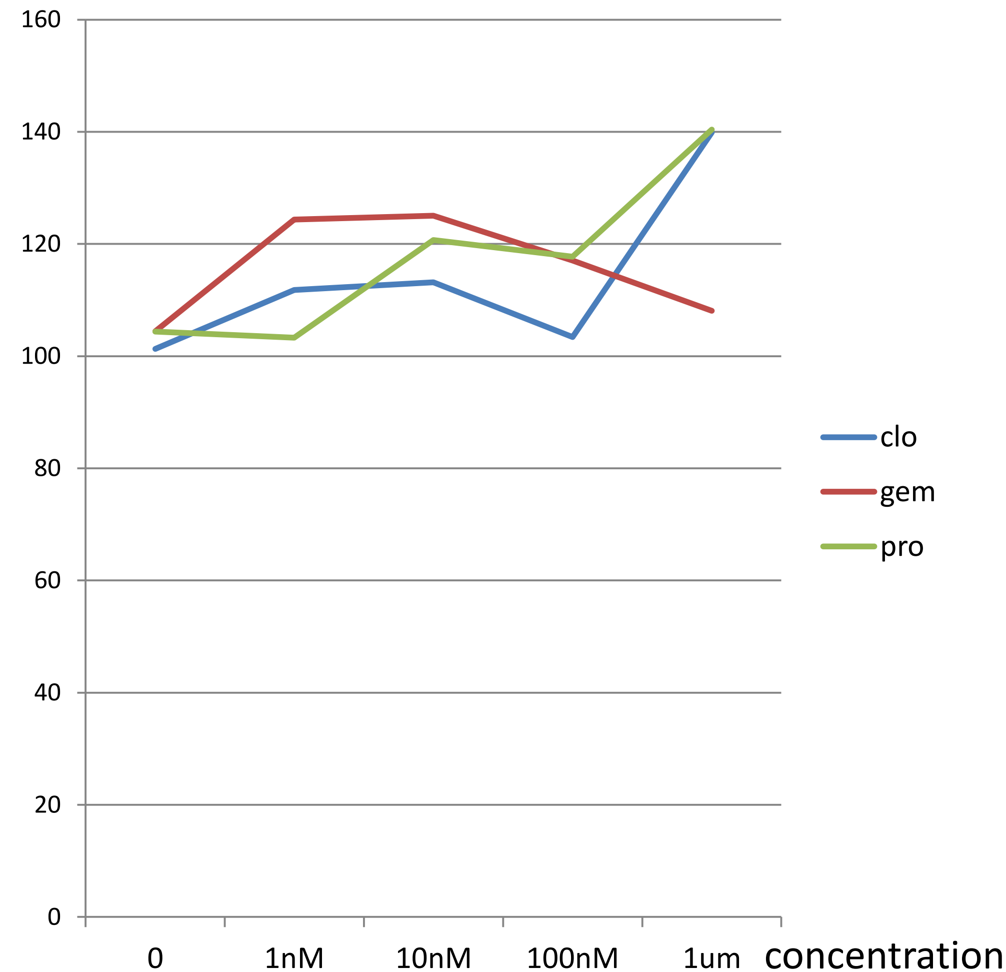


Quantifying yolk utilization

<u>Semi-automatic quantification</u>
Manually add 4 dots
Program calculates:
Area of yolk
Distance between eyes (orientation of fish)
Distances between eye and heart (size)

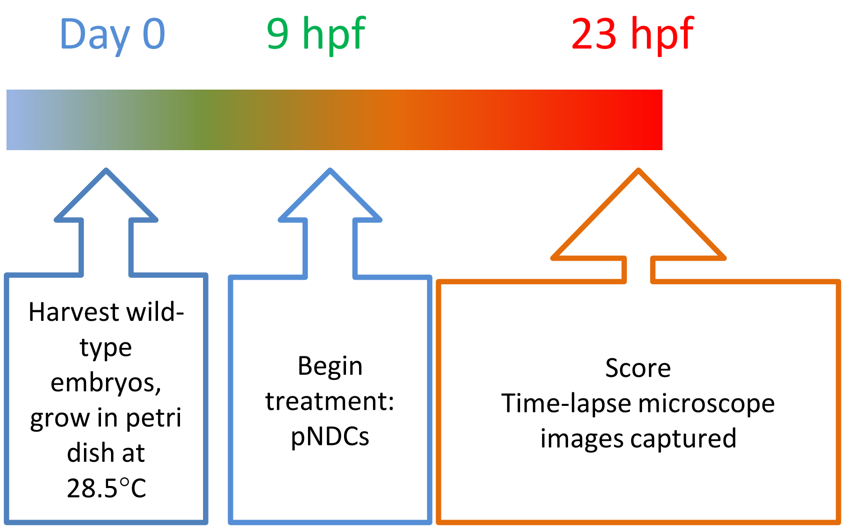
Clofibrate and Gemfibrozil: Fibrates used to prevent high cholesterol levels in humans.
Clofibrate increases lipoprotein lipase activity to promote the conversion of VLDL to LDL, and hence reduce the level of VLDL. It is proved that it can increase the level of HDL as well. Gemfibrozil is a PPARalpha ligand in mammals. Clofibrate and Gemfibrozil induce embryonic malabsorption syndrome (EMS) in fish (Raldua et al., Toxicology and Applied Pharmacology 228 (2008) 301–314).
Prochloraz is a fungicide that has been shown to affect yolk utilization (Domingues I et al., Environ Toxicol. 2011).
Imazalil and Pyridaben predicted to be vascular disruptors (Kleinstrauer, EHP 2011 Nov;119(11):1596-603)
MEHP, Monoethylhexylphthalate. Active metabolite of DEHP, plasticizer.

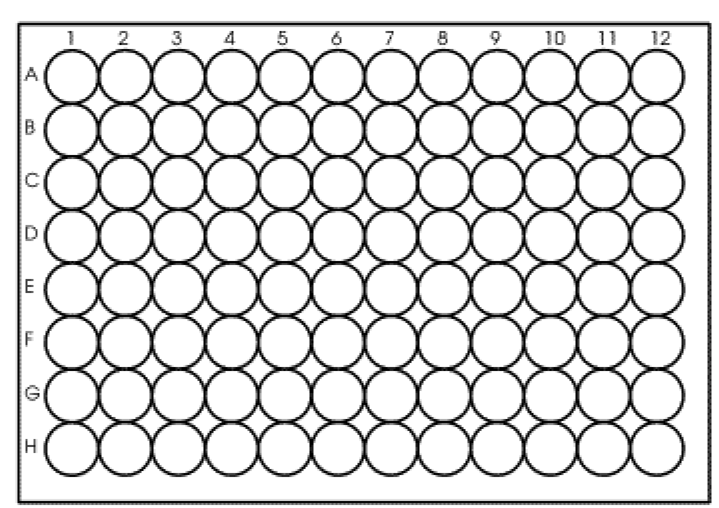
Yolk area (pixels)/Distance between eyes and heart

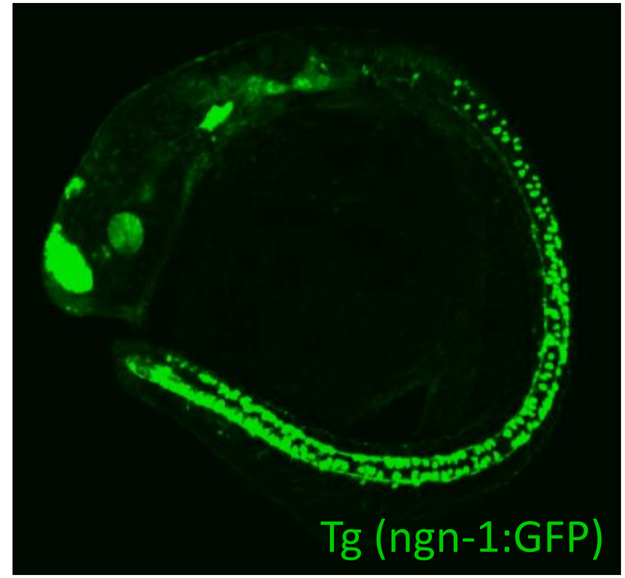


Eleni Zacharia

Tg (Ngn-1:GFP); Express GFP in neuronal precursors







Compounds tested so far:

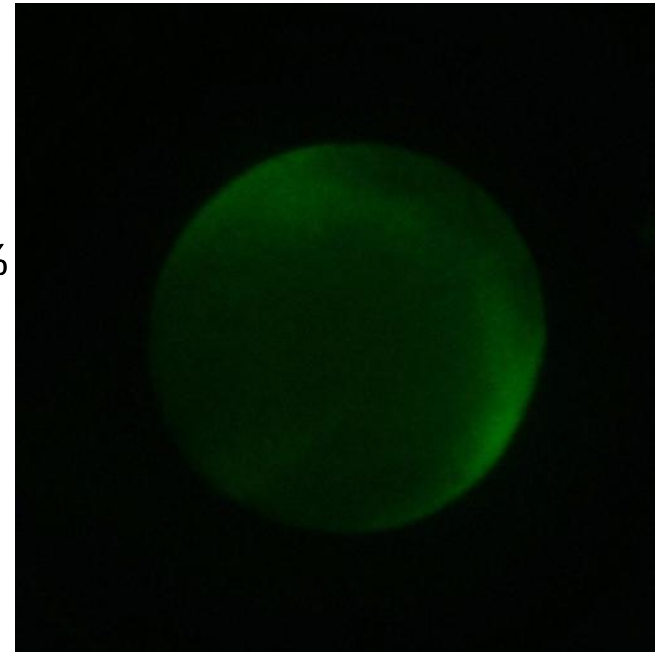
Atrazine
BPA
Endosulfan
Ethanol
Methoxychlor
Pentachlorophenol
Perfluorooctane Sulfonate
Perfluorooctanoic Acid
Retinoic Acid
VPA
DEHP

Anne Riu

Birth of neuronal system

Tg (Ngn-1:GFP); Express GFP in neuronal precursors

Control
DMSO 0.1%



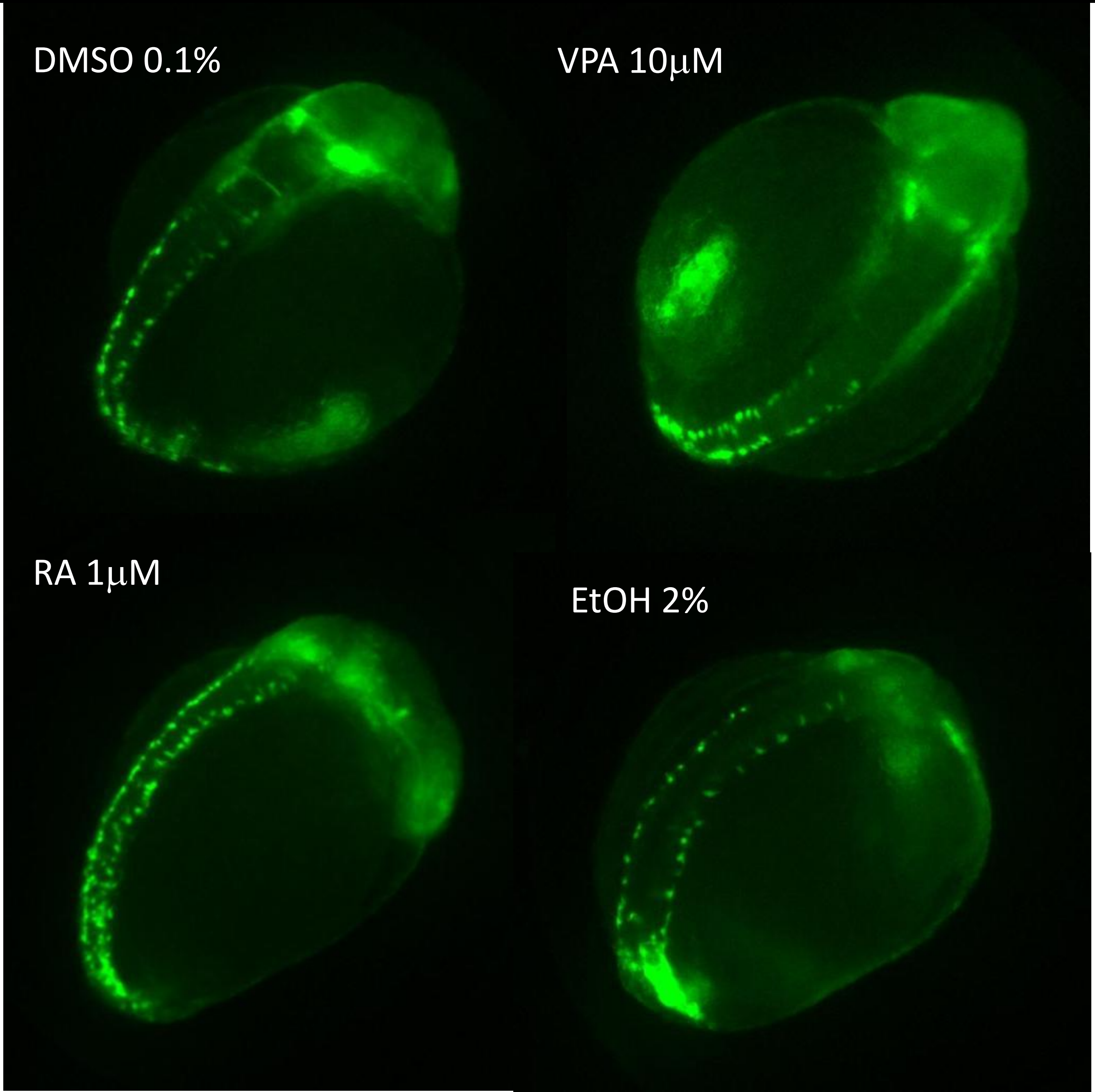
VPA 10μM

RA 1μM

EtOH 2%

Anne Riu

Toxic effects on neuronal precursor cells



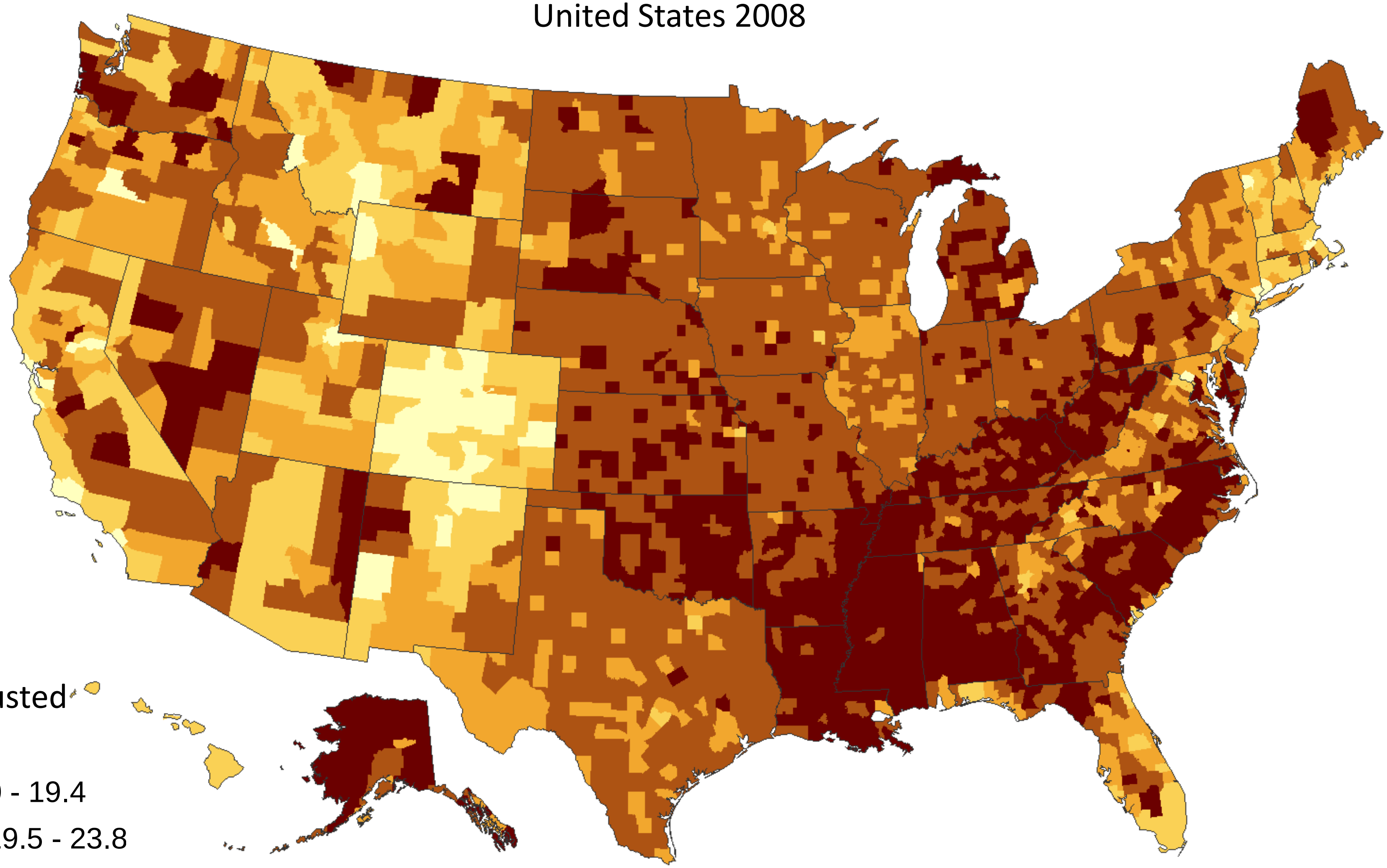
Toxic effects on neuronal precursor cells

Chemicals tested (concentration)	Effects observed
Atrazine (100nM - 10nM – 1nM)	No effect on ngn-1
Bisphenol A (10µM – 1µM- 100nM)	No effect on ngn-1
Endosulfan (1µM - 100nM - 10nM)	No effect on ngn-1
Ethanol (1%-2%-3%)	2%: development delayed - Low GFP expression in the head region 3%: development defect- Lethal
Methoxychlor (1µM - 100nM - 10nM)	No effect on ngn-1
Pentachlorophenol (1µM - 100nM - 10nM)	No effect on ngn-1
Perfluorooctane Sulfonate (10µM - 1µM- 100nM)	No effect on ngn-1
Perfluorooctanoic Acid (10µM – 1µM- 100nM)	No effect on ngn-1
Retinoic Acid (1µM - 100nM - 10nM)	1µM: development defect; ngn-1 expression stronger
Tetrabromobisphenol A (1µM - 100nM - 10nM)	No effect on ngn-1
Valproic Acid (1µM – 100nM – 10nM)	1µM: lethal – 100nM: no development (lethal) 10nM: Low GFP expression in the head region
DEHP (10µM – 1µM- 100nM)	100 nM Delayed migration of ngn-1 positive cells

Anne Riu

The obesity epidemic

County-level Estimates of Obesity among Adults aged ≥ 20 years:
United States 2008



www.cdc.gov/diabetes

Obesogen screening model experimental design

1. Chemical exposure → zebrafish → "Obesity"?

2. Do obesogens act through nuclear receptors?
3. Comparison between human and fish PPARgamma

Obesogen screening model experimental design

Day 0 | Day 1 | Day 3 | Day 6 | Day 11

Day 0: Harvest wild-type embryos, grow in petri dish at 28.5°C

Day 1: Add PTU

Day 3: Begin treatment

Day 6: Begin feeding high fat diet (AM) and daily solution changes (PM)

Day 11: Fix fish for staining with ORO

PTU = phenylthiouracil (inhibits pigmentation)

ORO = Oil Red O

TBBPA, TCBPA, TBT, DEHP, MEHP, PFOA

Image analysis to quantify lipid staining

Table I. Average value and the standard deviation of the lipid accumulation inside each zebrafish

Compound	***# of fish***	$P = redPixels / FishPixels$			
		Lean Fish (P<0.1)		**Obese Fish (P>0.1)**	
		% of fish	***average ± sd***	**% of fish**	***average ± sd***
DMSO only	5	100	0.0618 ± 0.0235	0	-
10 nM TBBPA	4	100	0.0607 ± 0.0253	0	-
100nM TBBPA	10	60	0.0524 ± 0.0109	40	0.2616 ± 0.1390
1 uM TBBPA	8	100	0.0446 ± 0.0141	0	-
10 nM TCBPA	9	78	0.0632 ± 0.0137	22	0.1677 ± 0.0095
100 nM TCBPA	8	62	0.0637 ± 0.0172	38	0.3525 ± 0.0457
1 uM TCBPA	4	100	0.0567 ± 0.0207	0	-

Eleni Zacharia, Ioannis Kakadiaris

Results lipid accumulation

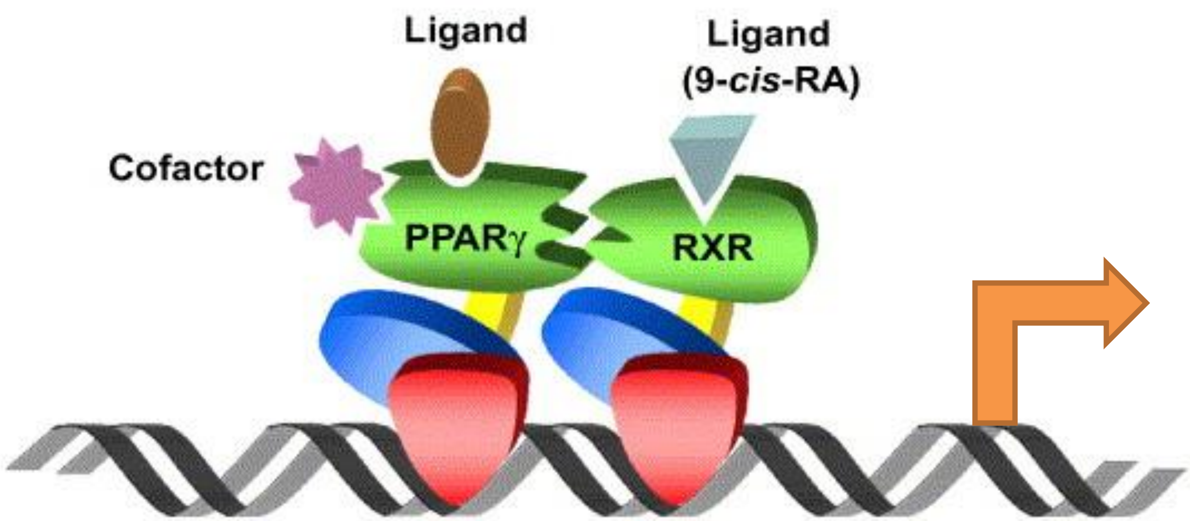
Percentage of fish with increased Oil Red O staining compared to control (DMSO only)

control	TBT	TBBPA	TCBPA
0.1% 0%	0.1 nM **17%**	10 nM **26%**	10 nM **5%**
	1 nM **40%**	100 nM **35%**	100 nM **15%**
		1 µM **5%**	1 µM **0%**

control	DEHP	MEHP	PFOA
0.1% 0%	100 nM **22%**	100 nM **50%**	1 µM **5%**
	1 µM **53%**	1 µM **26%**	10 µM **0%**
	10 µM **30%**	10 µM toxic	100 µM toxic

Catherine McCollum, Anne Riu

PPARγ is a master regulator of adipogenesis



aP2 (adipocyte lipid-binding protein, FABP)
ADRP (Adipose differentiation-related protein, lipid droplet associated)
LPL (lipoprotein lipase)
Adiponectin
C/EBP

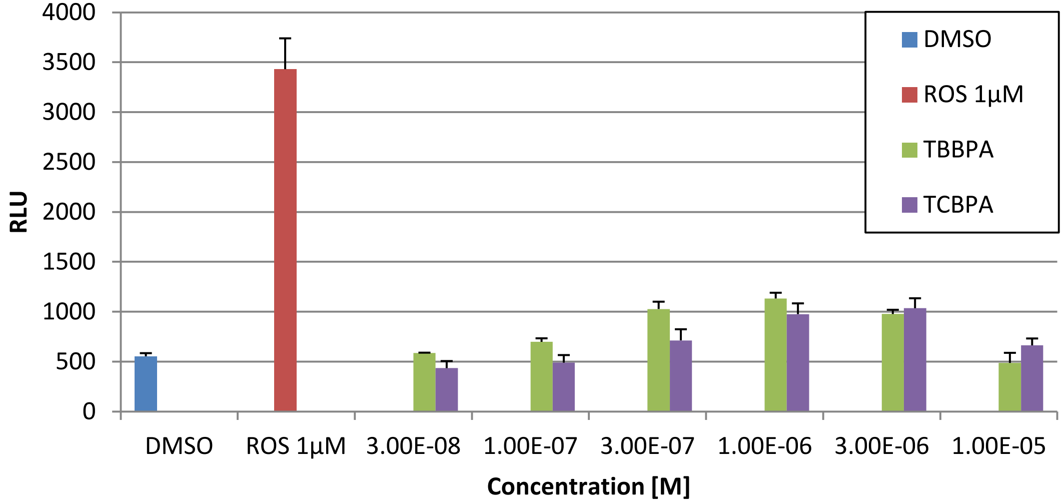
Adipocyte differentiation

Increased lipid storage

Potential obesogens activate PPARgamma in reporter cells

Gal4PPARgLBD → UAS-luciferase in HeLa cells

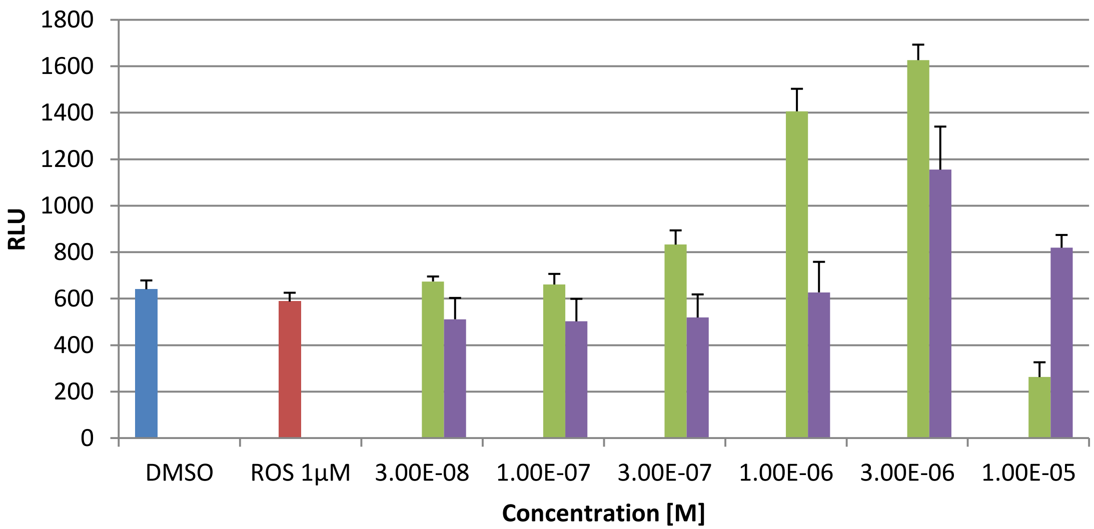
hPPARγ



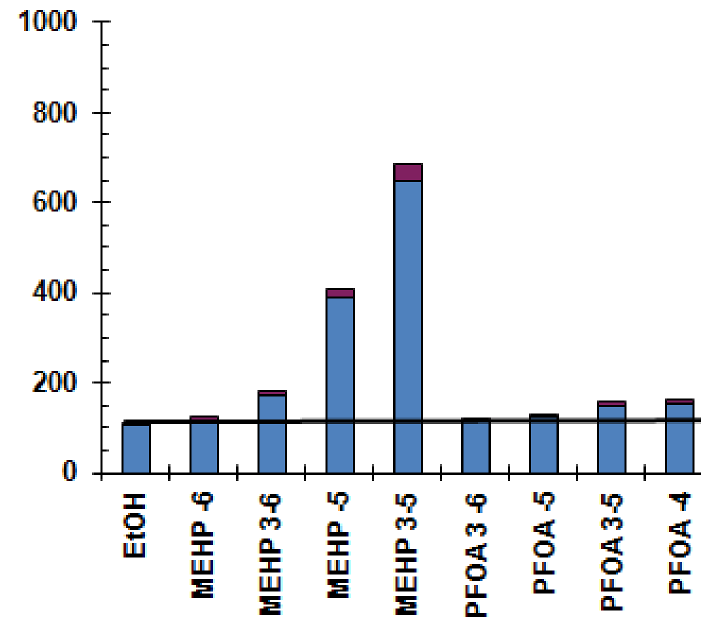
Rosiglitazone only activates hPPARγ

TBBPA ,TCBPA and MEHP activate zebrafish PPARγ

zfPPARγ



zfPPARgamma

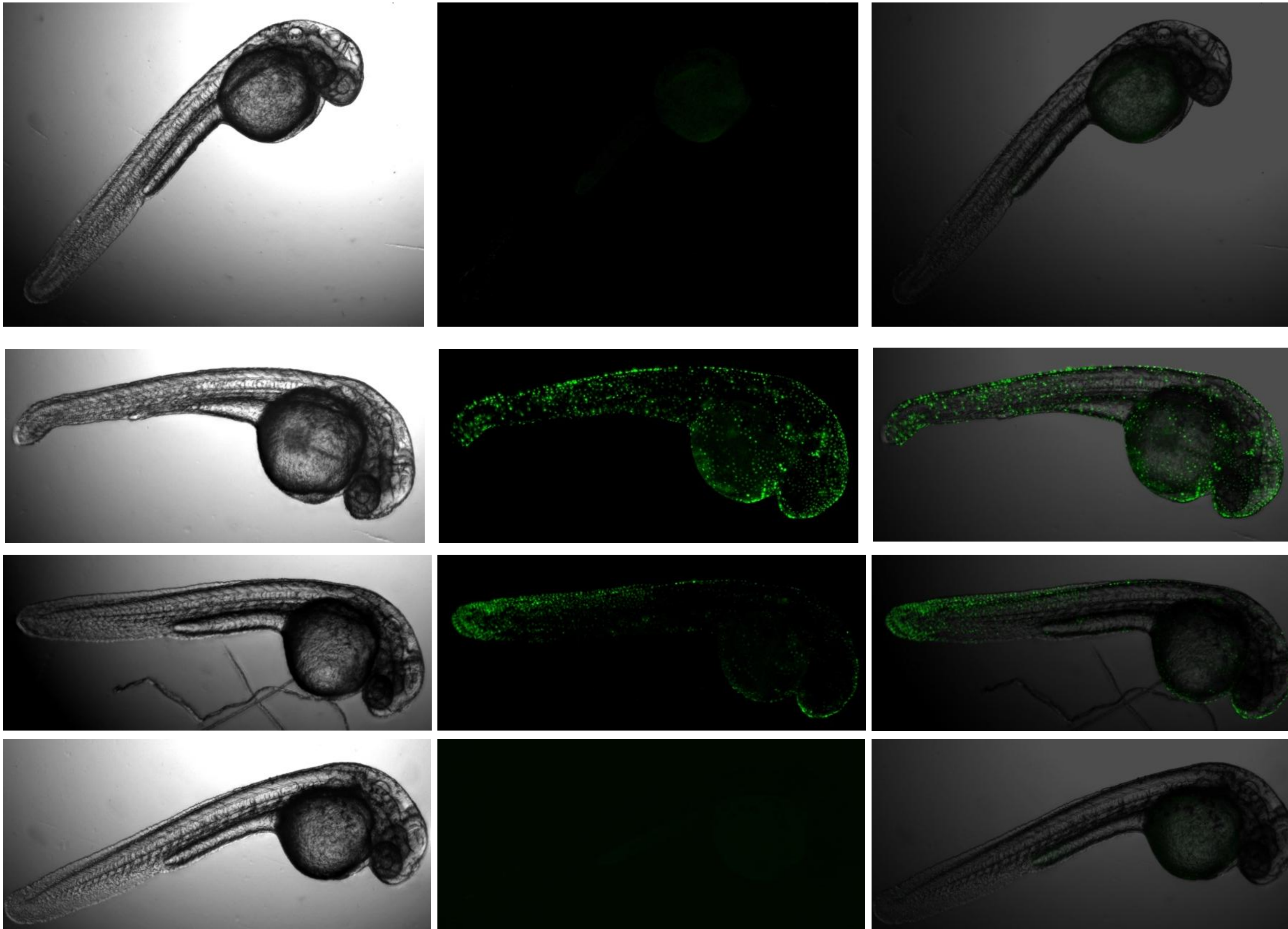


Caroline Pinto, Patrick Balaguer

TBBPA is a hPPARg agonist in vivo

Use of HS GAL4-HPPARγLBD-UAS-eGFP to screen PPARγ agonist *in vivo*

HS = 37°C/ 30 min at 13hpf – treatment during 24h – imaging at 37hpf



DMSO 0.1%

Rosiglitazone 1µM

TBBPA 1µM

TBT 1nM

Fish line from Tiefenbach J, et al. **PLoS One**. 2010 Mar 22;5(3):e9797.

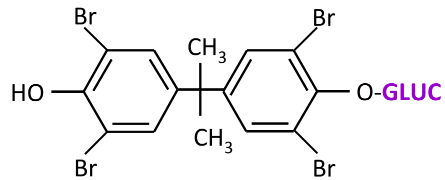
Anne Riu

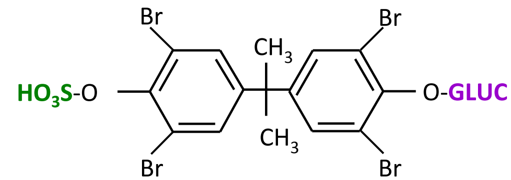
Metabolic fate of TBBPA

In vivo mammalian studies :

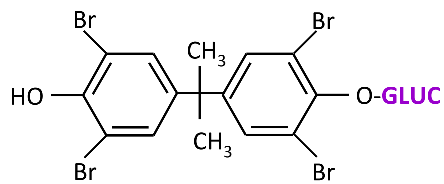
Mostly phase II biotransformation (conjugation)

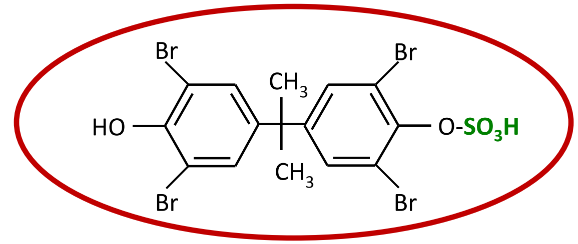
In Rat (2mg/kg bw)
Hakk et al. Xenobiotica 2000

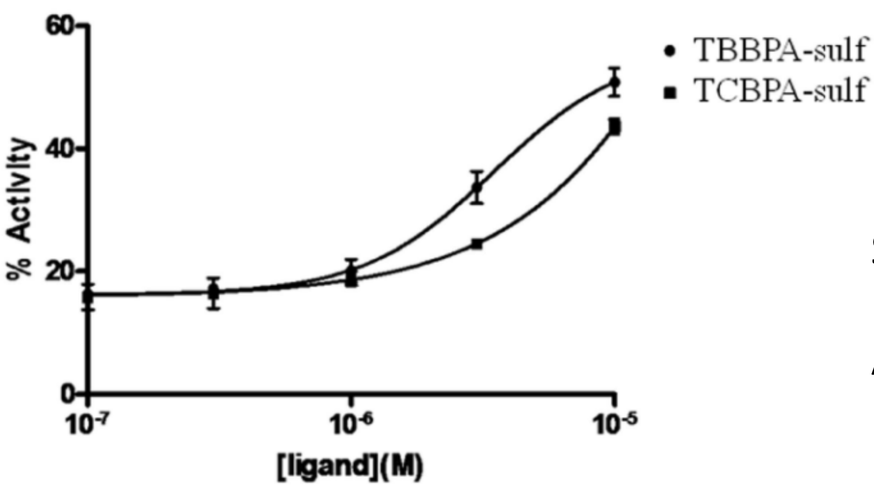




In Human (0.1 mg/kg bw)
Schauer et al. Tox Sci. 2006





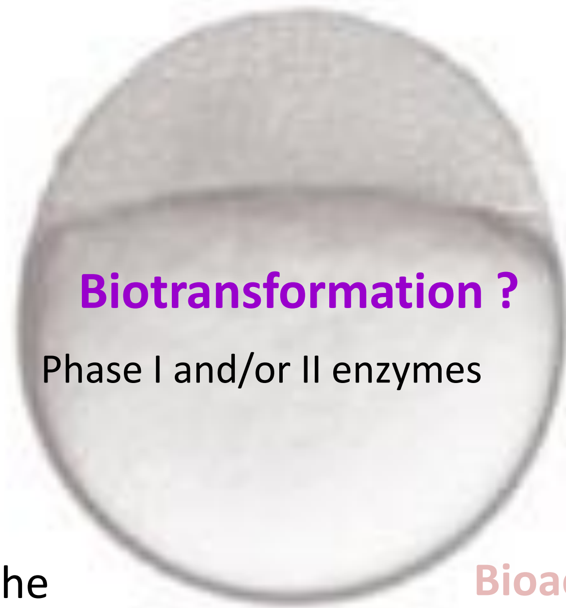


Sulfate conjugate are hPPARγ agonists but to a lesser extent than their parental compounds
Anne Riu et al. Toxicol Sci. 2011, 122(2):372-82

Could metabolism of TBBPA could be involved in the fat accumulation?

When are zebrafish embryos able to metabolize chemicals?

TBBPA → **Absorption ?**



Biotransformation ?

Phase I and/or II enzymes

→ **Elimination**

$TBBPA\text{-}SO_3H$

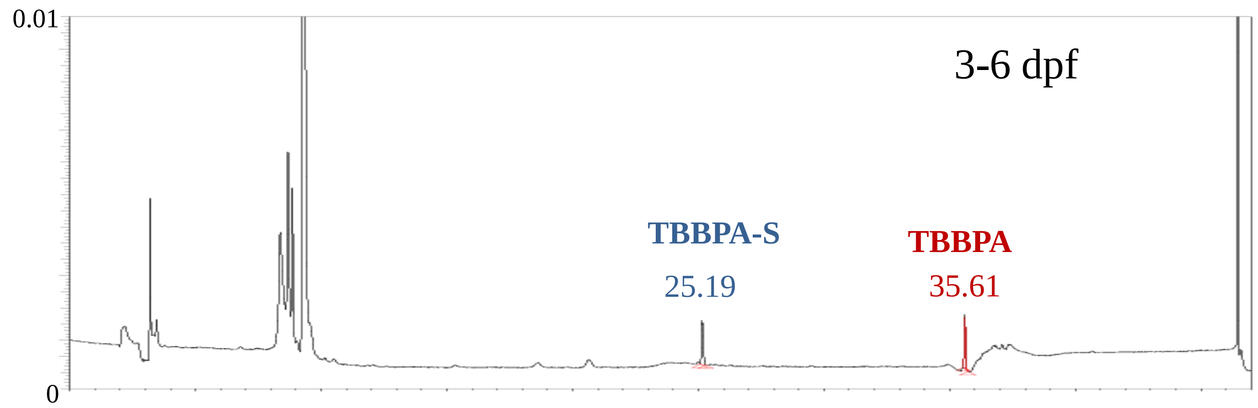
→ **Bioactivation** → **Reactive Metabolites**

Fat accumulation?

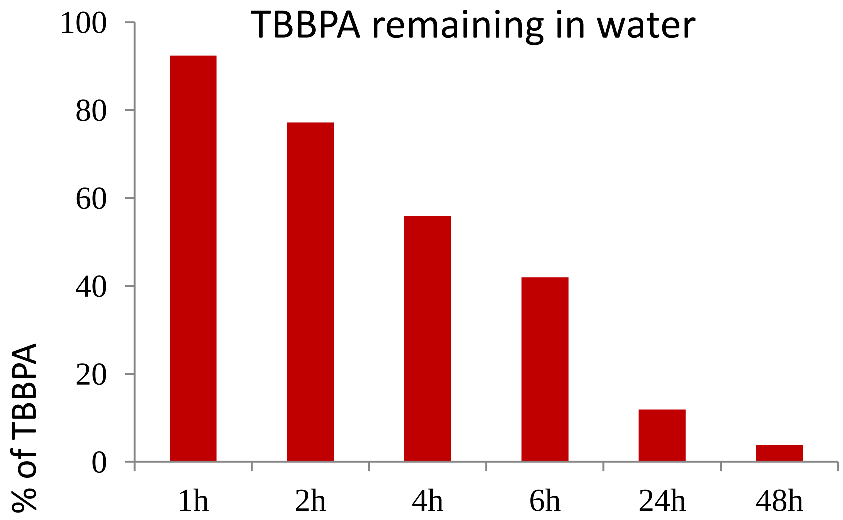
- 4 families of SULT in zebrafish (SULT1 to 4)
- SULT1 seem to be dedicated to the sulfation of xenobiotics
- Most of them are expressed at 3 dpf

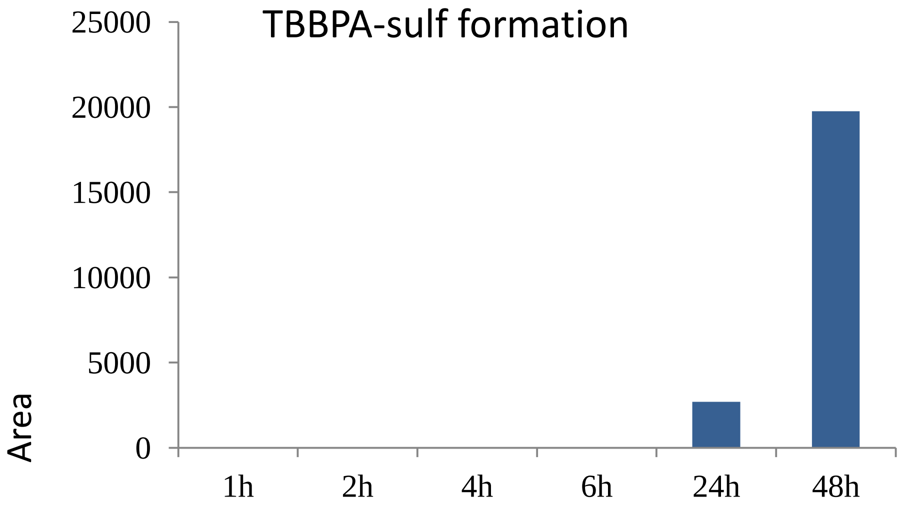
TBBPA uptake and sulfation by zf embryos

HPLC-UV



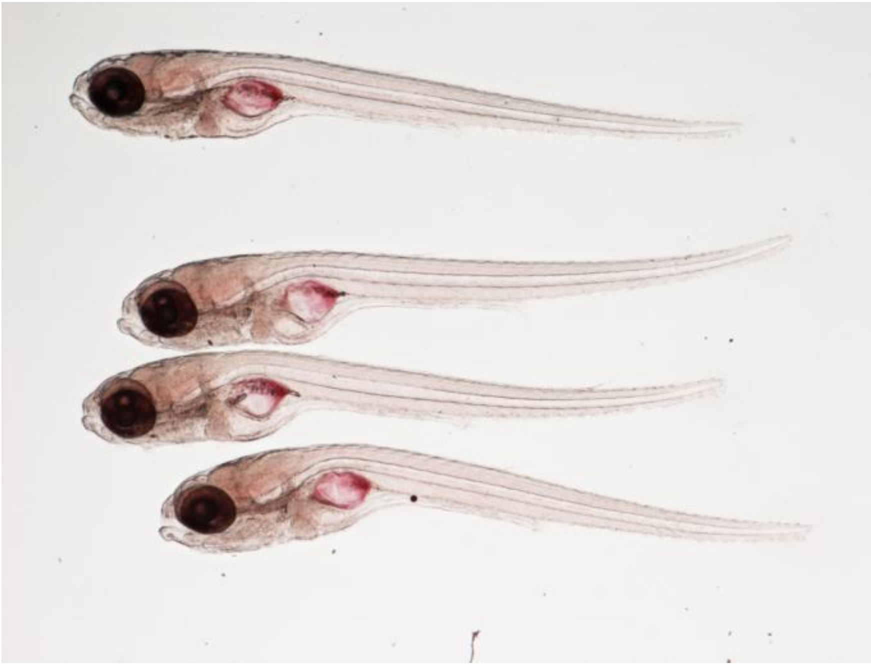
Absorption Kinetic (single treatment with TBBPA 1μM at 3dpf)





Does the metabolite acts as an obesogen?

Does TBBPA-sulf induce lipid accumulation?



Control

TBBPA-sulf
approx. 600 nM (11/19)- 58%

Anne Riu

Screening model for vascular development in ZF

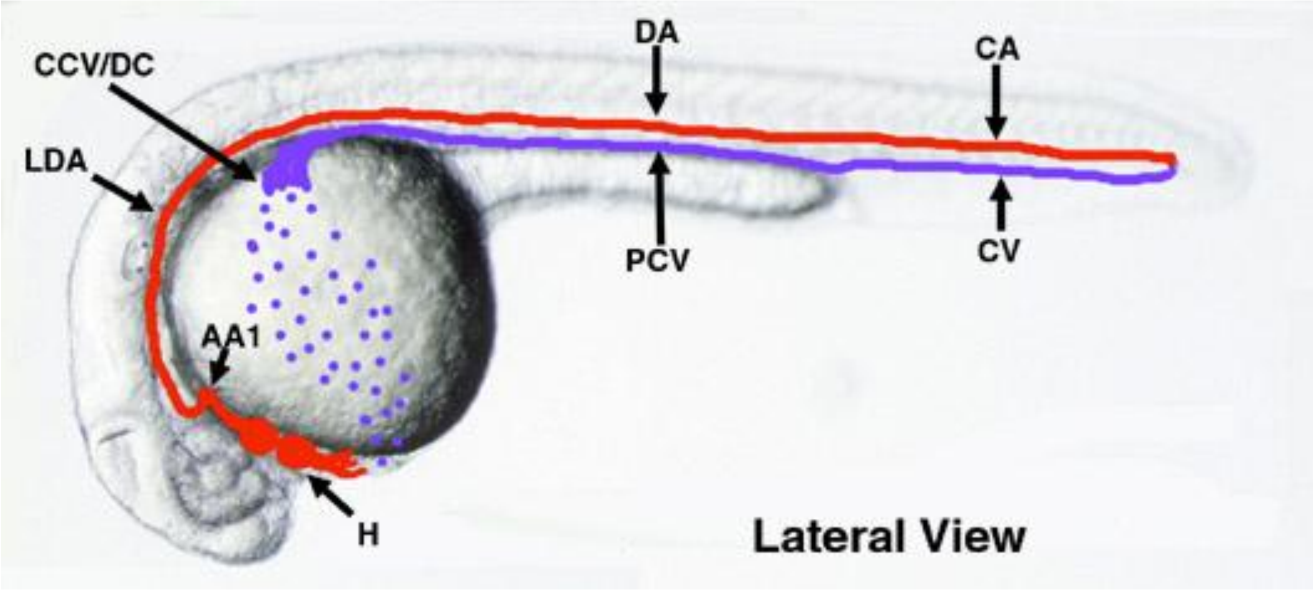
Vasculogenesis

the process of blood vessel formation occurring by a *de novo* production of endothelial cells

Angiogenesis

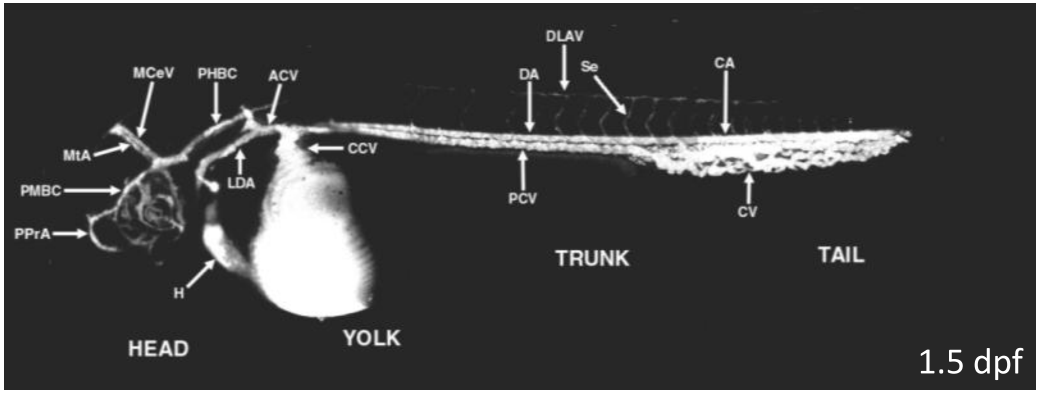
the physiological process involving the growth of new blood vessels from pre-existing vessels

24 hpf



http://zfish.nichd.nih.gov/zfatlas/FinalDesign1/DiagPage.html

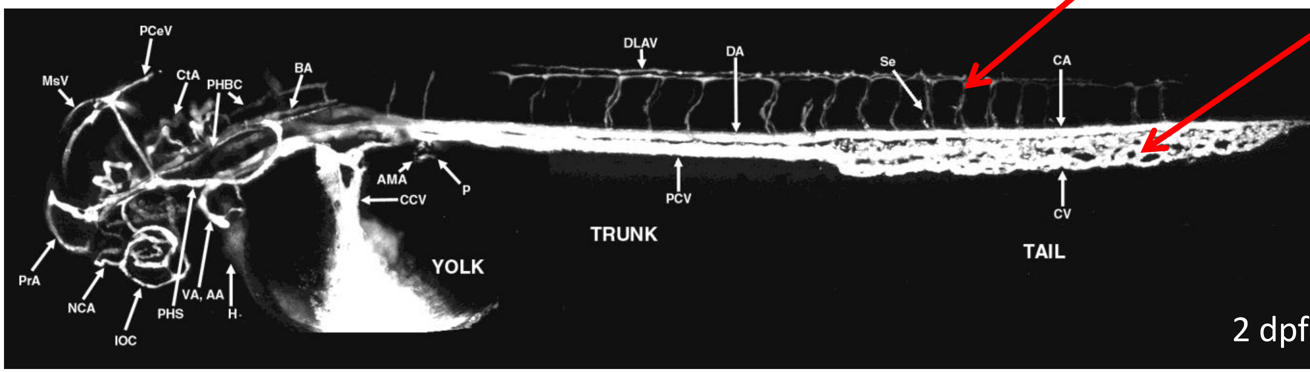
Angiogenesis



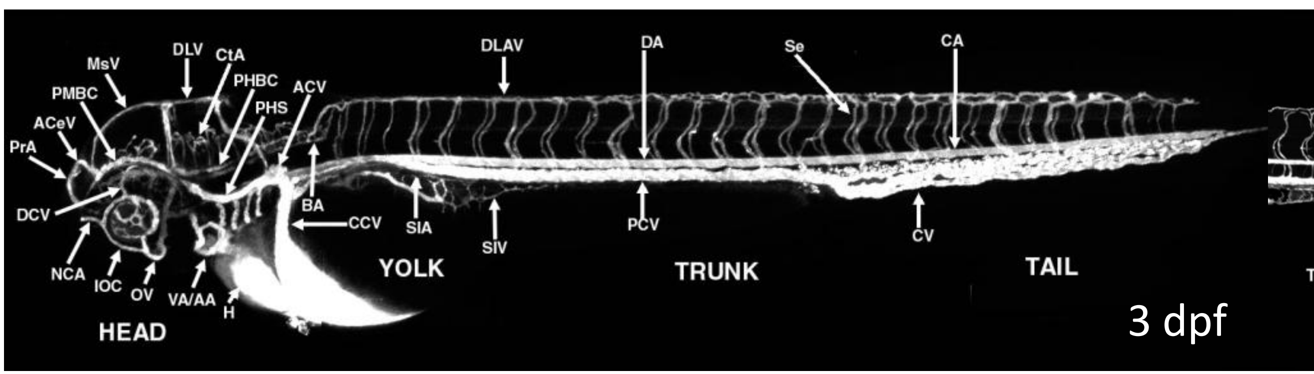
Score for:

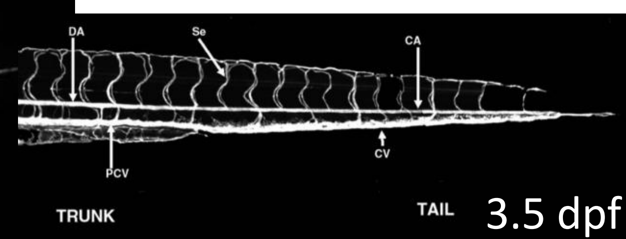
ISV sprouting and growth

Caudal vein formation



Flk-1 or Fli-1 transgenic fish

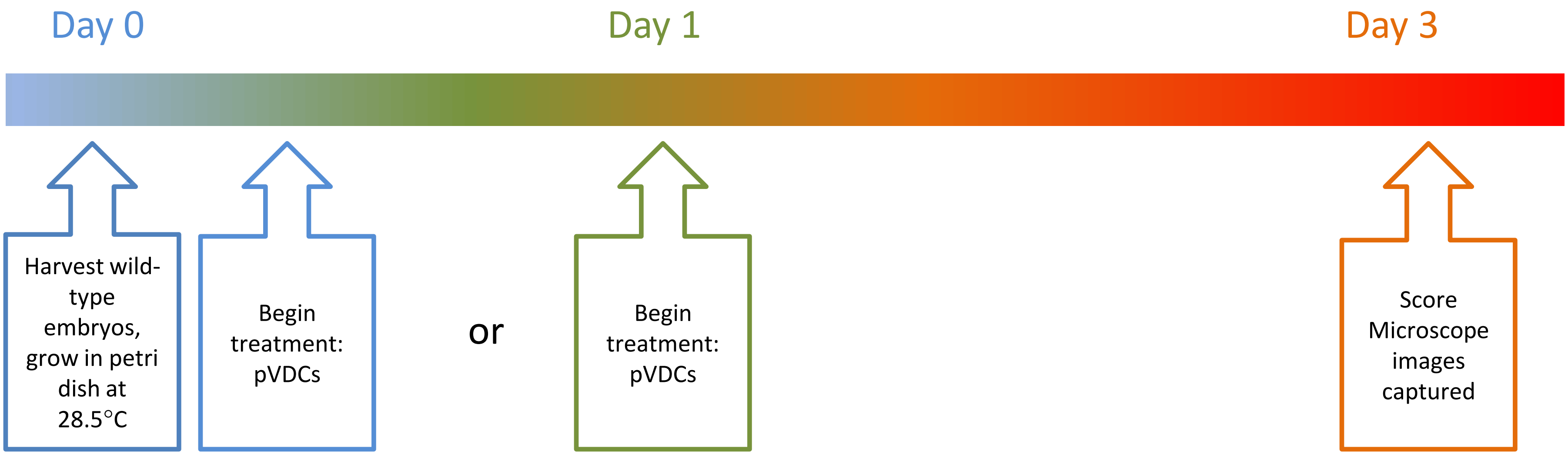


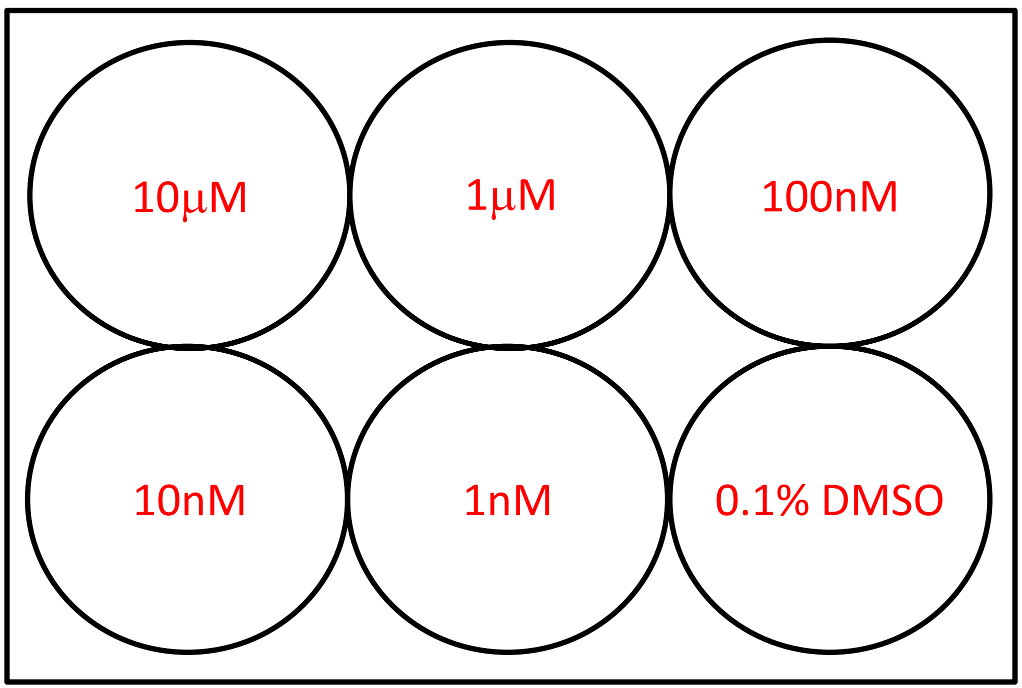


http://zfish.nichd.nih.gov/zfatlas/FinalDesign1/frame1a.html

ISV (Intersegmental Vessel); *DA* (Dorsal Aorta);*CV* (Caudal Vein); *DLAV* (Dorsal Longitudinal Anastomotic Vessel)

pVDCs experimental design





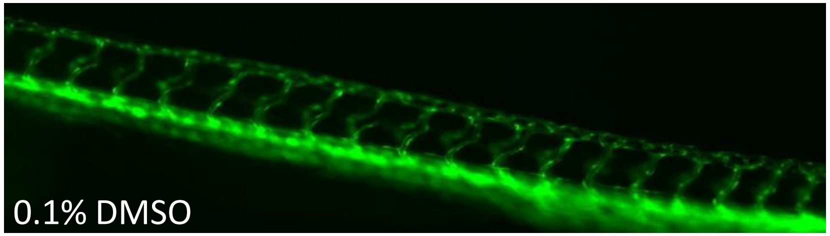
Compounds tested so far:

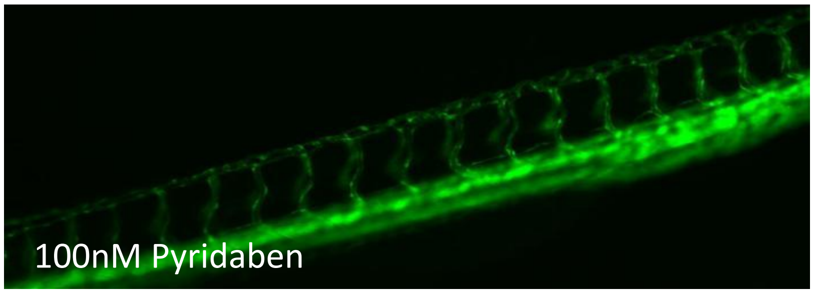
- Pymetrozin
- Butralin
- Imazapyr
- Pyridaben
- Imazalil
- 5HPP-33
- Emamectin benzoate

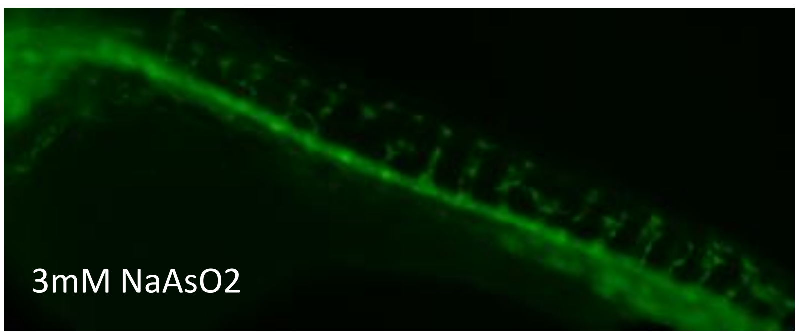
(Kleinstrauer, EHP 2011 Nov;119(11):1596-603)

pVDCs results

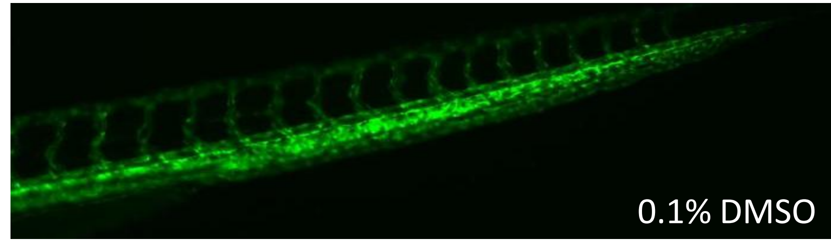
ISV sprouting and growth

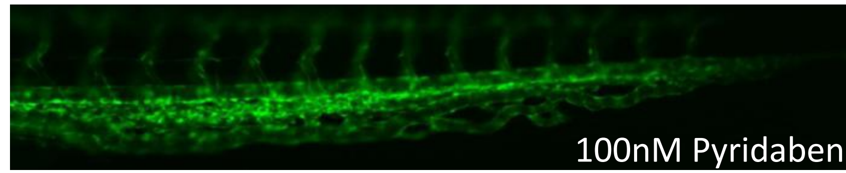


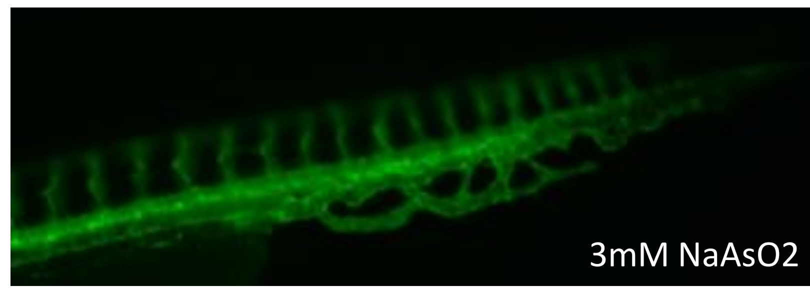




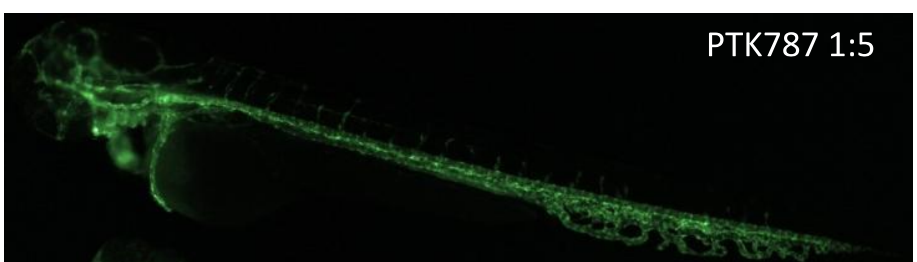
Caudal vein formation

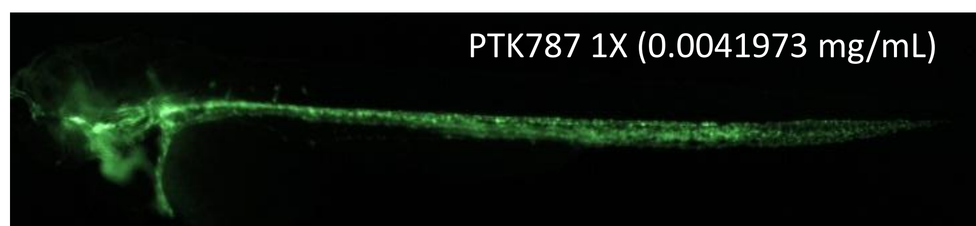






+ Control PTK787 (VEGFR Tyrosine Kinase Inhibitor)





Catherine McCollum

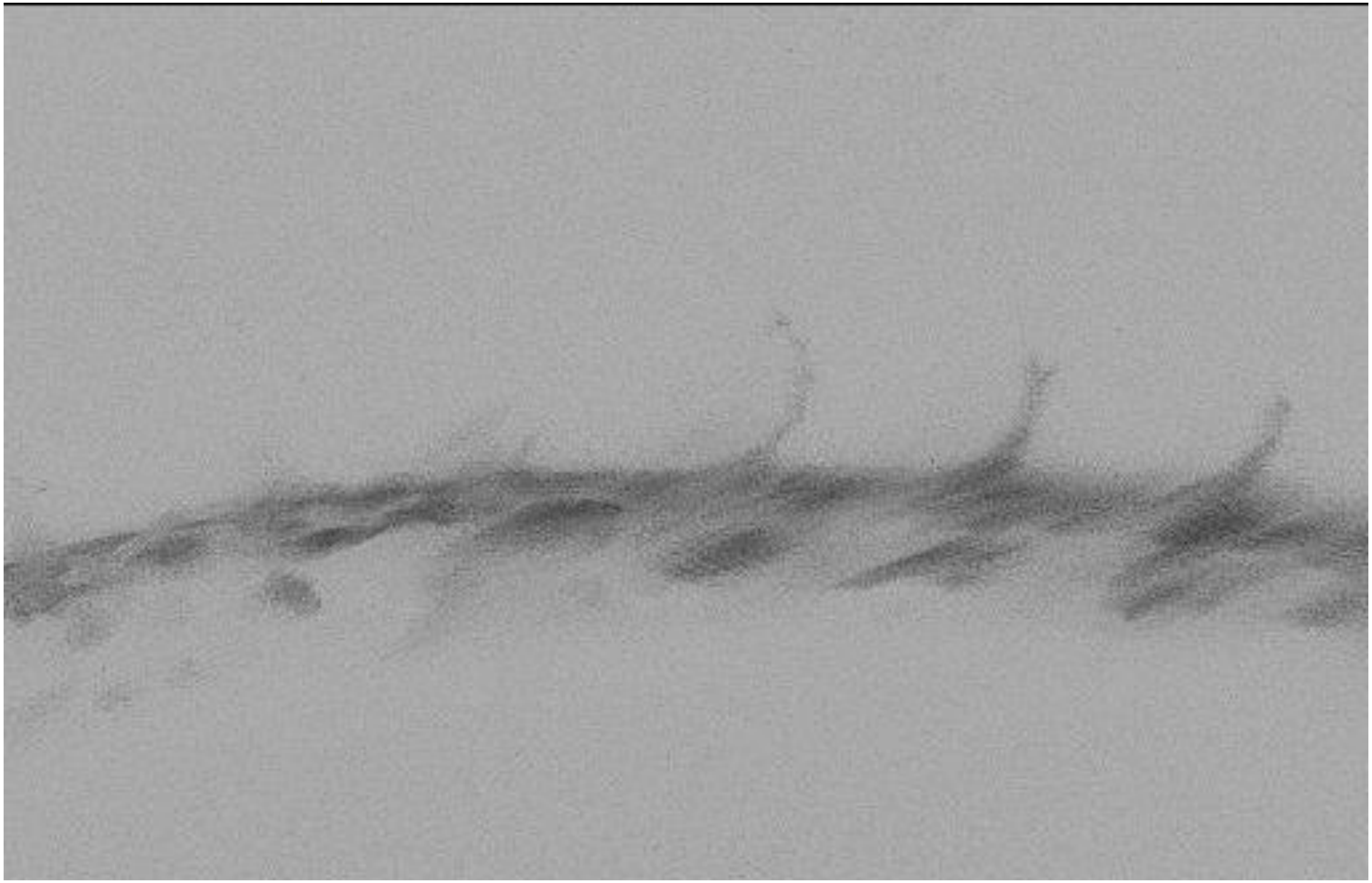
Summary of screening so far

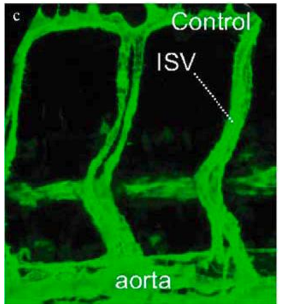
Chemicals	Lipid accumulation	Yolk absorption	Neuronal development	Vascular development ISV	Caudal vein	ERE-fish
TBBPA	y		n			n
TCBPA	y					n
TBT	y					n
DEHP	y		y			n
MEHP	y	n				n
PFOA						n
PFOS						n
prochloraz		y				
gemfibrozil		y				
clofibrate		y				
arsenic				y	y	
pyridaben		y		n	y	
imazalil		y		n	n	
EtOH			y			
VPA			y			
RA			y			
E2						y
EE2						y
5HPP-33				n	n	
emamectin benzoate				n	n	
imazapyr				n	n	
pymetrozin				n	n	
atrazine			n			
BPA			n			y
endosulfane			n			n
methoxychlor			n			y
pentachlorophenol			n			
BPC						y
BPAF						n

High information content models of ISV sprouting and growth using arsenite as a model compound

Understanding ISV sprouting
James Glazier's group,
Sherry Clendenon
Abbas Shirinifard
Indiana University

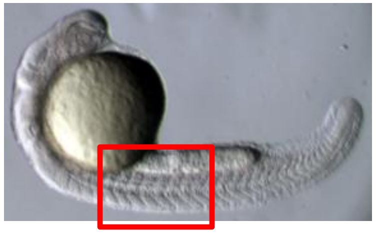
Modified from Isogai et al., 2003

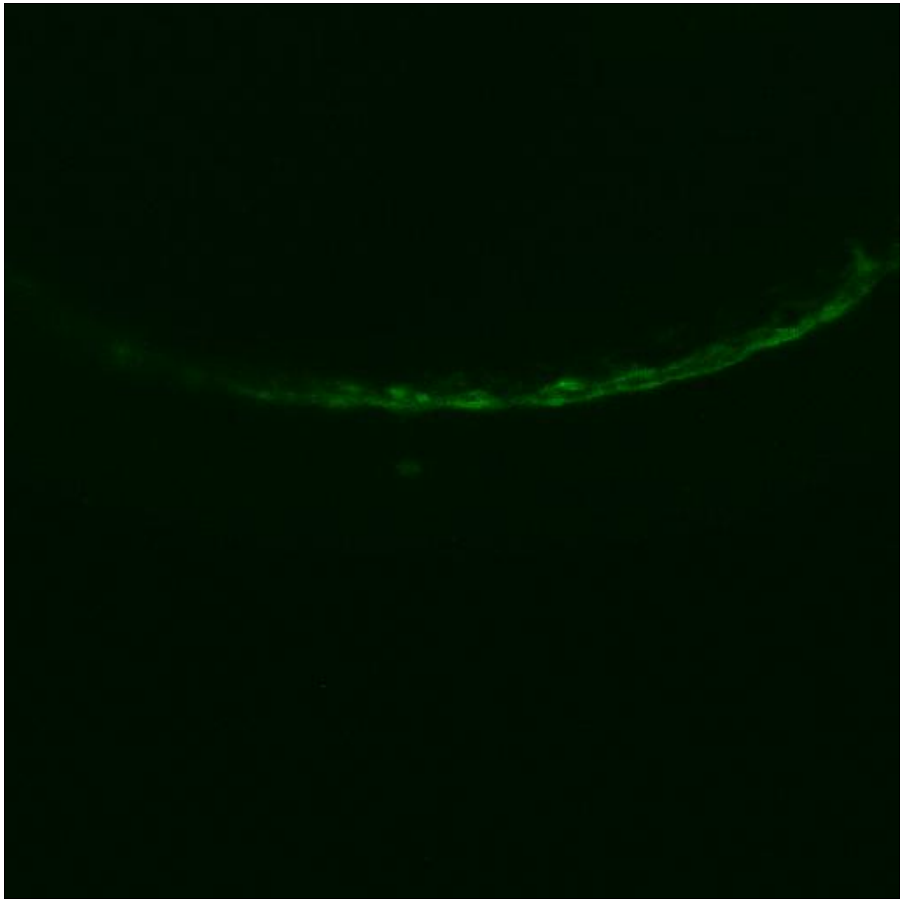




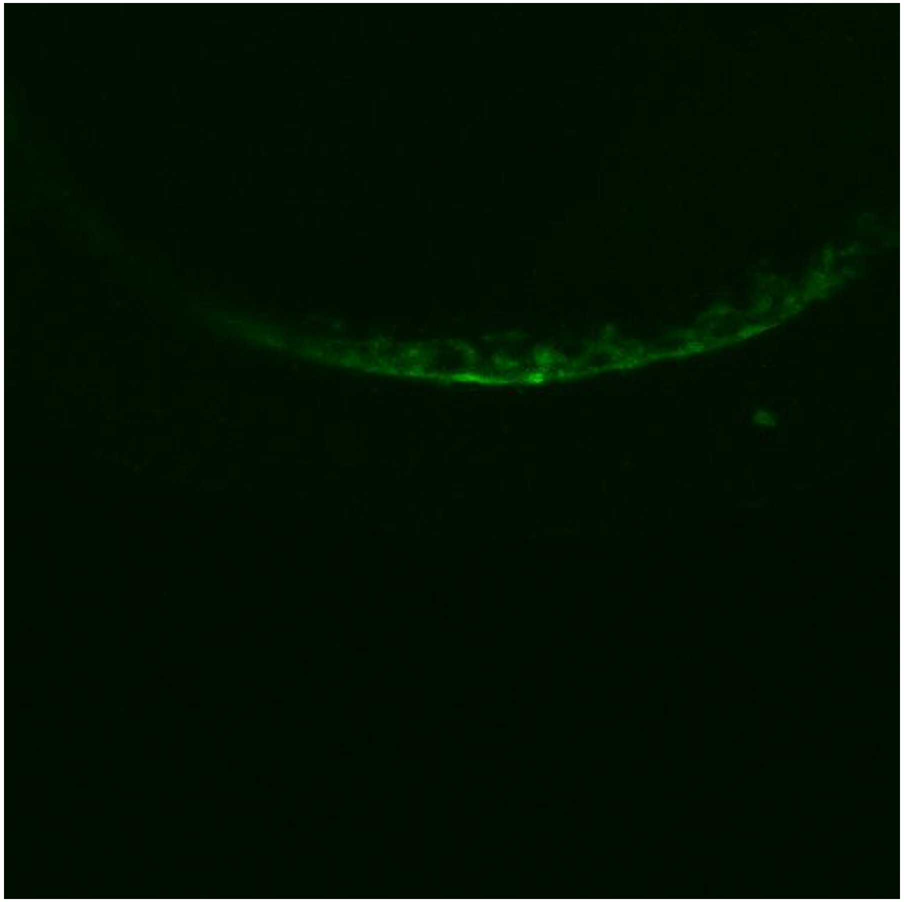
Confocal time-lapse for high information content models

Tg(Flk1:gfp) at 20X from approx. 20 hpf





untreated

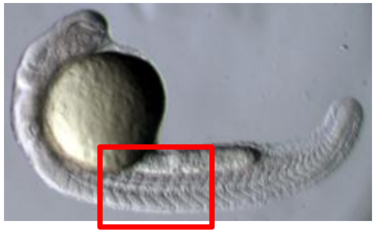


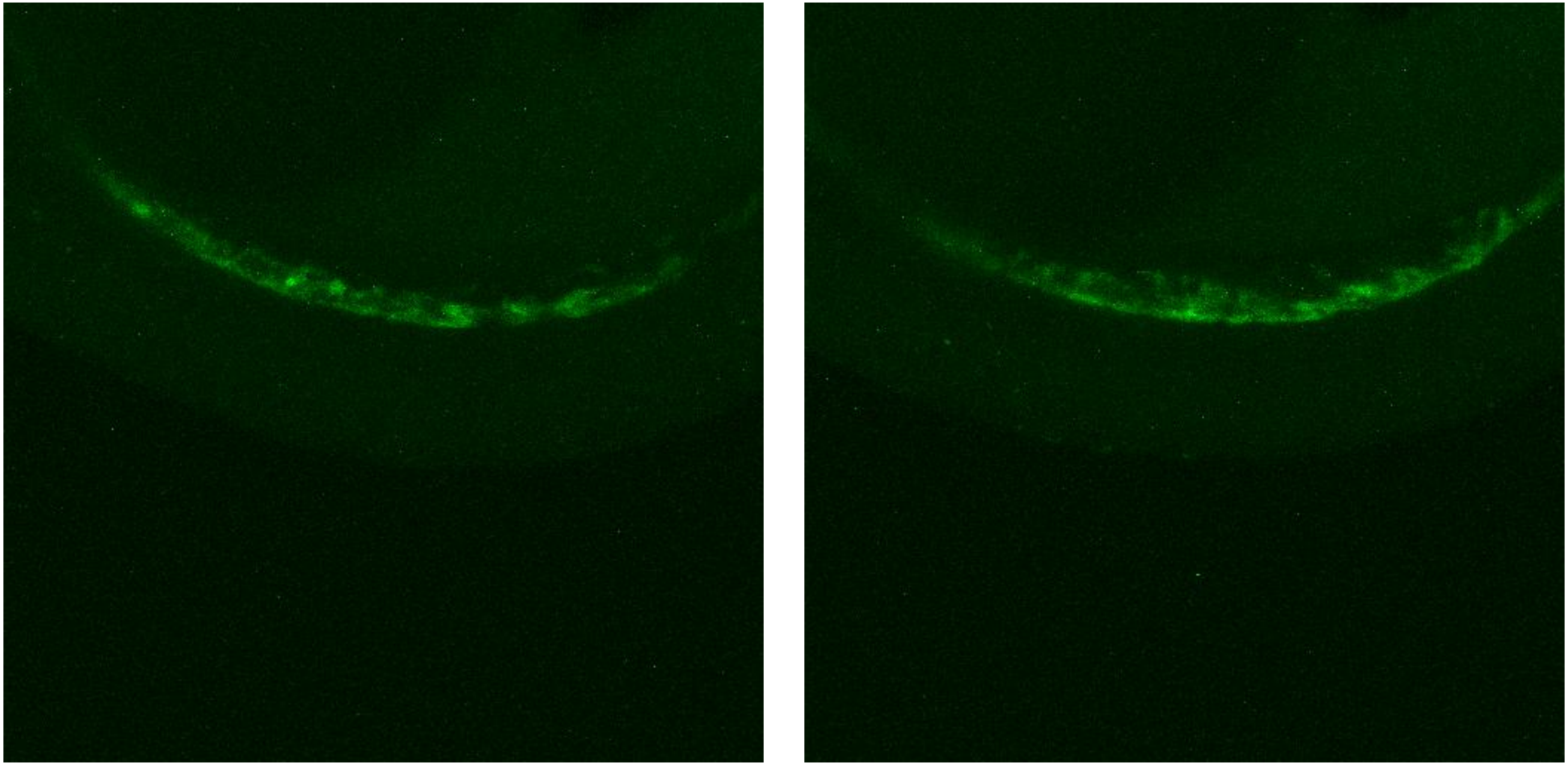
treated with 400 ug/mL sodium arsenite

Catherine McCollum, Fatima Merchant's group

Time lapse confocal microscopy of ISV growth with lower concentrations of arsenite

Tg(Flk1:gfp) at 20X from approx. 20 hpf

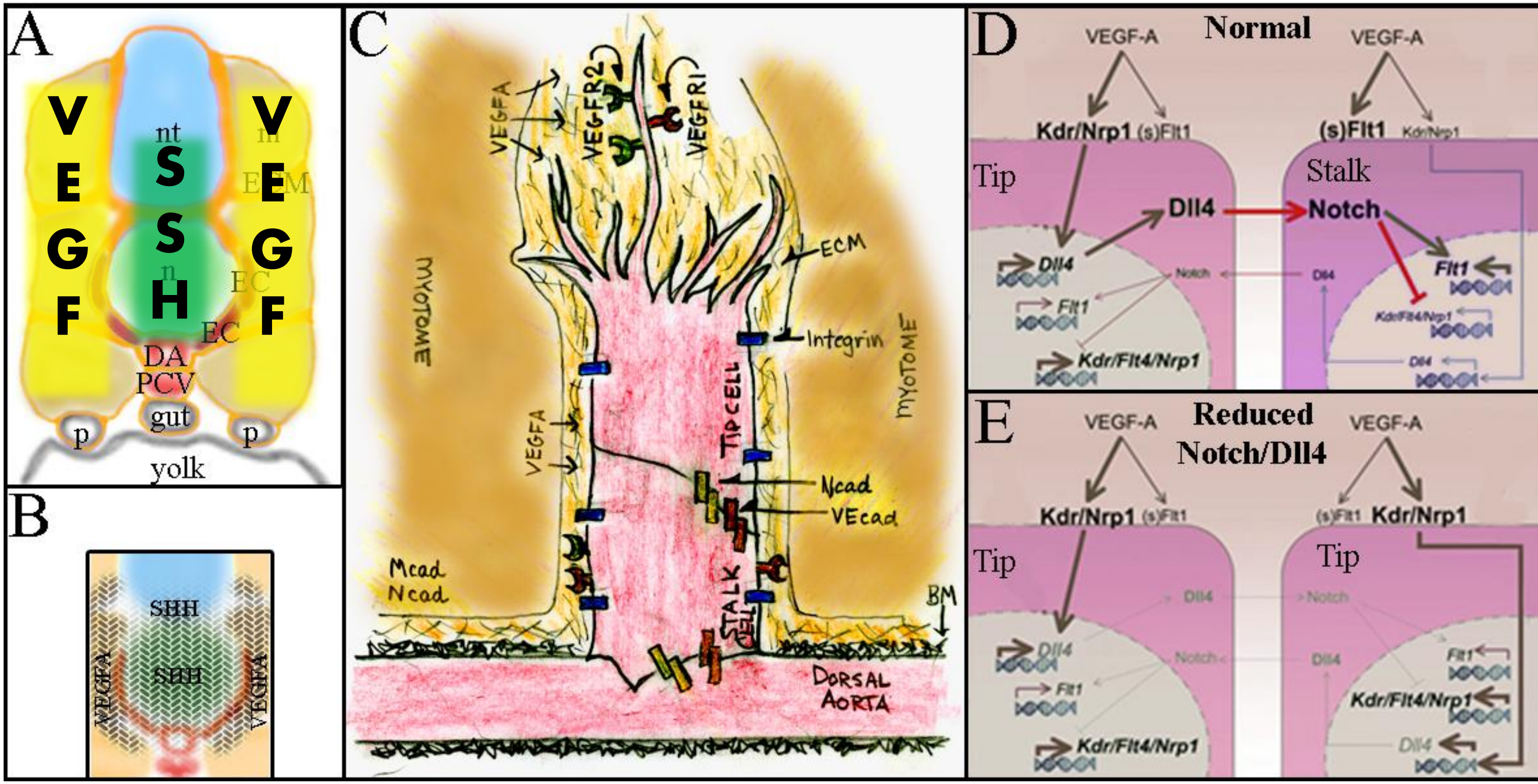




Treated with Arsenite 100 ug/mL

Multiscale Cell-Tissue Interactions

Sherry Clendenon
Abbas Shirinifard

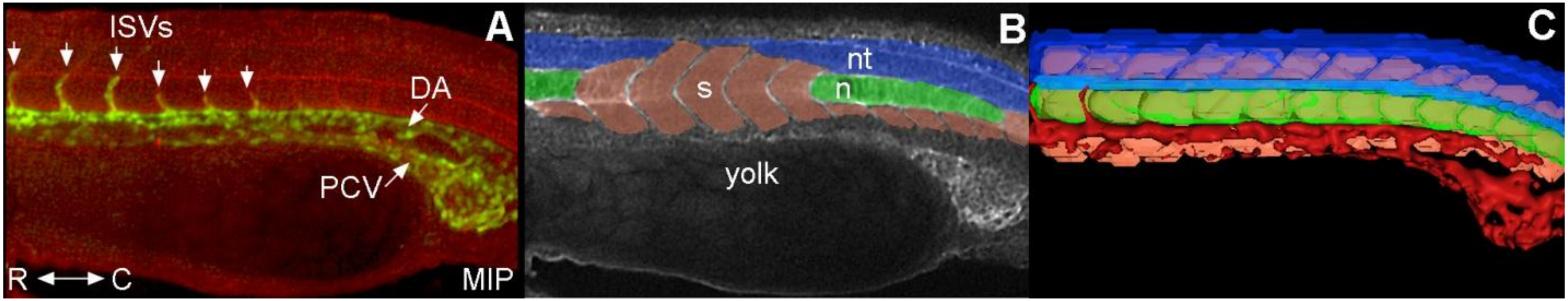


Shh>VEGF>Notch signals - Lawson and Weinstein. 2002. Nature Reviews: Genetics. 3:674.

Panels D,E – adapted from: Phng & Gerhardt. (2009) Angiogenesis: A Team Effort Coordinated by Notch, Developmental Cell. 16(2):196-208.

Quantitative Signatures: *3D Tissue Architecture Extraction from Experimental Data (Static)*

Sherry Clendenon
Abbas Shirinifard



Modeling requires accurate representation of 3D tissue geometry

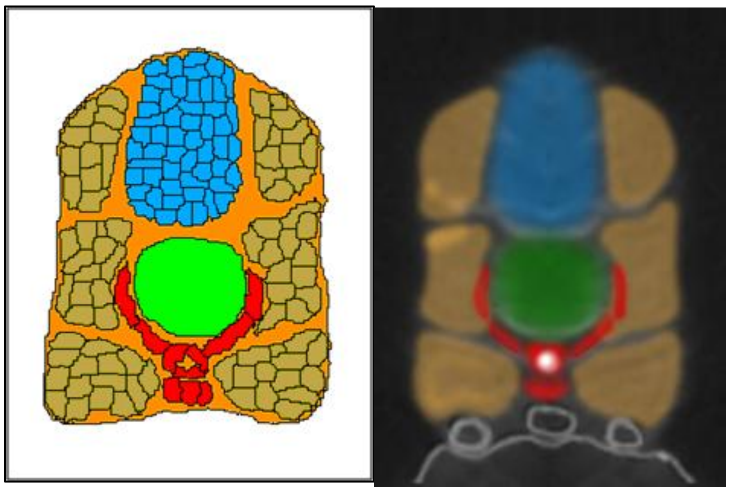
- VEGF distribution, which drives ISV development, depends on tissue geometry
- Tissue volume, dimensions and shapes change as the embryo grows and develops.
- This information is extracted from Experimental Data for model initiation and later from VEGFR2 inhibited and toxin treated embryos for model validation.

Flk1 expressing zebrafish embryos provided by Catherine McCollum (U of Houston)
Staining, microscopy, segmentation, rendering done by Sherry Clendenon (Indiana U)

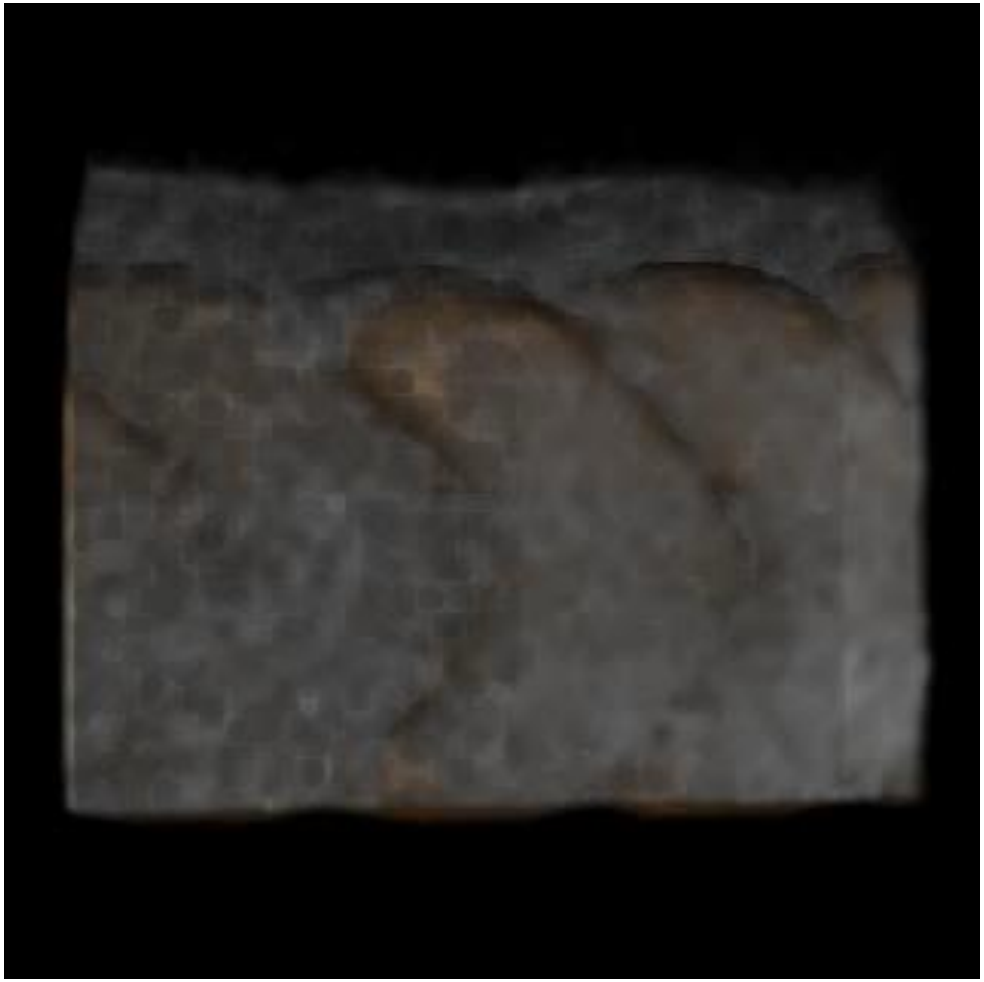
Quantitative Signatures: *3D Tissue Architecture Extraction from Experimental Data (Static)*

Sherry Clendenon
Abbas Shirinifard

CompuCell3D
(www.Compucell3D.com)

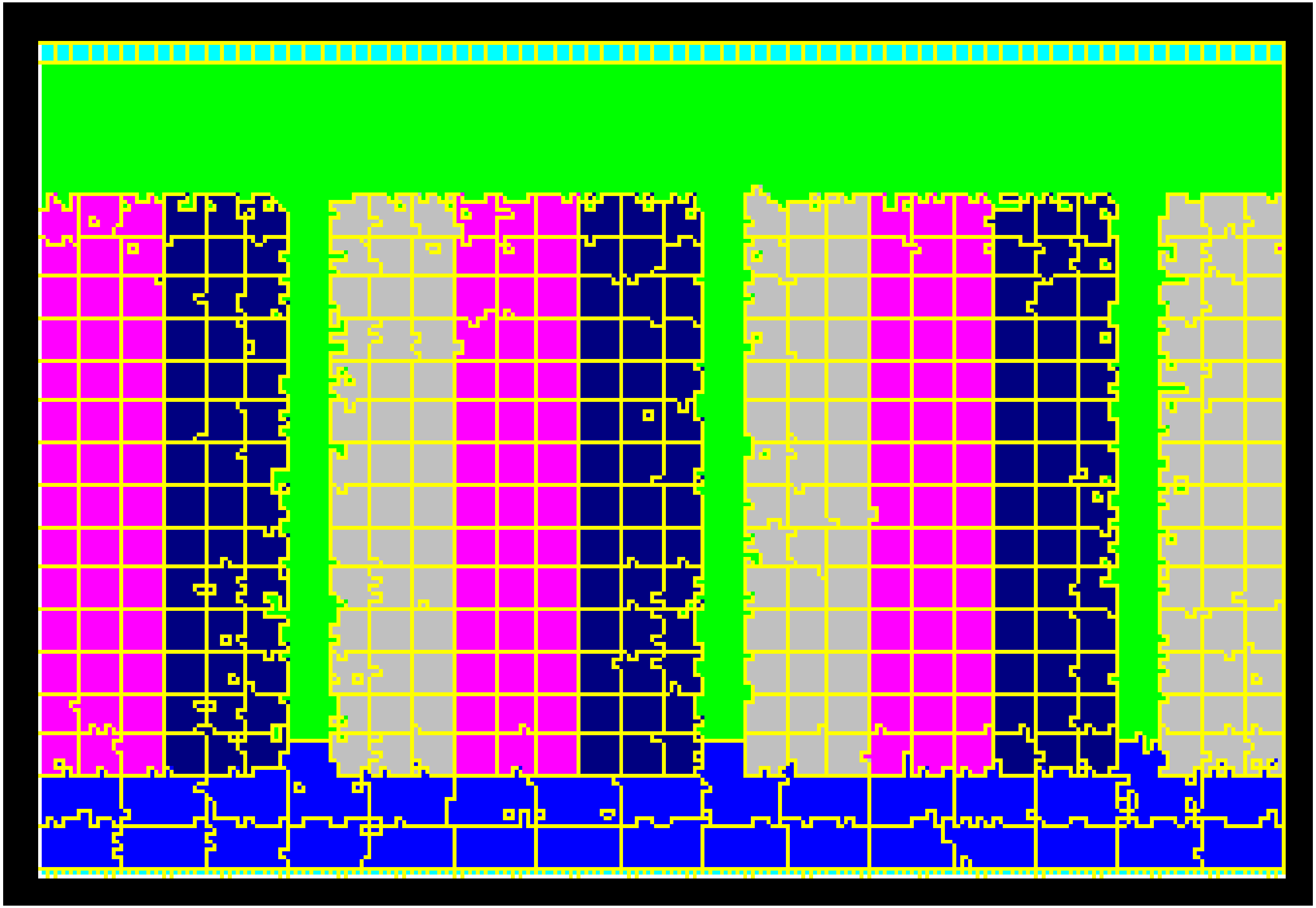


Brown=myotome/somite,
Green notochord,
Blue= neural tube
Orange=ECM



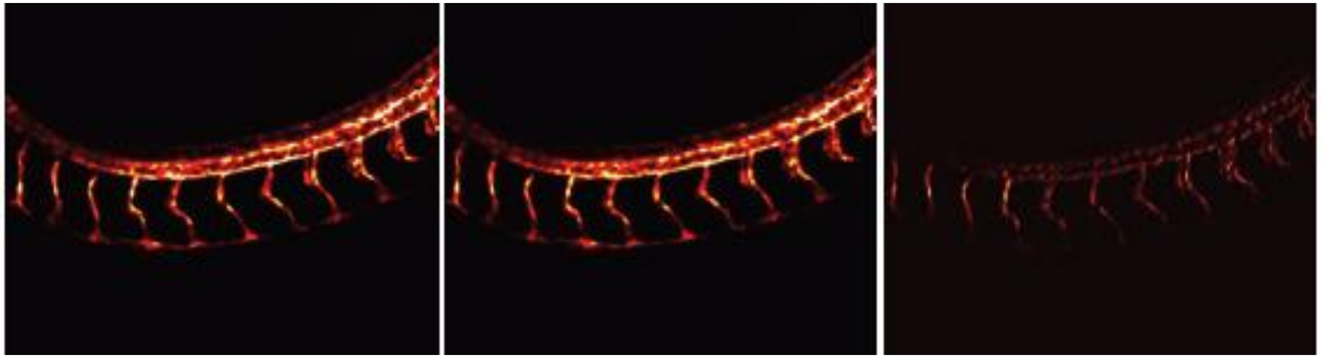
Segmentation, Rendering - Sherry Clendenon

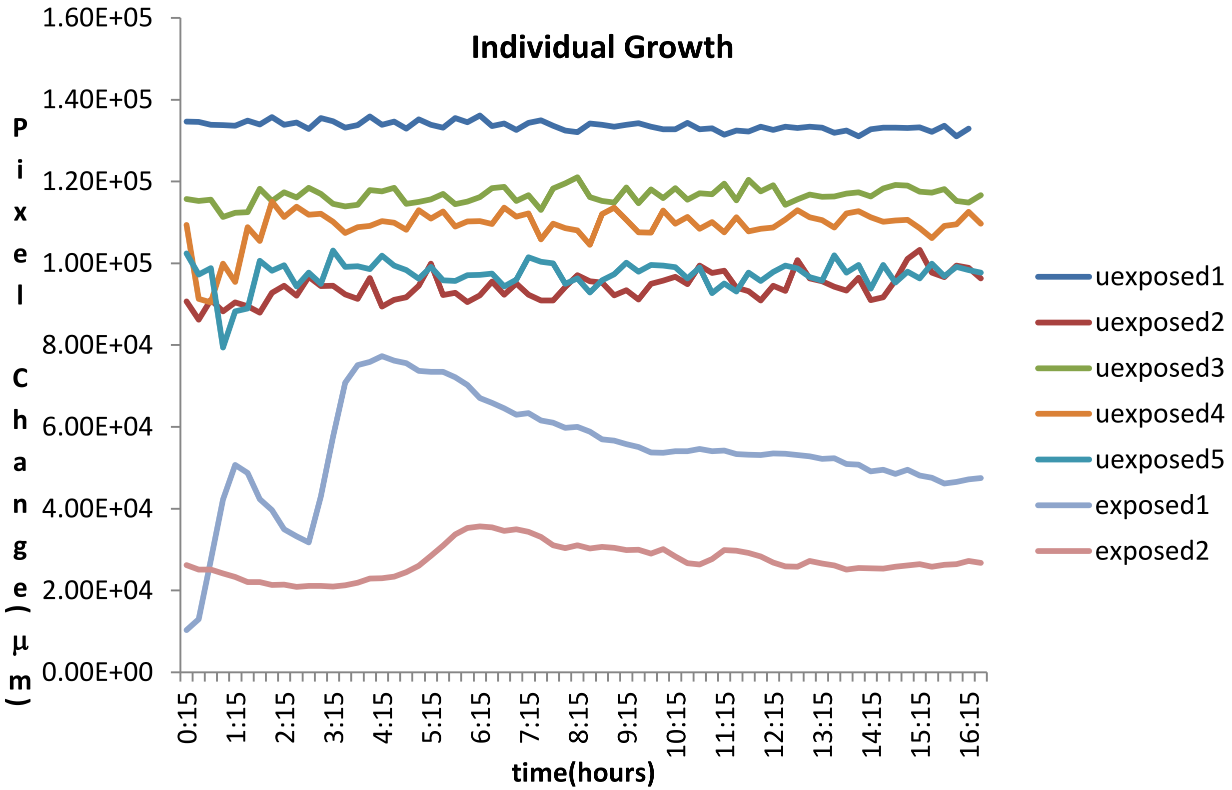
A Toy Model of ISV sprouting



Model - Abbas Shirinifard

Arsenic exposure decreases vascular system growth rate





The rate of change in vessel morphology over 16 hours for unexposed and arsenic exposed embryos.

7 embryos (5 untreated and 2 treated) were analyzed

unexposed1 – unexposed5 represents change in number of pixels for unexposed embryos growth, whereas exposed1 and exposed2 represents change in number of pixels for arsenic-treated embryos

Charu Hans, Merchant lab, UH

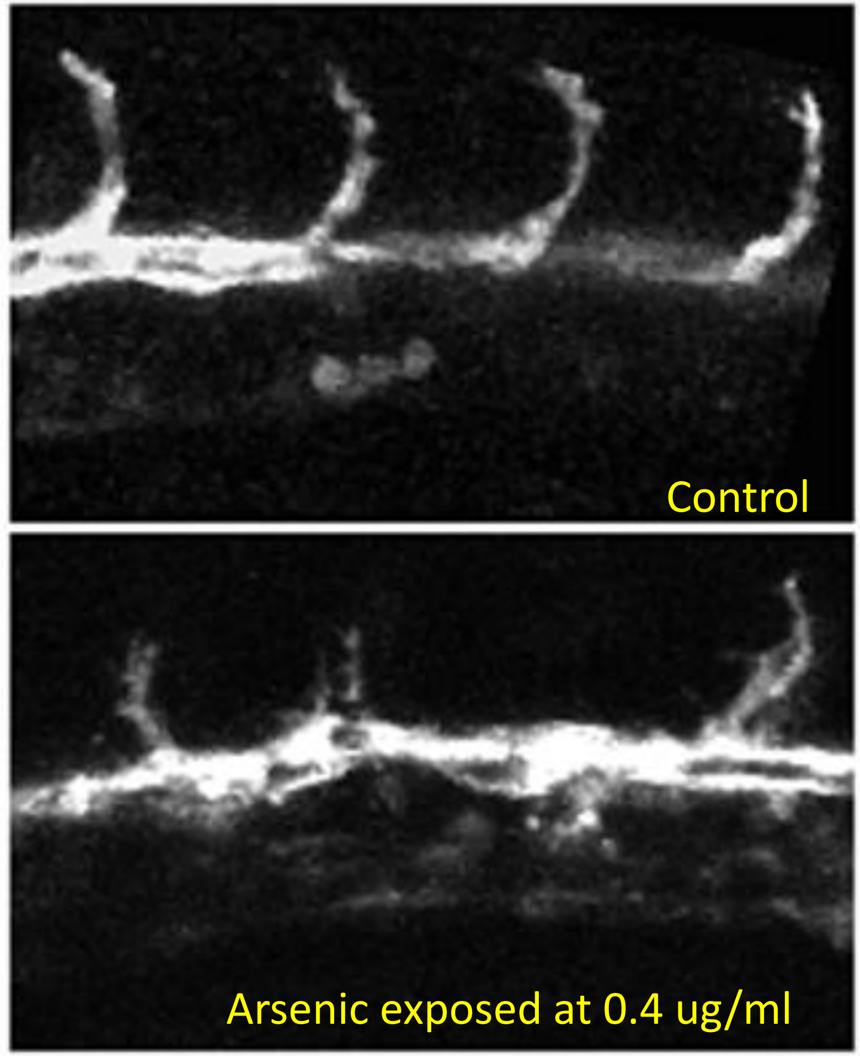
Experimental effects of arsenic exposure on ISV growth

Sherry Clendenon
Abbas Shirinifard

- Multiple tip-like cells
- Highly active filopodia in both tip cells and stalk cells
- Missing sprouts
- Sprout regression resulting in irregular spacing

Control and arsenic treated panels are 5hr after initiation of sprouting
one side of ISV shown

“Real model”



Data from: Catherine McCollum (U of Houston)

Acknowledgements

Collaborators
Leif Peterson, Methodist Hospital
Patrick Balaguer, IRCM, France
Daniel Zalko, INRA, France
Vincent Laudet, INRA, ENSL, France
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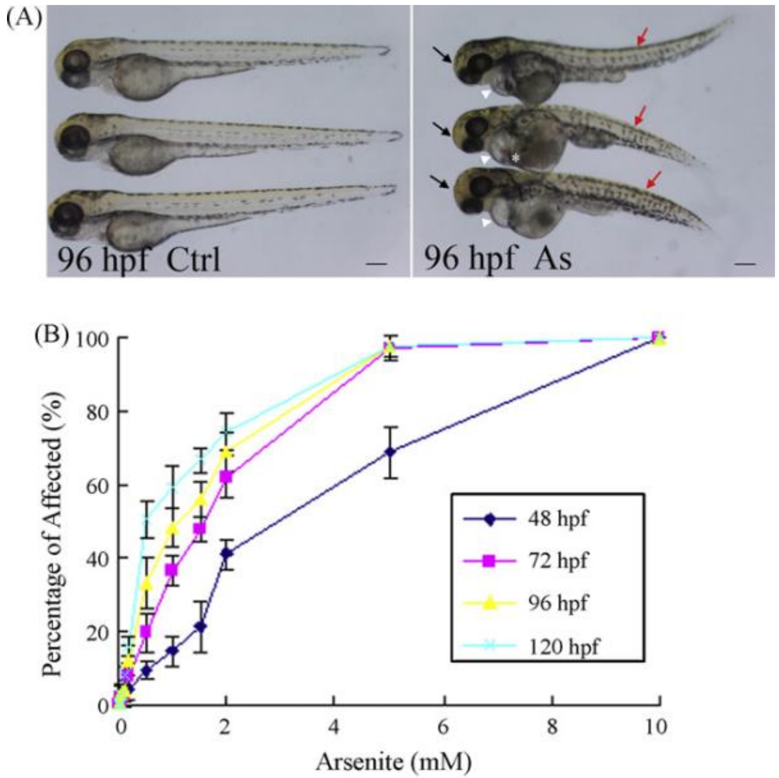
Ioannis Kakadiaris, UH
Eleni Zacharia, UH
Emilio Benfenati, IRFMN
James Glazier's group, Indiana University
Rick Finnell, UT Austin
Robert Cabrera, UT Austin
Tom Knudsen, EPA
Nicole Kleinstreuer, EPA
Amar Singh, EPA

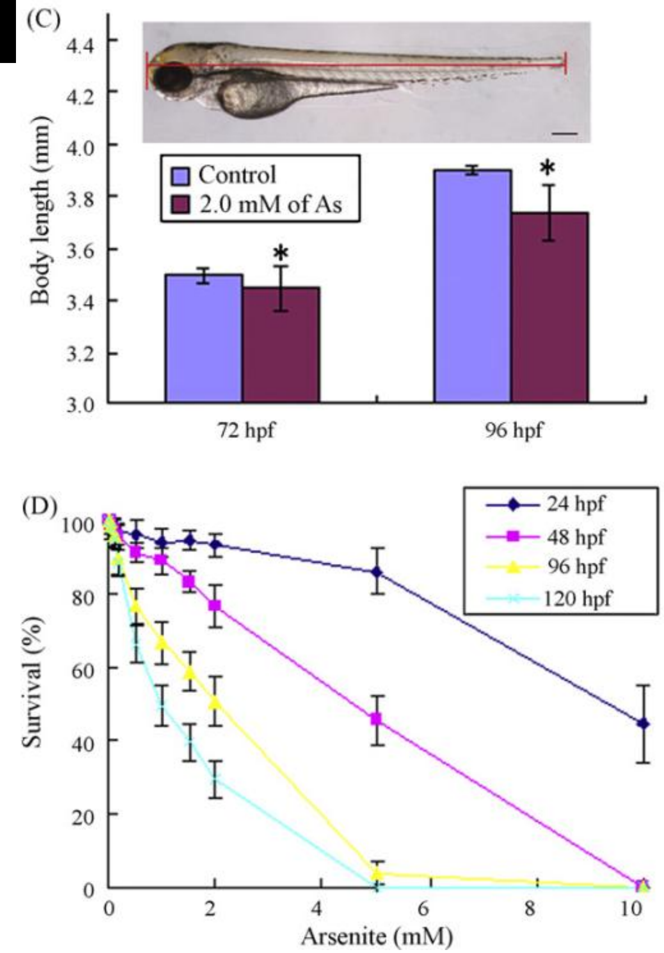
ZF/Mol. Tox. group at University of Houston
<u>Jan-Åke Gustafsson</u>
Maria Bondesson
Catherine McCollum
Nicole Ducharme
Anne Riu
Caroline Pinto
Ruixin Hao
Sharanya Maanasi Kalasekar
Triet Truong

Funding: US-EPA and CPRIT

UH CNRCS

Developmental effects of arsenite in ZF





From: Le et al. Aquatic Toxicology 91 (2009) 229–237

(A) Embryos exposed to arsenite (2.0mM) exhibited **morphological abnormalities**. White arrowheads show **pericardial edema**; red arrows show **dorsal curvature**; black arrows show **flat head**; white asterisk indicates **RBC accumulation**. (B) Percentage of affected embryos are plotted against the doses of arsenite exposed to embryos (mM) (n = 30 for each treatment). (C) Embryos treated with 2.0mMof arsenite showed reduced mean **body length** compared with the controls at both 72 and 96 hpf (n=10 for each treatment). Body length was measured along the body axis. Statistically significant differences are indicated by asterisks ($p < 0.05$, Student's t-test). (D) The percentage of **survival** plotted against the doses of arsenite (mM) (n = 30 for each treatment). Each treatment was replicated six times. Ctrl, control group; As, arsenite-treated group (2.0 mM). The scale bar represents 200m. Values are presented as mean±S.E.