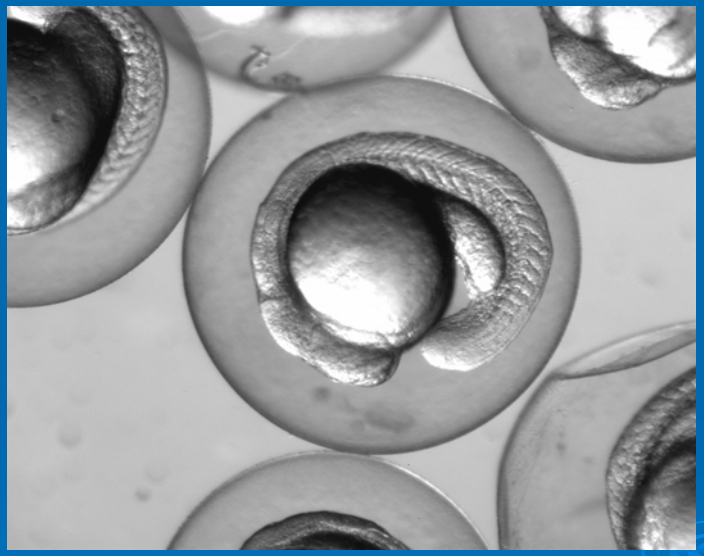
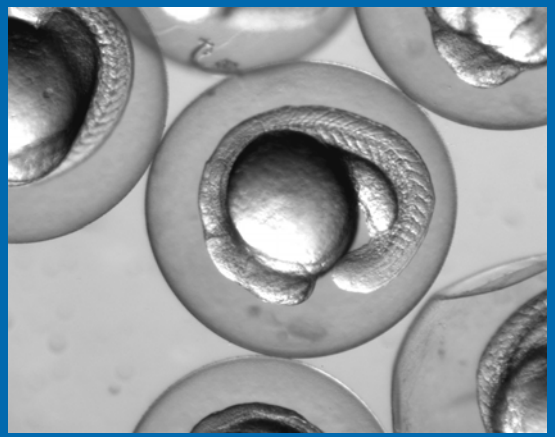
TOXICOLOGICAL ENDPOINTS EASILY MEASURED IN DEVELOPING ZEBRAFISH



Robert L. Tanguay
Environmental and Molecular Toxicology
Oregon State University

Why zebrafish for toxicity testing?

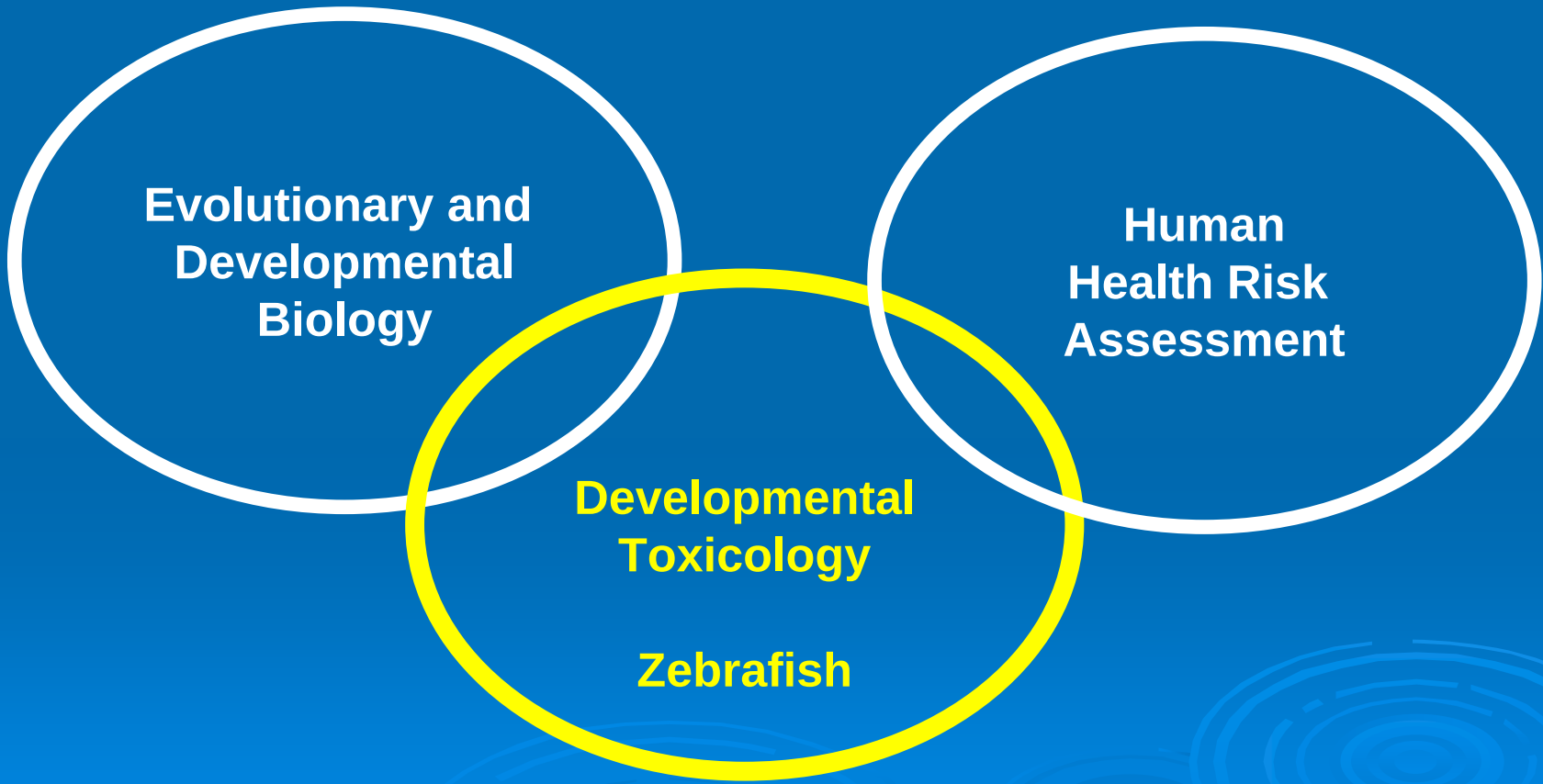


- Vertebrates
- ……..

Most importantly:

- Complex processes can be “probed” or interrogated by chemical exposures
- Experimental convenience of cell culture, with the added value of *in vivo* assessments
- Tools that permit mechanistic evaluations

Zebrafish: a tool to use developmental biology to protect and improve human health



Baer's Law

- "The general features of a large group of animals appear earlier in the embryo than the special features."

Karl Ernst von Baer, 1828.

In other words, early development is the period most well-conserved between species.

Why evaluate toxicity during early development?

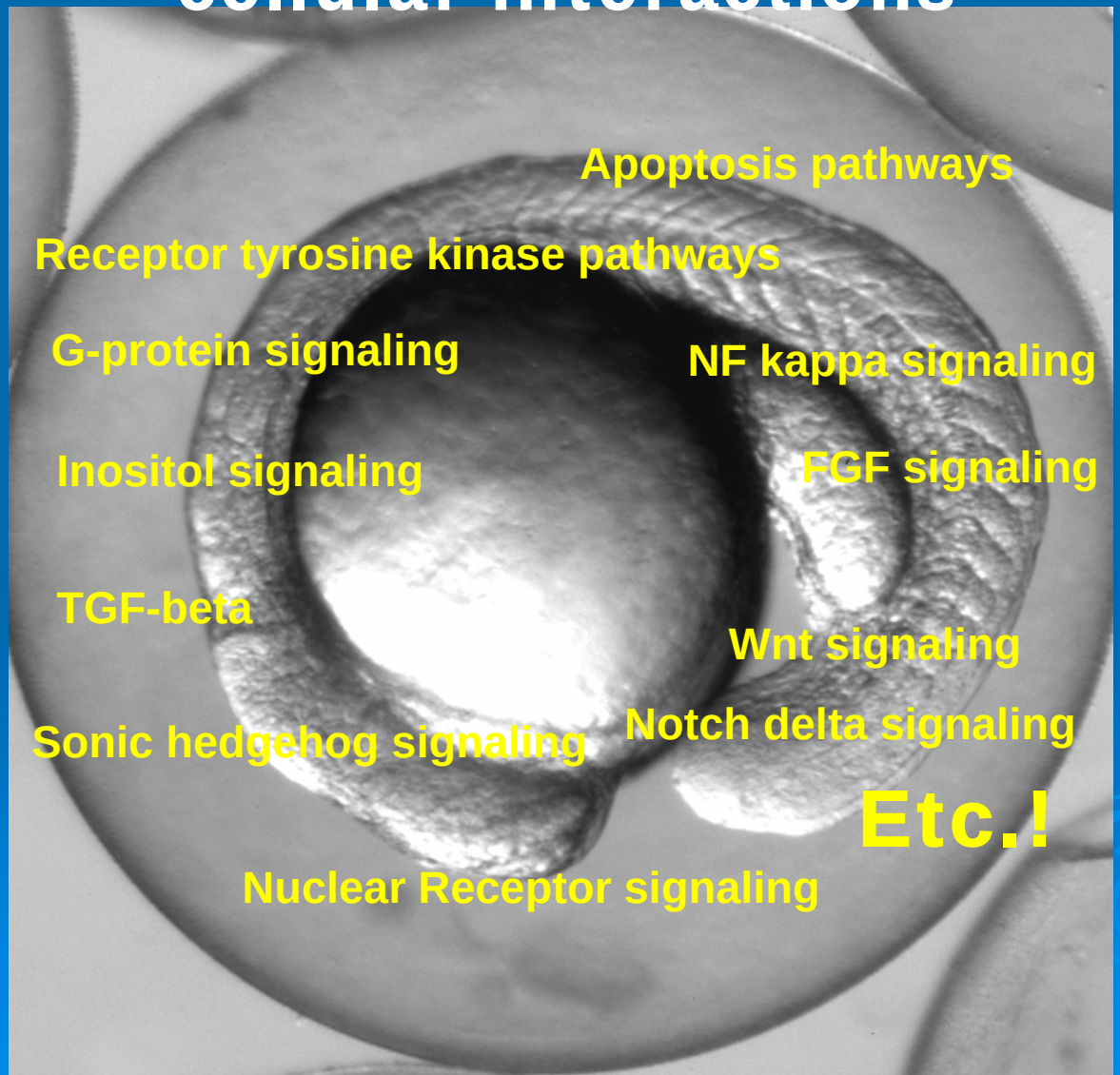
- Processes of development are remarkably conserved
    - Comparative genomics supports overall conservation of potential “targets”
- Generally more responsive to chemical insult

Why?

Signaling repertoire essential and most active

If a chemical is developmentally toxic it must influence the activity of a molecular pathway or process

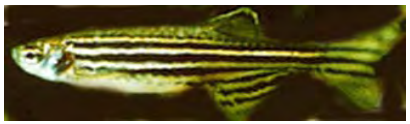
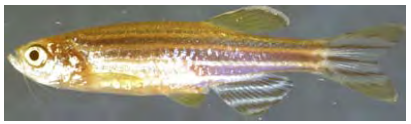
Embryonic Development: A platform to probe molecular signaling and cellular interactions

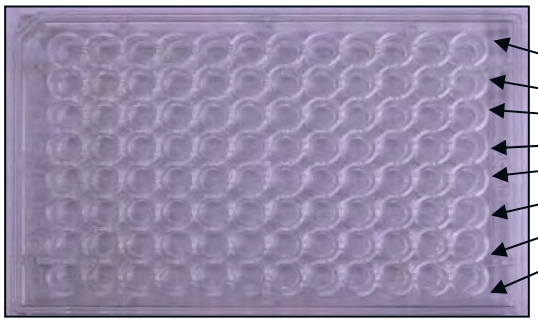


Consider startpoints - not endpoints

- Signaling pathways and molecular events are conserved
- ..But fish are not rodents or humans
- Consequences of disrupted signaling often species specific
- In other words, the mechanism by which a “target” is hit is likely conserved, but the consequence of the “hit” may be distinct

Tier 1 Testing

♀  X  ♂

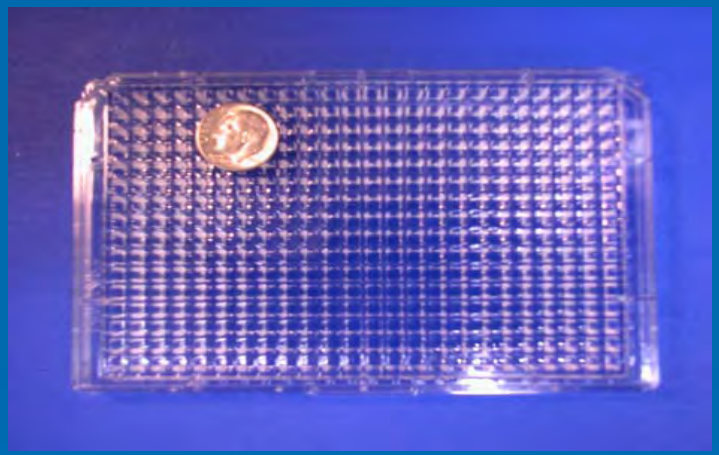


Test Chemicals

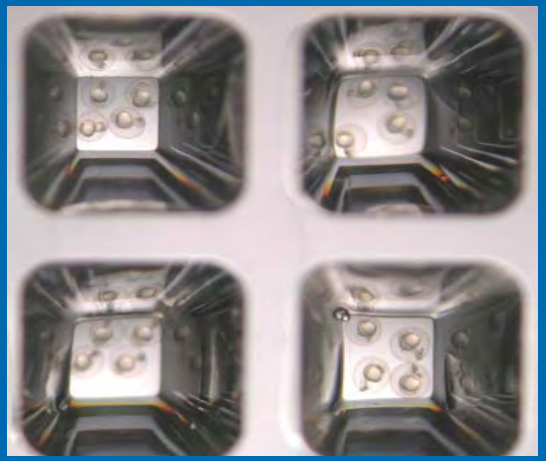
96 OR 384 well plates

Screening for TOXICITY 1-5 days

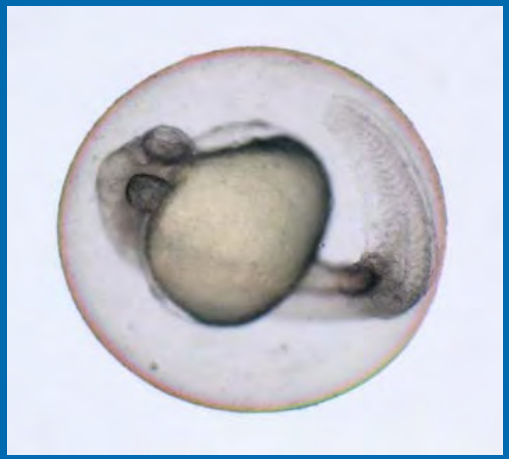
Amenable to rapid assessments
small amounts of chemicals



384 well plate



4 embryos in
Each well



23 hour
embryo

Assay Considerations

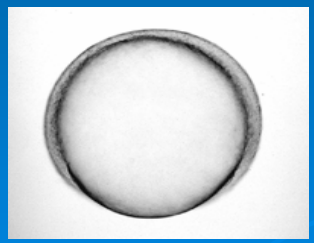
- **Efficient housing of adults for embryo production**

  Ex. 100 X 2 liter tank unit Produces up to 10,000 embryos/day.

- **Chorion considerations**

  We remove it to:

  Eliminate a potential chemical barrier
  Early assessments are facilitated

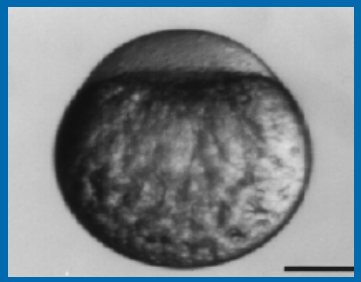


- **Start and duration of exposure?**

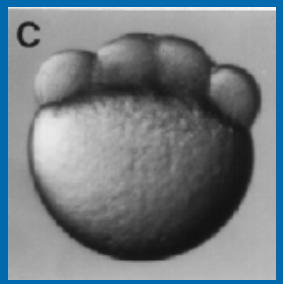
KEY DEVELOPMENTAL EVENTS
Zebrafish versus mammals

	Zebrafish	Rat	Human
Blastula/Blastocyst	2-5 hr	3-5 days	4-6 days
Neural Plate formation	10 hrs	9.5 days	18 days
First Somite	10-11 hrs	9-10 days	20-30 days
10 Somite Stage	14 hrs	10-11 days	25-26 days
Neural Tube Formation	18-19 hrs	9-12 days	22-26 days
First Branchial arch	24 hrs	10 days	20 days
Organogensisis	48 hrs	5-6 days	21-56 days
First Heartbeat	30 hrs	10.2 days	22 days
Birth/Hatching	48-72 hrs	21 days	253 days

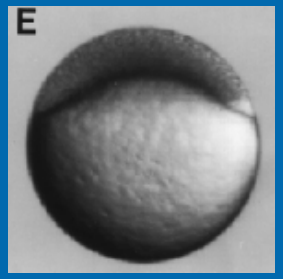
Stages Of Zebrafish Development



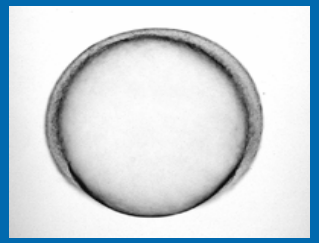
3 min



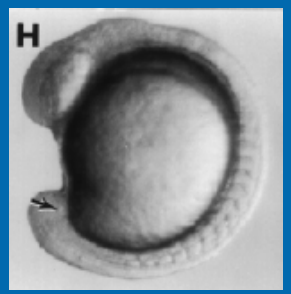
1. 25hr



4 hr



8 hr



19 hr

24hr

48hr

120 hr

Tier 1 Endpoints
(Assessed between 24 and 120 hpf)

Morphological

Malformations

i.e. pericardial edema, yolk sac edema, body axis fin malformations, eye diameter

Circulation

Heart beat (rate)

Pigmentation

Developmental progression (developmental delay)

Embryo viability

Behavioral

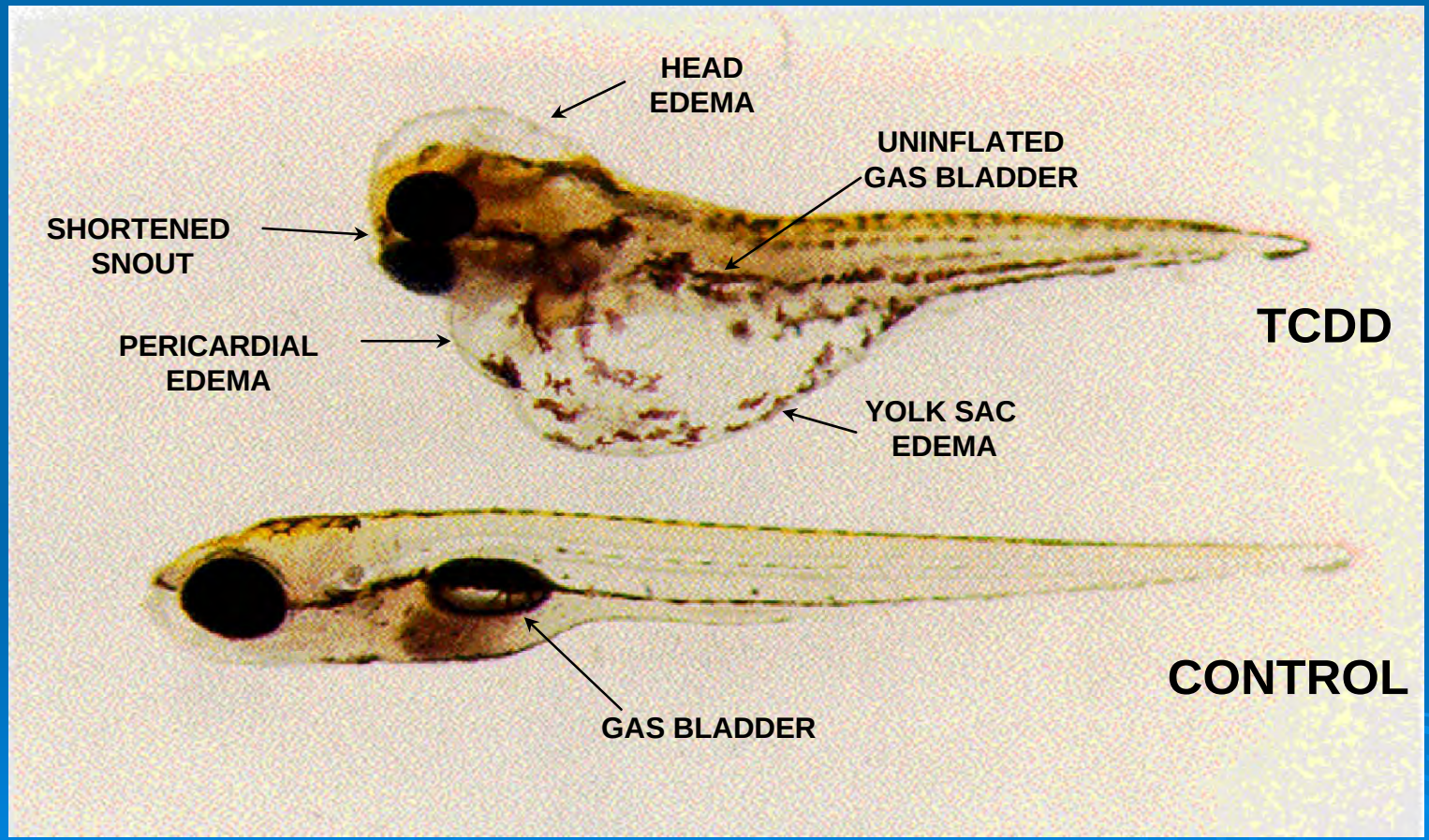
spontaneous movement (18-24 hpf) onset and frequency

touch response (27 hpf)

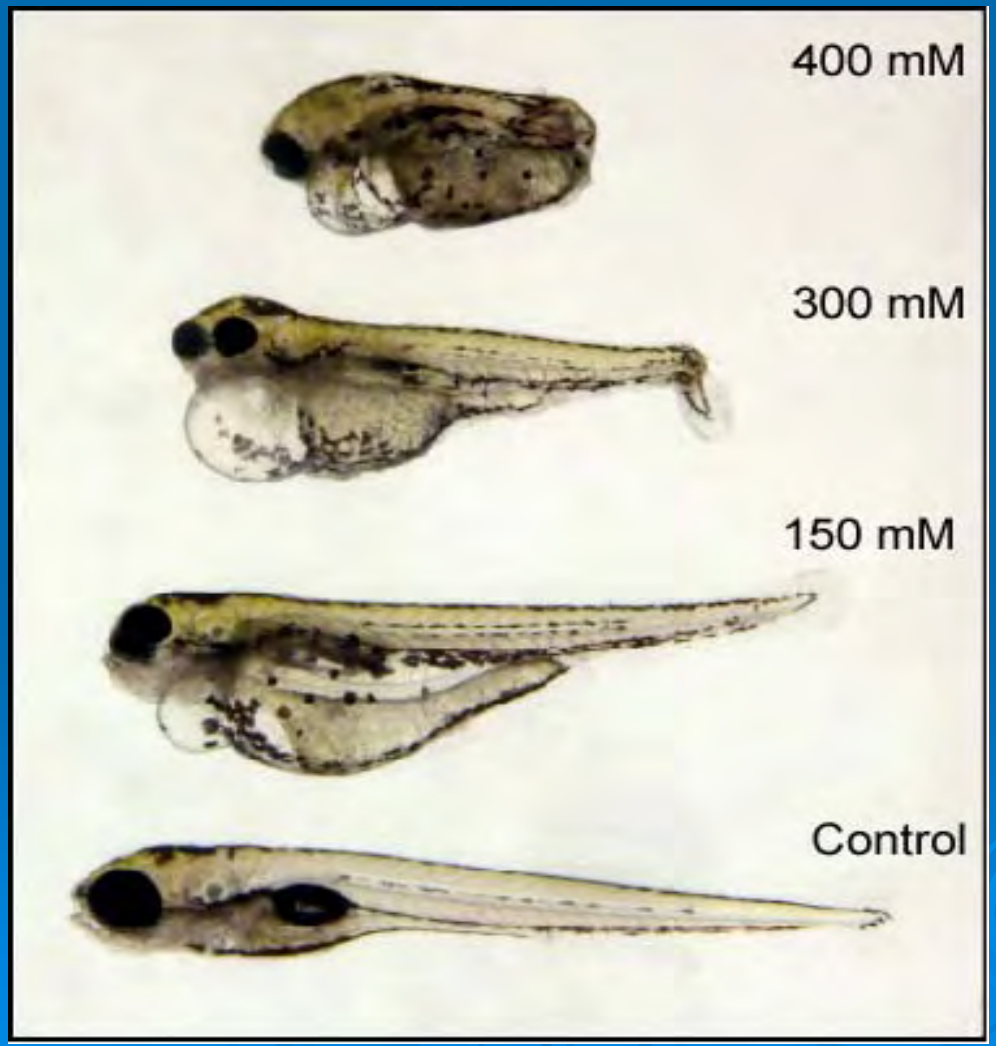
motility

Tier 1 examples

Early Life Stage Response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)



Early Life Stage Responses to Ethanol

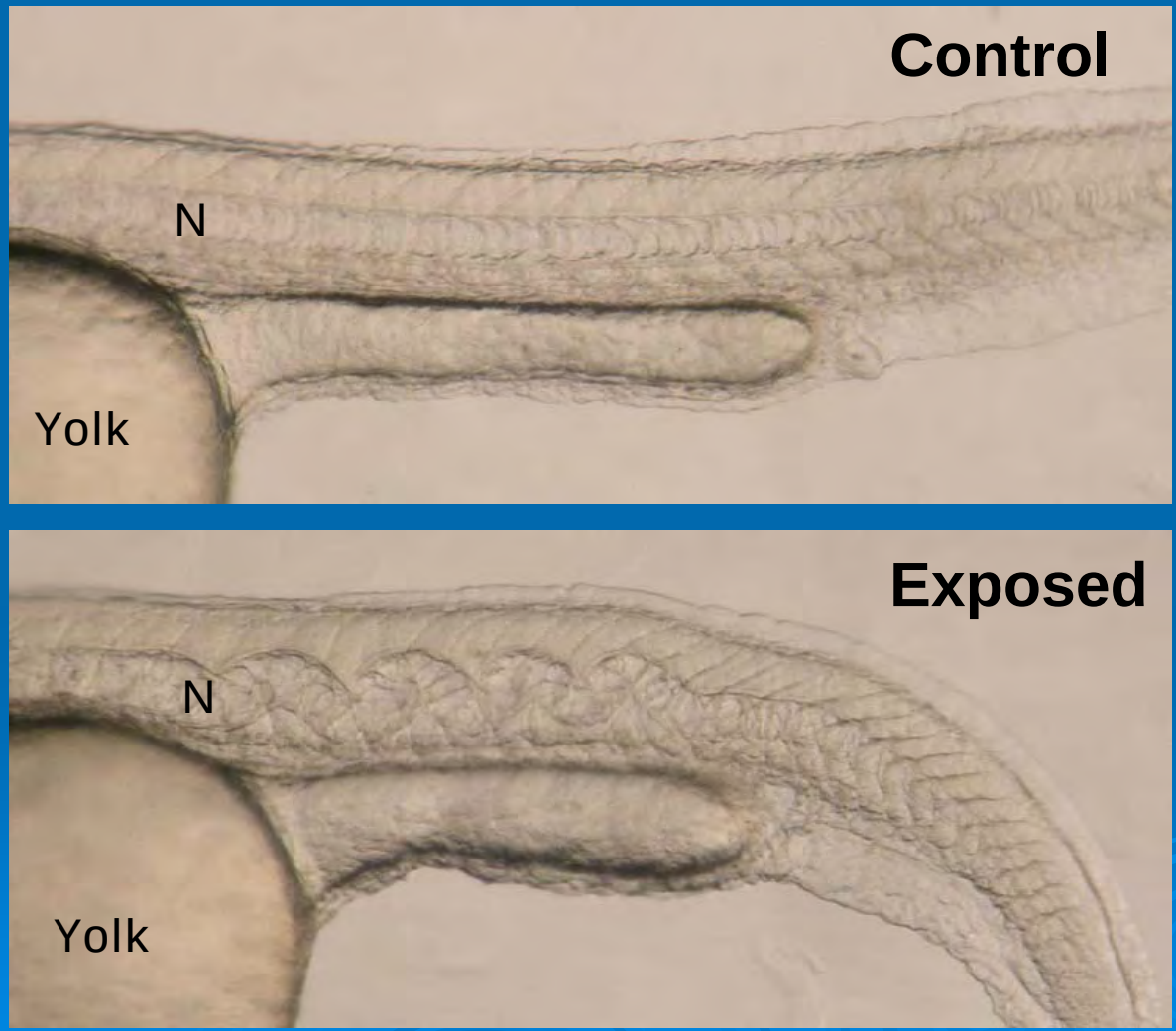


Early Life Stage
Responses to Nanoparticle Exposures

Control

Exposed

Early Life Stage
Responses to Dithiocarbamates (i.e Thiuram)



Tier 1 continued

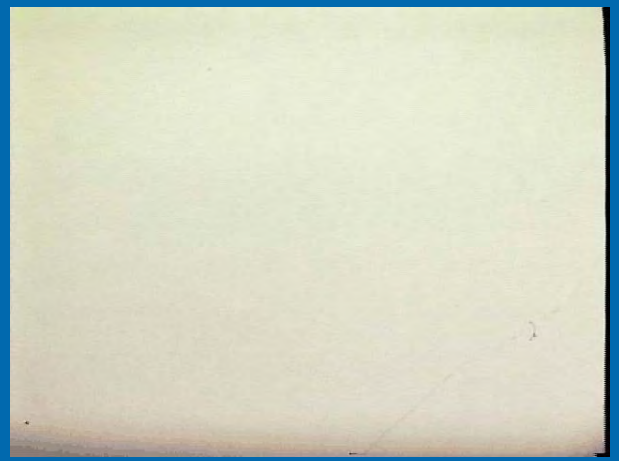
Early Behavioral Assessments

SPONTANEOUS BEHAVIOR

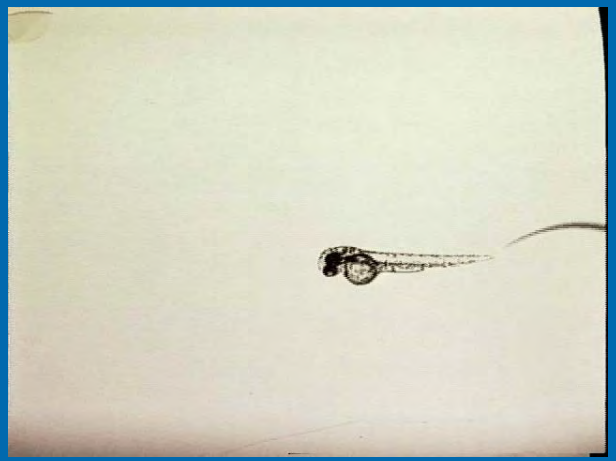
TOUCH RESPONSE

Touch Response Examples

Normal



Twitch



Spasms

Non-responsive

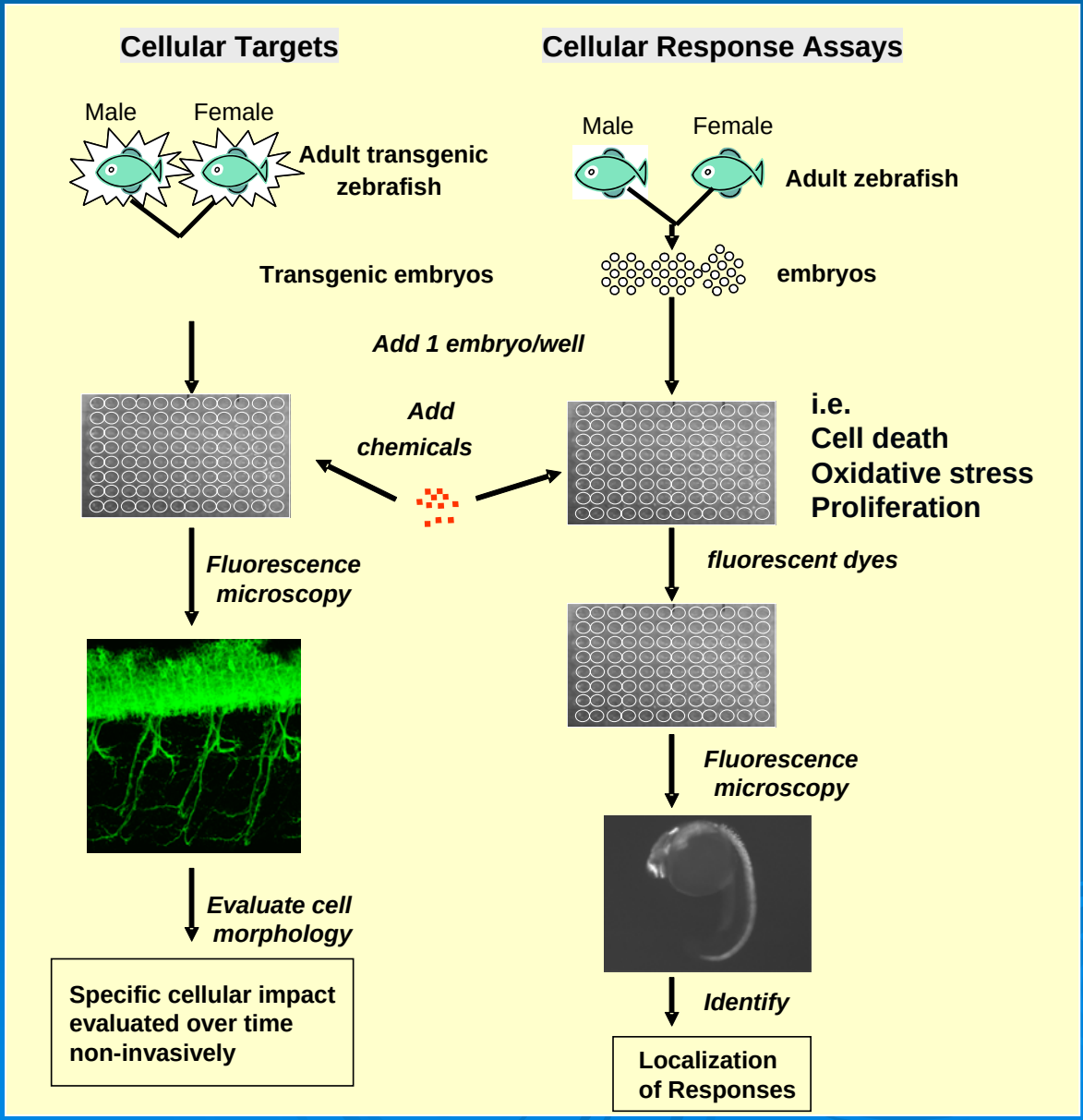
Drug Induced Behavioral Changes

CONTROL

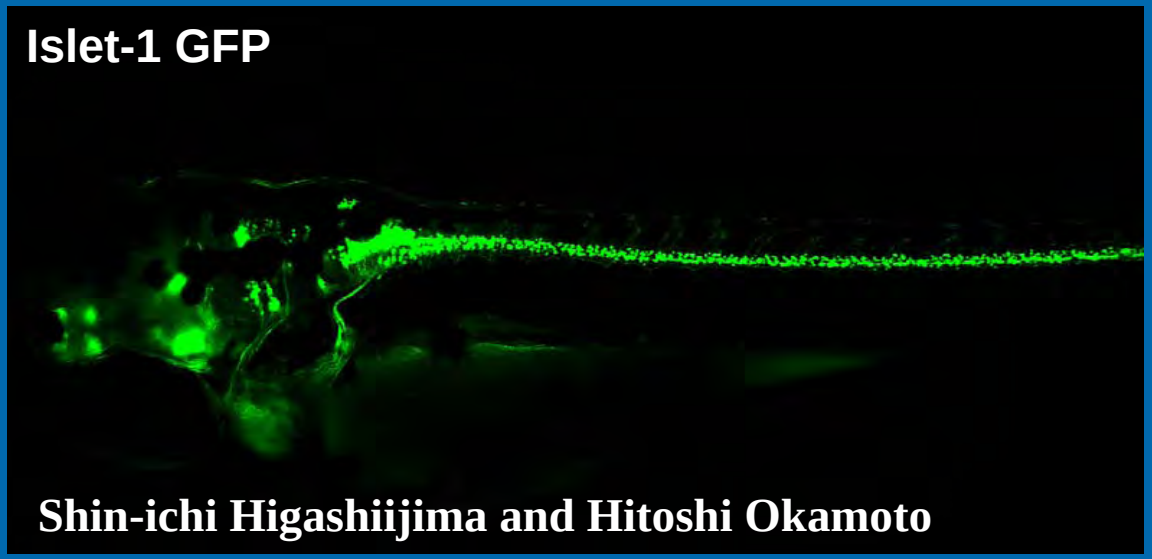
72 hpf

Exposed

Tier 2 Testing



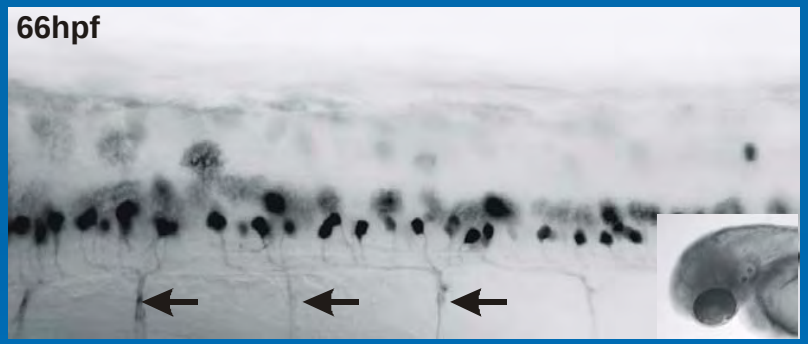
Transgenic Animals



Spinal Neuron Development

Live Transgenic Imaging

CONTROL

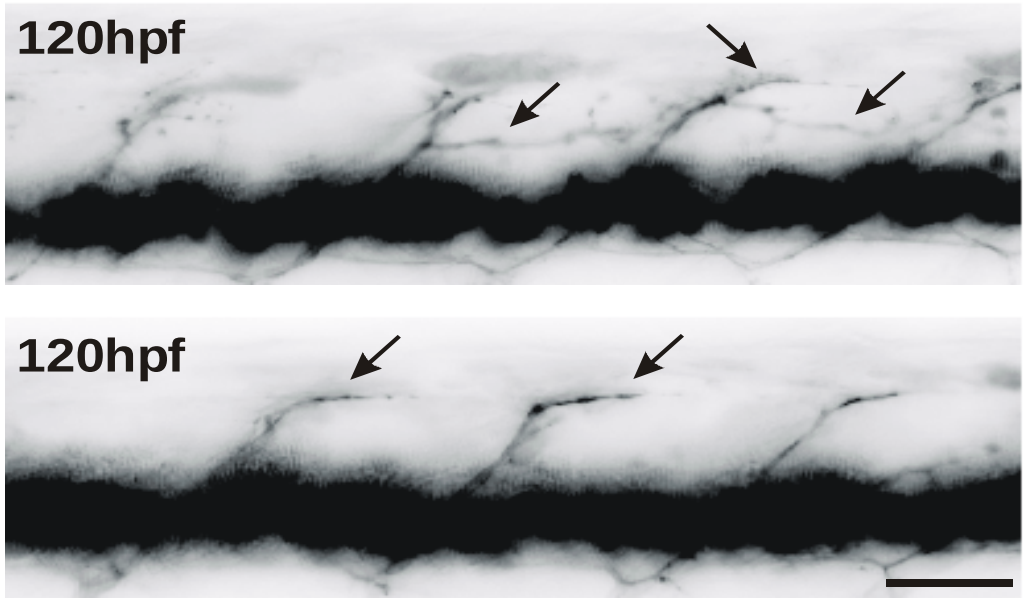


EXPOSED

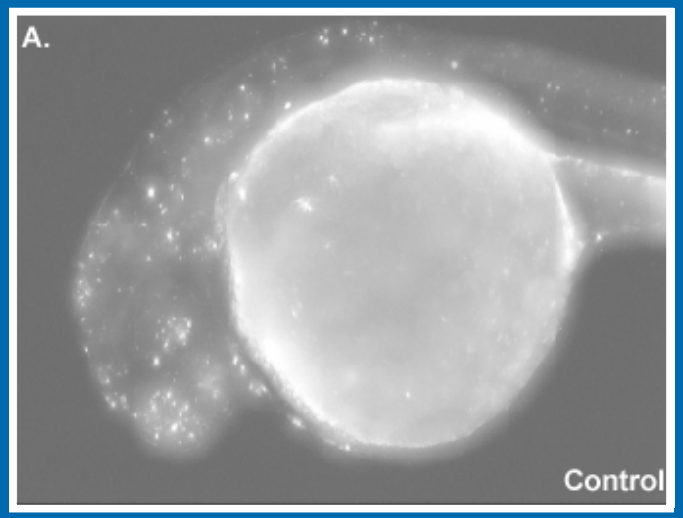
Islet-1 GFP

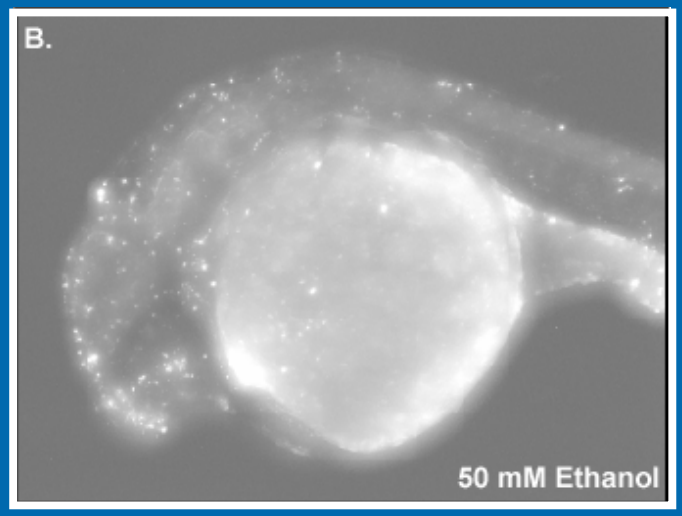
Persistent Neuronal Branching Deficits

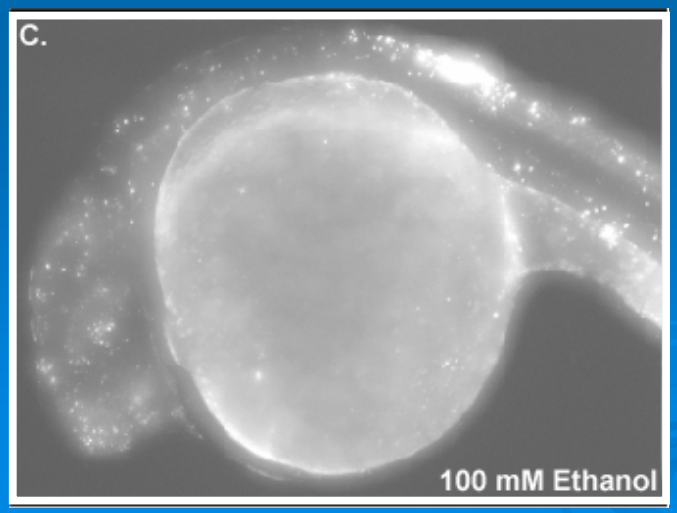
LIVE TRANSGENIC IMAGING

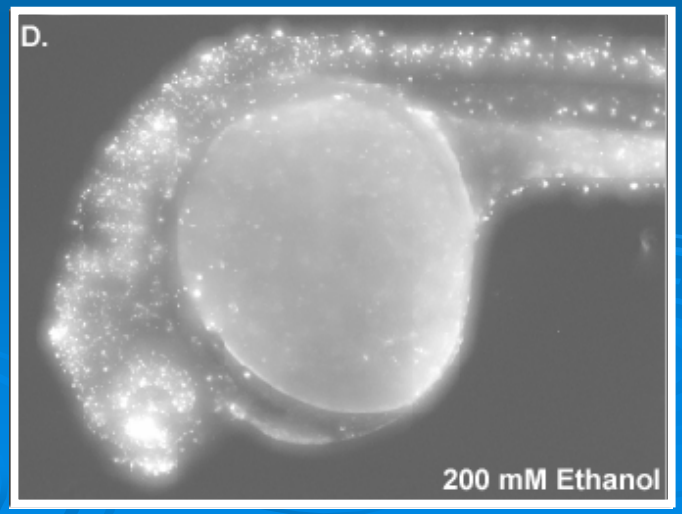


Ethanol Induced Cell Death
Live Animals Acridine Orange

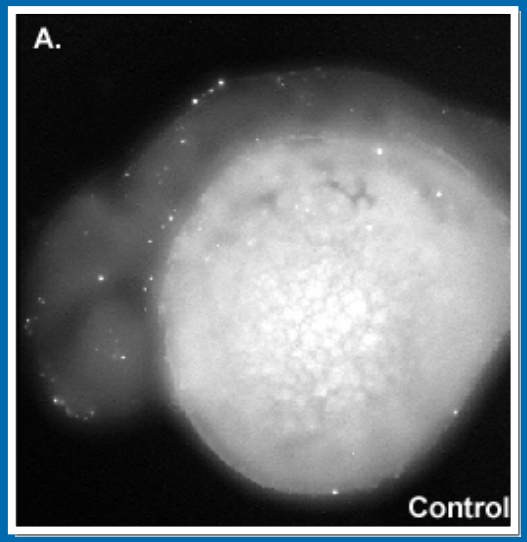


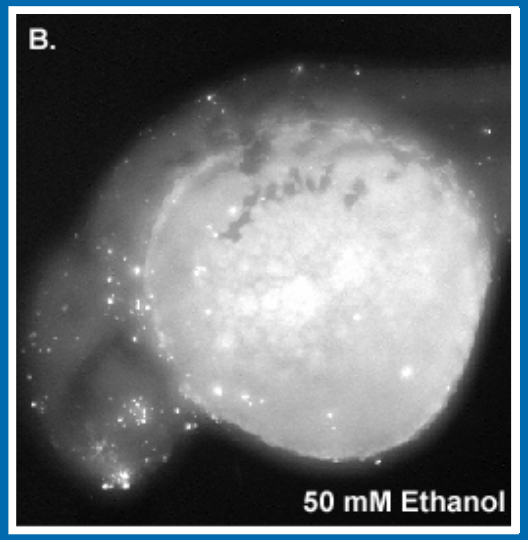


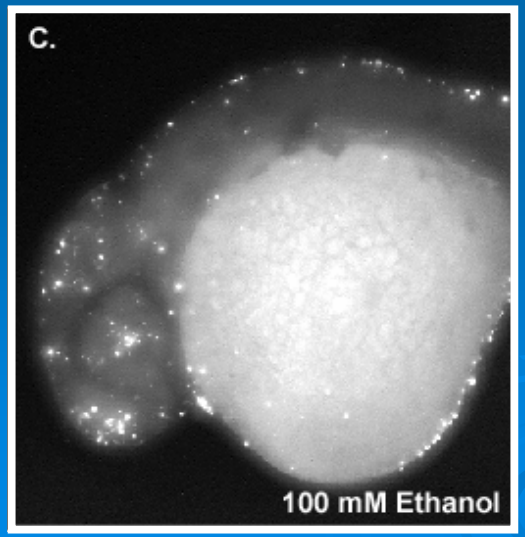


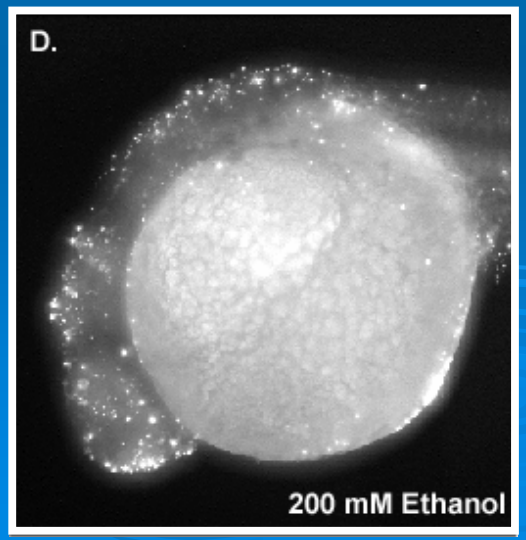


Ethanol Induced Apoptosis
TUNEL



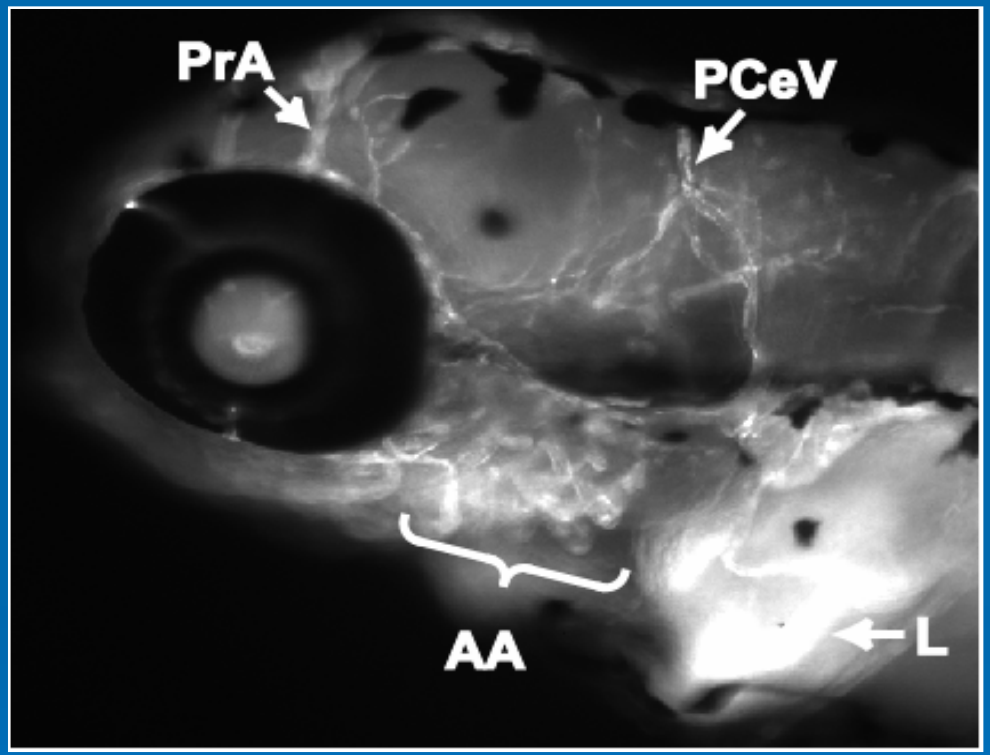




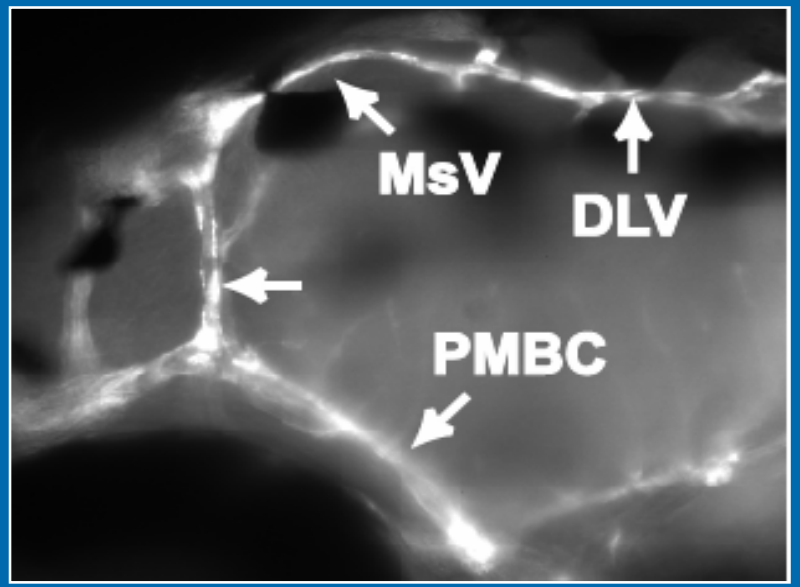


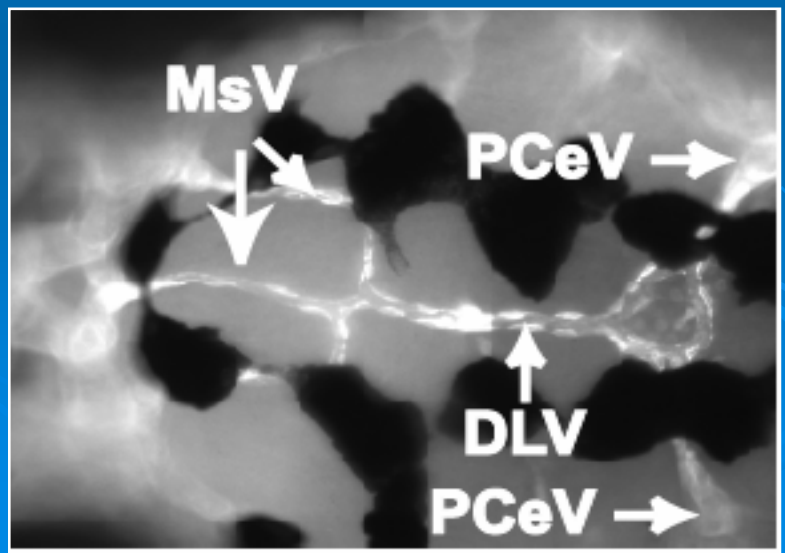
Vascular CYP1a Expression

Whole mount immunohistochemistry - 120 hpf



PrA - prosencephalic artery
PCeV- posterior cerebral vein
AA - aortic arches
DLV - dorsal longitudinal vein
MsV - mesencephalic vein
PMBC - primordial midbrain channel



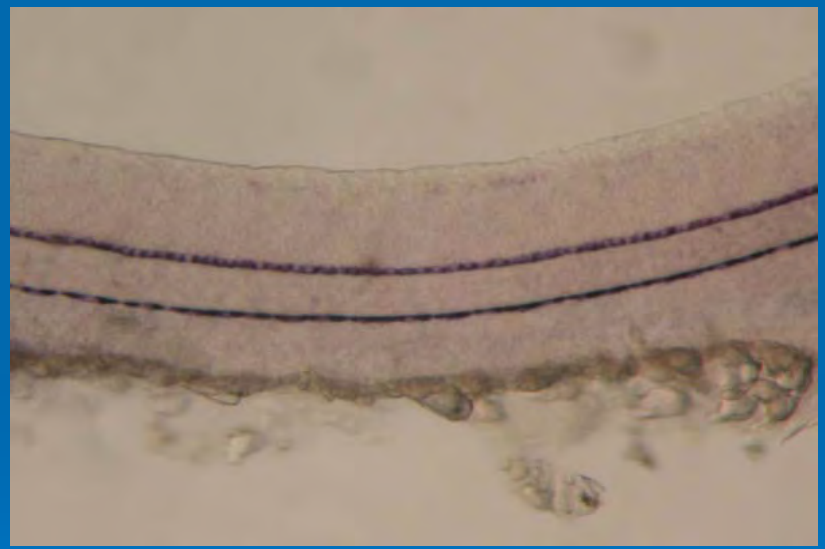


Alterations in gene expression
In situ hybridization

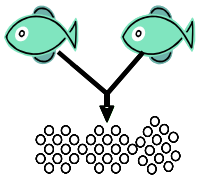
Control

Thiuram

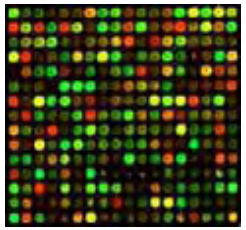
α-col2a



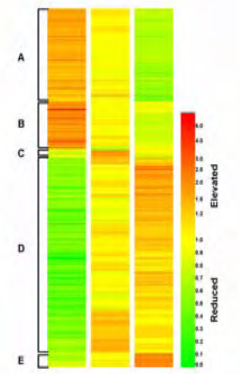
Tier 3 Testing



Chemical Exposures



Microarrays



Integrative and comparative studies

Investigate causality of gene expression changes

zebrafish toolbox:

transgenics, antisense, over expression, mutants chemical genetics

Define gene responses
- targets? Biomarkers of exposure, effect?

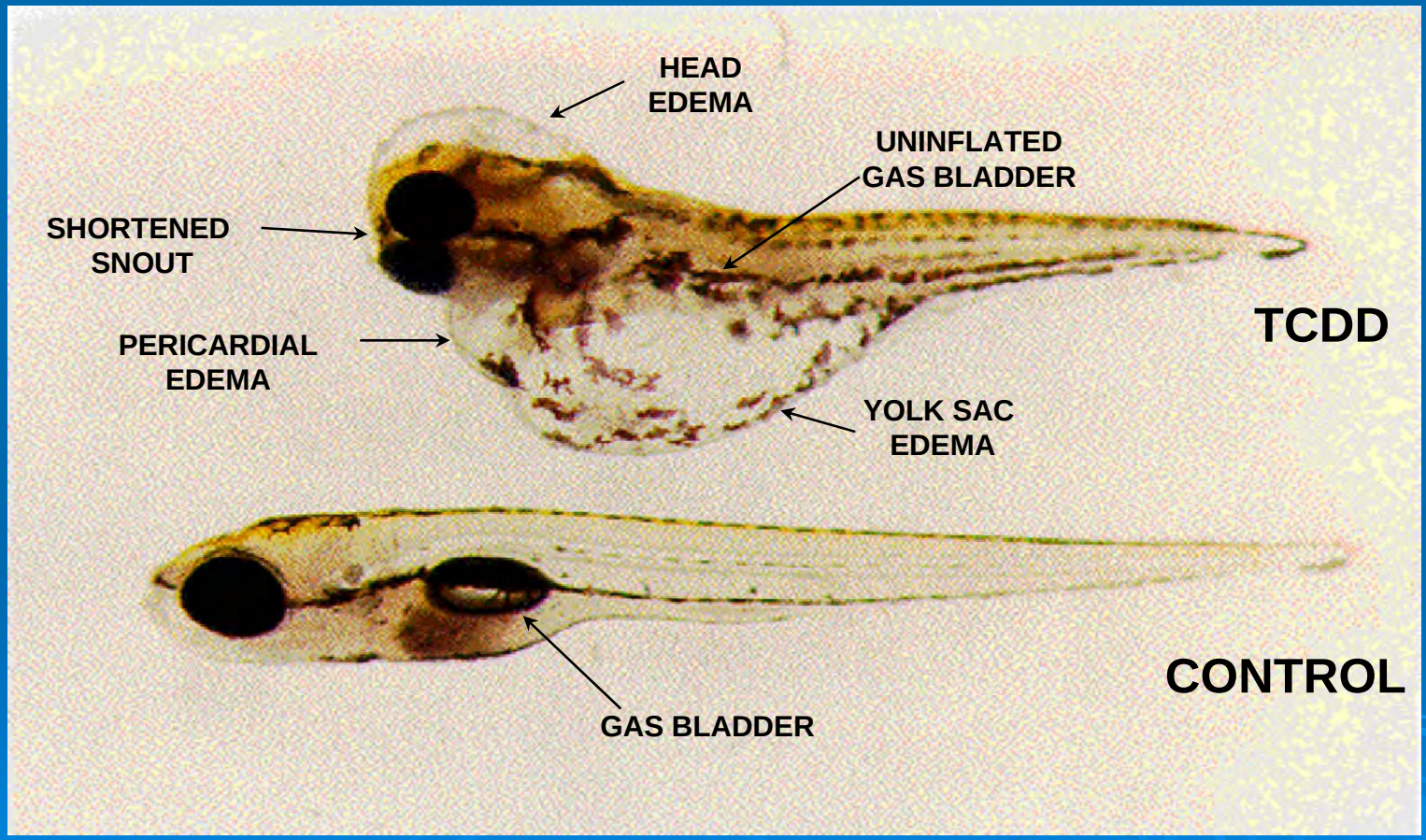
Interpreting Common Endpoints

Common endpoints revealed by chemical and genetic screening

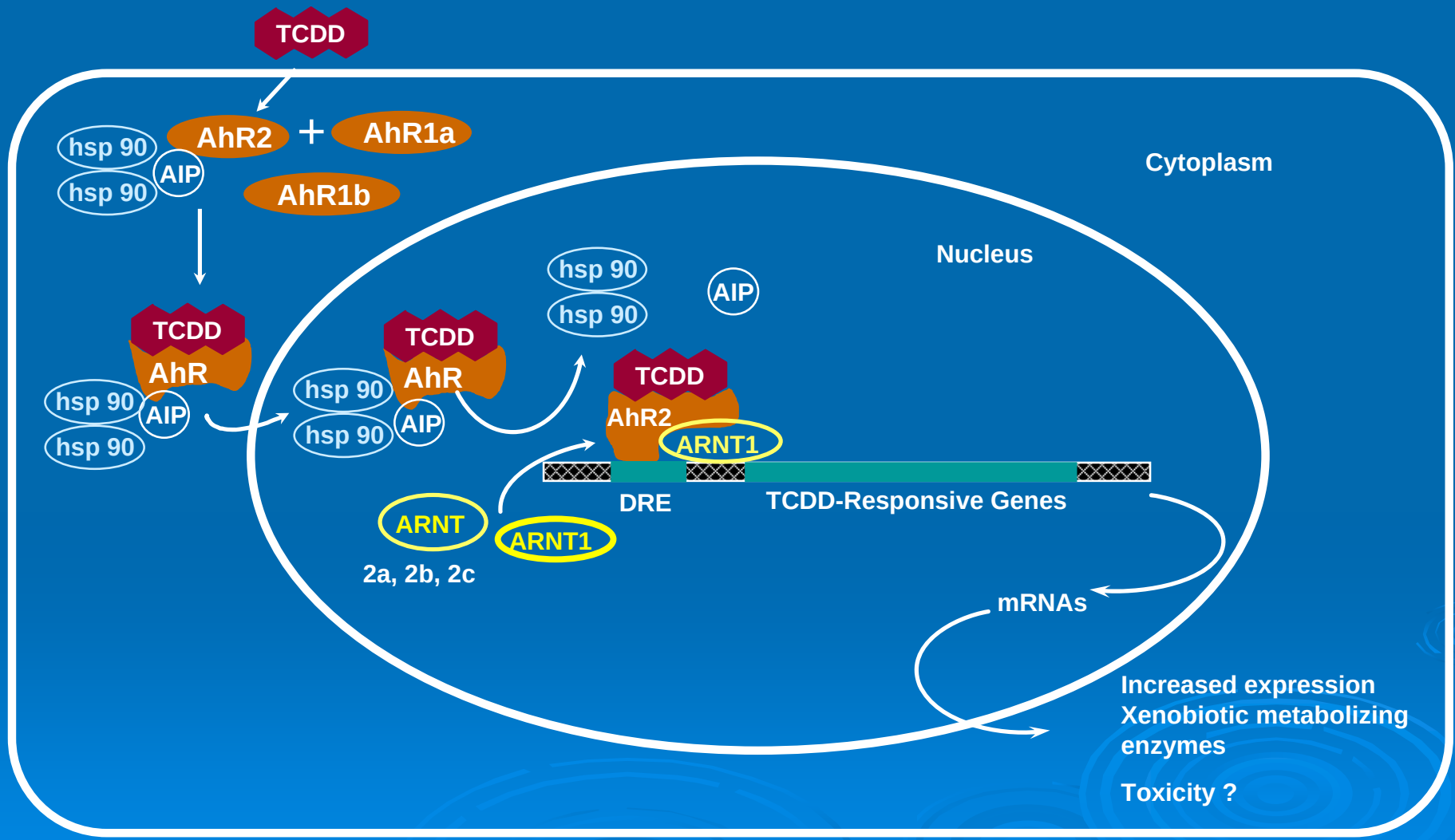
- **Pericardial edema**
- **Yolk sac edema**
- **Reduced growth**
- **Bent body axis**
- **Lack of swim bladder inflation**
- **Mortality**

Common **nonspecific**

Proof of Concept
“Startpoints”



FISH AHR SIGNALING PATHWAY



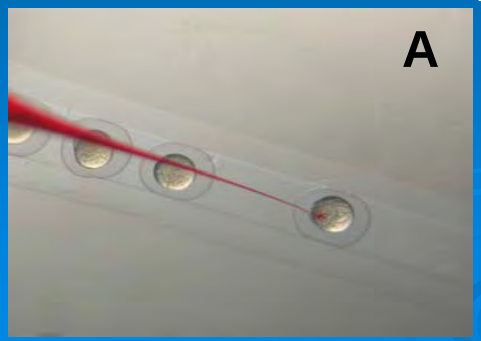
Assess gene functions - *in vivo*

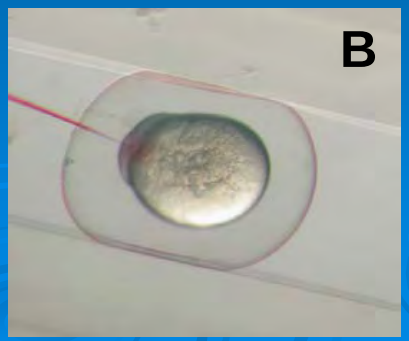
Morpholino Gene Repression –General approach

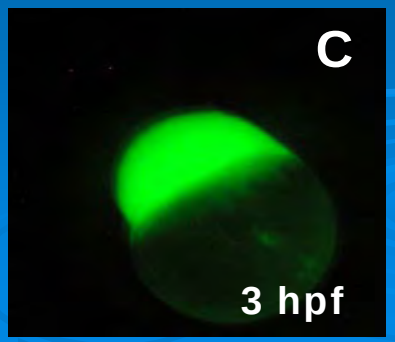
Translational Repression- Protein levels reduced

Since genome is sequenced, any gene can be targeted!

Morpholino knock-down

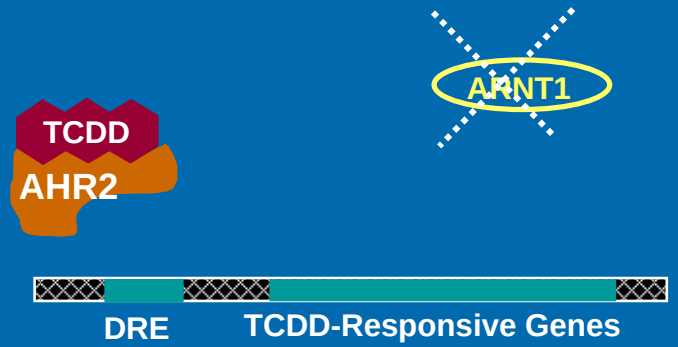




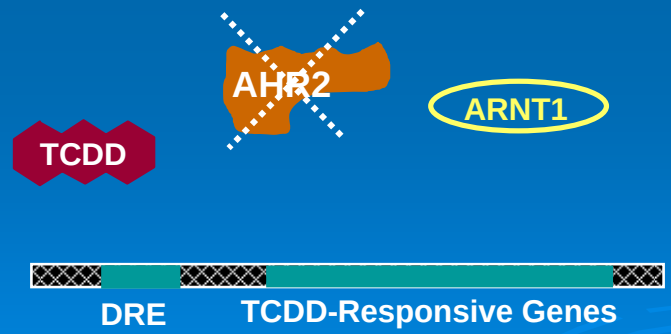


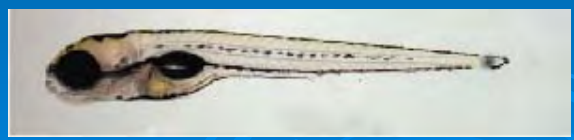
ARNT1 and AHR2 are Necessary for TCDD Toxicity

Repression of ARNT1 OR AHR2 Makes Fish Non-responsive to TCDD



No Toxic Response

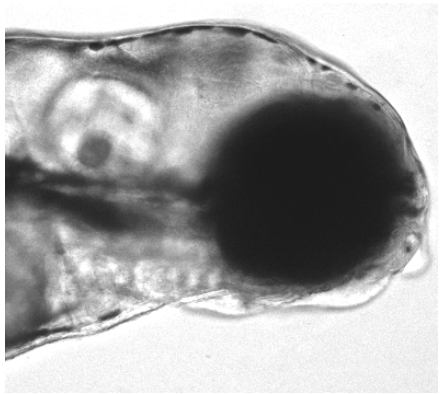




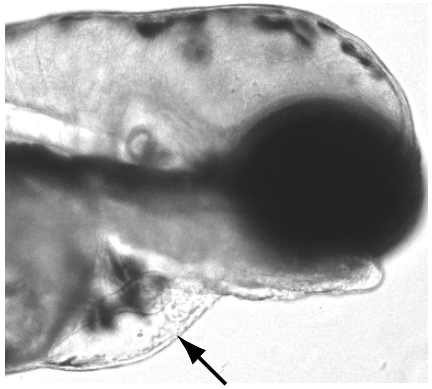
No Toxic Response

TCDD Toxicity is ARNT1 Dependent

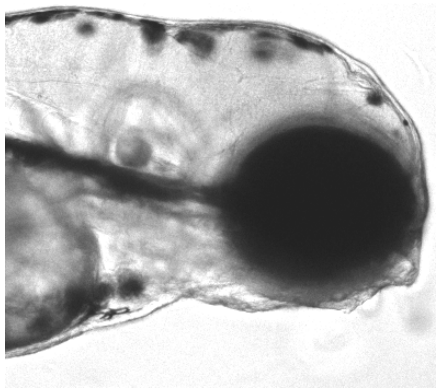
uninjected vehicle



uninjected TCDD



zfarnt1-MO TCDD



Prasch AL, Tanguay RL, Mehta V, Heideman W, Peterson RE: Identification of zebrafish ARNT1 homologs: 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity in the developing zebrafish requires ARNT1. Mol Pharmacol 2006;69(3):776-87.

Final Considerations

- No single model is sufficient to identify hazard or to assess risk.
- Zebrafish as a non-mammalian vertebrate model offers a number of powerful advantages
- Interpreting false negatives or false positives

Comparative studies will help to explain differential species responses

….Embryonic ADME differences

….No maternal contribution

….Molecular signaling well conserved (functionally), primary sequence not necessarily conserved!

Histone deacetylase 1 (HD1)

Identities = 382/442 (86%), Positives = 406/442 (91%)

```
Danio  5    SQGTKKKVCYYYDGDVGNYYYGQGHPMKPHRIRMTHNLLLNYGLYRKMEIYRPHKANAEE  64
            +QGT++KVCYYYDGDVGNYYYGQGHPMKPHRIRMTHNLLLNYGLYRKMEIYRPHKANAEE
Human  4    TQGTRRKVCYYYDGDVGNYYYGQGHPMKPHRIRMTHNLLLNYGLYRKMEIYRPHKANAEE  63

Danio  65   MTKYHSDDYIKFLRSIRPDNMSEYSKQMQRFNVGEDCPVFDGLFEFCQLSTGGSVAGAVK  124
            MTKYHSDDYIKFLRSIRPDNMSEYSKQMQRFNVGEDCPVFDGLFEFCQLSTGGSVA AVK
Human  64   MTKYHSDDYIKFLRSIRPDNMSEYSKQMQRFNVGEDCPVFDGLFEFCQLSTGGSVASAVK  123

Danio  125  LNKQQTDIAINWAGGLHHAKKSEASGFCYVNDIVLAILELLKYHQRVLYIDIDIHHGDGV  184
            LNKQQTDIA+NWAGGLHHAKKSEASGFCYVNDIVLAILELLKYHQRVLYIDIDIHHGDGV
Human  124  LNKQQTDIAVNWAGGLHHAKKSEASGFCYVNDIVLAILELLKYHQRVLYIDIDIHHGDGV  183

Danio  185  EEAFYTTDRVMTVSFHKYGEYFPGTGDLRDIGAGKGKYYAVNYPLRDGIDDESYEAIFKP  244
            EEAFYTTDRVMTVSFHKYGEYFPGTGDLRDIGAGKGKYYAVNYPLRDGIDDESYEAIFKP
Human  184  EEAFYTTDRVMTVSFHKYGEYFPGTGDLRDIGAGKGKYYAVNYPLRDGIDDESYEAIFKP  243

Danio  245  IMSKVMEMYQPSAVVLQCGADSLSGDRLGCFNLTIKGHAKCVEYMKSFNLPLLMLGGGGY  304
            +MSKVMEM+QPSAVVLQCG+DSLSGDRLGCFNLTIKGHAKCVE++KSFNLP+LMLGGGGY
Human  244  VMSKVMEMFQPSAVVLQCGSDSLSGDRLGCFNLTIKGHAKCVEFVKSFNLPMLMLGGGGY  303

Danio  305  TIRNVARCWTFETAVALDSTIPNELPYNDYFEYFGPDFKLHISPSNMTN----------  353
            TIRNVARCWT+ETAVALD+ IPNELPYNDYFEYFGPDFKLHISPSNMTN
Human  304  TIRNVARCWTYETAVALDTEIPNELPYNDYFEYFGPDFKLHISPSNMTNQNTNEYLEKIK  363

Danio  354  ----------------QMQAIPEDAV-QEDSGDEEDDPDKRISIRAHDKRIACDEEFSDS  396
                            QMQAIPEDA+ +E   ++EDDPDKRISI + DKRIAC+EEFSDS
Human  364  QRLFENLRMLPHAPGVQMQAIPEDAIPEESGDEDEDDPDKRISICSSDKRIACEEEFSDS  423

Danio  397  EDEGQGGRRNAANYKKPKRVKT  418
            E+EG+GGR+N++N+KK KRVKT
Human  424  EEEGEGGRKNSSNFKKAKRVKT  445
```

Transforming growth factor beta-1 (TGF-1)

Identities = 164/371 (44%), Positives = 229/371 (61%)

```
Danio  MSTCKTLDLEVVRKKRIEAIRGQILSKLRMAKEPESGADDDGQKIPDSLLSLYNSTVELS
       +STCKT+D+E+V++KRIEAIRGQILSKLR+A  P  G    G  +P+++L+LYNST +
Human  LSTCKTIDMELVKRKRIEAIRGQILSKLRLASPPSQGEVPPG-PLPEAVLALYNSTRD--

Danio  EEMKTKIVPVQDEDEDYFGKEVHKFVFQQAQN-------NTKHQ--MFFNVSEMKRSIPD
              P  + + DY+ KEV + +  +  N        + H   MFFN SE++ ++P+
Human  RVAGESAEPEPEPEADYYAKEVTRVLMVETHNEIYDKFKQSTHSIYMFFNTSELREAVPE

Danio  YRLLSQAELRLRIKNPTMDQEQRLELYRGVGDQA-RYLGTRFVSKDLSNRWLSFDVKQTM
          +  +   LR+    +  EQ +ELY+   + + RYL  R ++   S  WLSFDV   +
Human  --PVLLSRAELRLLRLKLKVEQHVELYQKYSNNSWRYLSNRLLAPSDSPEWLSFDVTGVV

Danio  IEWLQGSEDEETLELRLYCDCKA--NQQSTDKFLFTISGLDKQRGDTAGLADMMVKPYIL
        +WL    + E   L  +C C +  N    D   FT      +RGD A +  M  +P++L
Human  RQWLSRGGEIEGFRLSAHCSCDSRDNTLQVDINGFTTG----RRGDLATIHGMN-RPFLL

Danio  ALSLP-SNGNSLASVRKRRAVGTDETCDEKTETCCMRKLYIDFRKDLGWKWIHKPKGYFA
        ++ P      L S R RRA+ T+       + CC+R+LYIDFRKDLGWKWIH+PKGY A
Human  LMATPLERAQHLQSSRHRRALDTNYCFSSTEKNCCVRQLYIDFRKDLGWKWIHEPKGYHA

Danio  NYCMGSCTYIWNAENKYSQILALYKHHNPGASAQPCCVPAILDPLPILYYVGRQHKVEQL
       N+C+G C YIW+ + +YS++LALY  HNPGASA PCCVP  L+PLPI+YYVGR+ KVEQL
Human  NFCLGPCPYIWSLDTQYSKVLALYNQHNPGASAAPCCVPQALEPLPIVYYVGRKPKVEQL

Danio  SNMVVRNCKCS
       SNM+VR+CKCS
Human  SNMIVRSCKCS
```