Breakthroughs.

Partnerships.

Leadership.

Incorporating the Zebrafish Embryo Teratogenicity Assay Into the Drug Discovery Process

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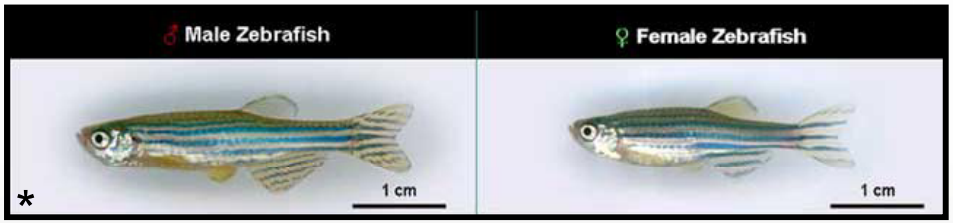
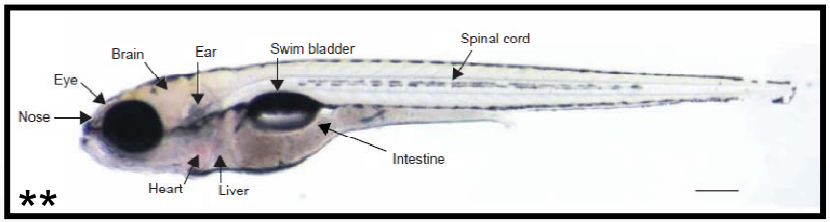
Bio International Convention

The Global Event for Biotechnology

Zebrafish as a Model of Development

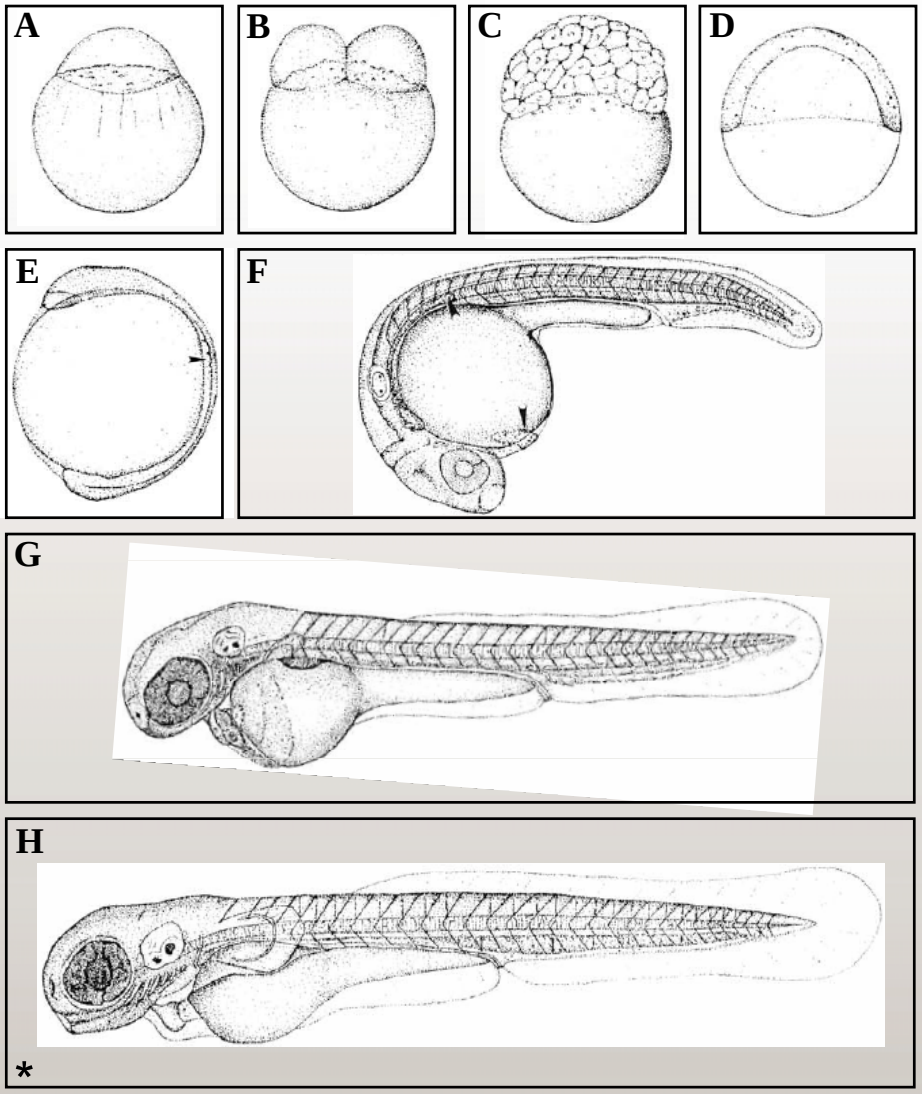




- Can be stimulated to breed year-round under proper photoperiod
- Fertilization and development occur *ex utero* and organogenesis takes only 2-3 days
- Embryos are small and therefore amenable to array screening
- Chorion/embryo are translucent, facilitating morphological assessment
- Good conservation of embryological processes and molecular pathways (possess orthologs to ~86% of human drug targets)
- Fully sequenced genome
- Model aligns well with the initiative to reduce, refine, and replace

*** http://www.naturalhistorymag.com/0606/images/zebrafish.jpg; ** Rubinstein, *et al*. 2003**

Developmental Staging Series



Stage Name	Timing (hpf)
Zygote	0-0.75
Cleavage	0.75-2.25
Blastula	2.25-5.25
Gastrula	5.25-10.33
Segmentation	10.33-24
Pharyngula	24-48
Hatching	48-72
Larval	>96

*** Kimmel *et al*., 1995**

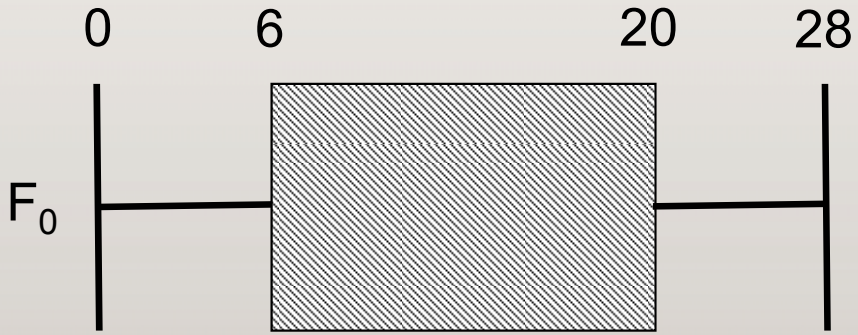
Drug Development: Opportunities for *In Vitro* Testing

- Suggested that for every 10,000 new molecular entities developed, only 1 will make it to market
  - Timeline from conceptualization to market: 10 years
  - R&D investment: $800 million - >$1 billion
- Teratogenicity findings are responsible for a significant portion of safety related pipeline attrition
- Teratogenicity studies typically occur at the end of preclinical safety studies or during Phase I clinical trials
- Opportunities exits to incorporate *in vitro* developmental toxicity studies early in the drug discovery process to proactively identify compounds with teratogenic liability

Developmental Toxicology: *In Vivo* Assays

- Mammalian studies:
  - Segment I: Assess fertility in males and females (rats)
  - **Segment II: Assess developmental toxicity/embryotoxicity (rats and rabbits)**
  - Segment III: Assess perinatal toxicity (rats)

- Segment II protocol example (rabbits)*:



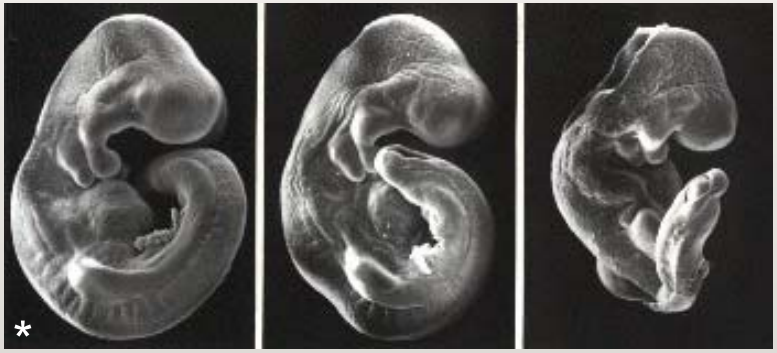
Maternal	Developmental
Body weight	Implantation
Food consumption	Resorption rate
Physical signs	Fetal weight
Gross lesions	External, visceral, skeletal alterations

*** Modified from Manson, 1981 (in Developmental Toxicology)**

Developmental Toxicology: *In Vitro* Assays

- Why consider *in vitro* alternatives for safety assessment?
  - Less expensive
  - Higher throughput
  - Compliance with REACH legislation
  - Alignment with 3 R's: Reduce, Refine, Replace

- Several rodent based assays:
  - Rodent whole embryo culture
  - Mouse embryonic stem cell test
  - Rodent micromass assay



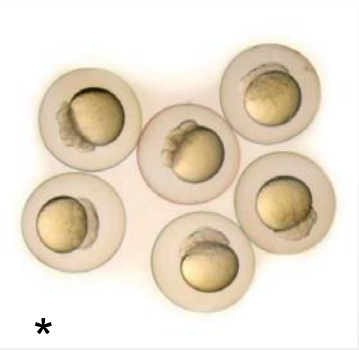
- Zebrafish, which have been used extensively in ecotoxicology and developmental genetics research, are gaining popularity as a model for developmental toxicity assessment

*http://www.medcellbiol.uu.se/research/ueresearche.html

Zebrafish as a Developmental Toxicology Model

- No harmonized method exists, although the several models that have been described share the following:
  - Compounds administered at same developmental stage as in mammalian teratology studies with morphology assessed at fetal-stage equivalent
  - Assessment of both viability and morphological alterations
  - Morphological assessment performed via quantitative and/or qualitative measures (i.e., score system)
  - Define a “teratogenic index”, typically a ratio between the concentration causing general toxicity and the concentration producing the lowest or no adverse effect
- Zebrafish can detect both direct acting teratogens and proteratogens that require metabolic activation
  - Bioactivation via cytochrome P450 enzymes
  - Addition of exogenous mammalian metabolic activation system (microsomes)

General Protocol



Array

Score

- Brannen, *et al*. 2010. Development of a Zebrafish Embryo Teratogenicity Assay and Quantitative Prediction Model. *Birth Defects Research (Part B)* 89: 66-77

- **Purpose**: Develop a zebrafish assay allowing for characterization of teratogenicity as it relates to specific abnormalities and concentration-response via screening of 31 known *in vivo* teratogens and non-teratogens

***http://www.unsolvedmysteries.oregonstate.edu/microarray_02; **http://www.kareldomansky.com/design-gallery/perfused-multiwell-plate-1**

Protocol – Brannen *et al*., 2010

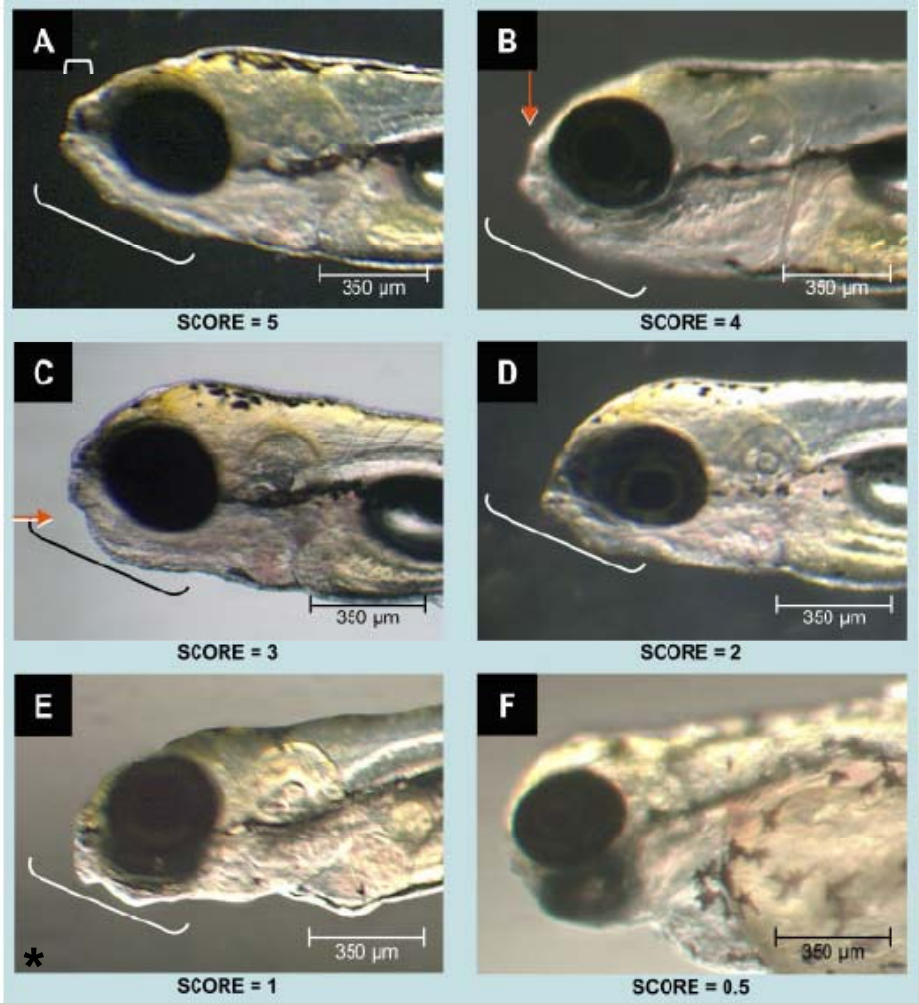
- Adult zebrafish are placed together in a 2:1 female:male ratio to facilitate breeding, and breeding is stimulated by photoperiod and addition of marbles to bottom of tanks → harvested early morning
- The outer membrane (chorion) is removed via protease treatment and microdissection to facilitate compound delivery
- At 4-6 hours post fertilization (hpf) embryos are cultured in the compound of interest along with a vehicle control
  - N = 12 embryos/dose
  - Typical dose range: 0.1, 1, 10, 100 µM (4 doses minimum)
- At 5 days post fertilization (dpf), viability is assessed (N = 12) and embryos are scored for developmental defects (N = 6)

Endpoints and Scoring

- Larval length/shape
- Motility
- Cardiovascular function
- Pigmentation
- Organs
- Morphology:
  - Body shape
  - Somites
  - Notochord
  - Tail
  - Heart
  - Facial structure
  - Neural tube
  - Arches/jaws

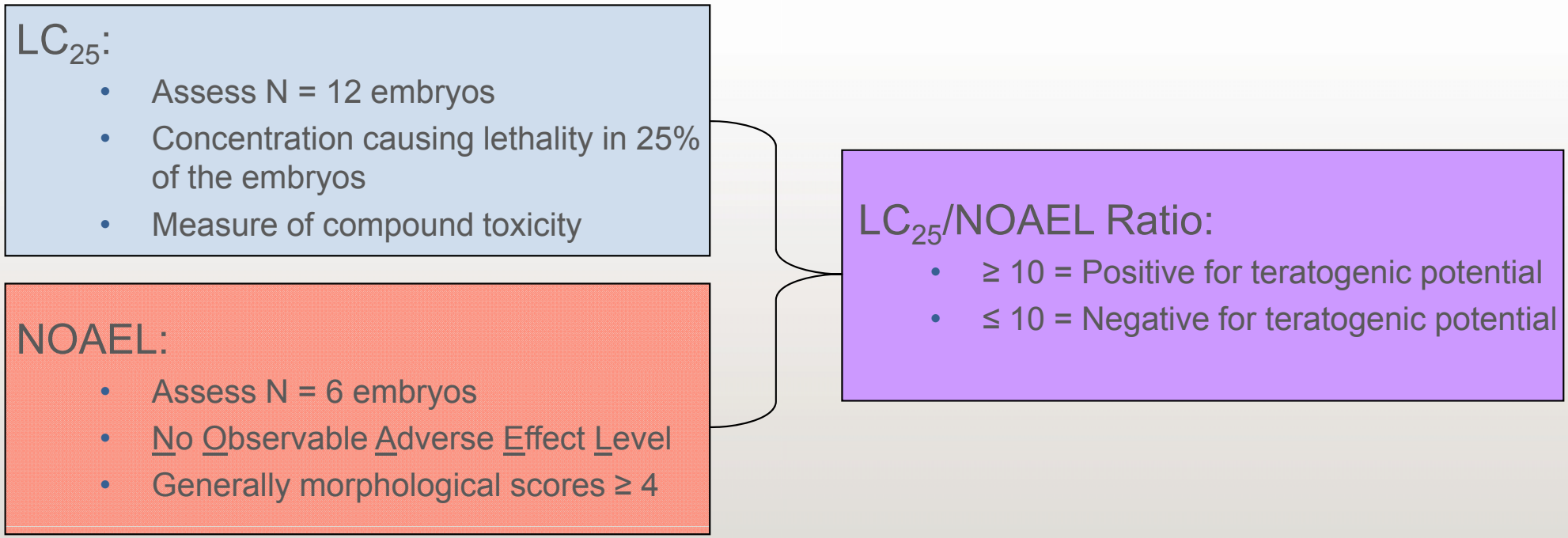
Score	Interpretation
0.5	Structure not evident
1	Severe malformation
2	Moderate malformation
3	Mild malformation
4	Subtle anomaly (growth delay or reversible)
5	Normal morphology

Morphological Scoring Example – Arches/Jaws



*** Panzica-Kelly *et al*. 2010**

Assessment of Teratogenic Liability

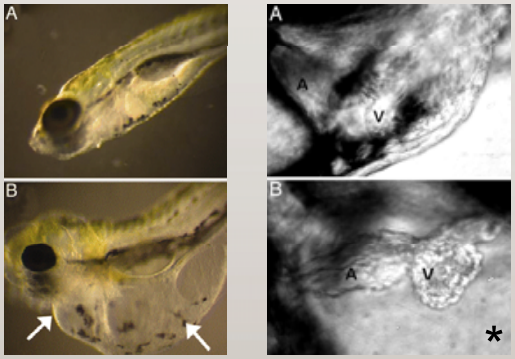


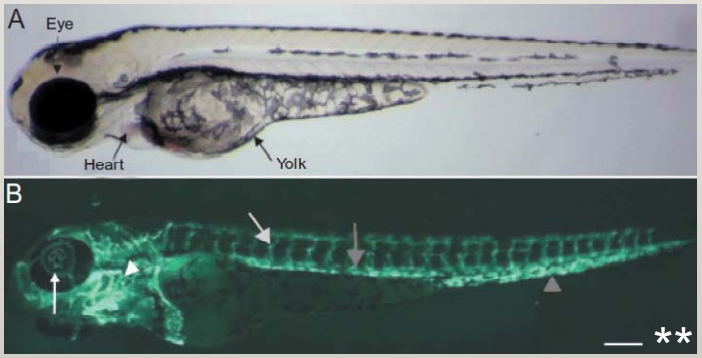
- **Results**: Excellent concordance (87%) for classifying *in vivo* outcome with only 2 false-positives and 2 false-negatives in 31 compounds tested

Additional Uses of the Zebrafish Model

- Hepatotoxicity
- Cardiotoxicity
- Ototoxicity
- Locomotor activity
- Seizures
- Neurotoxicity
- Nephrotoxicity
- Cytotoxicity
- Angiogenesis

- Disease Models:
  - Cancer
  - Epilepsy
  - Alzheimer's Disease
  - Diabetes
  - Huntington's Disease
  - Muscular Dystrophy
  - Amyotrophic Lateral Sclerosis
  - Leukemia
  - Cardiomyopathy
  - Thrombosis





*** Hamm *et al*., 2006; ** Rubenstein, 2003; *** http://content.usatoday.com/communities/sciencefair/post/2011/03/zebrafish-offer-skin-cancer-clues/1**

Conclusions / Future Directions

- Zebrafish teratogenicity assays offer a rapid, cost-effective, accurate assessment of teratogenic liability of discovery stage compounds
- Utilization of these assays could provide a crucial link between high-throughput *in vitro* screens and *in vivo* mammalian models
- Despite the zebrafish model gaining popularity in safety assessment research, there exists a continuing need for the following:
  - Testing of additional mammalian teratogens and non-teratogens as a means of assay validation
  - Assay harmonization
  - Incorporation of various imaging techniques capable of morphometry, etc. to facilitate high-throughput screening

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