Zebrafish Chemical Screens
Historical look at zebrafish chemical screens
The Effects of 2-Acetylaminofluorene on the Embryonic Development of the Zebrafish*

I. Morphological Studies

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II. Histochemical Studies*

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The effects of AAF on embryonic development in relation to its carcinogenic role
Figs. 1-4.—Camera lucida drawings, Mag. X40. Brachydanio rerio embryos reared at 26°C.

Fig. 1.—Embryo after continuous exposure to 0.06 per cent AAF from mid-gastrulation for 2 days. Heart beat but no circulation; xanthophores present; no melanophores.

Fig. 2.—Embryo after initial exposure during the early gastrula stage to 0.002 per cent AAF for 16 hours and subsequent transfer to aquarium water for 24 hours.

Fig. 3.—Embryo of 3 days after continuous exposure to 0.005 per cent AAF from time of blastulation; number and size of myotomes reduced; yolk protuberances; feeble heart beat; no circulation; epidermal hyperplasia; no visible melanophores.

Fig. 4.—Embryo after initial exposure of early cleavage stage to 0.005 per cent AAF for 16 hours and subsequent transfer to aquarium water for 24 hours. Eyes rudimentary; otic vesicle not visible; thickening of epidermis over head; number of myotomes reduced; no heart beat; no melanophores.

Abbreviations for all figures are as follows:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<td>AV</td>
<td>Auditory vesicle</td>
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<td>CF</td>
<td>Caudal fin</td>
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<td>E</td>
<td>Edema</td>
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<td>EH</td>
<td>Epithelial hyperplasia</td>
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<td>FV</td>
<td>Fourth ventricle</td>
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<td>Liver</td>
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<td>M</td>
<td>Melanophore</td>
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<td>N</td>
<td>Notochord</td>
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<td>OC</td>
<td>Optic cup</td>
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<td>OV</td>
<td>Otic vesicle</td>
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<td>PF</td>
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<tr>
<td>PM</td>
<td>Perioceleomic melanophores</td>
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<td>S</td>
<td>Somite</td>
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<td>T</td>
<td>Tail</td>
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<td>TV</td>
<td>Third ventricle</td>
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<td>V</td>
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<td>VF</td>
<td>Ventrail fin</td>
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<tr>
<td>Y</td>
<td>Yolk</td>
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<tr>
<td>YP</td>
<td>Yolk protuberance</td>
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CHART 1.—The inhibitory effect of 0.03 per cent AAF on the development of *Brachydanio rerio*. Initial exposures to AAF were made during six different developmental stages for short (4-32 hours) and prolonged (60-96 hours) periods.
Small molecule developmental screens reveal the logic and timing of vertebrate development

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Phenotypic screens

Developmental timing of small molecules

Small Molecule screening in Zebrafish
a model for drug/probe discovery

Zon and Peterson
Nat Rev. Drug Disc.
2005
Chemical Screens: An in vivo approach to drug discovery

- Yeast
- Drosophila
- C.elegans
- Stem cells
- Zebrafish
- Xenopus Laevis/Tropicalis
- Mouse
Current Screening Models

- *In vitro*

- Yeast/Cell Culture

- *C. elegans*

- *Drosophila*

- zebrafish

- Mouse

Throughput: $$/$$

Relevance: $$/$$$/$$/$$$
• **Zebrafish Advantages for small molecule screens**
  – Development is rapid: agents for organogenesis
  – Small embryos and large clutches. Screen large number of compounds
  – Multiple Signaling pathways are active
  – Whole organism: Not broken down to individual reactions
  – Whole organism: Address side effects.
  – Transgenic lines: Tailor screens for particular pathways and developmental processes

• **Bottlenecks**
  – Breeding large clutches
  – Transfer of embryos to multiwell plates
  – Read-out. Phenotypic, antibodies/in situ staining or fluorescent reporters.
  – Biological variability
  – Multiple Signaling pathways are active
Experiment III

- **Phenotypic effects of a FDA approved drug library on embryonic development**: Wild type AB* zebrafish embryos will be exposed to a collection of small molecules from a US Drug library for 24 hrs from 1K cell stage. Here, we demonstrate the logistics of transferring single embryos and compounds into multi-well plates and observe the phenotypic effects of chemicals on embryonic development.
Other screening assays:  
In situ based
In situ Screens

![Diagram showing in situ screens process]

In situ Screens

Incubate embryos with chemicals

\[ n = 2498 \]

In situ hybridization for \textit{runx1} and \textit{cmyb}

2416 unaffected

35 increased

47 decreased

2416 unaffected

35 increased

47 decreased

\text{runx1/cmyb}

Control

dmPGE2

Figure 2. Large-scale vertebrate chemical screening made possible by zebrafish. Embryos are incubated in groups of 5-10 with approximately 2,500 different chemicals. At 36 hours post-fertilization, in situ hybridization is conducted to analyze the expression of early hematopoietic markers such as \textit{runx1} and \textit{c-myb}. The embryos are then scored for a change in hematopoietic expression. We recently used this technique [45] to identify 82 compounds that influence hematopoietic stem cell differentiation, the most prominent of which was dimethyl prostaglandin E2 (dmPGE2). Modified with permission, from [45].

Discussion

• Pros
  – molecular marker(s) for cell types that are not apparent in phenotype
  – treated embryos can be fixed and stored for long periods of time

• Cons
  – labor intensive.
  – in situ takes time to process
  – scoring/variability
Live Reporter Screens

• A live molecular readout
• Experiment I & II
Transgenic reporters of Signaling: Screening for chemical modulators of pathways in vivo
Goal for today

• Compare and contrast agents that target FGFR and VEGFRs in two different reporter lines

• FGFR and VEGFRs are members of the receptor tyrosine kinase class. chemical structures of these compounds are similar.
Fig. 1. Architecture and domain organization for a variety of RTKs. The extracellular portion of the receptors is on top and the cytoplasmic portion is on bottom. Legend for the domain types is on the right side. The TK domain of the PDGF receptor contains a large insertion, represented as a break in the TK domain symbol. The lengths of the receptors are only approximately to scale.
Fig. 4. Mode of PD 173074 binding to FGFR1K. (A) Stereo view of the PD 173074 binding pocket in FGFR1K. The side chains of residues that interact with the inhibitor are shown as well as main-chain atoms that participate in hydrogen bonding. Split-bond coloring is used with carbon atoms orange (PD 173074) or green (FGFR1K), oxygen atoms red, nitrogen atoms blue and sulfur atoms yellow. The FGFR1K main-chain representation is colored light blue for the nucleotide-binding loop, purple for the segment connecting the two kinase lobes, yellow for the catalytic loop and orange for the activation loop. Hydrogen bonds are shown as dashed lines. (B) Superposition of PD 173074 and AMP-PCP (Mohammadi et al., 1996a) bound to FGFR1K. View is approximately perpendicular to the pyrido[2,3-d]pyrimidine/adenine rings. Bonds and carbon atoms are colored orange (PD 173074), green (FGFR1K) or gray (AMP-PCP). Other atoms colored as in (A) with phosphorus atoms black. Hydrogen bonds are shown as black (AMP-PCP) or orange (PD 173074) dashed lines. Due to disorder, the \( \gamma \) phosphate of AMP-PCP is not modeled. Prepared with GRASP (Nicholls et al., 1991).
Experiment I & II

• Experiment I
  – FGF reporter line - \( Tg(dusp6:EGFP)^{pt6} \)
    • Dusp6 inhibitor
      – BCI
    • FGFR kinase inhibitors
      – SU5402
      – PD173074

• Experiment II
  – VEGF reporter line - \( Tg(fli1a:EGP)^{y1} \)
    – SU4312
    – SU5402
    – Oxindole
    – PD173074
Experiment I

• Pipette $Tg(dusp6:EGFP)^{pt6}$ transgenic line embryos (~24 hpf) into first three columns (Columns 1-3 see plate format) of a 96-well plate and remove excess E3 using a micropipette (20-200 µl range). **This is important to avoid variability in the drug concentration we are adding to each wells.** Be careful not to damage the embryo.
  • Add 200µl of E3 solution to each well containing an embryo.
  • Using a micropipette, add 2 µl of DMSO to embryos in columns 1.
  • Add 2 µL BCI (2mM stock solution), SU5402 (1mM stock solution) and PD173074 (2mM Stock Solution) to embryos in columns 2, 3 and 4, respectively.
Mix each well by gentle pipetting.
Experiment II

• Pipette $Tg(fli1a:EGFP)^{y1}$ transgenic embryos at 22-somite stage into columns (columns 7-10) of 96-well plate from Experiment 1 (see plate format).
  • Remove excess E3 from each well as described in Experiment 1.
  • Add 200µl of E3 solution to each well.
  • Using a micropipette, add 2 µl of DMSO to embryos in column 7.
  • Then add 2 µL SU4312 (4mM stock concentration), SU5402 (1mM stock concentration) and Oxindole I (0.5mM stock solution) and PD173074 (2mM stock concentration) to embryos in columns 8-11, respectively. Mix by gentle pipetting.
  • Cover the plate with an aluminum foil and keep the plate with embryos treated with drugs in 28.5°C incubator.
Experiment I & II

Experiment I
• Between 4-6 hour remove plate from incubator and observe GFP expression under fluorescent stereo microscope.
• Note the changes in GFP expression in transgenic embryos treated with BCI, SU5402 or PD173074.
• Note any morphological changes.
• Cover with foil again and place into 28.5°C incubator.

Experiment II
• Remove plate from incubator on Saturday August 17th 10am. Observe the changes in GFP expression in vascular network in treated groups vs control under fluorescent stereomicroscope.
<table>
<thead>
<tr>
<th></th>
<th><strong>Tg(dusp6:EGFP)</strong></th>
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<th><strong>Tg(Fli1a:EGFP)</strong></th>
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<tbody>
<tr>
<td>A1</td>
<td>DMSO</td>
<td>A2</td>
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<td>F1</td>
<td>DMSO</td>
<td>F2</td>
<td>BCI</td>
<td>F3</td>
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FGF reporter line

$Tg(dusp6:EGFP)^{pt6}$

VEGF reporter line

$Tg(fli1a:EGFP)^{yt1}$

After 6hrs treatment

After 24hrs treatment
Experiment III

Procedures to follow for Experiment 3:

• Using a plastic transfer pipette, place the embryos (~1k-cell stage) to individual wells of a 96-well plate. The goal is to have about 3-5 embryos per well. Make sure you take minimum amount of E3 with the embryos, as each well of 96-well can hold only 300 µl volume.
• Using a micropipette, remove any excess E3 solution from the wells, without disturbing the embryos. This can be done with a multichannel pipette.
• Using a multi-channel pipette (50-300 µl) range, add 190µl of E3 solution to each well.
• Using a multichannel micropipette (5-50 µl range), dispense 10 µL each of US drug collection (final concentration 10µM) from the stock plate into their corresponding wells. Start at column 1 and go to column 12. Note the plate number.
• Cover with an aluminum foil and keep the plate with embryos treated with drugs for 24hrs in 28.5°C incubator. Saturday August 18th 9am-11am observe under the microscope for morphological changes and compare to the control (vehicle DMSO treated column 1 & 12).
# Phenotypic Screen with US drug collection

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Each column represents a well in a phenotypic screen, with DMSO (dimethyl sulfoxide) used as a control in some wells.
Other considerations

• Chemical libraries
  – Depends on what you are looking for:
    • small collection of known entities i.e. to determine if a specific pathway plays a role in the process you are studying (US drug collection)
  – To identify new novel compounds.
    • in house (chemistry depts.) vs store bought (microsource, timtec, chembridge etc...)
    • resupply
    • IP issues
Chemical Screens

• Zebrafish, an amazing model for chemical screens
• New transgenic reporter lines can be developed into novel assays
• Screen can be adapted for high throughput approaches.