Application of in situ Hybridization to Detect CYP19-A1 and -A2 Aromatase Gene Expression Patterns Along the HPG-axis at Different Organizational Levels in Japanese Medaka (Oryzias latipes)

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INTRODUCTION
Recent research on endocrine disruptors has focused on compounds that are direct hormone agonists or antagonists, especially those that interact with the estrogen receptor (ER). Because chemicals can cause both direct (receptor-mediated) and indirect effects through changes in signal transduction pathways, methods are needed that permit the screening of multiple effects. Furthermore, methods are needed that can screen for these effects simultaneously in a number of tissues, including during critical windows of development, when tissues may be small and the amount of material available for testing is small and difficult to remove from the organism. Therefore, we are developing a whole animal, in situ hybridization (ISH) test system to simultaneously screen for effects at multiple tissues in the same organism to better understand mechanisms of action of EDCs.

In situ hybridization (ISH):
- Sensitive method that can be used to detect as few as 10-100 nucleic acid molecules per cell
- A method to detect specific genes (RNA or DNA) within cells in tissues
- Provides information relative to temporal and spatial expression of genes (Innis et al. 1990)

Japanese medaka (Oryzias latipes):
- Suitable species to test for specific relationship between in gene expression patterns in different organs
- Allows screening for multiple effects simultaneously in a number of tissues due to its extensive physiological, and genetic backgrounds
- Represents an important test system for environmental research and is widely used for testing endocrine disrupters in ecotoxicology

METHODOLOGY

Whole Animal Histology
A. Cryosectioning
Whole fish were embedded in OCT and flash frozen in isocutane in a liquid nitrogen bath. Frozen blocks were cut into 10 micron sections on a Tissue Tek cryostat and thaw mounted onto Superfrost Plus slides.
- Not a viable method at this time
  - Undecalcified bones too sections; presence of skull destroyed brain morphology
  - Oil in body cavity
- Future development of technique: tape transfer methods may be necessary in some applications
- Success with comparable samples

B. Paraffin embedded section
- Gross dissection: Removal of skull roof, otoliths, and operculum; opening of body cavity
- Tissue Preparation for histology
  - Sections cut serially at 10 μm followed by floating on 40 °C water-bath
  - Sections picked up on Superfrost Plus slides
  - Slides dried at 40 °C overnight

Histological Evaluation: H&E staining
A. Hematoxylin and Eosin Staining
Figure 3. Paraffin embedding section slide shows good morphology

In situ Hybridization
A. Selection of genes
- Initially, three genes were selected to develop ISH techniques (CYP19-A1 & -A2, and β-actin)
- Entire list of genes to be analyzed in the project is given in Figure 1.

B. Synthesis of probes
Total RNA extraction
cDNA synthesis
PCR amplification (Table 1 & Fig. 5)
Insertion of amplified products into vector system

D. Digestion and in vitro transcription with DIG-labeled uracil
E. Purification of unincorporated DIG labeling mix
F. Estimation of labeling efficiency (Fig. 6 & 7)

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Table 1. Primer sequences for synthesizing probes

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<td>570646</td>
<td>5'-GCCCTTCATGCTTGCATTATT/5'-FATATGTTGCAACGTGTCTC</td>
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