Fluorescence in situ hybridization techniques (FISH) to detect changes in CYP19a gene expression of Japanese medaka (Oryzias latipes)

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A R T I C L E   I N F O

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A B S T R A C T

The aim of this study was to develop a sensitive in situ hybridization methodology using fluorescence-labeled riboprobes (FISH) that allows for the evaluation of gene expression profiles simultaneously in multiple target tissues of whole fish sections of Japanese medaka (Oryzias latipes). To date FISH methods have been limited in their application due to autofluorescence of tissues, fixatives or other components of the hybridization procedure. An optimized FISH method, based on confocal fluorescence microscopy was developed to reduce the autofluorescence signal. Because of its tissue- and gender-specific expression and relevance in studies of endocrine disruption, gonadal aromatase (CYP19α) was used as a model gene. The in situ hybridization (ISH) system was validated in a test exposure with the aromatase inhibitor fadrozole. The optimized FISH method revealed tissue-specific expression of the CYP19α gene. Furthermore, the assay could differentiate the abundance of CYP19α mRNA among cell types. Expression of CYP19α was primarily associated with early stage oocytes, and expression gradually decreased with increasing maturation. No expression of CYP19α mRNA was observed in other tissues such as brain, liver, or testes. Fadrozole (100 μg/L) caused up-regulation of CYP19α expression, a trend that was confirmed by RT-PCR analysis on excised tissues. In a combination approach with gonad histology, it could be shown that the increase in CYP19α expression as measured by RT-PCR on a whole tissue basis was due to a combination of both increases in numbers of CYP19α-containing cells and an increase in the amount of CYP19α mRNA present in the cells.

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Introduction

In recent years, an increasing number of genomic and/or proteomic techniques have been developed to identify mechanisms of toxic action in organisms exposed to environmental contaminants. Most of the methods that were developed to meet these objectives such as RT-PCR, Northern blotting, and RNase protection assays rely on relatively high yields of RNA extracted from whole tissues. However, the limitations of these techniques are that they often fail to detect gene expression of low-abundance mRNA in small tissues, or they do not allow localizing changes within certain tissues or cell types. Some genes are only expressed in certain tissues, while others are expressed in specific tissues at only certain times of development (Sanderson et al., 2001). Especially when using small laboratory animal model species, the limited amount of individual tissues available for study and the difficulty in excising them from the organisms has limited the efficacy of these techniques to determine effects during critical windows of time during ontogenesis. While advanced technologies such as laser capture microdissection-based PCR allow identification and quantification of expression of genes in small portions of tissues with great sensitivity, they are labor intensive and typically not feasible for higher throughput multi-tissue studies.

Many of the efforts in endocrine disruptor research have focused on individual endpoints such as receptor-mediated effects (Otsuka, 2002). However, such targeted screening methods may not be sufficient when disruptions are induced through indirect mechanisms. Some chemicals can act as direct agonists or antagonists to certain receptors while others act indirectly by modulating signal transduction, or affecting gene expression or substrate concentrations. For example, the triazine herbicide atrazine does not bind to the estrogen receptor (ER), but in vitro in a mammalian cell system,

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atrazine has been found to up-regulate the expression of aromatase (CYP19), the enzyme that transforms testosterone to estradiol. Although atrazine does not act like a typical estrogen via binding the ER, in mammalian cell systems it can, in some situations, at relatively great concentrations result in estrogenic effects by increasing endogenous estradiol production (Sanderson et al., 2000). As a result, it is important not only to develop methodologies that allow for evaluation of chemical-induced effects in multiple target tissues simultaneously, but also to determine subtle effects on multiple endpoints simultaneously within these tissues.

Whole-animal in situ hybridization (ISH) is a promising method for determining spatial changes in gene expression (Tompsett et al., in press; Zhang et al., 2008, in press). This methodology allows determination of effects on expression of multiple genes in multiple tissues simultaneously, and it can be used simultaneously with standard histology (Peterson and McCrone, 1993; Lichter, 1997; Hrabovszky et al., 2004; Jeffini et al., 2005; Jiiri et al., 2006). One of the major advantages of ISH is that it allows detection of changes in expression of mRNA for specific genes in organs, tissues, and/or cells of interest in a manner that is consistent with other methods that are used to detect lesions, including histopathology and immunohistochemistry (IHC) (Streit and Stern, 2001). The principle underlying ISH is the hybridization of specifically labeled probes to the complementary mRNA sequences in tissues or cells. A number of different visualization techniques can be applied to detect an ISH signal including radionucleotides, enzyme linked systems (e.g. biotin, digoxigenin), and fluorophores. Each label type has strengths and weaknesses depending on application. Radiolabeled probes have been widely used to detect specific mRNA sequence in tissues or embryos since the detection of mRNA in invertebrates and vertebrates was originally developed using the radiolabeled probe systems (Simeone, 1999). ISH utilizing radiolabeled probes have been found to be more sensitive and reliable than some other methods such as enzyme linked or fluorophore-based systems. However, radioisotope-based techniques have a number of disadvantages such as a relatively poor resolution, relatively long exposure times for autoradiographic visualization, and they are expensive and require special certifications in many institutions and extra precautions in the laboratory (Braissant and Wahli, 1998; Simeone, 1999; Pernthaler and Amann, 2004; Tompsett et al., in press). In contrast, enzymatic detection systems such as digoxigenin are very sensitive but tend to be variable. More recently, the application of fluorescent labeling techniques has considerably improved ISH due to the advantage of using different fluorescent tags to simultaneously detect different gene sequences (Wilkinson, 1999). However, application of fluorescence in situ hybridization (FISH) methods to detect specific mRNA in tissue sections has not been explored to the same extent as radiolabeled or enzyme-based methods due to issues with sensitivity and/or autofluorescence of tissues (Dirks, 1990; Wilkinson, 1999; Andreeff and Pinkel, 1999). Recent improvements in fluorescence labeling techniques render FISH techniques an increasingly useful tool. To effectively utilize FISH, however, a number of technical limitations needed to be overcome. Key issues include probe penetration of sections, auto-fluorescence of tissues, non-specific binding of probe, type of target tissues and species, and sample preparation (Wilkinson, 1999). Hence, there was a need for the development and optimization of FISH methods to overcome these issues.

The main objective of this study was to develop and optimize an ISH protocol that uses fluorophore-labeled probes to detect specific mRNA sequences in whole animal sections of Japanese medaka (Oryzias latipes). Specific goals of this study were: (1) develop and optimize methods to design fluorescent riboprobes for use in ISH; (2) develop and optimize methods to reduce auto- and background fluorescence in fish tissue sections by using a combination of chemical treatment and advanced confocal microscopy techniques; (3) validate the FISH methods developed in this study using Q RT-PCR; and (4) use the optimized FISH methods to examine changes in gonadal CYP19a gene expression in Japanese medaka exposed to a competitive pharmaceutical inhibitor of the aromatase enzyme, fadrozole. The physiology, embryology, and genetics of the Japanese medaka have been extensively studied in the past, and more recently, this species has been used as a model in endocrine disrupter research (Wittbrodt et al., 2002). The Japanese medaka has clearly defined sex chromosomes and sex determination (summarized in Wittbrodt et al., 2002). Cytochrome P450 aromatase, encoded by the CYP19 gene, is the key enzyme in estrogen biosynthesis from androgens (Simpson et al., 1994), and it has been extensively used as an endpoint to assess the exposure of endocrine disrupting compounds (EDCs) due to its relation with reproductive processes (Sanderson et al., 2000; Hayes et al., 2002; Rotchell and Ostrander, 2003; Hecker et al., 2006). Fadrozole has been reported to affect CYP19a gene expression (Villeneuve et al., 2006) but was also shown to result in other physiological effects in fish including altered plasma estradiol concentrations, gonadal pathologies, and fecundity (Alfonso et al., 1999; Ankley et al., 2002; Fenske and Segner, 2004).

Materials and methods

Test chemical

The fadrozole (CG5016949A; MW: 259.74 g) used in this research was provided by Novartis Pharma AG (Basel, CH).

Culture of Japanese medaka

Wild type Japanese medaka were obtained from the aquatic culture facility at the US EPA Mid-Continent Ecology Division (Duluth, MN, USA). Medaka were held in flow-through systems under conditions facilitating breeding (23–24 °C, 16:8 h light/dark). All procedures used during all phases of this study were in accordance with protocols approved by the Michigan State University Institutional Animal Care and Use Committee (IACUC).

Fadrozole exposure

Prior to initiation of exposure experiments, 12–14 wk old medaka were placed into 10 L tanks with 6 L of carbon filtered tap water and acclimated for 12 d under the same conditions as in the subsequent exposures. One fish died during acclimation. Each treatment group consisted of two replicate tanks, and each tank contained 5 male and 5 female fish. After the acclimation period, fish were exposed to 1, 10, or 100 μg fadrozole/L or carbon-filtered tap water as a control in a 7 d static renewal exposure. Every day one half of the water in each tank (3 L) was replaced with fresh carbon filtered water dosed with the appropriate amount of an aqueous fadrozole stock (5 mg/L). Fish were fed Aquatox flake food (Aquatic Ecosystems, Apopka, FL, USA) ad libitum once daily and held at 24 °C with a 16:8 h light/dark cycle. Water quality parameters were measured daily and values were within a normal range for water quality, as follows in all tanks: temperature (24 °C), pH (7.89–8.13), ammonia nitrogen (0.02–0.04 mg/L), nitrate nitrogen (0.02–0.3 mg/L), dissolved oxygen (4.3–6.9 mg/L), and hardness (370–480 mg CaCO3/L).

After 7 d of exposure medaka were euthanized in Tricaine S (50 mg/mL) (Western Chemical, Ferndale, WA, USA). Weight and snout length were recorded. Fish were separated into two groups, one group was for ISH and consisted of 2 fish per sex per tank, and a second group that was to be used for Q RT-PCR procedures and included three fish per sex and treatment group. Fish from the ISH group were fixed for ISH and histological investigations as described below. For the Q RT-PCR group, the brain, liver, and gonads were dissected from the fish and weighted individually. The liver somatic
(Table 1). The probes were designed to be approximately 500 bp long for conventional PCR with appropriate primers to amplify PCR products. In this study, RNA probes were synthesized by drying at 40 °C overnight. Slides were stored in RNase-free Superfrost Plus slides (Erie Scientific, Portsmouth, NH, USA) that had been cleaned and allowed to harden overnight at room temperature, and paraffin sections were cleared with melted Paraplast Plus paraffin (American Optical, Buffalo, NY, USA), 2% paraformaldehyde, and 0.05% lithium chloride precipitation (Ambion, Foster City, CA, USA). Fish were then gross dissected to remove fins, tail, skull roof, otoliths, and opercula. The body cavity was opened to improve the penetration of fluorescence-labeled riboprobe.

### ISH procedure

**Preparation of sections.** For ISH, fish were processed using methods adapted from Kong et al. (2008). Briefly, fish were gross dissected to remove fins, tail, skull roof, otoliths, and opercula. The body cavity was opened to improve the penetration of fixed (80% Histochoice MB (EMS, Hatfield, PA, USA), 2% paraformaldehyde, and 0.05% glutaraldehyde) for better internal organ fixation. Fish were then immersed in individual vials containing the fixative, and allowed to fix over night at room temperature. After approximately 22 h, fish samples were removed from the fixative, and were washed with 70% methanol and dehydrated through a graded methanol series (80%, 95%, and 100%), and then cleared in chloroform at 4 °C. Fixed and cleared samples were infiltrated with melted Paraplast Plus paraffin (McCormick Scientific, St. Louis, MO, USA) at 60 °C. The paraffin was allowed to harden overnight at room temperature, and paraffin blocks were stored under RNase-free conditions at 4 °C until sectioning.

Fish sections were sectioned on a rotary AO-820 microtome (American Optical, Buffalo, NY, USA) that had been cleaned and decontaminated with absolute ethanol and RNase-Zap (Sigma-Aldrich, St. Louis, MO, USA). Serial sections were cut at 7 μm. The sections were floated out onto a 40 °C water bath, and placed on Superfrost Plus slides (Erie Scientific, Portsmouth, NH, USA), followed by drying at 40 °C overnight. Slides were stored in RNase-free containers at room temperature until used for ISH.

**Fluorescence-labeled riboprobe synthesis.** In this study, RNA probes (riboprobes) were used for the detection of target mRNA. All mRNA/cDNA sequences used to design RNA probes were obtained from the NCBI database (www.ncbi.nlm.nih.gov). To synthesize riboprobes, reverse-transcribed first-strand cDNA was used as a template in a conventional PCR with appropriate primers to amplify PCR products (Table 1). The probes were designed to be approximately 500 bp long using Beacon Designer 2 software (PREMIER Biosoft Int., Palo Alto, CA, USA). Probe length was chosen based on a review of Wilkinson (1999) that reported that either too short or too long probes may give weaker signals possibly due to either low specificity to the target transcript or low penetration efficiency into tissue, respectively. The sequence of the riboprobe to detect CYP19a mRNA was compared with all sequences of known genes in Japanese medaka using Blast2 analysis (NCBI, Bethesda, MD, USA), and no sequence homogeneity was found except for the target gene. The PCR products were cloned into a pGEM T-Easy vector (Promega, Madison, WI, USA) following the manufacturer’s direction so that it was flanked by two different RNA polymerase initiation sites (T7 and SP6). The sequences of cloned amplicons were confirmed at the Research Technology Support Facility (RTSF) at Michigan State University using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and followed by a BLAST2 analysis with their corresponding sequences in GenBank. In order to synthesize sense and antisense probes for CYP19a, cloned plasmids were digested with Sall and Ncol (Invitrogen, Carlsbad, CA, USA), respectively. Complete digestion was confirmed with electrophoresis on agarose gel (data not shown).

Sense and antisense riboprobes for CYP19a mRNA were synthesized using in vitro transcription. Briefly, the sense riboprobes were transcribed with T7 polymerase with their respective plasmids, while the antisense probes were transcribed with SP6 polymerase using the manufacturer’s direction (Roche, Indianapolis, IN, USA). Sizes of synthesized riboprobes were confirmed by MOPS–formaldehyde gel electrophoresis (data not shown), followed by purification using lithium chloride precipitation (Ambion, Foster City, CA, USA).

Synthesized riboprobes were labeled with ULYSIS Nucleic Acid Labeling Kits (Alexa Fluor 488, Molecular Probes, Eugene, OR, USA). After labeling, the riboprobes were purified with a gel filtration-based spin column, Micro Bio-Spin 30 Columns in RNase-free Tris (Bio-Rad, Hercules, CA, USA) to remove excess, unincorporated fluorescent dyes. The quality of fluorescence-labeled riboprobes was confirmed by MOPS–formaldehyde gel electrophoresis without ethidium bromide (data not shown). The quantity of the riboprobes was measured using a spectrophotometer (260 nm). Riboprobes were separated into aliquots and stored at −80 °C. Probes were used within a few days after synthesis to minimize RNA degradation.

**Fluorescent in situ hybridization.** Slides on which whole histological sections of medaka had been replaced were incubated at 60 °C for 1 h to allow paraffin to melt and to fuse the sections to the slide (Fig. 1). The slides were then de-paraffinized and rehydrated as follows: washing with (a) xylene 3 times for 3 min, (b) 100% ethanol 2 times for 3 min, (c) PBS for 5 min, and (d) diethyl pyrocarbonate (DEPC)-treated water for 1 min. In order to reduce autofluorescence signal

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**Table 1**

<table>
<thead>
<tr>
<th>Probes</th>
<th>Primers (sense/antisense, 5′→3′)</th>
<th>Accession number</th>
<th>Amplicon size</th>
<th>Cycling condition</th>
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</thead>
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<td>CYP19a</td>
<td>CCTGTAATGCGCTGAGTCA/ GAAGAGCCTGCTTGAATCT</td>
<td>D82968</td>
<td>496</td>
<td>Denaturation 94/45, Annealing 55/30, Extension 72/90</td>
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**Fig. 1.** Brief steps of mRNA in situ hybridization with fluorescence-labeled riboprobe.
originating either from the tissues or from the fixative, slides were then treated 3 times for 20 min with 10 mg/mL sodium borohydride (SB) (Sigma-Aldrich, Saint-Louis, MO, USA) in PBS (Billinton and Knight 2001). Slides were then rinsed with PBS and DEPC-treated water and the sections were permeabilized for 20 min with 0.2 N HCl. To increase specificity of probes to target mRNA, slides were treated with 0.1 U/mL of RNase-free DNase I (Roche, Indianapolis, IN, USA), followed by inactivation of DNase I with DNase stop solution (10 mM Tris–HCl, 150 mM NaCl and 20 mM EDTA). Sections were acetylated in triethanolamine–HCl buffer plus 0.25% acetic anhydride (2 times) (Sigma-Aldrich, Saint-Louis, MO, USA), and then pre-hybridized with hybridization buffer (2× SSC, 50% deionized formamide, 1× Denhardt’s solution, 0.4 μg/mL of sonicated salmon sperm DNA, 1% SDS, 20% dextran sulfate, and DEPC-treated water to make 31.25 mL) without probes for 1 h in a humid box at 43 °C to reduce non-specific binding of probes. Probes were denatured at 90 °C for 10 min and chilled on ice. Sections were hybridized with the riboprobe (2 ng/μL) at 43 °C for 16 h. The negative control consisted of sections that were hybridized with equal amounts of sense probe under the same ISH conditions as described above. Following hybridization, the slides were washed in a SSC gradient (4× at room temperature (5 min), 2× at 37 °C (10 min), 2× at RT (5 min), and 0.2× at RT (5 min)). Following washing, slides were air dried, mounted with fluoromount G (EMS, Hatfield, PA, USA), and left in a dark chamber until the mounting medium was completely dry. Thereafter, slides were kept in the slide box to prevent photo-bleaching and photo-activation of fluorescent molecules until analysis by confocal microscopy. The optimum concentration of SB to quench auto- and background fluorescence signals was determined during preliminary studies with SB at 10 or 20 mg/mL, copper (II) sulfate at 10 or 100 mM, and a combination of SB and copper (II) sulfate.

Confocal laser scanning microscopy (CLSM) image analysis. Spectral distributions of fluorescence were determined with a Zeiss LSM 510 Meta confocal system (Carl Zeiss, Jena, Germany). Images were collected with a Zeiss EC Plan NEOFLUAR 10X (Carl Zeiss, Jena, Germany). Using the 458-nm line of an argon laser for excitation, 16

<table>
<thead>
<tr>
<th>Emission wavelength (nm)</th>
<th>Intensity</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
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<td>513</td>
<td>1356.1</td>
<td>869.3</td>
<td>733.2</td>
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<tr>
<td>524</td>
<td>1035.6</td>
<td>979.6</td>
<td>769.9</td>
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<td>534</td>
<td>1131.0</td>
<td>1087.1</td>
<td>818.7</td>
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</tr>
</tbody>
</table>

Fig. 2. Autofluorescence images of juvenile medaka ovary and emission spectra of the sections obtained after excitation with a 488 laser (A–C) of CLSM and autofluorescence image of section after applying linear spectral unmixing (D) of CLSM. (A) Section not subjected to ISH procedure; (B) Section in situ hybridized without probe and without SB treatment; (C) Section in situ hybridized without probe and with SB treatment; (D) Section in situ hybridized without probe and SB treatment after application of linear unmixing; (E) Autofluorescence intensities of the sections. Scale bar=100 μm.
s spectrally resolved images were recorded at 10.7 nm intervals from 475.5 nm–646.7 nm. Sections of ovary were imaged with a pinhole aperture corresponding to an optical thickness of <2.0 μm.

Several endogenous and/or fixative-induced fluorophores have broad band emission spectra that make separation from emission spectra related to riboprobe fluorophores particularly difficult. In this study, our effort focused on the separation of the individual spectral components associated with autofluorescence, background, and Alexa Fluor 488 dye. Autofluorescence and background spectral components were obtained from ovary sections (early stage oocytes) and other tissues, respectively, on slides hybridized without probe to account for these variables in the overall fluorescence associated with these sections. The specific spectrum of Alexa Fluor 488 dye was obtained directly from the dye reagent. Once the number of significant and independent sources of specific spectral components was defined by means of CLSM, linear spectral unmixing was used to separate the individual components, to remove autofluorescence signal in the recorded images (Fig. 2) and to isolate the specific fluorescence signal of the Alexa Fluor 488 dye. With each set of ISH experiments, a section with no probe added was included to control for alterations in the autofluorescence spectral shape by photo-bleaching that can result from consecutive laser scans.

Each set of ISH slides had at least one ISH section with sense probe that served as a negative control. Images of the ovary were collected at 10× magnification. For quantification, we randomly selected different portions of the ovary in each section hybridized with CYP19a antisense probe and then randomly selected 3 early stage oocytes (less than 100 μm in diameter) in each portion. Based on morphological criteria developed by Iwamatsu et al. (1988), those oocytes are classified into the previtellogenic stage. Then we applied the above described spectral unmixing method to quantify the intensities of the true Alexa Fluor signal in these oocytes. Due to the detection of low quantities of Alexa Fluor dye in the section hybridized with sense probe, we normalized the CYP19a antisense signal to the CYP19a sense signal in each set of ISH.

Q RT-PCR procedure

Total RNA isolation, cDNA synthesis, and Q RT-PCR were conducted as described by Park et al. (2006). Briefly, extracted total RNA from tissue samples was treated with DNase I, and then reverse transcribed to synthesize cDNA which was subsequently used as the template for the PCR reaction. To confirm complete removal of possible genomic contamination, a negative control (sample without reverse transcriptase) was run in parallel with each PCR experiment. That is, extracted total RNA was also treated with DNase I and no reverse transcriptase was applied. This sample was run in parallel with each PCR experiment, and which resulted in no amplification of the PCR product (data not shown). To improve sensitivity of PCR amplification, the cDNA:RNA hybrid molecules were removed by digestion with E. coli RNase H after first-strand cDNA synthesis. The expression level of CYP19 mRNA was normalized to an internal control gene, β-actin.

To determine the accumulation of the PCR product, SYBR Green I dye was used as a real-time reporter of the presence of double-stranded DNA. All cDNA sequences were obtained from the public GenBank database of NCBI. The primers were obtained using Beacon Designer 2 (Premier Biosoft International, Palo Alto, CA, USA). Primer sequences and conditions used for Q RT-PCR analysis are given (Table 2). Primer specificity was verified by a single distinct peak obtained during the melting curve analysis of the SYBR®-Green (bioMérieux, Marcy l’Etoile, France)-based real-time PCR system and by DNA sequencing of the PCR amplicons separated by gel electrophoresis (data not shown).

Histology

Histological changes in medaka ovaries were evaluated using H & E stained sections (Tompsett et al., in press). Briefly, slides were de-paraffinized in xylene and rehydrated through a descending ethanol series (100, 95, and 70%). Slides were then stained in Harris’ hematoxylin (EMS, Hatfield, PA, USA) for 3 min, processed through acid alcohol, ammonia, and ethanol washes, and then stained in 1% Eosin Y (EMS, Hatfield, PA, USA) in 80% ethanol for 1 min. Slides were then dehydrated through an ethanol series (70, 95, and 100%) and cleared in xylene. Slides were preserved under glass cover slips using Entellan mounting medium (EMS, Hatfield, PA, USA) and allowed to dry. Images of the gonad on each slide were recorded using a Camedia C-3040 ZOOM digital camera (Olympus, Center Valley, PA, USA) attached to an Olympus BX41 microscope (Optical Analysis Corporation, Nashua, NH, USA).

Statistics

Statistical analyses in this study were conducted using SAS (SAS Institute Inc. Cary, NC, USA). Prior to analysis, data sets were tested for normality using the Shapiro Wilk’s test. One-way ANOVA test was applied to test for differences of CYP19a gene expression across all treatment groups in both Q RT-PCR and ISH, followed by the Student–Newman–Keul’s test for multiple comparisons. The 2-tailed Spearman rank correlation analysis was used to evaluate the relationship between CYP19a gene expression levels by means of Q RT-PCR analysis and ISH analysis. The criterion for significance in all statistical tests was p < 0.05.

Results

Reduction of autofluorescence

Initial experiments revealed strong autofluorescence in non-treated sections (Figs. 2A, B). Of the treatments tested, only sodium borohydride (SB) and SB+copper (II) sulfate significantly decreased autofluorescence, but there were no significant differences in the signal intensity between the two treatments (data not shown).

<table>
<thead>
<tr>
<th>Targeted genes</th>
<th>Primers (sense/antisense, 5′-3′)</th>
<th>Accession number</th>
<th>Amplicon size</th>
<th>Cycling condition</th>
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<tr>
<td>CYP19a</td>
<td>GCCCGGTATGCTCCATTTGAG/ GAGTTCCGTGTTGCCCAGAG</td>
<td>DB2968</td>
<td>108</td>
<td>Denaturation 95/15, Annealing 59/50, Extension 72/30</td>
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<tr>
<td>Beta actin</td>
<td>GAGTTCCGTGTTGCCCAGAG/ TGATGCTGTTGTAGGTGGTCTC</td>
<td>S74868</td>
<td>89</td>
<td>Denaturation 95/15, Annealing 61/50, Extension 72/30</td>
</tr>
</tbody>
</table>
Therefore, SB was used in all further experiments. However, complete removal of autofluorescence to levels observed in the negative controls was not possible (Figs. 2C and E). When SB-treated sections were subjected to linear spectral unmixing of CLSM the background fluorescence signal previously observed in medaka ovaries was reduced or eliminated (Fig. 2D) relative to traditional excitation/emission filter system laser microscopy (Fig. 2C). Thus, analyses it was decided to treat all samples with 10 mg/mL SB to reduce and/or minimize the signal of autofluorescence, and then to subject each image to the above described spectral unmixing method.

**Tissue and cell specificity of CYP19a gene expression**

Longitudinally sectioned whole medaka hybridized with CYP19a antisense probe exhibited a fluorescence signal that was specific to ovary (Fig. 3A). The specificity of hybridization was demonstrated with negative controls using sense probe, which gave a very weak fluorescence signal (Fig. 3B). Sections hybridized in the absence of probe showed no signal (Fig. 3C). The greatest fluorescence was found in thecal cells, granulosa cells, and premature early stage previtellogenic oocytes. CYP19a expression in vitellogenic oocytes was much less and matured oocytes cells exhibited little fluorescence (Fig. 3). If present, CYP19a expression was found only in the outer layers of matured oocytes (Fig. 4). Furthermore, fluorescence specific for positive CYP19a staining was observed only in ovary and no CYP19a mRNA was detected by ISH in other tissues such as the brain or liver (Fig. 5). When measured by RT-PCR, CYP19a gene expression was greatest in ovary, while both brain and liver tissues expressed little CYP19a mRNA (data not shown).

**Fadrozole exposure**

**Gene expression**

Expression of CYP19a in ovaries of medaka exposed to fadrozole, as measured by both Q RT-PCR and ISH, was greatest in medaka exposed to 100 μg fadrozole/L. Q RT-PCR analyses showed that there was a significant, dose-dependent up-regulation of CYP19a gene expression in ovary of fadrozole-exposed females (Fig. 6 and supplementary Table 1). Exposure to 100 μg fadrozole/L caused a 14-fold up-regulation of CYP19a expression in ovaries, relative to that of controls. While there was also greater CYP19a expression measured by ISH, due to the relatively great variation and the semi-quantitative nature of the analyses these differences were not statistically significant. Furthermore, the 1.4 fold change observed by use of ISH was less than that demonstrated by use of Q RT-PCR (Fig. 7). While the up-regulation of CYP19a expression caused by 100 μg fadrozole/L showed the same increasing trend 1 that determined by Q RT-PCR, ISH was unable to demonstrate effects for lesser concentrations of fadrozole. Although trends in CYP19a expression were similar between quantifications of ISH and Q RT-PCR, no statistically significant correlation was observed between these two types of measurements ($r^2=0.0154, n=16, p=0.957$).

**Fadrozole exposure — mortality, morphometric and histological endpoints**

No mortality was observed in any of the exposure groups or the controls during the course of the experiment. Fadrozole caused no statistically significant changes in liver somatic (LSI) and gonadal somatic (GSI) indices of Japanese medaka (Table 1 in supplemental materials). However, as described in a parallel study (Tompsett et al., in press) exposure to 100 μg/L fadrozole caused histological effects. While female Japanese medaka from the control, 1, and 10 μg fadrozole/L treatments had oocytes in all stages of development, oocytes of females exposed to 100 μg fadrozole/L were predominantly in later vitellogenic stages, similar in size, and lacked a distinct yolk globule (stage VII and VIII of development as classified...
Fluor 488 dye was still obscured by the relatively strong auto- 

c foe, which indicates that aldehyde-induced 

escence signal when using traditional 

signi- 

Clancy and Cauller, 1998). While in our study treatment with SB 

fl- 

studies have reported that SB quenched the 

fi- 

generated after reactions of amines and protein molecules with 

borohydride (SB) is a known blocker of aldehyde groups that are 

covalently to any amino groups, resulting in aldehyde-induced 

reduce auto- 

microscopy techniques in ISH has been limited by the relatively great 

speci- 

sections that did and did not undergo ISH implies that the 

components of the ISH procedures used in this study did not 

significantly contribute to autofluorescence. The true dye signal 

could be detected after complete removal of the autofluorescence 

signal by use of linear spectral unmixing with CLSM to isolate the 

Alexa Fluor 488 dye signal from the remaining autofluorescence. The 

linear spectral unmixing technique is able to identify and separate 

specific spectral components in fluorescence images (Chorvat et al., 

2005). The application of this procedure allowed isolation of the dye-

specific signals that discerned between background and gene probe 

signal. The significantly greater fluorescence intensity in sections 

hybridized with antisense probe compared to those hybridized with 

the sense probe indicated the specificity of the developed ISH 

protocol. However, the optimization techniques applied here did not 

completely reduce the fluorescence signals in samples processed with 

sense probe. Possible reasons for this could be that probes were not 

completely removed from the tissue during post-hybridization 

washing steps, or that some of the tissue autofluorescence signal 

corresponded to the specific spectrum of Alexa Fluor 488 dye. 

Regardless of these artifacts, the FISH system developed in this 

study allowed for spatial determination of specific gene signals in 

medaka with high resolution and sensitivity, which represents a 

significant improvement compared to most of the previous FISH 

approaches.

Discussion

Optimization of FISH

Optimization of fluorescence ISH protocols, as described in this 

study, resulted in the development of a test system that allows for the 

identification of gene expression in paraffin embedded whole mount 

sections of small fish with relatively great sensitivity and spatial 

specificity when compared to other ISH approaches using DIG, Biotin 
or radiolabeled probe systems. To date, the application of fluorescence 

microscopy techniques in ISH has been limited by the relatively great 

autofluorescence of many tissue types or components (e.g. fixative) 

used in the assay (Szöllösi et al., 1995). Typically, the emission 

spectrum of autofluorescence is very broad compared to the spectra of 
exogenous sources such as a fluorescently labeled probe, complicating 
the use of fluorescent probes because autofluorescence cannot be 

avoided by simply choosing dyes with excitation and emission spectra 

out of the range of the spectra of autofluorescent molecules. Sodium 
borohydride (SB) is a known blocker of aldehyde groups that are 
generated after reactions of amines and protein molecules with 
aldehyde fixatives, and eventually these aldehyde groups combine 
covalently to any amino groups, resulting in aldehyde-induced 

fluorescence (Beischer et al., 1987). However, the capacity of SB to 
reduce autofluorescence seems to be species and/or tissue-specific. 

For example, attempts to reduce the autofluorescence in the aldehyde 
fixed-human bone marrow paraffin sections with SB failed, and even 
resulted in an increase of fluorescence (Baschong et al., 2001). Other 

studies have reported that SB quenched the fixative-induced auto-

fluorescence in brain tissue of mammals (Tagliaferro et al., 1997; 

Clancy and Cauler, 1998). While in our study treatment with SB 

significantly reduced autofluorescence, the specific signal of Alexa 

Fluor 488 dye was still obscured by the relatively strong autofluo-

rescence signal when using traditional fluorescent microscopy techni-

ques, which indicates that aldehyde-induced fluorescence was only 

one contributor to the observed autofluorescence. Furthermore, the 

small difference in the strength of the autofluorescence signal of 

sections that did and did not undergo ISH implies that the 

components of the ISH procedures used in this study did not 

significantly contribute to autofluorescence. The true dye signal 
could be detected after complete removal of the autofluorescence 
signal by use of linear spectral unmixing with CLSM to isolate the

Student Newman Keul’s test for multiple comparisons. Different letters indicate 
significant difference between treatment (p<0.05).
The Q RT-PCR analysis revealed differential expression of CYP19a mRNA in brain and ovary of juvenile female medaka (data not shown). Average Ct values for ovary and brain were 21 and 35, indicating a $2 \times 10^{14}$ difference in abundance of CYP19a transcript between these tissues. Significantly greater expression of CYP19a mRNA in ovary compared to brain is in accordance with findings of other studies with teleost fish (Callard et al., 2001; Kishida and Callard, 2001 (zebrafish); Villeneuve et al., 2006 (fathead); Liu et al., 2007 (catfish)). The results of the ISH revealed a clear signal for CYP19a mRNA only in the ovary. However, while no expression of CYP19a in the brain could be observed with ISH, expression was detectable at very low levels by use of Q RT-PCR. This result indicates that ISH on whole animal sections is relatively less sensitive than the use of Q-RT-PCR with excised tissue. This difference in sensitivity is likely due to the fact that CYP19a was amplified during the PCR process while the ISH signal is proportional to the absolute amount of mRNA present in the tissue. Furthermore, the RT-PCR method applied here utilized mRNA extracted from an entire gonad while the ISH procedure is limited to the visualization of a small section of a tissue. Now that the utility of the ISH method has been demonstrated for whole tissue mounts,
future work could increase tissue-specific sensitivity by use of advanced techniques such as in situ PCR.

While qualitative determinations of up-regulation of CYP19a expression by fadrozole could be detected both by the ISH on whole fish sections and RT-PCR with excised tissues the correlation between the magnitude of changes measured by the two methods was poor ($R^2 = 0.0154$). This result is in accordance with a parallel study that investigated the effects of fadrozole on CYP19a gene expression in the same fish using radionucleotide-based ISH (Tompsett et al., 2008).

Meanwhile, the increase in ovarian CYP19a expression after exposure to fadrozole compares well to findings of Villeneuve et al. (2006) in juvenile female fathead minnow. It has been hypothesized that this increase in gene expression, which was opposite to that of enzyme activity as has been reported by a different study (Villeneuve et al., 2006), is most likely due to a compensatory response through increased G6H from pituitary in response to decreased levels of E2 (Kim, 1998; Tompsett et al., in press). However, these findings indicate that the developed fluorescent ISH method is indicative of changes at the gene expression level that were previously reported in the same experiment using different analytical (RT-PCR) or detection (radionucleotide ISH) systems.

**Utilization of FISH**

The high resolution and integration into classical histological analysis suggests that FISH is a useful technique for use in studies aiming to elucidate mechanisms of effects of chemicals. However, while FISH allows the localization of mRNA in a number of tissues simultaneously with comparison to histological responses, FISH is less sensitive and more variable than Q RT-PCR. Thus, currently the results of FISH in whole animals sections are semi-quantitative. This is in accordance with a parallel study using radionucleotide-based ISH, which also reported relatively great variation between replicate slides and difficulties to quantify the ISH signal (Tompsett et al., in press). Utilization of ISH for localization and expression of mRNA in tissues or whole organisms are not novel, but the improvement made by reduction in autofluorescence has made it more useful. Specifically, the expression of the CYP19a gene has been studied in various teleost fish species previously (Goto-Kazeto et al., 2004; Kobayashi et al., 2004; Dong and Willett, 2007; Wang and Orban, 2007; Tompsett et al., in press). However, these studies have employed non-fluorescent visualization systems such as enzyme or radionucleotide-labeled probes. While such systems represent useful approaches to identify the spatial and tissue-specific expression of genes, they are associated with a series of issues that limit their applicability. Enzyme-based detection systems such as digoxigenin and biotin require immunological amplification steps to quantify and localize gene expression at the tissue and/or cellular level. Furthermore, while more sensitive than fluorescence-based systems, enzymatic or immunological amplification steps for probe visualization typically show a poor correlation between signal intensity and the abundance of mRNA (Day et al., 2007). One of the limitations of the use of radionucleotide detection methods is the limited resolution and sensitivity by which tissue-specific changes in gene expression can be determined (Day et al., 2007; Tompsett et al., in press). While there are alternative detection methods such as silver-grain-based techniques that are highly sensitive and provide good resolution (Hrabovzky et al., 2004), these techniques are labor intensive and very expensive.

The FISH approach applied in this study allowed specific, high resolution detection of CYP19a in whole mount female medaka sections. CYP19a was specific to the ooplasm of early stage oocytes that were less than 100 μm in diameter and possessed high expression of CYP19a, and the follicle cell layer of previtellogenic and vitellogenic stage of oocytes, albeit less expressed, which is consistent with the results of other studies with teleost fishes, such as killifish (Dong and Willett, 2007), zebrafish (Goto-Kazeto et al., 2004; Wang and Orban, 2007), and Atlantic croaker (Nunez and Applebaum, 2006). Overall, CYP19a mRNA gene expression gradually lessened with maturation of oocytes: early stage of oocytes > pre-vitellogenic oocytes > mature oocytes. Expression of CYP19a has been reported to be primarily localized in the ooplasm of primary growth staged oocytes of developing killifish (Fundulus heteroclitus) and gradually declined from stage I oocytes (previtellogenic follicle) to non-detectable levels at stage IV oocytes (mature follicles) (Dong and Willett, 2007). Expression of CYP19a mRNA was also observed in the follicular layer of developing oocytes in the medaka (Suzuki et al., 2004). In Atlantic croaker, CYP19a mRNA transcripts were most expressed in previtellogenic ovary (Nunez and Applebaum, 2006). In zebrafish, a greater abundance of CYP19a mRNA was observed in the mid-vitellogenic oocytes than in the primary stage of oocytes, which were embedded in connective tissue (Goto-Kazeto et al., 2004). This may be indicative of differences in synthesis of E2 and associated vitellogenesis among species. Overall, these findings indicate the validity of the FISH system used in our study as it reproduced the findings of other studies with teleost fish species. The utilization of the FISH could be further confirmed by the tissue specificity of CYP19a hybridization that was limited to the ovary.

**Comparison of FISH data to morphometric and histological results**

No significant effects on morphometric parameters, such as organ or body sizes, were caused by exposure to fadrozole, which is likely due to the relatively short duration of exposure. The histological analysis indicated that an effect on development of the ovary in response to fadrozole exposure occurred. This supports the hypothesis that aromatase is important in sexual development and gonadal maturation in medaka. This conclusion is supported by the results of studies that have shown that inhibition of aromatase by specific inhibitors caused a decrease in plasma E2 and vitellogenin levels, and ultimately inhibited oocyte growth in teleost fishes (Ankley et al., 2002; Suzuki et al., 2004; Sun et al., 2007).

One of the major advantages of FISH is that it allows the localization of a specific gene at the tissue and/or cellular level. This tissue or cell-specific response provides a clue for understanding the relationships between molecular and histological processes. While both molecular approaches utilized in this study revealed an increase in CYP19a gene expression at the greatest dose of fadrozole, the magnitude of the effect was very different, and based on this results the ISH data would not have allowed predicting the great increase measured by RT-PCR. By combining the ISH with standard histology, however, it was possible to demonstrate that there was a change in the number of earlier stage oocytes, the types of cells that are characterized by increased CYP19a expression. Given the relatively small change in CYP19a gene expression measured by ISH when compared to RT-PCR, therefore, it can be concluded that the change in tissue composition – increase in the number cells that have increased CYP19a expression – is likely to be the main factor resulting in the increase in gonadal aromatase expression in response to fadrozole exposure.

In summary, the optimized FISH method developed in this study allowed to detect CYP19a mRNA expression in the ovary of medaka. The method not only can provide useful information relative to temporal and spatial changes in gene expression, but also can aid in explaining molecular changes at the level of histological observation with the ultimate goal of being able to link histological changes to potential pathologies. Furthermore, FISH has the potential to be further optimized using multiple riboprobes with different emission spectrums simultaneously with the same section.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.taap.2008.06.012.

### References


