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Methodological approaches to cytochrome P450 profiling in embryos

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Abstract

Cytochrome P450 enzymes (CYPs) are heme thiolate proteins essential for vertebrate development, and also play important roles in toxicology as well as normal metabolic function. CYP enzymes catalyze the oxidative biotransformation of many endogenous and exogenous chemicals, including steroids and eicosanoids, and drugs and other xenobiotic toxicants. Many CYPs have known or potential roles in development, and many chemicals that cause developmental abnormalities are substrates for CYPs. The roles and regulation of most xenobiotic metabolizing CYPs during development are unknown, impeding understanding of mechanisms of developmental toxicity. The zebrafish (*Danio rerio*) has become one of the premier models in vertebrate developmental biology, in large part because of the logistical advantages of rapid external development, high adult maintenance density, and significant (bio)technological tool availability. The increasing use of zebrafish in drug discovery and mechanistic toxicology demands knowledge of CYP gene regulation and function. Here we present methods to examine CYP expression during early development in zebrafish, with an emphasis on developmental microarrays.

Keywords

Cytochrome P450; CYP; zebrafish; development; microarray; quantitative RT-PCR; qPCR

1. Introduction

The cytochrome P450 (CYP) superfamily includes enzymes that catalyze oxidative transformation of many endogenous and exogenous chemicals, with vital roles in normal physiology and disease processes, including development. The absolute embryo lethality upon knockout of the murine CYP oxidoreductase (Por) demonstrates the essential role of CYP enzymes in murine development (1). A number of CYPs have known roles in developmental specification. For example, CYP26 enzymes regulate levels of retinoids governing pattern formation during development (2–4). In addition, CYP isoforms that play essential roles in bile acid homeostasis, steroidogenesis, the vitamin D pathway, and the catabolism of many hormones may also have important roles in development (5–12).

Animal CYPs can be separated into two major classes. The first group includes those CYPs involved principally in synthesis or activation of endogenous regulatory molecules, including steroids and other lipoidal regulatory molecules. The second group includes those defense genes (13) involved in the metabolism of xenobiotics; many of these also function in the metabolism of endogenous compounds. Thus, CYP enzymes can determine the persistence and action of many drugs and other toxicants and teratogens, as well as endogenous regulatory molecules.

Many chemicals that cause developmental abnormalities are substrates for CYPs, and the oxidative biotransformation of such xenobiotics may determine the cellular and organ targets of those chemicals (12). CYP enzymes involved in xenobiotic metabolism could also function in producing morphogenic molecules or keeping regions free of them, creating temporal and spatial regions of morphogen action and supporting region-specific changes essential for successful development (12, 14, 15). The roles and regulation of most CYPs during development are unknown, impeding understanding of mechanisms of developmental toxicity. Few expression profiles for the full genomic complement of CYPs in any organism have been published (16). Despite the importance of understanding CYP expression during development, even fewer developmental expression profiles have been examined, including mouse (17), human (11), and zebrafish (18), only one of which (in zebrafish) is comprehensive.

The zebrafish (*Danio rerio*) has emerged as one of the most important vertebrate model species in embryology and developmental biology (19, 20), due largely to rapid development coupled with the possibility for genetic analysis and manipulation. The value of zebrafish as a model organism have been oft-repeated, including features that can be classified as phylogenetic, logistical, and technical in nature (21). Logistically, zebrafish exhibit external development of nearly transparent embryos, allowing direct observation of all stages of embryonic development. The rapid embryonic development (~60 hr from fertilization to hatching), and short generation time (4 mo) of zebrafish make it an ideal laboratory subject for developmental studies.

Several different methods have been described for developmental profiling of CYP expression, the most robust and technically straightforward of which is commercial oligonucleotide (60 base) microarray analysis. Microarrays, and in particular oligonucleotide or 'designed' cDNA microarrays provide the best compromise between broad coverage of an organism's CYP complement, analytical throughput, and cost. This chapter addresses the use of custom-commercial short oligonucleotide arrays in developmental profiling.

An alternative to commercial short oligonucleotide arrays for CYP profiling is medium-length (150bp) cDNA spotted arrays. These arrays were used with some success to investigate comprehensive CYP expression patterns in *Arabidopsis thaliana*, but have not been applied to animals (22, 23). cDNA oligonucleotides of this length exhibit the best theoretical (and experimental) compromise between individual probe specificity and microarray hybridization kinetics (24), properties that ideally are important for discriminating between closely related members of gene superfamilies. However, the increased labor costs and technical variability introduced by this method are a significant drawback.

Finally, quantitative PCR (qPCR), while highly specific and quantitative, is relatively expensive and labor intensive, although laboratory automation can reduce the amount of labor involved. However, new microfluidic technologies (e.g. the Fluidigm Biomark™ HD System (25) or Applied Biosystems OpenArray® System (26)) may provide cost effective parallelization of qPCR. These methods will be briefly noted where relevant.

2. Materials

2.1 Embryo rearing and sampling

1. Danieau's solution (10X) at 28.5 C (27). (see Note ¹)

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¹Unless stated otherwise, all solutions should be prepared in water that has a resistivity of 18.2 MΩ-cm and total organic content of less than five parts per billion. This standard is referred to as "water" in this text.

2. Dissecting microscope
3. Petri dishes, plastic or glass, depending on the use of chemical treatment
4. Egg and embryo handling pipettes (plastic or glass). We fashion our own egg samplers from Pasteur pipettes.
5. Liquid nitrogen. *Caution:* liquid nitrogen can cause severe frost-burns. Use appropriate protective equipment.
6. Cryogenic sample vials

2.2. RNA sampling

- 1 Aurum Fatty and Fibrous Tissue kit (Bio-Rad, Hercules CA) or equivalent (*see Notes* ^{2, 3})
- 2 Phase-Lok Gel (5 PRIME GmbH, Hamburg, Germany) (*see Note* ⁴)
- 2 Nano-Drop ND-1000 spectrometer (NanoDrop Technologies, Wilmington, DE) or equivalent
- 3 Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA) (*see Note* ²)

2.3 Microarray hybridization

1. Custom-commercial medium-oligonucleotide (60 base pair) microarray; store at room temperature, protected from light. Shelf-life is generally 12 months unhybridized (*see Note* ⁵).
2. Agilent Low-Input QuickAmp Labeling Kit (*see Note* ⁶)
3. Agilent In situ Hybridization Kit Plus
4. Agilent 10× Control Targets
5. Agilent In situ Hybridization Kit Plus
6. Agilent DNA Microarray Scanner or equivalent (*see Note* ⁶)

2.4 qPCR validation (or for examining fewer target genes)

1. Carefully designed qPCR probes (MWG-Operon, Huntsville, AL) (*see Note* ⁷)
2. Omniscript™ Reverse Transcriptase kit (Qiagen Inc., Valencia, CA) or equivalent
3. RNasin® RNase inhibitor (Promega, Madison, WI) or equivalent
4. Random hexamer primers or mixed oligo-dT and random hexamer (MWG-Operon) (*see Note* ⁸) for cDNA synthesis

²Total RNA quality must be excellent. Contamination with DNA may result in artifactual expression patterns, especially in early embryos where the relative amount of DNA may be large. Quality analysis using a gel-based or microfluidics-based technique (e.g. the Agilent microfluidic Bioanalyzer) is strongly suggested.

³We have employed the Aurum Total RNA Kit (Bio-Rad) and the RNeasy Kit (Qiagen Inc, Valencia, CA) with equal success. Other kits are likely to provide equivalent RNA quality.

⁴Use of Phase Lock Gel (5 PRIME GmbH, Hamburg, Germany) or a similar technique to more fully separate the organic and aqueous phases of the phenol-chloroform extraction may slightly lower the final total RNA yield, but importantly reduces the potential chloroform contamination of the RNA-binding column.

⁵Custom commercial here refers to the addition of custom probes to commercial datasets, and the printing of microarray slides 'to order' by a commercial company (e.g. Agilent Life Sciences, Roche NimbleGen, etc.)

⁶Manufacturer's protocols are followed, and specifically in the case of Agilent microarray protocols all materials and methods are from Agilent unless explicitly noted otherwise.

⁷Primer design for extremely similar CYPs may be challenging, and often requires the knowledge of complete sets of 5'- and 3'-UTR sequences to obtain regions differing enough to distinguish tandemly duplicated genes.

5. iQ SYBR Green Supermix (Bio-Rad) or equivalent
6. (iCycler Bio-Rad)

3. Methods

Cytochromes P450 are expressed at a wide range of levels in different tissues and at different times during development. In addition, there is a high degree of identity, even at the nucleotide level, between tandemly duplicated CYPs, which may be differentially expressed during development or experimental treatment. CYP gene coding regions exhibit from 30–99% nucleotide identity within a genome. Differentiating between most isoforms is relatively easy. However, mouse, human (28), zebrafish (18), and other species have tandemly duplicated CYPs that, due to their relatively recent origin, exhibit a high degree of identity. Another process that can significantly increase the sequence identity between two paralogous genes is gene conversion, which is an active process for some CYPs (e.g. (29)).

In the extreme case, it may not be possible to design either oligonucleotide probes (60-mer) or qPCR primers that are distinct enough to differentiate between duplicated CYPs, assuming that the relevant genomic assemblies and gene predictions are correct. This fundamental problem might be addressable by modern “next generation” high-throughput sequencing technologies such as Applied Biosystem’s SOLiD, Illumina’s Tru-Seq, or Helicos Direct RNA whole-exome sequencing. Thus, microarray probe and qPCR primer design becomes an important part of the methodological approach.

We have used various developmental stages as key sampling points, in some cases bracketing important developmental processes such as organ development, including blastula-1000 cell stage (3 hpf), segmentation-5–9 somites (12 hpf), pharyngula-prim5 (24 hpf), pharyngula-high-pec (42 hpf), hatching-long-pec (48–60 hpf) and larval-protruding-mouth (72 hpf). Cytochrome P450 expression appears to occur in waves during development, with different CYPs expressed at different times (FIGURE). This is likely the result of the developmental requirement for biosynthesis and catabolism of various endogenous signaling molecules, such as retinoids, eicosanoids, and steroids.

3.1 Microarray probe or qPCR primer design

1. Predicted or confirmed gene sequences for all CYP isoforms are collected. Ideally both 3’ and 5’ untranslated regions (UTRs) are included in the known or putative cDNA sequences.
2. The Agilent eArray microarray custom-commercial probe design software is used to design multiple 60-mer microarray probes per gene. This online tool ensures that the probes are unique to the submitted sequences and that the hybridization kinetics will be appropriate.
3. Alternatively, we have written custom computer scripts (available upon request) to design unique primers for highly similar gene families, such as the CYPs. These scripts are based on the well known software primer3 (30), and can be tuned for the design of qPCR primers or ‘designed cDNA’ microarray probes.
4. Custom-commercial microarrays are printed by Agilent, or ‘designed cDNA’ microarrays are produced by a microarrayer, following manufacturer’s guidelines.

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⁸We have observed that a 50:50 mix of random hexamers and anchored oligo-dT primers provides a better, unbiased primer set for cDNA synthesis.

3.2 Embryo sampling

1. Embryos are most readily obtained from group breedings of 30 female and 15 male fish (*see Note*⁹). Fertilized zebrafish eggs are collected from the breeding traps periodically after the traps are set. Zebrafish embryonic cell division is initially rapid (~ 20 minutes per division) and synchronized until approximately the 1000-cell stage (10 cell divisions, or approximately 3 hrs post fertilization (hpf) (27)). Thus collection times can be staggered by 20 minutes to obtain large numbers of staged embryos.
2. Embryos are rinsed from the breeding traps using pre-warmed (28.5 °C) 0.3X Danieau's solution through medium-mesh (100 µm) filters to remove detritus, including detrital eggs. Further sorting of eggs is performed using a dissecting microscope (*see Note*¹⁰).
3. Embryos are placed in 20 cm glass Petri dishes containing 50 ml of 0.3× Danieau's solution and incubated at 28.5 °C. At 24 hpf, the solutions are replaced with fresh 0.3× Danieau's solution and any dead embryos are removed; mortality is normally less than 4% per dish. For sampling, all embryos in a dish are pooled, frozen in liquid nitrogen, and stored at –80 °C (*see Note*¹¹).
4. Embryos are sampled at appropriate developmental times by removing incubation water from the petri dishes, pipetting embryos or ovolarvae into a cryogenic vial, and freezing in liquid nitrogen. Storage in liquid nitrogen is preferred; however storage in RNALater (Qiagen Corp, Valencia, CA).

3.3 RNA extraction

Standard methods of total RNA extraction and precipitation work well for zebrafish embryos, although we have had better results using column-based kits (*see Note*²). Here we describe the protocol for the Aurum Total RNA Kit (Bio-Rad Laboratories Inc, Hercules, CA), with modifications as noted.

1. Flash-frozen or fresh embryos (10–100; *see Note*¹¹) are homogenized with Trizol, Purzol, STAT-60, or Bio-Rad lysis solution for 20–30 seconds with a rotor-stator (*see Note*¹²).
2. Lysate is transferred to a pre-centrifuged Phase-Lock Gel (company) tube and incubated for 5 minutes. 0.2ml chloroform per 1ml Trizol is added, the tube is shaken for 30 seconds (*see Note*¹³), and centrifuged (12000 rcf for 10 minutes at 2–8 °C). The clear aqueous phase should be on top of the Phase Lock Gel.
3. The aqueous phase is transferred to a RNA binding column, and the manufacture's protocols for rinsing and RNA elution is followed. On-column DNase treatment ensures the elimination of sample contamination by genomic DNA, reducing or eliminating the need to design qPCR primers that cross exon-exon boundaries.

⁹All experimental procedures should be approved by the Institutional Animal Care and Use Committee.

¹⁰Unfertilized zebrafish eggs may still proceed through at least one round of cell division and thus sorting is most readily performed after several rounds of cell division (depending on desired sampling times) to remove unfertilized eggs.

¹¹Precise numbers of embryos per biological replicate unit depend on the age at which embryos will be sampled. For early (3–12 hpf) samples, we have found that 100 embryos per replicate are necessary to obtain sufficient material for both microarray analysis and qPCR confirmation of 10–12 genes. For later (48–96 hpf) embryos and ovolarvae, 50 individuals per replicate are sufficient.

¹²Trizol, PurZol, STAT-60, and similar solutions are composed of varying concentrations of phenol with enzyme inhibitors such as guanidinium chloride. We have not observed significant differences in RNA extraction using the different brands of phenol extraction solutions, but generally use the solutions appropriate to the kit being used.

¹³Do not vortex Phase Lock Gel, an irreversible emulsion may form.

4. RNA integrity is analyzed using microfluidic or gel analysis (e.g. Agilent BioAnalyzer or equivalent). (*see Note 2*)

3.4 Microarray hybridization

Standard methods of Agilent sample labeling and microarray hybridization are followed, and briefly noted here. We have generally used an academic core facility to perform microarray hybridization for data consistency. Single color microarrays are preferred when sample labeling and microarray processing can be automated to reduce technical errors. We have observed that biological variability is far larger than technical variability using these methods. If suitable laboratory automation is not available, then two color arrays should be used to control for analytical variability. In this case, a universal reference experimental design appears to be the best compromise that provides a robust statistical framework while also offering cost efficiency (minimizing the number of hybridizations) while minimizing the experimental complexity (31).

1. Following manufacturer's instructions, the Agilent Low-Input QuickAmp Labeling Kit is used for single-color (Cy3) labeling.
2. Samples are hybridized to a custom-commercial Agilent microarray using the Agilent In situ Hybridization Kit Plus.
3. Labeled cDNA is combined with the Agilent 10× Control Targets (to identify microarray corners).
4. Post-hybridization, microarray slides are washed as per the Agilent In situ Hybridization Kit Plus.
5. Arrays are scanned with an Agilent DNA Microarray Scanner. The use of standard Agilent Control Targets allows Agilent's Feature Extraction software to perform spatial and multiplicative background detrending, reducing technical variability.

3.5 Microarray data analysis

Detailed methods of microarray analysis have been published elsewhere. We will briefly note our methods here. Background-detrended microarray data sets are normalized using a non-linear scaling method based on rank invariant probes of Schadt et al. (32, 33), commonly performed for Affymetrix microarray data by the dChip software (34).

1. To determine the baseline microarray with the overall median Cy3 signal to which all other microarrays are normalized, any Cy3 values below 5 are set to 5, and an overall median Cy3 value is determined.
2. Each microarray is separately normalized to the baseline microarray by finding Rank Invariant probes between the two microarrays using the method outlined in Schadt et al. (32). A non-linear normalization curve is determined for each microarray, and a piecewise linear running median is used to normalize the individual data points. (*see Note 14*)
3. After normalization the average normalized signal is calculated for probes duplicated on the microarray. Probes are removed from consideration when they exhibited saturation in any instance among the microarrays and duplicate probes, or when signals are not above background, in all instances among the microarrays and duplicate probes.

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¹⁴Custom software programs may be required for the piecewise linear median calculation, but this can be programmed in common spreadsheet applications, or using Perl.

4. Standard ANOVA is not appropriate for data analysis due to auto-correlation between developmental time points, both on a theoretical and a practical basis. Thus Bayesian Estimation of Temporal Regulation (BETR; (35)) or a similar method should be employed. Normalized Cy3 values for each probe are log transformed, median-centered, and then can be analyzed using BETR relative to some standard sampling time.

3.6 qPCR validation

Quantitative real-time PCR (qPCR) validation of several target sequences is generally desirable to confirm the general pattern of expression, and to determine more precisely the magnitude of expression change.

1. Unique primers may be designed using standard tools, such as NCBI's PrimerBlast.
2. cDNA is prepared from an aliquot of total RNA using Omniscript™ Reverse Transcriptase kit or equivalent, a mix of random hexamers and anchored oligo dT, and RNasin® RNase inhibitor. (see **Notes** ^{2, 8})
3. Real time PCR is performed using standard methods.
4. To ensure that a single product is amplified, melt curve analysis is performed on the PCR products.
5. Further confirmation of the specificity of the CYP qPCR primer pairs is obtained by purifying and sequencing the cDNA products of the final qPCR reactions.

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