Microarray Gene Expression Analyses in Medaka (Oryzias latipes) Exposed to Hypoxia

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Overview of Experimental Plan

Hypoxia treatment
Tissue collection
Protein
RNA
Real-time PCR
Microarray
Probe preparation
Hybridization
Scan slides
Calculate transcription changes
Data mining
DIGE Analysis
Compare results
The Medaka Model

- Short generation times of 2-4 months.
- Genome size that is smaller than other models such as zebrafish and mammals.
- A large number of medaka ESTs generated through the Medaka Genome Initiative in Japan are publicly available (http://mbase.bioweb.ne.jp/~dclust/medaka_top.html).
- Collaborative efforts with the University of Southern Mississippi.
Medaka Oligonucleotide Microarray

- 60-mer oligonucleotide spotted on amine silane-coated slides.
  - Tm = 75±5°C.
  - Sequences within 1kb of 3' end of the coding region.
  - Maximum length of simple repeats: 6 bases.
  - BLAST searched to verify oligo specificity.
  - Yield: 8,046 ESTs meet criteria.

- (+) control is medaka cytoplasmic β-Actin.
- (-) control is a yeast tRNA.

- Oligos synthesized by Integrated DNA Technologies in 384-well plates.
  - Quality control ran on every oligo measuring full-length oligos provide targets.
Medaka Oligonucleotide Microarray

- 8,046 features (e.g. 60-mer oligo targets) plus controls spotted in duplicate to verify signal consistency across each slide.
- 337-338 features per sub-block (including controls).
- Spot quality is excellent:
  - Clean and even.
  - Minimum doughnut-shaped spots.
  - Allows feature data to be measured consistently.

[Image showing arrayed features]
## Gene Detection Capability

### Standard vs. Amplification Protocols

<table>
<thead>
<tr>
<th>Number of Genes Detected</th>
<th>Cy3</th>
<th>Cy5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>6602</td>
<td>5717</td>
</tr>
<tr>
<td>1</td>
<td>7136</td>
<td>7501</td>
</tr>
<tr>
<td>2</td>
<td>7580</td>
<td>7580</td>
</tr>
<tr>
<td>5</td>
<td>3297</td>
<td>2855</td>
</tr>
<tr>
<td>10</td>
<td>7571</td>
<td>7578</td>
</tr>
<tr>
<td>20</td>
<td>7471</td>
<td>93%</td>
</tr>
</tbody>
</table>

### Total RNA (ug)

- Standard Amplification Protocol
- Amplification Protocol

<table>
<thead>
<tr>
<th>Number of Genes Detected</th>
<th>0.5</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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</table>
Treatment Method

- Biospherix OxyCycler oxygen control system
Treatment Method

- Biospherix OxyCycler oxygen control system
  - Measures oxygen levels both in the water and headspace above the water.
  - Precision = ± 0.1 mg O₂/L
  - Adjusts gas infusions into the system via a feedback loop to achieve desired set point.
  - Real-time data monitoring & continual logging.
  - Allows manual or automatic control including recipes.
Oxygen Levels During Treatment

Dissolved Oxygen Over Experiment Duration

Control

Experimental

mg O2/L

0 24 48 72 96 120 144 168 192 216 240 264

Hours Elapsed

Oxygen Levels During Treatment
**Visual Medaka Responses**

- In aquaria:
  - Heavy respiration
  - Lethargic
  - Lack of appetite

- Upon dissection:
  - Very dark and enlarged gall bladders – a possible indication of liver stress.
aRNA Probe Synthesis

- 2 µg total RNA template amplification protocol

First strand synthesis
  - Random hexamer/primer (SuperScript III™
  - T7 (dT)24 primer

Second strand synthesis
  - DNA Ligase, DNA Polymerase I, RNase H, T4 DNA polymerase
  - Clean up with Qiagen Qiaquick PCR Purification kit

- In vitro transcription of amplified RNA (aRNA)
  - Cy-3 (fluoresces green) or Cy-5 (fluoresces red) labeled CTP
  - T7 RNA polymerase
  - Clean up with Sigmapro RNA Kit
Hybridization

- UV crosslinking to bind oligos to slide.
- Prehybridized slides with BSA to block background noise.

Hybridization:
- COT-1 DNA blocks highly repetitive, non-specific binding to targets.
- Poly d(A) blocks non-specific binding to any poly-T tails.
- Yeast tRNA binds to (-) control (has no fluorescent dye).
- Hybridization buffer, mostly formamide with SSC & SDS to aid in selecting for specific binding.
- Hybridized overnight, 16-20 hours.

Wash steps:
- 4 wash steps
- Stepping down concentrations of SSC and SDS
- Stepping down temperature
- Spin dry

Genomic Solutions Hybstation
Analysis

- Microarrays are scanned with an Axon GenePix 4000B scanner.
  - Red: Cy5 sample most abundant
  - Green: Cy3 sample most abundant
  - Yellow: Cy5 & Cy3 samples equally abundant
  - White: Feature is saturated with dye.

- GenePix software flags "bad" data:
  - Irregular shapes
  - < 70% of feature pixels are at least 2 std. deviations above background
  - Scratches, splotches, etc.

- GenePix software normalizes to the global mean.
  - Assumption that most genes' transcription levels are unchanged.
Analysis

- Repeats compared to one another as well as to their corresponding dye-flips.
  - Dye-flips counterbalance any potential biases in dye signal.
  - Recall: a minimum of 24 repeats (12 per dye-flip) are collected.

- Verify reproducibility via a Pearson correlation, r≥0.85.

- Intensity data subjected to a 1-sample t-test or SAM analysis to test for significance
  - p-value ≤ 0.05
  - q-value = 0

- Compute fold-increases

- Choose spots for verification via real-time PCR.
Analysis

- Differentially expressed features mined for re-annotation, gene ontology, and pathway:
  - Swiss-Prot
  - Database for Annotation, Visualization, and Integrated Discovery (DAVID)
  - Kyoto Encyclopedia of Genes and Genomes (KEGG)
Hypoxia Responsive Features by Tissue

A. Transcription up-regulated in medaka
q-value = 0

B. Transcription down-regulated in medaka
<table>
<thead>
<tr>
<th>GenBank Acc #</th>
<th>Match_id</th>
<th>Symbol</th>
<th>Description</th>
<th>e-value</th>
<th>BitScore</th>
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<tbody>
<tr>
<td>BJ005346</td>
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<td>EST</td>
<td>Hypoxia induced genes in medaka</td>
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<tr>
<td>BJ529972</td>
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<td>EST</td>
<td>Hypoxia suppressed genes in medaka</td>
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<tr>
<td>AB009569</td>
<td>Q9WVL4</td>
<td>GRK1</td>
<td>RK_MOUSE (Rhodopsin kinase (RK))</td>
<td>0</td>
<td>744</td>
<td>Signal transducer</td>
</tr>
<tr>
<td>AB023489</td>
<td>P18910</td>
<td>NPR1</td>
<td>ANPRA_RAT (Atrial natriuretic peptide A-type receptor))</td>
<td>0</td>
<td>1263</td>
<td>Signal transducer</td>
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<tr>
<td>AB041330</td>
<td>Oryzias latipes c GnRH-II mRNA for prepro-gonadotropin-releasing hormones-II</td>
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<tr>
<td>AU169186</td>
<td>Q8K097</td>
<td>FAIM2</td>
<td>FAIM2_MOUSE (Fas apoptotic inhibitory molecule 2 (Lifeguard protein))</td>
<td>1.00E-04</td>
<td>45.1</td>
<td>Anti-apoptosis</td>
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<td>AV669091</td>
<td>P70158</td>
<td>SMPDL3A</td>
<td>AS3A_MOUSE (Acid sphingomyelinase-like phosphodiesterase 3a precursor)</td>
<td>3.00E-33</td>
<td>139</td>
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<tr>
<td>D89724</td>
<td>Q8UW64</td>
<td>N/A</td>
<td>PSB9_ORYLA (Proteasome subunit beta type 9 precursor)</td>
<td>4.00E-114</td>
<td>409</td>
<td>Hydrolase</td>
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<tr>
<td>Y11252</td>
<td>P55260</td>
<td>ANXA4</td>
<td>ANXA4_RAT (Annexin A4 (Annexin IV))</td>
<td>1.00E-119</td>
<td>428</td>
<td>Binding</td>
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</table>
Gene Ontology Conclusions

- Gene ontology data was available for 424 of the identified spots.
  - The majority fell into various metabolism ontology groups, such as protein and mRNA, and were down-regulated.
  - Also represented groups include cell maintenance, transport, and the ubiquitin-cycle, among others.
- Overall, these results imply an energy conservation response and slow-down of general metabolic activities.
- Recall (lethargic fish, lack of appetite)
Real-time PCR Validation

R² = 0.7498
Conclusions

- Our medaka microarray yields reproducible data which correlates well with results from conventional PCR analysis, allowing for the calculation of changes in gene expression with high confidence levels.
- Over one thousand transcripts are found to vary their abundance with hypoxia exposure.
- Data mining offers glimpses into pathways and disorders associated with changes in environmental stimuli.
- More work is necessary to determine which transcripts are consistent over a set of experiments analyzing both RNA and protein. These will be pursued as potential biomarkers.
Future Plans

- Hypoxia exposures
  - Kinetic response
  - Episodic oxygen levels
- Heavy metal exposures
- Combinations of hypoxia and heavy metal exposures.
Microarray Gene Expression Analyses in Japanese Medaka (Oryzias latipes) Exposed to Hypoxia

Melissa Wells, Dr. Zhenlin Ju, Sheila Heater, and Dr. Ronald Walter

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Publications

Sources

- http://www.yd-g.co.jp/medaka.jpg
- http://mbase.bioweb.ne.jp/~dclust/medaka_top.html
Our Medaka Array Successfully Detects Xiphophorus Genes

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medaka</td>
<td>Undetected</td>
</tr>
<tr>
<td>Xiphophorus</td>
<td>Detected</td>
</tr>
</tbody>
</table>
Update – Current Processing & Techniques

• Employing an indirect labeling protocol of our aRNA probe:
  – Amino-allyl group acts as spacer, reducing crowding to facilitate dye-binding.

• Step-down hypoxia treatments:
  – Allows time for the fish to adjust to changing conditions.
  – Improves survival rates over time implying physiological adaptations.
<table>
<thead>
<tr>
<th>GenBank No.</th>
<th>Description</th>
<th>Fold Change (Log2)</th>
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<tbody>
<tr>
<td>BJ743818</td>
<td>Proteasome 26S subunit ATPase 1 (RPT2)</td>
<td>-1.9 0.19</td>
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<tr>
<td>D89724</td>
<td>Proteasome subunit beta type 9 precursor (P9beta)</td>
<td>-2.0 0.49</td>
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<tr>
<td>BJ716044</td>
<td>26S proteasome regulatory subunit (RPN8)</td>
<td>-1.9 0.40</td>
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<tr>
<td>BJ028102</td>
<td>Proteasome activator 28-beta subunit (PA28beta)</td>
<td>-1.2 0.16</td>
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</table>

Down-regulated genes involved in the proteasome pathway in medaka brain.
Sample Genes with Regulation Changes and Associated Diseases