6-6-2015

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Neurotranscriptome profiles of multiple zebrafish strains

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A R T I C L E   I N F O

Article history:
Received 31 May 2015
Accepted 1 June 2015
Available online 6 June 2015

Keywords:
RNA-sequencing
Brain
Gene expression
Sex
Zebrafish

A B S T R A C T

Behavioral displays or physiological responses are often influenced by intrinsic and extrinsic mechanisms in the context of the organism’s evolutionary history. Understanding differences in transcriptome profiles can give insight into adaptive or pathological responses. We utilize high throughput sequencing (RNA-sequencing) to characterize the neurotranscriptome profiles in both males and females across four strains of zebrafish (Danio rerio). Strains varied by previously documented differences in stress and anxiety-like behavioral responses, and generations removed from wild-caught individuals. Here we describe detailed methodologies and quality controls in generating the raw RNA-sequencing reads that are publically available in NCBI’s Gene Expression Omnibus database (GSE61108).

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1. Direct link to deposited data

The raw FASTQ files can be accessed through the Gene Expression Omnibus.

2. Experimental design, materials and methods

In this study we analyzed the whole-brain transcriptome profiles of male and female zebrafish (Danio rerio) in four different strains [1,2]. In brief, 17 week old zebrafish were quickly sacrificed and whole-brains were removed and processed for RNA-sequencing. Sequencing reads were subsequently aligned, analyzed, and quantified using open-source software. We also conducted technical and biological validation and replication of the RNA-sequencing results using quantitative reverse-transcriptase PCR (for overview of procedures, see Fig. 1).

2.1. Animal subjects

Zebrafish cohorts were generated and reared using previously described methods [3]. All fish were kept in mixed sex 100-liter tanks. Tanks were on a custom-built recirculating filtration system with water temperature kept at 28 °C and on a 12:12 light:dark cycle. Fish were fed twice daily with commercial feed (Tetramin). The AB and Scientific Hatcheries (SH) zebrafish strains originated from commercial suppliers (Zebrafish International Resource Center and Scientific Hatcheries, respectively). Although the AB and SH strains were bred in laboratory conditions for many generations at their respective stock centers, these strains were maintained in our laboratory for four and one generations, respectively. The two other strains (High Stationary Behavior (HSB); Low Stationary Behavior (LSB)) of zebrafish originated from approximately 200 wild caught individuals and were six generations removed from the wild (see [3] for additional selective breeding details).

2.2. Tissue collection

We collected whole brains from 160 individual zebrafish (n = 20 for each sex for each strain) that were 17 weeks post-fertilization. Between 09:00–12:00 we quickly removed fish from their home tanks, deeply anesthetized with tricaine methanesulfonate, followed by decapitation.

http://dx.doi.org/10.1016/j.gdata.2015.06.004
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Whole brains were removed within 3 min of being caught and placed in RNAlater (Ambion). After storing the samples at 4 °C overnight, we removed all RNAlater and stored brains at −80 °C until RNA extraction. Sex was assigned by observation of testes or ovaries on dissection.

2.3. RNA isolation

We extracted total RNA using column purification (RNeasy Plus Mini Kit, Qiagen). Brains were homogenized for 3 min at maximum speed with 50–100 μl of zirconium oxide beads (Bullet Blender, Next Advance) in 0.6 ml of Buffer RLT (Qiagen) with 2-mercaptoethanol (Sigma). We then added 100 μl of chloroform, mixed, and incubated at room temperature for 5 min. We subsequently centrifuged the samples at 12,000 × g for 15 min at 4 °C. The supernatant was transferred to the RNeasy genomic DNA column (Qiagen) and then we proceeded according to the manufacturer’s instructions. All samples were eluted with 30 μl of DEPC-treated water (Ambion).

2.4. RNA-sequencing library preparation and sequencing

For each strain we pooled 1 μg of total RNA from 10 same-sexed individuals into a biological replicate. This generated four biological replicates for each strain (two biological replicates for each sex). We analyzed the quantity and quality of the RNA for the 16 samples with a 2100 Bioanalyzer (Agilent). All samples were of high quality (RIN > 8.0, Table 1). Using 1 μg of total RNA from the pooled samples we generated cDNA libraries following the manufacturer’s protocol (TruSeq RNA Sample Prep V2, Illumina). We ligated a unique Illumina Index adapter to each biological replicate to allow for multiplexing. After cDNA library synthesis we submitted samples to the Genomic Sciences Laboratory at North Carolina State University for 72 bp single-end RNA sequencing (Illumina GAIIx). We followed a balanced block design [4] and multiplexed all 16 samples and ran them across 16 lanes.

2.5. Data processing

With reads that passed default quality controls (Illumina), we combined across lanes for each biological replicate. Total read counts varied between 34–65 million reads (Table 2). We utilized the open source software GSNAP [5] to align the reads to the zebrafish genome. We first built GSNAP genomic and GSNAP known and novel splice site databases using the Zv9 (release 71) D. rerio genome and gene sets, respectively (Ensembl). For each biological replicate we successfully aligned over 99% of the reads (assessed by SAMtools [6]) to the zebrafish genome using the default GSNAP parameters (Table 2).

2.6. Validation and replication with quantitative reverse-transcriptase PCR

We performed both technical validation of RNA-sequencing libraries and independent biological replication (HSB and LSB strains) through...
quantitative reverse-transcriptase PCR (qPCR). We quantified the reads for each protein-coding gene by using the “union” mode in HTSeq [7] in all of our RNA-sequencing libraries. Read counts were then normalized to the library size in edgeR [8]. We selected eight genes (msmo1, oxt, gabbr1a, comta, sell, prodra, hsd11b2, gapdh) for technical validation and 14 genes (msmo1, oxt, gabbr1a, comta, sell, prodra, hsd11b2, gapdh, cyp19a1b, dio2, pmchl, cfos, gabbri1b, igf1) for independent biological replication (see [1,2] for detailed primer characteristics and qPCR reaction parameters).

After normalizing each gene’s expression to ef1a, an endogenous reference gene [9], we confirmed a significant correlation between gene expression measured by RNA-sequencing and qPCR. Using the same material from cDNA libraries that were submitted for RNA-sequencing, we found a significant correlation between normalized read count (RNA-sequencing quantification) and cycle threshold (qPCR quantification) for the eight genes examined (technical validation; n = 64, Spearman’s \( \rho = -0.278 \) p = 0.026; Fig. 2). Using independent samples (n = 9 for each sex in each of the LSB and HSB strains), we similarly observed a significant correlation between expression measurements from the two techniques (RNA-sequencing and qPCR) for 14 genes (independent biological replication; n = 56, Spearman’s \( \rho = -0.406 \) p = 0.002). Of note, we also observed consistent patterns of differential gene expression between sexes and stress coping styles (see [1,2] for details).

## 3. Conclusions

Zebrafish are a model system utilized in many developmental, toxicological, neuroscience, and biomedical studies [10–15]. Understanding and accounting for genomic and transcriptomic variation will provide important additional insights. Here we describe in detail the procedures and methodologies in sequencing the whole-brain transcriptome of both male and female adult zebrafish in four different strains. The high quality RNA-sequencing results, which have been both technically and biologically validated, are available through the NCBI’s GEO database (GSE61108). This dataset should be of use to studies in a variety of contexts (e.g. evolution, neuroscience, genetics, bioinformatics, and biomedicine).

### Acknowledgements

We thank Brad Ring and John Davis for the assistance with fish husbandry. We are grateful to Cory Dashiel, Melissa Lamm, Melissa McLeod, Katie Robertson, Christopher Gabriel, and Nofisat Oki for the helpful discussions and technical assistance. We are grateful to Barrie Robison for the generous donation of the Scientific Hatcher’s line of zebrafish. This study received support from the National Institutes of Health (1R21MH080500) to JC. This study is a contribution of the W.M. Keck Center for Behavioral Biology at North Carolina State University. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

### References


