🌐 Comprehensive analysis methods for developmental GC exposed zebrafish V.2

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Abstract

This protocol describes methods for the molecular, behavioral, and bioinformatic analyses of GC-exposed zebrafish. All procedures were used for the study; Developmental Glucocorticoid exposure in zebrafish primes expression of genes linked to human psychiatric disorders (Choi et al., 2023)

Troubleshooting

Introduction

Fish husbandry and maintenance

Adult and juvenile zebrafish of the Tübingen (TU) strain were kept at 28°C on a 12:12 hour light/dark cycle and housed in either 3.5L or 11L tanks at a maximum density of 5 fish/L. Embryos were kept in egg water (3g/10L, Red Sea "Coral Pro Salt", RS 11230, Aqua Schwarz) or E2 medium[1] at a maximum density of 50 eggs per 30 mL until 5 dpf. Larval fish were kept in egg water at a maximum density of 50 fish per 500mL from 5-14 dpf. Larval/juvenile fish between 5-35 dpf were fed live paramecia once daily. Larval/juvenile fish were fed with brine shrimps (Premium Sander's Artemia-Eggs, FF 006425, Aqua Schwarz) once per day starting at 14 dpf and in addition flakes (TetraMin Flakes, T 769939, Aqua Schwarz) once per day from 56 dpf. Larvae up to 5 dpf were kept in dishes filled with egg water (60 ug/ml of sea salt in distilled water) or in 0.5X E2 medium (for injected embryos, 7.5 mM NaCl, 0.25 mM KCl, 0.5 mM $MgSO_4$, 75 µM KH_2PO_4, 25 µM Na_2HPO_4, 0.5 mM $CaCl_2$, 0.35 mM $NaHCO_3$, 0.5 mg/L Methylene Blue) at 28°C inside an incubator (RuMed 3101, Rubarth Apparate GmbH, Laatzen, Germany) with 12:12 light/dark cycle. All animal procedures were carried out in compliance with the ethical guidelines of the national animal welfare law and approved by the local government.

Procedure

Generation of transgenic line

1. Linearize pT2-2kbStAR:bPAC-2A-tdTomato (ZFIN ID: ZDB-GENE-001120-3) recombinant plasmids[2].
2. Inject linearized plasmid into 1-cell stage wildtype embryos of TU strain at 10-50 ng/mL in the presence of 10-50 ng/mL Tol2 transpose mRNA and 0.05% phenol red.
3. Screen the progenies of injected fish for interrenal gland-specific red fluorescent tdTomato expression to identify founders.
4. Maintain the established transgenic line, *Tg(star:bPAC-2A-tdTomato)*[uex300+/-] by crossing with wild-type TU fish and screening progenies for the presence of interrenal gland-specific red fluorescence (for bPAC+) or absence (for bPAC-) at 4 dpf. In the case of the usage of bPAC+ larvae before 4 dpf, use larvae from *Tg(star:bPAC-2A-tdTomato)*[uex300+/+] without screening.

Sample collection

1. Collect all samples within a 2-hour window during the morning (8:30 to 10:30) without feeding.
2. Immobilize larvae and fish by submersion into ice-cold system water before processing for histology, RNA, or cortisol extraction.
3. For the looming dots (LD)-exposed samples, return subjective fish to a holding tank for 15 minutes after LD exposure before being netted into ice-cold water, to wait for the GC induction.

Whole-body Cortisol assay

1. Place all samples immediately into a tube and then rapidly freeze using dry ice.
2. A single sample for the larval stage requires 12 larvae whilst from 28 dpf onwards, an individual fish constitutes one sample.
3. Samples can be kept at - 80°C until further analysis.
4. Thaw the sample at room temperature and add 150 µl or 1 ml/100mg (fish weight) of distilled water to each larval or adult fish sample, respectively.

5. For the larval samples, homogenize the sample for 20 seconds using a pestle and micro-tube homogenizer.
6. For the adult samples, homogenize the sample using KIMBLE Dounce Tissue Grinder (DWK Life Sciences, Mainz, Germany).
7. Place the homogenized samples on ice and add 1 ml and 8 ml of ethyl acetate (99.5%) to larval or adult samples respectively.
8. Vortex each sample vigorously for 2 minutes and centrifuge it at 5000 g at 4°C for 5 minutes or 10 minutes in the case of larval or adult samples, respectively.
9. Place the sample on dry ice for 30 seconds to freeze the aqueous layer.
10. Transfer the organic layer (ethyl acetate) to a new tube.
11. Evaporate the ethyl acetate layer in a vacuum dryer (Concentrator 5301, Eppendorf, Germany) for 30 minutes at 30°C.
12. After drying, resuspend the pellet in 20 µl of diluent before measurement.
13. Perform the competitive cortisol assay (Cisbio HTRF® Cortisol Kit, 62CRTPEG) following the manufacturer's protocol. ELISA signal can be detected using a CLARIOstar plate reader (BMG Labtech, Ortenberg, Germany).
14. For each assay, at least 6 samples per group will be required.
15. The data is presented as cortisol (pg) per larva and cortisol (ng) per weight of fish (g).

Behavior tests for adult fish

Female adult zebrafish (6-9 months old) should be moved into a designated behavior experimental room one week prior to testing and housed in groups of 10 fish in a 3 L tank. The behavior experimental room's water conditions, lighting schedules, and feeding routines should be the same as in the main facility. Experiments should be carried out between 09:00 and 14:00 and fish should not be fed on the morning of testing. Naïve fish should be subjected to multiple experiments on consecutive days. Immediately after testing, fish should be sacrificed for sampling. Fish should not be used in any repeat experiments. For each assay, the water will be changed, and the next experimental fish will be introduced to the tank. We rotated between wild type, bPAC- (where applicable), and bPAC+ fish for each trial.

Novel tank assay

The novel tank assay is a trapezoidal tank (25-28 cm (L), 6 cm (W) 9-10 cm (H)) filled with 1.1 L of system water. The light intensity should be maintained at 700 LUX at the water's surface. The tank should be positioned inside a photography dome. The trial will be recorded at 30 FPS using a camera (acA1300-60gm, Basler ace) with an infrared lens.

1. Place the tank in front of an infrared back panel to produce contrast.
2. Perform live-tracking using Ethovision (EthoVision XT 13, Noldus Information Technology), which generates X and Y coordinates for each frame.
3. Net a fish from its home tank into the center of the tank just above the water's surface.
4. Begin the trial as soon as Ethovision detects the fish and continue for five minutes.
5. At the end of the trial, remove the fish and sacrifice it for sampling as described in the sample collection procedure.
6. Calculate behavior parameters using the X and Y coordinates generated by Ethovision and a custom-built python script.
7. Calculate depth as the average distance from the bottom of the tank in cm for each minute.

8. Calculate average speed as the average distance moved from the previous frame to the current frame. Then, average for each minute and multiply by 30 to present as cm per second.
9. Calculate fast swimming as the percentage of frames out of the overall trial that fish spent swimming at over the 12 cm per second threshold.
10. Calculate immobility as the number of frames spent moving less than 0.1 cm per second for at least 10 consecutive frames, as a percentage of the total trial.

Social behavior test

The social assay is a transparent cuboidal clear acrylic tank (18 cm (L), 11 cm (W) 9 cm (H)) filled with system water (1.2 L). The walls are clear and 1/3rd of the floor is blue, and 2/3rds is white. At the white end is a white opaque divider that blocks the view of the visual stimulus. On the other side of the divider is the social cue tank, a cuboidal clear acrylic tank (13.5 cm (L), 6 cm (W), 9 cm (H)) filled with 0.7 L of system water that is positioned 2 cm from the main tank. Five naïve female wild-type conspecifics should be placed into the social cue tank 30 minutes before testing began to allow for habituation. The tanks should be placed on a platform inside a photography dome. An external light lit up the arena to 550 LUX (3200 Kelvin) at the water surface level.

1. Initiate the trial by netting a fish from the housing tank into the center of the blue and white tank.
2. Record fish for 60 seconds with the divider in a closed position, this is called the pre-social phase.
3. After 60 seconds, slide away the divider revealing the social cue. Continue recording for a further 120 seconds, this is referred to as the social phase.
4. Record the trials with two cameras at 30 FPS; a top camera positioned directly above the tank and a side camera positioned in front of the tank together with an infrared back panel to provide contrast.
5. Perform live tracking from both angles using EthoVision. Export and process the X and Y coordinates from each camera in python script to generate 3D coordinates for each frame.
6. Divide the tank into three zones for analysis, the blue zone, which encompasses the area above the blue floor, the white zone which covers the rest of the tank, and the interaction zone, which is a 'C' shaped area within the white zone and will be drawn to encompass the area in which wild-type fish spend 95% of their time in the white zone.
7. Calculate the time in each zone during the two stages of the trial using the 3D coordinates as a percentage of the phase.
8. Calculate the first entry to each zone in seconds from the beginning of the phase.
9. Measure social interactions from the top view coordinates. For this, calculate the swimming direction and turn events whilst the fish are in the interaction zone.
10. Social interaction refers to an event where a fish swims on the left (L) to the right (R) axis or R to L axis preceded and followed by either entering and leaving the interaction zone or a turn that changes the direction on the axis.
11. Measure immobility as the amount of time a fish spent swimming less than 0.1 cm per second for 10 or more consecutive frames.
12. Measure depth as the average distance from the bottom of the tank in cm.

Feeding assay

The feeding tank (16.9 – 18.7 cm (L), 8.7 cm (W), 10 cm (H)) is positioned inside a photography dome and in front of an infrared back panel. A camera positioned in front of the tank with an infrared filter will record the trial at 30

FPS.

1. Fish should not be fed for 24 hours before testing.
2. Net the Fish from their home tank and place it into the center of the feeding tank.
3. After a 10-minute habituation period, begin recording and live-tracking using Ethovision.
4. Record following an initial 30 seconds period in which baseline behavior is measured, place 25 pellets (Zebrafeed 200-400, SPAROS) onto the surface of the tank from an Eppendorf tube. The pellets will float and disperse across the surface of the water.
5. Record the fish for the remaining 9.5 minutes of the trial and count the number of pellets eaten.
6. Manually count each feeding event and define it as a fish eating the pellet when the pellet remains consumed until the end of the trial.
7. Transfer the X and Y coordinates for each frame into python script and calculate the duration each fish spent in the top 1 cm of the tank (feeding zone) as a percentage of the trial.

Fear conditioning assay

The fear conditioning assay will be performed using the Zantiks AD system (Zantiks, UK)[3]. The tank will be filled with system water. One fish will be placed into each one of the 4 compartments (20 cm (L), 3.5 cm (W), 15 cm (H)) (2 wildtype fish and 2 bPAC+ fish) that are separated by a white opaque wall. The floor of the tank is transparent and underneath the tank is a display. At each end of the tank are two electric shock plates.

1. The pre-programmed green aversion script will be immediately started, and the fish will be recorded using the built-in camera.
2. Following a 30-minute habituation period, the display will show a green background and blue on the other half of the display.
3. For the adaptation phase of the trial, the colors will be flipped every 5 minutes for 30 minutes to measure baseline preference.
4. In the conditioning phase, the screen will display the color blue for 9 seconds and the color green for 1 second. During the 1 second of green, 9 volts will be run through the electric shock plates. This will be repeated 6 times.
5. After the conditioning phase, in the test phase, the display will be returned to displaying a blue half and a green half. Each variation will be displayed for 1 minute.
6. The Zantiks system will provide the total amount of time spent in each colored zone for each phase of the trial. For the test phase, the time in the blue zone will be combined with each variation and the average time will be calculated as a percentage. Data from fish that did not move during the test phase will not be included in the analysis.

Locomotion assay

To measure locomotion, fish will be netted from their home tank into the center of a small tank (16.9 – 18.7 cm (L), 8.7 cm (W), 10 cm (H)) which should be positioned within a photography dome and the light intensity at the water's surface should be 350 LUX.

1. Record Fish using a camera positioned in front of the tank using an infrared lens and an infrared panel positioned behind the tank.
2. Perform recording and live-tracking using Ethovision. The fish will be recorded for 3 minutes.
3. Transfer the X and Y coordinates generated from Ethovision into python script where the average speed and turn frequency will be calculated.
4. Measure average speed as the average distance from the previous frame to the current frame across the trial and present it as cm per second.
5. Calculate turns as a change of direction event in which the heading angle of the fish changed from left to right or right to left.
6. Calculate the average number of turns per second by dividing the total number of turns by 180.

Acute stress by a predatory stressor (Looming dots)

1. Net a fish into a small tank (16.9 – 18.7 cm (L), 8.7 cm (W), 10 cm (H)) filled with system water and the tank will be immediately placed under a Raspberry Pi display (Raspberry Pi 3 7″ display 15.4 cm x 8.6 cm). The Raspberry Pi display runs a Linux operating system which is controlled with a custom-built graphical user interface (GUI) on a master PC.
2. Generate a txt file using the master PC and it will be transferred to the Raspberry PIs via WLAN (PLINK). The file is read by a custom-built script which generates a sequence of images that are presented on the display.
3. After 30 seconds the trial begins. Record the fish at 30 FPS via Ethovision. A white screen will be displayed for the initial 60 seconds to generate baseline locomotion data.
4. Subsequently, 10 consecutive expanding black dots will be displayed on a white background. Each dot starts from the middle of the screen and expands in 2.8 seconds to fill the screen. The dot remains on the screen for 1 second before the screen returns to white for 1 second. The duration of each event is 4.8 seconds and is repeated 10 times, for a total stress exposure time of 48 seconds.
5. Record fish for a further 60 seconds.

RNA preparation

Each sample for the larval stage contained 25-30 larval whole brains whilst from 28 dpf three whole brains constituted one sample.

1. For RNA isolation, dissect larval whole brain samples (at 6 or 13 dpf) in the RNAlater$^®$ (AM7021, Ambion, USA) after overnight incubation. For adult fish (at 120 dpf), dissect whole brains on ice in PBS, within 2 minutes, then snap freeze in liquid N_2 and keep them at -80°C until further processing.
2. For adult brains, homogenize each sample completely with a 7 mm stainless steel bead (69990, QIAGEN, Dusseldorf, Germany) in RNA Lysis buffer from the Quick-RNA miniprep Kit (R1055, Zymo Research, Irvine, CA, USA) in a round-bottomed 2 ml tube using a TissueLyser LT (QIAGEN, Dusseldorf, Germany) at 25 Hz for 1 minute and then 15 Hz for 2 minutes. For larval samples, homogenize for 30 seconds using a pestle and micro-tube homogenizer.
3. Isolate total RNA by following the manufacturer's protocol and keep it at -80°C until further use.
4. Measure the quality of RNA using RNA 6000 Nano kit and Agilent 2100 Bioanalyzer (Agilent, USA). Intact RNA samples (RIN>7) will be used for NGS analysis.

Quantitative PCR

1. Synthesize reverse-transcribed cDNA with 1ug of purified total RNA using High-Capacity RNA-to-cDNA™ Kit (4387406, Applied Biosystems, USA) following the manufacturer's protocol.
2. Design and synthesize primers for qPCR.
3. Dilute the cDNA with nuclease-free water (AM9937, Invitrogen, USA) in a 1:10 ratio (~100ng/ul).
4. Perform relative mRNA quantification by using real-time qPCR with PowerUp™ SYBR™ Green Master Mix (A25778, Applied Biosystems, USA) and specific primers.
5. Amplify the triplicates of each cDNA sample and normalize against *18s* gene and subject to the ΔΔCT method[4]. Normalize gene levels by dividing the absolute levels of each sample with the average of all wild-type samples.

mRNA sequencing

In the associated publication (Choi et al., 2023), mRNA-seq library preparation and sequencing on the Illumina NovaSeq6000 were performed by TRON gGmbH (Mainz, Germany). Briefly, construct paired-end TruSeq Stranded mRNA libraries (Illumina, CA, USA) and sequence it for over 20M of 50 bp reads/sample. For this study, a total of 60 samples were sequenced, consisting of 5 biological replicates at four different points in three groups (wildtype, bPAC+ and bPAC-).

Bioinformatic analysis

All used sequenced reads from RNA-seq can be downloaded from European Nucleotide Archive (ENA, PRJEB53713). Parameters, command lines, scripts, and version information are available in the GitHub repository (https://github.com/minkechoi/tx_star-bPAC_brain).

1. Check the quality of .fastq files using FASTQC[5]. If you find significantly low-quality reads or bias in your data, please remove or replace or process those data.
2. Perform fastp6 for sequenced reads of each sample to remove remaining adapter sequences, low-quality reads, and polyG in 3' ends.
3. Map cleaned reads to the zebrafish reference genome assembly (GRCz11) with Ensembl annotation version 107 using HISAT2[7].
4. Convert .sam file to .bam file using Samtools[8] and remove duplicate reads by using MarkDuplicates (Picard)[9].
5. Assemble RNA-Seq alignments into transcripts and estimate the expression of transcripts with Ensembl annotation version 107 using Stringtie[10].
6. Perform downstream and statistical analyses with in-house R scripts on and Rstudio (Build 485)[11]. Clone or download all folders in the repository and simply source the R scripts (mRNA-seq_analysis.R) in the code folder. The downstream process in R contains:
   - 6.1. Quantify the number of reads using edgeR[12] to identify DEGs that show more than absolute 2-fold changes (FC) in their expression with less than 0.01 False discovery rates (FDR). Apply a lower fold change threshold (absolute 1.5 FC) for 120 dpf samples because there is no elevation of cortisol in bPAC+ compared to the wild type at 120 dpf.
   - 6.2. For the functional analysis, use R packages including clusterprofiler[13], gProfiler2[14], enrichGO[13], and annotation platforms; Chea3[15], and DisGeNET[16].
   - 6.3. Perform cell-type enrichment test using Enrichr[17] with a single cell transcriptome-based cell type classification library, Tabula_Muris[18]. For this analysis, isolate human homologs of GC-primed genes and use

them as input. Check expressional specificity in Tabula_Muris with Brain Non-Myeloid tissue (https://tabula-muris.ds.czbiohub.org/images/Brain_Non-Myeloid-facs-cell_ontology_class-tsne.png)

- 6.4 Generate protein-protein interaction networks using the String database[19] and Cytoscape[20].
- 6.5. Generate the list of social genes (101 genes) and epigenetic modifiers which are associated with DNA modification (52 genes) and histone modification (365 genes) as well as RNA modification and processing (1886 genes) using the Gene Ontology Annotation (GOA) database. Files are available in the supplementary folder in the GitHub repository.

Immunohistochemistry

Immunohistochemistry will be performed following the protocol in the previously published papers[21] with minor modifications.

Modifications
1. Fix larvae at 5 dpf in fresh buffered paraformaldehyde fixative (4% paraformaldehyde in PBS) and permeabilize by incubation with proteinase K (10 mg/ml; 70663, Sigma, Germany) for 1 hour at room temperature.
2. Custom-made primary rabbit anti-oxt antibodies in 1:200 dilution[21] and Alexa Fluor® 488 goat anti-rabbit IgG (A11008, Invitrogen) as secondary antibodies (in 1:1000 dilution) will be used for the visualization of oxytocinergic neurons.

Imaging and analysis
1. For imaging, clear dissected larval head samples in 80% glycerol in PBS overnight at 4°C and mount samples in the proper position on the slide glass.
2. Record confocal stacks through the larval heads using a Zeiss LSM 880 with Airyscan FAST with PApo 10X/0.45 – dry objective with Airyscan mode from dorsal to ventral.
3. Analyze stacks using FIJI (ImageJ 2.1.0/1.53c)[22] to create maximum projections, quantify signal intensity, count the number of cells, and image rotation and cropping.
4. Select and crop neurosecretory preoptic area (NPO) for oxt + cells by Alexa 488+ signal and anatomical structure in the maximum intensity projection image. Create a rectangular box that can cover from the most right to left Alexa 488+ cells and place Alexa 488+ clusters into the middle of the box. The number of stacks is 30 to 35 in 1.3 μm intervals.
5. Identify the area of fluorescence+ cells by thresholding each image and creating ROIs in the maximum intensity projection.
6. Calculate Corrected Total Cell Fluorescence (CTCF) as integrated density - (Area of cells x Mean fluorescence of background reading) in a blind manner.

Statistics

In the associated publication (Choi et al., 2023), statistical analyzes were performed using R (version 4.2.1), and Prism9 (Graphpad Software Inc, San Diego, CA, USA). Before testing for statistically significant differences between groups, data were tested for normality and variance. We used unpaired t-tests (two-tailed) or Mann-Whitney tests for two group comparisons, ordinary one-way ANOVAs, or two-way repeated measures (RM) ANOVA with Dunn's or Tukey's multiple comparisons test as a post-hoc analysis for more than two groups, and

built-in statistical adjustments in the R packages for multiple comparisons including False Discovery Rate (FDR). Log-rank test was used for the social approach comparisons.

Associated Publications

Min-Kyeung Choi, Alex Cook, Helen Eachus, Anna Tochwin, Siyong Kim, Soojin Ryu. Developmental Glucocorticoid exposure in zebrafish primes expression of genes linked to human psychiatric disorders. *bioRxiv* (2023).

Helen Eachus, Min-Kyeung Choi, Anna Tochwin, Johanna Kaspareit, May Ho, Soojin Ryu. Elevated Glucocorticoid alters the trajectory of hypothalamic development and function. *bioRxiv* (2023).

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