Analysis of Brain Scans from Live Zebrafish

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Abstract - *Optical Coherence Tomography is a non-invasive technique useful in obtaining structural information on tissues and organs in animal models of human disease. The resolution of tissue scans are in the micrometer range, and this technique offers unique opportunities for biological experimentation, particularly as test subjects can be kept alive during and after imaging. OCT scanning is the optical analog of ultrasound imaging. This study demonstrates that this technique effectively assesses permanent physical changes to brain as a result of chronic ethanol ingestion. We describe the process of brain scanning, the ethanol treatment of test subjects, and the analysis of the data obtained. Adult zebrafish that were exposed to chronic levels of ethanol, followed by a significant washout period, exhibited changes in brain morphology consistent with hippocampal edema.*

Keywords: zebrafish, optical coherence tomography, brain scan, ethanol

1 Introduction

Damage to the CNS is a major complication of alcohol abuse. The health risks associated with excessive ethanol ingestion are many, including a greater prevalence (up to 5-fold increased risk) of post-operative complications, such as cognitive impairment [1]. Zebrafish is a useful model in studying alcohol-related disorders, and how alcohol impacts the effects of anesthesia. We previously proposed [2] that zebrafish is a potential animal model for post-operative cognitive dysfunction (POCD), a condition of serious concern for elderly patients [3]. The risk for POCD increases in individuals that excessively ingest ethanol [1], societally-accepted and potentially toxic non-nutrient agent. This study represents a preliminary examination of the effects of repeated ethanol exposure on adult zebrafish neurobehavior, using brain scans of live zebrafish. The brain scans were derived from optical coherence tomography (OCT), a non-invasive technology using interferometry of harmless light waves (λ=1,325nm). OCT can be used to scan zebrafish brain [4-6], which contains a forebrain structure with regions resembling those in humans (i.e. amygdala, hippocampus) [7]. This study examined the effects of chronic ethanol ingestion on telencephalon morphology, and describes the initial procedures that we used for analyzing the brain scans. We think that this technology can offer researchers a novel tool to ask, and answer, relevant questions regarding the function of an evolved brain.

1.1 Optical Coherence Tomography

OCT is a non-invasive imaging technique that uses light waves to take cross-section pictures of tissues. We used this technology to generate images of the brain in live zebrafish, namely the forebrain structure that contains the right and left hemispheres of the telencephalon and the olfactory bulbs. The axial resolution is 5.5 μm. OCT uses light from a matched pair of superluminescent diodes, which make up the reference and the sample beam, to obtain a reflectivity profile along the depth of the tissue. The use of near-infrared light of relatively long wavelength (i.e. λ = 1300nm) allows it to penetrate into the tissue. The backscattering of light waves from the tissue interferes with the reference beam, and the interference pattern is used to generate images to a depth of 3.5mm. The A-scan reflectivity profile of the light, derived from the reference and sample beams, scans one pixel width at a time down the Z-dimension incrementally along the X-dimension creating an image slice (i.e. B-scan). This process is repeated along the Y-dimension to generate a 3D image from stacked individual slices.

2 Methods and Results

Zebrafish (*Danio rerio*; wild-type AB) were bred and cared for using institutionally-approved standard protocols. Four female adult zebrafish were chosen from a cohort that was 537 days post fertilization (dpf) at the beginning of the study. Using the formula below, derived previously [2], where *x* is age of zebrafish in dpf and *y* is age equivalent in human years, we estimated the human age equivalent of the zebrafish used in this study to be 49 years old at the start of the study.

$$y = (0.425\chi^{0.757}) - 0.751 \quad (1)$$

Two groups (chronic ethanol ingestion and control), each with two zebrafish, were exposed (or, mock-exposed) to ethanol as described below. We will refer to these four zebrafish as test fish in this report. The test fish were kept on a Z-Hab Mini system (pentairaes.com) under standard conditions. During treatment, they were kept in vessels containing freshly-prepared and filtered (0.45μm cellulose membranes) environmental water (EvH_2O), which consisted of 60mg/L of

Crystal Sea® Marinemix, which is a typical sea salt mixture, and 87.5μM sodium bicarbonate (pH ≈ 7.3).

2.1 Chronic Ethanol Ingestion

The test fish were exposed to ethanol (1% for 60min at 28°C), or mock-exposed (water bolus), for five consecutive days followed by a brief two-day washout period and then another five consecutive days of ethanol exposure. Using the age-equivalent formula (equation 1), this 12-day treatment period represents approximately two human years of alcohol ingestion. The upper limit in the United States for legal purposes is 0.1% blood alcohol concentration, indicating that the 1% ethanol exposure is well above the expected dosage that is shown to significantly impair both motor [8] and cognitive [9] function in humans. Exposure of zebrafish to 1% ethanol is similar to that used by other researchers [10, 11]. Blood vessels of the gills and skin efficiently absorb ethanol [12, 13] and within about 40min of immersion of the test fish in the treatment vessel the levels of ethanol in blood and brain will reach equilibrium with the concentration in the vessel [14, 15]. Gerlai and coworkers [13] demonstrated that 1% ethanol for 60min inhibits several behavioral endpoints, including aggression. In our study the 1% ethanol exposure for 60min was repeated ten times over a 12-day treatment period to represent chronic exposure to ethanol, translatable to chronic alcoholism in humans.

Our ethanol treatment protocol was conducted as follows. The test fish were given their morning feedings. After at least one hour, all four of the test fish were moved from the Zebrafish Facility to the OCT Lab. Four 1.0L-size spoutless beakers were prepared with pre-warmed (28°C) 720mL EvH_2O. The test fish were transferred by net to the beakers, covered with a small glass plate and placed in a lab incubator and kept for 20min at 28°C to acclimate to their surroundings. Then, an 80mL volume of EvH_2O or 10% ethanol (1% final concentration) was added to the respective beakers (two controls and two ethanol test fish). The glass-covered beakers were returned to the lab incubator and kept for 60min at 28°C. Black shields were used to visually isolate each fish. Following exposure, all fish were first transferred to a rinse beaker with 500mL pre-warmed (28°) EvH_2O prior to return to each group tank. The test fish were given their afternoon feeding at least one hour after returning to the Z-Hab Mini system. Due to the limited number of test fish in the 1.5L group tank, greenery was added to minimize aggressive behaviors.

2.2 Anesthetization Chamber

All four test fish were anesthetized, immobilized and scanned on the same day, one fish at a time. The test fish was transferred by net to the anesthetization chamber, which was a beaker (size: 150mL) with pre-warmed (28°C) EvH_2O (90mL), containing freshly prepared tricaine methane sulfonate (TMS) at a concentration of 100mg/L. The beaker was placed in a 200mL water bath (Peltier-controlled benchtop cooler) that was set to 21°C. The opaque cover was placed over the water bath and the beaker was allowed to reach 22°C (6min). The room temperature (rt) of the OCT Lab was 20°C. By visual observation, the test fish was immobile after approximately 1min. Upon reaching the designated temperature (22°C) in the anesthetization chamber, the test fish was quickly transferred by carefully decanting into the immobilization chamber.

2.3 Immobilization Chamber

The apparatus for positioning and immobilizing the test fish was setup as shown in Figure 1. EvH_2O (40mL) was added to the chamber and allowed to reach rt. After transferring to the immobilization chamber, the test fish was gently positioned manually into the spaces between the bristles (Figure 1), ensuring that the gill plates are unobstructed and submerged. The level of the water is adjusted to make sure that the entire head is just barely submerged for optimal OCT scanning.

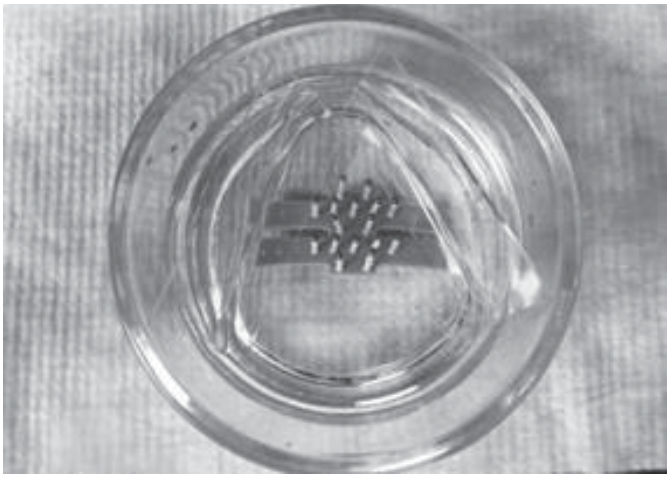

Figure 1: Positioning Live Zebrafish

This photo of the immobilization chamber shows a glass culture dish (250mL-size) on a standard lab mat. In the center, there is a modified plastic base with smooth bristles that was custom cut from a commercial cosmetic product (i.e. hair detangler). A knotted rubber band kept the plastic base angled slight up from the bottom of the dish, to compensate for the downward curvature of the fish's head. Two large-sized microscope glass slides are placed to keep the plastic base submerged. The head of the test fish was positioned just past the bristles (the open lower area of the image).

2.3.1 Maintenance of Anesthesia

Maintaining optimal levels of anesthesia during OCT scanning ensures that the fish remains immobile and breathing calmly. EVH_2O and a concentrated solution of TMS were used by pipette to control water level and anesthesia.

2.3.2 Recovery from Anesthesia

Following scanning, the anesthetized test fish was transferred to a recovery beaker containing 120mL plain EvH_2O at rt. The beaker was place in a lab incubator (set to 28°C). The test fish was monitored for normal swim movement that should occur within two minutes of exposure to fresh EvH_2O. After a 40min acclimation period the test fish was then placed back into the group tank and returned to the Z-Hab Mini system.

2.4 Brain Scanning

The OCT device used in this study was purchased from ThorLabs (thorlabs.com), a Telesto series spectral domain OCT imaging system (TEL 1300V2-BU), equipped with a base unit, or engine, that consists of a superluminescent diode light source, scanning electronics, and a spectrometer for detection. The bandwidth is over 170nm with a 5.5μm axial resolution at an imaging depth of 3.5mm. The scanner is mounted on a focus block attached to an aluminum base with a fixed stage allowing for XY linear translation as well as rotational positioning capability. OCT scanning is the optical analog of ultrasound imaging.

The software allows for real-time 3D sectional imaging of the tissue simultaneously showing a standard, also real-time, video image of the subject, allowing for the experimenter to make adjustments to the stage in order to locate the target area. The image, as see in Figure 2, is brought into focus using coarse and fine knobs that control *z*-axis travel (40mm and 225μm per revolution, respectively) of the rigid scanner. Using this image, respiration rate can be determined by counting the number of gill openings per unit time.

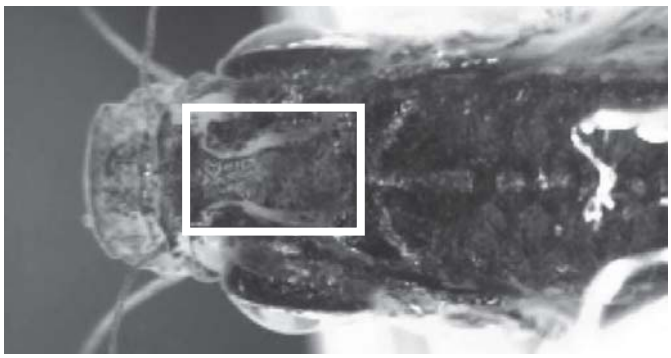

Figure 2: Locating the Target Telencephalon
This photo of a test fish shows the positioning of the head prior to the live brain scan with OCT. The *white box*, which defines the scan parameters, is drawn as a virtual marker above the area to be imaged. The zebrafish brain, which is elongated and segmented, exists with the forebrain (i.e. telencephalon/olfactory bulb), our target, situated between the eyes. The dimensions of the *white box* are kept constant, and a duplicate *white box* was utilized for each test animal. These photos were also used to determine head size.

The virtual rectangular box is drawn above the target area in the video image provided. As illustrated in Figure 3, the box is click-started at the lower left and click-ended at the upper right. The illustration in Figure 3 depicts the XYZ volume that is to be imaged. Operationally, once the box is drawn, its precise size can be adjusted and fixed. In our studies the X-, Y- and Z- dimensions were the same in all scans. The number of pixels along the XYZ dimensions was also set, and the same numbers were duplicated for each subject: 651, 769, and 640 pixels for X-, Y-, and Z-dimension, respectively. The intensity of the light source is adjusted to an optimal setting after choosing the target scanning speed: 28, 48, or 76 Hz. Next, the reference beam is adjusted to bring the 3D sectional image into proper focus. Once the 3D image is presented with the simultaneous video image of the subject's head, we determined the positional accuracy of the white box (Figure 2) and fine adjustments, using the translation stage, are performed to center the target telencephalon keeping both hemispheric lobes in view. Once all settings are defined and the image is optimized, the scans are acquired for permanent data collection. The scan acquisition time varies depending on the parameters set, particularly the pixel number and scan speed chosen. In our experiments the acquisition time varied between one and four minutes.

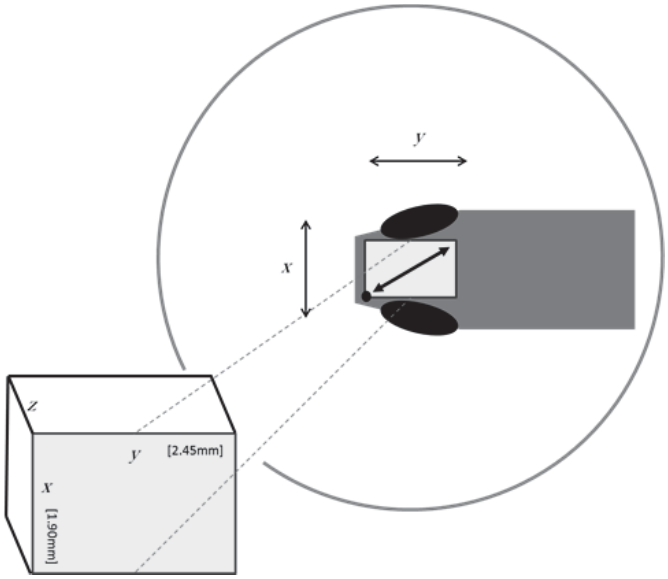

Figure 3: Target Zone for Scanning
The illustration schematically depicts a test fish (*dark gray polygon* with two *black beveled ovals*) representing the area of the body and eyes visible in the live video (see Figure 2) during preparation of the subject. The view here is looking down from the perspective of the optical device. The circle indicates the immobilization chamber that is initially manually adjusted, then fine-adjusted using the stage controls. The test fish is positioned left-to-right (the rostral-most point, or nose, is towards the left) in the immobilization chamber. The box is 1.90mm by 2.45mm, for X- and Y-dimensions respectively.

2.5 Resolution versus Image Stability

Resolution is a function of the chosen pixel density along one or more of the XYZ dimensions, such that the greater the number of pixels, the higher the resolution. Scan speed is another factor affecting image clarity, whereas the slower speeds (i.e. 28Hz) generate clearer images. Both of these parameters (high pixel density and low hertz setting) dramatically increase the length of time it takes to complete a single scan. Since we used live breathing zebrafish in our study, while the respiration rate was low in the anesthetized subjects, the repetitive gill movement caused a slight but disruptive shift in head (and therefore brain) position during the longer scans. This breathing-related head movement was less noticeable in the faster, less resolved, scan. This situation is a trade-off in balancing resolution over image stability, and remains an important issue going forward, as this technique is further utilized in experimentation.

2.6 Post-Scanning Analysis

We used the scans obtained at the highest speeds (i.e. 76Hz) in order to analyze the images with the least amount of instability due to head movement.

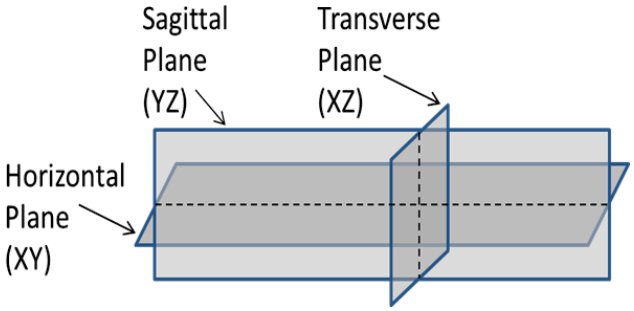

Figure 4: Anatomical Planes
The illustration defines the anatomical planes that represent the stacked slices of multiple 2D images acquired during scanning. Since we set the pixel density for the Y-dimension to be 769 (see discussion of Figure 3), we acquired 769 'transverse plane' slices. Likewise, the number of slices defined by the other two planes is identical to the corresponding number set as the pixel density. The transverse plane shows images proceeding in the rostral to caudal (nose to tail) direction. The horizontal plane shows images proceeding from the top to the bottom of the fish.

The length of the forebrain region (i.e. telencephalon and olfactory bulb) was calculated by examining sagittal plane images (Figure 4). To do this, we first determined the slice number (1 to 769) that defines the beginning of the olfactory bulb, the neuronal tissue just rostral to the telencephalon. And then, we determined the slice number corresponding to the junction between the end of the telencephalon and the beginning of the optic tectum. We then calculated the total number of slices that make up the forebrain, and then calculated the length in mm, using the relationship, 2.45mm per 769pixels in the Y-dimension.

We then determined the specific 'transverse plane' slice number that is one-quarter the total length of the forebrain, from the telencephalon-optic tectum junction. This target 'transverse plane' slice represented the center slice for further analysis (Figure 5). The group of stacked XZ slices was further processed, by measuring the width of the telencephalon lobes. The location chosen to measure the width was calculated by multiplying 25% of the forebrain length, and measuring down from the inner region of the skull. The measurements were performed by two different experimenters and then averaged. Additionally, the widths of the two control fish (and two ethanol-exposed) were averaged, normalized and compared.

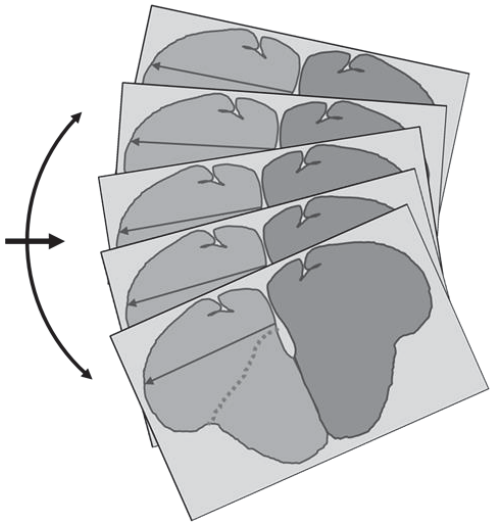

Figure 5: Designated 'transverse plane' XZ Slices
This illustration shows the center target XZ slice (*left horizontal arrow*) as well as the additional contiguous slices (minimum of 12 in each direction, shown by *curved arrow*) in the rostral and caudal directions. The bi-lobed telencephalon is schematically shown in *medium and dark gray*. The *dotted line* demarcates the upper pallial and lower sub-pallial regions. The upper pallial area consists of regions similar to that found in humans, namely the amygdala, isocortex and hippocampus [16]. The *small arrows* shown in the left lobes in the stacked slices indicate distances that were measured and compared.

2.7 Normalization of Data

Post-scanning analysis involved normalizing the lobular telencephalon widths for each subject. To do this, we measured the head size using the points immediately rostral to

the optic socket from the video image provided during scanning (see Figure 2). The two control head sizes were averaged and then all data was normalized to that value. The head sizes were accurately assessed since each recorded video image contained the 'white box' described in Figure 2, which was 1.9mm in the orientation of the head size measurement.

2.8 Comparison of Lobular Widths

The zebrafish telencephalon is made up of two lobes as shown in the schematic slices in Figure 5. In each of these slices, the *small arrows* represent the measured distance defined as lobular widths. The normalized data of the lobular widths of the pallial telencephalon are shown in Figure 6. Control zebrafish are compared with those chronically exposed to ethanol. The data were compared using a paired *t*-test, which demonstrated a significant difference between the two groups ($p < 0.001$; two-tailed).

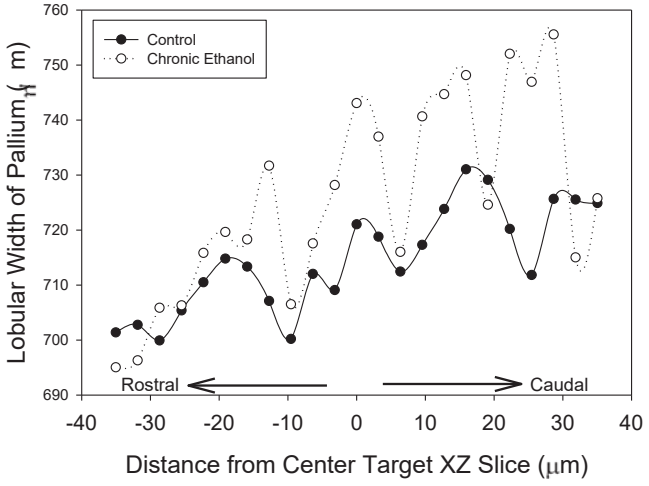

Figure 6: Telencephalon Lobes from Control Zebrafish and those Chronically Ethanol-Exposed The graph compares the lobular widths measured from the brain scan XZ slices of control zebrafish (*solid line* and *filled circles*) with those measured from brain scan XZ slices of zebrafish chronically exposed to ethanol (*dotted line* and *open circles*). Each symbol represents the mean from two zebrafish (after first averaging the measurements obtained from two independent experimenters). The data points were linked using spline lines to better visualize the surface contour of the telencephalon. The center target XZ slice (*vertical dashed line*) is given the value zero, and the distance of each slice from the center target is given in μm, progressing in the rostral and caudal directions.

The data was also compared using an *f*-test to compare variances between the two groups. The results also indicated a significant difference ($p < 0.01$). These observations suggest that the telencephalon in the zebrafish that were chronically exposed to ethanol was edematous and exhibited an abnormal uneven surface contour.

3 Conclusions

Since this ethanol-induced physical change to the region of the telencephalon that contains hippocampal and amydala nuclei, we would expect that the zebrafish chronically exposed to ethanol would behavioral challenges associated with spatial navigation and emotional responses to novel environment or presence of a predator. Permanent behavioral issues, such as aberrations in navigating through a familiar or unfamiliar tank or environment, would be expected. We intend to examine these neurophenotypic behaviors. Our findings indicate that the telencephalon in the zebrafish that were chronically exposed to ethanol was edematous, consistent with the long-standing hypothesis of overhydration [17] that occurs during the withdrawal period of alcoholism. Our subjects were tested 11 days after alcohol withdrawal. Curiously, the hippocampus appears particularly susceptible to the damaging effects of chronic ethanol ingestion, as evidenced by a significant volume loss in active alcoholic human subjects relative to controls [18]. The area of the telencephalon that we measured contains the hippocampal region. We think that there was an alcohol-induced pathology to this area that proceeded to experience an overhydration during the withdrawal period, resulting in an edematous-structure that exhibited an abnormal uneven surface contour.

4 References

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