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A novel method for automated tracking and quantification of adult zebrafish behaviour during anxiety

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Highlights

- Custom-made economic imaging setup for distraction-free behavioural recording.
- ImageJ based algorithm for fast and automated tracking of Zebrafish without plugins.
- Mathematical workflow for extraction of behavioural endpoints.
- New representation for spatio-temporal nature of choice-based behaviours.

Abstract

Background

Behavioural neuroscience relies on software driven methods for behavioural assessment, but the field lacks cost-effective, robust, open source software for behavioural analysis.

New Method

Here we propose a novel method which we called as ZebraTrack. It includes cost-effective imaging setup for distraction-free behavioural acquisition, automated tracking using open-source ImageJ software and workflow for extraction of behavioural endpoints. Our ImageJ algorithm is capable of providing control to users at key steps while maintaining automation in tracking without the need for the installation of external plugins.

Results

We have validated this method by testing novelty induced anxiety behaviour in adult zebrafish. Our results, in agreement with established findings, showed that during state-anxiety, zebrafish showed reduced distance travelled, increased thigmotaxis and freezing events. Furthermore, we proposed a method to represent both spatial and temporal distribution of choice-based behaviour which is currently not possible to represent using simple videograms.

Comparison with Existing Method(s)

ZebraTrack method is simple and economical, yet robust enough to give results comparable with those obtained from costly proprietary software like Ethovision XT.

Conclusion

We have developed and validated a novel cost-effective method for behavioural analysis of adult zebrafish using open-source ImageJ software.

Keywords:

Automated Tracking; Zebrafish; ImageJ; Anxiety Behaviour; Open field test; ZebraTrack; DanioJ 1.0.

1 Introduction

Behaviour is defined as "the internally coordinated responses (actions or inactions) of whole living organisms (individuals or groups) to internal and/or external stimuli" (Levitis et al., 2009). Manual scoring of behavioural endpoints, which often consists of motor output, is laborious and subjective (Jhuang et al., 2010). To overcome these limitations, computational approach for automated behavioural analysis is preferred over manual one. Computational based approach for behavioural analysis consists of three distinct steps: a) Acquisition; b) Tracking; c) Extraction. Acquisition starts with video recording of live behaving animals in distraction-free condition. In the tracking step, software-driven digitization of acquired videos is done. The main purpose of this step is to determine the precise spatio-temporal location (2D and 3D coordinates) of the animal in each video frame. Finally, in the extraction step, mathematical calculations on the animal's 2D or 3D coordinates are done to extract several behavioural endpoints (e.g. total distance travelled by animal, number of freezing bouts, turn angle etc.).

Currently, several proprietary software programs are available for automated tracking and analysis of animal behaviour e.g. Ethovision (Noldus), Zebralab (Viewpoint), Visiotracker (TSE systems), ANY-maze (Stoelting Company), Matlab/plugins, iPhone app etc (Blaser and Rosemberg, 2012; Cachat et al., 2010; Delcourt et al., 2006; Grossman et al., 2010; Kabra et al., 2013; Mueller et al., 2011; Pittman and Ichikawa, 2013; Rosemberg et al., 2011; Zhong et al., 2014). Unfortunately, not only are these software programs closed-source (not amenable to modifications by end users), but many of the popular and robust ones are also very expensive.

Several groups have made their own software/plugins for automated tracking of live animal and made them available in a license-free agreement (Colwill and Creton, 2011; Creton, 2009; Gomez-Marin et al., 2012; Kato et al., 2004; Perez-Escudero et al., 2014; Ramazani et al., 2007; Wang et al., 2014). But some of these options are either lab specific and have never been validated by other groups or require specific computer configuration/robust coding skills. A cost-effective customizable option for automated tracking and behavioural analysis of adult zebrafish is not yet widely available.

In this report, we propose a method which we called as ZebraTrack. It includes cost-effective imaging setup for distraction-free behavioural acquisition of adult zebrafish in the open field test tank, automated tracking of adult zebrafish using open-source ImageJ software and workflow for extraction of behavioural endpoints. We have validated our method by demonstrating spatio-temporal assessment of novelty induced “state-anxiety” behaviour in heterogeneous population of adult zebrafish. Results obtained by our ZebraTrack method are comparable with the results obtained from leading commercial software like Ethovision XT.

2 Materials and Methods

2.1 Animals and their maintenance

We purchased 30 adult zebrafish (*Danio rerio*) of heterogeneous genetic makeup of mixed gender from a local vendor. Zebrafish expressed red fluorescent protein in their muscles similar to that mentioned elsewhere (Gong et al., 2003; Ji et al., 2005; Snekser et al., 2006; Vick et al., 2012). Zebrafish were maintained in rectangular tanks as described previously (Nema and Bhargava, 2016). Zebrafish underwent at least four weeks of acclimatization period in the laboratory environment before being used for the behavioural studies. All animal handling and experiments were performed in accordance with the approved protocols and guidelines prescribed by the Committee for the Purpose of Control and Supervision of

Experiments on Animals (CPCSEA), Government of India. No animals were killed at the end of the experiment.

2.2 Setup for behavioural acquisition

Behavioural acquisition was performed in a custom-made imaging setup (Figure 1A). The setup consisted of light weight wooden ply pieces installed in a U-shaped arrangement to minimize the distraction from the sides. ZebraTrack method required high contrast between the background and the zebrafish for automated tracking. Therefore, the background was illuminated (1000 – 1200 Lux) using white LED light sheet (an array of white LED lights made by gluing 15 – 20 white LED light strips each containing 20 – 25 individual LED lights on a plastic sheet). A translucent white plastic sheet was always placed on top of white LED light sheet to diffuse the light emanating from the LED. This light diffuser sheet also served as a platform for placing open field test (OFT) tank for behavioural measurement. This arrangement acted like a light box and produced the desired contrast between background, which appeared bright and zebrafish which appeared dark. Our OFT tank was a transparent plastic chamber with dimensions 29 x 37 x 18 (W x L x H in cm) containing 5 cm of water (Figure 1B). The size of the tank was similar to that used in other studies (Stewart et al., 2010; Stewart et al., 2012). Water in the OFT tank was always replaced with fresh water before each behavioural measurement. The video acquisition was done using a cost-effective webcam (Zebion Inc, India) placed in “top scan configuration” (i.e. camera facing down) at the height of ~100 cm relative to the bottom of the OFT tank so as to cover its complete dimension. The video files were recorded in AVI format at 10 frames per second (fps) using H264 (Native) video encoder of basic free version of “Debut video capture” software v2.02 (NCH software) installed on Windows 7 PC. H264 encoding was preferred because it resulted in small sized output of AVI files with better graphic quality.

2.3 Treatment and Behavioural test

A total of twenty one zebrafish (mixed gender) were used in behavioural recordings during four trials. Each zebrafish was naïve and used only once. Zebrafish were transferred from their housing tanks to a holding tank (made of translucent plastic) an hour before the start of experiment. From the holding tank zebrafish were individually transferred to the OFT tank by gentle netting. It is important to mention that between each experiment OFT tank was thoroughly rinsed and filled with fresh, three-stage purified water at a temperature of 28 ± 2 ^{o}C. Exploratory behaviour of zebrafish in brightly lit OFT tank (~1000 Lux) was recorded for a little more than 6 minutes so as to record all the events including the transfer of zebrafish to the OFT tank. The transfer time of zebrafish from the holding tank to the OFT tank was kept minimal (~2 – 5 s). At the end of experiment no fish were killed. For the validation of ZebraTrack method, behavioural endpoints of adult zebrafish observed during the first three minutes (pre) were compared to the last three minutes (post).

2.4 Method for automated 2D tracking of adult zebrafish using ImageJ

This method involved video processing steps and using them for automated tracking of zebrafish.

2.4.1 Video processing: Segmentation step

The current version of ImageJ (v1.50a, 64 bit) could not recognize H264 video encoded AVI files. Therefore, open source Virtual Dub software (v1.10.4, 64 bit) was used in order to open and segment each recorded video into pre (first three minutes) and post (last three minutes) video sets. Before videos were segmented to pre and post sets, editing of zebrafish transfer process was required. For this editing time of zebrafish in the tank was assumed only when the fish transfer net was out of the video frame. In the virtual Dub software, the next immediate frame in which the fish transfer net was not visible was considered as the start frame for pre video segments (t = 0 s). The segmentation was done using the ***select range*** option available in the ***video*** tab located in the main menu bar of the Virtual dub software. After the range assignment for segmentation, the video was saved as uncompressed AVI file which was significantly bigger in size (e.g. the size increased by more than 100 times).

2.4.2 Video processing: Cropping and video filtering

The segmented AVI video files were individually opened as virtual stack with grey scale/8 bit filtering option using ImageJ software. The video was cropped using a user defined region of interest (ROI) by placing it close to the water level around the wall of the OFT tank. The cropped videos were saved again as AVI files with JPEG compression at 10fps using the ImageJ software. The cropping significantly reduced the video size (file size from several GB reduced to few MB).

2.4.3 Automated tracking: thresholding and tracking

A short script was written using ImageJ software to automatically track the centroid of adult zebrafish. We called this short script as “DanioJ 1.0”. It involved steps for brightness and contrast adjustment, blurring the frames using Gaussian blur and applying the unsharp mask. It also involved threshold adjustment to create a binary stack so as to separate zebrafish as white pixel from the uniform dark background. Finally, particle analyzer step was used to automatically track the centroid of zebrafish in each video frame. To use DanioJ 1.0 as ImageJ plugin/macro, the following DanioJ 1.0 script should be copied and pasted in a blank notepad and be saved it as DanioJ1.0. The ***Install*** option located in the ***Plugin*** tab of the ImageJ main menu should be used to save the DanioJ1.0 notepad file in any Plugin folder.

```
//Start of DanioJ 1.0 script//
title=getTitle(); run("8-bit"); run("Invert", "stack");
run("Brightness/Contrast..."); waitForUser("Please move <Minimum> slider rightward before
pressing OK."); selectWindow("B&C"); run("Apply LUT", "stack"); run("Gaussian Blur...",
"sigma=2 stack"); run("Unsharp Mask...", "radius=5 mask=0.90 stack"); run("Gaussian
Blur...", "sigma=2 stack"); run("Enhance Contrast...", "saturated=0.4 normalize process_all");
run("Threshold..."); setAutoThreshold("Intermodes dark"); setThreshold(255, 255);
setOption("BlackBackground", true); run("Convert to Mask", "method=Intermodes
background=Dark black"); selectWindow("Threshold"); run("Close");
run("Set Measurements...", "area centroid stack redirect=None decimal=3"); run("Analyze
Particles...", "size=50-Infinity display summarize stack");
//End of DanioJ 1.0 script//
```

Users can modify/troubleshoot DanioJ 1.0 script to suit their need by changing values of Sigma e.g. 2 – 5 at Gaussian Blur step, Radius e.g. 2 – 10 at Unsharp Mask step and white pixel Size e.g. 0 to Infinity at Analyze Particles Size step. The default particle size to track in particle analyzer listed in DanioJ 1.0 is 50 – Infinity, meaning the DanioJ 1.0 script will not track any white pixel of area below size 50. It should be noted that after adjusting the “minimum” slider of brightness and contrast pop up, the “apply” button need not be pressed to apply the adjustment. The script automatically does that. It is also important to note that the slice numbers should always match with the serial numbers in the result output window produce by this script. If any discrepancy is observed between them, users are encouraged to modify/troubleshoot DanioJ 1.0 script as suggested above.

Although 32 bit and 64 bit versions of ImageJ were found to be equally capable of automated tracking, the 64 bit ImageJ version was found to be better than the 32 bit version because it offers larger memory size and can handle more threads. This matters especially when working with huge files. Complete tracking of a single fish from video segmentation to automated tracking using DanioJ 1.0 script is possible within ~10 – 25 minutes depending on the computer hardware.

2.5 Method for extraction of behavioural endpoints

2.5.1 Total distance travelled by zebrafish

Automated 2D tracking of zebrafish using ImageJ resulted in a list of X and Y locations of zebrafish centroid in all the video frames. Total distance travelled by zebrafish in a video was calculated by summing up the distance travelled by zebrafish in each successive frame as shown in equation 1 – 3.

$$Total\ distance\ travelled = \sum D2P \qquad \text{Equation 1}$$

$$D2P = \sqrt{(\Delta X)^2 + (\Delta Y)^2} \qquad \text{Equation 2}$$

$$(\Delta X = X_{next} - X_{previous}) \text{ and } (\Delta Y = Y_{next} - Y_{previous}) \quad \text{Equation 3}$$

In these equations, D2P represents the distance travelled by zebrafish between successive frames, and ΔX and ΔY are the differences between locations of X and Y in successive frames respectively. X_{next}, $X_{previous}$ and Y_{next}, $Y_{previous}$ are successive X and Y frames respectively.

2.5.2 Choice-based behaviour of zebrafish

To quantitatively determine the choice (wall or central area) of zebrafish in OFT tank, we first determined its actual location in the tank with respect to the four walls in each video frame. Actual wall distance (AWD) of zebrafish in each video frame was determined using equation 4.

$$(AWD) = (R_{OFTwall}) - \left(R_{fish}\right) \qquad \text{Equation 4}$$

where, $R_{OFTwall}$ and R_{fish} represent the resultant vectors of video frame and fish respectively. These were calculated using equations 5 – 8.

$$R_{OFTwall} = \sqrt{(X_{max})^2 + (Y_{max})^2} \qquad \text{Equation 5}$$

where, X_{max} and Y_{max} represent the maximum width and height of the video frames along the X and Y axes respectively. For example video frame of size 300 x 250 px would have X_{max} = 300 and Y_{max} = 250.

$$R_{fish} = \sqrt{(\Delta X_f)^2 + (\Delta Y_f)^2} \qquad \text{Equation 6}$$

$$\Delta X_f = \left(X_{auto} - (X_{max} - X_{auto})\right) \qquad \text{Equation 7}$$

$$\Delta Y_f = \left(Y_{auto} - (Y_{max} - Y_{auto})\right) \qquad \text{Equation 8}$$

where, X_{auto} and Y_{auto} represent the X and Y locations of zebrafish in each video frame obtained through automated tracking using DanioJ 1.0. Therefore, the overall equation for calculation of Actual Wall Distance (AWD) of zebrafish in each video frame could be written as:

$$AWD = \left(\sqrt{(X_{max})^2 + (Y_{max})^2}\right) - \left(\sqrt{\left(X_{auto} - (X_{max} - X_{auto})\right)^2 + \left(Y_{auto} - (Y_{max} - Y_{auto})\right)^2}\right) \qquad \text{Equation 9}$$

2.5.3 Thigmotaxis time of zebrafish

Thigmotaxis represents movement of an organism close to the wall. We asked how much distance from the wall should be considered as close to the wall. It was essential to determine because the OFT tank in our study was rectangular rather than a square box. It means that the distance from the centre to the walls would be different for X and Y axis walls. Thus, we calculated the Near Wall Distance (NWD) using equation 10.

$$(NWD) = (AWD) - (AWD_{mean}) \qquad \text{Equation 10}$$

where, AWD is the Actual Wall Distance of zebrafish calculated using equation 9. AWD_{mean} denotes the mean value of AWD of each fish. By doing this simplification we could reduce the location of zebrafish (centroid) into two mutually exclusive choices either close to the walls or close to the centre.

The fish was considered to be thigmotactic in all the frames in which the AWD value of zebrafish was lower than its mean AWD value (i.e. negative NWD values as per equation 10). Automated extraction of negative NWD values from the NWD column for each fish was done using "if" operator in the worksheet-query option of Origin 8.6. Following this extraction, statistical analysis was done to count the total number of extracted frames. Finally, the thigmotaxis time was calculated by converting total number of extracted frames into time using video acquisition rate (in our case the videos were acquired at 10 frames per second).

2.5.4 Shuttling frequency of zebrafish between wall and centre of the tank

A short script in Origin 8.6 was used to determine the number of transitions a zebrafish made between the walls and the central zone of the OFT tank. This script automatically counted the number of times a transition occurred in NWD values (as determined from equation 10) from negative to positive (walls to centre) and positive to negative (centre to walls).

```
//Start of shuttling frequency Script//
ncols=wks.ncols; for(ii=1; ii<=ncols; ii++)
{range rr=wcol(ii); nrows=rr.getsize(); nneg=0; npos=0;
for(jj=2; jj<=nrows; jj++)
{if(wcol(ii)[jj-1]<0 && wcol(ii)[jj]>0) npos++;      //count up positives
else if(wcol(ii)[jj-1]>0 && wcol(ii)[jj]<0) nneg++;  //count up negatives
}
type -a col:$(ii) colname:%(wks.col$(ii).name$):
Pre sets (Move to centre = Count –ve to +ve): $(npos):
Post sets (Move to wall = Count +ve to -ve):  $(nneg);
}
//End of shuttling frequency Script//
```

2.5.5 Calculation of freezing behaviour: freezing bouts

A displacement in the centroid of the fish by 0.1 cm or less per frame was considered as freezing/inactivity. D2P values satisfying this freezing criterion were extracted along with their corresponding frame numbers using “if” operator on the D2P column using the worksheet-query option of Origin 8.6. Accordingly, a period of inactivity for one or more frames in continuation was termed as the freezing bout. To test the continuity of the extracted frames, equation 11 was run on the extracted D2P values as "set column value" command.

((i==1)?:(col(1)[i]-col(1)[i-1]-1)!=0)?i:col(2)[i-1] Equation 11

This equation would mark all the extracted D2P values in the same freezing bout if the continuity of their corresponding extracted frames were maintained. If the continuity of the extracted frames was found to be broken, the remaining extracted D2P values were assigned as next freezing bout till the continuity of their corresponding extracted frames was maintained and so on. Therefore a freezing bout could consist either of a single frame or several frames. The total number of freezing bouts was calculated by counting all the discrete bouts of inactivity.

2.5.6 Calculation of freezing behaviour: freezing duration

Total freezing duration for each zebrafish was calculated by summation of total number of extracted frames in which the fish satisfied the freezing criterion (see above). Number of extracted frames were appropriately converted into time in seconds using the video acquisition rate (i.e. 1 frame = 0.1 s or 10 fps).

2.5.7 Assessment of angle-based behaviour

The turn angle of zebrafish between frames was calculated as theta (θ) degree from the slope using equation 12. The slope was determined as the ratio of ΔY and ΔX (as determined using equation 3).

$$(\theta) = \frac{\left(\left(\tan^{-1}\left(\frac{\Delta Y}{\Delta X}\right)\right) * 180\right)}{\pi} \qquad \text{Equation 12}$$

The absolute turn angle as the sum of all the turn angles was calculated for each zebrafish without considering the sign of angular direction (positive and negative signs denote clockwise and counter-clockwise turns by zebrafish between successive frames respectively) (Rosemberg et al., 2011). Meandering was calculated as the absolute turn angle taken by zebrafish per meter of locomotion. Angular velocity was calculated as degree of turn per second.

2.5.7 Spatio-temporal representation of motor behaviour

Videogram is a superimposed representation of all the locations of zebrafish acquired in certain duration. In the ImageJ, we created videogram by using minimum intensity option of Z-projection which was accessed through ***stack*** option of ***Image*** tab in the menu bar. Minimum intensity option was used because in our measuring conditions zebrafish appeared dark over light background. Heat-map based videograms were generated using "heat-map from stack" plugin (Pean, 2012). We applied the thermal LUT on 2x2 grids and used the absolute with log10 transformation as calculation method for these panels. Absolute calculation method was preferred over relative because of high density superimposition of zebrafish in the videogram.

Dyadic plot is a simplified spatio-temporal representation of the centroid of zebrafish in mutually exclusive locations i.e. walls or central zone of the OFT tank. Dyadic plots were constructed in following manner. First, using "if" operator, all the positive (centre location) and negative (walls location) NWD values (as determined using equation 10) were converted into 1 and 0 (binary values) respectively. Next, these binary NWD values were then converted into 1 and -1 values through simple mathematical function using "set column values" option e.g. [((Column of binary NWD values) * 2) -1]. Finally, these 1 (centre) and -1 (walls) values were plotted along the time as histogram.

2.6 Statistics

All data were analysed using Graphpad prism version 5. Normality distribution of the data was analysed using normality test in Graphpad prism. For parametric data, paired two tailed t-test was used. For non-parametric data, Wilcoxon signed rank test was used for paired pre (first three minutes) and post (last three minutes) values. P value of <0.05 was considered significant, where * denotes P<0.05; ** denotes P <0.005 and *** denotes P<0.0001. Appropriate conversion of pixels to meter or cm was done before doing statistical analysis in every measurement.

3 Results

3.1 Automated tracking for determination of 2D coordinates of zebrafish in the OFT tank

The pre-requisite for automated tracking is good contrast between the animal and its background. Sometimes, background intensity fluctuation, fast movement of animals and video acquisition at low frame rates could resulted in errors like frame dropping (software failure in recognizing the animal), erroneous reporting of animal's centroid etc. These issues are already reported with many commercial software programs (Cachat et al., 2011). One of the issues related to erroneous reporting of animal's centroid is highlighted in the figure 2, where ANY-maze (trial version 4.99m) could recognize the centroid of the fish in the first frame, but failed to consistently locate the fish in all video frames. In many commercial software programs, these issues can be best resolved by adjusting the threshold intensity between the animal and its background. However, finding the single threshold intensity for the entire video could be an issue especially with videos with non-uniform background. In contrast, DanioJ 1.0 consistently tracked the fish by locating its centroid in each video frame virtually at zero cost compared to costly commercial software programs.

3.2 ZebraTrack workflow for extracting information about mean distance travelled by zebrafish

Mean distance travelled by zebrafish (in meter) during exploration in the OFT tank is shown in figure 3A. We found that as zebrafish spent more time in OFT tank, their locomotary profile was reduced, as indicated by a significant reduction in their mean distance travelled during the last three minutes as compared to the first three minutes.

3.3 Determining choice-based behavioural features

In the OFT tank, the walls and central area represent two distinct zones. To quantitatively determine choice of zebrafish between walls and central area, we determined the Actual Wall Distance (AWD) as described previously in the methods section. It is a measure of spatial distance of the zebrafish from walls of the OFT tank. Figure 3B shows the average AWD of zebrafish. We found that as fish spent more time in the tank, AWD decreased significantly. As the OFT tank was rectangular, the distance between centre to all four walls was not symmetrical, therefore, using Near Wall Distance (NWD) we reduced the spatial location of zebrafish in two mutually exclusive zones *viz.* walls or central zone. NWD is a measure of thigmotaxis behaviour. Figure 3C shows the average time spent by zebrafish closer to the walls as thigmotaxis time. It is clear from the results that zebrafish showed time dependent preference towards walls as compared to the centre of the OFT tank. This is also evident in figure 3D where average shuttling frequency towards the centre is plotted. An inverse relation between the time spent in the OFT tank and the average shuttling frequency towards the centre was observed. Thus, our results suggest that the choice of zebrafish shifted towards walls as they spent more time in the OFT tank. Therefore increased thigmotaxis behaviour by zebrafish in our study indicates an increase in anxiety in them (Cachat et al., 2010).

3.4 Determination of freezing behaviour

Here we determined freezing bouts and percent freezing time as freezing behaviour. Number of freezing bouts shown by zebrafish during the pre and post video sets is plotted in figure 4A. We found time dependent increase in freezing bouts. The fraction of time zebrafish showed freezing behaviour is plotted as percent freezing time in figure 4B. Although, time dependent marginal increase in freezing duration was observed, this increment was found to be statistically insignificant.

3.5 Assessment of angle-based behavioural endpoints

Angle-based behavioural endpoints also provide vital information about the state of zebrafish e.g. zebrafish in anxiety show sudden and erratic change in swim angles (Cachat et al., 2010). The turn angle of each zebrafish between frames was calculated as theta (θ) degree from the slope using equation 12. The average absolute turn angle of zebrafish is plotted in figure 5A. We found virtually no change in absolute turn angles of zebrafish over time. Similarly, meandering (absolute turn angle taken by zebrafish per meter of locomotion) and angular velocity (degree of turn per second) remained unchanged even if zebrafish spent more time in the OFT tank (Figure 5B and C respectively).

3.6 Videogram and dyadic plot representations

Figure 6A and B show representative videograms of one of the zebrafish exploration during first and last three minutes respectively. Same brightness adjustment was done in both these figures to reduce noise from the background. Unfortunately, behavioural interpretation is very difficult using such videograms because neither the information related to spatial preferences nor the temporal distribution of zebrafish locations is possible to extract. Figure 6C and D are representative heat-map videograms of the same zebrafish shown in figure 6A and B respectively. Heat-map resulted in colour-coded spatial distribution of zebrafish. Red colour indicates high occurrence of zebrafish in that location and is linked with spatial preference. These "hot-spots" are called homebase. These figures clearly show time dependent changes in the spatial preference i.e. this zebrafish preferred the left-hand side wall of the tank in the last three minutes of exploration (Figure 6C and D). Heat-map based videograms have good spatial resolution of choice-based behaviour of zebrafish, but they lack temporal resolution of such behaviour. Therefore, we created "dyadic plots" to represent spatio-temporal nature of choice-based behaviour of zebrafish between two mutually exclusive events. Figure 6E and F show representative dyadic plots of the same zebrafish as shown in figure 6A and B respectively. Based on these results we can infer that especially during the last three minutes of measurement, anxiety of this zebrafish increased as it showed diminished movement towards the central zone of the OFT tank.

3.7 Comparison between behavioural endpoints obtained through Ethovision XT and our ZebraTrack method

Ethovision XT is the preferred behavioural analysis software among neuroscientists; therefore, we decided to compare the output of our ZebraTrack method with that of Ethovision XT. With the assistance of an expert from Noldus IT Inc., the behavioural endpoints of a single zebrafish for the pre and post videos were determined. Table 1 lists output of behavioural endpoints obtained from Ethovision XT and ZebraTrack method. It was found that both these methods reported similar trend in behavioural endpoints produced by the zebrafish during the first and the last three minutes *viz.* reduced distance travelled and reduced velocity with marginal increment in angular velocity in the last three minutes compared to the first three minute. The data on freezing bouts and freezing duration was also found comparable among these methods. Furthermore, the qualitative assessment of choice-based behaviour of the zebrafish between the first and the last three minutes also resulted in identical observation. Based on these results, it appears that our method is robust and produces comparable results as those obtained through commercial software programs like Ethovision XT.

4 Discussion

We tested ImageJ potential for automated behavioural analysis because it is an open-source, scientific image processing software with vast end user base (Bearer, 2003; Hartig, 2013). Apart from this, it is maintained and constantly improved by an active. However, the application of ImageJ for automated tracking of adult zebrafish is still limited, possibility due to the bigger size of adult zebrafish, their fast locomotion, bigger arena and complex behavioural repertoires as compared to their larval stage (Kalueff et al., 2013). Even the macros written in ImageJ for zebrafish larvae (Clift et al., 2014; Colwill and Creton, 2011; Wyeth et al., 2011) are complex and not usable for the behavioural analysis of adult zebrafish. This motivated us to develop ZebraTrack, a cost effective method for automated behavioural analysis involving the use of ImageJ.

Our ZebraTrack method consists of three major steps a) Distraction-free behavioural acquisition using custom-made cost-effective behavioural imaging setup; b) Rapid and robust automated tracking of adult zebrafish using open source, license free ImageJ software. The power and speed of ImageJ based automated tracking is only limited by the computer resources at hand; c) The use of mathematical workflow for handling large amount of data and rapid extraction of several behavioural endpoints which are otherwise only possible using specially developed or licensed expensive commercial software programs like Ethovision XT etc. We have validated our method by demonstrating behavioural insight into adult zebrafish when exposed to novelty induced “state-anxiety”. Furthermore a direct comparison of behavioural endpoints produced by Ethovision XT and ZebraTrack method showed comparable outcome. We therefore believe, to many labs, such an economical and reliable method may be more appealing in comparison with the methods that use expensive commercial software.

4.1 The choice of OFT for behavioural analysis in adult zebrafish

OFT has been used to assess behaviours like emotionality, motivation, fear, exploration and anxiety in rodents (Blizard et al., 2007; Walsh and Cummins, 1976). In the OFT, the animal is placed in either round or rectangular arena. Round arena is often preferred over rectangular one to avoid potentially modulating animal’s behaviour because of the presence of corners. However, recent studies showed that animals’ behaviour has been found to be independent of arena geometry (Kulikov et al., 2008; Stewart et al., 2012). Once placed in the arena, the animal is tracked for varying lengths of time (e.g. 2 minutes to 1 hr). Several studies have used 30 minutes as standard recording time but the choice of such longer durations over shorter one has never been discussed in detail. Recently, a study on zebrafish unravelled the occurrence of robust oscillations in their several behavioural endpoints every 5 – 7 minutes (Stewart et al., 2012). Interestingly, this study also reported that the repeated nature of behavioural endpoints was independent of the size of OFT arena, thus, indicating that behavioural investigation using zebrafish could possibly be valid on shorter duration measurements. In this study, we used the OFT tank similar to tank 3 in their study (Stewart et al., 2012) and measured the novelty induced anxiety behaviour in zebrafish for 6 minutes duration. We assessed changes in their locomotion behaviour between the first and the last three minutes of their exploration in the OFT tank.

4.2 Custom designed economic imaging setup

Distraction-free behavioural acquisition of subjects in a uniformly lit environment was made possible in our study by the use of a custom-made behavioural imaging setup for zebrafish as described in the methods section. Our imaging setup design has several advantages: 1) it offered easy access to the testing tanks for zebrafish placement and replacement, 2) the covered side walls minimized environmental influence on the behaviour of zebrafish, 3) both top and side configuration of camera placement is possible, 4) the unique placement of LED array and white sheet served as a light-box and a platform for holding behavioural tank, while also providing the desired contrast between the background and the animal, 5) the same design can be used for zebrafish larval behavioural recording and 6) unlike the tall cabinet used in larval studies (Colwill and Creton, 2011), our design offers the ease of working coupled with natural aeration and ambient distraction-free surroundings for animals under observation.

4.3 Validation of ZebraTrack method using several behavioural endpoints

Our results showed that social separation and placement of adult zebrafish in the brightly lit novel arena of the OFT tank was sufficient to induce "state-anxiety" as indicated by reduced mean distance travelled coupled with significant reduction in the frequency to visit central region of the OFT tank (Figure 3). Zebrafish preferred to stay close to the walls over visiting central region of the tank in a time dependent manner. The reduction in total distance travelled after the first three minutes of stay in the OFT tank is attributed to the significant increase in the freezing bouts (Figure 4), which is a hallmark feature of "state-anxiety"

(Godwin et al., 2012). This raised a question if novel environment in our study had induced mild or severe anxiety in zebrafish. It is reported that chemical cues liberated by injured or severely stressed fish could modulate anxiety in zebrafish as shown by increased erratic movements (Jesuthasan, 2012; Mathuru et al., 2012). Increased erratic movements are intrinsically linked to increase in angular parameters. However, in our experimental setup, we overruled the possibility of chemical build-up of fearful cues liberated (if any) by zebrafish under acquisition by thorough rinsing of the OFT tank and changing tank water for each fish before measurement. Therefore, to test the severity of anxiety in zebrafish, we measured angle-based behavioural endpoints. Interestingly, we found no change in behaviour involving angular parameters (Figure 5), thus indicating that placement of zebrafish in the novel arena induced "state-anxiety" of mild nature. It is important to note that our OFT tank was transparent which could motivate them to explore options for escape; hence they preferred to stay close to the walls or corners. These results are in agreement with the ethologically relevant behaviour of animals that they prefer to stay close to the walls or corners when exposed to novelty induced "state anxiety" (Eilam and Golani, 1989; Stewart et al., 2010). We also realized that representation of zebrafish locomotary profile via videogram lacks temporal resolution. To this end, we proposed the use of dyadic plots. Dyadic plots are useful in representing both spatial and temporal nature of choice-based behaviour of any subject under investigation. We therefore expect to see more applications of dyadic plots by behavioural neuroscientists as these plots are easy to generate and spatio-temporal extraction of behaviours is possible through the single graph.

Based on several behavioural endpoints scored by Ethovision XT and our ZebraTrack method, comparable behavioural trends were observed (Table 1). Both these methods reported time dependent increase in the anxiety of the zebrafish under investigation. Despite the fact that this comparison between Ethovision XT and our ZebraTrack method was based on the outcome of a single, randomly selected zebrafish, several behavioural endpoints produced by these methods resulted in consistently similar outcomes. The initial comparison between Ethovision XT and our ZebraTrack method produced encouraging results with a suggestion that our method is also robust and reliable for behavioural determination. However, it remains to be seen how our method would fare in terms of its frequency of erroneous reporting of centroid of fish and frame dropping (if any) under various recording conditions. Considering the open source nature of ImageJ, further development of DanioJ 1.0 by community driven contribution would certainly enhance capabilities of ImageJ in behavioural neuroscience.

Finally, it has been reported in various studies that zebrafish shows characteristic anxiety like behaviour e.g. wall preference, dwelling at the bottom of the tank, reduced distance travelled, more freezing etc when placed in a novel environment. However, if allowed to stay in the environment without any external threat, the intensity of such anxiety-like behaviour would show time dependent reduction e.g. increased distance travelled, less freezing and more visit to central zone etc (Cachat et al., 2011; Rosemberg et al., 2011). Interestingly, zebrafish in our behavioural studies showed a contrasting behaviour which was also validated by Ethovision XT. We found increased anxiety-like behaviour if fish were allowed to stay in the test tank. In our lab, we have consistently observed this behaviour in control zebrafish used for other studies (data not shown). We propose two possible reasons for this deviation. First could be related to illumination of arena. As a normal practice, zebrafish in our lab are maintained in housing tanks with illumination from the top to mimic natural light conditions, whereas, during the behavioural acquisition, we illuminated the test tank from the bottom side. This sudden change in the illumination conditions may have induced “state anxiety” in adult zebrafish in a time dependent manner. The other possibility for the observed behavioural deviation in our study could be attributed to the type of water used for behavioural testing. It is now well documented that fresh water which has not been exposed to the fish could induce anxiety behaviour in them as opposed to the aquarium water which has been shown to reduce the anxiety (Parker et al., 2012; Tran et al., 2016). Since we used fresh water for behavioural measurements in our study, and it was changed for every fish before the start of new experiment, it might have also induced the anxiety behaviour in the fish. Thus, our results seem to be in agreement with the previously published findings.

4.4 Future of ImageJ as behavioural analysis software

We believe that ImageJ capabilities for behavioural analysis could be further enhanced with the help of its active community. For example, in our experiments, we had to rely on Debut video capture software to record the video in H264 (native) video encoded format. This software also provided an option for video acquisition at user defined frame rates. However, ImageJ currently lacks the support for H264 AVI format. Due to this, an extra step for conversion of H264 AVI to raw AVI using Virtual dub software was required. Although ImageJ offers a plugin for webcam but video capture and adjustment of frame-rate features are missing in that plugin. Apart from this, if incorporation of colour correlogram feature of idTracker is possible in the ImageJ, it would help to track unmarked subjects in groups for extended period of time (Perez-Escudero et al., 2014). We believe that incorporation of these important features as plugins would certainly enhance the capabilities and dimensions of ImageJ to be a leading open-source, license free software for behavioural analysis.

Author contributions

Idea and conceptualization by AB and YB.; Experimental validation by SN, WH and YB.; Formal data analysis SN, AB and YB.; Writing – Original Draft by YB.; Writing – Review &

Editing by SN, WH, AB and YB.; Data visualization, supervision, project administration and funding acquisition by YB.

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Figure Legends

Figure 1: Imaging setup. A) Dimensions of the imaging setup. The U-shaped walls were designed to minimize the distraction of zebrafish and simultaneously allow for a convenient access to top or front side for behavioural imaging. The yellow paper on the U-shaped wall was used for ambient light scattering. The tough white sheet placed above the array of LED lights served as a platform for placing OFT tank. **B)** Dimensions of the transparent plastic tank used as the OFT tank.

Figure 2: Comparing ANY-maze and DanioJ 1.0 based method for detection of centroid of zebrafish during automated tracking. The first column shows time lapse images (t = 0 s to 90 s) of the original video acquired at setup. The second column shows time lapse images of same video analyzed by ANY-maze software. The fish is visible as dark elongated structure. Circles and arrows indicate location of fish centroid as computed by ANY-maze. The third column shows time lapse images of the same video analyzed by DanioJ 1.0 script. Arrows indicate location of centroid of zebrafish in various frames.

Figure 3: Distance and choice-based behavioural endpoints. A) Mean distance travelled by adult zebrafish in meters. **B)** Mean of actual wall distance (AWD) (cm) of the adult zebrafish during exploration in the OFT tank. **C)** Mean time (minutes) of thigmotaxis shown by the adult zebrafish. **D)** Average number of times the adult zebrafish visited the walls and the central zone (shuttling frequency). All data are presented as mean ± SEM of 21 subjects. ** denotes $P < 0.005$ and *** denotes $P < 0.0001$.

Figure 4: Freezing behavioural. A) Average number of freezing bouts shown by the adult zebrafish in the OFT tank. **B)** Average time (in percent) the adult zebrafish showed freezing behaviour. All data are presented as mean ± SEM of 21 subjects. * denotes $P < 0.05$.

Figure 5: Angle-based behavioural endpoints. A) Mean absolute (Abs) turn angle made by adult zebrafish during the first and the last three minutes. **B)** Meandering as degree of turn per meter for adult zebrafish. **C)** Mean angular velocity as degree of turn per second. All data are presented as mean ± SEM of 21 subjects.

Figure 6: Spatio-temporal assessment of anxiety behaviour: A,B) Representative videogram of one of the zebrafish under investigation during first and last three minutes respectively. **C,D)** Representative heat-map based videogram of the same zebrafish during the first and the last three minutes respectively. The colour represents value of pixel from 0 (minimum) to 100 (maximum). **E,F)** Representative dyadic plots of the same zebrafish showing choice-based exploratory profile between the walls and the central zone of the tank during the first and the last three minutes of the total exploration time respectively.

Figure 1

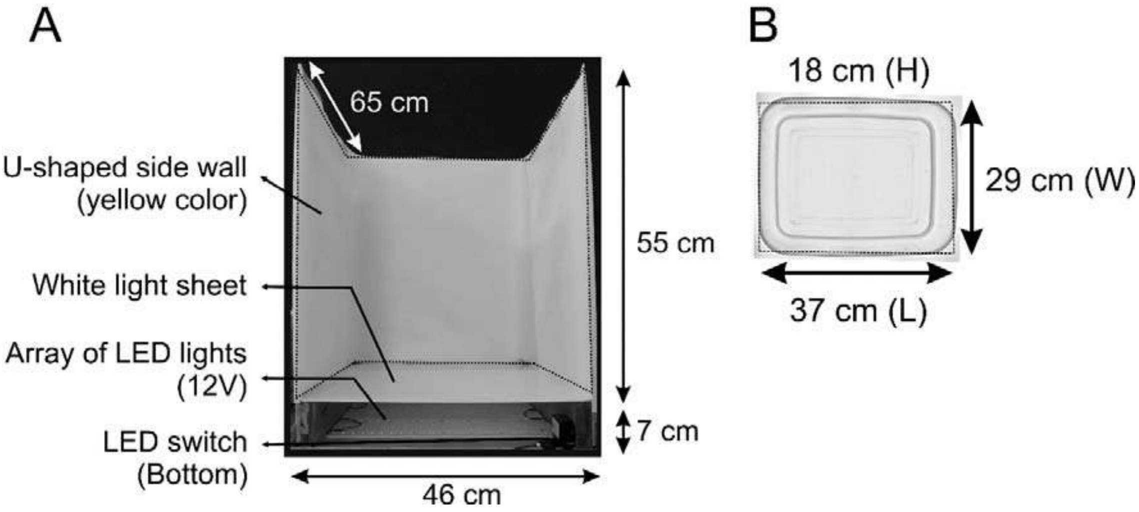
A
65 cm
U-shaped side wall
(yellow color)
White light sheet
Array of LED lights
(12V)
LED switch
(Bottom)
55 cm
7 cm
46 cm
B
18 cm (H)
29 cm (W)
37 cm (L)

Figure 2

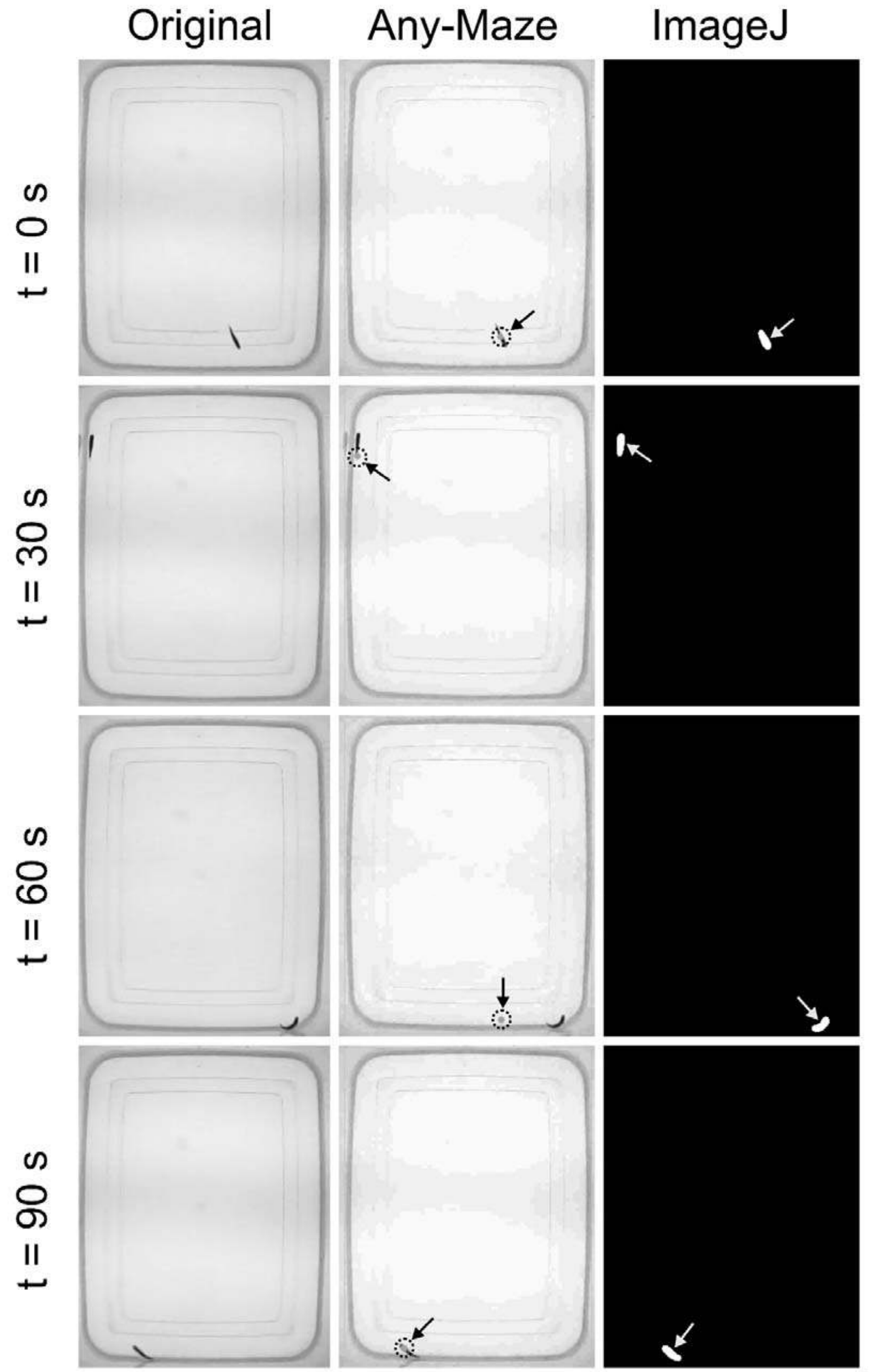

Figure 3

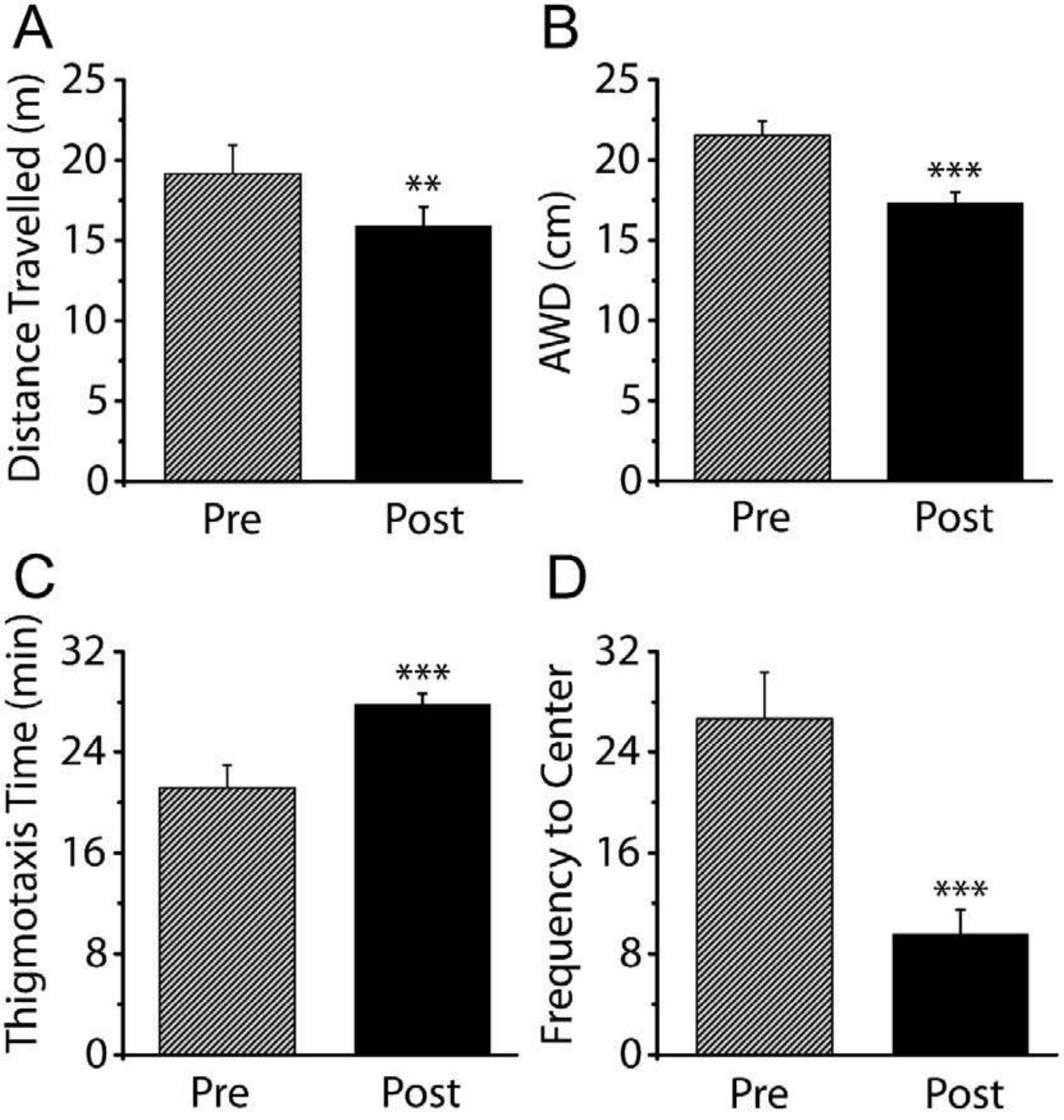
A
Distance Travelled (m)
0
5
10
15
20
25
**
Pre
Post
B
AWD (cm)
0
5
10
15
20
25
***
Pre
Post
C
Thigmotaxis Time (min)
0
8
16
24
32
***
Pre
Post
D
Frequency to Center
0
8
16
24
32
***
Pre
Post

Figure 4

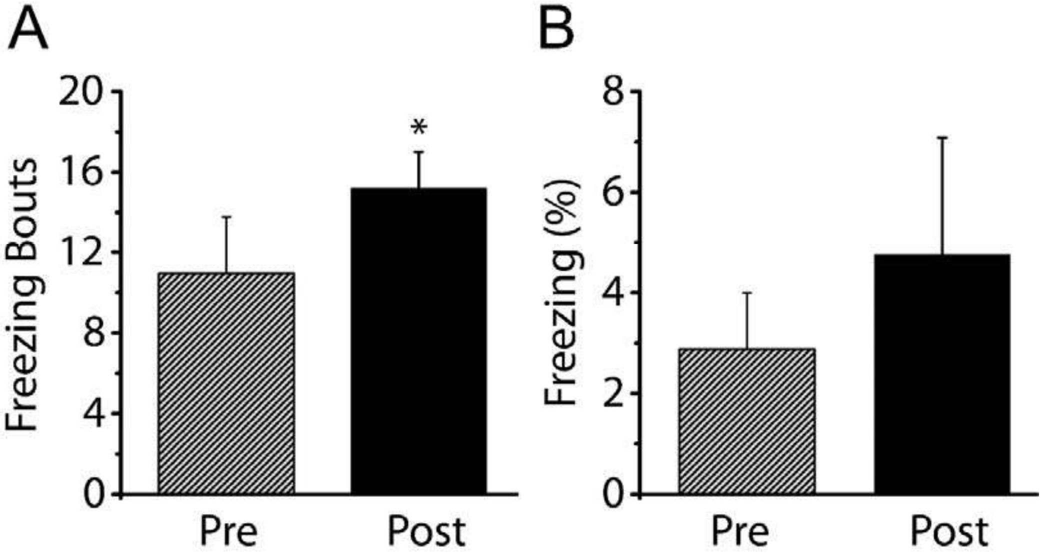
A
B
Freezing Bouts
20
16
12
8
4
0
*
Pre
Post
Freezing (%)
8
6
4
2
0
Pre
Post

Figure 5

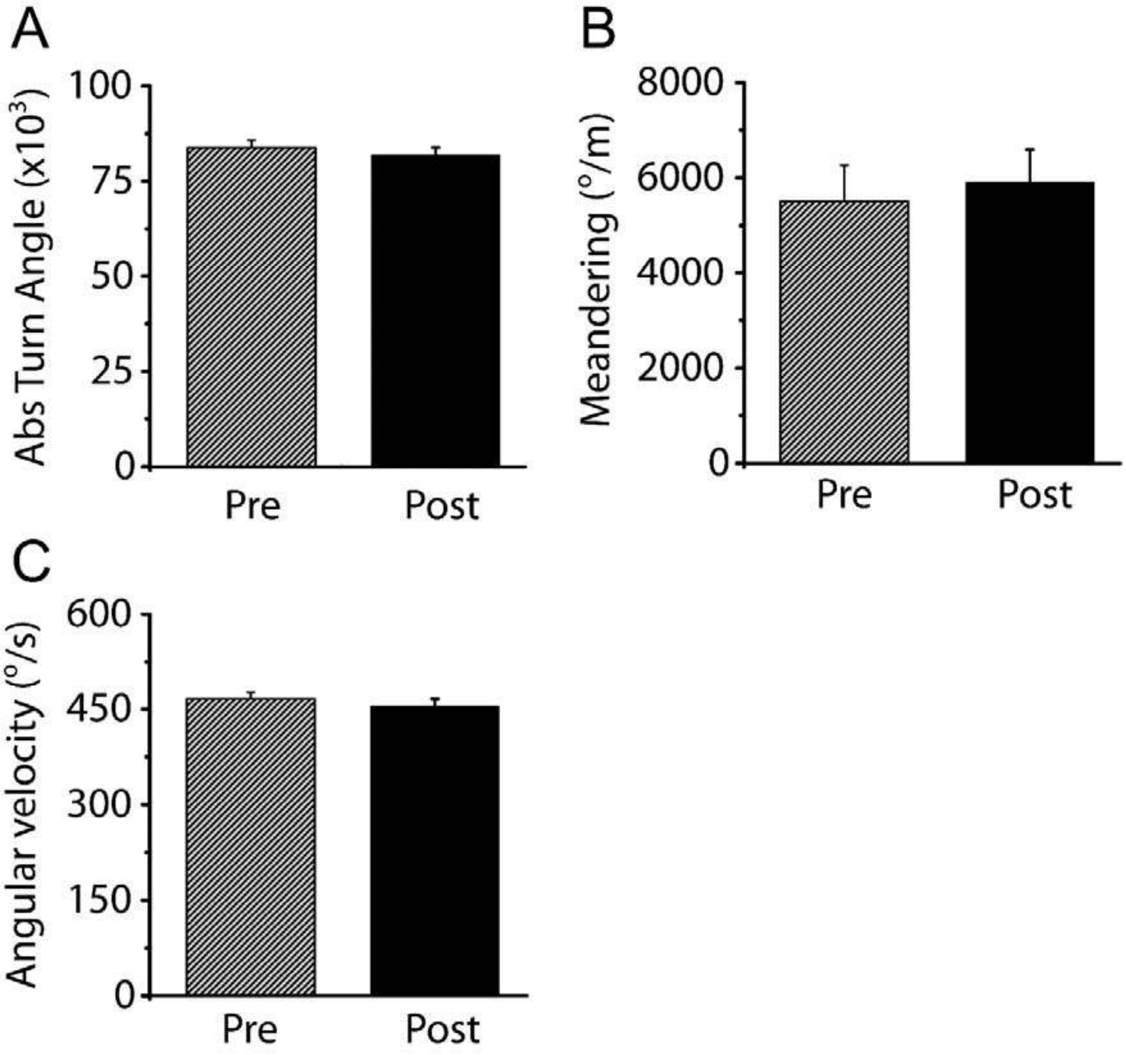
A
Abs Turn Angle (x10³)
100
75
50
25
0
Pre
Post
B
Meandering (°/m)
8000
6000
4000
2000
0
Pre
Post
C
Angular velocity (°/s)
600
450
300
150
0
Pre
Post

Figure 6

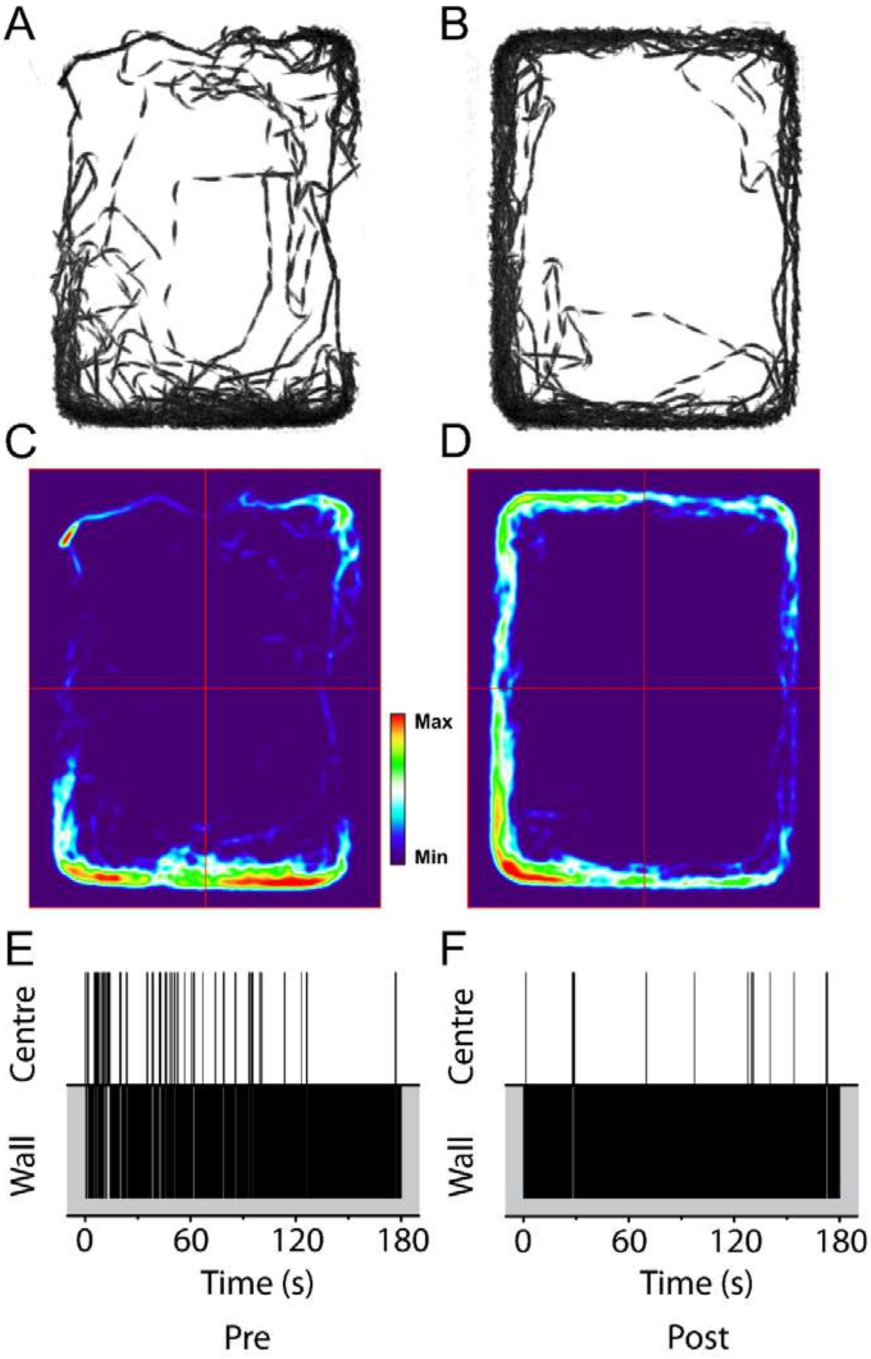
A
B
C
D
Max
Min
E
F
Centre
Wall
0
60
120
180
Time (s)
Pre
Centre
Wall
0
60
120
180
Time (s)
Post

Table 1: Comparison between Ethovision XT and our ZebraTrack method for determination of behavioural endpoints of adult zebrafish.

S. No.	Behavioural Endpoints	Analysis Method	Pre (Fish 1) (First 3 min)	Post (Fish 1) (Last 3 min)	Remarks (% Change between Pre and Post)
1.	Total Distance Travelled (cm)	Ethovision XT	2140	1650	77.10 %
		ZebraTrack	3535	2556	72.30 %
2.	Velocity (cm/s)	Ethovision XT	12	9	75 %
		ZebraTrack	19.6	14.2	72.4 %
3.	Angular Velocity (Degree/s)	Ethovision XT	137	147	107.29 %
		ZebraTrack	462	498	107.79 %
4.	Freezing Bouts (Frequency of inactivity)*	Ethovision XT	0	3	
		ZebraTrack	0	3	
5.	Freezing Duration (s)*	Ethovision XT	0	37.2	
		ZebraTrack	0	39	
6.	Preference Based Behaviour (Qualitative)	Ethovision XT	Less left-side wall	More left-side wall	Fish preferred left-side wall during last 3 minutes
		ZebraTrack	Less left-side wall	More left-side wall	Fish preferred left-side wall during last 3 minutes

*Freezing behaviour was considered if the centroid of fish remained immobile or showed a maximal displacement of 0.25 cm/s.