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.

ARTICLE INFO

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.

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.).

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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 Zebra-Track. 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 (Fig. 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 lx) 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 $29 \times 37 \times 18$ (W $\times$ L $\times$ H in cm) containing 5 cm of water (Fig. 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 $28 \pm 2\,^{\circ}$C. Exploratory behaviour of zebrafish in brightly lit OFT tank (~1000 lx) was recorded for a little more than 6 min 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 min (pre) were compared to the last three min (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 min) and post (last three min) 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