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Automated measurement of zebrafish larval movement

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Abstract The zebrafish is a powerful vertebrate model that is readily amenable to genetic, pharmacological and environmental manipulations to elucidate the molecular and cellular basis of movement and behaviour. We report software enabling automated analysis of zebrafish movement from video recordings captured with cameras ranging from a basic camcorder to more specialized equipment. The software, which is provided as open-source MATLAB functions, can be freely modified and distributed, and is compatible with multiwell plates under a wide range of experimental conditions. Automated measurement of zebrafish movement using this technique will be useful for multiple applications in neuroscience, pharmacology and neuropsychiatry.

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Abbreviations dpf, days post-fertilization; PTZ, pentylenetetrazole.

Introduction

The zebrafish has become increasingly useful as a model system for understanding the molecular basis of movement and behaviour. Phylogenetic conservation of many molecular, cellular and morphological features suggests that the zebrafish CNS provides a representative model for studying vertebrate neurophysiology, and potentially an appropriate setting in which to model neurological disease (Bandmann & Burton, 2010). Genetic manipulation of zebrafish is straightforward, allowing determination of the physiological effects of altered gene function and elucidation of the roles of specific neural circuits by genetically targeting neuronal populations for inactivation (Koide *et al.* 2009) or ablation. A variety of motor responses can be measured in zebrafish larvae (Orger *et al.* 2004), including spontaneous and evoked movements; the latter range in complexity, from reflex reactions following acoustic or cutaneous stimuli (Burgess & Granato, 2007*b*) to complex behaviours relating to assessment of visual cues (Gahtan *et al.* 2005) or social behaviour. For many of these applications, a means of quantifying motion is an essential component of methodology. The purpose of this study was to develop experimental techniques and software allowing straightforward quantification of zebrafish larval movement in multiwell plates commonly used to house larvae for experimental manipulations.

Installation files for the latest versions of LSRtrack and LSRanalyze are available on request from the corresponding author.

Methods

Ethical approval

Experiments were carried out in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee as meeting the standards for humane animal care stipulated by the US Animal Welfare Act.

Zebrafish (*Danio rerio*)

Strain AB zebrafish were raised in 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, pH 7.4 at 28.5°C in a 14 h:10 h light:dark cycle. Larvae were transferred into multiwell plates using a large-bore Pasteur pipette with a flame-polished aperture of at least 4 mm to avoid mechanical damage and were acclimatized to wells for 30 min at 28.5°C prior to recording.

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Video and analysis

Video recordings were made using the following camera and light source combinations: (i) a consumer-grade camcorder (Sony HDR-HC5) and fluorescent light X-ray viewing box (PerfectView 1L64, Leicester, NY, USA) (Fig. 2*C* and *D*); (ii) a similar camcorder, modified to remove infrared filters, and LED infrared light source (BL46-880, Spectrum, Montague, MI, USA) (Fig. 2*A* and *B*); or (iii) a high-speed camera (Dragonfly Express, Point Grey, Richmond, BC, Canada) with infrared illumination (Fig. 2*E*). 96- and 24-well plates (CoStar, Corning, Lowell, MA, USA) were milled to a depth of 10 mm to reduce shadow artefacts, and were trans-illuminated for maximal image contrast. Camcorder video was digitized using Kino (http://www.kinodv.org/); high speed video was captured using FlyCapture (Point Grey). Recordings were analysed offline using custom software written in the MATLAB programming environment (MathWorks, Natick, MA, USA).

Results

We developed a MATLAB function, *LSRtrack*, which analyses video images of zebrafish moving in the wells of multiwell plates (Fig. 1*A*; the source code and manual are provided in the Supplemental material, available online only). *LSRtrack* identifies the wells of common multiwell plate formats in the first frame of the video and at user-determined intervals thereafter. This enables detection of larval objects to be restricted to a user-specified proportion of each well during analysis, improving tracking performance. Pixel dimensions are automatically scaled to distances in millimetres using conversion factors derived from the standard dimensions of common plate formats. *LSRtrack* detects the frame rate of the video allowing conversion of frame number to time. Each subsequent frame of the video recording is opened and analysed sequentially, so that the maximum size of the video file is not limited by available memory. The video frame is converted into a binary image using threshold settings that can be adjusted for optimal performance. Larval objects are detected as contiguous areas of occupied pixels within the tracking area of each well in the binary image. The centroid of each object is calculated and its coordinates and displacement from the previous frame written to an output file. *LSRtrack* was designed to detect a single zebrafish larva in each well, because this is the most common experimental configuration allowing unequivocal identification of each larva for subsequent calculation of displacement and velocity. When more than one object is detected in a single well, larval coordinates are assigned to the largest object. When no objects are detected in a well, the last known coordinates of the larva are assigned. In both cases, errors are reported allowing analysis of tracking performance and subsequent rejection of wells where tracking did not reach designated standards.

We tested *LSRtrack* under a variety of conditions using several different types of video recordings. Figure 1*B–E* and Supplemental Video 1 illustrate experiments in which a camcorder was positioned beneath a trans-illuminated 96-well plate within an incubator for temperature control and isolation from extraneous stimuli. Manual observation of many hours of recordings showed that the centroid assigned by *LSRtrack* for each larva was nearly always located just caudal to the larval head, even during rapid larval movement (Fig. 1*B*). Reconstruction of the paths traversed over a recording confirmed that movement of each larva was detected only within its respective well (Fig. 1*C*). We verified reported tracking errors manually (Fig. 1*D*) – overall these occurred in substantially less than 1% of video frames for each animal. In a 3000 frame video segment of 24 larvae, in which 185 'no object' errors were reported, manual redirection of the tracking algorithm at each error increased measured larval displacement by 0.33%. In a separate 10,000 frame video segment of 24 larvae, 780 'too many object' errors were reported; *LSRtrack* returned the coordinates for the wrong object in 99 of the 780 reported errors, so the overall object misidentification rate was 0.04%. The frequency of 'no object' and 'too many object' errors is dependent on the parameters chosen for *tracking threshold* (the threshold pixel grey value that becomes black when the image is converted to a binary format) and *tracking area scale factor* (the proportion of each well area in which potential larval objects are identified). These can be adjusted 'live' during tracking in the user interface of *LSRtrack* allowing optimization for a particular experimental setting. Registration of frame-to-frame displacement, and categorical assignment of each frame as showing movement or no movement for subsequent analysis, is dependent on exceeding a threshold value for *minimum displacement*. This is adjusted in the user interface and is optimized for specific experimental conditions to eliminate centroid displacements attributable to pixel noise without discarding movements; its validation for the studies reported here is shown in the Supplemental material. Overall, *LSRtrack* performs well in detecting and tracking zebrafish larval movement under a variety of conditions.

After completion of tracking, a second function, *LSRanalyze*, discards wells whose combined 'no object' and 'too many object' errors exceed a critical threshold (set at >5% as default) then performs simple calculations to determine: the total displacement of each larva and its mean velocity, active velocity, the proportion of time it spent moving and mean rest and active durations (Fig. 1*E*). *LSRanalyze* also draws graphical outputs to illustrate how these parameters vary between larvae, and writes results into a spreadsheet for further analysis.

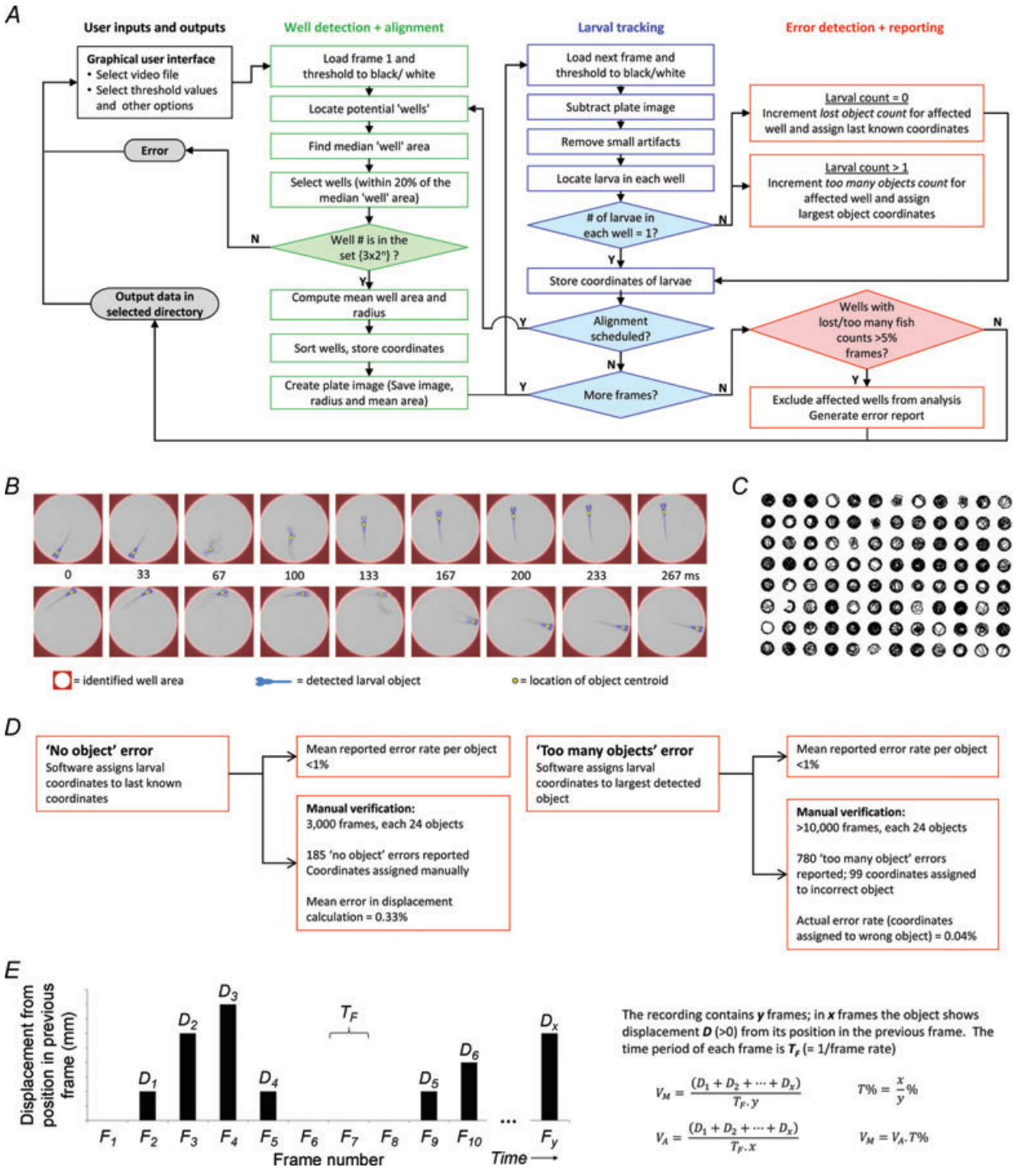

Figure 1. Software functions for measuring and analysing zebrafish movement
A, the flowchart provides an overview of the algorithm used by *LSRtrack* to track zebrafish movement and report errors. The source code is shown in the online Supplemental material. *B*, two series of consecutive video frames at 30 frames s^{-1} are shown, illustrating zebrafish larval movements in individual wells of a 96-well plate. *LSRtrack* identified the area surrounding the well (red), the larval object (blue) and the location of the object centroid (yellow circle). Supplemental Video 1 shows a movie segment of the tracking process in operation, using the same colour scheme. *C*, the locus traversed by each zebrafish larva in a 96-well plate over a 10 min period is shown as a black line; detected movements are confined to individual wells. *D*, the flowchart summarizes manual verification of tracking errors reported by *LSRtrack*. *E*, simple calculations were performed by *LSRanalyze*, using larval tracking data, in order to determine displacement and to derive values for mean velocity (V_M), active velocity (V_A) and the proportion of frames during which a movement occurred ($T\%$).

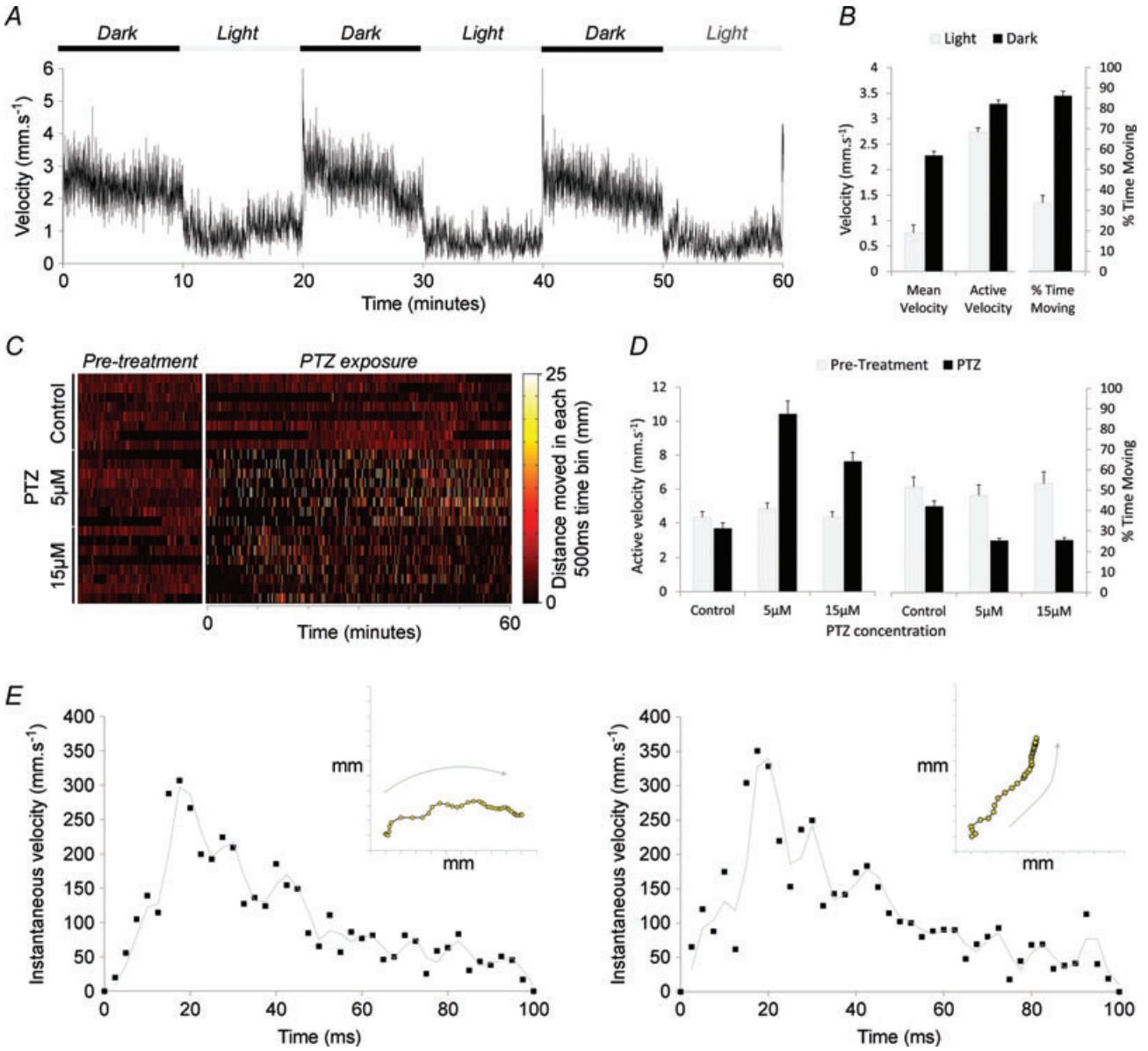

Figure 2. Three example applications of *LSRtrack* and *LSRanalyze*
A, *LSRtrack* was used to determine the activity of a population of 24 zebrafish larvae, housed in a 96-well plate, in response to transitions between light and dark at 7 dpf. Video was recorded using a modified camcorder under infrared illumination at a frame rate of 2 s^{-1}. The graph shows mean larval displacement in each consecutive frame of the video, the axes are scaled to show the equivalent mean velocity in mm s^{-1} and time in minutes. Ambient illumination is indicated above the graph. *B*, *LSRanalyze* was used to determine V_M, V_A and *T*% (see Fig. 1*E* for calculations) of the population shown in panel *A*, and to determine how these parameters changed under different illumination conditions. Error bars show the standard error of the mean ($n = 24$). *C*, the individual movement profiles of 24 larvae at 5 dpf are shown before and after application of PTZ at the concentrations shown. The displacement in each consecutive 0.5 s time bin is shown using a colour scale (the scale is shown to the right of chart), illustrating alterations in movement patterns following PTZ exposure. *D*, *LSRanalyze* was used to determine how active velocity (V_A) and proportion of time moving (*T*% – see Fig. 1*E* for calculations) changed following PTZ exposure. Error bars show the standard error of the mean ($n = 8$). *E*, following a mechanical stimulus, the escape response of two different larvae at 9 dpf was recorded using a high speed camera at a frame rate of 400 s^{-1}. The main graphs show larval displacement in each consecutive video frame as black squares. The axes are scaled to show the equivalent instantaneous velocity in mm s^{-1} and time in ms; the trend line was calculated as a moving mean. The inset panels show how the position of the larvae (yellow circles) changed in each successive frame of the video; the axes show distance in mm and the arrows indicate the direction of movement.

Figure 2 demonstrates three different experimental applications using *LSRtrack* and *LSRanalyze*. Each example was recorded using different cameras and experimental conditions so that the resulting video segments differed in temporal and spatial resolution, illustrating the flexibility of this approach. First, alterations in population activity in response to changes in ambient illumination were determined at 7 days post-fertilization (dpf) using infrared videography at 2 frames s^{-1}. Characteristic changes in larval activity occur during light–dark cycling (Burgess & Granato, 2007*a*) which have recently been exploited for neuropharmacological drug profiling (Rihel *et al.* 2010). Mean frame-to-frame larval displacement changed with ambient illumination (Fig. 2*A*); at the light–dark transition, a burst of activity was seen that slowly declined over time (after prolonged dark periods >15 min, larvae enter an inactive state). Analysis of individual larval movements (Fig. 2*B*) showed that the increased movement recorded in the first 10 min after the onset of darkness was attributable to (i) a >2-fold increase in the proportion of time that larvae spent moving; and (ii) a modest increase in frame-to-frame displacement. Second, we exposed zebrafish larvae at 5 dpf to a convulsant compound, pentylenetetrazole (PTZ), which induces paroxysmal motor and electrographic activity in zebrafish larvae (Baraban *et al.* 2005); the resulting model has been used in preclinical anticonvulsant development (Goldsmith *et al.* 2007). Using a simple camcorder, we recorded larval movements in bright light at 30 frames s^{-1} before and after exposure to PTZ. A colour scale velocity–time representation of each individual larva illustrates the transition from continuous low velocity movement to paroxysms of high velocity movement after PTZ exposure (Fig. 2*C*). Quantitative analysis showed that PTZ reduced the proportion of time larvae were moving, but increased their velocity by 2-fold during episodes of movement (Fig. 2*D*). Finally, we used a high-speed camera to record individual zebrafish movements at a frame rate of 400 s^{-1}, following a mechanical stimulus (Fig. 2*E*). The escape response has been used to isolate mutants with abnormalities in the formation of circuits mediating spinal contralateral inhibition (Wolman & Granato, 2011) and to investigate sensorimotor gating (Burgess & Granato, 2007*b*). The recordings illustrate the stereotyped larval escape response at 9 dpf, in which an initial turn is followed by displacement at peak velocities of over 300 mm s^{-1} occurring 18–20 ms after the start of the movement. Later components of the instantaneous velocity curve show how propulsion occurs in a series of waves corresponding to tail beating movements.

Together these examples illustrate the flexibility and potentially broad range of applications for video analysis using *LSRtrack* and *LSRanalyze*.

Discussion

Measurement of zebrafish larval movement has multiple experimental applications in physiology, pharmacology and experimental neurogenetics. Several proprietary solutions for quantifying zebrafish movement are available, but the method we report here may offer advantages. First, some commercially available packages include hardware and software components, such that adaptation for new applications might be limited by the suitability of the hardware. Since *LSRtrack* analyses videos from any source, almost any camera can be used to make recordings under a multitude of different experimental conditions, provided the video is of suitable quality for tracking and can be read by MATLAB. Second, open source software encourages modification of the code for new applications, which is straightforward using the MATLAB programming language, whereas the source code for available commercial packages is proprietary and so development of new assays or analyses is potentially more difficult. Finally, in many situations, measurement of zebrafish activity would be a useful complement to the analysis of mutants, transgenic lines and chemical modifiers, but is limited by the expense of acquiring proprietary packages. *LSRtrack* allows larval activity to be determined using basic apparatus and will greatly broaden access to simple motor measurements.

Currently there are two potential limitations to this technique. First, the automated well-detection subroutine of *LSRtrack* is only compatible with video recordings made in plates where round wells appear light on a dark background and the total number of wells is in the set $\{3 \times 2^n\}$ (n is a positive integer). This encompasses most of the common plate formats, although use of plates with square wells or other formats such as linear swimming tracks will require modification to the code. Second, *LSRtrack* detects a single larva in each well of a multiwell plate. Methods for resolving individual objects after collisions are still under development and are not provided in any of the currently available packages. The most reliable method for unequivocally identifying individual animals for calculations of velocity and displacement is to separate them physically, facilitated in zebrafish larvae by the use of multiwell plates.

In conclusion, we report source code MATLAB functions allowing tracking of zebrafish larval movement for measurement of kinetic parameters and determination of behavioural patterns. The tracking function is accurate, using video recordings captured with a variety of cameras under different experimental conditions. It is likely that this technique will be useful for numerous applications.

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Author contributions

C.L.C. wrote and verified software; T.C.F. and C.M. designed and carried out experiments; E.A.B. conceived the study, interpreted data and wrote the manuscript. All authors agreed the final version of the manuscript.

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