**Supplementary Figures and Legends for Videos**

***Supplementary Figure 1 – Manual optimization of minimum displacement threshold***

The graph shows manual verification that the minimum displacement threshold of 1 pixel (used for the 2 frames.s-1 recordings in figure 2A and B) adequately discarded frames in which larval centroid displacement was caused by stochastic pixel noise rather than larval movement. A 1200-frame video segment of 24 larvae (10 minutes of movement, or 28,800 data points) was manually analysed; for each frame, each larva was annotated as having moved or not moved from the previous frame. These manually-determined categorical assignments were then compared with automated tracking data at a range of minimum displacement thresholds. The % of frames that showed centroid displacement > minimum threshold, but which were judged manually to have shown no larval movement, was calculated for each *minimum displacement* threshold value. Similarly, for each *minimum displacement* threshold value, the % of total recorded displacement contributed by frames that were judged manually to have shown no movement was calculated. With *minimum displacement* threshold set to 0, 4.5% of frames showing centroid displacement did not show visible movement of the larva. By increasing the threshold to 1 pixel.frame-1, inappropriate registration of movement occurred in 2.2% of frames, contributing 0.5% to total displacement. In this hardware configuration, a larval zebrafish is >15 pixels in length and so the minimum displacement threshold for registering movement allows detection of any movement greater than 1/15 larval length occurring during the 500ms interval between frames.

***Supplementary Figure 2 – Automated optimization of minimum displacement threshold for distance recorded***

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The graph shows automated verification of the *minimum displacement* threshold for a higher frame rate recording that could not practicably be validated manually. 24 control and 24 tricaine-exposed larvae were recorded simultaneously under the same conditions, at 30 frames.s-1, for 30 minutes (48 larvae x 54,000 video frames, or 2.6 x 106 data points). The x-axis shows frame-to-frame centroid displacements grouped into 0.04 pixel bins. The y-axis shows total displacement over the 30-minute recording accounted for by frame-to-frame displacements in each bin, expressed as % of the total distance recorded for control larvae. Note that the x-axis is truncated; >65% displacement for control larvae over the recording occurred at frame-to-frame displacements >1.12 pixels. Tricaine anaesthesia causes immobility, such that apparent movement recorded in the tricaine-exposed population arises from centroid displacements attributable to stochastic pixel noise or other non-active forms of larval movement (drift, convection, vibration). With the *minimum displacement* threshold set to 0, measured displacement in tricaine-exposed larvae was 5.0% that of controls. Increasing the *minimum displacement* threshold to 0.16 pixels.frame-1 decreased measured movement attributable to pixel noise to 2.2% of total displacement, but retained 97% of displacement in controls (presumably some of the 3% discarded movement was attributable to the same artefacts affecting immobilized larvae). 0.16 pixels corresponds to approximately 1% larval length under these experimental conditions, so over 7 – 8 frames (≈250ms) these settings would capture any movement of >10% larval length. These data confirm that the minimum displacement threshold setting used in figures 2C and D discarded most centroid movement attributable to pixel noise, but the assay remained sensitive to small displacements of the larva. Consequently, the calculation of VM is likely to be accurate.

***Supplementary Figure 3 – Automated optimization of minimum displacement threshold for % frames registered as showing movement***

91.0

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55.6

Data are shown from the same experiment as supplementary figure 2. The x-axis shows frame-to-frame centroid displacements grouped into 0.04 pixel bins. The y-axis shows the number of frames over the 30-minute recording accounted for by frame-to-frame displacements in each bin, expressed as % of the total number of frames. The x-axis is truncated, since 9.2% frames for control larvae and 0.23% frames for tricaine-exposed larvae showed >1.12 pixel displacements. The y-axis is discontinuous to show the large % of frames in which the larval centroid did not move. With the *minimum displacement* threshold set to 0, pixel noise and other artefacts caused 8% of frames for tricaine-exposed larvae animals to be categorized as showing movement. Increasing *minimum displacement* threshold to 0.16 pixels.frame-1 decreased the proportion of frames in which movement was inappropriately registered in the tricaine-treated larvae to 4%. This did not change the categorical assignment of frames showing movement in the control larvae in 93% of the frames designated as ‘moving’ when the *minimum displacement* threshold was 0 (and presumably many of the discarded small displacements were also artefacts). These data confirm that the minimum displacement threshold setting used in figures 2C and D correctly categorized the majority of frames as showing larval movement or not, and was sensitive to small authentic larval movements. Consequently the calculation of T% is accurate. In conjunction with the data shown in supplementary figure 2, these data also confirm that the calculation of VA is accurate.

**Supplementary Video 1**

The video shows an example of *LSRtrack* tracking larval movement. A single well of a 96-well plate is shown at high magnification. Regions of the video frame identified by the software as well edge, well area and larva are coloured as shown in figure 1B. The calculated position of the larval centroid is shown as a yellow circle, illustrating the accuracy of the tracking algorithm.

**Supplementary Videos 2 and 3**

An example of *LSRtrack* tracking larval movement is shown, in which the same video segment is analysed with the *minimum displacement* threshold set at 0 (video 2) or 1 pixel (video 3). The calculated position of the larval centroid is shown as a red circle, which changes to yellow when the *minimum displacement* is exceeded. This is identical to the live tracking window in *LSRtrack*. This illustrates visually how altering the *minimum displacement* threshold affects the categorical assignment of video frames as showing movement or not with respect to the previous frame. Stochastic pixel noise causes small movements in the larval centroid. With the *minimum displacement* threshold set at 0, these are registered as movements, whereas they are not when the *minimum displacement* threshold is set at 1 pixel. For a given magnification, frame rate, level of illumination and camera/hardware set up, minimum displacement should be optimized, either empirically using the live tracking function of *LSRtrack*, or by similar experiments as shown in supplementary figures 1 – 3.