Supporting information:

Quantification of functional recovery in a larval zebrafish model of spinal cord injury

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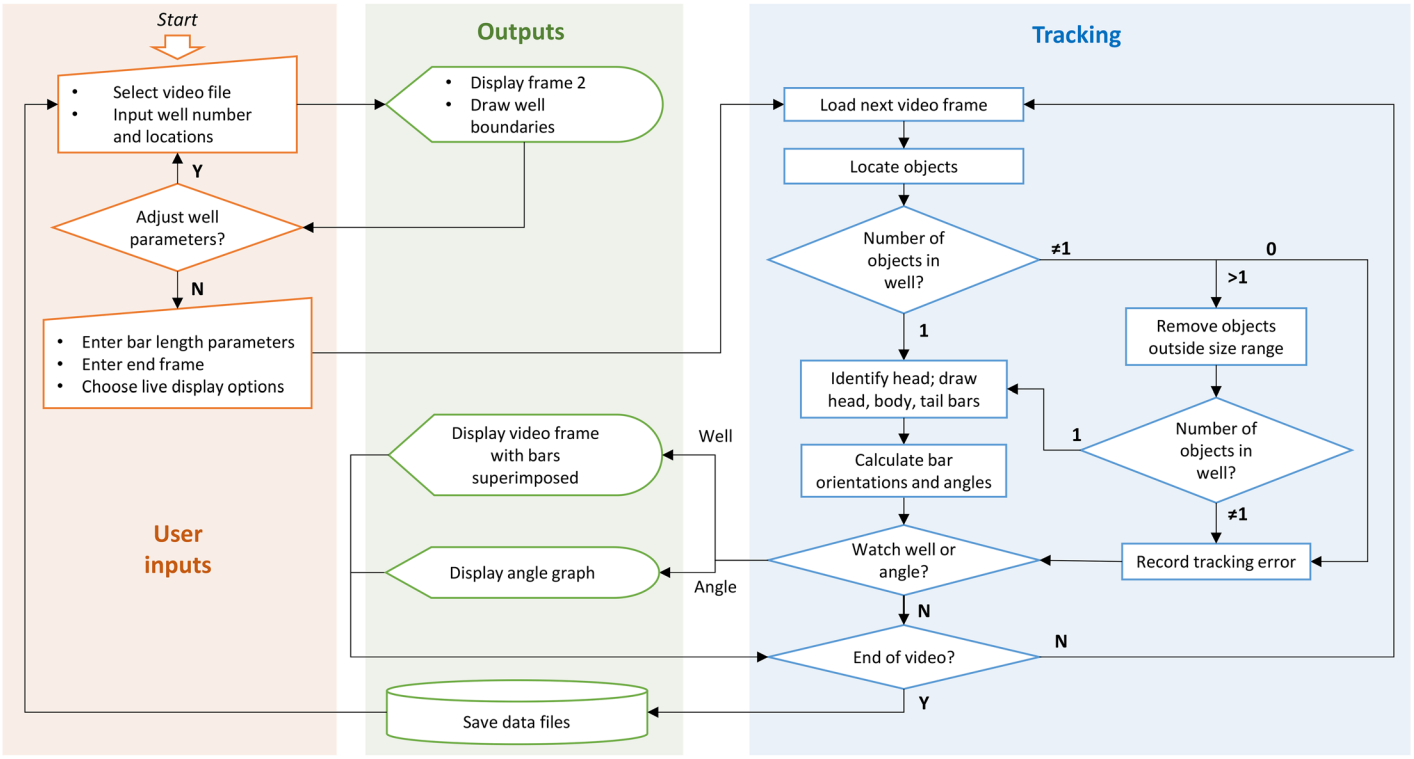
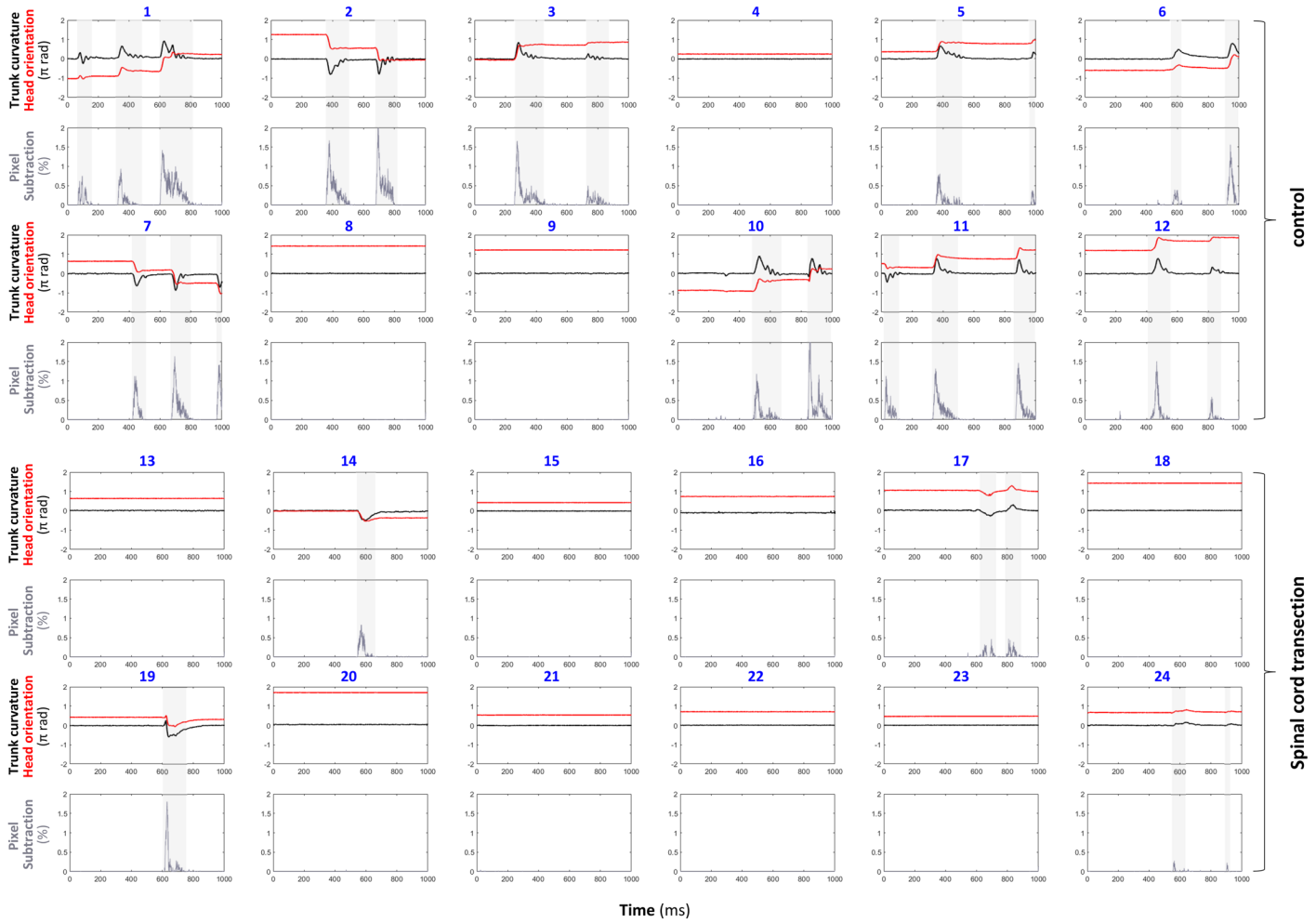
Supplemental figure 1: Logic of tracking software



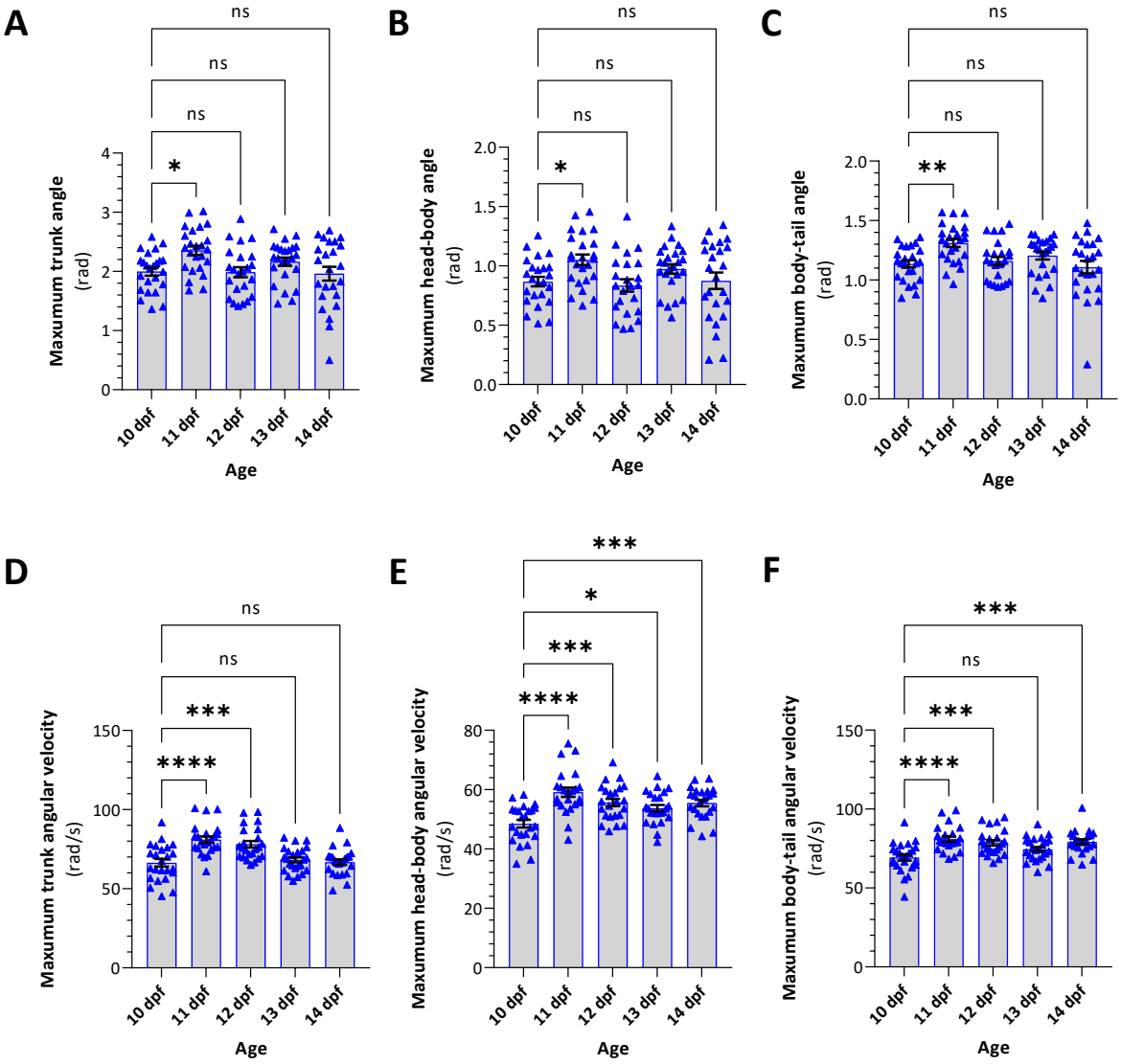
The flow chart depicts the logic of the software algorithm used to detect motion and measure truncal curvature for figures 2 – 5. The MATLAB source code/installation files are provided in the accompanying .zip archive.

Supplemental figure 2: Example tracking outputs for control and SCI zebrafish immediately post-injury



The figure shows tracking outputs for 12 control uninjured sibling zebrafish (numbered 1 – 12 in blue) and 12 SCI zebrafish immediately after the injury at 10 dpf (numbered 13 – 24). For each zebrafish the top graph shows trunk curvature (4 head-tail; black) and head orientation (angle of head bar with respect to x axis; red) with angles in radians shown as multiple of π. The lower graph of each pair shows the result of a framewise image subtraction used as a sensitive measure of motion (units are % pixels that changed at each video frame transition, within a ROI corresponding to the well housing the zebrafish). Movement events are shaded grey. All control zebrafish except #4, 8 and 9 responded to this stimulus; only SCI zebrafish #14, 17, 19, 24 showed responses, which are greatly attenuated with respect to the controls.

Supplemental figure 3: Changes in VMR kinematics in control zebrafish 10 – 14 dpf (secondary analysis)



Kinematics of motor responses to sudden ambient light-dark transition were quantified in control zebrafish (blue, triangles; same data as figure 5 reanalyzed). Data points represent individual zebrafish and show the mean of all elicited responses for each zebrafish at a particular time point (all zebrafish that showed at least one response are included at each time point). Bars show group mean ± SE. A – C show maximal angles: (A) ∢head-tail; (B) ∢head-body; (C) ∢body-tail. D – F show mean angular velocity: (D) head-tail; (E) head-body; (F) body-tail. Data were analyzed by one-way ANOVA with age as a variable, and pairwise comparisons made between 10dpf and each other time point by Dunnett *post hoc* test ($p<0.05$*, 0.001**, 0.0001***, 0.00001****).

Supplemental table 1: ANOVA table to accompany figure 5A

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	16.255	1.16666E-07	****	Yes
Time post-injury	14.728	5.46542E-07	****	Yes
Experimental group	4.403	0.000935667	***	Yes

ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
Interaction	10.015	4	2.5039	F (4, 190) = 10.4313	P=0.000000116665512
Time post-injury	9.074	4	2.2686	F (4, 190) = 9.4511	P=0.000000546541728
Experimental group	2.713	1	2.713	F (1, 190) = 11.3029	P=0.000935667496024
Residual	45.606	190	0.24		

Difference between column means	
Predicted (LS) mean of Control	2.093
Predicted (LS) mean of SCI	1.8476
Difference between predicted means	0.2454
SE of difference	0.073
95% CI of difference	0.1014 to 0.3894

Data summary	
Number of columns (Column Factor)	2
Number of rows (Row Factor)	5
Number of values	200

Supplemental table 2: ANOVA table to accompany figure 5B

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	7.692	0.001576844	**	Yes
Time post-injury	4.223	0.044349271	*	Yes
Experimental group	5.176	0.000584344	***	Yes

ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
Interaction	1.831	4	0.4576	F (4, 192) = 4.544	P=0.001576844206423
Time post-injury	1.005	4	0.2512	F (4, 192) = 2.494	P=0.044349270545758
Experimental group	1.232	1	1.232	F (1, 192) = 12.23	P=0.000584343755853
Residual	19.34	192	0.1007		

Difference between column means	
Predicted (LS) mean of Control	0.9204
Predicted (LS) mean of SCI	1.085
Difference between predicted means	-0.1647
SE of difference	0.0471
95% CI of difference	-0.2576 to -0.07182

Data summary	
Number of columns (Column Factor)	2
Number of rows (Row Factor)	5
Number of values	202

Supplemental table 3: ANOVA table to accompany figure 5C

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	11.24	7.53644E-07	****	Yes
Time post-injury	9.708	5.66909E-06	****	Yes
Experimental group	31.81	<0.000000000000001	****	Yes

ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
Interaction	2.354	4	0.5885	F (4, 192) = 9.240	P=0.000000753643871
Time post-injury	2.033	4	0.5084	F (4, 192) = 7.982	P=0.000005669088093
Experimental group	6.664	1	6.664	F (1, 192) = 104.6	P<0.000000000000001
Residual	12.23	192	0.06369		

Difference between column means	
Predicted (LS) mean of Control	1.184
Predicted (LS) mean of SCI	0.8006
Difference between predicted means	0.3831
SE of difference	0.03746
95% CI of difference	0.3092 to 0.4570

Data summary	
Number of columns (Column Factor)	2
Number of rows (Row Factor)	5
Number of values	202

Supplemental table 4: ANOVA table to accompany figure 5D

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	5.928	0.004672	**	Yes
Time post-injury	19.42	3.43E-09	****	Yes
Experimental group	8.597	4.05E-06	****	Yes

ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
Interaction	0.003556	4	0.000889	F (4, 189) = 3.885	P=0.004672171159318
Time post-injury	0.01165	4	0.002912	F (4, 189) = 12.73	P=0.000000003431203
Experimental group	0.005157	1	0.005157	F (1, 189) = 22.54	P=0.000004054707134
Residual	0.04324	189	0.000229		

Difference between column means	
Predicted (LS) mean of Control	0.07214
Predicted (LS) mean of SCI	0.06143
Difference between predicted means	0.01071
SE of difference	0.002256
95% CI of difference	0.006262 to 0.01516

Data summary	
Number of columns (Column Factor)	2
Number of rows (Row Factor)	5
Number of values	199

Supplemental table 5: ANOVA table to accompany figure 5E

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	2.46	0.199535	ns	No
Time post-injury	16.29	2.26E-07	****	Yes
Experimental group	8.07	1.43E-05	****	Yes

ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
Interaction	0.000404	4	0.000101	F (4, 186) = 1.515	P=0.199535278429522
Time post-injury	0.002674	4	0.000669	F (4, 186) = 10.03	P=0.000000225797247
Experimental group	0.001325	1	0.001325	F (1, 186) = 19.88	P=0.000014265607023
Residual	0.0124	186	6.67E-05		

Difference between column means	
Predicted (LS) mean of Control	0.05452
Predicted (LS) mean of SCI	0.04902
Difference between predicted means	0.005499
SE of difference	0.001234
95% CI of difference	0.003066 to 0.007933

Data summary	
Number of columns (Column Factor)	2
Number of rows (Row Factor)	5
Number of values	196

Supplemental table 6: ANOVA table to accompany figure 5F

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	5.543	0.001427	**	Yes
Time post-injury	21.5	1.96E-12	****	Yes
Experimental group	28.24	<0.000000000000001	****	Yes

ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
Interaction	0.001966	4	0.000491	F (4, 185) = 4.611	P=0.001427349824331
Time post-injury	0.007626	4	0.001907	F (4, 185) = 17.89	P=0.000000000001958
Experimental group	0.01002	1	0.01002	F (1, 185) = 93.98	P<0.000000000000001
Residual	0.01972	185	0.000107		

Difference between column means	
Predicted (LS) mean of Control	0.07655
Predicted (LS) mean of SCI	0.06146
Difference between predicted means	0.0151
SE of difference	0.001557
95% CI of difference	0.01202 to 0.01817

Data summary	
Number of columns (Column Factor)	2
Number of rows (Row Factor)	5
Number of values	195

Instructions for installing and operating HiSpeedTracking

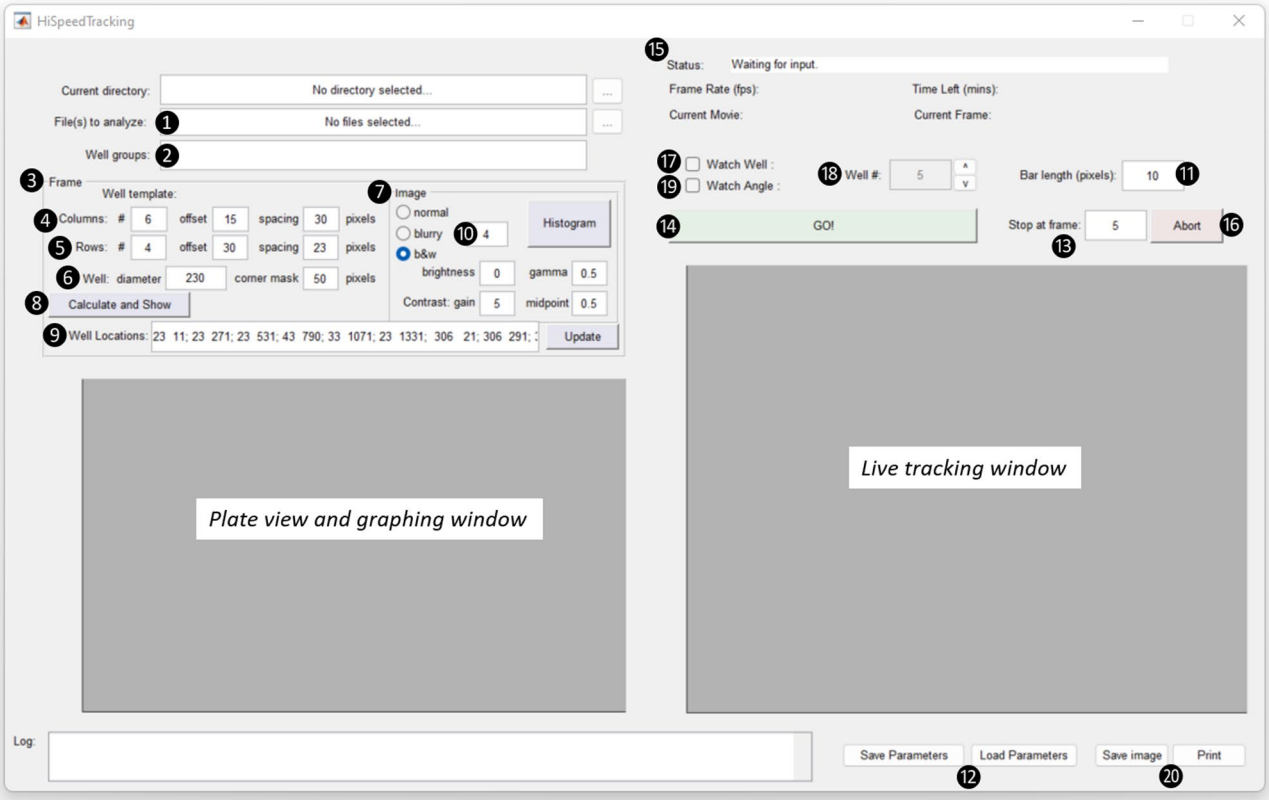
Introduction: *HiSpeedTracking* is a MATLAB application that finds and records the position, orientation, and body curvature of multiple larval zebrafish, located in separate behavioral arenas, viewed from above or below in a video recording. The video is acquired at a high frame rate (1000fps) to capture movement details. The zebrafish must be the only dark object within each region of interest in the video image for the software to work as intended. The application has been tested with Windows 10 and 11, and with MATLAB versions 2019a, 2020b. The source code and MATLAB files are open-source and provided to the academic community for use, modification, and distribution.

Installation: The software files can be found in the accompanying .ZIP archive labeled *Software files*. To install the software, extract the files into an uncompressed folder.

Operation:

1. Open *HiSpeedTracking*:

    a. Open MATLAB, change the current directory to the folder containing *HiSpeedTracking*
    b. In the command window type **`run HiSpeedTracking.m`** and hit **`<Enter>`**
    c. The main program window will appear (numbers in black circles refer to the instructions below):



2. Select video files. To select video files for the analysis, click on the **`<…>`** button adjacent to the **`File(s) to analyze`** box ❶. A new window with a file browser will open. Navigate to the folder containing the video files, select the files, and click the **`<OK>`** button to confirm selection.

3. Define experimental groups. The **`Well groups`** box ❷ allows the data outputs to be divided into separate files for each experimental group (such as control, injury, chemical exposure etc.) for convenience during subsequent analysis. Enter the well numbers in the format **`[aa:bb];[cc:dd]`**, where aa and bb are the first and last wells in the first group, cc and dd are the first and last wells in the second group, etc. Alternatively list the well numbers for each group

separately with spaces between each well number. For example **[1:12];[13:24]** indicates an experiment in which wells 1 to 12 belong to one experimental group and wells 13 to 24 belong to a second experimental group. **[1 3 4 6 8];[2 5 7 9 10];[11:14]** indicates three groups with wells 1, 3, 4, 6 and 8 in the first group, wells 2, 5, 7, 9 and 10 in the second group, and wells 11, 12, 13, and 14 in the third group. If this field is left blank, all wells are included in one group; in the example below, a blank field and **[1:24]** yield the same results.

4. Define plate layout in the **Frame** panel ❸:

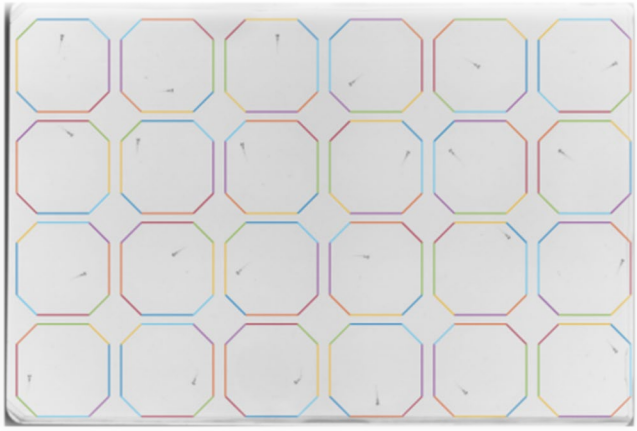
   a. In the **Columns** ❹ and **Rows** ❺ sections, enter the number of columns and rows of wells into the **#** fields.

   b. In the **Columns** section ❹, enter the distance (in pixels) of the first column of wells from the left side of the video frame into the **offset** field and the distance between columns (in pixels) into the **spacing** field.

   c. In the **Rows** section ❺, enter the distance (in pixels) of the first row from the top of the video frame into the **offset** field and the distance between rows (in pixels) into the **spacing** field

   d. Enter the diameter of the wells into the **Well Diameter** field ❻ and the size of the corner mask into the adjacent **corner mask** field (both in pixels). Removing the corners from each region of interest improves tracking performance. The optimal amount of corner masking is determined empirically; ¼ of the well diameter is often a good starting point.

   e. In the **Image** panel ❼, check the **Normal** option, then click the **Calculate and Show** button ❽ in the **Frame** panel ❸. The first frame from the first video file is displayed in the plate view and graphing window, with borders of each region of interest superimposed (see image to right).

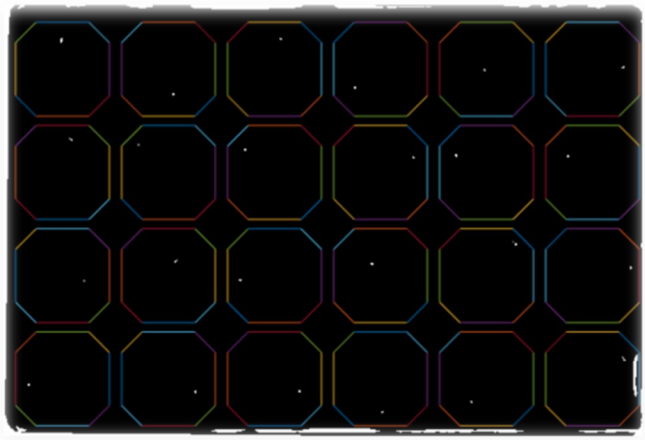


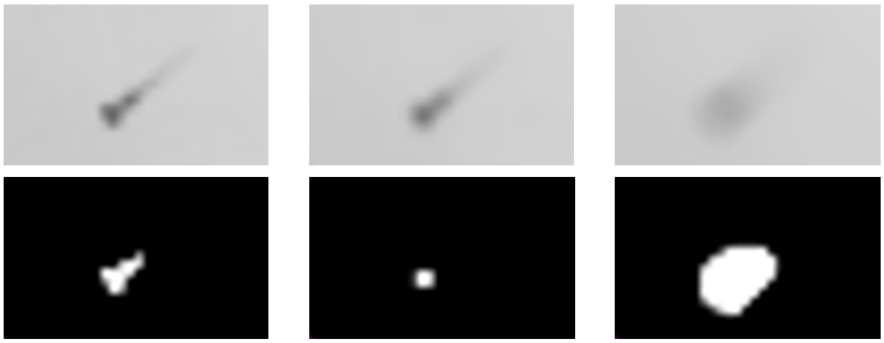
   f. Adjust the well offset, spacing, diameter and corner masking until every well is fully contained within a different region of interest on the image. After each adjustment, click **Calculate and Show** ❽ to see the results.

   g. The plate template can be adjusted manually as needed by entering the pixel coordinates of the top left corner of each region of interest in the **Well Locations** field ❾ followed by pressing the adjacent **Update** button.

5. Adjust image processing parameters in the **Image** panel ❼.

   a. Image blurring is used to ensure that the darkest parts of the zebrafish larva in the image (eyes and swim bladder region) appear together as a single area after image thresholding, to ensure the centroid position that marks the origin of the head bar (see below) is assigned correctly. Blur is adjusted empirically. Select **b&w** mode in the Image panel ❼ to show the thresholded image, adjust the image blurring factor ❿, and click the **Calculate and Show** button ❽ to display the results. Optimal blurring yields a single small object whose centroid is readily assigned. Insufficient blurring leads to multiple objects whereas excessive blurring leads to inaccurate centroid location (see figure below).





Blur = 1
Insufficient
Too many objects

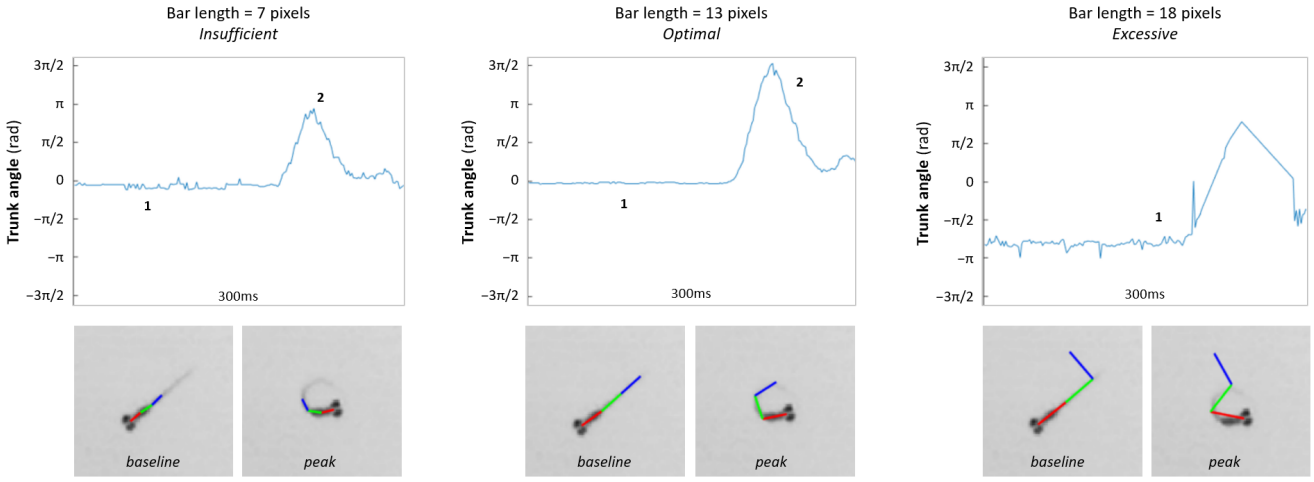
Blur = 4
Optimal
Accurate centroid location

Blur = 10
Excessive
Centroid location approximate

b. Image **brightness**, **gamma**, and **contrast** (**gain** and **midpoint)** can be adjusted to ensure that the zebrafish appear as dark objects on a uniform light background and a single object is detected in each well.

c. Pressing the **Histogram** button displays the image histogram in the *Plate view and graphing window* to assist in selecting appropriate parameters for detection and tracking of the zebrafish.

6. Adjust bar length in the tracking panel. The program fits three connected bars of equal length from the centroid of the head region along the long axis of the zebrafish to allow calculation of truncal curvature. The length of these body segment bars is usually ⅓ – ¼ the length of the zebrafish. Enter bar length in pixels into the **Bar length** field ⓫. The length of the bar depends on the pixel dimensions and magnification of the images and is determined empirically. Bars that are too short tend to under-estimate peak curvature and cause disproportionate angular changes in the presence of stochastic pixel noise in the video recording. Bars that are too long prevent the program from correctly identifying the zebrafish body axis. Optimal bar lengths give rise to smooth traces that accurately capture the peak curvature (see figure below):



1. Excessive fluctuations caused by pixel noise
2. Maximum curvature under-estimated

1. Smooth tracing reflecting true curvature
2. Maximum curvature estimated correctly

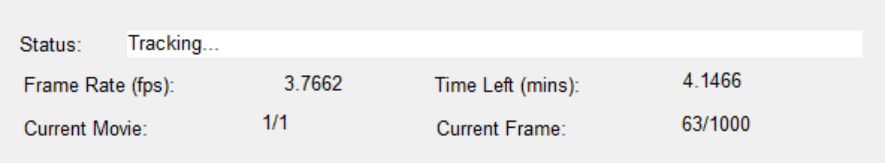
1. Errors in locating zebrafish body axis prevent calculation of correct angles

7. *Optional: storing tracking paraments for subsequent use.* The well layout, image processing, and body segment bar settings can be saved using the **Save parameters** button ⓬. This enables parameters to be standardized between experiments and easily loaded during the next session using the adjacent **Load parameters** button. By default, the program loads the last saved or loaded parameters on startup.

8. Set the number of video frames to analyze. The program will start at frame #1 of the video and analyze the video file until the frame number set in the **`Stop at frame:`** field ⓭. To track the entire video, set this number to the total number of video frames in the video segment, for example 1000 in a 1s segments at 1000 frames/s.

9. Start tracking. Click **`GO!`** ⓮ to start tracking.

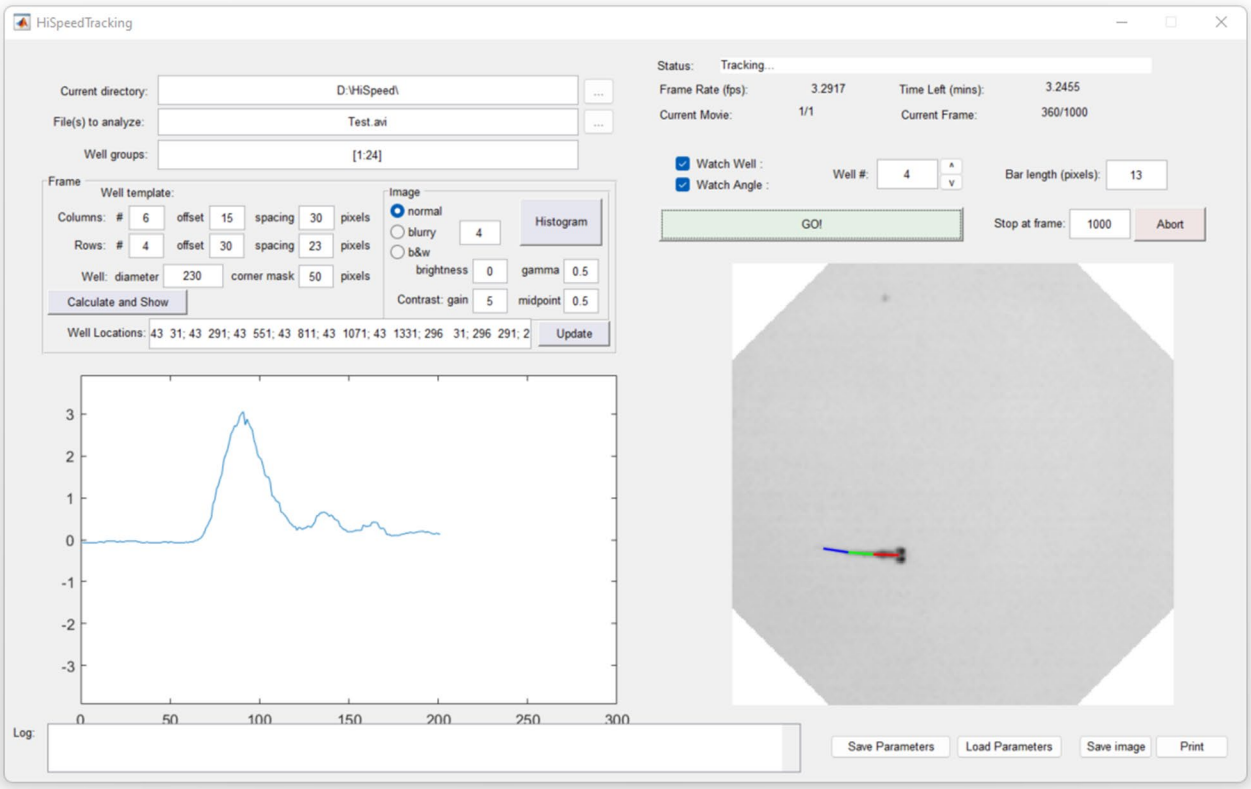
   The status monitor ⓯ indicates that the program is currently working and gives a live update of the number of frames it is analyzing every second, the time left until the current analysis is completed, the video file currently being analyzed (during batch video file processing), and the current frame number:



10. Stop tracking. Tracking can be stopped manually at any time by clicking the **`Abort`** button ⓰. This is useful to re-start analysis after adjusting tracking parameters, especially when the video is very long.

11. Live tracking window. Image analysis outputs can be viewed live as the program is running. This enables a quick visual check to ensure that tracking parameters are optimal so that the zebrafish segments are located correctly. Click the **`Watch Well`** checkbox ⓱ and then enter the well number of interest in the **`Well #:`** field ⓲. Alternatively, the index increment and decrement buttons to the right of the **`Well #:`** field ⓲ can be used to cycle conveniently through each well in turn. An animated image of the selected well with body segment bars superimposed in the zebrafish appears in the *Live tracking window*.

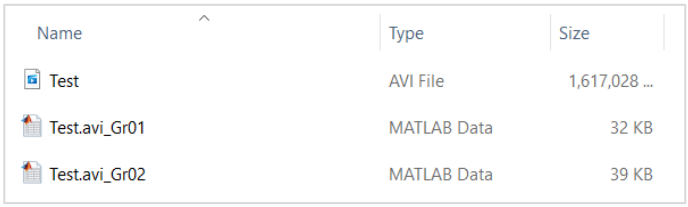
12. Live angle window. The calculated angle of body curvature for the selected well can be viewed as a dynamically updating graph during tracking. This is useful to monitor for excessive angular noise or intermittent body segment assignment errors that might necessitate adjustments to the tracking parameters. Click the **`Watch angle`** button ⓳. The dynamically updating graph replaces the plate image in the *Plate view and graphing window*.



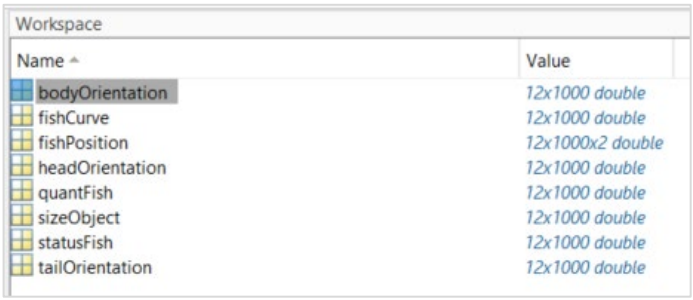
13. Saving and printing screenshots. A screenshot of the program window can be saved as an .EPS file by clicking the **`Save image`** button or printed by clicking the **`Print`** button.

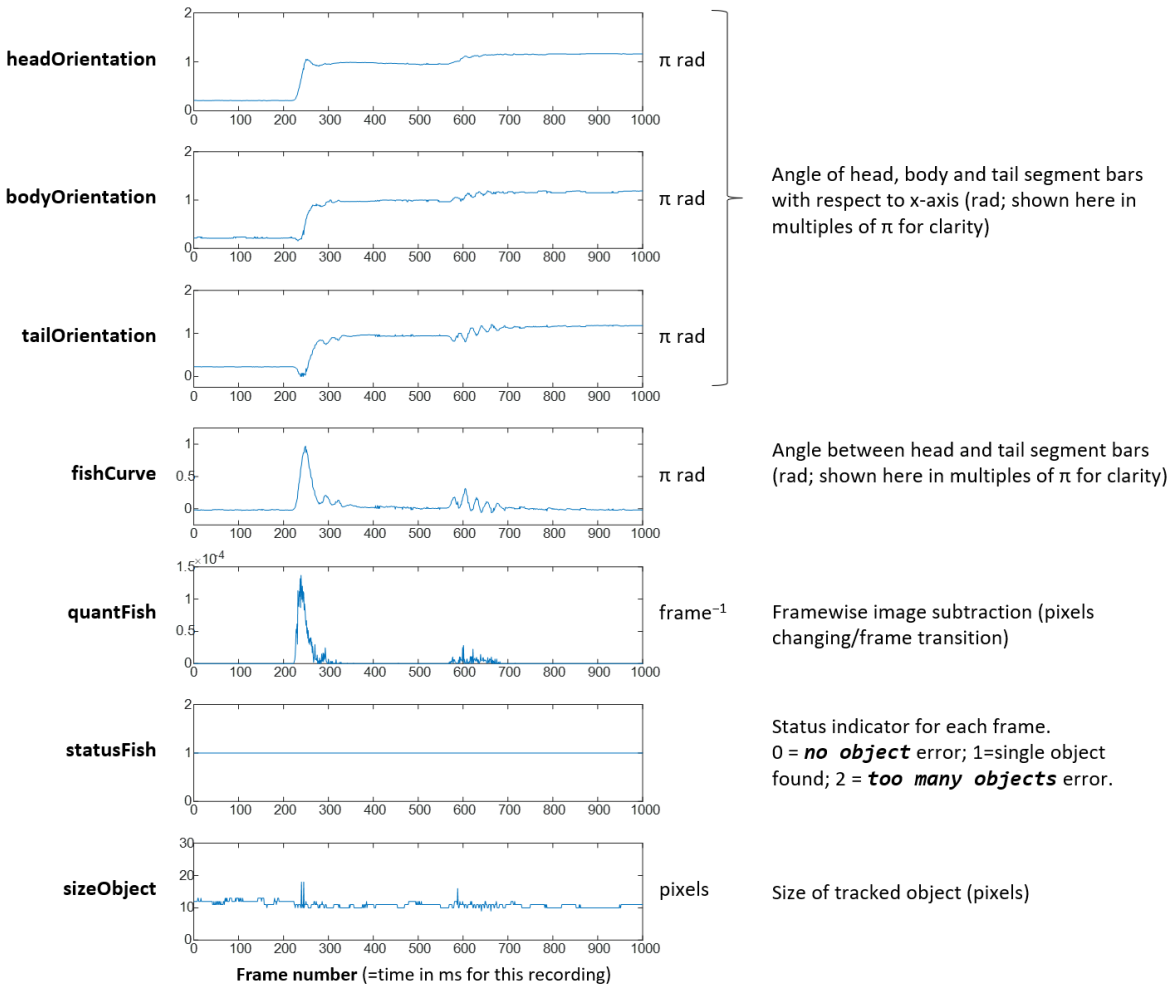
14. Results. Once all video files have been analyzed, the status monitor ⓯ displays a message to indicate that tracking is completed.

Tracking data are stored in .mat files in the same directory as the video file. A separate file is generated for each experimental group for convenience in downstream analysis steps.

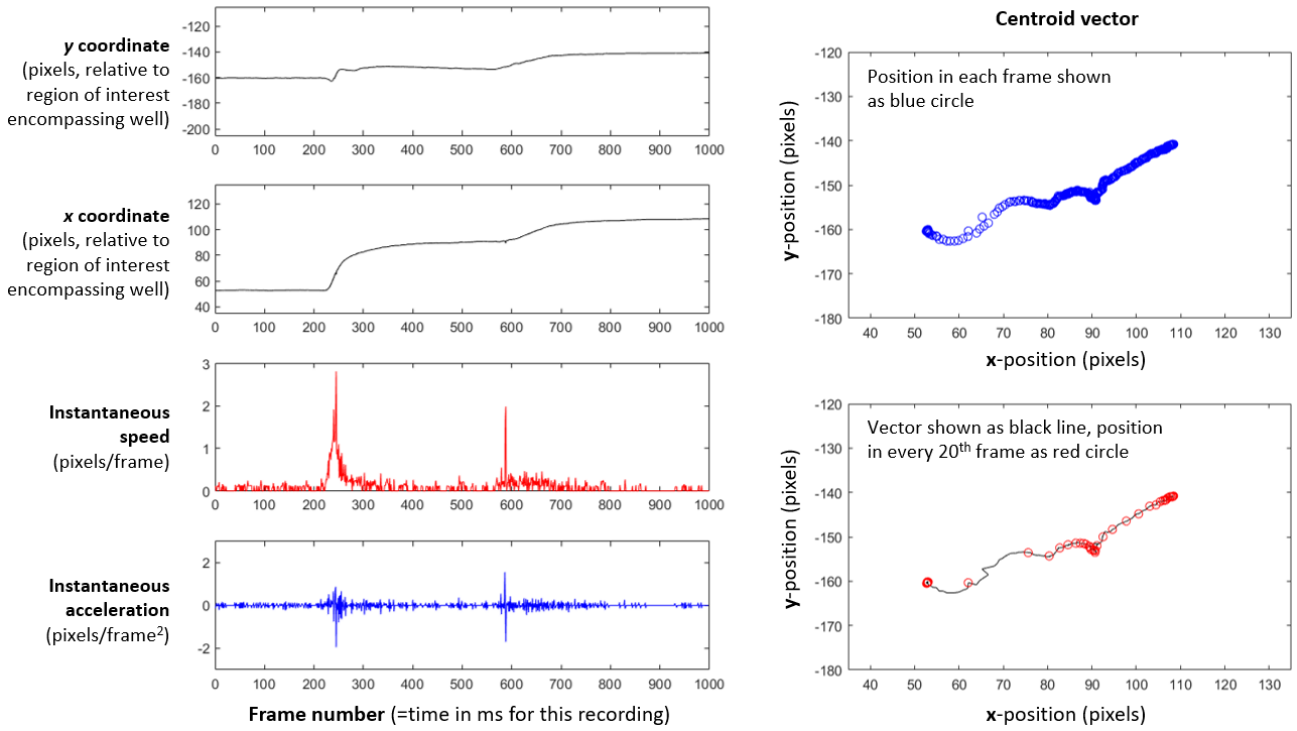


Each output file contains a series of variables describing different aspects of zebrafish movement. 7 of the 8 output variables have dimensions [number of zebrafish] x [number of video frames]. These are shown below to illustrate the data outputs. The zebrafish shown in the example made an 'O'-bend movement followed by a small propulsive swimming movement





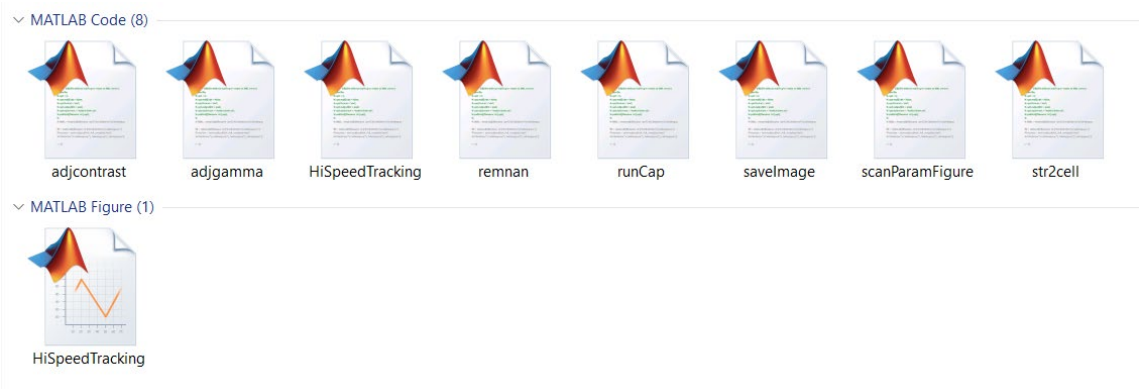
The last of the 8 variables has dimensions [number of zebrafish] x [number of video frames] x 2. This contains the (x, y) coordinates of each zebrafish centroid relative to the origin (top left corner) of its well region in every frame of the video. This can be used to plot the centroid vector and to calculate instantaneous speed and acceleration:



15. Further analysis: The data are formatted as MATLAB matrices that simplify the design and implementation of additional downstream analyses. It is recommended that the framerate of the camera, coupled with a pixel to mm calibration, are used to convert the data to SI units (mm, s, etc.) instead of pixels and frames.

Tracking software

The attached .zip archive contains the MATLAB files necessary to run the tracking analyses reported in this manuscript.



Data set

Numerical data shown in the figures are provided in the attached Excel workbook.