

Chapter 16

Deconstructing Adult Zebrafish Behavior with Swim Trace Visualizations

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Abstract

Three-dimensional reconstruction is a method of qualifying the behavioral activity of several animals including mice, rats, and zebrafish. This method allows for measuring behavioral endpoint data on two types of tracking planes (temporal and spatial). Temporal tracking measures the activity of a subject across time while spatial tracking measures the activity of a subject in a specific area of the experimental arena as such. Data representation over 3D visual trajectory reconstruction is a robustly advantageous method of behavioral phenotyping. Digital video-tracking and analysis eliminates the inaccuracies of manual tracking and allows for improved investigation of behavioral activity at specific points in time or specific areas of the tracking arena.

Key words: Zebrafish, behavioral endpoints, novel tank test, video-tracking technology, affective behaviors, 3D reconstruction.

1. Introduction

The use of video-tracking software in neuroscience research has markedly advanced neurobehavioral phenotyping by permitting rapid, more objective quantification of the animal activity. Video-tracking programs standardize and automate behavioral endpoints, promoting reproducibility of phenotypic studies and allowing for multiple endpoints to be recorded at once (1) (Figs. 16.1, and 16.2).

Three-dimensional (3D) trajectory reconstruction is a method of behavioral analysis that has been performed in multiple animal models ranging across insects, rodents, and primates

A																B															
1	A	B	C	D	E	F	G	H	I	J	K	L	M	1	Time	X	Y	Area	AreaAch	Elongat	Direction	Distance	Velocity	In zone	TurnAng	TurningI	TurningII				
2	Experiment	3												2	0	-0.040	-0.057	0.0001	0	0.9170	-15.0662	5.5E-06	0.00076	0	43.3179	1473.55	1473.55				
3	System	Independent	Variable											3	0.033	-0.040	-0.057	0.0001	0	0.9170	-15.0662	5.5E-06	0.00076	0	4.91725	1343.22	1343.22				
4	Train	Time	7											4	0.066	-0.040	-0.057	0.0001	0	0.9178	-15.0662	3.7E-06	0.00071	0	43.3179	1473.55	1473.55				
5	Train	ID	6											5	0.099	-0.040	-0.057	0.0001	0	0.9170	-15.0662	4.7E-06	0.00074	0	44.3175	1343.22	1343.22				
6	Area	Area	1											6	0.133	-0.040	-0.057	0.0001	0	0.9170	-15.0662	6.4E-06	0.00079	0	19.6196	588.576	588.576				
7	Area	Area	2											7	0.166	-0.040	-0.057	0.0001	0	0.9170	-15.0662	8.1E-06	0.00084	0	19.6196	588.576	588.576				
8	Subject	Subject	1											8	0.199	-0.040	-0.057	0.0001	0	0.9170	-15.0662	9.8E-06	0.00089	0	19.6196	588.576	588.576				
9	Object	Object	1											9	0.233	-0.040	-0.057	0.0001	0	0.9170	-15.0662	1.1E-05	0.00094	0	19.6196	588.576	588.576				
10	Area	Area	Settings	1										10	0.266	-0.040	-0.057	0.0001	5.3E-07	0.91706	-19.198	1.1E-05	0.00093	0	315166	105.411	105.411				
11	Detect	Detection	Settings	1										11	0.299	-0.040	-0.057	0.0001	3.6E-07	0.91682	-24.3461	1.2E-05	0.00095	0	2.52026	84.9628	84.9628				
12	Track	Track	Settings	1										12	0.333	-0.040	-0.057	0.0001	0	0.91682	-16.5131	1.2E-05	0.00097	0	2.37666	71.2985	71.2985				
13	Track	Track	Settings	2										13	0.366	-0.040	-0.057	0.0001	0	0.91682	-16.5131	1.8E-05	0.00054	0	3.56837	91.7659	91.7659				
14	Track	Track	Settings	3										14	0.399	-0.040	-0.057	0.0001	0	0.91682	-16.5131	1.8E-05	0.00057	0	4.17034	125.951	125.951				
15	Record	Record	Settings	1										15	0.433	-0.040	-0.057	0.0001	1.2E-05	0.91937	-16.5131	1.9E-05	0.00056	0	2.8022	84.067	84.067				
16	Record	Record	Settings	2										16	0.466	-0.040	-0.057	0.0001	0	0.91937	-16.5131	1.7E-05	0.00051	0	1.34737	31.4206	31.4206				
17	Track	Track	Settings	4										17	0.499	-0.040	-0.057	0.0001	0	0.91937	-16.5131	1.5E-05	0.00044	0	0.90841	2.94245	2.94245				
18	Track	Track	Settings	5										18	0.533	-0.040	-0.057	0.0001	0	0.91937	-16.5131	1.2E-05	0.00036	0	3.23406	97.0227	-97.0227				
19	Video	Video	Settings	1										19	0.566	-0.040	-0.057	0.0001	0	0.91937	-16.5131	3.2E-06	0.00029	0	2.49715	74.6123	74.6123				
20	Video	Video	Settings	2										20	0.599	-0.040	-0.057	0.0001	0	0.91937	-16.5131	5.7E-06	0.00027	0	4.46713	445.723	-445.723				
21	Train	Train	Settings	1										21	0.633	-0.040	-0.057	0.0001	0	0.91937	-16.5131	3E-06	0.00025	0	19.2284	576.858	576.858				
22	Train	Train	Settings	2										22	0.666	-0.040	-0.057	0.0001	0	0.91937	-16.5131	2.2E-06	0.0002	0	21.4606	643.805	643.805				
23	Track	Track	Settings	6										23	0.699	-0.040	-0.057	0.0001	0	0.91937	-16.5131	2E-06	0.00015	0	14.181	312.356	312.356				
24	Video	Video	Settings	3										24	0.733	-0.040	-0.057	0.0001	0	0.91937	-16.5131	5.4E-06	0.00016	0	16.0712	452.34	452.34				
25	Sync	Sync	Settings	1										25	0.766	-0.040	-0.057	0.0001	0	0.91937	-24.3461	3.2E-06	0.00028	0	35.2001	959.301	-959.301				
26	Reference	Reference	Settings	1										26	0.799	-0.040	-0.057	0.0001	0	0.91937	-16.5131	7.5E-06	0.00022	0	11.9137	483.401	-483.401				
27	Reference	Reference	Settings	2										27	0.833	-0.040	-0.057	0.0001	0	0.91937	-16.5131	8.5E-06	0.00025	0	158986	47.4562	47.4562				
28	Set	Set	Settings	1										28	0.866	-0.040	-0.057	0.0001	3.6E-07	0.91951	-11.5943	3.3E-06	0.00028	0	0.34146	10.4238	10.4238				
29	File	File	Settings	1										29	0.899	-0.040	-0.057	0.0001	0	0.91937	-16.5131	1.8E-05	0.00034	0	6.12509	165.743	165.743				
30	File	File	Settings	2										30	0.933	-0.040	-0.057	0.0001	1.3E-05	0.90706	-3.88821	1.1E-05	0.00032	0	3.39664	119.3	-119.3				
31	File	File	Settings	3										31	0.966	-0.040	-0.057	0.0001	0	0.90706	-3.88821	3.2E-06	0.00028	0	5.52617	85.73	-85.73				
32	File	File	Settings	4										32	0.999	-0.040	-0.057	0.0001	0	0.90706	-3.88821	7.6E-06	0.00023	0	7.95882	226.73	-226.73				
33	File	File	Settings	5										33	1.033	-0.040	-0.057	0.0001	0	0.90706	-3.88821	5.9E-06	0.00019	0	11.943	332.871	-332.871				
34	File	File	Settings	6										34	1.066	-0.040	-0.057	0.0001	0	0.90706	-3.88821	4.5E-06	0.00013	0	14.7203	441.614	-441.614				
35	File	File	Settings	7										35	1.099	-0.040	-0.057	0.0001	0	0.90706	-3.88821	6.5E-06	0.00019	0	19.627	466.85	-466.85				

Fig. 16.1. An example of a track data sheet exported by Noldus EthoVision XT7. **a** – Initial, unprocessed track data obtained from the software. **b** – Formatted, processed track data (after removing identification information, removing spaces from column headers and null values) ready for import into RapidMiner for 3D visualization.

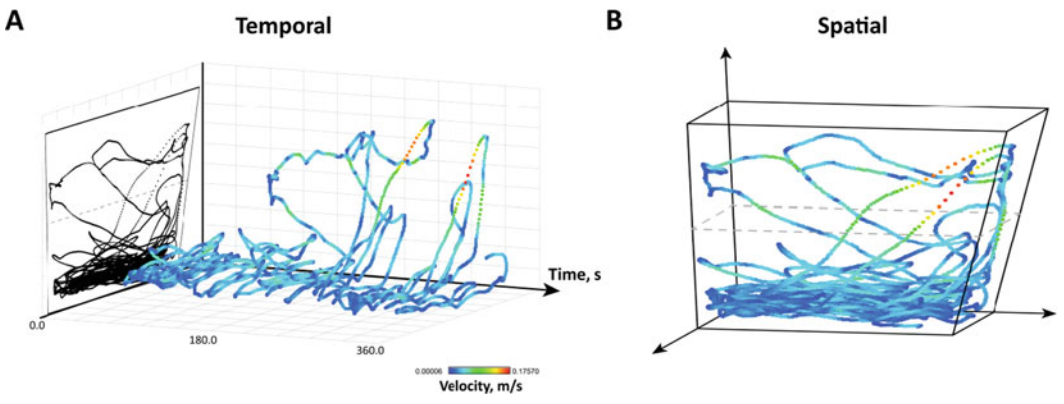


Fig. 16.2. Swim Path 3D Reconstructions. **a** – Temporal swim trace reconstruction of wild-type control fish in standard 6-min novel tank test. **b** – Complete (spatial) reconstruction of same wild-type control fish. Note that time is used as the third axis for temporal reconstructions (**a**) and Cartesian coordinates form the framework for spatial reconstructions (**b**).

(2–4). Video analysis has also been used in zebrafish models to determine specific behavioral endpoints such as velocity, total distance traveled, angular characteristics, and mobility (5). However, previous studies using 2D video-analysis have not been able to fully characterize the behavioral activity of zebrafish, indicating that alternative approaches, such as 3D-based analyses, may be needed to address this problem (6, 7).

Motion-based information in 3D video capture can be obtained through video-tracking programs that occlude human error and inter/intra-rater reliability, and consequently avoid subjective misinterpretation (8). Collectively, this indicates the importance of 3D-based behavioral analyses in neuroscience

research as a more precise method that characterizes zebrafish behavior more comprehensively (e.g., (9)).

Three-dimensional trajectory reconstruction described here involves two major approaches, including spatial and temporal modeling. Temporal reconstructions (**Fig. 16.2a**) visualize zebrafish activity across experimental time, while spatial reconstructions map behavioral changes in real spatial regions of the arena (**Fig. 16.2b**). The tracks produced by plotting the temporal activity allows for visualization of the behavior of the zebrafish at specific points in time. The tracks produced by measuring the spatial activity allows for visualization of the behavior of the zebrafish in specific areas of the arena.

The goal of this chapter is to introduce 3D trajectory reconstruction as a method of tracking zebrafish behavior and to explain how the data collected from the 3D reconstruction can be used to complement the measurement of specific zebrafish behaviors.

2. Materials

Adult zebrafish (~50:50 male:female) can be obtained from a commercial distributor or from the growing availability of genetic mutants at the Zebrafish Information Network (ZFIN.org). Generally, all fish should be given at least 10 days to acclimate to the laboratory environment and be experimentally naïve, although these conditions may vary with the nature of each investigation. Fish can be housed in groups of 20–30 fish in a 40-L tank with water and room temperature maintained at 25–27°C. Illumination can be provided by ceiling fluorescent light bulbs on a 12-h cycle (06:00–18:00 h).

3. Experimental Setup

There are a number of behavioral paradigms for adult zebrafish, with unique experimental specifications thoroughly described within each respective chapter of this volume. For video-tracking and swim track analysis, several universal precautions should be considered. To achieve a precise subject detection, sharp contrast between the subject(s) and the arena background is required. For example, laminated white sheets of paper can be placed behind or below the behavioral apparatus. Reflections from ambient lighting on both the experimental tank and the water line should also be

minimized. Care should be taken to ensure that the camera position does not shift between experimental trials. Notably, video recording does not require premium resolution camera equipment. Our group has found that lower-resolution (320×240 or 640×480), high frame rate (30 fps) videos are preferable due to smaller file size and subject-to-background pixel representation.

3.1. Time Requirement

Time requirement varies according to behavioral paradigm and track data processing requirements. A typical experiment involving 10 control and 10 experimental fish will require ~ 2.5 h for capturing the videos and ~ 2.5 h to analyze the videos. If the laboratory has the ability to record and analyze videos “live” (which requires a video-digitizing system), this time can be reduced to only 2.5 h. Exporting and formatting the data, as well as visual reconstruction of traces in RapidMiner, will require additional 2–3 h.

3.2. General Procedure

There are several available video-tracking software packages including both commercial and open source software. Our group has successfully used both LocoScan (CleverSys, Inc. Reston, VA) and EthoVision XT7 (Noldus Information Technologies, The Netherlands) for adult zebrafish behavioral analysis. This chapter will focus on Ethovision XT7, although the protocol can easily be applied to any video-tracking software, provided time-series and spatial coordinate data can be exported.

4. Video and Track Analysis

Since the procedure for video-aided analysis of zebrafish behavior is detailed in another chapter ([Chapter 1](#)) of this volume, this protocol will focus on supplementary procedures pertinent for swim track visualization.

For each experiment, an arena and respective zones are established over the experimental tank in order to focus detection at the location of swimming activity. While calibrating each arena, specific attention should be given to the placement of the calibration axes, which specify x -, y -coordinate values. By default, EthoVision XT7 places the origin of the calibration axes at the center of the image. However, for different behavioral paradigms, it may be beneficial to customize the origin location. For example, in a standard novel tank test the origin axis is placed along the dividing middle line, denoting the center of the tank as $(0, 0)$. Although customizing the calibration axes is not required, knowing the origin location relative to the testing arena is critical for visual reconstructions.

After all videos are acquired for an experiment, tracks should be smoothed (across 10 samples) and each trial examined for any

abnormalities (i.e., bad detection or wrong arena). Smoothing is required to eliminate “noise” or small bodily movements that are irrelevant to the data collection and could lead to an over-estimation of, for example, the total distance traveled. In the event of major issues, it is recommended to reacquire afflicted videos with properly revised detection settings. Ethovision XT7 includes a basic track visualization feature in which 2D swim paths are plotted within the arena and can be saved as an image file for future reference. For details on troubleshooting, refer to Notes 1–4.

Following a full examination of the behavioral analysis export, tracks need to be interpolated for each trial in order to complete track data across the trial duration. This step replaces missing spatial coordinates by a linear interpolation of the nearest neighbor detection points or the previous and most recent valid detection coordinates. In EthoVision XT7, interpolation is performed within the track editor. At this point, rogue track points (i.e., brief jumps outside of the arena) should be removed prior to interpolation.

4.1. Track Processing

1. Export swim path data as an Excel file from EthoVision XT7 by selecting “Export”, “Raw Data...” and then checking “Track & dependent variables” option. Track data exported from EthoVision XT7 provides spatial coordinates and end-point values for each trial across a time scale broken down into fractions of a second. Based on the frame rate of the acquired video, values are typically provided at 0.033 or 0.024 of every second.
2. In Excel, open each export file and save a copy to a separate folder, naming the file based on the subject’s ID (i.e., Controll). This is to ensure that the original export files are not manipulated and each subject’s swim path can be identified in the future. Once a copy is saved with necessary identification information in the file name, delete all cells above the raw data positioning the column headers (i.e., Trial time, Recording time, X center...) in the first row (**Fig. 16.1**).
3. Rename each column header, or dependent variables, to remove all spaces. For example, “Recording time” to “RecordingTime” or “X center” to “X”.
4. In order for tracks to be properly imported into RapidMiner, there cannot be null (“-”) values in the first time point for each variable. If found, null values should be replaced by the first valid data point or the entire time point(s) (i.e., entire row) can be deleted. This corrective procedure should be limited to the first 0.5 s. All remaining null values “-” need to be replaced as blank cells. To change all “-” to blank,

perform a “find and replace all” procedure with the “match entire cell contents” option checked.

5. Depending on research interests, specific dependent variables can be removed by selecting and deleting the entire column. Additionally, discrete dependent variables (i.e., Movement, Elongation), exported as binary values (0 = false, 1 = true), can be merged with custom “Find and Replace” as well as “concatenate” templates or macros in Excel.
6. For complete 3D reconstructions, raw track data from both side and top views should be processed and corrected separately. Following, the spatial coordinates from the top view can be merged into the side view excel sheet as “Xtop” and “Ytop”, respectively, using the recording time, trial time, and/or unique video time stamps (i.e., the fish being placed into the tank) for proper synchronization. Note that larger arenas (i.e., open field tests) may require more advanced calibration procedures to synchronize the spatiotemporal data of two videos.
7. After initial processing steps, each track file is subsequently saved as a comma separated value (CSV) file.

4.2. RapidMiner

1. Obtained track files are then imported into RapidMiner 5.0 Community Edition, an open-source data analysis and mining system (Rapid-I GmbH, Germany). The corrected track files are imported as separate CSV example sources within a single process based on the goal(s) of the reconstruction process. This permits each track to be explored in a variety of plots after the process is run. Each column is designated as either a real or integer value type based on its contents and no special attributes are assigned. For details on troubleshooting, refer to Notes 5–6.
2. Temporal 3D reconstructions (**Fig. 16.2a**) are created with a Scatter 3D Color plotter, in which the X center, Trial time, and Y center are plotted on the x -, y -, and z -axes, respectively. Complete (spatial) 3D reconstructions (**Fig. 16.2b**) are also produced with a Scatter 3D Color plotter, but X center (side), X center (top), and Y center (side) are plotted on the x -, y -, and z -axes, respectively. Subsequently, select dependent variables (i.e., velocity, elongation, direction) are actively cycled across the reconstructed trace as the Color attribute. In order to compare across fish and/or experimental cohorts, the bounds of each axis are standardized. Images and videos of these reconstructions are exported or captured using screen capture software (Debut Video Capture, NCH Software, Australia). For details on troubleshooting, refer to Notes 7.

5. Anticipated/ Typical Results

Swim trace visualizations are beneficial to all aspects of behavioral research. In the novel tank test, 3D trajectory visualization renderings allow for color-based separation of microscopic behavioral activity across a real-time depiction of fish movement (Figs. 16.3 and 16.4). Such an interactive, investigative environment offers a significant advantage when comparing multiple behavioral endpoints. Typically, behavior between control and experimental cohorts are assessed by comparing bar, column, or line graphs of each quantified endpoint. As the number of valuable behavioral endpoints increase, these methods of data representation are less practical because comparing 20+ bar graphs between or across experimental trials is incomprehensible.

With 3D reconstructions, we have the capacity to both manipulate resolution and orientation of the real-time virtual movement model and view geometric trends occurring over time. With advances in computational neuroscience, 1D graph and 2D trace modeling of behavioral parameters have become methods of extrapolating the same information conveyed in a 3D model, except to a higher volume. Printouts of 3D Model traces can be easily viewed side by side to compare any physically defined trend, such as bouts of freezing and erratic movement (Figs. 16.1–16.3). Software-mediated manual recording of behaviors can be compared for reliability to automated software-run recordings. Furthermore, each reconstruction can easily be saved in a file for later review.

Finally, by cycling various behavioral endpoints across the same swim track reconstruction, we can compare manually registered activity with automated endpoints (Fig. 16.3). Performing this procedure across multiple endpoints and several experimental challenges will improve customization settings in video tracking software. Eventually, such comparisons will advance automated event detection to allow software to register endpoints once previously limited to manual quantification.

6. Notes

1. *Video-tracking software*: For issues involving arena settings, subject detection and/or behavioral variables, please consult the troubleshooting [Section 11](#) of the video-tracking [Chapter 1](#) by Cachat et al. in this book.

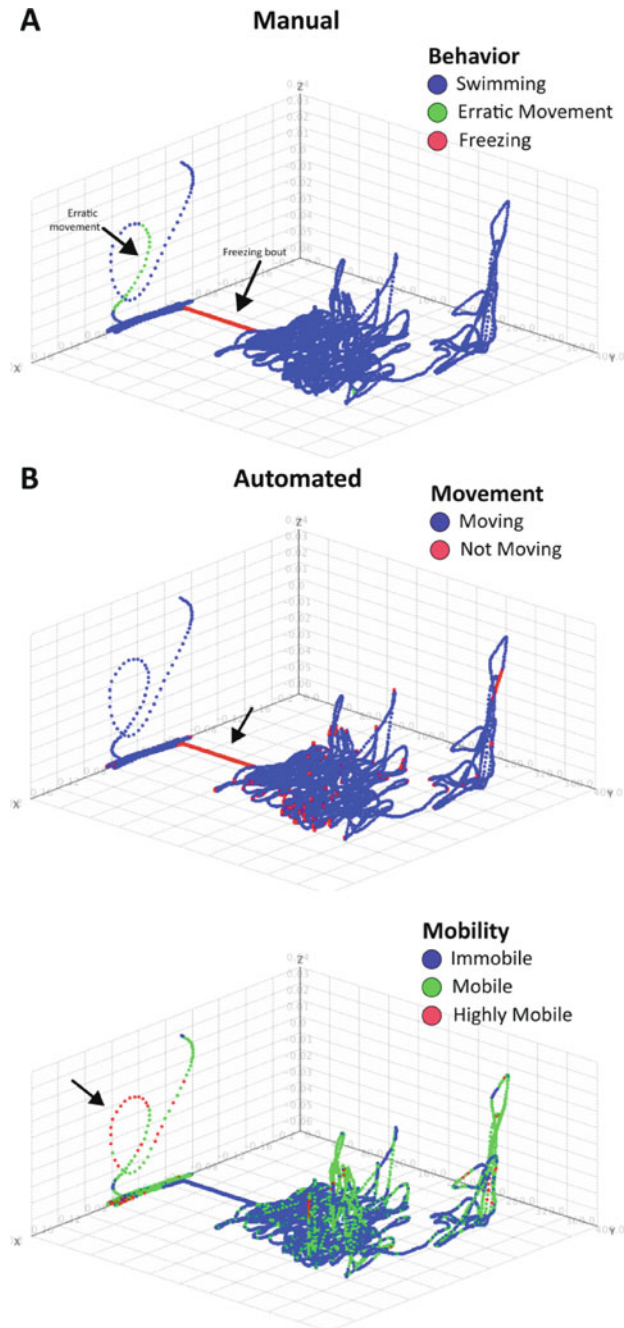


Fig. 16.3. Comparison of manual and automated behavioral endpoints for temporal reconstructions of a typical wild-type control fish tested in a standard 6-min novel tank test. Manual behavior (a) was registered during video acquisition using EthoVision XT7. Automated quantification of “Movement” was applied to behavioral endpoints (note correlation of *not moving* here with *freezing* in panel a), and “Mobility” (note correlation of highly mobile here with erratic movement in Panel a).

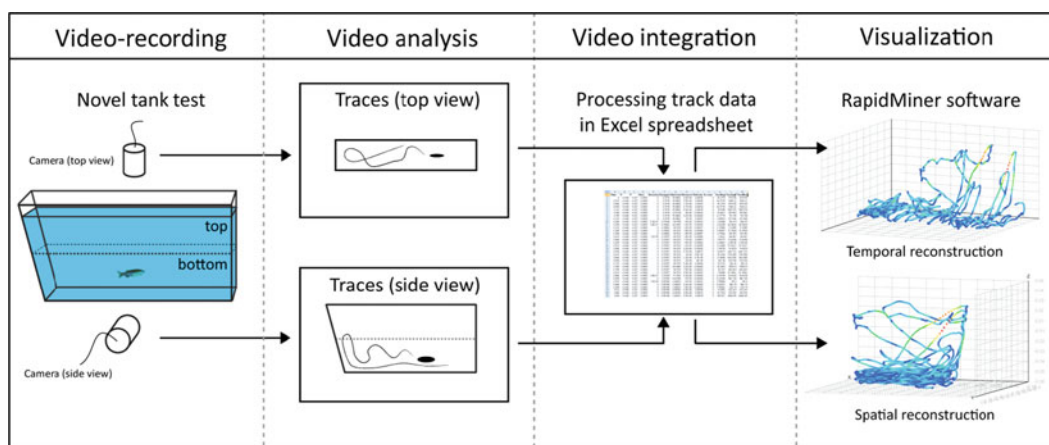


Fig. 16.4. A summary of the swim path reconstruction process.

2. *Trial ID and Subject ID lost*: After removing the identification information from the raw track data export, if the new file is not named with a unique subject ID, the swim path cannot easily be identified as a specific fish. By saving the processed track data as a new file, in a separate folder, the original export file can always be consulted for identification information. As a last resort, the original video analysis experiment can be reviewed in EthoVision to correlate Trial IDs to specific Subject IDs.
3. *Swim path points outside of arena*: Irregularities in the swim path should be noticed and corrected within the EthoVision XT7 track editor. For example, EthoVision will occasionally detect background spots as the subject causing the swim track to jump outside of the experimental tank. Such points should be removed by smoothing the track, but can be manually removed within the track editor prior to interpolation. However, sound judgment should be practiced during such manipulation. If these rogue points are frequent across all experimental trials, it is recommended to reanalyze the videos with revised detection settings.
4. *Swim path points fixed or congregated abnormally*: If there is an unusual point within the arena where the swim track repeatedly “jumps” to or centers around, this is typically the result of a reflection in the video. Especially in top view videos, video-tracking software can easily confuse the movement of a surface reflection for that of the subject. In most cases, this requires rerecording the videos after all reflections have been eliminated or minimized.
5. *RapidMiner shifting column headers*: Open the affected CSV file(s) in Excel and check that each column header is labeled without spaces.

6. *RapidMiner incorrectly importing CSV data:* Generally, errors importing data into RapidMiner occur because the CSV file is not formatted correctly. Additionally, make sure that the first value of each variable is not null and contains a real number, whereas all remaining null “-” values have been replaced as empty cells. All columns must also contain the same number of rows. In a few cases, we have found that the last row of some dependent variables is reported as null. This can be corrected by copying the last valid value or removing the time point entirely for all endpoints. If these corrective measures have been taken and import issues remain, it is possible that a single dependent variable is causing a nonspecific import issues. Consider limiting the track file to Time, X and Y coordinate values, resaving the CSV file and performing the import procedure. Provided the import processes correctly, dependent variables can then be included one at a time as necessary.
7. *Swim path visualization appears abnormal:* As mentioned previously, noting the placement of the calibration axes when establishing the arena settings is critical. Initial 3D scatter plots can appear incorrect because the RapidMiner automatically chooses the max/min range for each axis of the scatter plot. Standardizing the calibration axes during video analysis and manipulating the scatter plot axis ranges will improve the swim path visualization.

7. Conclusion

Three-dimensional trajectory reconstruction offers important opportunities to improve behavioral phenotyping of phenotyping of adult zebrafish. It is also possible that similar approaches can be applied to behavioral phenotyping of larval zebrafish, complementing traditional high-throughput 2D-based screens in small wells with more complex analyses of their spontaneous, unrestricted locomotion in larger 3D environments. The data acquired through 3D reconstruction (**Figs. 16.1–16.4**) can provide extensions to pinpointing measurements of specific behavioral endpoints that are not measurable by simple video-tracking (i.e., erratic movements). Data collected for average velocity indicate a correlation that can be used in the video-tracking software to set range of predetermined values where a specific behavioral endpoint will occur. Finally, 3D trajectory reconstruction also allows for manipulation within the tracking software to better characterize the behavior of the zebrafish.

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