

Supplementary Manual: Applying behavioral studies to the ecotoxicology of corals: a case study on *Acropora millepora*

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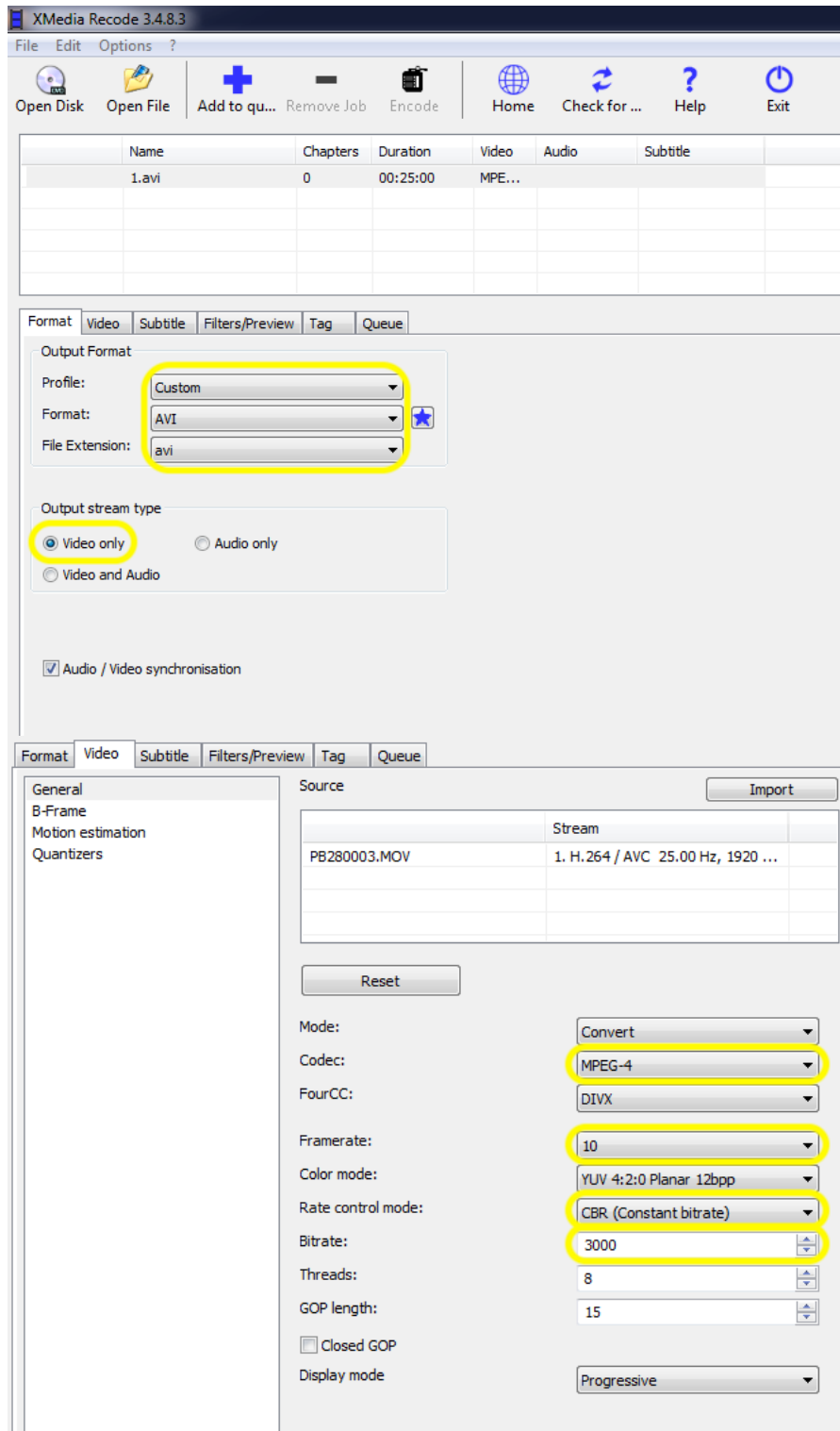
Supplementary Manual

The track analysis of *Acropora millepora* coral larvae across antifouling (AF) coated surfaces encompassed two steps:

1. Post-processing of videos to enhance the contrast between larvae and background using XMedia Recode (version 3.4.8.3) (Dörfler, 2019).
2. Analysis of larvae tracks using the video tracking software EthoVision[®] XT (version 10.1.856) (Noldus, Wageningen, Netherlands), hereafter “EthoVision XT”.

XMedia Recode Settings

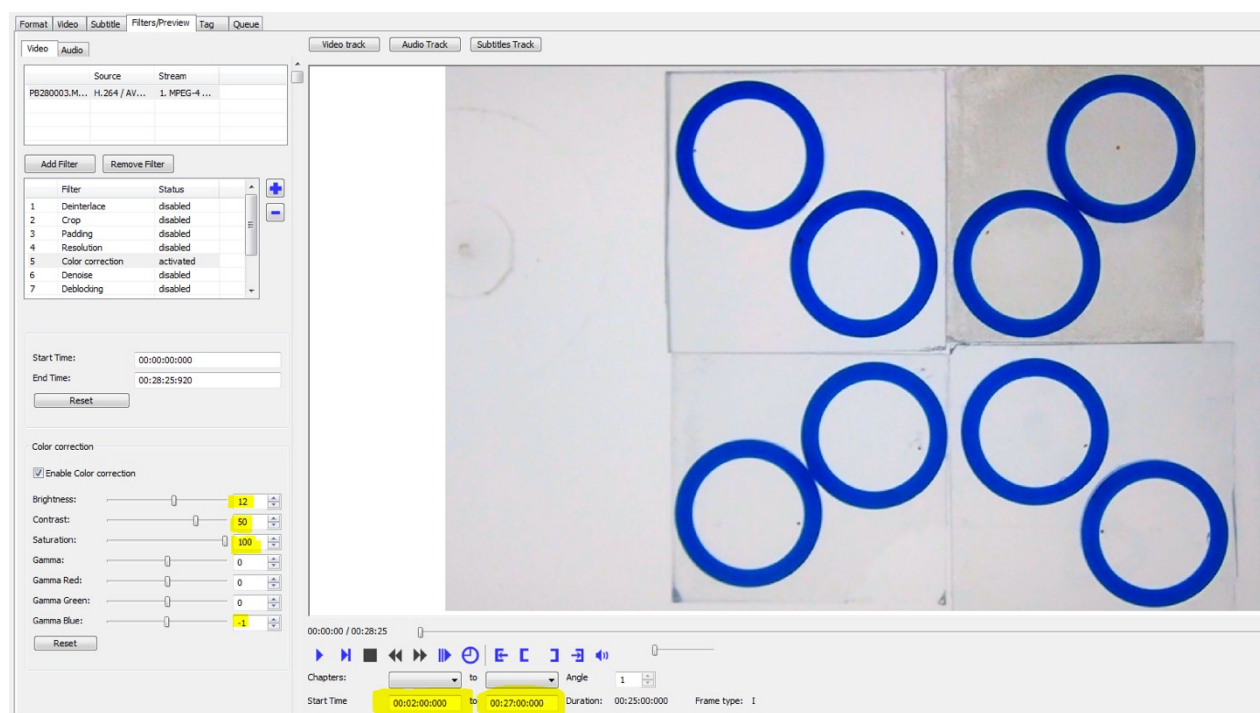
Videos were converted from the original format (.mov) to .avi video-container-format for further application in EthoVision XT. In the video tab, MPEG-4 was chosen as video codec and a constant bitrate of 3000 bit/sec was assigned. The framerate was lowered from the original 25 FPS to 10 FPS. Other settings remained default (**Supplementary Figure 1**).



Supplementary Figure 1. Format and video options in XMedia Recode. Yellow rectangles highlight settings changed from default.

Filters to enhance the contrast between larvae and background were adjusted to the following:

- a. “Color correction” (+12 *Brightness*, +50 *Contrast*, +100 *Saturation*, -1 *Gamma Blue*)
- b. “Denoise” (default settings)
- c. “Color Curves” (preset: strong contrast)
- d. “Hue/Saturation” (+12 *Saturation*) (**Supplementary Figures 2, 3**)



Supplementary Figure 2. XMedia Recode *filters/preview* options. The *Color correction* filter was activated and its settings are shown here (yellow markings).

	Filter	Status
1	Deinterlace	disabled
2	Crop	disabled
3	Padding	disabled
4	Resolution	disabled
5	Color correction	activated
6	Denoise	activated
7	Deblocking	disabled

Start Time: 00:00:00:000
End Time: 00:28:25:920
Reset

Denoise

☒ Enable Denoise

Spatial Chroma Strength: 4.0

Temporal Chroma Strength: 3.0

Spatial Luma Strength: 6.0

Temporal Luma Strength: 5.0

Reset

A

	Filter	Status
4	Resolution	disabled
5	Color correction	activated
6	Denoise	activated
7	Deblocking	disabled
8	Color Curves	activated
9	Grayscale	disabled
10	Hue / Saturation	activated

Start Time: 00:00:00:000
End Time: 00:28:25:920
Reset

Color Curves

enable

☒ Preset: strong contrast

☐ Photoshop curves file: ...

B

	Filter	Status
4	Resolution	disabled
5	Color correction	activated
6	Denoise	activated
7	Deblocking	disabled
8	Color Curves	activated
9	Grayscale	disabled
10	Hue / Saturation	activated

Start Time: 00:00:00:000
End Time: 00:28:25:920
Reset

Hue / Saturation

enable

Saturation: 12

Brightness: 0

Hue: 0

Reset

C

Supplementary Figure 3. XMedia Recode filters/preview options. The *Denoise* filter (A), the *Color Curves* filter (B), and the *Hue/Saturation* filter (C) were activated. Yellow markings indicate settings.

Track analysis in EthoVision XT

EthoVision XT was used to analyze the tracks of the recorded *A. millepora* coral larvae. This section shows which settings of the software were set for the analysis.

By using silicone rings, the larvae were contained in small “pools” filled with filtered seawater on top of PMMA tiles on uncoated controls and 3 different antifouling coatings: CeO₂ nanoparticle coating, DCOIT coating and antiadhesive coating. Each tile contained 2 silicone rings, each with one larva. 4 tiles were filmed (one tile from each treatment incl. control) with one of two identical camera settings, resulting in 8 larvae recordings per video session. The movements of the larvae in the “pools” on the coatings and control were recorded for 28 minutes (file size of four gigabytes: limit of FAT32 formatted SD cards). The first two and the last one minute of each video were cut to acquire clips with a duration of 25 minutes. This procedure ensured a steady video quality without any effects from camera handling and the start. In total, 32 larvae per treatment and control were recorded, resulting in 16 videos to be analyzed.

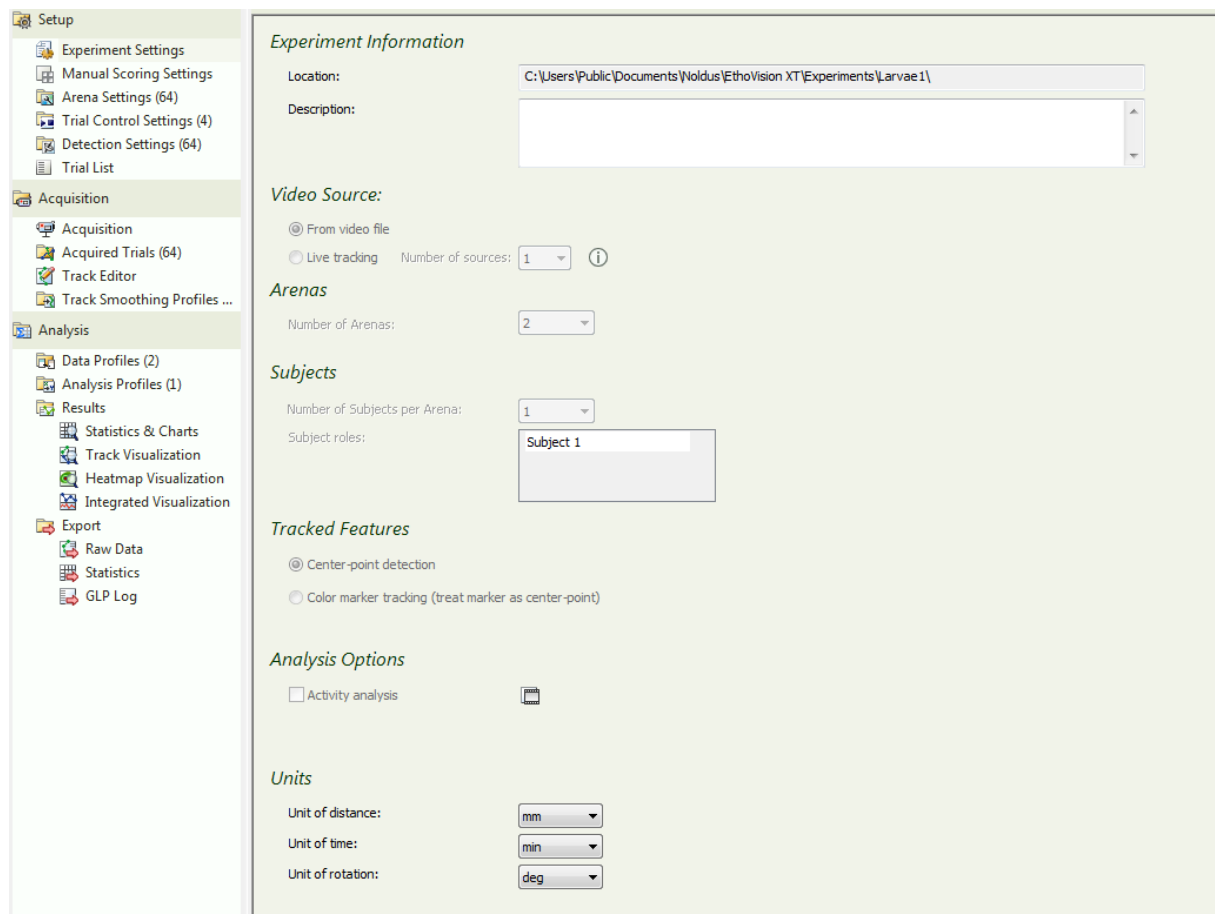
Experiment Settings

The basic settings were adjusted to the following parameters (**Supplementary Figure 4**):

- a. An experimental design with two arenas (= inner diameter of silicone rings) on one tile and one subject (= larva) per arena was chosen. Hereby, the clear separation of treatments was ensured and detection settings were easier to find within one treatment.
- b. Center-point detection was used as tracking method.
- c. “mm” was chosen as unit of distance and “min” as unit of time.

Arena Settings

Each video contained recordings of 8 open-field arenas, with two arenas belonging to one treatment/control (and PMMA tile), respectively. Each arena contained one subject (= one larva). Detection parameters were set on a per treatment basis. Hence, each video was analyzed in 4 separate trials (3 treatments and control), in which each trial contained 2 arenas of the same treatment. The arenas were drawn slightly larger than the inside area of the silicone ring to avoid missed larval recordings by minimal shading effects of the silicone rings. The arenas were calibrated by setting the inner diameter of all arenas (= inner diameter of silicone rings) to the inside diameter of the silicone rings (15 mm) at two points (**Supplementary Figure 5**).



Supplementary Figure 4. Basic experimental settings in EthoVision XT.

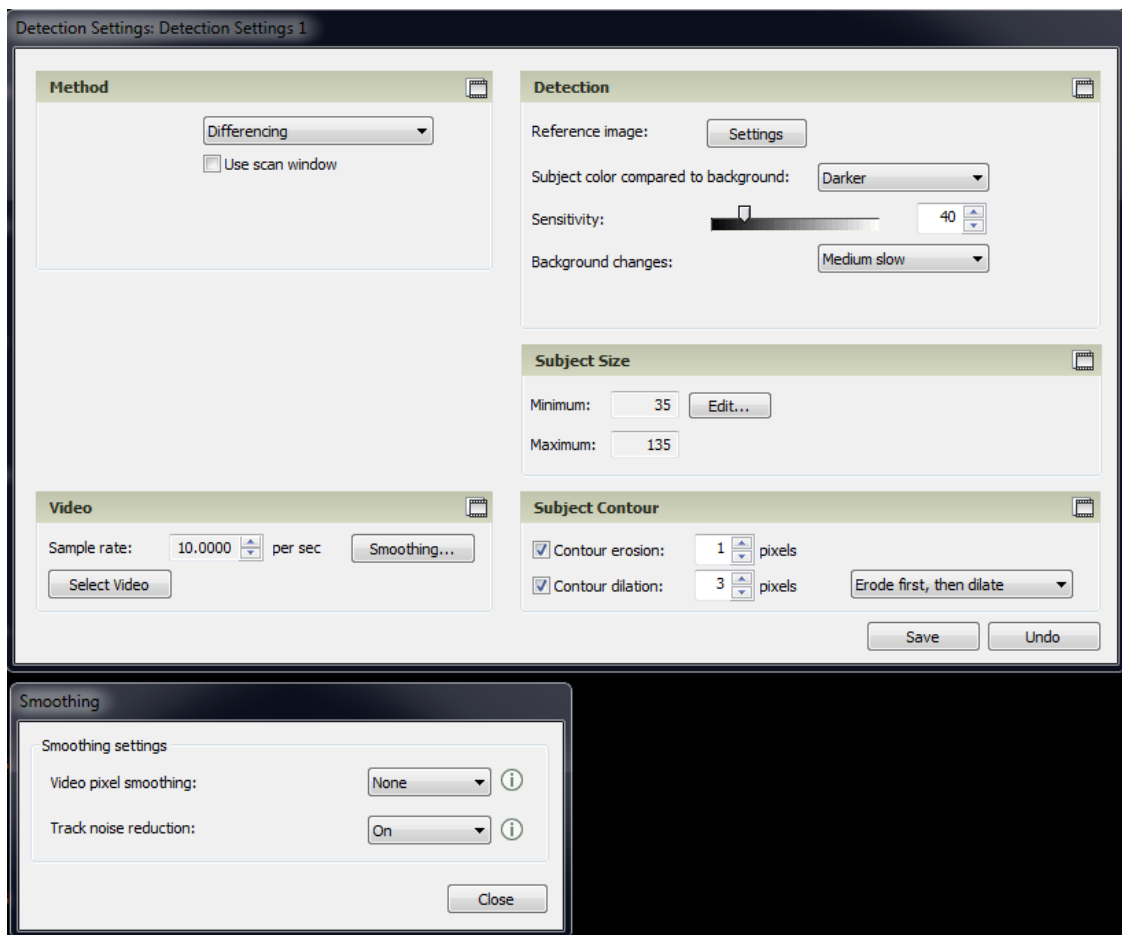


Supplementary Figure 5. Arena settings in EthoVision XT. Arenas for tracking were drawn to be slightly larger than the area of the water surface. Arenas were calibrated by drawing two calibration lines in each arena (silicone rings had an inner diameter of 15 mm).

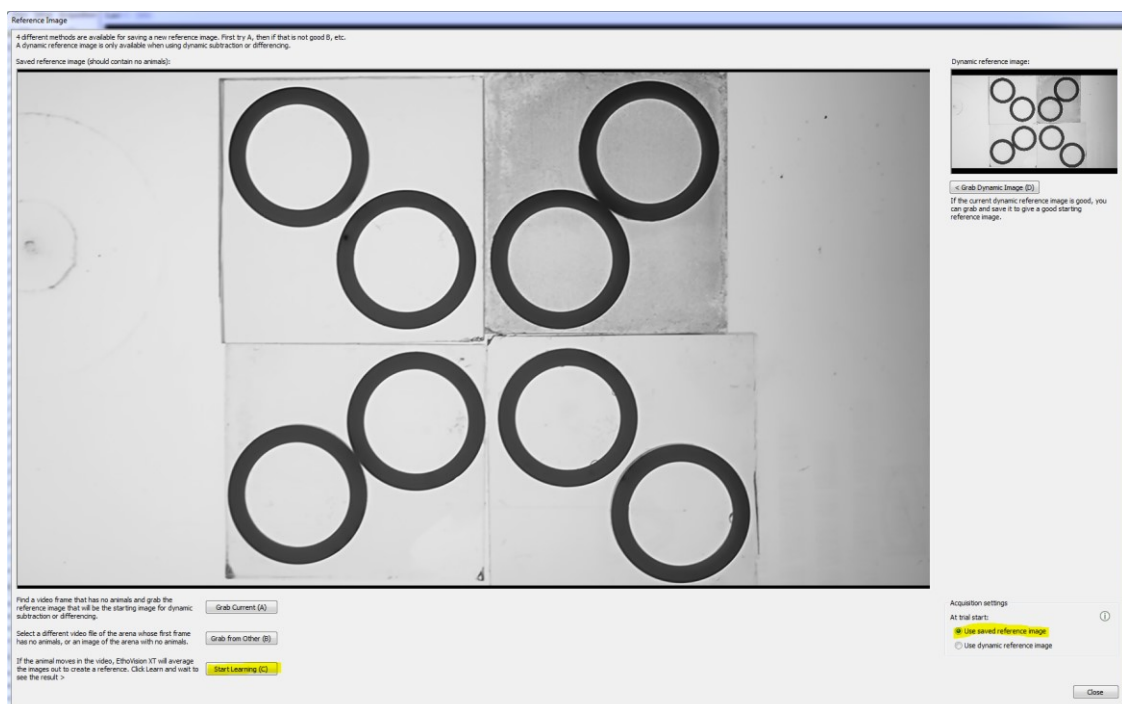
Detection Settings

In the *detection settings* tab, the parameters used to track subjects inside the arena can be manipulated to ensure good detection and low noise. The following points demonstrate how the *detection settings* were manipulated to track the larvae (**Supplementary Figure 6**):

- a. A new *detection setting* was produced for each two arenas on one tile (64 in total).
- b. “*Differencing*” was used as detection method for all videos.
- c. Under “*smoothing*”, “*Track noise reduction*” was activated for all videos to eliminate noise in the detection.
- d. A reference image was produced by averaging the videos using the “*start learning*” option in the reference image settings. If ghosts remained in the automatically acquired images, they were removed using Gimp (Version 2.10.14)(The GIMP Development, 2020) and the corrected images were imported using the “*grab from other*” button. “*Use the saved reference image*” was chosen upon track acquisition under the *acquisition settings* (**Supplementary Figure 7**).
- e. “*Subject color compared to background*” was defined as darker.
- f. “*Background changes*” was set to different values ranging from very slow to very fast, depending on detection.
- g. “*Sensitivity*” was adjusted for each arena setting separately to ensure good detection.
- h. “*Minimum subject size*” was defined as 35 and “*maximum subject size*” as 135.
- i. “*Erosion and dilation*” were usually set to 1 and 2, respectively. However, *erosion* was sometimes set to 0 or *dilation* was set to 1 or 3 if continuous detection would fail.



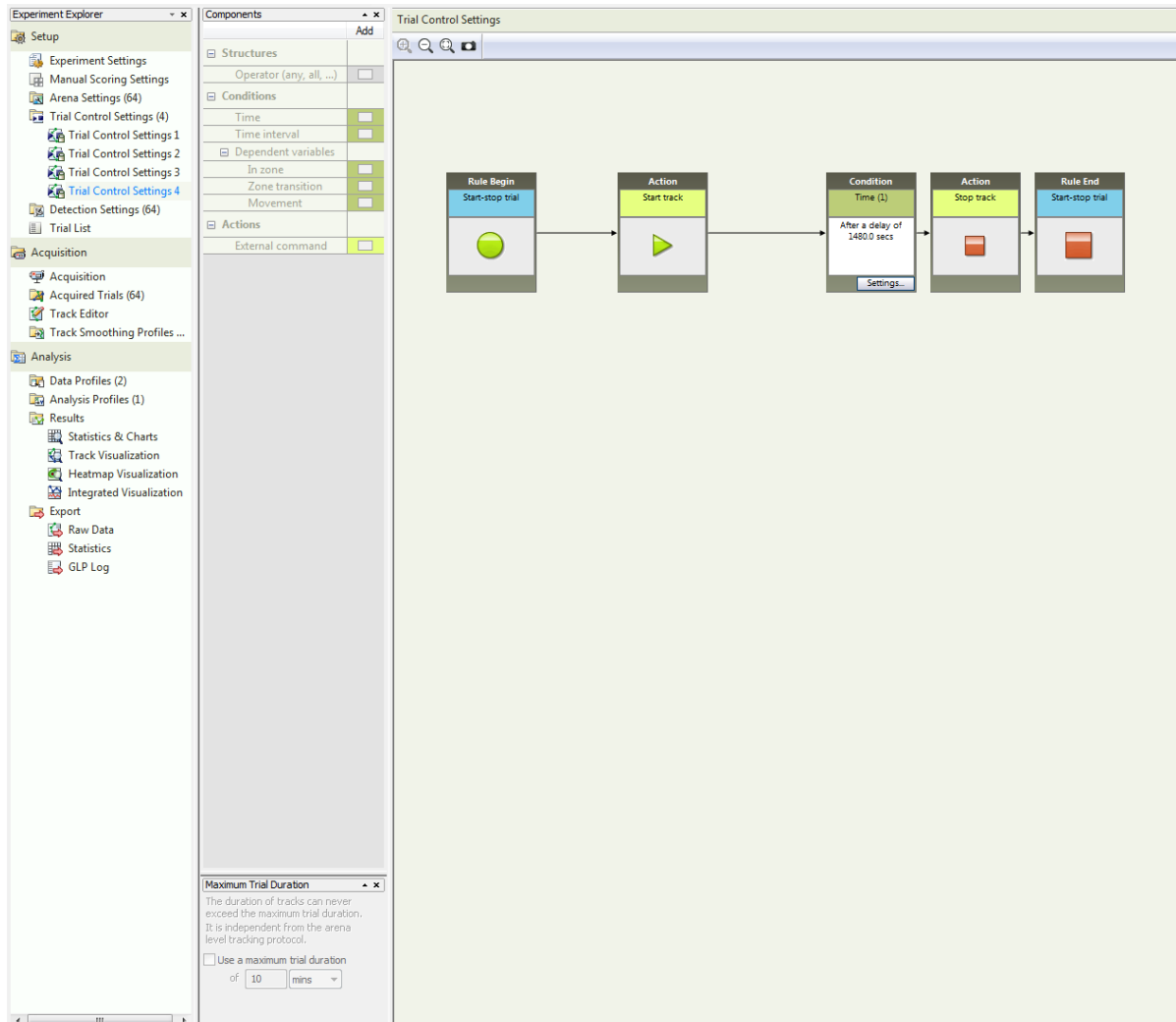
Supplementary Figure 6. *Detection settings* in EthoVision XT.



Supplementary Figure 7. *Reference image* window in EthoVision XT. Chosen settings are marked in yellow.

Trial Control Settings

In the *trial control settings* tab, the sequence of each trial can be adjusted. Each of our trials was set identically. Each trial was terminated after exact 1480 seconds (**Supplementary Figure 8**). 64 trials (128 acquired tracks) were set up, one for every tile (with 2 arenas each) of each treatment and control. The video files, *arena settings*, *detection settings* and the treatment/control were set herein for each trial to enable each track acquisition run.

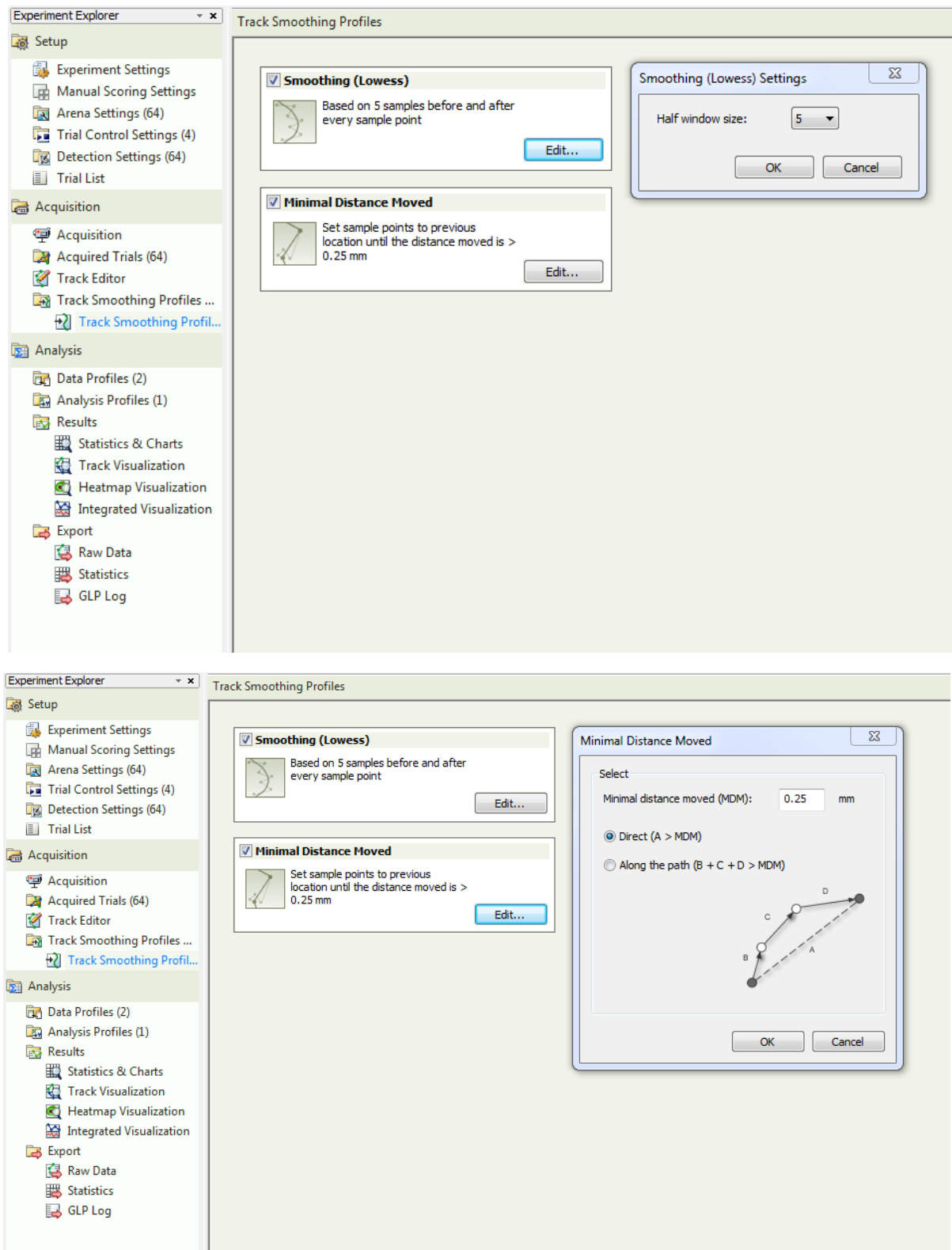


Supplementary Figure 8. Trial Control settings.

Track Smoothing Profile

Track smoothing can be enabled to get rid of detection noise, or the undesired detection of small movements (**Supplementary Figure 9**):

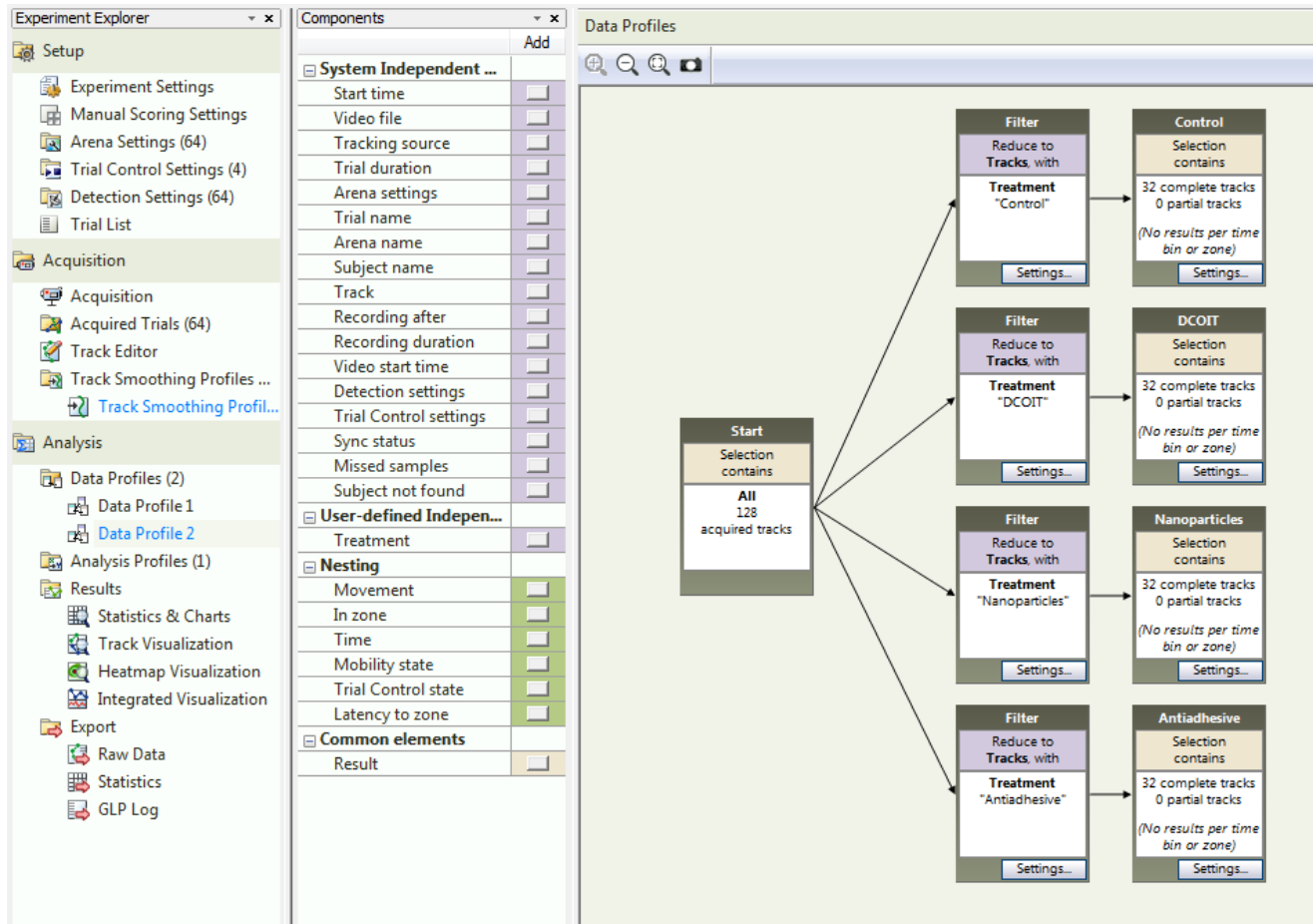
- “*Lowess smoothing*” was set to smooth the track by taking 5 samples before and after each detection point into consideration.
- “*Minimal distance moved*” was set to record track changes only when the larvae moved more than 0.25 mm (direct distance).



Supplementary Figure 9. Track smoothing profile window in EthoVision XT.

Data Profile

The *data profile* can be used to group trials by treatment and to determine how they should be analyzed after track acquisition. Here, the *data profile* was set for statistics to be calculated per treatment (**Supplementary Figure 10**).



Supplementary Figure 10. Data profile window in EthoVision XT with filters set for each treatment.

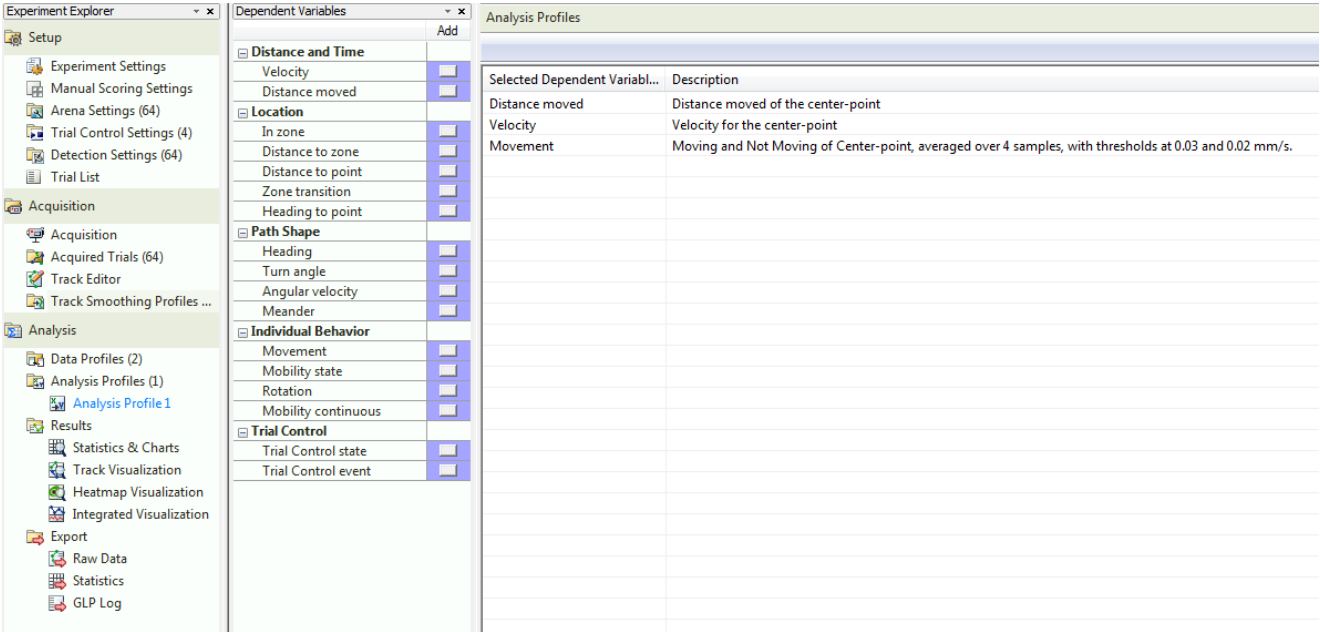
Analysis Profile

The *analysis profile* can be used to set the parameters to be estimated from the acquired tracks (**Supplementary Figure 11**).

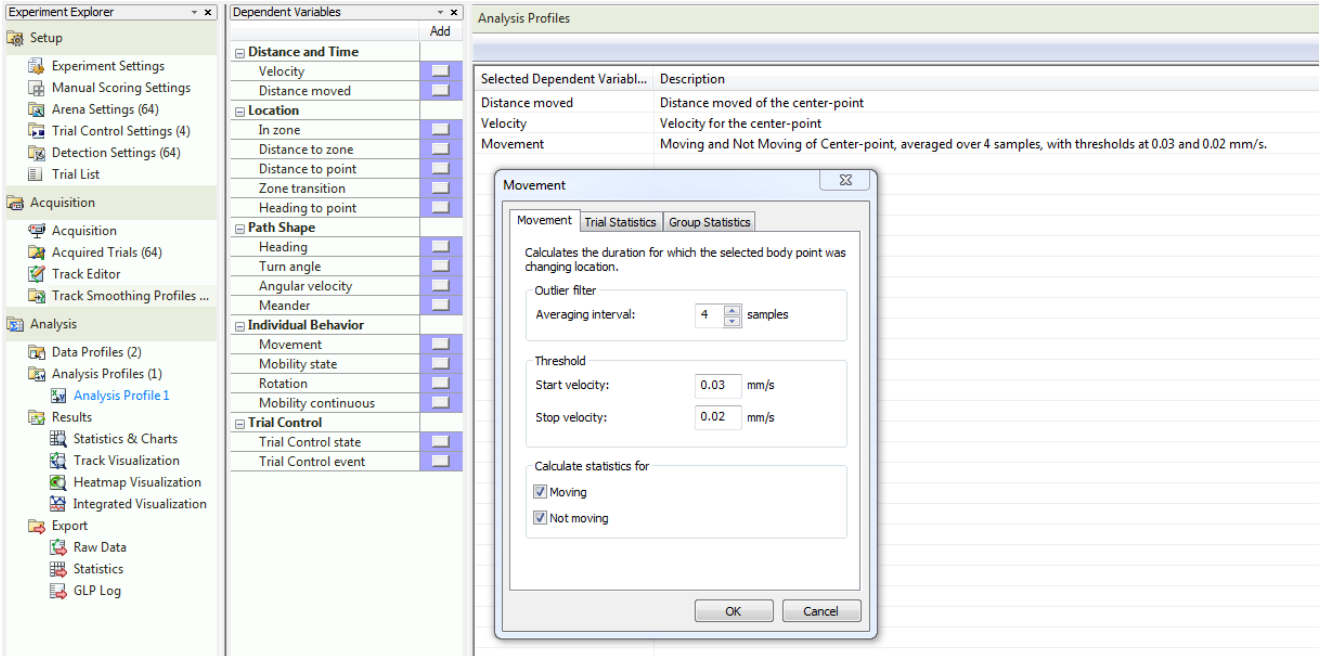
The Analysis Profile was set to calculate:

- Total distance moved.
- Mean velocity.
- Activity time (moving/not moving).
- The threshold velocity of the larvae considered “moving” was set to 0.033 mm s^{-1} ($\sim 1.98 \text{ mm min}^{-1}$), the threshold for “not moving” was linked to a velocity below 0.02 mm s^{-1} ($\sim 1.21 \text{ mm min}^{-1}$). If a larva travelled more than 1.98 mm min^{-1} initially, but lost speed below this threshold, “moving” was still detected. Below 1.21 mm min^{-1} , however, no movement was measured. These settings suppressed noise by ensuring recordings of

actual larval movements and no “jitter of detail” video effects, that could have biased the behavior (Supplementary Figure 12).



Supplementary Figure 11. Analysis profile window in EthoVision XT. The total *distance moved*, the *velocity*, and the *activity* were chosen as parameters of interest.



Supplementary Figure 12. Movement settings in the analysis profile window.

Acquisition of Tracks

The *acquisition* tab can be used to acquire tracks from subjects in video recordings using the previously determined *arena*, *trial control* and *detection settings* (**Supplementary Figure 13**):

- The *acquisition* settings were set to the previously determined *arena*, *trial* and *detection settings* for the corresponding trial. Tracks were acquired one after another.
- Successful detection was monitored throughout the analysis.
- If detection of tracks was not continuous, the *detection settings* were altered to improve detection.
- Speed of acquisition was determined by detection.

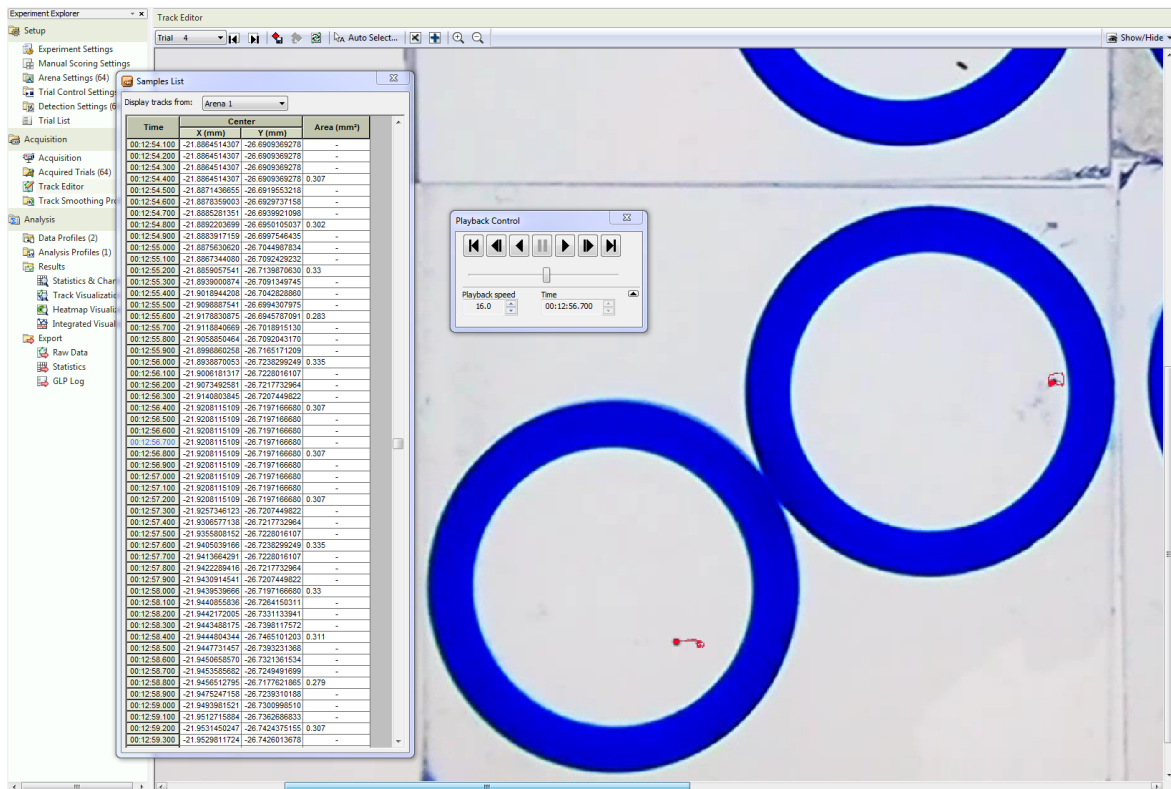


Supplementary Figure 13. *Acquisition* window in EthoVision XT. Each recording was processed individually.

Track Editor

The *track editor* can be used to correct acquired tracks, if detection briefly failed (Supplementary Figure 14):

- Successful detection of tracks was analyzed in the trial editor by watching the detected tracks at higher speed (up to 16x, depending on the activity of the larvae).
- Errors in acquisition were corrected.
- Missing points were interpolated.



Supplementary Figure 14. *Track editor* window in EthoVision XT with each trial sighted and corrected.

Statistics

Trial statistics and group statistics were calculated per treatment group. Statistics were exported to Microsoft Excel 2019 for later analysis in R version 4.1.1 (R Core Team, 2021) (Supplementary Figure 15).

The screenshot displays the 'Statistics & Charts' window in EthoVision XT. The left sidebar shows the 'Analysis' section with 'Statistics & Charts' selected. The main window shows a table of trial statistics for two treatment groups: Antiadhesive and Control. Each group has multiple trials, each with two arenas (Arena 1 and Arena 2). The table columns include Trial, Treatment, Distance moved (Total, mm, mm/min), Velocity (Center-point, mm/min), and Movement (Moving / Center-point, Cumulative Duration, Cumulative Duration (%), Not Moving / Center-point, Cumulative Duration, Cumulative Duration (%)).

An 'Export Statistics' dialog box is open, showing the following settings:

- Export: ☒ Trial statistics, ☐ Group statistics
- Destination folder:
- File type:
- Data formatting: ☐ Swap rows and columns, ☒ Merge column headers

The table data is as follows:

Trial	Treatment	Arena	Distance moved		Velocity	Moving / Center-point		Not Moving / Center-point	
			Total	mm	mm/min	Cumulative Duration	Cumulative Duration (%)	Cumulative Duration	Cumulative Duration (%)
Antiadhesive	Trial 6	Arena 1	56.2250	2.2799	1.4667	5.9455	23.2000	94.0477	
		Arena 2	1.5112	0.0613	0.0400	0.1622	24.6267	99.8311	
	Trial 10	Arena 1	5.0845	0.2556	0.1333	0.6702	19.7600	99.3214	
		Arena 2	0.5030	0.0253	0.0133	0.0670	19.8800	99.9246	
	Trial 14	Arena 1	128.1410	5.1949	2.2233	9.0129	22.4433	90.9803	
		Arena 2	10.0897	0.4090	0.2667	1.0810	24.4000	98.9122	
	Trial 18	Arena 1	4.5538	0.1862	0.1200	0.4865	24.3450	98.6893	
		Arena 2	101.6758	4.1228	2.4100	9.7696	22.2567	90.2236	
	Trial 22	Arena 1	247.0175	10.0142	4.7833	19.3906	19.8833	80.6027	
		Arena 2	27.4107	1.1112	0.7133	2.8917	23.9533	97.1015	
	Trial 26	Arena 1	2990.1637	121.9397	23.4867	95.2098	1.0450	4.2362	
		Arena 2	117.8985	4.7806	2.4050	9.7493	22.2617	90.2439	
	Trial 30	Arena 1	404.3007	16.3906	8.5100	34.4977	16.1567	65.4956	
		Arena 2	1769.0350	71.7176	20.3283	82.4066	4.3383	17.5866	
	Trial 34	Arena 1	14.6682	0.5948	0.3867	1.5675	24.2800	98.4258	
		Arena 2	39.1466	1.5873	1.0067	4.8088	23.6600	95.9124	
	Trial 38	Arena 1	10.6796	0.4383	0.2883	0.9455	24.3450	98.6893	
		Arena 2	1.0069	0.0530	0.0333	0.1267	24.6267	99.8311	
	Trial 42	Arena 1	503.3312	21.3556	9.5944	47.8333	19.3906	19.8833	
		Arena 2	5.5310	0.2556	0.1333	0.6702	19.7600	99.3214	
	Trial 46	Arena 1	88.8038	3.7917	1.8958	7.9722	22.4433	90.9803	
		Arena 2	43.2832	1.7667	1.1067	4.5833	19.8800	99.9246	
	Trial 50	Arena 1	2.7631	0.1146	0.0733	0.2883	24.4000	98.9122	
		Arena 2	91.3337	3.7917	1.8958	7.9722	22.4433	90.9803	
Trial 54	Arena 1	1265.3242	52.7142	24.3333	95.2098	1.0450	4.2362		
	Arena 2	527.1426	21.5467	10.7833	52.7142	24.4000	98.9122		
Trial 58	Arena 1	55.5046	2.2799	1.4667	5.9455	23.2000	94.0477		
	Arena 2	845.9122	35.0833	16.7000	84.5500	19.8800	99.9246		
Trial 62	Arena 1	1285.8307	53.5750	24.3333	95.2098	1.0450	4.2362		
	Arena 2	1920.0316	76.6667	37.6667	95.2098	1.0450	4.2362		
Trial 66	Arena 1	2859.9301	115.4083	57.7083	95.2098	1.0450	4.2362		
	Arena 2	963.7368	39.7833	19.8833	19.8833	19.8833	19.8833		
Control	Trial 1	Arena 1	618.2441	24.7529	6.4383	25.7757	18.5383	74.2177	
		Arena 2	58.4422	2.3698	1.5200	6.1617	23.1467	93.8315	
	Trial 7	Arena 1	2051.2730	83.1766	20.9767	85.0348	3.6900	14.9584	
		Arena 2	1850.7182	75.0443	18.4867	74.9409	6.1800	25.0524	
	Trial 11	Arena 1	1775.3797	71.9894	19.4550	78.8663	5.2117	21.1270	
		Arena 2	545.1143	22.1037	11.4200	46.2942	13.2467	53.6991	
	Trial 15	Arena 1	2321.0607	94.1161	18.5517	75.2044	6.1150	24.7889	
		Arena 2	161.8356	6.5622	3.6100	14.6341	21.0567	85.3591	
	Trial 19	Arena 1	246.8574	10.0098	5.7050	23.1268	18.9617	76.8664	
		Arena 2	2380.6589	96.5328	16.4467	66.6712	8.2200	33.3221	
	Trial 23	Arena 1	1969.2981	79.8526	23.7317	96.2030	0.9350	3.7903	
		Arena 2	1923.0745	77.9994	21.1833	85.8726	3.4817	14.1139	
	Trial 27	Arena 1	842.8314	34.1758	8.5867	34.8085	16.0800	65.1848	
		Arena 2	3218.6749	130.5133	24.3300	98.6285	0.3367	1.3648	
	Trial 31	Arena 1	1876.6029	76.0939	17.2200	69.8061	7.4467	30.1871	
		Arena 2	1032.8143	41.8793	11.8500	48.0373	12.8167	51.9559	
	Trial 35	Arena 1	1087.9167	44.1137	11.8550	48.0576	12.8117	51.9357	
		Arena 2	1907.8818	77.3622	19.6233	79.5487	5.0433	20.4446	
	Trial 39	Arena 1	25.1562	1.0201	0.6600	2.6755	24.0067	97.3177	
		Arena 2	125.1174	5.0737	2.9133	11.8100	21.7517	88.1765	
	Trial 43	Arena 1	2926.0085	118.6460	22.6350	91.7573	2.0317	8.2359	
		Arena 2	2688.6521	109.0510	23.0233	93.3315	1.6417	6.6550	
	Trial 47	Arena 1	1040.5383	42.1925	15.3900	62.3877	9.2767	37.6056	
		Arena 2	1001.0173	40.5818	13.3500	54.1180	11.3167	45.8753	
Trial 51	Arena 1	3627.4722	147.0597	23.8200	96.5610	0.8467	3.4322		
	Arena 2	2122.2422	86.0543	22.2183	90.0682	2.4483	9.9250		
Trial 55	Arena 1	1408.9572	57.1315	19.6167	79.5217	5.0500	20.4716		
	Arena 2	1607.4233	65.1790	14.4967	58.7663	10.1700	41.2269		
Trial 59	Arena 1	2387.9777	96.8295	20.4717	82.9876	4.1950	17.0056		
	Arena 2	3140.4998	127.3176	23.8400	96.6421	0.8267	3.3511		

Supplementary Figure 15. Export *statistics* window in EthoVision XT with export settings visible.

References

- Dörfler, S. (2019). XMedia Recode 3.4.8.3. Available at: <https://www.xmedia-recode.de/en/version.php>.
- R Core Team (2021). R: A Language and Environment for Statistical Computing. Available at: <https://www.r-project.org/>.
- The GIMP Development (2020). GIMP (GNU Image Manipulation Programm). Available at: <https://www.gimp.org>.