

Video Article

High-resolution Quantification of Odor-guided Behavior in *Drosophila melanogaster* Using the Flywalk Paradigm

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Abstract

In their natural environment, insects such as the vinegar fly *Drosophila melanogaster* are bombarded with a huge amount of chemically distinct odorants. To complicate matters even further, the odors detected by the insect nervous system usually are not single compounds but mixtures whose composition and concentration ratios vary. This leads to an almost infinite amount of different olfactory stimuli which have to be evaluated by the nervous system.

To understand which aspects of an odor stimulus determine its evaluation by the fly, it is therefore desirable to efficiently examine odor-guided behavior towards many odorants and odor mixtures. To directly correlate behavior to neuronal activity, behavior should be quantified in a comparable time frame and under identical stimulus conditions as in neurophysiological experiments. However, many currently used olfactory bioassays in *Drosophila* neuroethology are rather specialized either towards efficiency or towards resolution.

Flywalk, an automated odor delivery and tracking system, bridges the gap between efficiency and resolution. It allows the determination of exactly when an odor packet stimulated a freely walking fly, and to determine the animal's dynamic behavioral reaction.

Video Link

The video component of this article can be found at <http://www.jove.com/video/53394/>

Introduction

The overarching goal of any neuroethological research is to establish a causal link between the activity states of single neurons or neuronal circuits and the behavior of an organism. To achieve this goal neuronal activity and behavior should be monitored under identical stimulus conditions and these stimulus conditions should ideally be similar to those the nervous system under scrutiny evolved to make sense of. Particularly when it comes to behavioral bioassays, these requirements have historically proven quite demanding in *Drosophila melanogaster* olfactory neuroethology.

Once released from the source, odor plumes rapidly break up into thin filaments with turbulent diffusion caused by air movement being the main determinant of odor distribution¹. As a result, an insect navigating towards an odor source experiences intermittent stimulation with odor packages interspersed with variable intervals of clean air. Both walking and flying insects - including *Drosophila* - have been demonstrated to exploit this intermittent stimulation regime for navigation by surging upwind upon plume encounter and predominantly moving cross-wind in the absence of odors²⁻⁵. Whereas stimulation procedures in physiological experiments largely mimic those an insect may experience in its natural environment by either providing single puffs of odors interspersed with extended periods of clean air or dynamic stimulation sequences⁶⁻¹¹, many behavioral bioassays used in *Drosophila* neuroethology such as trap assay, open-field arenas or T-maze rely on odor-gradients¹²⁻¹⁵. However, because odor gradients by definition are variable in concentration depending on the distance from the odor source, a particular behavior cannot be attributed to a precise odor concentration using these paradigms. In addition, the slope of an odor gradient critically depends on the physicochemical properties of the odorant. A gradient of a highly volatile compound will be shallower than that created by a less volatile compound and therefore also harder to track for an organism relying on measuring concentration differences in space as the only means of navigation¹⁶⁻²⁰, which may lead to a misinterpretation of olfactory preferences particularly in choice assays. This effect is also highly detrimental when investigating behavior towards odor mixtures because it leads to different blend component ratios at every point in space and therefore again precludes a clear correlation between physiology and behavior.

While vinegar flies tend to aggregate on fermenting fruit, they are solitary in their navigation towards food sources and oviposition sites. Nevertheless, rather than testing individual animals many behavioral paradigms used in *Drosophila* neuroethology examine the odor-guided behavior of cohorts of flies and attraction is scored as the fraction of flies choosing the odor over a control stimulus. These cohort experiments have contributed greatly to the understanding of fly neuroethology and many of the observations made by using them could be confirmed in single-fly experiments. However, it has been observed that flies can influence each other's decision²¹ and in extreme cases the evaluation of an odor can switch from indifference to avoidance depending on population density²². Additionally, results from these kinds of experiments often provide only the endpoint of a sequence of behavioral decisions rather than observing what the fly is doing while it is doing it, which would be

desirable when attempting to correlate behavior with neuronal activity. These rather low-resolution cohort experiments are contrasted by high-resolution single-fly methods such as tethered flight arenas and treadmills which allow for a direct observation of behavioral responses at the time the stimulus is presented^{20,23,24}. Nevertheless, cohort experiments are still popular, because they are very efficient and provide robust results even at comparably low sample sizes because inter-individual and inter-trial variability are partially averaged out due to the observation of populations over extended periods of time. While tethered flight and treadmill probably provide the gold standard concerning stimulus presentation and temporal resolution, the arenas used are designed for single animals and it is therefore time-consuming to obtain sample sizes necessary for a statistical analysis. Several other approaches have recently been developed that allow an efficient acquisition of high-resolution behavioral data in combination with a well-defined stimulus regime. These include unsupervised 3D-tracking of multiple vinegar flies in a windtunnel in combination with an accurate 3D-model of the odor plume⁵, tracking of multiple individual flies in choice chambers supplied with airstreams from both sides²⁵ and the Flywalk paradigm²⁶.

In Flywalk, 15 individual flies are situated in small glass tubes and continuously monitored by an overhead camera under red-light conditions. Odors are added to a continuous airstream of 20 cm/sec and travel through the glass tubes at a constant speed. The airstream is humidified by passing it through 250 ml bottles containing distilled water (humidifiers) before entering the odor delivery system. The flies' positions are recorded within a square region of interest (ROI) encompassing most of the length of the odor tubes (but excluding the outer edges of the tubes (approximately 5 mm at each side) where the flies cannot move further up- or downwind) around the time of odor presentation (**Figure 1A, B**). Fly identities are kept constant by the tracking system throughout the experiment on the basis of their Y-positions (*i.e.* their glass tube limits). Odor stimulation is achieved using a multi-component stimulus device which allows the presentation of up to 8 single odors and all possible mixtures thereof^{26,29} (**Figure 1B**). The course of an experiment is controlled by a computer regulating the odor delivery system and collecting temperature and humidity information (computer 1, **Figure 1C**). This computer also controls a datalogger (start/stop recording) on a second computer which continuously tracks fly positions at 20 frames per sec (computer 2). Fly positions, odor valve status (*i.e.* time-point of valve opening), odor ID, temperature and humidity around odor stimulation cycles are logged on computer 2. This way information on odor and fly positions are synchronized and exported as .csv-files which can be further processed and analyzed using custom-written analysis routines. Because the whole system is computer-controlled, no human intervention is necessary during an experimental session.

Protocol

The construction and technical details of Flywalk have been described elsewhere²⁶ (in case of any problems of establishing this set up, further information can be obtained from MK). Here we focus on detailed instructions on the handling of the paradigm that will help to obtain reliable results.

1. Fly Handling

1. Rear flies in low to medium density cultures on food medium²⁷ under a 12 hr:12 hr light:dark regime at 23-25 °C and 70% relative humidity. To this end, allow 20-30 newly emerged adult flies to reproduce in a big food vial for 1 week, then discard adult flies and wait for offspring to emerge.
2. Collect 30-40 newly emerged (age <24 hr) adult flies and age them on in new a vial containing food medium²⁷ for 3-5 days.
3. Twenty-four hr before the start of the behavioral experiment: Transfer all 30-40 previously collected 3-5 d old (see 2.2) flies to a new vial containing a moist rubber foam plug or a moist tissue paper using an aspirator.
NOTE: Do not anaesthetize flies using CO₂.

2. Preparation of the Flywalk Setup

1. Use 250 ml bottles as humidifiers. Fill humidifiers with 100 ml of distilled water.
2. Prepare odor vials.
 1. Prepare 500 µl 10⁻³ dilutions of the pure odors ethyl acetate, ethyl butyrate, isopentyl acetate and 2,3-butanedione in the solvent mineral oil.
 2. Attach two ball check valves per odor vial. Note that check valves allow for uni-directional airflow only. Therefore, connect check valves in such a way that air can enter the vial on one side and leave it on the other side.
 3. Remove the lid of a 200 µl PCR reaction tube. Pipette 100 µl of every odor dilution into a separate reaction tube and place the tubes in separate odor vials. Also prepare one odor vial containing only the solvent mineral oil.
 4. Tightly seal the odor vials by closing them using stainless steel plugs and rubber gaskets.
 5. Connect the 5 odor vials (4 containing odors and 1 containing mineral oil) to the odor delivery system. Make sure to connect them in the right flow direction. A wrong connection will not only compromise the planned experiment, but it may also contaminate the delivery system.
3. Check for leaks by sealing the outlet of the mixing chamber of the stimulus device. Make sure, that all airflows before the stimulus device now progressively drop to zero. If not, check for leaks which can now be identified by the hissing sound of air leaving the system.
4. Carefully transfer 15 individual flies to 15 individual glass tubes using an aspirator and close glass tubes on both sides using the corresponding adapters.
Note: Because the system must be hermetically sealed for successful experiments, ensure that adapters fit glass tubes tightly and note that glass tubes may break during this step. Take care to avoid injuries by wearing protective gloves and goggles.
5. Connect the glass tubes to the Flywalk setup and, from here on, wait for at least 15 min before starting the experiment to allow flies to habituate to the new environment.
6. After attaching glass tubes: check the readout of the downstream digital flow meters on computer 1 if the 16 airflows after the glass tubes add up to the airflow entering the system. Also check on computer 1, if humidity is between 60% and 80%.

7. Design stimulus protocol controlling the sequence and timing of odor stimuli presented to the flies. To obtain e.g. the described data, present 4 odors and the control (mineral oil) singly and all possible ternary and the quaternary mixtures of the odors simultaneously for 40 times each. Set pulse duration to 500 msec at an interstimulus interval of 90 sec and randomize stimulus sequence.
8. Switch on the light source (LED-cluster; $\lambda = 630$). Make sure to provide enough light for efficient tracking without increasing the temperature inside the glass tubes.
9. Set up a region of interest of the tracking system by dragging a frame across the area to be monitored in such a way that all 15 glass tubes are included and approximately 5 mm of the edges of the tubes are excluded.
10. Set up 14 parallel separation lines between individual tubes in the tracking system by changing their Y-positions in the corresponding script to keep individual flies identifiable throughout the experiment. Make sure to position them in such a way that there is always one glass tube between two such separator lines, because only one fly will be tracked between any set of two lines.
11. Make sure to set camera parameters in such a way that flies are reliably tracked throughout the glass tubes. If flies are lost at the edges of the region of interest, increase brightness or gain of the tracking software. Avoid mechanical vibrations of the tracking system. Track using commercial software according to manufacturer's protocol.
12. Start experiment by starting the stimulus protocol. Record flies' XY-coordinates at 20 fps (frames per sec) and log in combination with the odor valve status in text files.

3. Data Analysis

NOTE: The following steps in the data analysis are automatized using custom-written routines programmed in R. Because these steps are crucial to obtain meaningful results the analysis will nevertheless be presented in a step-by-step manner. The raw data for the analysis are .csv-files containing synchronized information on odor valve status, pulse number in the experiment and 15 fly x-positions in cm on a common time axis for one odor stimulation cycle. Custom code for data analysis can be provided upon request.

1. Open .csv-file, find time-point of valve opening signified by a change in the column representing the valve status.
2. Calculate the linear function of odor position of the form $f(t) = s \cdot t + i$ where t is time in the stimulation cycle, s is the wind speed (here 20 cm/sec) and the intercept i can be calculated using the time-point the odor enters the tubes at position 0 (valve opening plus delay).
3. Find the time point at which odor and fly x-position intersect for every fly and set this time-point to 0. Note: This way fly positions are aligned to each individual's encounter with the odor.
4. Exclude flies sitting at the very edges of the region of interest.
5. Calculate speed from X-positions by dividing displacement along the x-axis by the time interval (100 msec) and repeat procedure for every stimulation cycle.
6. To obtain speed time-courses as shown in **Figure 2E** calculate mean speed time-course for every fly and odor and from those the mean time-course for a given odor.
7. To obtain net displacement as shown in **Figure 3C** calculate the net displacement within 4 sec after the odor pulse for every tracking event and afterwards the mean net displacement per fly and odor.

4. Cleaning Procedure

1. Clean Glass Tubes
 1. Remove flies and adapters from glass tubes and soak glass tubes in detergent.
 2. Rinse glass tubes under running distilled water and dry them using pressurized air.
 3. Heat glass tubes at 200 °C for 8 hr.
2. Clean Odor Delivery System
 1. Remove all odor vials and tubing from the central mixing chamber.
 2. Remove tubing adapters from the mixing chamber.
 3. Clean mixing chamber by rinsing it with laboratory cleaning solution and solvents (e.g. ethanol, acetone). Perform these steps under the laboratory hood.
 4. Dry mixing chamber using pressurized air and heat it at 200 °C for 8 hr.
3. Clean Odor Vials and Check Valves
 1. Remove steel plug (discard rubber gasket) and check valves from odor vials and soak all components in laboratory cleaning solution.
 2. Sonicate components in an ultrasound bath and rinse them with distilled water.
 3. Clean all components except check valves with ethanol and acetone. Perform these steps under the laboratory hood.
 4. Dry components using pressurized air and heat them at 200 °C for 8 hr.
 5. Clean check valves on the inside by flushing them with ethanol and acetone using a syringe (consider flow direction). Perform these steps under the laboratory hood wearing laboratory goggles. Because acetone attacks rubber parts, immediately dry check valves by flushing them with pressurized air.
 6. Remove residual odors by pulsing air through check valves for several days. Use an incubator at 60 °C and a 1 sec air on/1 sec air off regime for this cleaning step.

Representative Results

Because flies are allowed to distribute freely within their glass tubes between odor pulses and the odor pulse travels through the glass tubes at a constant speed flies encounter the odor at different times depending on their x-position at the time of stimulation. As a result, the onsets of the

upwind trajectories evoked by a 500 msec pulse of an attractive 10^{-3} dilution of ethyl acetate are delayed by about 1 sec for flies at the downwind end of their glass tubes compared to those of flies sitting closer to the upwind end at a wind speed of 20 cm/sec and 20 cm glass tube length (**Figure 2A**). Correcting for the temporal difference in odor encounter for every individual based on its x-position at the time of odor presentation reveals that response delays towards ethyl acetate are consistent across individuals (**Figure 2B**).

Correspondingly, the average upwind trajectory without correction for odor travel is delayed by approximately 0.5 sec compared to the average trajectory with correction (**Figure 2C**; note that correction for the time the odor needs to enter the upwind end of the glass tubes was performed for both trajectories). In addition, the corrected mean upwind trajectory for a single odor pulse also displays a steeper slope (*i.e.* a higher walking speed) than the uncorrected one (**Figure 2D**). Similar to the observations for a single odor pulse, omitting the correction for odor travel leads to an increased delay and lower response amplitude in a complete dataset consisting of two experimental sessions (*i.e.* 30 flies) with 40 presentations of odor pulses each (**Figure 2E**).

Repeated stimulation with 500 msec pulses of attractive 10^{-3} dilutions of ethyl butyrate (EtB), isopentyl acetate (IAA), ethyl acetate (EtA) and 2,3-butanedione (BEDN) elicits upwind surges upon odor encounter in starved female flies, whereas stimulation with the solvent mineral oil (MOL) evokes no or only weak responses. Mechanical stimulation alone has previously been shown to induce increased movement in a similar paradigm²⁸. However, because odor stimulation in the Flywalk paradigm does not alter total airflow and increased movement is mostly absent in the control situation using MOL, these upwind surges reflect true odor responses. Mean response time-courses are stereotyped across individuals (**Figure 3A**) and odor-specific in latency, amplitude and duration (**Figure 3A, B**). Responses to ethyl acetate display a sharp onset, high amplitude and a short duration. In contrast, responses to 2,3-butanedione typically display a slightly later onset, a lower amplitude and a longer duration. Ethyl butyrate and isopentyl acetate elicit similar temporal dynamics as ethyl acetate, but responses are lower in amplitude. Correspondingly, all 4 odors elicit a higher upwind displacement within 4 sec after odor encounter than does the solvent and negative control mineral oil (**Figure 3C**).

Using the same 4 attractants, it was previously shown that binary mixtures of attractants are at least as attractive as the more attractive mixture constituent²⁹. Here, this observation is extended by testing all possible ternary mixtures and the full blend of all 4 attractants. Similar to the previous observation with binary mixtures, all of these more complex blends are at least as attractive as the most attractive single compound (**Figure 4A**). The most attractive blends are those containing both ethyl acetate and 2,3-butanedione. Responses to these 3 blends do not differ significantly from each other and also the response kinetics are strikingly similar (**Figures 4A, B**). In contrast, omitting ethyl acetate from the full blend leads to a decrease in the maximum upwind speed, while omitting 2,3-butanedione shortens the response (**Figure 4C**). Because ethyl acetate elicits short high-amplitude responses whereas 2,3-butanedione elicits responses of lower amplitude but longer duration (**Figures 3B, 4D**), these observations are reminiscent of our previous finding, that responses time-courses towards blends of attractants tend to follow an optimum response time-course created from blend constituent response time-courses²⁹. In this dataset the optimum of all 4 attractants can be constructed on the basis of response time-courses towards ethyl acetate and 2,3-butanedione. Ethyl butyrate and/or isopentyl acetate are necessary in addition to reach the maximum walking speed observed in responses towards the full blend (**Figure 4D**). Hence, the increase in mixture complexity from 2 to 3 or 4 components increases the attractiveness of the mixture even further than what would be expected from the previous observation, that responses towards mixtures of attractants represent an optimum of the responses towards mixture constituents. Nevertheless, the general conclusion that constituent valence is conserved in odor mixtures remains valid also for these more complex mixtures²⁹.

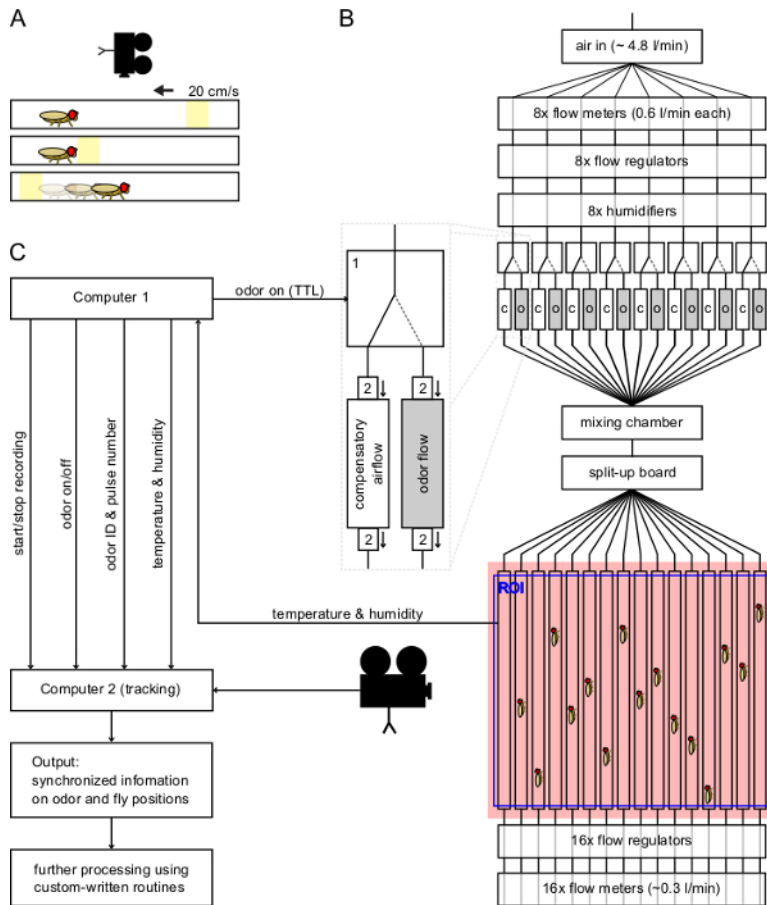


Figure 1. Principle and layout of the Flywalk setup. (A) Schematic drawing of the principle. Yellow square: odor stimulus moving through the tube and resulting in upwind movement of the fly; black object: camera to track behavioral responses. (B) Schematic of the airflow through the setup with charcoal-filtered air being humidified and split into 8 channels, before entering the odor delivery system^{26,30}. Blow-up Figure: 1, three-way solenoid valve passing the airflow either through an empty vial (c; compensatory flow) or through a vial containing the odor source (o; odor flow); 2, ball check valves to restrict air flow in one direction and to avoid system contamination; mixing chamber: custom-built box, that collects air from all solenoid valves and transfers to split-up board, where air is split for 15 glass tubes loaded with individual flies and 1 tube equipped with temperature and humidity sensors. Note: flow regulators and flow meters after glass tubes guarantee identical flow in all tubes. Blue square denotes region of interest (ROI) of the tracking system. (C) Schematic of the information flow between tracking camera, tracking computer, and odor delivery system. [Please click here to view a larger version of this figure.](#)

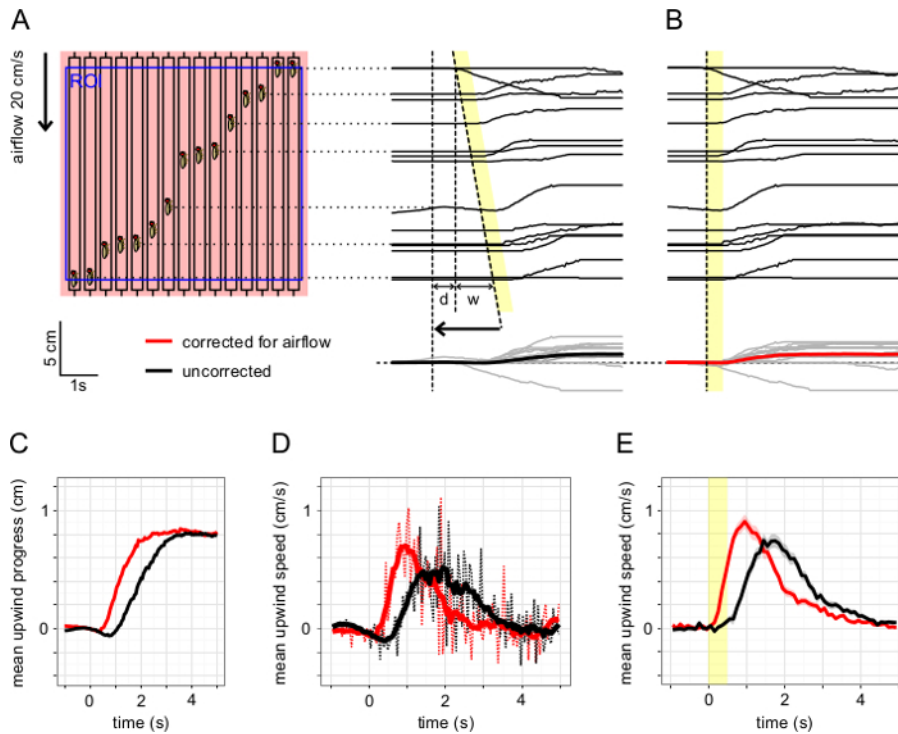


Figure 2. Procedure of and rationale behind data analysis. (A) Individual flies encountering the odor at different positions and, therefore, different times. Left panel: Schematic of possible fly positions at the time of odor valve switching. Right panel: Raw data of x-positions of 15 flies around the presentation of a 500 msec pulse of a 10^{-3} dilution of ethyl acetate. Note upwind walks of individual flies at different times depending on their x-positions. Left dashed line: time of odor valve switching. Odor encounter of individual flies is shifted by a system-intrinsic delay for the odor to reach the glass tubes (d) and the wind speed (w). Therefore, odor encounter is calculated individually for each fly based on its x-position. Bottom right: aligned x-positions of the 15 flies (grey) and mean x-position (bold black). (B) Same data as in A, but corrected for delay and wind speed. Bottom: aligned x-positions of the 15 flies (grey) and mean X-position (bold red) after correction. (C) Comparison of mean upwind progress of 15 flies elicited by one 500 msec pulse of ethyl acetate with and without correction for odor travel. Note that the uncorrected (black) trace is corrected for the delay, but not for odor travel. (D) Mean upwind speed of 15 flies elicited by one 500 msec pulse of ethyl acetate with and without correction for odor travel. Dashed lines indicate speed calculated from upwind progress values shown in C, bold lines display upwind speed after smoothing using a 1st order 9-point Savitzky-Golay filter. (E) Unfiltered mean upwind speed with and without correction for odor travel for 30 flies and 40 pulses of ethyl acetate each (*i.e.* a complete data set). [Please click here to view a larger version of this figure.](#)

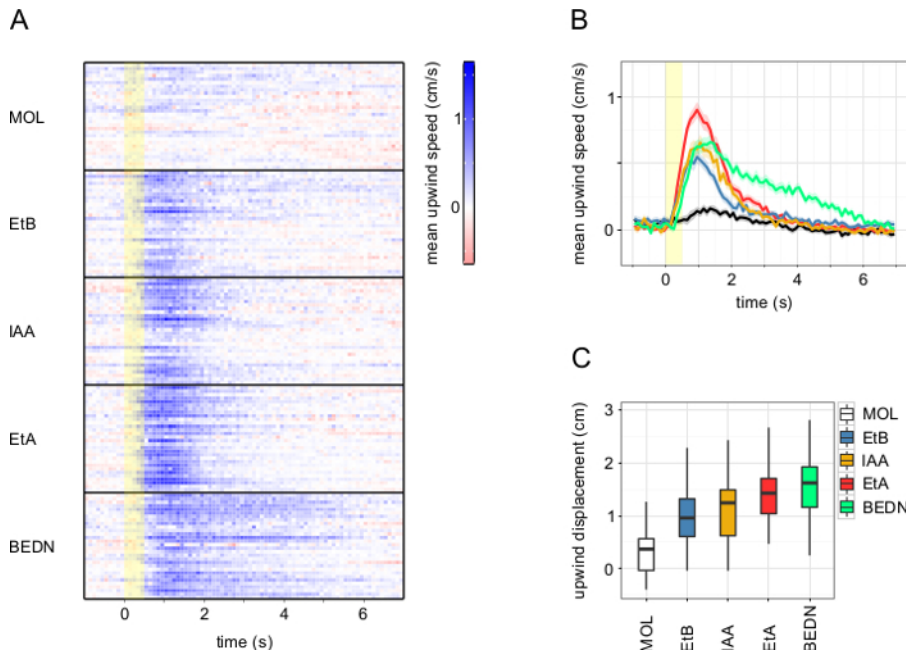


Figure 3. Example responses for 10^{-3} dilutions of 4 attractive odors. (A) Color-coded mean response time-courses of 30 individual flies to 500 msec pulses of 4 attractants and the solvent mineral oil (MOL; EtB: ethyl butyrate; IAA: isopentyl acetate; EtA: ethyl acetate; BEDN: 2,3-butanedione). Each row represents the mean response time-course of one individual fly. Each fly was presented with each odor for 40 times and - because flies are allowed to distribute freely and may leave the region of interest of the tracking system - mean response time courses are calculated from all complete trajectories per fly ($n = 7$ -39 trajectories per fly and odor). Yellow bar represents the odor pulse. (B) Response time-courses to 4 attractants and the solvent mineral oil ($n = 30$ flies; mean $\pm$ s.e.m.). (C) Net upwind displacement within 4 sec after odor encounter (same data as in (A) and (B), $n = 30$ flies). Filled boxes indicate statistically significant upwind movement compared to the negative control mineral oil ($p < 0.05$; Wilcoxon signed rank test). [Please click here to view a larger version of this figure.](#)

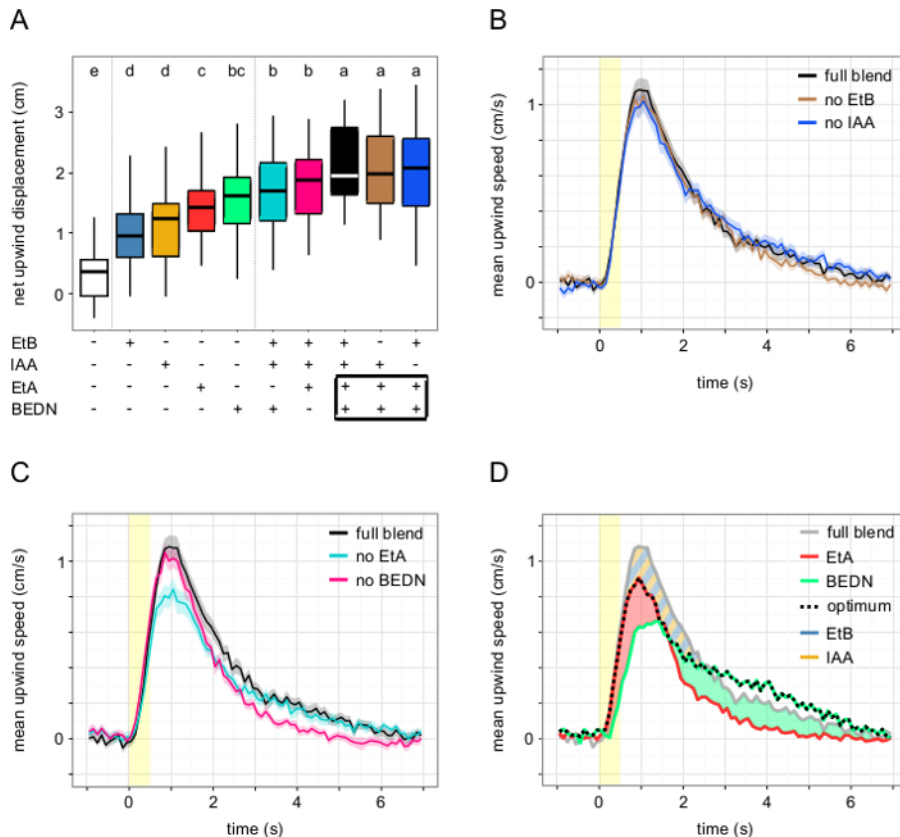


Figure 4. Responses towards ternary and quaternary mixtures of attractants. (A) Net upwind displacement for 4 attractants and all ternary and quaternary mixtures thereof sorted by their median response. Different letters indicate statistically significant differences between responses ($p < 0.05$; Kruskal-Wallis rank sum test and *post hoc* Wilcoxon signed rank test; $n = 30$ flies). Black box below indicates mixtures containing ethyl acetate and 2,3-butanedione. (B) Response time-courses of mixtures containing ethyl acetate and 2,3-butanedione (mean $\pm$ s.e.m.; $n = 30$ flies). Note similar response-kinetics. (C) Comparison of response time-courses of mixtures without ethyl acetate or 2,3-butanedione and response kinetics evoked by the full blend (mean $\pm$ s.e.m.; $n = 30$ flies). Note lower amplitude without EtA and shorter response without BEDN. (D) Comparison of optimum time-course (dashed) constructed from EtA (red) and BEDN (green) and the full blend. Shadings indicate parts of the time-course explained by different mixture constituents. Note that in a previous study it has been shown that responses towards binary mixtures of attractants can be predicted from an optimum time-course created on the basis of mixture constituent time-courses²⁹. This optimum time-course for EtA and BEDN is shown as a dashed line. [Please click here to view a larger version of this figure.](#)

Discussion

Although the Flywalk system appears rather sophisticated at first glance, once set up and running it is easy to use and produces very robust results. To stress the consistency of the results produced with the bioassay it may be said that the representative results shown here were obtained almost 2 years after some of the results shown in a previous study²⁹ with a modified setup using a new tracking software and light source. Nevertheless, the attractant responses are - despite slightly higher response amplitudes - very similar to those previously published concerning their dynamics.

There are some critical aspects which should be considered in particular in order to obtain high-quality results using Flywalk. Importantly, humidity should not drop below 60% during the course of an experiment. A typical experimental session lasts for approximately 8 hr. Reasonably-sized female CS wild-type flies starved for 24 hr before the experiment easily survive for at least 12 hr in the experimental setup provided humidity is high enough. To avoid problems related to humidity it is advisable to install a humidity sensor in an empty glass tube (Figure 1B) and to avoid placing flow regulators in the airflow anywhere between humidifiers and glass tubes. It is also absolutely essential for data quality that the system is hermetically sealed. The airflows leaving the system after the glass tubes should sum up to the airflow entering the system. Most failed experiments can be attributed to leaks in the system and great care should be taken before the start of the experiment to assure that the system is airtight. Finally, as with any setup used in olfactory research, one of the major everyday issues is to avoid contamination. Most of the parts coming into contact with the odors are made of glass, steel, Teflon or PEEK and can therefore be heated up to at least 200 °C, which is sufficient to remove most odors except for those with particularly high boiling points such as long-chained pheromones. Because check valves contain rubber parts they cannot be heated as high and therefore are the major source of contamination, which is why a particular cleaning protocol was devised for them. Nevertheless, it is advisable to keep track of the odors a particular check valve has come into contact with. In case of doubt regarding its cleanliness replace it.

As a compromise between tethered assays and cohort experiments, Flywalk of course also has some disadvantages compared to other methods. The paradigm is very efficient when behavior towards a multitude of different stimuli has to be assessed and compared. Notably, because the response time-course of 15 individuals to just one pulse of a given odor strongly resembles response time-courses obtained in

a full data set (*i.e.* 30 flies and 40 presentations each; **Figures 2D, 2E**), it may be possible to further increase the number of different stimuli presented in a given experiment by using different stimulation methods such as coupling the system to a gas chromatograph. However, because of the duration of an experimental session only one experiment is possible per day and a given experiment should at least be repeated once to obtain reliable results. Therefore, trap assay or T-maze are more efficient when *e.g.* the hedonic valence of just one odor needs to be examined. Also, the temporal resolution of visual tracking systems is often lower than that of the fastest bioassays such as tethered flight or treadmill. The shortest response delays reported in *Drosophila* odor-guided behavior are well below 100 msec after odor encounter in tethered paradigms^{20,23} and thus fall within a time window which cannot be resolved when analyzing the data at 10 Hz. However, attractant responses in Flywalk typically begin within the first 100-300 msec (**Figure 3B**), which is well in line with upwind surge delays observed in free-flying flies⁵. It therefore remains to be determined whether this difference in response delays in tethered paradigms compared to wind tunnel and Flywalk is caused by differences in spatial and/or temporal resolution in visual tracking paradigms or by a higher arousal state of flies in the tethered situation.

In summary, Flywalk is a no-choice bioassay, which combines the highly controlled stimulus presentation of tethered assays with the efficiency of cohort experiments regularly used in *Drosophila* neuroethology. Because the same set of individuals can be challenged with a multitude of different stimuli, its particular strength lies in the statistical power when comparing responses towards different stimuli. Additionally, it exploits the fact that flies surge upwind upon encounter of an attractive odor and this way uncouples odor evaluation from odor source localization without the need for a gradient as a directional cue. It should therefore be ideally suited to exploit the optogenetic toolbox available in *Drosophila*.

Disclosures

The authors declare that they have no competing financial interests.

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