Video Article

High-Throughput Method for Measuring Alcohol Sedation Time of Individual *Drosophila melanogaster*

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Abstract

Drosophila melanogaster provides an excellent model to study the genetic underpinnings of alcohol sensitivity. In contrast to studies in human populations, the *Drosophila* model allows strict control over genetic background, and virtually unlimited numbers of individuals of the same genotype can be reared rapidly under well-controlled environmental conditions without regulatory restrictions and at relatively low cost. Flies exposed to ethanol undergo physiological and behavioral changes that resemble human alcohol intoxication, including loss of postural control, sedation, and development of tolerance. Here, we describe a simple, low-cost, high-throughput assay for assessing alcohol sedation sensitivity in large numbers of single flies. The assay is based on video recording of single flies introduced without anesthesia in 24-well cell culture plates in a set-up that enables synchronous initiation of alcohol exposure. The system enables a single person to collect individual ethanol sedation data on as many as 2,000 flies within an 8 h work period. The assay can, in principle, be extended to assess the effects of exposure to any volatile substance and applied to measure effects of acute toxicity of volatiles on other insects, including other fly species.

Video Link

The video component of this article can be found at https://www.jove.com/video/61108/

Introduction

The National Institute on Alcohol Abuse and Alcoholism reports that in 2015 excessive alcohol consumption, designated as "alcohol use disorder", affected an estimated 16 million people in the United States. Alcohol abuse causes a wide range of adverse physiological effects and is a major cause of death in the U.S. In humans, decreased sensitivity, or a low level of response to alcohol, has a strong genetic component and is associated with a higher risk of developing alcohol use disorders[1,2,3,4]. Genetic risk studies on human populations are challenging because of population admixture, diverse developmental histories and environmental exposures, and reliance on self-reported questionnaires to quantify alcohol-related phenotypes, which are often confounded with other neuropsychiatric conditions.

Drosophila melanogaster provides an excellent model to study the genetic underpinnings of alcohol sensitivity[5,6,7,8]. The *Drosophila* model allows strict control over genetic background, and virtually unlimited numbers of individuals of the same genotype can be reared rapidly under well-controlled environmental conditions without regulatory restrictions and at relatively low cost. In addition to publicly available mutations and RNAi lines that target a majority of genes in the genome, the availability of the *Drosophila melanogaster* Genetic Reference Panel (DGRP), a population of 205 inbred wild-derived lines with complete genome sequences, has enabled genome-wide association studies[9,10]. Such studies have identified genetic networks associated with effects on development time and viability upon developmental exposure to ethanol[11,12]. Evolutionary conservation of fundamental biological processes enables translational inferences to be drawn by superimposing human orthologs on their fly counterparts.

Flies exposed to ethanol undergo physiological and behavioral changes that resemble human alcohol intoxication, including loss of postural control[8], sedation, and development of tolerance[13,14,15]. Alcohol induced sedation in *Drosophila* can be quantified using inebriometers. These are 122 cm long vertical glass columns with slanted mesh partitions to which flies can attach[16,17,18]. A group of at least 50 flies (sexes can be analyzed separately) are introduced in the top of the column and exposed to ethanol vapors. Flies that lose postural control fall through the column and are collected at 1 min intervals. The mean elution time serves as a measure of sensitivity to alcohol intoxication. When flies are exposed to alcohol a second time after recovering from the first exposure, they can develop tolerance, as evident from a shift in mean elution time[13,15,19,20]. Whereas inebriometer assays have led to identification of genes, genetic networks, and cellular pathways associated with alcohol sedation sensitivity and development of tolerance[12,13,14,21], the assay is time consuming, low-throughput, and ineffective for measuring alcohol sensitivity in single flies.

Alternative ethanol sedation assays that do not require the elaborate inebriometer set-up allow for more convenient measurements but are still limited in throughput and generally require analyses of groups of flies rather than individuals[21,22,23,24,25]. Assessing single flies minimizes the

potential for confounding effects due to group interactions, such as those stemming from social behaviors. Here, we present a simple, low-cost, high-throughput assay for assessing alcohol sedation sensitivity in large numbers of single flies.

Protocol

1. Construction of the testing apparatus

1. Create a cardboard template the size of a 24-well cell culture plate by tracing around the plate on cardboard and cutting out the designated area.
2. Cut a piece of small insect screen mesh the size of the cell culture plate using the cardboard template from step 1.1.
3. Prepare a 24-well cell culture plate by placing a small line of hot glue around the perimeter of the top of the plate using a hot glue gun and affixing the screen mesh on top of the open wells.
4. Secure a wooden craft stick to each of three sides of the same cell culture plate from step 1.3 using a hot glue gun. The modified cell culture plate should now resemble the plate diagram shown in **Figure 1A** and the experimental setup shown in **Figure 2**.
   NOTE: Prepare at least as many cell culture plates as will fit in the filming chambers (see below).

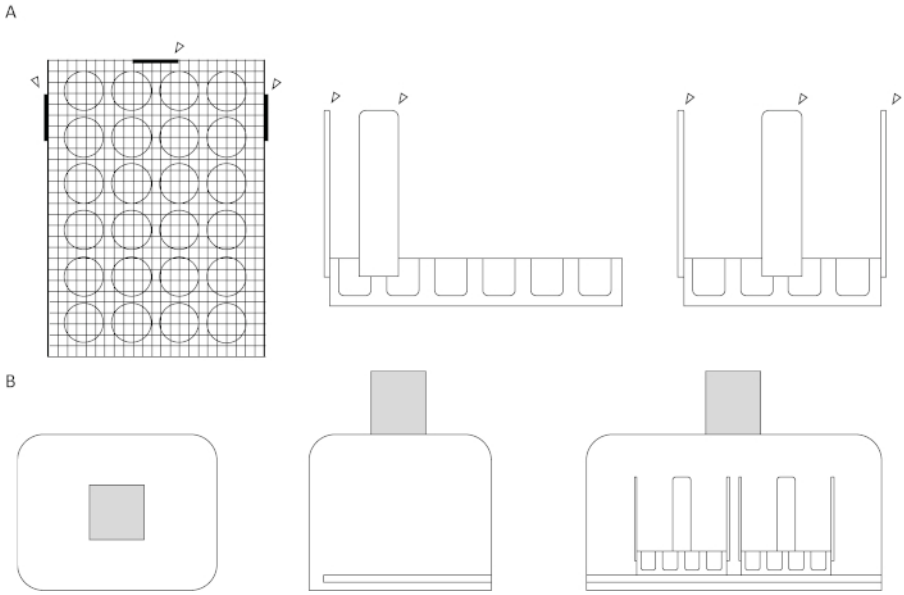

Figure 1: Diagram of the testing apparatus and filming chamber. (**A**) Upper Diagrams. The top, side, and front views of the testing apparatus are shown, respectively. A screen mesh lays flat on top of a 24-well cell culture plate. The wooden craft sticks, represented by the arrowheads, are attached to three adjacent sides for stability and alignment aid, two on the side of the well plate with six wells and one on the side of the plate with four wells. All attachments are hot glued onto the apparatus. (**B**) Lower Diagrams. The top, side, and front views of the assay set-up are shown, respectively. A slit is cut in the right side of the box, from the opening for the lid to the back of the opening, with the bottom of the slit level to the inner surface. The hole on the top of the box, the surface parallel to the ground, is centered for maximum video exposure. The shaded box represents the video camera. Please click here to view a larger version of this figure.

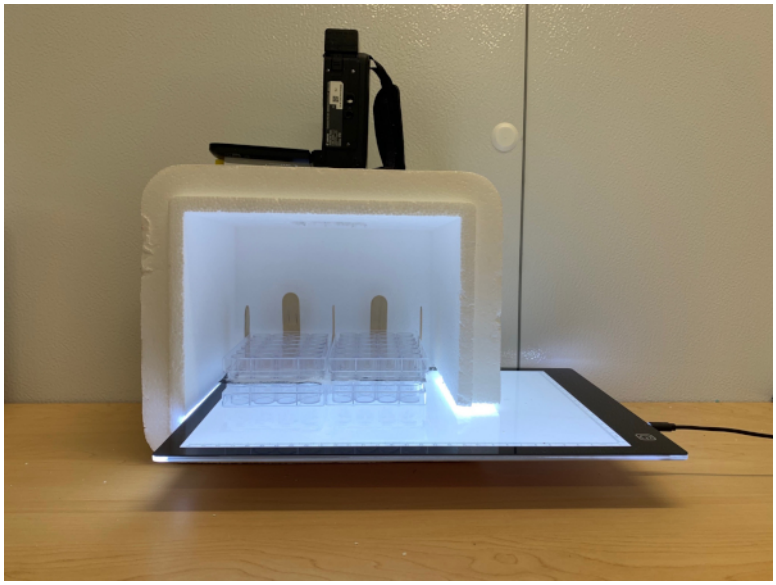

Figure 2: Photograph of the assay system. The video camera is placed on top of the polystyrene chamber, with the lens inserted in the cut-out hole, illustrated in the diagrams of **Figure 1B**. Two sets of modified 24-well cell culture plates rest on top of an illumination pad that is inserted in a slit through the side of the chamber. Please click here to view a larger version of this figure.

2. Construction of the filming chamber

1. Create a filming chamber by cutting a hole the size of the video camera lens on the side of a polystyrene box. Cut an additional slit the width of the illumination pad in the opposite side of the polystyrene box. The filming chamber should resemble the filming chamber shown in **Figure 1B** and **Figure 2**.
2. Prepare the filming chamber for use by inserting the illumination pad into the slit and positioning the camera in the lens hole above the illumination pad.
3. Place all materials and perform all subsequent testing in a controlled environment, preferably a behavioral chamber with approximately 30% humidity, 25 °C temperature, uniform airflow, and noise levels less than 65 dB.

3. Preparation of the testing apparatus and flies

1. Pipette 1 mL of 100% ethanol through the screen mesh into each well.
2. Dry the screen mesh with a piece of cheesecloth.
3. Cut two pieces of cheesecloth the dimensions of the cell culture plate using the cardboard template created in step 1.1. Place them on top of the dry screen mesh of the modified cell culture plate containing ethanol from step 3.2.
4. Create a small piece of thin, flexible plastic cutting board by tracing around the cardboard template created in step 1.1 as a general guide and expanding the traced area by 1–2 cm on one of the short sides. Cut out the expanded traced area from the thin, flexible plastic cutting board. After cutting, ensure that the plastic still fits between the three wooden craft sticks on the testing apparatus, but hangs off one end by 1–2 cm.
5. (Optional) If an aspirator needs to be created, assemble an aspirator like the one shown in **Figure 3** by first cutting a P1000 pipette tip in half. Insert the piece with a larger diameter into one end of a ~30 cm piece of flexible tubing to serve as a mouthpiece.

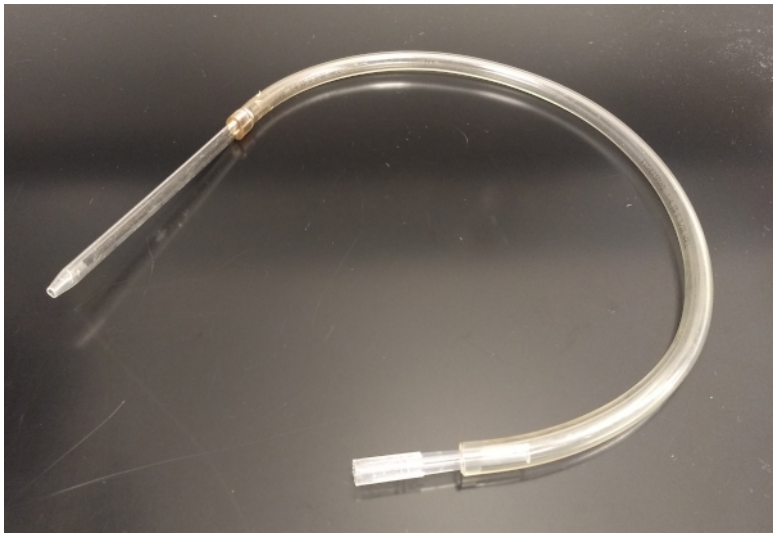

Figure 3: A fly aspirator in which flies are collected with an interchangeable mouthpiece attached to flexible tubing and a wide bore serological pipette with a cotton gauze stopper. The operator can aspirate a single fly into the pipette for transfer without anesthesia. Please click here to view a larger version of this figure.

6. (Optional) To complete the aspirator assembly, cover the wide end of a 10 cm piece of serological pipette with gauze to prevent flies from getting into the tubing and insert the pipette, gauze first, into the open end of the tubing to serve as a fly chamber. The aspirator should resemble that shown in **Figure 3**.
7. Using an aspirator (**Figure 3**, steps 3.5 and 3.6), aspirate one fly per well into a separate 24-well cell culture plate. Use the flexible plastic to cover any wells containing previously aspirated flies. Record the well position and any relevant genotype or phenotype information of each fly.
8. Hold the flexible plastic flush with the top of the cell culture plate containing the flies to prevent their escape and invert the plate onto the top of the modified cell culture plate with the ethanol. The sheet of flexible plastic should be resting on top of the sheets of cheesecloth. Align the inverted cell culture plate containing flies using the craft sticks to ensure each well with ethanol aligns with each well containing a fly.
9. The experimental setup should resemble **Figure 2**.

4. Testing the flies

1. Ensure the illumination pad is lit at full brightness for maximum visual contrast. Start recording with the video camera.
2. To expose the flies to ethanol, carefully remove the plastic from between the well plate and testing apparatus, taking care not to dislodge the cheesecloth.
3. Terminate the video recording once all flies have lost postural control. Once it is suspected that all flies have lost postural control, tap firmly in the center of the plate to ensure that all flies have complete loss of postural control. If there is movement, continue to record. Continue to tap periodically (every 1–2 min) until no movement occurs.
4. (Optional) To quickly recover the flies, remove only the top plate from the testing apparatus, revealing sedated flies resting on the cheesecloth. Aspirate individual flies into chosen containers for recovery.
5. Replace the ethanol in the modified cell culture plates with 1 mL of fresh 100% ethanol at least 1x every hour to control for evaporation and humidification of the ethanol and to maintain consistent ethanol exposure throughout the assay. Dry the screen mesh with cheesecloth.
6. Repeat for as many samples as desired.
   NOTE: For highest throughput, aspirate the next round of flies into new cell culture plates during the video recording. The protocol can be paused here, as the video recording can be reviewed later.

5. Determination of fly sedation time

1. Record sedation time for each individual fly by watching the video recording. Sedation time is defined as the moment a fly loses complete postural control and locomotor ability. It is recommended to watch the film in reverse and record the time that the fly begins to move to ensure accuracy.

Representative Results

Two 24-well microtiter plates could generate data simultaneously on 48 individual flies within as little as 10 min. **Table 1** lists measurements of ethanol sedation times for 48 individual flies, males and females separately, of two DGRP lines with different sensitivities to alcohol exposure on development time and viability[13]. Flies of line RAL_555 were less sensitive than line RAL_177 (**Figure 4**, **Table 2**; $p < 0.0001$, ANOVA). Males and females of RAL_177 showed no sexually dimorphic effect (**Figure 4**, **Table 2**; $p > 0.1$, ANOVA), whereas females of line RAL_555 were less sensitive to ethanol exposure than the males (**Figure 4**, **Table 2**; $p < 0.006$, ANOVA). The large number of flies that can be measured simultaneously and the ability to measure sexes and different lines contemporaneously can increase accuracy by reducing error due to environmental variation.

A.	Ethanol Sedation Time (s)						B.	Ethanol Sedation Time (s)					
	Females			Males				Females			Males		
	414	365	477	423	568	309		937	742	622	460	331	498
	201	384	498	411	523	626		791	619	197	467	455	562
	228	364	333	440	403	267		504	744	513	570	582	506
	440	416	404	408	422	384		970	540	369	865	533	492
	888	283	285	322	369	287		595	550	606	392	544	345
	1079	519	315	393	376	284		418	709	553	308	477	388
	718	287	432	275	206	411		366	564	558	385	576	377
	598	337	398	279	631	372		437	692	578	460	511	412
	241	398	364	347	374	808		665	729	484	532	425	354
	229	423	534	386	396	628		312	576	305	334	531	506
	388	488	451	523	322	533		682	638	420	560	548	379
	252	529	375	427	330	540		1045	741	708	832	509	472
	674	401	303	401	307	311		394	675	381	477	449	784
	303	453	351	429	525	262		540	690	520	556	495	226
	258	483	302	389	562	319		356	615	336	454	524	590
	346	426	385	416	596	287		626	678	840	634	677	509

Table 1: Measurements of ethanol sedation times (s) of individual flies of (A) DGRP lines RAL_177 and (B) RAL_555 for separate sexes (n = 48). See also **Table 2**, **Figure 4**.

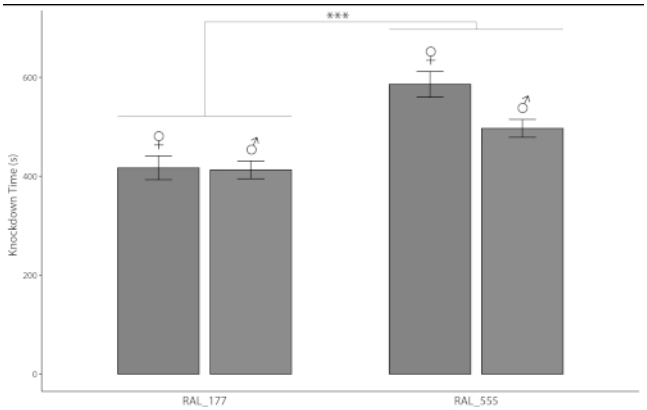

Figure 4: Alcohol sedation times of DGRP lines RAL_177 and RAL_555. The bars represent means and the error bars SEM (n = 48). Sedation times for RAL_177 flies were less than those for RAL_55 flies ($p < 0.0001$, ANOVA). Individual data points are indicated in **Table 1**. Additional statistically significant differences between sexes and lines are indicated in the text and in **Table 2**. Please click here to view a larger version of this figure.

Analysis	Source of Variation	df	SS	F-Value	P-value
Full Model Pooled	Line	1	769627	34.869	<0.0001
	Sex	1	105001	4.757	0.0304
	Line x Sex	1	86021	3.897	0.0498
	Error	188	4149491		
Reduced Model Females	Line	1	685126	23.58	<0.0001
	Error	94	2730718		
Reduced Model Males	Line	1	170522	11.3	0.0011
	Error	94	1418774		
Reduced Model RAL_177	Sex	1	473	0.023	0.8800
	Error	94	1943741		
Reduced Model RAL_555	Sex	1	190549	8.12	0.0054
	Error	94	2205751		

Table 2: Analyses of variance for sedation time across sex and DGRP line. The model used was $Y = \mu + L + S + LxS + \varepsilon$, where μ is the overall mean, L is the fixed effect of the DGRP line (RAL_177, RAL_555), S is the fixed effect of sex (male, female), LxS is the interaction term (fixed), and ε is the error term. The models $Y = \mu + L + \varepsilon$ and $Y = \mu + S + \varepsilon$ were used for the reduced models. Line, Sex, and the Line x Sex interaction term were all significant in the full model at $\alpha < 0.05$. Reduced models by sex and DGRP line RAL_555 were also significant at $\alpha < 0.01$. See also **Table 1**, **Figure 4**. df = degrees of freedom, SS = Type I Sums of Squares.

Discussion

Here, we present a simple, inexpensive, and high-throughput method for assessing sedation time due to ethanol exposure in *Drosophila melanogaster*. Unlike many current methods, which require group analyses, this assay enables a single person to collect individual sedation time data for ~2,000 flies within an 8 h work period. We found that a single person can score 48 flies for sedation time in about 5 min. At this rate, 2,000 flies can be scored in approximately 4 h, though scoring can be conducted later. With our assay, the recorded sedation time for most flies ranges from 5–15 min at an exposure to 1 mL of 100% ethanol. Lower concentrations of ethanol or smaller delivery volumes will result in longer sedation times.

Current methods for assessing sedation time require testing large numbers of flies without readily enabling measurements on single individuals[15,16,17,18,19,20,21,22,23,24,25,26]. Many current sedation and sensitivity assays rely upon ST50[22,23,24], the timepoint at which 50% of the flies are sedated as a result of ethanol exposure. Although obtaining the ST50 for groups of flies was not the primary motivation for developing this assay, the video recordings demonstrate higher utility compared to current methods, as the recordings can be used to ascertain the ST50 for groups of individually tested flies and to measure the percentage of flies that satisfy a given criterion (e.g., loss of postural control) at any time point. It should be noted that such video analyses would require additional time.

Unlike current inebriometer assays, the method we describe does not require specialized tools to set up and can be performed in any laboratory using common materials. Using this method, we have obtained reliable and consistent sedation times for individual flies. The assay can, in principle, be extended to assess the effects of exposure to any volatile substance. The assay can also be applied to measure effects of acute toxicity of volatiles on other insects, including other fly species. Individual sedation time data can be used to assess the extent of phenotypic variation within a population, such as the DGRP.

We used small insect screen mesh to prevent direct contact with the ethanol solution while allowing adequate quantities of ethanol vapors to reach the fly. The layer of white cheesecloth on top of the screen mesh provides visual contrast between the fly and the surface below and ensures that flies do not get caught in the screen mesh, which could lead to ambiguous determination of loss of postural control. Commercially available membranes that are porous to water and air gave inconsistent results and were insufficiently penetrable to ethanol vapors. We intentionally used small insect screen mesh because it is a uniformly porous material that minimizes variation in ethanol exposure as a result of fly position within a well. Modifications can be made to this protocol based on available materials, although we recommend a controlled behavioral chamber, access to 90%–100% ethanol close to the fly, and uniform ethanol exposure.

Fly position within the cell culture plates should be randomized between replicates to avoid positional bias. For larger experiments that require use of this assay across multiple days and are therefore subject to environmental variation that could influence assay results (e.g., changes in barometric pressure)[27], we strongly recommend that flies be tested at the same time each day and randomized both within and across days, especially if different lines and/or sexes are to be compared against one another.

The method we developed is best suited for measuring the effect of acute alcohol exposure but is not suitable for obtaining consumption data or modeling addiction. Alcohol sedation sensitivity data obtained from this assay can, however, be integrated with other measures of alcohol-related phenotypes. One limitation of the system is that the vertical height of standard cell culture plates allows for vertical fly movement that cannot be readily tracked by video for detailed assessment of overall activity or locomotion. However, this limitation does not affect accurate assessment of sedation time. When using flies of different genotypes (e.g., in DGRP-derived outbred populations[28]), this assay also enables retrieval of individual flies to collect pools of flies with contrasting phenotypes for bulk DNA sequencing and extreme QTL mapping[29,30]. Overall, this assay permits rapid, inexpensive collection of alcohol sedation data on large numbers of single flies.

Disclosures

The authors have nothing to disclose.

Acknowledgments

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