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Circadian Entrainment of Drosophila melanogaster

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TITLE:

Circadian Entrainment of Drosophila melanogaster

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KEYWORDS:

Drosophila, circadian entrainment, immunofluorescence, protein extraction, fixation, neurobiology

SUMMARY:

Here, we detail how to synchronize Drosophila to a circadian day. This is the first, and most important step necessary for studying biological rhythms and chronobiology.

ABSTRACT:

Nearly universal among organisms, circadian rhythms coordinate biological activity to earth's orbit around the sun. To identify factors creating this rhythm and to understand the resulting outputs, entrainment of model organisms to defined circadian time-points is required. Here we detail a procedure to entrain many Drosophila to a defined circadian rhythm. Furthermore, we detail post-entrainment steps to prepare samples for immunofluorescence, nucleic acid, or protein extraction-based analysis.

INTRODUCTION:

Almost all organisms on Earth, from the largest down to single celled, have an internal biological clock with a cycle of about one day. This is known as the Circadian rhythm (coined in 1953 by Franz Halberg from the Latin terms circa = about/approximately and "dies" = day)[1]. Although components of the core clock are known and their rudimentary mechanisms of function conceptualized, there is still much to understand about how biological rhythms are maintained throughout the body. Importantly, misregulation of biological rhythms is associated with poor health outcomes including poor memory formation, sleep disorders, seasonal affect disorder, depression, bipolar disorder, diabetes, obesity, neurodegeneration, and cancer[2-5].

Drosophila is a well-established model for investigation of circadian biology. Genetically and biochemically tractable, large numbers are easily entrained (as will be shown). In fact, all seven publications cited as key publications of support in awarding the Nobel Prize for the discovery of circadian rhythms leveraged these strengths of the Drosophila model[6-12].

Additionally, we show effective strategies for collecting entrained flies for the purposes of either immunofluorescence, nucleic acid, or protein extraction-based analysis. Using these strategies, one may process and store larger amounts of samples for analysis in the future. These methods are very advantageous in that they are reproducible and can yield hundreds of entrained flies that can be a part of a large data pool.

PROTOCOL:

1. Fly food production

1.1. Per every 1 L of water, prepare fly food consisting of 4.69 g of dried molasses, 19.70 g of dry active yeast, 87.22 g of corn meal and 7.83 g of agar.

1.2. Combine the contents listed above in a crockpot and turn the heat to 250 °F. Mix well as ingredients are added.

1.3. Keep the lid on the crockpot as the fly food is heating while also mixing the contents every 10 min until it reaches a rolling boil. Allow the rolling boil to continue for 20 min before turning off the heat.

1.4. Add 83.60 mL of water and keep the lid of the crockpot off as the food cools.

1.5. Mix and record the temperature of the food every 10 min with a glass thermometer. Avoid letting a layer of film settle on top of the food.

1.6. Once the food has cooled to 60 °C, add 10.44 mL of Tegosept and 5.51 mL of propionic acid per liter of water added (see step 1.1).

1.7. Mix well and turn heat up to 60 °C to prevent the food from becoming too cool.

1.8. Pump the fly food as needed using the Droso-filler.

1.9. For narrow vials, pump 10 mL and for 6 oz square bottom bottles pump 60 mL.

2. Collecting flies of defined age

2.1. Monitor bottles of wild type flies stored at 25 °C for 5-7 days until large amounts of pupa (about 200 pupa) are attached to the side of the bottle. The bottles used are 6 oz Drosophila stock bottles with square bottoms.

2.2. Clear existing adults from the bottle. Either tip the adults into a new bottle or place them in 70% ethanol. Use the dull end of a #0 paintbrush to push any remaining adults into the fly food. Be sure to wipe the paint brush down with 70% ethanol before and after uses.

2.3. Allow cleared bottles to sit for 3 days in a 25 °C incubator to allow for the next generation to eclose. These flies will be between 0 and 3 days old.

3. Fly separation

3.1. After 3 days, separate males from females and collect the desired number for each sex for each of the four time points. Flies can be differentiated by sex by examining genitalia; males have dark rounded genitalia whereas females have lighter, more pointed genitalia. Females are also much larger than male flies.

3.1.1. Use a CO_2 anesthesia pad to effectively separate the flies and differentiate between the sexes. Move the flies with paint brushes to avoid killing them.

3.1.2. Collect 100 males and 100 females for each time point, with 50 males per vial and 100 females per vial. Males tend to be more aggressive and their social interactions lead to a lot of deaths when there are 100 individuals in a vial. The females-up to 100 individuals- are unaffected while contained in the vial.

3.2. Perform the collections at the following time points: ZT1, ZT7, ZT13 and ZT19 (**Table 1**). Note that collections done in the dark (ZT12-ZT24) are light sensitive whereas ZT0-ZT11 collections are done during lights-on times and room lights can be on. Please note that two separate incubators are used with inverse 12 hour light patterns to allow for all collections to occur during the day and not overnight.

3.3. Use excess flies to create new bottles of flies for future entrainment. Place 25 females with 5-7 males in each new bottle and place in the incubator at 25 °C.

4. Fly incubation

4.1. Allow the flies to stay in light regulated incubators for 3-5 days to allow circadian entrainment to occur. Ensure that incubators are light-tight because even small amounts of light pollution will disturb entrainment.

5. Immunofluorescence fixation

5.1. After entrainment, prepare new vials of fixation solution for samples that will be used for immunofluorescence. Prior to removing the flies from the incubator, add 4.8 mL of 4% formaldehyde diluted in 1x PBS + 0.1% Tween-20 to each new narrow vial for every 100 flies that are to be fixated. Each vial will house 100 male or 100 female Circadian entrained flies. Place the narrow vials in ice.

6. Immunofluorescence collection

6.1. When collecting the flies from the incubator for immunofluorescence, remove the bottle cap and quickly invert the bottle into the funnel. Gently tap the flies into the solution via the funnel to help guide the flies into the vial; combine two tubes of the 50 males for a total of 100 males in one vial and use a single tube of 100 females for the other vial.

6.2. Perform ZT13 and ZT19 collections in the dark; use a red light in order to see as drosophila are far less sensitive to these light wavelengths and are therefore less prone to light pollution[13]. Cryptochrome protein, in particular, is especially sensitive to blue light, which must be avoided[14].

6.2.1. To reduce light exposure, ensure the room of collection is light tight with any sources of light blocked out or covered. Wrap these vials in foil so that ZT13 and ZT19 flies are not exposed to light when placed on the nutating mixer in the following step. ZT1 and ZT7 flies are not light sensitive and can be placed on the mixer without foil covering.

7. Nutating mixer and storage

7.1. After the flies have been collected, tape the top of the vials containing fixative to avoid spillage and place them on the nutating mixer at 165 RPM at 4 °C for 4 h. The flies are no longer light sensitive after this fixation step, so foil may be removed to verify the solution is moving and flies are being submerged in the fixative.

7.2. After removing the fly containing vials from the nutating mixer remove the formaldehyde and wash three times with 3,000 µL of 1x PBS, inverting the vial with each wash.

7.3. Store the vials bearing immunofluorescence samples at 4 °C to await future immunofluorescence[15].

8. Collection for protein extraction

8.1. Prepare four 50 mL tubes and a Dewar containing liquid nitrogen for preservation of samples for protein extraction

8.2. For collecting flies for protein or nucleic acid extraction, transfer flies from the bottles to the tube in the same manner as in step 6.1 and quickly cap the tube to prevent the release of flies and place the tube into liquid nitrogen to snap freeze.

9. Storage for protein extraction

9.1. Store snap frozen samples for protein extraction at -80 °C. These can be processed according to the protein extraction protocol appropriate for downstream analysis, including immunoblotting[16].

REPRESENTATIVE RESULTS:

Controlled circadian entrainment allows researchers to examine biology at specific time points throughout the circadian day using the ZT1-ZT19 timing schedules or to add time-points as necessary. Here we use light and darkness to entrain flies to circadian cycles and verify entrainment by immunoblotting and immunofluorescence analysis of the period protein, a marker for circadian entrainment (**Figure 1**). Upon correct entrainment, period proteins should have a characteristic intensity and mobility pattern (**Figure 1A**) and should be visible at specific locations in the ZT1 brain (**Figure 1B**). Although other variables, including food and temperature, can influence circadian entrainment, light is most simple and reliable to control[17]. For the purpose of these methods, incubator temperatures are kept constant, relying on cerebral clock neurons that are influenced by light for entrainment[18].

FIGURE AND TABLE LEGENDS:

Figure 1: Verification of entrainment. (**A**) Immunoblotting of whole cell extracts prepared from heads of entrained flies shows canonical patterns of period protein mobility and intensity[19]. 1.4 female heads from each of the indicated Zeitgeber times (ZT) were analyzed using an anti-Per antibody. (**B**) Immunofluorescence of entrained brains collected at ZT, where the Period protein is found in a characteristic pattern (bottom panels, recreated from Helfrich-Forster[20]). Shown are images taken from different sections of the brain, capturing all neurons expected to contain Period protein at ZT1. Scale bar is 40 μm.

Table 1: ZT1-ZT19 Circadian Rhythm Timing Schedules

DISCUSSION:

Researchers utilize this entrainment protocol with success and consistency. This procedure allows the fixation of a large sampling pool that can be stored for future analysis. Additionally, this strategy preserves the neurological patterns induced by entrainment for future examination.

Fixation for storage is a major component of the entrainment process as it helps to stabilize brain tissue and it allows for more time to analyze each brain from the data pool thus minimizing waste from brains that lose viability due to age[21]. The main goal is to circadian entrain as many flies as

possible so that there is continuous inventory available for head dissections and ultimately immunofluorescence or protein extraction to observe the findings and determine if results are of high confidence. To ensure that circadian entrainment is preserved through fixation, it is integral that any source of light pollution is eliminated. The fixation process allows for *Drosophila* to be stored while maintaining its neurological "timestamp" so that they can be dissected later and analyzed with no noticeable differences to flies that are dissected and have undergone immunofluorescence immediately after entrainment. For the purposes of fixation prior to immunofluorescence, the lab has determined with consistency that flies are viable at least up to 1 month. Fixations for western blot protein extraction render the brains viable indefinitely when stored at -80 °C.

Another critical protocol step is the sexing of the flies. It is important that this step is done accurately as having both sexes in the same jar prior to fixation can lead to mating which will yield new flies that are of younger age and corrupt protein analysis if males are accidentally examined instead of females or vice versa. Additionally, when sexing it is important to remove larvae specimens that are at times attached to females. This prevents the development of new progeny inside the female vial that could potentially corrupt results.

The next step for the entrainment protocol may be with items related to data analysis. The focus of the protocol is protein localization, but if there are other variables that are impacted by circadian entrainment, they must be explored through new avenues, often requiring protein or nucleic acid extraction. Additionally, there are other proteins of the brain that may still be analyzed via this protocol. The experiments associated with the protocol analyzed certain proteins but the list of genes and proteins that play a role in circadian biology has not been exhausted. The protocol is effective in accomplishing the goal of establishing a circadian rhythm, however, the applications are wide-ranging.

ACKNOWLEDGMENTS:

Special thanks to the University of Missouri-Kansas City and the Jeffrey L. Price laboratory.

DISCLOSURES:

None.

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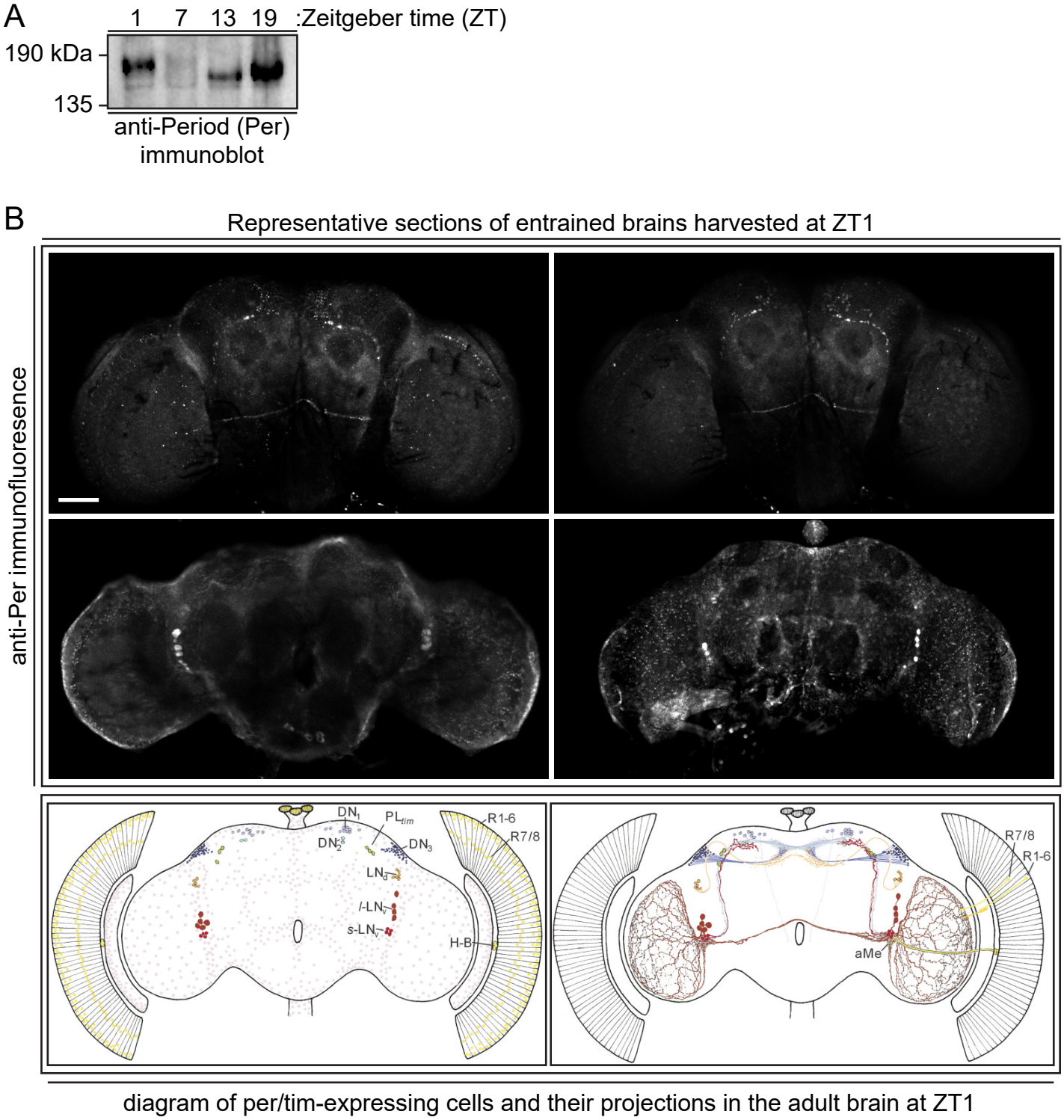
Figure 1



Table

ZT1	ZT7	ZT13
Light between 10 am - 10 pm	Light between 10 am - 10 pm	Dark between 9 am - 9 pm
Collected at 11 am after 1 hour in light	Collected at 5 pm after 7 hours in light	Collected at 10 am after 1 hour in dark

ZT19
Dark between 9 am - 9 pm
Collected at 4 pm after 7 hours in dark

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
100-1000uL pipette	Eppendorf	ES-1000	
10-100uL pipette	Eppendorf	ES-100	
16% Paraformaldehyde Solution	15710 Caisson		
1X PBS	Labs	PBL01-6X100ML	
Agar	Fisher Scientific	BP1423500	
Anesthesia Filter Connection Kit	World Precision Instruments	EZ-251A	
Corn meal	Genesee Scientific	62-100	
Dried Molasses	Food Service Direct	OT280504	
Droso-filler Food Pump	geneseesci .com	59-169	
Drosophila Stock bottles, 6 oz square bottom w/ Flugs	geneseesci .com	32-130BF	
Drosophila vials, Narrow K-Resin super bulk	Geneseesc i.com	32-118SB	
Dry active yeast	Genesee Scientific	62-103	
Ethanol	IBI Scientific	IB15720	

EZ Basic Anesthesia System	World Precision Instruments	EZ-175		
Falcon Centrifuge tubes	Corning		352097	
Falcon round bottom tubes	Corning		352057	
Fine point Sharpie marker	Sharpie		30001	
Fisherbrand Nutating Mixer	Fisher Scientific	88-861-043		
Flugs-Narrow Plastic Vials	Genesee Scientific	49-102		
Glass Thermometer	Cole-Palmer	EW-08008-12		
Liquid nitrogen hose	Thermo Scientific		398202	
Liquid nitrogen tank-Dewar	Cooper Surgical Inc	900109-1		
Liquid nitrogen transfer vessel	Electron Mircoscopy Sciences	61891-02		
Paintbrushes(Red Sable) Size #0	Electro Microscopy Sciences	66100-00		This is used to separate the flies via sex without causing injury.
Plastic funnel	Plews and Edelmann	570-75-062		
Polarizing light microscope	Microscope Central		1.1001E+12	Used to more clearly view Drosophila during sexing

ProPette Pipette Tips	MTC Bio Incorporated	P5200-100U		
ProPette Pipette Tips	MTC Bio Incorporated	P5200-1M		
ProPette Pipette Tips	MTC Bio Incorporated	P5200-5M		
Propionic Acid	Sigma Aldrich	P1386-1L		
Rayon Balls	Genesee Scientific	51-100		
Reynolds wrap standard aluminum foil	Staples		1381273	
Roaster Oven (Crockpot)	Hamilton Beach		32950	
Scotch 810 Magic Tape	Electron Microscopy Sciences		77300	
Spray bottle with trigger	US Plastic		66446	Used to spray ethanol to clean work bend areas
Tegosept	Genesee Scientific	20-258		
Thermo Scientific Drosophila Incubator	Thermo Scientific	3990FL		
Thermo Scientific Revco 4 degree Lab fridge	ThermoFisher Scientific	REL7504D		
Thermo Scientific Revco Lab Freezer	ThermoFisher Scientific	REL7504A		
Tween 20	Anatrace	T1003-1-GA		

School of Biological Sciences
Division of Cell Biology and Biophysics

February 6, 2020

Editors
Jove

Regarding: Manuscript Number: **JoVE61176**

Dear Editors;

We read the reviewer comments for " Circadian entrainment of *Drosophila melanogaster*" with great interest and excitedly embarked on edits to satisfy their requests. We believe all reviewer comments are addressed and are pleased to submit this revised manuscript for your consideration.

A detailed response to reviewers can found below.

Sincerely,

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Dear Reviewers;

Thank you for your thoughtful and constructive comments. We have engaged in a series of edits to address the points made during initial review. Here, we list the changes executed. We believe these edits result in a better, more clear representation of circadian entrainment and the protocols necessary to achieve success in this experimental field. Our responses are in blue text.

ESSENTIAL REVISIONS:

Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

This is done

2. Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg).

This is done

3. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

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4. Please include volume and issue numbers for all references.

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7. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

This is done

8. Please revise the title to remove superfluous words such as: “For the purposes of”. Please be more direct with the title.

This is done

9. Please provide affiliations and email addresses for each author.

This is done

10. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

This is done

11. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

More clarity has been added to the protocol steps including:

1. The addition of a protocol on fly food preparation
2. Citing additional sources that further explain certain protocol concepts to help readers understand why our lab did our experiment a particular way.
3. Addition of more detailed protocol steps to eliminate possible confusion

12. 1.1: What is the strain of the fly? How many flies are used in a bottle? What are the conditions for growth? Please quantitate large amounts of pupa. How big is the bottle?

The protocol steps were edited to be more detailed to answer these questions

13. 1.3: What is used for fly food?

This question is addressed in the first section of our protocol.

14. 2.1: How are males differentiated from females? What is the desired number and the desired time point? How is the CO2 anesthesia done? What are the collection times? The ZT1/ZT7/ZT13/ZT19 labels must be explained. Cite the table.

These have been done

15. 3.1: What is the light parameters? Dark:Light schedule?

This has been specified

16. 4.1: Presumably, the formaldehyde solution in PBS is the fixation solution? Please clarify.

This step has been clarified

17. Please specify all tube sizes.

This is done in the protocol steps

18. As western blot and microscopy are shown as results, please add the details to perform these characterization steps in the protocol as well.

We added references for these techniques as a complete protocol would necessitate an entire article. Please see :

Kelly, S. M., Elchert, A. & Kahl, M. Dissection and Immunofluorescent Staining of Mushroom Body and Photoreceptor Neurons in Adult Drosophila melanogaster Brains. Journal of visualized experiments : JoVE. 10.3791/56174 (129), 56174, (2017).

And

Au - Eslami, A. & Au - Lujan, J. Western Blotting: Sample Preparation to Detection. JoVE. doi:10.3791/2359 (44), e2359, (2010).

19. Please number all figures and tables. Please provide a title and short caption as well.

This has been done

20. Western blot image: Please include a space between the number and the unit: 245 kDa.

This figure has been remade and this change is included in the new figure.

21. Microscope image: Please provide a scale bar.

This has been done

22. Please reference the figures in the manuscript text.

This has been done

23. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

These topics are now addressed in the discussion section

MAJOR CONCERNS:
Protocol should be reorganized to focus on Fixation/collection/storage for samples destined to be used for immunofluorescence or protein/nucleic acid extraction. Currently, it all runs together and someone trying to follow the protocol for an experiment could easily get them mixed up.

The protocol section has been edited to address these concerns

- Authors should specify in 1.1 how many days after mating a researcher would expect to see this. 5-7 days at 25 deg C would be a good place to start. Also, no note is made about how to store bottles of flies at this point. Previous articles about food, storage, and husbandry of Drosophila should be linked.

These have been done

- Using excess flies to create new crosses as listed in 2.2 is entirely dependent on the crosses originally made. For wild-type or true-breeding stock lines, this is fine. However, if genetic crosses were made and the entrained flies are in an F1 generation/heterozygotes, section 2.2 is entirely inappropriate, and new crosses will need to be made. The "excess" flies could thus be continued to be used in the protocol as described, since its primary purpose is to create large quantities of flies for study.

We make note of entraining wild type drosophila in the protocol

- Authors need to specify more into the incubation step. Light/Dark cycles of 12 hours lights on:12 hours lights off on an automated timer is common, but the timing of the beginning of a cycle can be set to whenever is convenient for a researcher. Authors should specify that it takes 3-5 days for flies to become entrained to that light schedule over one they were previously on (say, that of the research lab or another incubator) and cite proper evidence for this. It is also crucial that the incubators themselves be in a light controlled room, as related to section 5.2. To open the incubator to collect the flies, the room's surroundings must also be light tight. You can create light-tight spaces by using black out curtains, red light, and ensuring that any electronic switches with LED lights are covered with electrical tape and black construction paper.

More specification was given to this section to inform the reader of the following:

1. ZT time zones were solely used as reference to our lab's particular experiment
2. Two separate incubators are utilized in order to allow for light and dark collections to occur during the day to avoid overnight collections
3. Explained the idea that drosophila are insensitive to red light and supplemented that claim with a research paper

- Similarly, authors should describe the ZT system and define the light/dark cycle. The timing schedule listed in the table is only one example of how a light schedule can be displayed. In a 12 hour light/dark cycle, ZT0 is lights on and ZT12 is lights off. It should also be clarified that in that table, two different incubators are being used so that dark-collected flies can happen during a normal workday to avoid overnight time points.

This was done via the edits done to correct the previous concern

- The authors specify that these techniques can be used to store samples for prolonged periods of time, however show no results attesting to the stability of the samples. They do show a western blot of the period protein but the following experiments would add more confidence into their entrainment and storage protocol:
o Take two batches of flies and entrain them to inverse light cycles. Repeat the western blot on both and show inverse staining of the period protein.
o Immunofluorescence staining of the period protein over the individual time points to show specificity and circadian rhythmicity.
o Either a western blot, nucleic acid extraction, or immunofluorescence experiment on day of fixation, 2 weeks after fixation, and 2 months after fixation to show that the samples have not degraded. Authors

should also give a "shelf-life" of stability for each of the sample types.

Information has been included based on our lab's trial and error experiments on brain viability limits to reflect the stability of samples after entrainment and fixation for both immunofluorescence and protein extraction.

MINOR CONCERNS:

- Authors should emphasize in the abstract and the last paragraph of the introduction

We believe that proper emphasis has been given to each of these parts to address the main objective of our manuscript protocol.

- Authors should specify vial and bottle sizes within the protocol - this is important because 50-100 flies in a smaller vial will not be entrained as easily due to social interactions and may introduce sleep deprivation artifacts to the experiments.

Vial and bottle sizes have been specified

- Section 1.3 is unnecessary or could be combined with step 1.2. Simply emphasize that all adults need to be cleared to prevent contamination of offspring with adult flies.

This has been done

- Paint brush size should be specified. It is best to use a size 00 or 000 paintbrush.

This has been done

- Authors should specify that with females collected, many, if not most, may not be virgin. When doing collection from female vials, one should be careful not to contaminate the sample with larvae specimens, especially for samples destined for protein or nucleic acid extraction.

This concern has been addressed

- In section 5.1, it would be more accurate to describe exactly how to transfer the flies without anesthesia, or a link to another protocol on the handling of flies should be provided. It is not so much of a "guiding" method, which sounds gentle and would result in the release of many flies. A suggestion: "When collecting… immunofluorescence, tap the flies down to the bottom of the bottle gently to not embed them in the food, remove the cap, and invert the bottle into a funnel over the vial with fixative solution. Tap flies from bottle into vial."

This protocol section has been specified

- Also in section 5.1, researchers should specify that flies for protein or nucleic acid extraction should be transferred in a similar manner into the frozen Falcon tube mentioned in section 4.1.

This protocol section has been edited to address this concern

- In section 6.2, the vial is removed from the mixer, not the flies. The current wording implies that the flies were removed from the tube.

The wording has been changed

- Authors do not need to specify which time points the researcher should collect flies. The experiment the researcher is doing will determine which time points are necessary. Authors should say that for their experiments, ZT 1, 7, 13, and 19 were typical, but that collection may vary depending on the aim of the experiment. Specify that ANY collection done in dark (ZT12-24) should be considered light-sensitive.

This concern has been addressed

1. The keywords are odd. What is Ataxia?

Keywords have been edited to better reflect the manuscript

2. Abstract: "circadian rhythms coordinate biological activity to earth's orbit around the sun". "earth's orbit around the sun" may be "earth's rotation"? Please check it.

This has been checked

3. The sampling protocol is mainly for immunohistochemistry, but the part of the method can be used for protein/RNA extractions. However, it is sometimes confusing to separate the two things. For example, in "4. Fixation" a way to collect many files in Falcon tubes is described, but we would not call "Fixation" for collecting the samples for protein/RNA extractions. So, I would suggest that the authors should first write only about the sample collections for immunohistochemistry from "1. Collecting flies of defined age" to "6. Nutating mixer and Storage". After that, write 1-3 steps are exactly the same for sample collection for protein/RNA extractions and add the part of step 4 and 6.3.

The protocol section has been reorganized to address this concern

4. step 2. Fly separation: Why do the authors separate 50 males per vial and 100 females per vial to collect 100 males and 100 females? Please explain it.

This step has been explained

5. step 5. Collection: Actually, Drosophila can see the red light and the circadian clock can be reset by it (e.g. Hanai et al., 2008, Neuroreport). However, they are relatively insensitive to the red light and short exposure of it does not affect the clock.

This step has been reworded and now is linked to the above-mentioned research paper

6. In step 5. Collection: It would be important to mention that CRY is extremely sensitive (Vinayak et al., 2013, PLOS Genet) and experimenter should be extra careful with blue light.
The protocol section explains the importance to block out all other sources of light outside of red light, thus effectively addressing this concern

This is an excellent point and has been added.

7. In the western blot result, there are three bands. Which one is the PERIOD protein?

This figure has been remade and a reference included to unambiguously identify Period.

Edery, I., Zwiebel, L. J., Dembinska, M. E. & Rosbash, M. Temporal phosphorylation of the Drosophila period protein. Proceedings of the National Academy of Sciences of the United States of America. 91 (6), 2260-2264, (1994).

Thank you again for these thoughtful comments. It was a pleasure to develop this manuscript and we believe that it is now stronger after revision.

Sincerely,

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