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Analysis of circadian photoresponses in Drosophila using locomotor activity

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Abstract:	Circadian rhythms are only beneficial to animals if they can be synchronized by changes of ambient conditions. Light and temperature are two dominant environmental parameters that synchronize animal circadian clocks. In Drosophila circadian photoresponses are mediated by a solely blue light photoreceptor named CRYPTOCHROME (CRY). Upon photoreception, CRY changes its conformation and initiates the proteosomal dependent degradation of TIMELESS (TIM). TIM is an important pacemaker protein, thus degradation of TIM will reset the circadian clock. Under constant light conditions (LL), wild type flies quickly become arrhythmic because of the constant degradation of TIM, while flies bearing defects with circadian photoresponses will still be rhythmic. Thus LL triggered arrhythmicicty has been used for screening of components in circadian light input pathways. A brief short light pulse in the night can also dramatically shift phases of circadian rhythms. As expected, this phase shift response is reduced in flied with defects in circadian photoresponse. Thus analyzing locomotion behavior rhythmicity under LL or phase changes after short light pulse in constant darkness (DD) are two major methods to study circadian photoresponse. Here we describe how to design and analyze LL and phase response experiments. LL arrythmicity is suitable for screening light input pathways mutants, whereas phase response validates the results and provide further information for light sensitivity.
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TITLE:
Analysis of circadian photoresponses in Drosophila using locomotor activity

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SHORT ABSTRACT:
Drosophila locomotor activity is a robust and quantitative measurement of circadian photo-responses. We describe protocols for designing behavior experiments for circadian photo-responses and analyzing the data. Studying the circadian photo-responses is important for dissecting the neuronal and molecular mechanisms of light entrainment.

LONG ABSTRACT:
Circadian rhythms are only beneficial to animals if they can be synchronized by changes of ambient conditions. Light and temperature are two dominant environmental parameters that

synchronize animal circadian clocks. In *Drosophila* circadian photo-responses are mediated by a solely blue light photoreceptor named CRYPTOCHROME (CRY). Upon photoreception, CRY changes its conformation and initiates the proteosomal dependent degradation of TIMELESS (TIM). TIM is an important pacemaker protein, thus degradation of TIM will reset the circadian clock. Under constant light conditions (LL), wild type flies quickly become arrhythmic because of the constant degradation of TIM, while flies bearing defects with circadian photo-responses will still be rhythmic. Thus LL triggered arrhythmicity has been used for screening of components in circadian light input pathways. A brief short light pulse in the night can also dramatically shift phases of circadian rhythms. As expected, this phase shift response is reduced in flied with defects in circadian photoresponse. Thus analyzing locomotion behavior rhythmicity under LL or phase changes after short light pulse in constant darkness (DD) are two major methods to study circadian photoresponse. Here we describe how to design and analyze LL and phase response experiments. LL arrhythmicity is suitable for screening light input pathways mutants, whereas phase response validates the results and provide further information for light sensitivity.

INTRODUCTION:

Most organisms, from cyanobacteria to mammals, use circadian clocks to anticipate daily environmental changes. Circadian clocks synchronize most bodily functions of animals, from metabolic level, to rest/activity cycles and other behaviors[1]. Circadian rhythms are self-sustained, which can be maintained even in constant conditions for several days. Circadian rhythm is generated by a molecular pacemaker, which is highly conserved among organisms. In fruit flies, the core of this circadian clock, is a transcriptional-translational feedback loop[2,3]. Two transcription factors, CLOCK (CLK) and CYCLE (CYC) form a heterodimer and generate rhythmic transcriptions of down stream clock controlled genes. Among these genes, PERIOD (PER) and TIM are two main transcriptional repressors. PER/TIM undergo post-translational modifications, and accumulate in the cytoplasm, then enter the nucleus to repress their own transcription by blocking CLK activity.

Circadian pacemakers are self-sustained, but environmental cues determine their phase of oscillations. Light and temperature are the most crucial cues for synchronization of the circadian clock[4]. Compared to temperature, entrainment by light is much better understood. Circadian photo-responses are mainly mediated by CRY input pathways in flies. CRY is the blue light photoreceptor, which changes its conformation after receiving light, and then it is able to bind TIM[5,6]. After binding with CRY, TIM undergoes proteosomal dependent degradation through E3 ubiquitin ligase JETLAG[7-9] (JET). Thus, the degradation of TIM resets the circadian pacemaker.

Flies become arrhythmic in constant light conditions, because of the constant degradation of TIM by CRY. However flies with defects in the CRY pathway still remain rhythmic. Based on this observation, the behavioral rhythmicity in LL is often used to demonstrate circadian photo-responses in flies. A short light pulse in the night will cause transient degradation of TIM, thus shifting the phase of circadian pacemaker[5,6]. Light pulse at early night will mimic a delayed day, thus called phase delay; while a light pulse at late night will mimic an advanced dawn, which is named as phase advance. This phase response is sensitive to light intensity, which is an important parameter for circadian photo-responses. Phase response is almost abolished in mutants of CRY input pathways. Measuring the phase response by light pulse is also extensively used to examine

circadian photo-responses. Here we describe how to perform these two experiments as well as methods to analyze the behavior data.

PROTOCOL:

1. Constant light (LL) Experiments

1.1) Preparation of experimental flies

1.1.1) Raise flies in incubators or rooms that have regular light: dark cycles on standard fly food at 25°C. Collect female and male virgin flies for crosses.

NOTE: Unlike mated flies newly emerged virgin female flies have larger white abdomens. There is also a dark dot on their abdomen, which is only for virgin flies.). For RNAi screen, collect driver lines with GAL4 driven by circadianly regulated promoter (e.g. *tim-GAL4* for all circadian neurons) and RNAi responder lines for crosses.

1.1.2) Put approximately 5 virgins and 2 males in a vial for each cross to ensure enough progenies for behavioral analysis. Collect cry^b or other mutants in CRY input pathway as positive control.

1.1.4) Eclosion takes around 14 days at room temperature. In 25°C incubator, it takes about 10 days for flies to eclose. Use CO2 to anesthetize flies and use small fine tipped paintbrush and collect 1-5 days old male progeny with right genotype on fly pad with CO2 flows for following LL behavior assay.

1.2) Prepare fly activity tubes.

1.2.1) Autoclave glass activity tubes before use. Use 121°C, 29 psi for 45 minutes for autoclave. After autoclave, put activity tubes vertically into 500ml glass beaker before pouring the liquid fly food.

1.2.2) Prepare white fly food for behavior (5% sucrose, and 2% bacto-agar). Heat deionized water with sucrose and bacto-agar and boil for at least 5 min. Pour liquid white food into beaker (1.2.1) to make approximately 1 cm food in each tube. Seal the activity tubes from the food side by plastic caps. Store the fly food in sealed box at 4°C.

1.3) Set up LL experiment.

NOTE: Before this experiment, make sure to have activity monitor systems set up as described[10].

1.3.1) Load a single male fly using a small brush into each activity tubes. Seal the tube with cotton strips (about 0.5 cm long, made from regular cotton).

1.3.2) Load activity tubes into activity monitors. Use at least 8 flies for each genotype. Write down the number (i.e 1-8, 9-16) for each genotype. Once inserted into the monitors, use rubber bands to prevent the activity tubes from falling out.

1.3.3) Put the loaded activity monitor-2 (such as DAM2) in incubators with light control. Connect the activity monitor-2 to the data collection system with telephone wires.

1.3.4) Set the incubator temperature at 25°C, 60% humidity, and light intensity around 1,000 lux.

1.3.5) Use the DA activity monitor-2 software to set the incubator light cycle as: 5 days of light:dark, and then 6 days constant light.

1.4) LL experiment data analysis

1.4.1) After the experiment, collect the raw data from the activity monitor-2 software. Use activity monitor-2 software to process the raw data. Sum the behavior data into 30 minute bins. Set the light on/off parameter as the same for the incubators.

1.4.2) Use a spreadsheet program to assign different genotypes. Analyze the data with FaasX software (downloaded from F. Rouyer lab, CNRS, France). In FaasX, click "open experiment" in the main menu. From the "Fly group selection", choose the genotype to analyze, and name the fly group, then click "proceed".

1.4.3) Then from the "Analysis" menu, choose "period, cycle_p" option. For analysis of rhythmicity in LL, choose the data from 5^{th} day for 5 days. Define the rhythmic flies with following criteria: power$\geq$ 20, width $\geq$ 2.

NOTE: In certain circumstances, a power of 10 could also be used, it the robustness of the rhythm is not strong. Use a signal processing toolbox for a computing program such as Matlab to generate behavior Actograms. Wild type flies should show very low rhythmicity, flies with defects in circadian photoresponses show high rhythmicity in LL (50%-100%).

2. Phase responses Experiments

NOTE: The major part of preparation of this phase response experiment is the same as LL, except the behavior program and data analysis. Here we will only describe in detail the steps that diverge between the two experiments.

2.1) Prepare experimental flies as described in Section 1.1 and 1.2.

2.2) Load flies into activity tubes. Prepare at least 48 male flies for each genotype. Use 16 flies each for non-light pulse (NLP), light pulse at ZT15 (15hours after light on), and light pulse at ZT21 (21 hours after light on).

2.3) Set up three sets of activity monitors with same genotypes and number of flies. Label monitors as NLP, ZT15, and ZT21.

2.4) Use telephone wires to connect monitors with the data collection system. Put different sets of monitors onto different shelves in the incubator. Use a light meter to measure and set the light intensity at 1500 lux.

2.5) Set incubator temperature at 25°C. Set up the incubator light:dark cycles as: 5 days of light:dark, then 6 days of constant darkness.

2.6) Perform light pulse on the last day of the light: dark cycle. Expose flies (set ZT15) to 1500 lux for 5 mins at ZT15 using light source or a separate incubator. Carefully put the monitors back

and connect them. Repeat at ZT21. Perform this light pulse in the dark.

NOTE: 5 min light pulse is sufficient to shift the phase of circadian clock, and this acute phase shift is mediated by CRY pathway.

2.7) Collect data and transform with activity monitor-2 software. Assign the genotypes and treatment in table. Analyze the data (from 2nd in DD for 5 days) with FaasX using the function of "Phase".

2.8) Use two methods to calculate the phase shift. First, compare the "phase" differences of same genotype ZT15 and ZT21 versus NLP calculated in FaasX.

2.8.1) In FaasX, click "open experiment" in the main menu. From the "Fly group selection", choose the genotype to analyze, and name the fly group, then click "proceed". Then from the "Analysis" menu, choose "phase" option.

2.8.2) Choose the data from 1st day of constant darkness for 5 days for "Data Range". Check "at least through the data range requested" for "Fly survival". Define the phase point with "peak" or "valley". For the output, check "Phases" as "Text files"; check "Plot group mean waveform" for "Graphic".

2.8.3) Then click "Run". Observe the FaasX generate a text file with periods and phases of each genotype in each treatment fly group. Compare the phase difference between ZT15 and NLP, as well as ZT21 and NLP.

NOTE: A brief light pulse in the early night mimics delayed dusk thus causes a phase delay, while light pulse at late night mimics advanced dawn and causes an advanced phase. Light pulse at ZT15 or ZT21 causes phase delay or advance. Use negative numbers to represent phase delay and positive number for phase advance. Sometimes, there are multiple peaks of activity; it becomes tricky to use the software. Then it is recommended to analyze the data by visual observation.

2.8.4) Repeat the same analysis except that for the "Graphic" option, check "Plot individual waveforms". Based on the waveforms, define the time at which the subjective night activity drops 50% as a "phase" marker for single fly of each genotype. Then compare the phase differences at both ZT15 and ZT21 with NLP and plot the phase changes.

REPRESENTATIVE RESULTS:

Under constant light, wild type flies become arrhythmic because of constant degradation of TIM, while circadian photo-response mutants remain rhythmic. Figure 1 shows behavior actograms of flies under constant light. The results can also be presented in a quantitative manner. Table 1 shows the percentage of rhythmic flies, and period in constant light. Normally, very few wild type flies show rhythmicity (0-25%), while majority of flies with defects in circadian photo-response remains rhythmic (75-100%). By comparing the phase changes after light pulses to non light pulse control, phase responses are represented as "negative or positive" values separately (Figure 2). *cry* mutant flies have minimal phase responses, which are close to zero. If under LL, *cry*

mutant flies are less than 75% rhythmic, usually that means the experiment fails. Make sure not to include dead flies in your analysis.

FIGURE LEGENDS:

Figure 1: Locomotion behavior under constant light.

Double plotted behavior actograms of *y w* control flies, and cry^b mutants. Flies are entrained for three full days of light: dark (12:12hr), and then released into constant light for six days. cry^b mutant flies are still rhythmic while wild type flies become arrhythmic. Grey area indicates dark phase. n=16 for each fly genotype.

Figure 2: Phase responses after light pulse at ZT15 and ZT21.

Flies are entrained for three days of standard light: dark cycles, and release into constant darkness for six days. A 5 min 1,500 lux light pulse is performed at ZT15 or ZT21 on the last night of light: dark cycle. Phase advance (left panel) and phase delay (right panel) are showed by negative and positive numbers compared to non light pulse on two separate graphs. Error bars represent SEM. Figure 1 and 2 are modified from Lamba *et al.* 2014 with permission.

Table 1: Percentage of Rhythmicity under constant light.

Modified from Lamba *et al.* 2014 with permission.

DISCUSSION:

Circadian rhythms exist in most organisms on earth. Animals utilize circadian clocks to coordinate their bodily functions with daily changes. Since environmental conditions are variable, the circadian clock is only beneficial if it can be adjusted by different changes. For flies, light is the primary environmental cue used to synchronize and shift the circadian clock. Studying circadian photo-response is important for understanding how light is processed and regulates circadian behavior. Disruptions of circadian rhythms are also associated with depression, anxiety and many psychiatric disorders, such as bipolar disorder[11].

Constant light and phase response behaviors are widely used to study circadian photo-responses. Recently, constant light has been successfully used to screen and identify genes involved in CRY input pathways[12]. It is an efficient, and reproducible method for behavior screening. Same as other behavioral assays, there is always some variability among individual flies.

It is important to have good sample size to get interpretable data. Based on experimental aims, the number of tested flies can be different. At least 8 flies are required for a LL screen, and 16 flies are suggested to confirm the results later. A brief light pulse in the night is sufficient to shift the circadian phase of fly behavior. ZT15 and ZT21 are the two typical time points to do light pulse, since they generate strong phase response. Pulsing with different light intensities, phase response experiments can also be useful for determining circadian photosensitivity. A 5 min 1500 lux pulse is sufficient to generate full response. 200 lux or even lower light pulse can be used to determine photosensitivity depending on different research purpose. For both LL and phase response experiments, light intensity and duration of light pulse are critical parameters.

Both constant light and light mediated phase responses are standard methods for measurement of circadian photoresponse. Constant light is suitable for screening, while phase response is typically

used to delicately dissect the photo-response of a particular genotype. It is always useful to perform both of these experiments to validate circadian behavior phenotype.

Finally it is important to consider the genetic background of the strains when designing circadian photo-response experiments. In nature, there are two alleles of *tim*: *s-tim* and *ls-tim*, which have different light sensitivity[13]. Flies carrying *s-tim* allele is more sensitive to light, since s-TIM binds to CRY in a higher affinity way than ls-tim. It is recommended to check the *tim* allele of the strains when doing the circadian photo-responses experiments, thus to exclude background effects. The study of circadian photo-response may provide clues to investigate seasonal effects on sleep, mood and other psychiatric diseases.

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DISCLOSURES:
The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

REFERENCES:
1. Mohawk, JA, Green, CB, Takahashi, JS. Central and peripheral circadian clocks in mammals. *Annu Rev Neuroscience*. **35**, 445– 462, doi: 10.1146/annurev-neuro-060909-153128 (2012).
2. Hardin, PE, Panda, S. Circadian timekeeping and output mechanisms in animals. *Curr Opin Neurobiol*. 5, 724-731, doi: 10.1016/j.conb.2013.02.018 (2013).
3. Zhang, Y, and Emery, P. Molecular and neural control of insect circadian rhythms. *Insect Molecular Biology and Biochemistry.* **15**, 513-551, (2012).
4. Dubruille, R, and Emery, P. A plastic clock: how circadian rhythms respond to environmental cues in Drosophila. Mol Neurobiol, **2**, 129-145, doi: 10.1007/s12035-008-8035-y (2008).
5. Emery, P, So, WV, Kaneko, M, Hall, JC, Rosbash, M. CRY, A Drosophila clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. *Cell.* **95**, 669-679, doi: 10.1016/S0092-8674(00)81637-2 (1998).
6. Stanewsky R., et al. The cryb mutation identifies cryptochrome as a circadian photoreceptor in Drosophila. *Cell.* **95**, 681-692, 10.1016/S0092-8674(00)81638-4 (1998).
7. Koh, K, Zheng, X, Seghal, A, JETLAG resets the Drosophila circadian clock by promoting light-induced degradation of TIMELESS. *Science*. **312**, 1809-1812, doi: 10.1126/science.1124951 (2006).
8. Peschel, N, Chen, KF, Szabo, G, Stanewsky, R. Light-dependent interaction between the Drosophila circadian clock factors cryptochrome, jetlag, and timeless. *Curr Biol*. **19**, 241-237, doi: 10.1016/j.cub.2008.12.042 (2009).
9. Lamba, P, Bilodeau-Wentworth, D, Emery, P, Zhang, Y. Morning and evening oscillators cooperate to reset circadian behavior in response to light input. *Cell Rep*. **7**, 601-608, doi: 10.1016/j.celrep.2014.03.044 (2014).

10. Chiu, JC, Low, KH, Pike, DH, Yildirim, E, Edery, I. Assaying locomotor activity to study circadian rhythms and sleep parameters in Drosophila. *J Vis Exp*. **43**. Pii:2157, doi: 10.3791/2157. (2010).
11. Abreu T, Bragança M. The bipolarity of light and dark: A review on Bipolar Disorder and circadian cycles. *J Affect Disord*. **185**:219-29. doi: 10.1016/j.jad.2015.07.017. (2015).
12. Dubruille, R, Murad, A, Rosbash, M, Emery, P. A constant light-genetic screen identifies KISMET as a regulator of circadian photoresponses. *PloS Genet*, doi: 10.1371/journal.pgen.1000787, (2009).
13. Peschel, N, Veleri, S, Stanewsky R. Veela defines a molecular link between Cryptochrome and Timeless in the light-input pathway to Drosophila's circadian clock. *Proc Natl Acad Sci USA.* **103**, 17313-17318, doi: 10.1073/pnas.0606675103 (2006).

Figure
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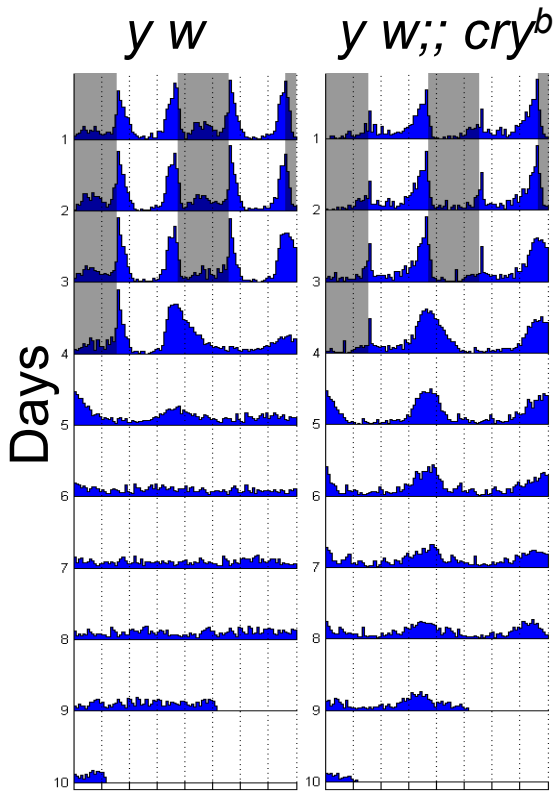

Figure 1

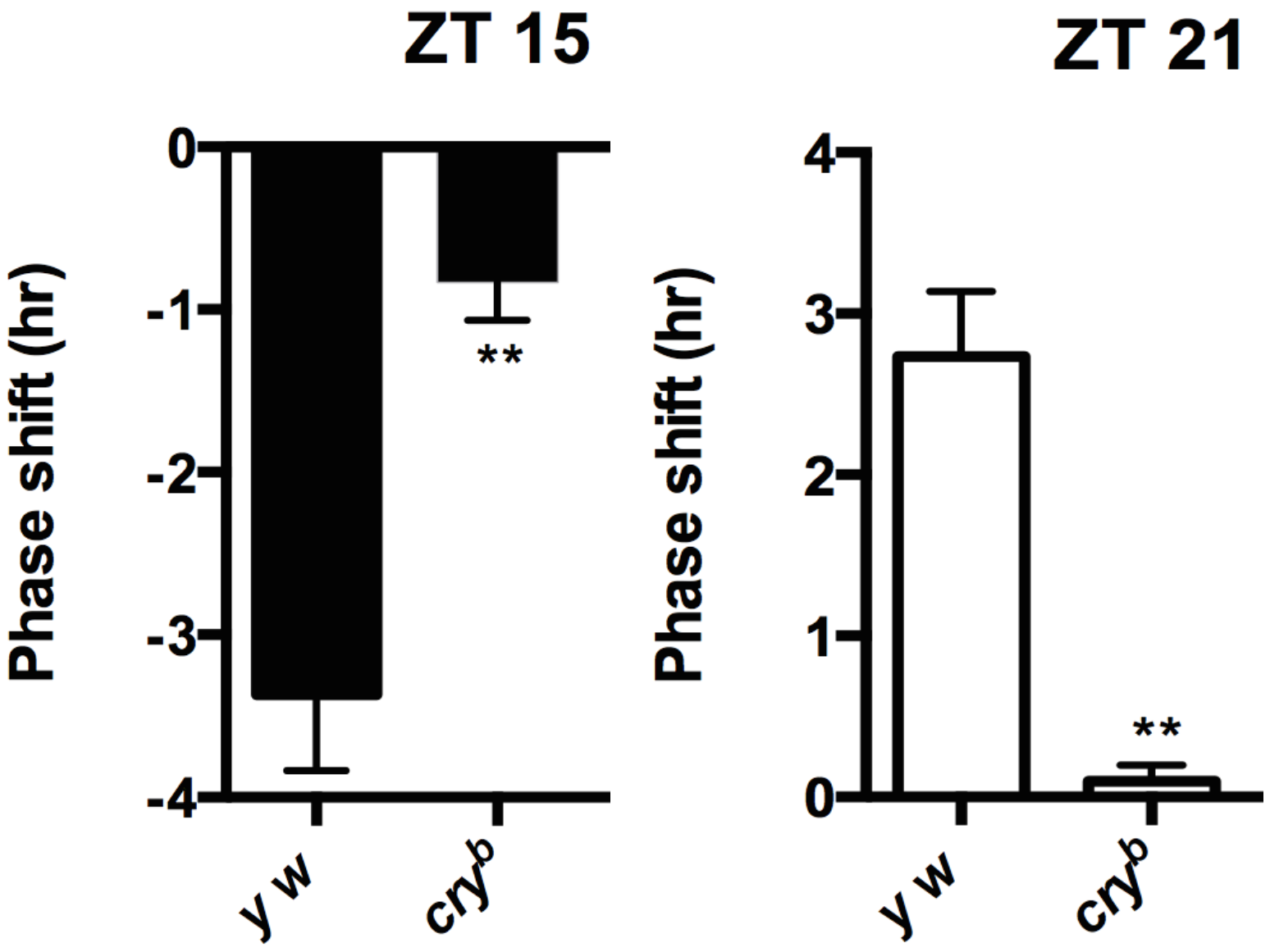
ZT 15
ZT 21
Phase shift (hr)
0
-1
-2
-3
-4
**
y w
cry^b
Phase shift (hr)
4
3
2
1
0
**
y w
cry^b

Figure 2

Table

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Genotype	Number of flies (n)	% of rhythmic flies	Period average (±SEM)
y w	32	3 (1/40)	20.5
cryb	32	91 (29/32)	23.9±0.11

Power average (±SEM)

11.5

63.2±4.49

Excel Spreadsheet- Table of Materials/Equipment

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Incubator with light and temperature control	Percival Scientific	I-36LL	
Drosophila Activity Monitor (DAM)	Trikinetics	DAM2	activity monitor-2
Pyrex glass activity tubes	Trikinetics	PGT5	autoclavable
DAM system software for data acquisition	Trikinetics	DAMSystem308	free download
File Scan software	Trikinetics	DAMFileScan110X	activity monitor-2 software
FaasX software	Centre National de la Recherche Scientifique		Data analysis, Rouyer lab
Sucrose	Fishersci	S2-500GM	making fly food for behavior
Bacto Agar	BD Biosciences	214010	making fly food for behavior

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Article Title: Analysis of circadian photoresponses in Drosophila using locomotor activity

Signature: [signature] Date: 6/25/2015

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Response to editor and reviewer comments

Note: All revisions are marked with red font and highlighted with yellow in the text.

Editorial comments:

•Please copyedit the manuscript for grammatical and typographical errors throughout. This editing should be performed by a native English speaker. Some examples are listed below.

A: We thank the editor and reviews for pointing out the errors. We thoroughly read the manuscript again and also had Matthew Gruner in our department edit the English in the text for us.

-Short abstract – should be “Study of the…”
-1.1.4 – Please use imperative tense, and delete one instance of “paintbrush.”

A: We did the changes as suggested.

-1.2.3 – Please correct the grammar in the first sentence. It is unclear.

A: We changed the sentence and made it clear as “Seal the activity tubes from the food side by plastic caps”.

-1.3.5 – Please correct the verb tense.
-1.4.3 – “FassX” should be “FaasX”
-1.4.1 – Please correct the grammar in the second sentence.
-Section 2 note: Please correct the grammar throughout the note.
-2.2 – Please use complete sentences.

A: We did the changes as suggested

•Please note that while many of the editorial comments have been addressed, there are still some concerns regarding the language and grammar in your article. Our editors will not copy-edit your submission to correct these issues. Additionally, we cannot proceed with a final review and proceed to production until these errors are corrected. Please thoroughly review the language and grammar of your article text prior to resubmission. Failure to address will delay us from proceeding with the review process of your article.

•Formatting:
-The long abstract should focus more on the protocols.
A: We moved some first two sentences to the introduction part and added several sentences about the protocols to address this problem.

-2.2 – Please define ZT15 and ZT21.

A: We defined ZT15 and ZT21 in the text.

-2.5 – Please use the same font throughout the manuscript.

A: We used the same font for the text, except for title of each section.

•Additional detail is required:
-1.2.1 – For what? Last sentence is incomplete.
A: This step is to prepare fly vials for making fly food. We added "before pouring the liquid fly food" in the last sentence to make it clear. We also mentioned again in the next step.

-2.8 – How are the phases compared? Please provide details of software usage. What is clicked on to achieve this?
A: We added enough details of software usage now.

-2.4 – How is light intensity measured?
A: We added "use light meter to measure". We also added the catalogue number in the table.

-2.6 – Please clarify what is meant by "This light pulse is done at dark, except for the 5 min light. Use a flashlight covered with red filter in case if needed."
A: We deleted "except for the 5 min light" to make it clear.

-Figure 2 – Please define the error bars (SD, SEM, etc.).
A: We added "Error bars represent SEM" in Figure 2 legend.

•Unnecessary branding should be removed from the protocol text; specific supplier or manufacturer information can be given in the Materials table:
-1.3 note – Drosophila Activity Monitor
-Substeps of 1.3, 1.4.1, 2.3 – DAM2, DAM; Please use a general term for this equipment.
-1.4.3, 2.7 - Excel
-1.4.3.1 – Math Works
-2.7 - DAM2 Filescan 110X
A: We changed "Drosophila Activity Monitor" into "activity monitor". For "DAM2", "Excel", "Math Works", "DAM2 Filescan 110X", we were not able to find a general term to replace.

•Discussion: The discussion should be extensively edited for grammatical errors. Please discuss the future applications of the protocol as well as the significance of the protocol with respect to other methods. Please specify which other methods are compared to.

A: We asked a native speaker Matthew Gruner to extensively check the grammatical issues in the manuscript. So far these two methods mentioned in the standard published protocols for measuring circadian photoresponse. We were not able to identify other methods to compare to. But we do discuss the strength and weakness of each method.

•Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

•If your figures and tables are original and not published previously, please ignore this

comment. For figures and tables that have been published before, please include phrases such as “Re-print with permission from (reference#)” or “Modified from..” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes.

A: We obtained the permission from Cell press. We also put “figures modified from Lamba et al 2014 with permission” in the legend.

- JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

A: We added DOIs to the references, except for No.3, which is a book chapter.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This protocol describes two widely used and important assays to examine circadian photoresponses. These protocols will be very handy for new researchers as well as for training undergraduate researchers and new graduate students.
Besides the editorial changes I am suggesting below, I believe this article is ready for publication.

Major Concerns:
N/A

Minor Concerns:
Editorial changes suggested:
(1)In the Abstract, line 53: "A brief short light pulse in the night can also dramatically shift phases of circadian rhythms, which are decreased in flies with circadian photoresponses defects" should be changed to ""A brief short light pulse in the night can also dramatically shift phases of circadian rhythms. As expected, this phase shift response is reduced in flies with defects in circadian photoresponse".
(2)Line 70: "entry into" should be changed to "enter"
(3)Line 70: "block" should be changed to "blocking"
(4)Line 76: it should be "Circadian photoresponses are mainly mediated……"
(5)Line 77: "receives" should be changed to "receiving"
(6)Line 84: The first "rhythmicity" should be changed to "rhythmic" instead
(7)Line 84: "is well used" should be changed to "is often used"
(8)Line 90: I am not sure what "almost diminished" means. Do the authors mean "almost abolished"?
(9)Line 105-106: Perhaps the authors should change this sentence to "collect driver lines with GAL4 driven by circadianly regulated promoter (i.e. tim-GAL4 for all circadian neurons) and RNAi responder lines for crosses".
(10)Line 108: "in a vial to make cross" should be changed to "in a vial for each cross"
(11)Line 109: "behavior" should be "behavioral analysis"
(12)Line 113: the hyphen between anesthetize and flies should be removed
(13)Line 114: the word paint brush is repeated

(14)Line 115: The word "following" should be removed
(15)Line 119: The word "for" at the end of the sentence should be removed
(16)Line 121: Why 4L? Do you recommend that the users of the protocol make 4L of food every time?
(17)Line 150: The word "can" should be removed
(18)Lines 155-156: "FassX" should be "FaasX"
(19)Lines 170: The word "part" should be removed
(20)Line 170-171: This sentence should be changed to "Here, we will only describe in detail the steps that diverge between the two experiments".
(21)Line 191: "red filter in case if needed" should be changed to "red filter if necessary".
(22)Line 203: "constantly" should be changed to "constant"
(23)Line 204: "photoresponses" should be singular
(24)Lines 205-206: "The assay can also be used to analyze in quantitative way" should be changed to "The results can also be presented in a quantitative manner".
(25)Lines 212-213: "Check out any dead flies to reanalyze" should be changed to "Make sure not to include dead flies in your analysis"
(26)Line 218: "control flies" should be "yw control flies". It is better to specify the genotype.
(27)Line 226: Please change this to "Phase advance (left panel) and phase delay (right panel)….."
(28)Line 227: "non light pulse separately" should be changed to "non light pulse on two separate graphs".
(29)Line 236: "Study circadian …" should be "Studying circadian….."
(30)Line 239: "photoresponses" should be singular.
(31)Line 240: Psychiatric is misspelled
(32)Line 243: "screen genes" should be changed to "screen and identify genes"
(33)Line 245: "viability" should be "variability"
(34)Line 246: "good number of flies" should be changed to "good sample size"
(35)Line 247: it should be "Based on experimental aims, the number ….."
(36)Line 261: it should be "Another factor to consider when designing……"
(37)Line 265: tim needs to be italicized.

A: We thank the reviewer for corrections. We did the changes as suggested.

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
Pang and colleagues describe a detailed protocol for analyzing circadian photo response in Drosophila, which is quite helpful for those interested in studying the effects of light on circadian rhythm in flies. The authors delineate two protocols: one for analyzing locomotor rhythms in LL and the other for quantifying the magnitude of phase shifts in response to short light pulses. While the former is efficient for screening mutants that demonstrate defects in circadian photo response, the latter is a more sensitive method for further characterizing alterations in the photo response.

As the authors pointed out, there are intricate connections between circadian rhythm and mood. Moreover, light exerts profound influences on both circadian rhythm and mood. Therefore, studying the circadian photo response may advance our understanding of

how light modulates circadian rhythm and mood, thus facilitating the development of potential treatments and therapies for psychiatric disorders.

Overall, the manuscript is conceptually sound, although I do have a few comments and suggestions.

Major Concerns:
1) When analyzing LL data, the authors only focused on rhythmicity, but LL period values could also be informative. Based on the velocity response curve, the period in LL reflects summed (i.e. phase-delaying and phase-advancing) effects of light. Presumably a mutant with alterations in phase delay and/or phase advance would exhibit altered period in LL. This may be further characterized in detail by measuring PRC using the second method described by the authors.

A: We thank reviewer#2 for the useful comments. Compared to period in constant darkness, LL period is much more complicated. It does not necessarily indicate the photoresponse differences (Dubruille et al 2009). For example, *cry*^{b} and *jet*^{set} mutants which show severe defects in photoresponse, the LL period are normal (Lamba et al 2014). In Drosophila, so far it is standard to compare the rhythmicity for detection of photoresponse.

2) The authors discussed about the relationship between circadian rhythm and psychiatric disorders, but given that the focus of this manuscript is circadian photo response, it may be more relevant to discuss about the connections between light, circadian rhythm and psychiatric disorders. Also appropriate references are needed.

A: We added a review article as reference of “bipolar disorder and circadian clocks”

Minor Concerns:
1) There are a number of grammatical errors and typos in the manuscript:
Ln37 Change "Study" to "Studying".
Ln38 Change "to dissect" to "for dissecting".
Ln70 Change "entry" to "enter" and "block" to "blocking".
Ln76 Add "are" between "photoresponses" and "mainly"
Ln77 Change "receives" to "receiving".
Ln78 Add "it" between "then" and "is", and change "bound" to "binding".
Ln86 Delete "the" before "transient degradation" and change "shift" to "shifting".
Ln98 Change "in standard" to "on standard".
Ln105 Change "relative" to "relevant".
Ln109 Change "progeny" to "progenies".
Ln112 Change "At" to "In".
Ln113 Delete the hyphen.
Ln114 Delete "paint-brush" and change "progeny" to "progenies".
Ln115 Change "following" to "subsequent".
Ln119 Delete "for".
Ln126 Change "with" to "by".
Ln133 Change "in" to "into".
Ln136 Change "Put" to "Use".
Ln145 Change "using" to "use".
Ln146 Add "and" before "then".
Ln150 Delete "can".

Ln157 Change "at" to "in".
Ln169 Add "The" before "Major".
Ln170 Add "we" between "Here" and "only".
Ln171 Change "of this" to "between these".
Ln182 Change "set" to "sets", add "the" between "in" and "incubator", and change "Measure" to "Set".
Ln190 Change "at" to "in the".
Ln191 Delete "in case".
Ln203 Change "constantly" to "constant".
Ln204 Change "photoresponses" to "photoresponse".
Ln205 Delete "that".
Ln206 Add "a" before "quantitative".
Ln219 Add "and" before "then".
Ln224 Change "of" to "to", and "release in" to "released into".
Ln225 Change "mins" to "min" and "at the last" to "on the last".
Ln227 Change "separately" to "respectively".
Ln236 Change "Study" to "Studying" and "to understand" to "for understanding".
Ln238 Change "psychiatry" to "psychiatric".
Ln245 Change "viability" to "variability".
Ln252 Change "mins" to "min".
Ln261 Add "that" between "thing" and "needs".
Ln263 Change "carry" to "carrying".

A: We did the changes as suggested.

2) In 2.4 the authors indicate that light intensity used is 1000 lux for the PRC experiment, but later on in the text the light intensity used appears to be 1500 lux. Please make appropriate changes.

A: We changed the light intensity to 1500 lux.

3) The authors mentioned transforming data with DAM2 Filescan in 2.7, but not for the LL protocol. If this procedure is also carried out in LL, it may be good to state it.

A: It is the same procedure. We did mention about it in 1.4.1

4) Both Fig1 and 2 demonstrate results for yw flies, whereas w1118 is used in Table 1. It would be better to be consistent throughout the paper and show results for yw in Table 1 instead of w1118.

A: *cry*^{b} is in *y w* background. It was a typo. So we corrected the genotype in Table 1.

Additional Comments to Authors:
N/A

Reviewer #3:
Manuscript Summary:
This article explains how circadian locomotor behavior is recorded and analyzed. In particular, the authors focus on constant light induced arrhythmicity and phase-shifts induced by a brief light pulse in the dark, which can be used as a tool to screen for

mutants of the light input pathway to the clock.

Major Concerns:
The article is poorly written and has too many grammar mistakes in addition to phrases that are not scientific (such as: "do the same thing" or "check out any dead flies". This should be addressed.

A: We thank the reviewer for this and we checked throughout the manuscript for grammar and spellings.

There are many recent reviews on how locomotor behavior is recorded and analyzed (e.g. Chiu and Edery, Jove 2010). The techniques used for this assay have not changed in the last decade. Therefore, explanation of how you prepare a fly for the typical locomotor monitoring is redundant. Instead, the authors should focus on the specific details of how you analyze circadian arrhythmicity induced by light or phase-shifts induced by light pulses.

A: Explanation of how we prepare flies is required by editors. We think it might be helpful for biologists without fly pushing experience.

A section on the history of phase-shifts should be included to give the readers a sense of how this works. Also, authors should clearly define what is used as the phase-marker and why and under which condition (Depending on the quality of the raw data, phase-marker can be the peak, trough or mid-point of the offset of activity).

A: We added a brief introduction of the history of phase-shift in flies. We also mentioned about what should be used for phase markers at different conditions.

Also, there are various softwares available and they should be explained within a coherent paragraph with proper citations.

A: We agree with the reviewer that various softwares are useful for analysis. But this is beyond the scope of this method paper. We will not discuss in this manuscript.

Use of the raw traces of periodograms should be explained for determining arrhythmicity and should be included in the figures.
Analysis of such an important part of circadian biology requires a section of its own and it's the heart of the paper and is currently missing in the literature. This would make the paper a nice addition to the aforementioned reviews which don't explain phase-analysis.

A: We appreciate the reviewer comments. We added some sentences explain how phase shift experiment works in Drosophila.

Minor Concerns:
-Figures 1 and 2 are cropped versions of the figures from the Lamba et al. (2014 Cell. Rep,ref #9). The legend should state that these figures are adapted from this paper.

A: Thanks for pointing it out. Before submission, we consulted with editors in Jove. We already got the permission of Cell Press and stated in the legends.

-In the introduction, there are more recent reviews on the Drosophila molecular clock

available and they should be added since the field is progressing rapidly.

A: As the reviewer said the field is progressing rapidly, it is almost impossible to cover all the progress of molecular clock. We just chose the classical reviews for this manuscript, which covered the essential components in the field.

-It should be noted that the reason for light pulses being only 5 minutes or so is to avoid the additional light input pathway from the eyes. Also, the relationship between intensity and time should be discussed since it is not a linear integration (e.g. Hirsh 2013 Plos Genetics). In addition, lower light intensities could reveal whether if a mutant is hyper- or hyposentisitive.

A: We added the note that why 5 min of light pulse is used. In Hirsh 2013 Plos Genetics, they used a very different method “6 hours extremely low light intensities”. It is an elegant study, but for the scope of this manuscript, we did not discuss that.

-ZT and CT should be explained.
-line104: dark dot should be explained

A: This is too detailed, so we did not explain.

-113:% of co2 should noted.
A: 100% of CO2 was used.

-humidity plays a strong role in the robustness of flies and should be mentioned

A: 60% humidity was used.

-162:depending on robustness of the rhythm, a power of 10 could also be used.

A: We added that in the manuscript.
-191:wavelength for red filter should be noted
A: We deleted the sentence about red filter since it is not necessary.

-197-200 not clear. See above

A: We made some corrections to try it make it clear.

-210:advances are positive and delays are negative. This should be explained in the introduction in the history part.

A: we added one sentence for explanation of phase delay and advance for 2.8

-The length of the LD entrainment before each experiment is not stated consistently. This should be checked throughout the paper. For example, line 185 says 5 days LD while line 224 says 3 days.

A: We made the LD entrainment consistent to 5 days LD.

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