**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, *cryb* and *jetset* 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: *cryb* 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.