**Reviewer #1:**

*Major Concerns:*

1. The w1118 line contains a mutation in the white gene and should not be considered as wild-type. The authors could name it as "control" instead of "wild-type".

**Changed to “control” in all instances.**

2. What stages of larva (1st, 2nd, or 3rd instars) did the authors use in their experiment? Can the system detect all 3 stages or just the largest 3rd instar larvae?

**We present additional data from first and second instars.**

3. The larva is trapped insider the monitor tube that is sealed with agar in both ends. Does the lack of aeration affect the behavior of the larva? Does the larva show any changes in behavior during the 20min assay?

**Activity over the 20 minutes was analyzed in one-minute bins, and shows no change. This will be presented as a separate figure.**

4. Figure 1 and 2 seems switched in the pdf file.

**Corrected.**

*Minor Concerns:*

1. Line 142: need to define what PSIU is.

**Term defined.**

2. Any speculation why there is a sudden reduction in larval activity at 25oC, which should be the usual culturing temperature for Drosophila.

**Conjecture is offered.**

3. Table of materials: "Flugs for plastic fly bottle" - typo "Plugs"? Materials used or general fly husbandry (e.g. vials, corn meal, Tegosept… etc) may not need in this section since they are not specific to this experimental setup.

**Spelling is correct for the product name “flugs”, husbandry materials left to editor’s discretion**

**Reviewer #2:**

*Major Concerns:*

1) In Figure 1, the move/minute data for w1118 larvae at temperatures of 5°C-35°C show a consistent upward trend with the exception of either the 20°C or 25°C data point. It is surprising that steadily increasing temperature would cause larval activity to fluctuate in this manner. Is this pattern consistent between experimental runs? Is it possible that the 20°C and 25°C data were reversed or that some anomaly during the assay resulted in an abnormal reading?

**Issue discussed. This is indeed what we actually observed.**

2) If, as speculated above, either the 20°C or 25°C data point shown in Figure 1 is incorrect, is the 20°C moves/minute reading for w1118 still significantly different than that for iav1 shown in Figure 2?

**Issue discussed.**

3) Does this assay system provide as meaningful a representation of movement rates as other available methods? Video 1 shows an unidentified larva in an assay tube. A visual count of body wall contractions (a common low cost method used to quantify larval movement) during this video indicates ~12 in a 22 second period. This can be extrapolated to a rate of approximately 33 moves/minute, a rate nearly three times that of the highest rate shown in Figure 1. The two assay methods are obviously not directly comparable in terms of raw counts but this does reveal that the authors' assay system involves a significantly higher threshold in order to score a movement. The authors also note that their assay method may be unable to detect circumferential movements that do not involve breaking a beam. They feel that this is not a serious limitation since it would affect all test samples equally, however, combined with the issue of a higher movement threshold, it suggests that this method could miss small but significant differences in movement rate between test groups or decrease the apparent significance of larger differences.

**We admit that the device may not be sensitive to small-scale movements.**

*Minor Concerns:*

1) The labels on Figures 1 and 2 are reversed compared to the figure legends.

**Corrected.**

2) Italics are generally used for both complete names of Drosophila genes and their abbreviations.

**All instances italicized.**

3) Formatting of Materials/Equipment Table could be improved to fit on one page and improve readability.

**Now fits on one page and striped for readability.**

4) The method used to humidify the assay tubes prior to larval insertion should be improved. Breathing into each tube introduces variability in the degree of condensation achieved based on differences between operators and environmental conditions. It also increases the possibility of sample contamination.

**Great comment. The breathing method has been replaced with a method using a hot water bottle.**

5) Which components of the assay system were located inside the incubator during testing (activity monitor, power supply, power supply interface, etc)? Are there any potential concerns with the stability of incubator temperature due to heat generation by the power supply (if it is located inside the incubator) or the necessity of running cords out of the incubator (if power supply is located outside)? A diagram describing the experimental setup would be helpful.

**A diagram has been supplied as Figure 5.**

**Reviewer #3:**

For what time frame is the assay useful for?

**Some analysis and discussion of the time frame used, as well as conjecture about how to increase the length of an assay, is provided**

Do wildtype animals show changes since they are put in the assay? This is a key criterion since adults show no change in activity and thus the assay can be well used to monitor activity/sleep/rhythm.

**We are not sure to what situation we are being asked to compare larval activity?**

The proposed method uses Agar plugs at both sides of the tube. This would not allow a regular gas exchange to the chamber and thus could rapidly result in hypoxic conditions. This should be addressed by using an alternative plug at one side. Similarly the lack of food will affect the behavior of animals.

**Behavior was analyzed over the 20-minute period and no change in locomotion was observed. See Figure 4.**

Since both larvae and adults show circadian rhythms it could be tested if this assay can be used to monitor circadian activity.

**We have offered discussion of this possibility, which would require modifications that allow for feeding and probably increased gas exchange.**

**Reviewer #4:**

*Minor Concerns:*

I have one minor concern: The term "data" is plural and not singular (datum is the singular and is generally not used). I would urge the authors to go through the manuscript and change sentences in which the word data is used to reflect that the term is plural.

**Good point. Done.**

**Reviewer #5:**

*Minor Concerns:*

1. As the company Trikinetics provides a wide variety of monitoring devices, a brief discussion (in the introduction or discussion sections) on why the MB5 MultiBeam Activity Monitor was chosen over their other lines of products could be useful. That way, investigators unfamiliar with these devices know that the high number of IR beams in the MB5 (17) (and other benefits) versus their other products (DAM2 or LAM10 with 9 IR beams) provide the necessary resolution and special software to detect the small motions by the larvae.

**We have added a phrase that makes this comparison.**

2. A graphical representation (an actogram) of the larval activity over time of day would be a helpful addition. While the larvae are not in the devices for a long time, it would be an easy way to visualize high or low activity points over the course of the experiment, rather than only investigating the total movement counts at the end. The Trikinetics website provides free activity analysis software from other sources.

**This has been added.**