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## Recording and analysis of circadian rhythms in running-wheel activity in rodents

--Manuscript Draft--

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Abstract:	<p>When rodents have free access to a running wheel in their home cage, voluntary use of this wheel will depend on the time of day<sup>1-5</sup>. Nocturnal rodents, including rats, hamsters, and mice, are active during the night and relatively inactive during the day. Many other behavioral and physiological measures also exhibit daily rhythms, but in rodents, running-wheel activity serves as a particularly reliable and convenient measure of the output of the master circadian clock, the suprachiasmatic nucleus (SCN) of the hypothalamus. In general, through a process called entrainment, the daily pattern of running-wheel activity will naturally align with the environmental light-dark cycle (LD cycle; e.g. 12h-light:12h-dark). However circadian rhythms are endogenously generated patterns in behavior that exhibit a ~24h period, and persist in constant darkness. Thus, in the absence of an LD cycle, the recording and analysis of running-wheel activity can be used to determine the subjective time-of-day. Because these rhythms are directed by the circadian clock the subjective time-of-day is referred to as the circadian time (CT). In contrast, when an LD cycle is present, the time-of-day that is determined by the environmental LD cycle is called the zeitgeber time (ZT). Although circadian rhythms in running-wheel activity are typically linked to the SCN clock<sup>6-8</sup>, circadian oscillators in many other regions of the brain and body<sup>9-14</sup> could also be involved in the regulation of daily activity rhythms. For instance, daily rhythms in food-anticipatory activity do not require the SCN<sup>15,16</sup> and instead, are correlated with changes in the activity of extra-SCN oscillators<sup>17-20</sup>. Thus, running-wheel activity recordings can provide important behavioral information not only about the output of the master SCN clock, but also on the activity of extra-SCN oscillators. Below we describe the equipment and methods used to record, analyze and display circadian locomotor activity rhythms in laboratory rodents.</p>
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August 8, 2012

Dr. Nandita Singh  
Senior Science Editor  
Journal of Visualized Experiments (JoVE)

Dear Dr. Singh,

We are submitting a revised version of our manuscript: **Recording and analysis of circadian rhythms in running-wheel activity in rodents** . We have responded to the referees comments and trust that the paper is now suitable for publication.

Sincerely,

Shimon Amir, PhD, FRSC



## **Recording and analysis of circadian rhythms in running-wheel activity in rodents**

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### **Keywords:**

Suprachiasmatic nucleus, locomotor activity, mouse, rat, hamster, light-dark cycle, free-running activity, entrainment, circadian period, phase shift

**Short Abstract:**

Circadian rhythms in voluntary wheel-running activity in mammals are tightly coupled to the molecular oscillations of a master clock in the brain. As such, these daily rhythms in behavior can be used to study the influence of genetic, pharmacological, and environmental factors on the functioning of this circadian clock.

**Long Abstract:**

When rodents have free access to a running wheel in their home cage, voluntary use of this wheel will depend on the time of day<sup>1-5</sup>. Nocturnal rodents, including rats, hamsters, and mice, are active during the night and relatively inactive during the day. Many other behavioral and physiological measures also exhibit daily rhythms, but in rodents, running-wheel activity serves as a particularly reliable and convenient measure of the output of the master circadian clock, the suprachiasmatic nucleus (SCN) of the hypothalamus. In general, through a process called entrainment, the daily pattern of running-wheel activity will naturally align with the environmental light-dark cycle (LD cycle; e.g. 12h-light:12h-dark). However circadian rhythms are endogenously generated patterns in behavior that exhibit a ~24h period, and persist in constant darkness. Thus, in the absence of an LD cycle, the recording and analysis of running-wheel activity can be used to determine the subjective time-of-day. Because these rhythms are directed by the circadian clock the subjective time-of-day is referred to as the circadian time (CT). In contrast, when an LD cycle is present, the time-of-day that is determined by the environmental LD cycle is called the zeitgeber time (ZT).

Although circadian rhythms in running-wheel activity are typically linked to the SCN clock<sup>6-8</sup>, circadian oscillators in many other regions of the brain and body<sup>9-14</sup> could also be involved in the regulation of daily activity rhythms. For instance, daily rhythms in food-anticipatory activity do not require the SCN<sup>15,16</sup> and instead, are correlated with changes in the activity of extra-SCN oscillators<sup>17-20</sup>. Thus, running-wheel activity recordings can provide important behavioral information not only about the output of the master SCN clock, but also on the activity of extra-SCN oscillators. Below we describe the equipment and methods used to record, analyze and display circadian locomotor activity rhythms in laboratory rodents.

## **Protocol Text:**

### **1) Animal housing**

**1.1 Cage:** In order to record the running-wheel activity of an individual rodent, each cage should house a single rodent and running-wheel.

Because running wheels can be considered a form of enrichment, all rodents in any study should have similar access to a running wheel.

**1.2 Bedding changes:** Animal handling as well as changes in cages or bedding can all have non-photic effects on circadian rhythms<sup>21-23</sup>, so, cages with mesh-flooring are ideal because they minimize contact with the animal. Notwithstanding the availability of such a tray-system, bedding changes should be avoided during critical phases of an experiment.

Alternatives include using longer-lasting bedding, which would allow for

more infrequent cage changes, or changing cages on a pseudo-random schedule.

**1.3 Isolation Boxes:** Cages should be kept in isolation boxes that are sound-attenuated, light-controlled, and well-ventilated. Depending on the size and configuration of the isolation boxes, the number of cages within each box will typically range from 1-8. When housing multiple cages in a single isolation box, one should be aware that various odors and sounds coming from other animals can have confounding effects on the circadian behavior of individual animals. To avoid these problems, one should attempt to house one cage per isolation box.

**1.4 Ventilation:** Adequate air flow is imperative to making the boxes a comfortable home environment for rodents. The fan in each box should be hooded, so as to prevent light from outside the box from reaching the inside. Also, fans will typically remove air from the box and blow it into the room. Small light-tight vents allow air to enter the isolation boxes from several points, and help to avoid uncomfortable breezes. In order to verify that there is adequate ventilation, the temperature inside the isolation box (when closed for several hours, with the lights on) should be virtually identical to the temperature in the room where it is housed.

**1.5 Lighting:** Environmental light intensity should be the same in all cages. Arrange a single light at a similar location above each cage, and always use the same brand/type of bulb. Use moderate intensity illumination (100-300 lux) at cage level. Avoid excessively high illumination levels,

which are more likely to produce direct changes on behavior attributable to the light rather than the circadian system, *per se* (e.g. masking).

**1.6 Darkness/Dim red lighting:** If it is necessary to handle or treat animals in the dark (e.g. in constant darkness or nighttime), night vision goggles should be used. Alternatively, because the circadian system is relatively insensitive to red-wavelengths, dim red lighting can be used. The specific red light you use should be tested to ensure it does not alter running-wheel activity (e.g. positive or negative masking) or adjust the circadian clock (e.g. produce a phase shift).

## **2 Data Collection (See Figure 1 – Vitalview Hardware Configuration)**

**2.1 Running wheels:** The diameter and ergonomics of the running wheel will change the amount of use<sup>24</sup>. Thus, use smaller and lighter wheels for mice, and larger heavier wheels for rats. When washing and re-installing wheels, ensure that the wheels are able to spin unobstructed, do not “wobble”, and that the recording micro-switches are activated by each turn of the wheel.

**2.2 Micro-switches:** Each revolution of the running wheel should activate a magnetic or mechanical micro-switch. Information from the micro-switch is transmitted via a single channel and recorded by a computer which can bin the data over time (e.g. every 2, 5, 6, or 10 min).

**2.3 Computer hardware:** Our running-wheel recordings are made with Vitalview, a hardware and software platform developed by Mini Mitter ([http://www.minimitter.com/vitalview\\_software.cfm](http://www.minimitter.com/vitalview_software.cfm)). However, there are

other recording platforms such as ClockLab, developed by Actimetrics (<http://www.actimetrics.com/ClockLab/>). Both platforms bring together data from many single-channel sources (e.g. a single micro-switch activated by a single running wheel) into a single computer file. Data from individual channels can then be graphed and analyzed separately at a later date.

### **3 Data recordings**

**3.1 Files:** The above mentioned software platforms can be used to separate out single channels so that individual files are created for each running-wheel record. Such data are best visualized and graphed with specially designed programs such as Actiview (Minimitter, Bend, OR), Circadia, or Clocklab (Actimetrics, Wilmette, IL) which can all produce periodograms and actograms. However, single-channel files can also be opened and analyzed using general spreadsheet programs such as Excel (Microsoft, Redmond, WA).

**3.2 Calculating Circadian Time (CT):** CT 12 is, by definition, the onset of running-wheel activity in nocturnal rodents. In parallel with the 24h day, by convention, one circadian day is broken into 24 circadian h. Accordingly, if the circadian free-running period is 24h 30 min as measured by the wall clock, CT 0 will occur approximately 12h 15 min after CT12.

### **4 Representative Results:**



**4.1 Computer programs:** Specialized computer programs are typically used in the generation of actograms and the calculation of circadian period.

These programs include, but are not limited to, ActiView (Minimitter, Bend, OR) and Circadia.

**4.2 Actograms:** Actograms provide a graphic illustration of the daily patterns of running-wheel activity. There are single-plotted (x-axis = 24h) and double-plotted (x-axis = 48h) actograms. Both methods plot sequential days from top to bottom, but double-plotted actograms plot two days on each horizontal line. Specifically, double-plotted actograms show the “second day” on the far right of each line, as well as at the start of the second horizontal line, and so on. Double-plotting is especially helpful to visualize non-24h rhythms.

**4.3 Periodogram:** A periodogram is constructed from a spectral analysis of the running wheel activity over time. Periodograms show the relative power for a range of pre-set periods, and are commonly used to determine the free-running period.

**4.4 Results:** In the laboratory, rodents are usually housed under a 24h LD cycle. Under these conditions the rhythm of activity is entrained, such that the daily pattern of running-wheel activity is aligned with the precise 24h LD cycle. In Figure 2A, the double-plotted actogram on the left shows the running-wheel activity of a rat that became active at the same time each day, soon after the environmental lights were turned off. The periodogram on the right shows a strong peak at 24h, consistent with entrainment to a

precise 24h LD cycle. Figure 2B illustrates the running wheel use of a rat that was housed in constant darkness. In this case, the daily onset of running-wheel activity occurred slightly later each day, creating a rightward “drift”. This rightward “drift” indicates that the endogenous circadian period is greater than 24h, but it is the peak in the periodogram that quantifies this period. According to the periodogram, the maximum power is observed at 24.33h. In contrast to the automated periodogram analysis, Figure 3 illustrates a method to manually calculate the free-running period using the time of onset of running-wheel activity. It is important to realize that calculating the period by hand and calculating it with a periodogram may yield slightly different results.

The daily patterns of running-wheel activity can be disrupted in several ways. Figure 4 illustrates an arrhythmic pattern of running-wheel activity, produced by an electrolytic lesion of the SCN. This type of experiment provided some of the first evidence that the SCN contained the “master” circadian clock<sup>7,8</sup>. The periodogram on the right confirms this arrhythmic pattern of activity by showing equivalently low power for all periods in the circadian range (20-30h). The circadian pattern of running-wheel use can also be disrupted by housing rats in constant light. Figure 5 shows an actogram from a rat exposed sequentially to several of the lighting conditions already described. First, the rat was housed in constant darkness and exhibited a running wheel activity rhythm of approximately 24.33h. Second, the environmental light was kept on and

the rat was housed in a constant light environment. Constant light is known to disrupt the SCN-based clock and produce arrhythmic patterns of running-wheel activity, similar to an SCN lesion. This disruption by light, however, occurs gradually over the course of 2-3 weeks. Therefore, when the running-wheel record is analyzed after the initial 3 weeks in constant light, the periodogram does not yield a peak. Finally, in the third phase the rat was put back on a 12h:12h LD cycle and the running wheel activity rhythms recover nearly immediately.

The amount of running, and the time of day it occurs, can also be manipulated by environmental factors. For instance, if rodents are fasted and given a temporally-restricted meal each day, this restricted feeding schedule will induce a daily bout of food-anticipatory activity. It is termed “anticipatory” because it occurs prior to the arrival of the daily meal, and is especially obvious when the meal is given in the middle of the day, a time when nocturnal rodents are relatively inactive. For instance, if an experiment provides a single 2h meal each day, food can be added to the cage at ZT 4 (4h after lights turned on) and removed at ZT 6 (2h later). Moreover, wire mesh flooring in the cages is also advantageous for this type of experiment because it makes it impossible for the rats to hide food and store it for later, thus ensuring that the rat is actually consuming all the food within the prescribed mealtime. Finally, one of the main advantages of an accurate running-wheel activity recording is that it allows for correlations to be made between running-wheel activity and daily

oscillations in the expression of circadian clock gene expression throughout the brain and body.

#### **4.5 Common pitfalls:**

- 4.5.1** Many software platforms automatically adjust for daylight saving time changes. When conducting an experiment at the time of yearly time changes, be sure this option is turned off in the recording software as well as the computer operating system software. This safeguard should help avoid discrepancies between the recording and the external light cycle.
- 4.5.2** To be able to check on discrepancies in the data or unexpected changes in behavior, keep a text file with the precise time and date of every box opening, feeding and watering, bedding changes, experimental manipulations, and any other disruption that might occur. The precise start and end times of the data recording should also be noted in this file.
- 4.5.3** It is important to check regularly that the lights are turning on and off at the expected times. Many problems can occur, including power outages and burnt out light bulbs. Some running-wheel platforms are equipped with light-sensors, but others do not verify lighting conditions.

## 5. Figures

**Figure 1:** The Vitalview hardware configuration begins with a rodent running wheel, which is designed to activate a micro-switch with each revolution. This information then travels to the QA4 modules and is relayed to the DP24 dataport and finally is recorded by the Vitalview-equipped computer. The computer sums running-wheel revolutions from each channel every 10 min; these data can be viewed later as an actogram or periodogram. Depending on how the lights are set up, they can be controlled remotely either by the same Vitalview-equipped computer or by wall timers purchased from any electronics store.

**Figure 2:** Representative actograms and periodograms for male Wistar rats housed in a 12h:12h LD cycle (A) and in constant darkness (B). Double-plotted actograms (left column) illustrate the lighting conditions along the top, 48h of running-wheel activity along the X-axis, and plot sequential days from top to bottom. Periodograms (right column) perform a spectral analysis on the running wheel data illustrated in the actograms. Figure 1A illustrates the behavior of a rat that was housed under a 24h LD cycle. Under these conditions the rat becomes active at the same time each day, showing an exact 24h peak in the periodogram. Figure 1B illustrates the behavior of a rat that was housed in constant darkness. Under these conditions the rat was active slightly later each day, hence the rightward drift in the actogram and 24.33h peak in the periodogram.

**Figure 3:** Period can also be extrapolated by hand. First draw a line of best-fit based on the daily onset of activity (red line). Next calculate the slope in h/day, remember that if the rhythm is <24h the slope will be a negative value, and finally add 24h. This procedure will provide an estimate of the circadian period for that animal. In this case, 4h/10 days suggests that the animal is becoming active approximately 0.4h later each day (slope 0.4h/day). Therefore, the circadian period is approximately 24.4 h (or about 24h and 24 min). Any manipulation that is scheduled according to CT requires the free-running period to make an accurate prediction of CT.

**Figure 4:** Electrolytic lesions of the SCN will produce arrhythmic patterns of running-wheel activity. In this case the rat is housed under constant darkness and, because the “master” circadian clock has been lesioned, the rat fails to show an endogenous circadian rhythm in running-wheel activity. The periodogram on the right confirms that there is no significant rhythm in the circadian range.

**Figure 5:** Lighting conditions have strong effects on the patterns of running wheel activity. In this record, the rat is initially housed under constant darkness (DD), as denoted by the shaded portion of the record. Under this condition, the circadian clock drives a circadian rhythm in running-wheel activity with a period of 24.33h, shown by the periodogram (top right). Next, the rat is housed under constant light (LL), as denoted by the white portion of the actogram. Under this condition the endogenous circadian clock is disrupted gradually over 2-3 weeks, and as shown by

the periodogram (middle right) the rat becomes arrhythmic. Finally, the normal 12h:12h LD cycle was reinstated and the running-wheel activity rhythm was restored with a precise 24h rhythm as shown by the periodogram (bottom right).

## **6. Discussion**

Monitoring daily activity rhythms using running wheels is the most commonly used and reliable method for assessing the output of the master circadian clock in nocturnal rodents. Wheel-running activity, however, is only one of many aspects of behavior and physiology that can be monitored continuously. Indeed, although the vast majority of running-wheel activity occurs during the night<sup>25</sup>, over 30% of the total wakefulness occurs during the daytime<sup>26</sup>. Other endpoints can be used to assess circadian rhythms, including general activity, food-bin approaches, drinking, sleep, and body temperature. Thus, depending on the nature of the study, researchers may record several rhythms simultaneously.

### **Acknowledgements:**

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### **Disclosures:**

The authors have nothing to disclose

**Table of specific reagents and equipment:**

<b>Name of the reagent</b>	<b>Company</b>	<b>Catalogue number</b>	<b>Comments (optional)</b>
Vitalview Card & Software	Mini Mitter (Bend, OR, USA)	#855-0030-00	
DP24 Dataport	Mini Mitter (Bend, OR, USA)	#840-0024-00	
QA4-Module	Mini Mitter (Bend, OR, USA)	#130-0050-00	
Magnetic Switch	Mini Mitter (Bend, OR, USA)	#130-0015-00	
C-50 Cable assembly	Mini Mitter (Bend, OR, USA)	#060-0045-10	
Rat running wheel assembly	Mini Mitter (Bend, OR, USA)	#640-0700-00	
Cage and tray support	Mini Mitter (Bend, OR, USA)	#640-0400-00	
Useable cut away cage	Mini Mitter (Bend, OR, USA)	#664-2154-00	
Grid floor for cage	Mini Mitter (Bend, OR, USA)	#676-2154-00	



Waste tray	Mini Mitter (Bend, OR, USA)	#684-2154-00	
Lamp housing	Microlites Scientific (Toronto, ON, Canada)	#R-101	
4W Fluorescent lamps	Microlites Scientific (Toronto, ON, Canada)	#F4T5/CW	
Isolation chambers	Custom built	n/a	28"H x 20"W x 28"D ½" Black Melamine.

## References

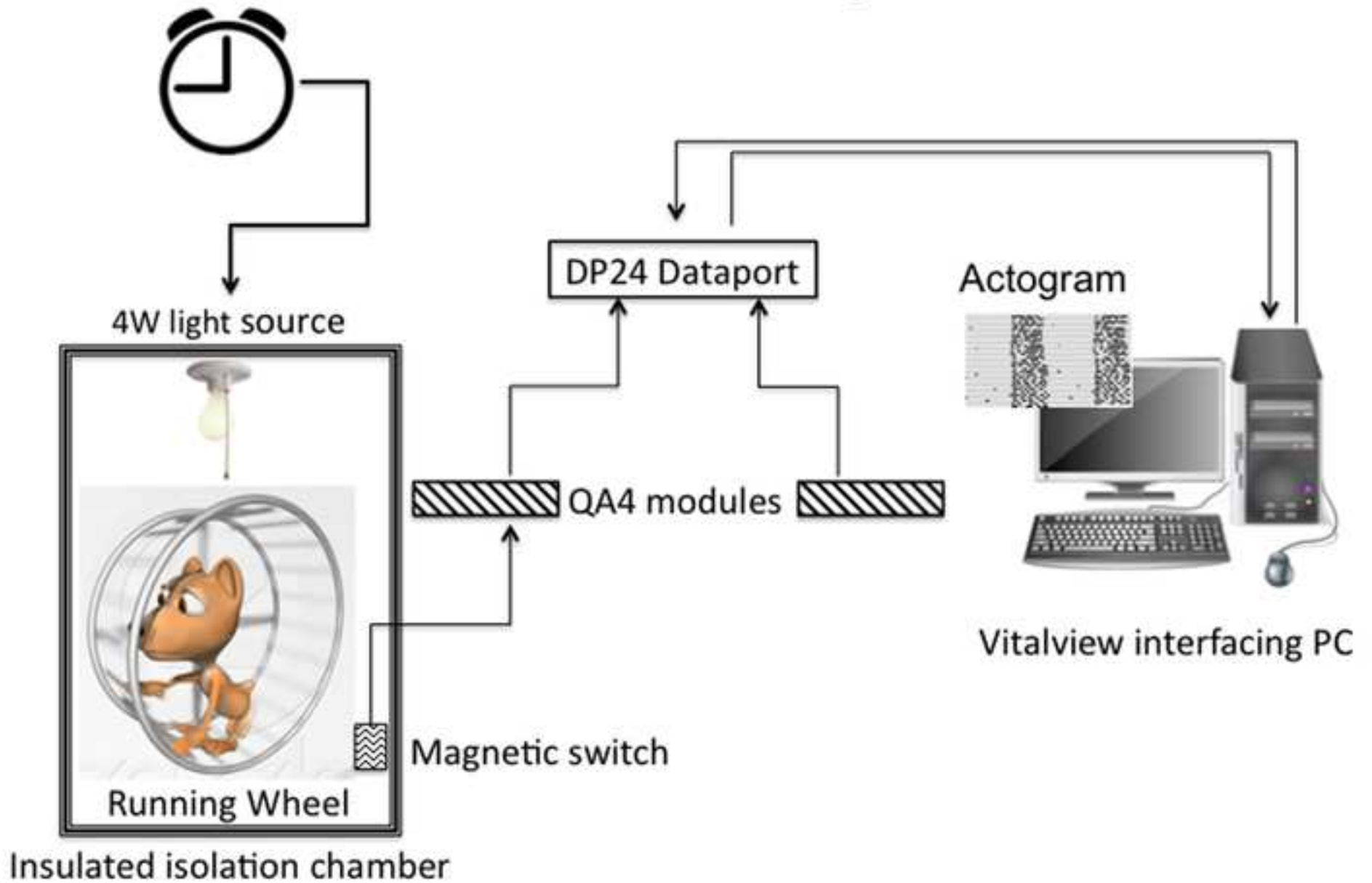
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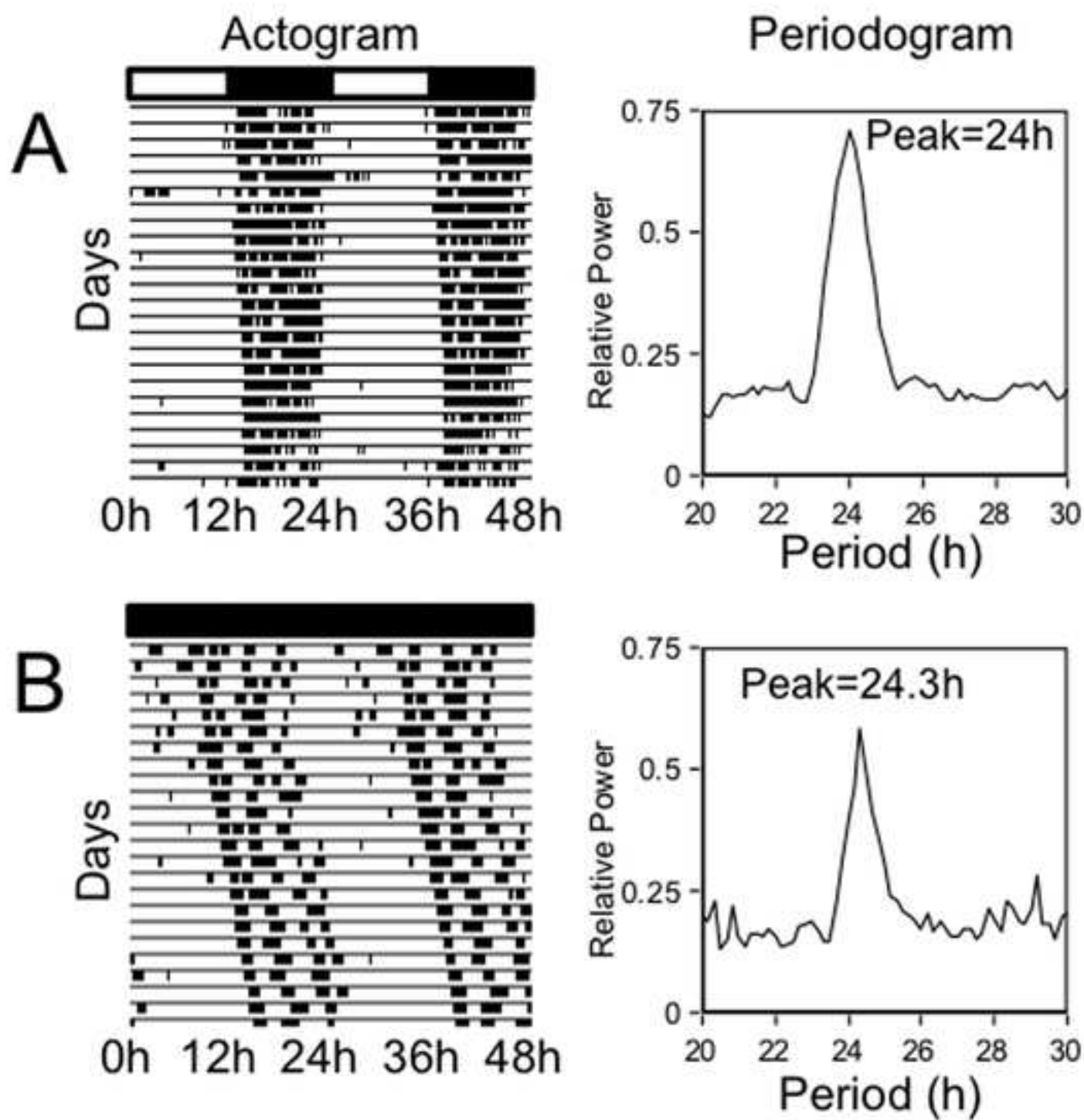
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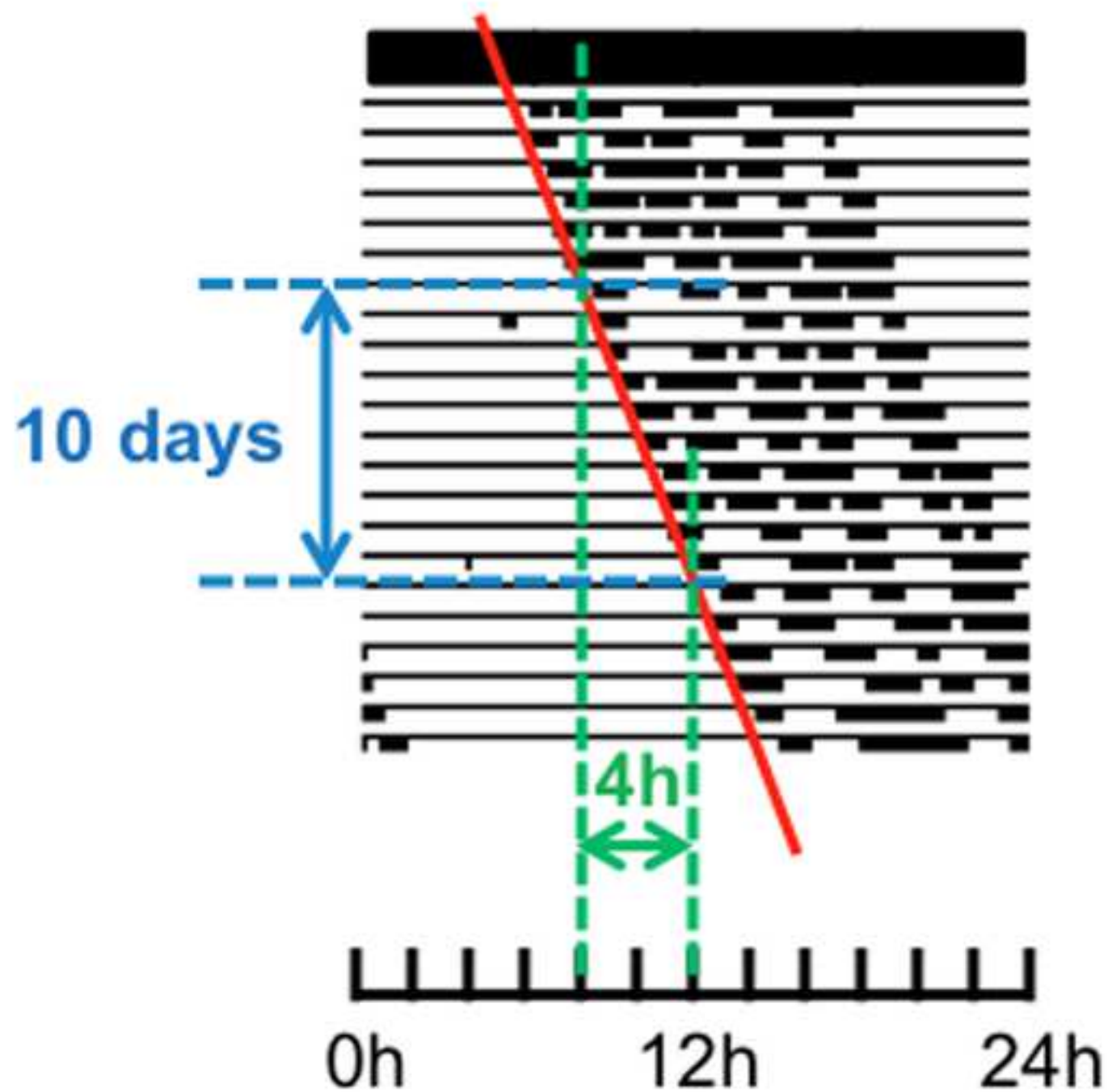
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## Vitalview hardware configuration

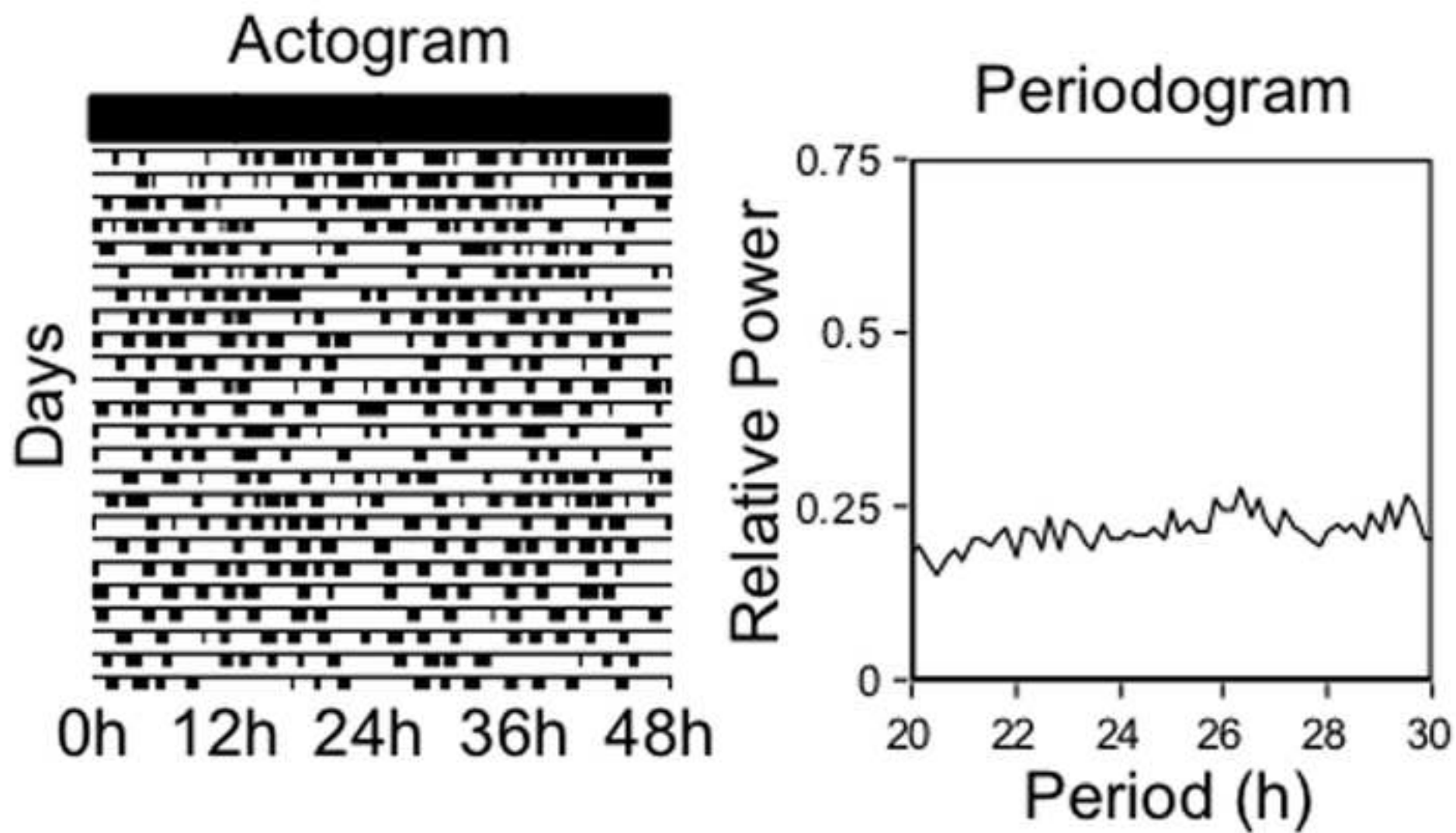




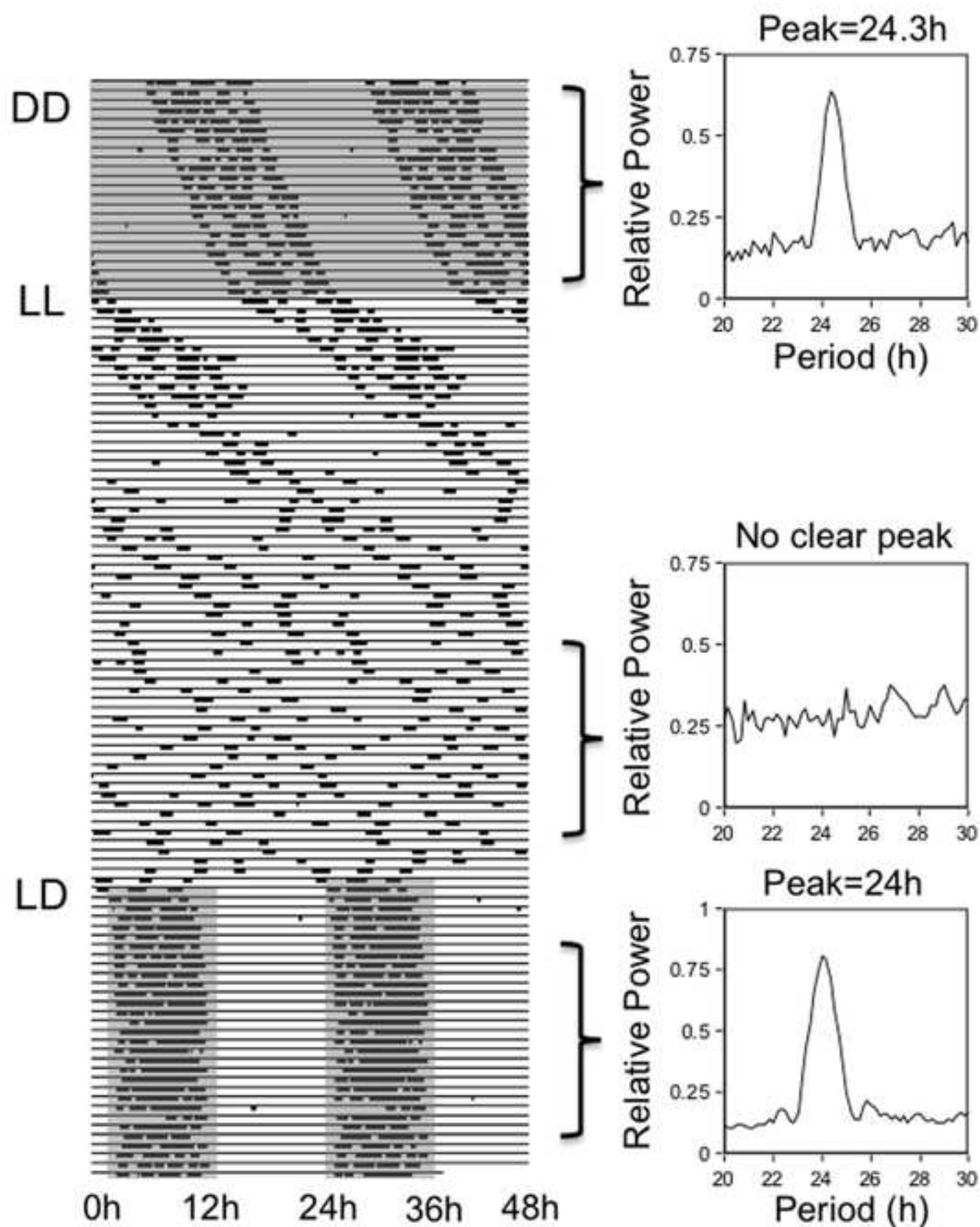
\*Figure 3  
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\*Figure 5  
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**Table of specific reagents and equipment:**

<b>Name of the reagent</b>	<b>Company</b>	<b>Catalogue number</b>	<b>Comments (optional)</b>
Vitalview Card & Software	Mini Mitter (Bend, OR, USA)	#855-0030-00	
DP24 Dataport	Mini Mitter (Bend, OR, USA)	#840-0024-00	
QA4-Module	Mini Mitter (Bend, OR, USA)	#130-0050-00	
Magnetic Switch	Mini Mitter (Bend, OR, USA)	#130-0015-00	
C-50 Cable assembly	Mini Mitter (Bend, OR, USA)	#060-0045-10	
Rat running wheel assembly	Mini Mitter (Bend, OR, USA)	#640-0700-00	
Cage and tray support	Mini Mitter (Bend, OR, USA)	#640-0400-00	
Useable cut away cage	Mini Mitter (Bend, OR, USA)	#664-2154-00	
Grid floor for cage	Mini Mitter (Bend, OR, USA)	#676-2154-00	
Waste tray	Mini Mitter (Bend, OR, USA)	#684-2154-00	

Lamp housing	Microlites Scientific (Toronto, ON, Canada)	#R-101	
4W Fluorescent lamps	Microlites Scientific (Toronto, ON, Canada)	#F4T5/CW	
Isolation chambers	Custom built	n/a	28"H x 20"W x 28"D ½" Black Melamine.



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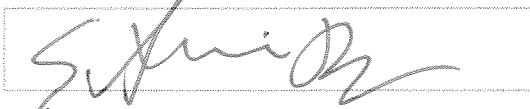
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**Response to Reviewers (Re: Manuscript by Verwey, Robinson & Amir entitled Recording and analysis of circadian rhythms in running-wheel activity in rodents; Submitted to JoVE)**

We would like to start by thanking both reviewers for their valuable feedback on this manuscript. We are confident that we have been able to address most of their concerns, and that these changes have produced many tangible improvements in the paper. These changes are detailed below, itemized according to the points of each reviewer.

**REVIEWER #1**

**Point #1-1:** It would be helpful to give at least some details on the light-tight boxes used to house the animals, as there obviously must be a certain sophistication in ventilation system to avoid light leaks. Some type of schematic would do it, just to sensitize the reader that the ventilation system needs proper consideration.

**Authors' response to point #1-1:** We have added a section entitled "Ventilation", which appears as section 1.4 in the revised manuscript and reads as follows. "Ventilation: Adequate air flow is imperative to making the boxes a comfortable home environment for rodents. The fan in each box should be hooded, so as to prevent light from outside the box from reaching the inside. Also, fans will typically remove air from the box and blow it into the room. Small light-tight vents allow air to enter the isolation boxes from several points, and help to avoid uncomfortable breezes. In order to verify that there is adequate ventilation, the temperature inside the isolation box (when closed for several hours, with the lights on) should be virtually identical to the temperature in the room where it is housed."

**Point #1-2:** The authors should mention which specific software they used to calculate the periodograms

**Authors' response to point #1-2:** We have added a section entitled "computer programs", which appears as section 1.4 in the revised manuscript and reads as follows: "Computer programs: Specialized computer programs are typically used in the generation of actograms and the calculation of circadian period. These programs include, but are not limited to, ActiView (Minimitter, Bend, OR) and Circadia."

**Point #1-3:** "The periodogram on the right confirms that this arrhythmic pattern exhibits roughly equivalent power for all circadian periods." The authors should change to something like "The periodogram on the right confirms the observed arrhythmic behavior showing equivalently low power for all periods in the circadian range (20-30hrs)"

**Authors' response to point #1-3:** This statement was changed to "The periodogram on the right confirms this arrhythmic pattern of activity by showing equivalently low power for all periods in the circadian range (20-30h)."



## **REVIEWER #2**

**Point #2-1:** When applied to the real world, some statements and strategies are oversimplified and require a better description. Specifically the strategies for analyzing data are very basic and will be useful for a baseline, however it is not explained how to analyze running-wheel activity after pharmacological or other environmental modifications. It is important to include advice and strategies for specific manipulations, specifically it will be useful to advise how to analyze behavioral changes elicited by food anticipatory activity, which is mentioned in the abstract, but is not explained further on in the text.

**Authors' response to point #2-1:** Because of the relatively brief JoVE formatting requirements, a lot of text had to be cut away from our first draft. We have limited the manuscript to the scope of this single technique and, as much as possible, avoided talking about "other" manipulations and techniques that might warrant their own JoVE articles. That being said, because of our keen interest and previous experience with restricted feeding schedules, we have added a small section on food-anticipatory activity at the end of the results section. It reads: "The amount of running, and the time of day it occurs, can also be manipulated by environmental factors. For instance, if rodents are fasted and given a temporally-restricted meal each day, this restricted feeding schedule will induce a daily bout of food-anticipatory activity. It is termed "anticipatory" because it occurs prior to the arrival of the daily meal, and is especially obvious when the meal is given in the middle of the day, a time when nocturnal rodents are relatively inactive. For instance, if an experiment provides a single 2h meal each day, food can be added to the cage at ZT 4 (4h after lights turned on) and removed at ZT 6 (2h later). Moreover, wire mesh flooring in the cages is also advantageous for this type of experiment because it makes it impossible for the rats to hide food and store it for later, thus ensuring that the rat is actually consuming all the food within the prescribed mealtime. Finally, one of the main advantages of an accurate running-wheel activity recording is that it allows for correlations to be made between running-wheel activity and daily oscillations in the expression of circadian clock gene expression throughout the brain and body."

**Point # 2-2:** ABSTRACT: Some sentences are oversimplified and suggestive; however they are not accurate and are not supported by the cited references: The statement: "running-wheel activity serves as a particularly reliable and convenient measure of the output of the master circadian clock, the suprachiasmatic nucleus (SCN) of the hypothalamus" is partially true, as indicated by the authors in lines 5-7 Page 4. There are other examples that clearly show that voluntary wheel-running can be a response to external stimuli and does not exclusively report the activity of the SCN (See Redkin and Mrosovsky 1999; Beyond the suprachiasmatic nucleus Mrosovsky 2003; and others).

**Authors' response to point #2-2:** We now mention masking directly in section 1.6 "Darkness/Dim red lighting", which now includes the sentence "The specific red light you use should be tested to ensure it does not alter running-wheel activity (e.g. negative masking) or adjust the circadian clock (e.g. produce a phase shift)." We also now mention non-photic phase shifts directly in section 1.2 "Bedding changes:" and state that "Animal handling as well as changes in cages or bedding can all have non-photic effects on circadian rhythms".

**Point #2-3:** Authors state that "circadian oscillators in many other regions of the brain and body<sup>9-14</sup> could also be involved in the regulation of daily activity rhythms" Non of the studies cited here have proven that brain or peripheral oscillators can determine daily activity rhythms; all of them have evidenced the existence of extra-SCN oscillators. Likewise references 17 to 20 have evidenced that food related cues can entrain extra-SCN oscillators, however they have not reported that such oscillators can regulate anticipatory activity.

**Authors' response to point #2-3:** This distinction is certainly correct. This is why we state that those putative oscillators "could be involved...." instead of using the more causal statement that they "are involved....". Moreover, this statement has no references because the evidence that we are aware of, like the reviewer points out, is largely correlative. References 17-20, are again correlative, and so we have changed the sentence to read "...are correlated with changes in the activity of extra-SCN oscillators 17-20"

**Point #2- 4:** In the last paragraph it is suggested that running-wheel activity can be used to study the output of extra-SCN oscillators, however further on no strategies for this analysis are provided.

**Authors' response to point #2-4:** We have added a new paragraph at the end of our results section discussing food-anticipatory activity. We now end this paragraph with: "Finally, one of the main advantages of an accurate running-wheel activity recording is that it allows for correlations to be made between running-wheel activity and daily oscillations in the expression of circadian clock gene expression throughout the brain and body."

**Point # 2-5:** 1.2 Bedding changes: a good suggestion to possibly avoid entraining signals by handling due to bedding cleaning is to perform this at random hours and to use special long lasting bedding that will allow sporadic cleaning.

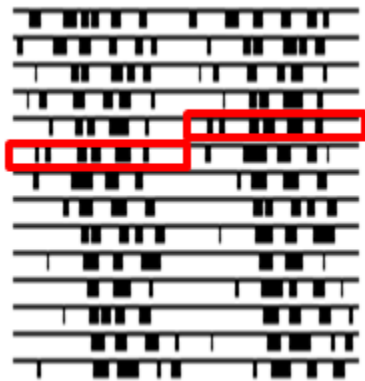
**Authors' response to point #2-5:** This section now includes a sentence that reads "Alternatives include using longer-lasting bedding, which would allow for more infrequent cage changes, or changing cages on a pseudo-random schedule."

**Point #2-6:** 2.2 Computer hardware: are there other companies providing hardware and software for running-wheel analysis? If so, they should be mentioned in order to avoid a biased advertisement.

**Authors' response to point #2-6:** Both Vitalview and Clocklab were already listed in Section 2.3 "Computer hardware: ". In the Software section we mention both Actiview (Vitalview product) and Circadia, but have also added another reference to Clock lab. So, Section 3.1 "Files" now has a sentence that reads "Such data are best visualized and graphed with specially designed programs such as Actiview (Minimitter, Bend, OR), Circadia, or Clocklab (Actimetrics, Wilmette, IL) which can all produce periodograms and actograms".

**Point #2-7:** 4.1 Actograms: the double plotted actogram represents in the same line day1 followed by day 2. The second line represents day 2 and day 3 and so on. A double plotted actogram does not "illustrate the same day twice" as stated in the manuscript.

**Authors' response to point #2-7:** Double-plotted actograms are simply two single-plotted actograms put side-by-side and offset by 1 day. The actogram on the left contains the only plot of the first day, and the actogram on the right contains the only plot of the last day. Otherwise, all of the other data is duplicated in a double-plotted actogram. This duplication of a single day is what we were referring to by "illustrate the same day twice", and is highlighted in red in the schematic below. In attempt to solve some of this confusion, we have rephrased the sentence to read "but double-plotted actograms plot two days on each horizontal line"



**Point #2-8:** 4.3 Results: (page 8 line 2) "the double plotted actogram on the left shows a rat?" needs to be rephrased: the actogram illustrates the ACTIVITY (running.wheel activity) of a rat?

**Authors' response to point #2-8:** As suggested, this sentence has been rephrased to read "In Figure 2A, the double-plotted actogram on the left shows the running-wheel activity of a rat..."