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An automated tracking system for *Caenorhabditis elegans* locomotor behavior and circadian studies application

Sergio H. Simonetta *, Diego A. Golombek

Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Buenos Aires and National Research Council (CONICET), R.S. Peña 352, B1876BXD Bernal, Buenos Aires, Argentina

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Abstract

Automation of simple behavioral patterns, such as locomotor activity, is fundamental for pharmacological and genetic screening studies. Recently, circadian behaviors in locomotor activity and stress responses were reported in the nematode *Caenorhabditis elegans*, a well-known model in genetics and developmental studies. Here we present a new method for long-term recordings of *C. elegans* (as well as other similar-sized animals) locomotor activity based on an infrared microbeam scattering. Individual nematodes were cultured in a 96-well microtiter plate; we tested L15, CeMM and *E. coli* liquid cultures in long-term activity tracking experiments, and found CeMM to be the optimal medium. Treatment with 0.2% azide caused an immediate decrease in locomotor activity as recorded with our system. In addition to the validation of the method (including hardware and software details), we report its application in chronobiological studies. Circadian rhythms in animals entrained to light–dark and constant dark conditions ($n = 48$ and 96 worms, respectively) at 16 °C, were analyzed by LS periodograms. We obtained a 24.2 ± 0.44 h period (52% of significantly rhythmic animals) in LD, and a 23.1 ± 0.40 h period (37.5% of significantly rhythmic animals) under DD. The system is automateable using microcontrollers, of low-cost construction and highly reproducible.

Keywords: Locomotor activity; Automation; *Caenorhabditis elegans*; Circadian

1. Introduction

Circadian rhythms are ubiquitous in nature and are found from bacteria and fungus to humans, exhibiting circa 24 h rhythms in most behavioral and physiological parameters. In many animal species circadian rhythms are controlled by biological clocks located in their central nervous system; moreover, most species share a common molecular clockwork mechanism which consists of a series of transcription/translation feedback loops which are consistently conserved among evolution (Dunlap et al., 1999).

Chronobiological experiments usually depend on the accurate measurement of output variables controlled by the biological clock. Indeed, wheel-running in rodents, locomotion and eclosion in *Drosophila*, conidiation in *Neurospora* or melatonin secretion in humans are examples of variables useful for understanding the underlying temporal mechanisms responsible for circadian control (Dunlap et al., 2004). Therefore, precise recordings of circadian parameters are crucial for an accurate description of the biological clock.

The use of invertebrate systems has been very successful in elucidating the *bona fide* molecular components of the circadian clock. In *Drosophila melonagaster* in particular, the development of specific hatching and locomotor tracking systems, such as the one by Trikinetics (Waltham, Massachusetts, United States), allowed the simultaneous recording of individual animals in real time. This system is useful for a wide range of experiments ranging from circadian to pharmacological, developmental and neurological studies (Cirelli et al., 2005; Dimitrijevic et al., 2004; Dzitoyeva et al., 2003; Rosato and Kyriacou, 2006; Ye et al., 2004). On the other hand, circadian studies of many small organisms are only possible by indirect measurement such as luciferase reporter, metabolite production or oxygen consumption (Dunlap and Loros, 2005; Hardeland and Poeggeler, 2003; Kondo et al., 1993), video tracking (Hirayama et al., 2005; Tanakadate et al., 1985), or even visual inspection. Multitracking of locomotor behavior for screening and high throughput studies usually involves high-cost processing systems capable

* Corresponding author. Tel.: +54 11 4365 7100x154; fax: +54 11 4365 7132.
E-mail address: ssimonetta@unq.edu.ar (S.H. Simonetta).

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of analysis of large data series, so that the development of new technologies in the area might be useful in overcoming these limitations.

The nematode *Caenorhabditis elegans* is one of the best-known models for genetic and developmental experiments (Hodgkin, 2005). In addition, since neural connections are extensively mapped, it is also used for the study of the neurobiological basis of behavior. The relative lack of circadian rhythms studies in this nematode responds, at least in part, to the absence of an accurate recording system for locomotor behavior which could allow for long-term, robust recordings, necessary for genetic screenings. Previous reports have used video-tracking and eye inspection recordings (Kippert et al., 2002; Saigusa et al., 2002) but they were not applicable for high throughput studies.

The aim of the current work was the design of a system capable of tracking locomotor activity of *C. elegans* and similarly sized organisms in real time, with potential applications in circadian rhythms and genetic screenings. The approach was based on an infrared microbeam system that detects light refraction through the animal body. Digital signal processing enables the system to perform long-term recordings of the locomotor activity state of the worm, with at least 1-min temporal resolution. Along with the mechanical device, data acquisition, animal culture conditions, and signal conditioning were determined. The method was tested in a long-term study of *C. elegans* circadian behavior.

2. Materials and methods

2.1. General description

The locomotor activity recording system counts infrared photo-beam interruptions ("bins") in a fixed time lapse. Our system senses transient analogical changes in order to detect how individual worms move across a microbeam of infrared light. Worms were cultured in 96-well microtiter plates (U shape), and locomotor activity was recorded for more than 2 weeks, under different experimental conditions. Data was analyzed and processed to study circadian activity rhythms, and plotted as actograms.

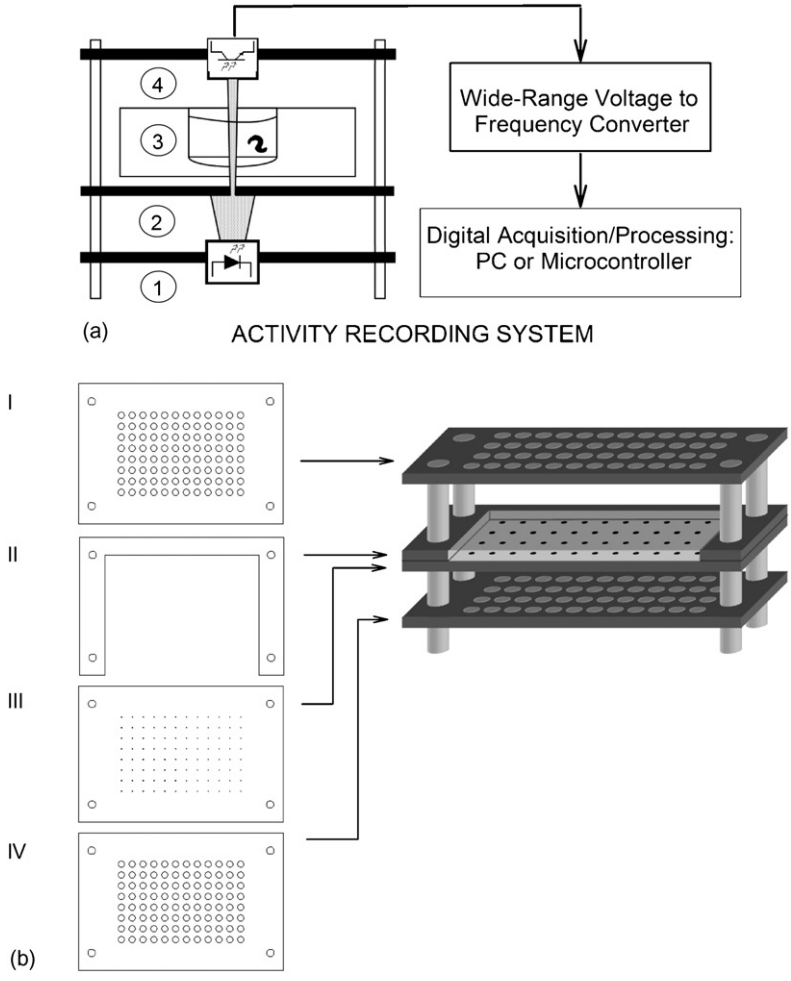

Fig. 1. Detection of worm locomotion. (a) Schematic diagram of the actometer: (1) bottom acrylic plate, where the infrared led is located; (2) middle plate, with 100 μm microholes for the light microbeam; (3) worm liquid culture receptacle; (4) top plate, where the phototransistor is located. The analogical output of the phototransistor is converted to a direct proportional (linear) frequency and this frequency is captured by a PC sound board or microcontroller for digital processing. (b) Mechanical design and assembling scheme. *Left*: diagram of the acrylic plates. From top to bottom: (I) plate for phototransistor mounting, with 5 mm holes; (II) guiding plate; (III) plate with microholes; (IV) plate for infrared diodes mounting. *Right*: scheme of the assembled apparatus.

2.2. Locomotor activity tracking system

A tracking system was designed as shown in Fig. 1a. The detection of activity is based on infrared microbeam interruptions. This microbeam crosses a microhole array within an acrylic filter, passes through the worm receptacle and is finally sensed by a phototransistor. When worms move across the light beam, a transient fluctuation in the signal received by the phototransistor is generated; movement is detected by digital analysis of the phototransistor output.

2.2.1. Mechanical elements

Ninety-six holes were perforated in three black acrylic plates (165 mm × 110 mm × 2 mm) with a laser system so that the holes would correspond with 96-well microtiter plates. The top and bottom black plates were cut with 5.0 mm diameter holes, while the middle plate was cut using the minimum laser diameter (approximately 100 μm). All plates were also perforated at the four corners for fixation with 5 mm screws. The distance between the plates was of 10 mm from bottom to middle and 22 mm from middle to top. A fourth plate was inserted in the middle as a guide to insert the microtiter plate in the correct position. Fig. 1b presents a diagram of the plates and the device.

2.2.2. Signal transducers and analog to digital (A/D) conversion

For each well we used one 940 nm infrared (IR)-diode (P_{max} = 1 mW/cm^2, model TLN105A Toshiba Semiconductor or equivalent) and one phototransistor (940 nm with white-light filter, model SFH300FA Siemens Semiconductor or equivalent) inserted into the opposite holes of the bottom and top acrylic plates. These kind of emitter/receiver diodes are normally used for TV remote control devices, and are easily available.

The amplification and analog-to-digital conversion of the phototransistor output was performed in a single step with a linear wide-range voltage-to-frequency converter (LM331 National Semiconductor) as described in the integrated circuit datasheet (http://www.national.com/pf/LM/LM231.html, application number DS005680-9), obtaining a frequency signal which was proportional to light detection within a range of 0 Hz (darkness) to 100 kHz (bright light). The power of the IR-diode was regulated with resistors in order to obtain a frequency output in the converter in the audible range (500–1000 Hz) when no movement was detected through the microbeam (the system was calibrated before inserting the microtiter plates). We got a frequency range which resulted low enough as to sample with low resolution PC sound cards (11 kilo samples per second (Ksps)) or with low range microcontrollers (e.g., PIC16 Microchip^{TM} series).

2.2.3. Data acquisition

The frequency output of the A/D converter was acquired with a standard PC audio board at 11 Ksps. This output data was multiplexed and sampled at 20 s for audio recording plus 10 s for audio processing for each channel per minute. With a stereo input the system allows for the recording of four channels per PC soundcard.

We programmed a simple algorithm to convert frequency changes into activity events (Visual Basic Source code algorithm in supplementary data) as described below. When there is no movement through the microbeam the period of the audio signal is constant. However, the basal signal, dependent of the optical density of the microbeam pathway, could change as a consequence of different culture conditions, medium consumption, products of metabolism, evaporation rate, etc. We removed the basal audio period value using a direct current (DC) blocker filter (http://ccrma.stanford.edu/~jos/filters/DC_Blocker.html) consisting in a one-pole infinite impulse response filter (IIR). In brief, the mathematical calculation for this filter is

$$Y_n = X_n - X_{n-1} + RY_{n-1}$$

being X_n is the actual period value, X_{n-1} the last period value, R the parameter of the transfer function (we fixed R at 0.95), Y_n the actual function output value, and Y_{n-1} is the last function output value.

If the value of the filtered audio signal period results higher than a threshold determined empirically as the 6% of the last period value (X_{n-1}) (which is enough to detect movement, but not for noise interference), then the activity event is valid and an activity counter is incremented.

This algorithm is repeated every time a peak of the sine audio signal is detected, until the acquisition lapse time of the "bin" concludes. Fig. 2 shows an example of data conversion. An implementation of the algorithm is described in supplementary data.

2.3. Software

We developed two specific computer programs; one for data acquisition, and the other for data processing and analysis. The acquisition program allows saving 20 s of raw audio data in the memory, which is converted into locomotor activity bins and saved in ASCII format.

The second program reads the ASCII data, which is transformed into a user-defined bin lapse (we chose a 30 min bin size). This data is now ready for actogram plotting or data filtering (the software is available at http://cronos.unq.edu.ar/simonetta.htm).

Converted ASCII data was analyzed for circadian rhythmicity with χ^2, Lomb–Scargle and autocorrelation periodograms.

2.4. Experimental testing

2.4.1. Culture conditions

All animals used in these experiments were of the species *C. elegans*, N2 Bristol wild type strain. Worms were synchronized to the same developmental stage by the chloride method and cultured in NGM medium with thick bacterial lawns of *E. coli* OP50 strain (Brenner, 1974). Stock plates were maintained and grown at 20 °C. At L4 stage, one hermaphrodite was picked to a well of a 96 microtiter plate with 50 μl of CeMM liquid medium (Cellgro, generously donated by N.J. Szewczyk, University of Pittsburgh), plus 25 μM fluorodeoxyuridine (FUdR) to prevent self-reproduction and 1× Antibiotic/Antimicotic (AB) (Gibco, Rockville, MD). The well was sealed with tape to prevent desiccation and two small holes were perforated in the border with a

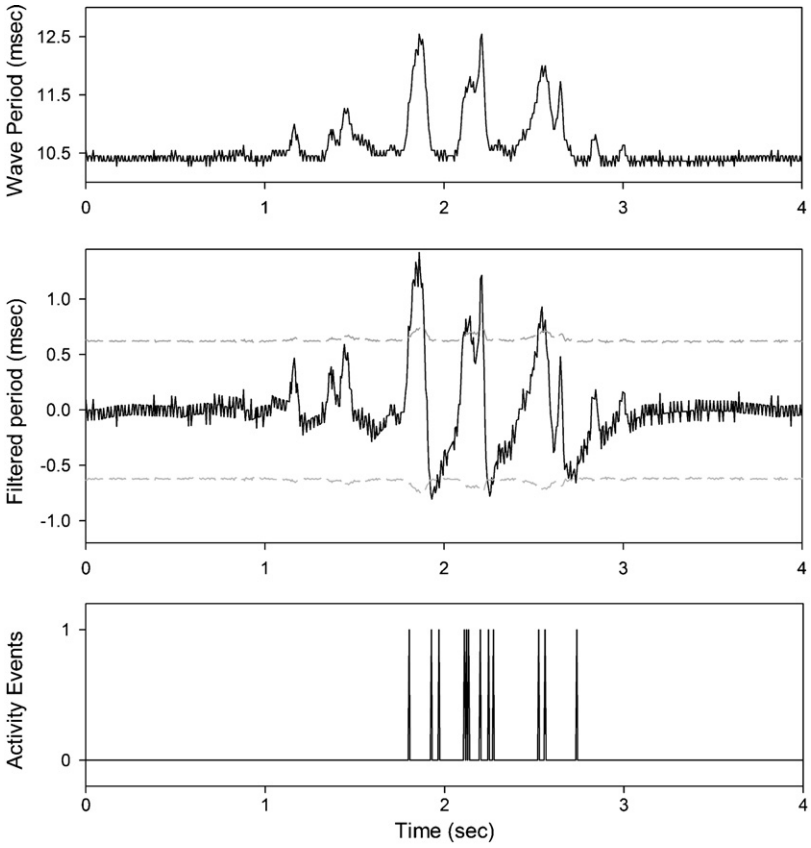

Fig. 2. Treatment of frequency output for detection of locomotor activity. Top panel: raw wave period, showing the fluctuation in the signal when a worm is passing through. Middle panel: filtered period, obtained by removal of basal signal through a high-pass filter (IIR dc blocker). If the transient variation of the filtered signal passes a threshold of 6% of the period (dashed line), then locomotor activity is computed. Bottom panel: result of the activity detection protocol (measured as locomotor activity events).

needle to allow for oxygen interchange and to prevent water condensation, as described (Iijima-Ando and Yin, 2005). If contamination and culture medium evaporation are avoided, it is possible to register worm locomotor activity for more than 21 days.

2.4.2. Video tracking recordings

In order to determine whether the animals moved adequately in the wells and caused interruptions of the light microbeams, we videotracked the swimming behavior of the animals before performing the tests in our data acquisition system. For video recording, 10 worms were transferred to a 96-well microtiter plate and placed on the acrylic plate with microholes (as indicated before). A standard red laser pointer (<1 mW power) regulated to the minimal intensity was fixed in a plastic basement below this plate. A videocamera (MOTICAM 2000, Motic Instruments Inc., BC, Canada) was mounted on a binocular microscope (OLYMPUS SZ30) and images were recorded at a rate of 15 frames per second with a 640 × 480 pixel resolution. Videos were converted to grayscale and the pixel intensity at the light spot was analyzed using ImageJ NIH processing software (Bethesda, MD).

2.4.3. Digital recordings

The microtiter plates in the acquisition system were placed in an anti-vibration incubator (whose refrigerator motor was separated) at 16.0 °C. Stability of the incubator temperature was checked with an IButton sensor model DS1921H-F5 (Maxim Integrated Products, Inc. Sunnyvale, CA), programmed to take one sample every 5 min. This sensor provides a resolution of 0.125 °C.

As a negative control, 1–10 worms were picked per well as described before, and after 2 h they were treated with 0.2% azide to stop the respiratory chain.

In order to determine whether the medium influenced locomotor activity recordings, worms were picked from agar into CeMM, Leibovitz L-15 (Saigusa et al., 2002) or S basal medium + *E. coli* OP50 strain (OD600 = 1.0) (Fabian and Johnson, 1994). Total activity levels were calculated daily.

For circadian rhythms analysis we recorded one worm per well. Animals were subjected to light–dark cycles (LD 12:12, 400 lx) for 8 days, followed by continuous darkness (DD).

2.4.4. Time-series filtering and circadian analysis

The presence of an unstable or dampened baseline, high frequency noise and different basal levels of individual locomotor activity patterns led us to prepare the data for posterior analysis. Data was first detrended as described previously (Levine et al., 2002). In brief, detrending and normalization were accomplished by dividing each data point by the corresponding point of a trend curve (fitted by a 72 h low-pass Butterworth filter). Since

direct elimination of high frequencies through the use of low pass filters may change the overall pattern of circadian activity and mask useful information we used a different approach by superimposing the low-pass filtered data on the detrended raw data.

With this purpose, high frequency values were removed by a 4 h low-pass Butterworth filter. The above-average values of this data series were smoothed with a 3 h point moving average (Saigusa et al., 2002). These data represent the time block when worms move more actively. We combined the filtered data with the original detrended set accepting as valid the values with a filtered activity greater than zero (equivalent to a logical binary "AND" function). This procedure yielded clear actograms which were useful for both visual inspection and periodogram analysis. Long-term data recordings were analyzed with Lomb–Scargle and χ^2 periodograms. Autocorrelation analysis (Dowse and Ringo, 1994) was applied on the 3 h moving average data. In order to control for potential artifacts due to analytical manipulation, the original data were randomly shuffled twice and subjected to the same filters and analysis.

Activity recordings under light–dark (LD, $n = 48$) and constant dark (DD, $n = 96$) conditions were filtered and analyzed as described before. The means and standard errors for period scores and the percentage of animals exhibiting a significant circadian rhythm were calculated.

3. Results

3.1. Detection of worm locomotion

Although the nematode *C. elegans* is transparent, it is still possible to detect its movement under a dissecting microscope with a bright background. Indeed, a swimming worm passing through a photic microbeam causes a light scattering which is possible to detect with a CCD camera, as shown in the light spot quantification of the video frames by ImageJ (Supplemmentary Fig. 1, Suppl. Movie 1)

This same movement recording can be automated by our digital tracking system. As described in Section 2, the output of the phototransistor (Fig. 1a) was converted to frequency and recorded in a PC. Digital analysis of these frequency changes (proportional to light intensity) allowed us to detect the movement of the worm passing through the light beam. When an animal appears near (or passes through) the light beam, the intensity of light received by the sensor is decreased, and is transduced as an increment in the audio signal period (Fig. 2, top panel). Moreover, if the basal signal (obtained when the worm is not moving through the light microbeam) is digitally removed, then the output signal represents the transient changes corresponding to worm movement (Fig. 2, middle panel). The bottom panel of Fig. 2 shows the detection of activity events when transient variations in locomotion cross the activity detection threshold.

As shown in Fig. 3a, activity signals ceased completely after azide administration, both in single and 10-worm recordings. We also investigated the optimal culture conditions for activity detection. The use of L15 medium or S basal medium plus *E. coli* appears to be suitable for short-term recordings; however, in long-term experiments (i.e., more than 10 days) activity levels decreased to less than 30% with respect to the first day of recording. This decrease was avoided by using CeMM medium for liquid culture (Fig. 3b). Taking into account that this nematode is hermaphrodite, if no FUdR (a drug that inhibits DNA replication) is added to the medium, larvae are born, which causes a significant increase in total activity levels (Fig. 3b).

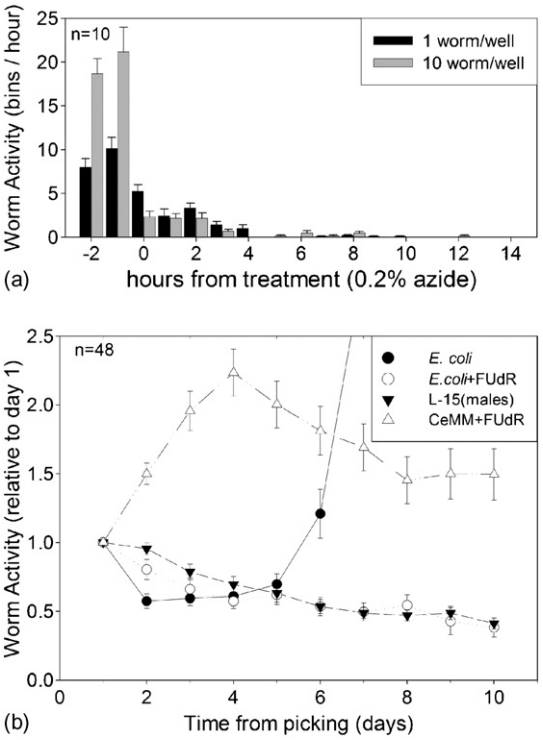

Fig. 3. (a) Negative control of locomotor activity. One or 10 worms per well were cultured in liquid medium and after 2 h they were treated with 0.2% azide. The plot shows the decay of worm locomotor activity after azide treatment. Zero hours represents the activity average of the first hour after treatment. (b) Culture medium determination. One worm (N2 strain) per well was cultured with 50 μl of S basal medium plus *E. coli* (OP50 strain $OD_{600} = 1$) with or without FUdR, Leibovitz L-15 or CeMM medium for 10 days. The activity in bins/day was calculated for each worm and the average ± S.E.M. of 48 worms was plotted for each culture condition. Locomotor activity was increased by larvae born in OP50 without FUdR, and the best culture medium was CeMM.

4. Circadian studies

Visual inspection or periodogram analysis of raw data corresponding to long-term records of worm locomotor activity under LD or DD cycles did not reveal clear circadian rhythms, although trends towards cycles of activity and rest were evident. Indeed, high basal levels of activity and sharp spikes in the apparent phases of rest make the interpretation of this data difficult. However, the preconditioning of the records by several layers of filtering (i.e., superimposition of detrending and low-pass filtering) gives a clear plot for the detection and detailed analysis of circadian rhythms (Fig. 4a). Random data shuffling eliminated circadian rhythmicity (only 2% of 96 shuffled actograms showed a significant rhythm as analyzed by LS periodogram or visual inspection). It is important to state that data filtering did not alter

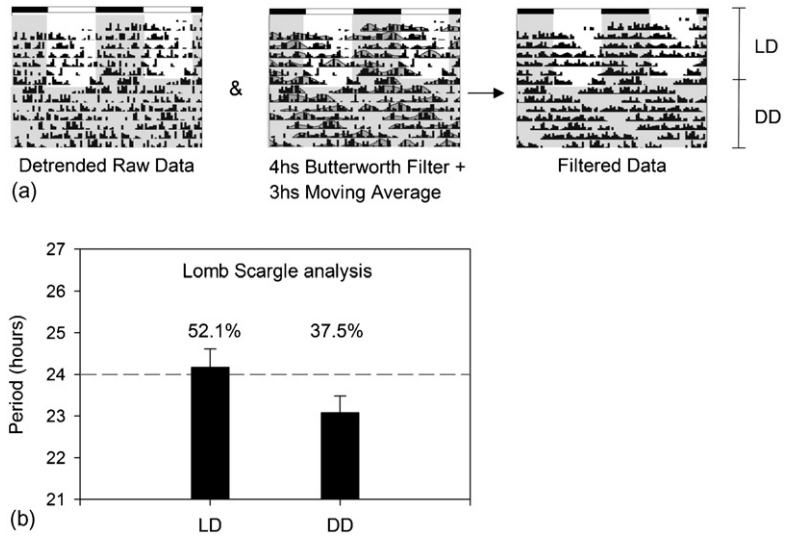

Fig. 4. (a) Data filtering for circadian analysis. Double-plotted actograms represent 48 h per line; each subsequent 48 h cycle is plotted beneath the previous one. Gray shadows indicate darkness conditions (initial photoperiod is depicted by black and white bars at the top of each actogram). Left panel: long-time raw locomotor activity detrended using a LP Butterworth 72 h filter normalization. Middle panel: treatment of detrended data with low pass filter. Right panel: combination of detrended AND filtered data ("&" represents a logical binary "AND" function through which values with a filtered activity greater than zero are accepted as valid). This data was used to evaluate circadian behavior through periodograms and visual inspection. (b) Circadian period under LD and DD conditions. One worm per well was cultured in 96-well microtiter plates with 50 μl CeMM + 25 μM FUdR + 1× antibiotic–antimicotic. Animals were entrained to LD (12:12, 400:00 lx) or DD conditions. Long-time recordings were subjected to pre-filtering as described above. The filtered data was analyzed by LS periodograms. Average circadian periods ± S.E.M. are shown. The percent of animals with statistically significant circadian rhythms according to LS periodogram is indicated on top of each bar.

the spectrum of frequencies in the circadian range (Suppl. Fig. 2).

We calculated circadian parameters for worms entrained to an LD cycle (400 lx direct light, 40 lx indirect light beneath the recording chamber) or under constant dark conditions, by applying autocorrelation, χ^2 and Lomb–Scargle periodograms. The LS periodogram was the best method for statistical analysis of filtered data, and correlated with visual inspection of the actograms. As shown in Fig. 4b, at least 52% of worms in LD and 37.5% of animals in DD showed significant circadian rhythms. Visual inspection of actograms by trained observers blind to experimental conditions indicated a 56% of rhythmic animals under LD and 42% under DD. The average period for locomotor activity was 24.2 ± 0.44 h in LD and 23.1 ± 0.40 h in DD (individual animals under constant dark conditions exhibited a wider distribution of periods, ranging from 21 to 25 h).

χ^2 periodogram analysis yielded results that were not compatible with visual inspection of the actograms (less than 5% rhythmic animals), while autocorrelation was found to be particularly unsensitive to randomization, with statistically significant peaks evident in the autocorrelograms even after data shuffling (Suppl. Fig. 2).

5. Discussion

The nematode *C. elegans* has become a widely studied model for many behaviors controlled by the nervous system. However, long-term records of many individuals have been difficult to obtain and have been performed mainly through visual inspection (e.g., in aging studies) or by filming the behavior of individual worms (as in quantitative analysis of mutant phenotypes) (Baek et al., 2002).

Previous reports indicate the existence of circadian rhythms in *C. elegans* (Kippert et al., 2002; Saigusa et al., 2002), assessed by video tracking or visual inspection. Notwithstanding, these methods are not suitable for the recording of the number of individuals usually used for circadian studies (e.g., $n = 20$ to over 60 in *Drosophila* studies) (Rosato and Kyriacou, 2006). Therefore, no screening protocols have been performed to study putative circadian mutants in this model.

Light beam interruptions of *C. elegans* have only been studied in agar media in order to determine laser heat avoidance (Wittenburg and Baumeister, 1999). However, it is remarkable that there is no data regarding light scattering by worms and its possible applications.

In this paper we report the development of a new method to track locomotor activity of *C. elegans*, or other organisms with similar size. This method is suitable for circadian locomotion recording, effect of toxic compounds and research in aging mechanisms, with the possibility of high-throughput automation.

Movement detection was performed by sensing the changes in light microbeams, followed by amplification and frequency conversion of this signal. Data was acquired and processed digitally in order to detect transient changes caused by the worm passing through the microbeam.

Since the detected light goes across a liquid medium culture, changes in the absorbance characteristics of the medium could interfere with the measurements, so that system calibration would be necessary for every experiment. However, we avoided this problem through the application of a dc blocker digital filter (a special version of a high-pass IIR digital filter), allowing us to analyze just transient (fast) changes in the signal to detect locomotor activity.

An alternative acquisition method could take advantage of analog to digital converters (ADC) PC cards without the need for a voltage to frequency converter; however, an amplification step previous to data acquisition is still needed (as stated in methods, in our system we have performed this amplification with the voltage-to-frequency converter integrated circuit LM331). Although we have used sound cards successfully for the acquisition of locomotor activity data, digital microcontrollers or multichannel ADC cards might perfectly serve the same function when monitoring several worms simultaneously through the same PC. Moreover, digitalization of data allows the use of electronic systems based on low-range microcontrollers (e.g., PIC microchip series), without the need of more costly and complex analogical high resolution inputs, making it possible to emulate the actual kind of locomotor systems used in *Drosophila* laboratories.

We could detect microbeam light scattering as a transient variation in the light signal sensed by a phototransistor (as shown in Fig. 2). Also, microbeam light scattering is easily detected by a video camera (as appreciated in Supplementary Fig. 1 and Suppl. Movie 1). However, we obtained different light variation patterns in both cases. Infrared photobeam interruptions induce a decrease in the phototransistor signal variation, while the appearance of an animal near the light spot from the laser increases the light intensity at the videocamera detector. This apparent contradiction could be explained by the fact that, taking into account that laser energy received by CCD videocamera has a saturating value, since the incident light on the moving worm is diffused (by a combination of scattering and refraction), the camera will sense an increment in the number of pixels detecting the laser light (quantified as signal increment) each time the animal moves through the beam. On the contrary, the phototransistor detection system is not under a saturated level, so that when the worms interrupt infrared beams, this light interruption or scattering is interpreted as a reduction in the signal.

A note of caution must be called regarding the avoidance of contamination in the culture plates. Since axenic culture medium CeMM is used, special care has to be taken at the moment of picking the worms. As we have described, AB and FUdR addition plus the use of very clean stocks partially prevent this issue but it is still possible for the cultures to become contaminated by resistant fungus or bacterial strains.

Another drawback is the possible desiccation of the culture media. The desiccation rate of the wells is higher than 0.5 mg/(cm^2 h) and is difficult to prevent (see http://www.labcor.com/techinfo/print.asp?htmlfile=384-wellplate.htm). We have partially solved this problem by using at least 50 μl of medium and adhesive plastic sheets to seal the plates. However, we occasionally found a significant decrease (up to 50%) in the volume of the culture medium. This problem is of particular importance when recordings are performed for more than 15 days.

The system is also highly sensitive to mechanical vibration. Since worm movement is detected through scattering of the light beam, when the setup is subjected to mechanical vibrations it may cause false positive activity values. We have prevented this problem by performing recordings in a quiet and isolated room, or even better, inside an incubator, and also by using styrofoam for setup attachment. Since there is absolutely no activity detected by the system when no worms are picked into the well, we conclude that the potential movement of the liquid culture medium does not affect light refraction (indeed, liquid vibration could be dampened by superficial tension or, alternatively, light intensity fluctuation in this control situation is underneath the detection threshold).

In most circadian experiments (including real time recording of luciferase activity as a reporter for rhythmic gene expression), adequate data filtering is needed in order to visualize and analyze rhythmicity. Indeed, two of the main applications for digital filters are locomotor activity moving average and the de-trending of data in the analysis of luciferase activity rhythms (due to the progressive depletion of the luciferin substrate).

Our results indicate that the LS periodogram analysis was the best suited for our studies. This type of analysis is commonly used in *Drosophila* research, along with the χ^2 periodogram. Notably, autocorrelogram analysis did not yield consistent results, since many significant peaks were found after data shuffling which do not allow the automatic determination of a "real" periodicity (see Levine et al., 2002). In addition, other methods might be applied for the automatic detection of significant rhythmicity. For example, Rosato and Kyriacou (2006) have recently reported the use of the "CLEAN" program (developed for astronomical research) for circadian studies, which might be useful in the further analysis of worm locomotor activity records. Indeed, as stated by Klemfuss and Clopton (1993) the best way to estimate circadian parameters depends on the nature of the data and on research expectations, so it might be useful to test a range of available methods for analysis, which is outside the scope of the present paper.

Ongoing studies in our laboratory are aimed to determine whether other environmental entraining stimuli (*zeitgeber*) (such as temperature cycles) are capable of inducing stronger rhythms in terms of synchronization and amplitude.

In summary, our system is optimal for long-term recordings of circadian locomotor activity rhythms in the nematode *C. elegans*, allowing the simultaneous assessment of a large number of worms and, therefore, screening protocols for putative mutations that disrupt the molecular clock in this species, whose location and mechanism is presently unknown. The use of such a powerful animal model in terms of the knowledge of its genetics and developmental characteristics will certainly be useful not only for chronobiologists looking for common features in the circadian clock but also to neuroscientists in need for a robust and reliable system for the recording of behavior in very small animals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jneumeth.2006.11.015.

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