Video tracking and analysis of sleep in *Drosophila melanogaster*

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Published online 26 April 2012; doi:10.1038/nprot.2012.041

In the past decade, *Drosophila* has emerged as an ideal model organism for studying the genetic components of sleep as well as its regulation and functions. In fruit flies, sleep can be conveniently estimated by measuring the locomotor activity of the flies using techniques and instruments adapted from the field of circadian behavior. However, proper analysis of sleep requires degrees of spatial and temporal resolution higher than is needed by circadian scientists, as well as different algorithms and software for data analysis. Here I describe how to perform sleep experiments in flies using techniques and software (pySolo and pySolo-Video) previously developed in my laboratory. I focus on computer-assisted video tracking to monitor fly activity. I explain how to plan a sleep analysis experiment that covers the basic aspects of sleep, how to prepare the necessary equipment and how to analyze the data. By using this protocol, a typical sleep analysis experiment can be completed in 5–7 d.

INTRODUCTION

Overview

D. melanogaster locomotor activity follows a tight circadian regulation: fruit flies are most active in the early hours of the morning and at dusk, and less active in between, with prolonged nearly continuous inactivity at night. This so-called crepuscular pattern of activity has been known since the early days—the very name *Drosophila* is Greek for 'lover of the dew'—and the first systemic reports about the alternation of periods of activity and quiescence in adult *Drosophila* date back to 1956 (ref. 1). However, despite a long-standing interest in the circadian regulation of *Drosophila* activity, little was known about the biological characteristics of the mainly nocturnal resting state until the year 2000, when two independent laboratories showed that the prolonged inactivity observed in *Drosophila* fully satisfies the five behavioral characteristics that define a sleep-like status[2]: (i) consolidated circadian periods of immobility, (ii) a species-specific posture and/or resting place, (iii) an increased arousal threshold, (iv) reversibility to wakefulness and (v) a homeostatic regulatory mechanism[3,4]. We now know that sleep loss has similar detrimental effects on flies as it has on mammals[5,6] and that social experience modulates sleep requirements in *Drosophila*, as it does in vertebrates[7,8]. Conservation of sleep from humans to flies extends beyond behavior to pharmacology (e.g., caffeine and amphetamines promote wakefulness[3,4], whereas sedatives like hydroxyzine promote sleep[4]) and flies, similarly to vertebrates, show distinct electrophysiological correlates of wakefulness and sleep in the brain neuronal activity[9]. Together, these findings show an evolutionary conservation between *Drosophila* and human sleep at the behavioral and molecular levels, making flies an ideal system for studying the mysterious functions of sleep and all its connections to pathological conditions in humans.

Because sleep is associated with sustained physical inactivity, the easiest way to record sleep in flies is to measure their locomotor behavior. Traditionally, this has been done using tools that are already used to measure circadian rhythms, such as by detecting when a single fly breaks an infrared light beam transecting the midline of a small recording chamber[10] (**Fig. 1**). Conceptually, this system dates back to the 1970s when Benzer[11] adopted it in his mutagenesis screen for flies with defective circadian rhythms and for the past 40 years this approach has indeed proved efficient for circadian research[12]. Infrared-based monitoring of *Drosophila* locomotor activity does, however, carry drawbacks when applied to sleep: above all, low spatial resolution and the inability to record interacting flies. The former is particularly important, as detecting movements only when the flies are crossing the midline has been reported to strongly overestimate sleep in some conditions[13]. Here I recommend using an automated motion-tracking system based on computer vision analysis, as it offers important advantages over traditional systems (see **Table 1** and below). Automatic analysis of behavior is an exciting emerging field in *Drosophila* neuroscience, and several systems for computer-assisted monitoring of sophisticated behaviors are currently available (**Supplementary Table 1**). In this protocol I concentrate on a system developed by our laboratory that was expressly designed to be used for sleep detection and analysis; it carries specific features that other software with different aims do not provide. pySolo[14] is a software program for data analysis that is characterized by an easy-to-use, powerful interface; its current alternative would be to analyze and plot data using elaborate spreadsheets or scripts, such as Excel (Microsoft) macros and MATLAB (MathWorks) scripts (**Supplementary Table 1**). pySolo-Video translates video-detected motion into activity data: it is integrated with pySolo, and it is highly scalable and relies on inexpensive hardware components.

Comparing video tracking and infrared beam split

Differences between video tracking and the traditional system are summarized in **Table 1**. The main advantages of video tracking over infrared beam-split monitors are (i) a more faithful read out of locomotor activity and, consequently of sleep, (ii) very affordable costs, (iii) freedom in choosing the recording environment and (iv) the added value of the data recorded. The increase in spatial and temporal resolution of video tracking over the beam-split system is not due to an intrinsic weakness of the latter, but merely a consequence of the fact that traditional infrared monitors rely on only one beam per tube. In the single-beam system, the infrared sensor intersects the midpoint of the walking chamber and movements can be detected only if the fly is walking across (**Fig. 1a**). When the

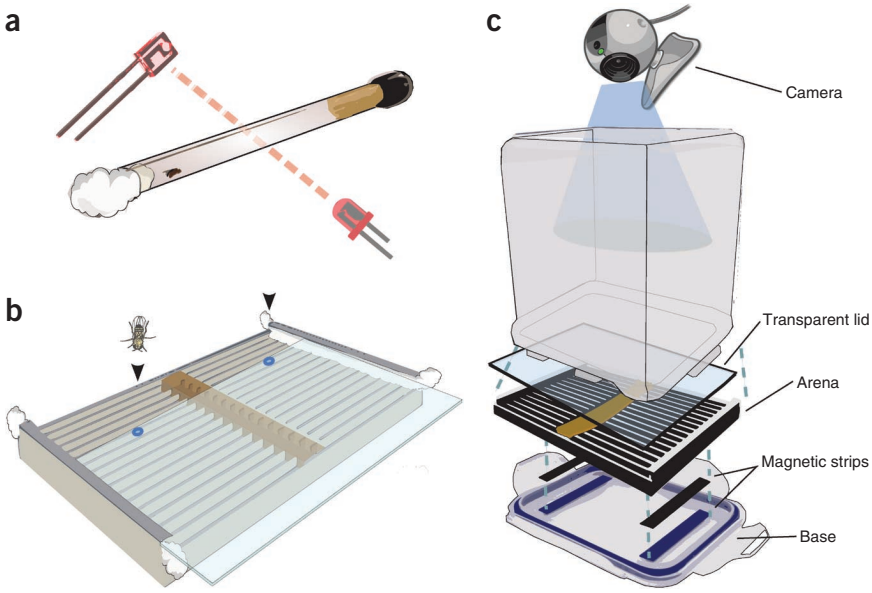

Figure 1 | Principles of locomotor detection. (**a**) Infrared beam-split measurement. A single fly is hosted in a glass tube, secured between food and cotton. A beam of infrared light crosses the midline of the tube (dashed red line) and a collector connected to a computer can record and count whenever the fly is breaking the beam by passing through it. If the fly moves away from the beam or if the fly sits right in the middle of it, too little or too much movement may be detected. (**b**) Arena for video recording. Arenas of different shapes and complexities can be produced using 3D printing technology. The cartoon in this figure shows an arena hosting up to 32 flies, which are housed in parallel walking grooves covered with a lid of transparent plastic. On one end, flies are in contact with cotton and on the other end with food. Flies can be transferred to the arena either sedated or unsedated; in the latter case, a mouth pipette is used to blow single flies through holes in a transferring lid (blue circles). An STL file ready to be used for 3D printing is provided as **Supplementary Data** (see MATERIALS). (**c**) An expanded view of a video recording chamber. A food-grade plastic container can conveniently house an arena similar to the one depicted in **b**. The arena is secured to the inner side of the lid through a pair of adhesive magnetic strips. A sheet of clear plastic slides onto the top of the arena and keeps the flies from escaping. The camera looks at the flies through a hole in the opposite side of the container. Lighting can be provided either externally or with LEDs placed inside the container (not shown). A chamber built in this way will record flies in a closed environment and will be resistant to shaking and manipulations.

fly wanders on one side of the chamber, no movement is detected and sleep may be overestimated (Zimmerman *et al.*[13] and G.F.G., unpublished data). To overcome this drawback, new monitors were recently commercialized that detect activity using 3, 9 and up to 16 infrared beams per tube (LAM and MB5, TriKinetics). Data from these new high-resolution monitors are not yet available in the scientific literature, but it is easy to predict how an increase in spatial resolution will result in a more faithful readout. Video tracking, however, still offers a much higher level of resolution (an arena of 110 × 65 mm imaged at 640 × 480 pixels corresponds to a definition of less than 0.2 mm). Difference in costs between the two systems will vary according to the number of monitors required and the level of resolution desired. A system of 30 video-tracking monitors will cost around $3,000, whereas the infrared split-beam alternative may range between $15,000 and $100,000, depending on the desired spatial resolution. The price difference is modest for a small installation but considerably more relevant for a bigger setup (**Supplementary Table 2**). However, the economic gain of adopting video tracking comes at the expense of time investment: commercially available infrared beam-split monitors (TriKinetics) were engineered to be ready to use. The only assembly required is the connection of the recording monitors to the computer, which can be successfully done in a few hours of work or less[15]. The system for video tracking described here, on the other hand, requires the experimenter to assemble, test and adapt all the hardware equipment, an effort that may require days, depending on the experience and ability of the experimenter. A final important advantage of video tracking over infrared beam-split monitors is the ability to host and record flies in an environment different from the traditional glass cuvette. Owing to engineering design, beam-split recording must be performed in sealed vials of limited size (**Fig. 1a**); video tracking, on the other hand, can detect movement independently of the size and complexity of the environment in which the fly is located, allowing the researcher to adopt arenas that are more convenient to use—like the ones described in this protocol—or that are suitable for complex experiments. For instance, video tracking is commonly used to monitor flies interacting with each other in an open environment, and it is technically possible to detect activity and sleep of several flies walking freely in a Petri dish. However, unequivocally resolving the identity of single flies over prolonged times is still not possible using computer vision and experiments of this type should either be treated as 'population readings' (rather than 'individual readings') or else be performed by marking flies beforehand with paints of different colors. Finally, video tracking provides richer data: by analyzing the Cartesian coordinates of a fly at any given time, the researcher can obtain information on a number of kinematic aspects such as speed, directions, preference in local positions.

Experimental workflow

A typical sleep experiment consists of three phases: a preparative phase; a recording phase; and a data analysis phase (**Fig. 2**). In the preparative phase, flies are prepared for the experiment, with stocks or crosses expanded as required. At the appropriate age, flies are transferred to the arena that will host them throughout the recording and provide them with the necessary nutrients. Recording typically lasts 5–7 d and requires little or no human intervention. To score their baseline sleep needs, flies are first monitored in a controlled environment for two or three consecutive days; sleep deprivation can then be performed, followed by a third phase of recovery sleep to study rebound. Data collected during the recording phase can be analyzed at any time and from any computer.

Experimental design

Defining sleep. Flies that are sleeping are not moving, but flies that are not moving are not necessarily asleep. The best way to distinguish

TABLE 1 | Main differences between video tracking and split IR beam system.

	Split IR beam		
	Single beam	**Multiple beams**	**Video tracking**
Spatial resolution	Low	Medium to high	High
Movement resolution	Binary (crossed/did not cross)	Up to 3 mm	Can detect up to submillimeter movements
Interactions among flies	Flies must be isolated	Flies must be isolated	Flies can be interacting, with caveats
Scalability	Limited by cost	Limited by cost	High if using webcams
Scalability (time)	Limited by tube preparation	Limited by tube preparation	High if using custom arena
Number of monitors per computer	Up to 120	Up to 120	Between 30 and 120[a]
Cost of a single monitor	$500–$800	$800–$3,200	$10–$50
Promptness of usage	Ready to use on arrival	Ready to use on arrival	Need custom building, testing
Adoption in the field	Popular with circadian and sleep researchers	New	New

[a]Technical specifications of the USB protocol allow for up to 127 devices to be connected to a single computer. However, we have not yet tested more than 30 devices connected to the same machine and we take 30 as a 'safe limit' in this protocol and when estimating total costs.

locomotor rest from sleep is to measure arousal threshold. When flies are asleep, their arousal threshold increases and it is therefore possible to reliably infer whether a fly is asleep by actively challenging it with a sensory stimulus, such as tapping[3,16] or vibrating[4] the vial. The likelihood that a fly will respond to a complex stimulus levels off after 5 min of immobility[16] and for this reason sleep in flies is commonly defined as a period of inactivity lasting for at least five consecutive minutes. This definition implies that our estimate of sleep is a linear mathematical derivation of the measure of activity and explains why a more careful reading of activity will result in a more faithful estimate of sleep (**Fig. 3**; see **Box 1** for a glossary providing definitions of other aspects of sleep and sleep monitoring).

Choice of computer. In ideal conditions, the experimenter will have access to at least two computers: one dedicated to video tracking and located in the laboratory where the experiments are taking place, and one for data analysis. The video tracking part of the software is written in Python using the Open Source Computer Vision Library (opencv, Intel) and will run on any major operating system (Ubuntu Linux 10.10 is the recommended choice). A consumer business desktop PC, such as HP Compaq 8200 or Dell Vostro 460, will handle 30 video monitors and possibly more (we have not tested our system with more than 30 monitors). There are no special requirements for the computer used to analyze data.

Choice of the video camera. A setup for video analysis relies on four main components: a video camera, a recording arena where flies are housed, software to collect images and analyze movements, and software to analyze data (**Fig. 1b**). Virtually any video camera that can be connected to a computer will work with pySolo-Video given that there is no need for high refresh rates or high resolution. The only requirement is the ability to capture images under infrared light conditions during the dark hours. We routinely use inexpensive cameras that are normally commercialized for video conferencing (webcams, see MATERIALS): for less than $5 they offer enough image resolution (640 × 480 pixels), a more-than-sufficient refresh rate (approximately 10–20 frames per second) and the ability to record under infrared illumination. A single camera can record over 30 flies simultaneously from a distance of about 20 cm. **Figure 3a** shows a snapshot of a video collected using a similar camera.

The recording arena. In experiments measuring circadian rhythms, flies were traditionally housed in small glass cuvettes, containing fly food on one end and cotton wool on the other (**Fig. 1a** and **Supplementary Fig. 1**). However, video analysis offers added freedom in the choice of recording arena[17]. We use and recommend custom-designed plates obtained through 3D printing techniques (**Fig. 1b** and **Supplementary Fig. 1b**,**c**) because they provide more consistency between samples, are inexpensive and, most

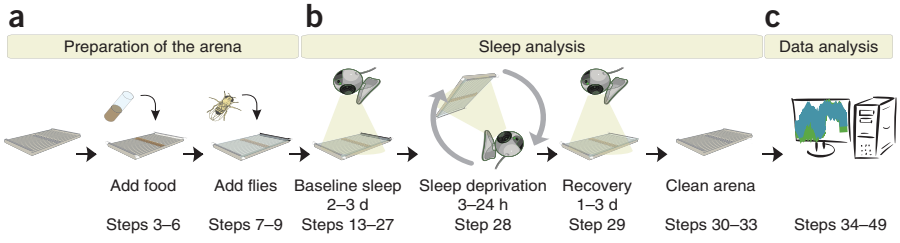

Figure 2 | Experimental workflow. The three main phases of the sleep assay. (**a**–**c**) During the preparative phase (**a**), flies of the correct age are inserted into a monitoring arena previously loaded with food. Typical recording (**b**) may last for 4–7 d. At the end of the recording, flies are discarded, the arena is cleaned for next use and the data are analyzed (**c**). The relevant PROCEDURE steps are listed under each drawing.

importantly, their preparation requires less time (minutes versus hours or days). Custom recording arenas can be virtually modeled using 3D modeling software and then printed in-house or using third-party printing services. Alternatively to 3D printed models, the experimenter still has the choice to use traditional glass tubes or multiwell plastic containers that are normally adopted for cell cultures (see EQUIPMENT).

Illumination. Flies must be illuminated using two different sources of light: visible light during the subjective day, and infrared light during the dark hours. In both cases, direct illumination of the recording arena may result in reflective glare that affects the quality of the image acquisition; this is a particularly frequent issue when flies are illuminated by an infrared light located above the arena, for instance when using the LEDs integrated in the camera itself. For best results, disable or remove the LEDs in the camera and place an independent infrared LED strip underneath the arena, illuminating the flies from below; this will minimize the glare and increase contrast (see **Fig. 3a** for a snapshot of a nocturnal video taken using back illumination).

For both visible and infrared illumination, we recommend using low-voltage LED strips as they are easy to wire and install, and they provide controlled intensity of light without noticeably raising the temperature in the recording chamber (see MATERIALS).

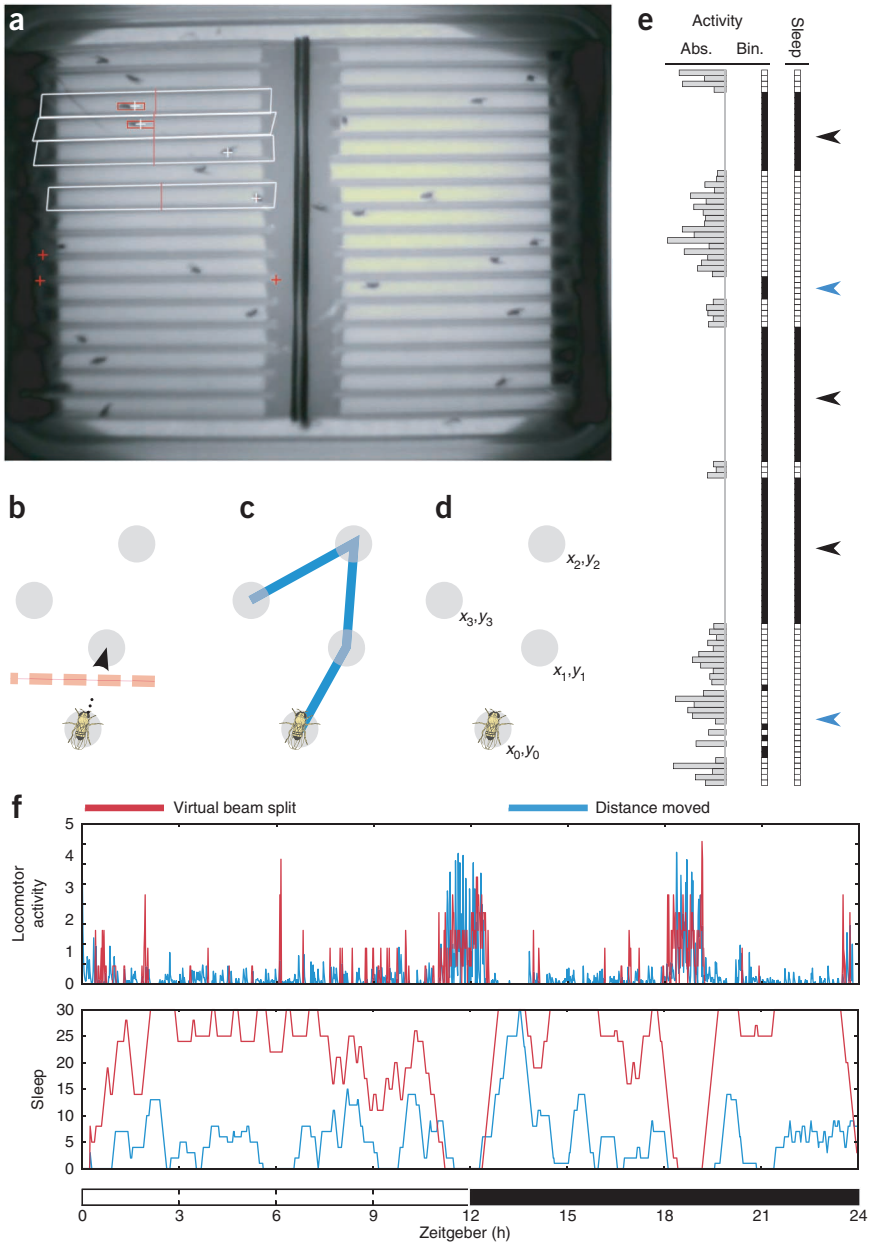

Figure 3 | Translating motor activity to sleep. (**a**) Snapshot of pySolo-Video in action. Nocturnal view of flies in an arena, as seen by the webcam. Flies are in total darkness and infrared illumination comes from underneath the arena through infrared LEDs. Some of the grooves have an active recording mask delimiting the area where flies move (white rectangles). Each mask shows a midline point that is used by the program to emulate the beam-split system (vertical red line). Moving flies are highlighted by pySolo-Video with a red rectangle whose size is proportional to the extent of movement measured. A white cross on the fly indicates immobility. Rectangular masks can be drawn holding the left mouse button while dragging the cursor on the image or can be drawn by clicking to indicate four corners to be connected for higher flexibility in drawing shapes (red crosses). (**b**–**d**) Three different ways of recording motion. Cartoons showing a fly walking through four different points in space. The fly's motion can be recorded in three different formats. (**b**) Emulation of the infrared beam split. The position of the fly is calculated at every video frame (about 15–25 times a second), but motion is recorded only if the fly is crossing the transversal midline of the chamber (red line in **a** and **b**). In this example, motion would be recorded only when the fly moves from the lower point to the next. (**c**) Measuring distance. The position of the fly is calculated at every frame and activity is measured by calculating the distance walked by the fly at each minute (blue line). (**d**) The position of the fly is calculated at every frame, then saved with resolution of 1 s. This latter format is to be considered as the raw data from which the other two can be derived. Additional custom analysis may also provide information on the activity of the fly, including the position held at any single time, as well as speed and directions. (**e**) Schematics of how sleep is estimated and processed. Locomotor activity of the fly is plotted as function of time (left, gray rectangles): each rectangle represents the distance walked by the fly in one minute. To estimate sleep, activity data are first flattened to a binary matrix in which flies can be only inactive (black) or active (white; middle bar); episodes of inactivity that last for at least five consecutive minutes are considered sleep (right bar). In this example, the fly experiences three sleep bouts (black arrowheads) and two episodes of simple inactivity (blue arrowhead). Abs., absolute; bin., binary. (**f**) Real activity versus virtual split-beam. Activity (top) and sleep (bottom) of a single fly recorded for 24 h and plotted calculating the actual distance of the flies as shown in **c** (light blue) and the virtual beam-split as explained in **b** (pink). Both methods show clearly the daily peaks of activity (two, in this case), which are used for circadian analysis. However, only by analyzing distance can the real activity and sleep be properly estimated, and beam-split can overestimate sleep amount by more than 100% (see also ref. 13).

Box 1 | Glossary

Arousal stimulus: a sensory stimulus that will induce motion in an otherwise immobile fly. It can be a mechanical stimulus such as a vibration induced by tapping a finger against the recording chamber.

Arousal threshold: the threshold beyond which a certain stimulus will promote motion. When the fly is asleep, the arousal threshold is higher than when it is awake but immobile. Also, longer periods of inactivity correlate with even higher arousal threshold, suggesting that *Drosophila* sleep can also be divided into light sleep and deep sleep[16].

Sleep episode or *bout*: an episode of continuous immobility lasting for at least five consecutive minutes. Its duration is measured in minutes.

Sleep latency: the time passing between turning lights off and the start of the first sleep episode at night. It is used as a readout of the mechanisms underlying sleep initiation.

Sleep cycle: interval from the beginning of one sleep episode to the beginning of the subsequent sleep episode.

Sleep fragmentation: indicates the number of sleep episodes in a sleep cycle.

Sleep duration: the cumulative amount of sleep in a 24-h period measured in minutes.

Baseline sleep: the sleeping pattern in undisturbed conditions.

Sleep deprivation: the act of forcefully keeping the fly awake.

SNAP (sleep-nullifying apparatus): a machine that sleep-deprives flies by rotating on one axis and interfering with their geotactic sense.

Sleep loss: the amount of sleep lost upon sleep deprivation, measured in minutes and calculated against baseline sleep.

Recovery sleep or *sleep rebound*: the amount of sleep recovered at the end of sleep deprivation. It is frequently calculated looking at sleep during the 6, 12, 24 or 48-h period following the end of sleep deprivation, using baseline sleep as reference value.

Activity count: an absolute measure of the locomotor activity of the flies. When activity is measured using the infrared beam-split system, the activity count equals the number of beam crosses per time unit. When activity is measured using video analysis, activity count equals the distance per time unit.

Activity index (AI): a relative measure of locomotor health. AI is calculated by dividing the activity count by the total time spent awake in a 24-h period. This is a way to normalize activity among flies with different total sleep durations.

Planning the experiment. A typical experiment may be composed of three successive phases (**Fig. 2**): baseline sleep (2–3 d; **Fig. 2a**); sleep deprivation (6–24 h; **Fig. 2b**); and recovery sleep (2–3 d; **Fig. 2c**). During baseline sleep, flies are left undisturbed and each individual fly is monitored to set its baseline sleep need. On the third or fourth day, flies may undergo sleep deprivation using a protocol of choice (see 'Interfering with sleep' below). A homoeostatic response to sleep deprivation should manifest in the days following sleep deprivation, during which flies are tested for their ability to recover sleep loss.

Interfering with sleep. The ability to interfere with sleep is as important as measuring sleep itself. Rebound following sleep deprivation is a hallmark of sleep, and sleep deprivation has important consequences on a multitude of conditions. Flies, similarly to vertebrates, show both cognitive and physical impairments upon prolonged sleep deprivation, with consequences that range from learning deficits to death[6,18]. There are several types of sleep deprivation in *Drosophila*: pharmacological, mechanical, social, and starvation-induced. **Table 2** provides a summary of strengths and weaknesses of each of them, but details are only provided below for mechanical and pharmacological manipulation of sleep, as they are most relevant to a broader audience.

- *Pharmacological manipulation of sleep*. Many pharmacological compounds that modulate sleep in vertebrates act on insects too (**Table 3**). Sleep or sleep loss can be induced by feeding flies with

TABLE 2 | Comparison of methods of sleep modulation.

Method	High throughput	Specificity of action	Controlled	Strength
Mechanical (manual)	– – –	+ + +	+ + +	Allows you to specifically target only flies that are asleep and leave awake flies undisturbed
Mechanical (automatic)	+ + +	+ +	–	Highly scalable and quite specific
Pharmacological	+ + +	–	–	Useful for testing whether a gene is involved with sleep regulation through a given pathway
Social	+	?	–	Useful for testing links between sleep and social learning
Starvation-induced	+ + +	?	–	Useful for testing physiological regulation of the sleep-wake cycle

TABLE 3 | Drugs used to manipulate sleep.

Compound	Approximate effective dose (mg ml^{-1})	Reported effect	Reference
Methamphetamine	0.6–1.25	W	27
Octopamine	5–10	W	23
Caffeine	0.2–5	W	3, 4, 28, 29
CPT	2.5	W	28
DPMX	2.5	W	28
Cocaine	0.5	W	30
Modafinil	0.3–2.5	W	31
Cyclohexyladenosine	0.5	S	3
Carbamazepine	0.2–1.2	S	32
Hydroxyzine	2.5–200	S	4
5-Hydroxytryptophan	1	S	33
3-Iodo-tyrosine	5	S	26

CPT, 8-Cyclopentyll-1,3-dimethlxanthin; DPMX, 3,7-Dimethyl-1–2-propynylxanthine; W, increases wake time; S, increases sleep time.

food containing a sleep-altering compound. It is important to keep in mind that drugs may have side effects that extend beyond sleep control and that can complicate the readout of the experiment; however, there are cases when the experimenter may want to assay the response of a given genotype to specific compounds, for instance to test whether a gene acts in a specific sleep-regulating pathway.

- *Mechanical disruption of sleep*. It is possible to obtain robust sleep deprivation through mechanical stimulation of flies: for instance, by tapping the vials where the flies are housed whenever they stop moving[3], by spinning the flies upside down to interfere with their geotactic instincts[6,18], or by placing flies on a laboratory orbital rotator[19]. Manual sleep deprivation is efficient but incompatible with high-throughput experimental conditions; automatic sleep deprivation provides a good compromise and can be achieved by placing the monitor in a rotating device, which is relatively easy to build[18], or on a common laboratory orbital shaker[19].
- *Socially induced suppression of sleep*. Sleep loss occurs when a socially naive experimental fly is suddenly disturbed by another fly introduced into the arena. Socially induced sleep suppression may have greater ecological relevance than mechanically induced sleep suppression, but it will work only when using male-male coupling (ref. 7 and G.F.G., unpublished data); other kinds of interactions may still induce changes in sleep behavior that may be interesting to study[8]. Locomotor activity during social interaction must be measured using video-tracking techniques; however, completely automatic tracking of multiple flies interacting in an open arena for prolonged times is still difficult for computers and may require substantial corrections by a human observer[20,21].
- *Starvation-induced sleep suppression*. This behavior is conserved across phyla and may be particularly important given that sleep-feeding interactions have been linked to diseases including obesity, diabetes and metabolic disorder. Switching the food source from standard fly food to agar, where they attain their water needs with no nutritional benefit, results in robust suppression of sleep[17,22–24].

Controls. Experimental groups should be carefully matched to reduce any influence of uncontrolled factors, such as the genetic background of the flies. A good description of this problem and how to address it was discussed by Rosato and Kyriacou[12], and I refer to them for more details. In particular, male and female flies show radically different sleep patterns, with males undertaking a markedly longer post-meridian nap (also called siesta[24]) than females. Sleep patterns also change with age[25], social experience[8] and feeding status[17,23,24]. Therefore, it is crucial to compare flies of the same sex, age and with similar previous experience. Genotype should also be considered and comparisons within *Drosophila* stocks with dissimilar genetic backgrounds should always be avoided. When comparing flies expressing transgenes under the control of the GAL4-UAS system, it is advisable to start by crossing heterozygous parental stocks so that the progeny will be composed of experimental flies and their sibling controls, which are distinguishable by eye color or, post hoc, by PCR genotyping.

Sample size should vary as a function of statistical power; that is, the difference in the mean values between experimental and control groups and the internal variability within each group. Many sleep parameters (e.g., sleep duration) tend to be relatively homogeneous within an isogenic population; others (e.g., sleep deprivation efficiency) will vary substantially even within sibling flies. Therefore, sample sizes should be empirically determined on the basis of the experimental conditions and may vary from as few as 16 flies per group to a few hundred[12,26].

Environmental conditions. Temperature, humidity and light intensity should be kept constant with time and within groups. Any external stimulus, including noise, should be avoided, and experiments should be run in a dedicated environment, preferably in dedicated incubators.

MATERIALS

REAGENTS

- Flies: commonly used control lines are CantonS, OregonR or BerlinW (Bloomington *Drosophila* Stock Center; http://flystocks.bio.indiana.edu/)
- Fly food: The Bloomington *Drosophila* Stock Center provides methods and recipes for cooking different kinds of fly media (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/media-recipes.htm)

EQUIPMENT

- Vials and plugs for raising flies (plastic flat-bottom tubes 75 mm × 23.5 mm, Regina industries, cat. no. P1014L; Regina industries, cat. no. 49-102)
- Organic cotton wool (Genesee Scientific, cat. no. 52-101)
- Narcotic pad (Genesee Scientific, cat. no. 59-114)
- Fine artist's paintbrushes (for handling flies)

- A computer for recording data. Computer specifications will vary depending on the number of cameras recording simultaneously in the system. For up to four cameras, a 'deskbook-like' computer will suffice (e.g., Acer Aspire Revo, Atom D525 1.8 GHz processor, 2 GB ram, 250 GB disk). A more powerful system will handle many more cameras (e.g., HP Compaq 8200, Intel i7 2.8 GHz processor, 4 GB ram, 500 GB Disk). The number of USB ports is not crucial as they can be easily expanded using USB hubs (see below). The computer should be connected to the network for data accessibility
- Recording and analysis software (pySolo and pySolo-Video; http://www.pysolo.net/)
- One or more video cameras with USB connection ▲ **CRITICAL** The camera must be able to see under IR light conditions. Many cameras carry an IR filter that would have to be removed. Cameras that provide in-built IR-LEDs do not have such a filter and are therefore preferred (we routinely use http://www.ebay.com/ to find inexpensive ($5) cameras with integrated IR LEDs, such as the Kinamax WCM-6LNV).
- One or more USB hubs (D-Link DUB-H7) ▲ **CRITICAL** The hub should be powered by an external power supply or cameras may not receive enough current to work properly.
- Visible LED lights (http://www.ledlighting.co.uk/, cat. no. 154798)
- Infrared LED lights (http://www.ledlightsworld.com/, 850-nm Tri-Chip LED). Both visible and infrared LED strips are sold by the 5-m reel, but they can be conveniently cut to fragments of 5 cm and wired directly to a power supply
- A digital mains timer socket (Munro, via http://www.amazon.co.uk/, cat. no. B000TBGWIO)
- Adhesive magnetic strips (RS components, cat. no. 297-9093)
- Food-grade plastic container (1.6-liter medium container, Lock & Lock Storage)
- One or more recording arenas to host flies. A purposely designed arena can be custom made using 3D printing (see EQUIPMENT SETUP)
- PETG clear co-polyester sheet (RS Components, cat. no. 334-6444)
- Multiwell cell culture plates (BD Falcon, cat. no. 353503) or glass tubes (Trikinetics). These are ready-to-use alternatives to the 3D-printed arena
- Optional: a *Drosophila* incubator (Percival Scientific)
- Optional: a sleep deprivator. A rotation device can be custom built or can be adapted from a laboratory orbital rotator (Cute Mixer CM-1000, EYELA or VWR VX2500 with a TriKinetics adaptor)

REAGENT SETUP

Fly maintenance Grow flies in vials, keeping them in constant light, temperature and humidity conditions. An artificial light-dark regimen of 12:12 (h) is considered standard; temperature should be constant, between 21 °C and 25 °C; humidity should be kept above 60%. Avoid overcrowding by removing adult parent flies 3–5 d after setting up the vial.

Preparing food for the arenas Place a vial with fresh food in a microwave oven and heat it for 10 s or until melted. Stir the food with a pipette to remove clumps and heat it for a few more seconds. For experiments using drug-induced sleep suppression, substitute fly food with a 1% (wt/vol) agar-water solution or food laced with the desired drug.

EQUIPMENT SETUP

Computer and software Place a computer next to the incubator where experiments will run. Any major operating systems will work, including Microsoft Windows and MacOSX, but for a brand new installation we recommend using Linux Ubuntu, version 10.10 (http://www.ubuntu.com/). To Install pySolo-Video on Windows or Linux, point your internet browser to the pySolo website (http://www.pysolo.net/), go to the Download section and select the latest packaged version. Double-click on the file to install it. On Ubuntu Linux, subscribe to the pySolo repositories to have automatic updates of new releases. **Supplementary Video 1** offers step-by-step instructions on how to install pySolo and pySolo-Video on Linux Ubuntu 10.10.

Print the recording arena The arenas described in this protocol are obtained using 3D printing technology. We use an Eden 250 (Objet) using VeroWhite FullCure830 as printing material. With our printer, the cost of printing an arena similar to the one shown in **Supplementary Figure 1b** is around $20. An STL file ready to print is provided as **Supplementary Data** and can be opened and visualized using the free software meshlab (http://meshlab.sourceforge.net/). Many third-party services offer remote 3D printing for comparable prices (for example, http://www.shapeways.com/). Whether printed in-house or by third parties, we recommend stereolithography, as the final product will be smoother and easier to clean under running tap water.

Prepare the lids for the recording arena Cut the co-polyester sheet to fit as a sliding lid for the arena (63 mm × 100 mm, **Fig. 1b** and **Supplementary Fig. 1b**). Cut a larger (>80 mm × 100 mm) portion of the co-polyester to be used as a transferring lid. The transferring lid should be wider than the normal lid and have two holes of about 1 mm in diameter, through which flies will be blown in (**Fig. 1b**).

Build a recording box To build a recording box, drill a hole through the bottom side of the food container (that is, opposite to the lid) and place the camera so that the lens will look through the hole. Use glue or screws to secure the camera to the surface so that it will not move even when the container is shaken. Position the recording arena with flies on the internal side of the lid. Use self-adhesive magnetic strips on both sides to fasten the arena to the inner surface of the lid. Cut a small strip (5 cm) of visible LED lights and place it inside the container to illuminate the flies during subjective day. Regulate the timing of light transitions using a digital timer connected to the power supply. See **Figure 1c** for an illustration of the recording box and **Supplementary Video 2** for a detailed description of how a recording box is built. Connect the camera to the computer through the USB port, using an intermediate USB hub if necessary. Place the recording box in an environment with controlled light, temperature and humidity (ideally in an incubator or, if an incubator is not available, in an undisturbed cabinet in the fly room).

PROCEDURE

Preparing the experimental flies ● TIMING 13 d at 25 °C

1| Approximately 2 weeks before the planned start of the experiment, prepare one or more vials with the lines to be analyzed and compared.

2| Allow flies to mate and lay eggs for 3–5 d, and then remove parent flies and wait for progeny to develop to the imago stage.

Preparing the activity arena ● TIMING 20–30 min

▲ **CRITICAL** See **Supplementary Video 3** for a step-by-step description of how to prepare an arena, as described in Steps 3–6 below.

3| Open the arena by sliding the transparent lid and fit the separation walls around the food chamber to avoid spillage.

4| Prepare the food to be transferred to the arena (see REAGENT SETUP).

5| Use a syringe to transfer melted food to the inner chamber of the arena and then wait for it to solidify (about 10 min at 25 °C).

6| Remove the separation walls, place some cotton wool on the external edges of the arena and slide the lid in from the side.

■ **PAUSE POINT** Multiple arenas can be prepared at once and then stored at 4–10 °C for a few days.

Loading the activity arenas ● TIMING 20 min

▲ **CRITICAL** See **Supplementary Video 3** for a step-by-step description of how to load an arena, as described in Steps 7–9 below.

7| Collect flies 1 or 2 d after pupal eclosion and place them in new vials in small groups of ~10 individuals of the same genotype, to avoid overcrowding and to make sure that all the flies are of the same age. If flies must be sorted to separate different genotypes, you may need to use CO_2 anesthesia on a narcotic pad.

▲ **CRITICAL STEP** Compare flies of the same sex and age and make sure flies are of normal size with fully expanded wings. Sick flies will show strong differences in sleep/wake regulation.

▲ **CRITICAL STEP** If you are using females, make sure to collect virgins; mated female flies will lay eggs and larval development in the recording environment should be avoided because crawling larvae will interfere with motion detection and consume the food.

? TROUBLESHOOTING

8| When flies are at the correct age (normally 5 d old), transfer them to the arena. Flies can be transferred to the arena using two systems: transferring sedated flies (using CO_2 or cold; option A) or transferring the awake flies by mouth pipetting (option B). **Supplementary Video 3** contains a detailed explanation of both procedures.

? TROUBLESHOOTING

(A) Transferring flies using CO_2 sedation

(i) Sedate flies using a narcotic pad connected to 100% CO_2.

(ii) By using a small paintbrush, transfer each fly from the pad to a single groove in the arena (one fly per groove).

(iii) Close the lid by sliding in the clear plastic.

? TROUBLESHOOTING

(B) Transferring flies using a mouth pipette (fly sucker)

(i) Slide the transferring lid into the arena so that the portion with the two holes covers the first groove.

(ii) Insert the tip of the mouth pipette into the vial containing the flies through an opening in the cotton wool stopper.

(iii) Suck a single fly into the tip of the pipette.

(iv) Transfer the fly into the arena by blowing it through the hole in the transferring lid; transfer one fly to each side of the arena.

(v) Gently slide the transferring lid to cover the next grooves in the arena.

(vi) Repeat the process from Step 8B(ii) until all the required flies have been transferred.

(vii) Replace the lid with another that has no holes in it and that is of the correct size.

▲ **CRITICAL STEP** When comparing experimental and control flies, it is always preferable to host both in separate grooves in the same arena, mixing 16 control flies to 16 experimental flies, either randomly or by placing 16 flies of the same genotype on each side of the arena. This will reduce the operational variability. Remember to keep a record of fly genotypes and location in the arena.

? TROUBLESHOOTING

9| Place the arena with flies in the recording box and place the box in the incubator.

▲ **CRITICAL STEP** Let the flies recover and habituate for at least one night before starting any experimental recording, especially if CO_2 sedation was used to load the arena. Social experience does have an effect on sleep amounts and regulation, so allow 1 or 2 extra days of habituation if flies loaded in the arena were previously kept in a socially enriched environment.

Preparing the pySolo software for data fetching ● TIMING 30 min

▲ **CRITICAL** Steps 10–12 are configuration steps, and are required only once at the beginning of the experiment or whenever the location where the data are saved changes.

10| **Supplementary Video 4** provides a step-by-step guide on how to set up automatic formatting and collection of data using the *datafetcher* component of pySolo. Briefly, navigate to the directory where the *datafetcher* is installed.

11| By using your favorite text editor, open the configuration file *copyfiles.cfg* and change the relevant parameters. An explanation of each variable is given in **Supplementary Video 4**: *inputpath* is the path of the rawdata; *outputpath* is the path to the processed data that will be used by pySolo for analysis; *zippath* is the path where a backup copy should be saved. For the latter, it is recommended to use an external hard drive or a network disk for the sake of data safety.

12| Add the script *copyfiles.py* to your scheduled tasks so that it will run daily, moving the data into a directory tree organized by date and monitor numbers.

Preparing the pySolo-Video software for data recording ● TIMING 1 h

13| **Supplementary Video 5** provides step-by-step instructions on how to get started with pySolo-Video. Briefly, the first time you start the program, open the *options* panel to customize the total number of available webcams and monitors, as well as the path where raw data will be saved (same as *inputpath* in Step 11).

▲ **CRITICAL STEP** this is a configuration step and is required only once at the beginning of the experiment or whenever the location where the data are saved changes.

? TROUBLESHOOTING

14| Restart the program to reload the new settings.

15| At the beginning of each experiment, associate each camera to its own monitor by clicking on the thumbnail in the *thumbnail* panel and selecting the appropriate source from the dropdown menu found in the 'Select Video Input' box.

16| Move to the *live view* panel in the program interface.

? TROUBLESHOOTING

17| Use the mouse to draw a mask to delineate and separate the area in which a single fly will move.

18| To create a mask, either drag the cursor on the screen while holding down the left button of your mouse, or click on four arbitrary corners to draw a polygonal irregular area. Click on the middle mouse button to confirm the selection and activate the mask. The first mask you draw will be channel one of your monitor, the second mask will be channel two, and so on. The *live view* panel will show real-time analysis of the flies' activity as you draw to mask (**Fig. 3a**).

▲ **CRITICAL STEP** Each mask should cover only one groove or channel. Pay special attention in drawing accurate borders. If you make a mistake, select the mask by clicking inside it and delete it by clicking the right mouse button.

19| Save the mask with a chosen name.

20| Go back to the *thumbnail* panel.

21| For each monitor, decide the format in which to save the data (see **Fig. 3b–d** for an explanation of the different possibilities) and associate the mask you just created, then hit the Apply button.

22| Save all your settings in a configuration file.

23| Close the pySolo-Video GUI.

Recording data and interfering with sleep ● TIMING 1 week

24| Start the pySolo-Video Acquire component.

25| Click on Browse and locate the configuration file you saved in Step 22. Click on Open.

26| The Acquire window will show a table with a list of all monitors previously configured.

27| Click on Start to begin tracking.

28| Advantages and characteristics of the different methods to alter sleep are discussed in the Experimental design section. Here follows a more detailed description of the commonly used kinds of sleep interference: mechanical disturbance (option A) and pharmacological modulation (option B).

(A) Mechanically induced sleep deprivation ● TIMING 3–24 h

(i) Record baseline sleep for 48–72 h.

(ii) The day the disruption is scheduled to start, place the recording chamber on the rotating device.

(iii) Switch the orbital rotator on.

▲ **CRITICAL STEP** High rotation speed, especially if maintained for long stretches of time (>3 h), may be damaging and kill the flies. Optimal speed settings may vary depending on the orbital shaker and should be empirically calibrated. Start at very low speed and increase if necessary. A sleep deprivation period of 6 h is normally sufficient to induce noticeable effects. Sleep deprivations that extend beyond 24 h may result in death. Sick fly stocks may die even earlier[19].

(iv) After the required time has elapsed, end the sleep deprivation period and place the flies back in the incubator.

? TROUBLESHOOTING

(B) Pharmacologically induced sleep deprivation ● TIMING 12–48 h

(i) Pharmacological sleep deprivation can be achieved by manually transferring flies from an arena with control food to another with drug-enriched food. To do this, prepare two arenas: one filled with control food and one with laced food (either 1% (wt/vol) agar or food with chemicals for pharmacological manipulation. **Table 3** provides a review of all pharmacological manipulations shown to have an effect on *Drosophila* sleep).

(ii) Measure baseline sleep, keeping flies in the control food for 48–72 h.

(iii) At the beginning of the manipulation time, use a mouth pipette to move flies from the arena with regular food to the one with laced food. Place the arena back in the recording chamber.

(iv) Record treated flies for 24 h, and then transfer flies back to the arena with untreated food using a mouth pipette (as described in Step 28B(iii)).

! CAUTION Sleep is an evolutionarily conserved phenomenon and some drugs may have neurological effects on humans as well as on flies. Handle all drugs with care.

? TROUBLESHOOTING

29| Measure the rebound after sleep deprivation for at least 48 h.

Cleaning the arena and discarding the flies ● TIMING 10 min

30| At the end of the experiment, remove the arena from the recording box.

31| Fill a container with warm water and place the arena inside. Open the lid while it is still in the water and use a small brush to remove flies, food and cotton wool.

32| Gently brush the arena under warm tap water, then rinse it in deionized laboratory water and let it dry.

33| Optionally, the arena can be washed using 70% (vol/vol) ethanol or, depending on the material adopted for printing, it can be placed in a dishwasher or autoclaved.

! CAUTION Wash the arena soon after each use. With time, food will dry inside and will be more difficult to clean.

? TROUBLESHOOTING

Preparing the pySolo software for data analysis ● TIMING 30 min

34| **Supplementary Video 6** and the **Supplementary Manual** provide a detailed description of how to configure and use pySolo for data analysis. Start by installing pySolo on your office computer or laptop.

35| The first time you start the program you will be asked to provide some basic parameters in the *options* panel. This configuration step is only required once, the first time the program is started. You will have access to the option panel at any given time later on, but three options must be specified immediately for pySolo to work: the path where the program will find the daily data (the same as *outputpath* from Step 11); the extension of the files containing the daily

data (*txt* by default), and the format of the input data (**Fig. 3d**). pySolo must have access to the folder where the daily data are located in order to work.

Analyzing data using pySolo ● TIMING 30 min to 2 h

36| In the database window of the program, load your experimental database file or create a new one.

37| Click the button with a green plus sign to add new rows to your database.

38| Fill in data about your experiment in each row: starting monitor, starting channel, end monitor, end channel, genotype, comment and the days of the experiment. See also the documentation on the pySolo website for more information (http://www.pysolo.net/).

39| Save your file.

40| Select the lines of the experiments you want to analyze by marking their corresponding check box on the left side of the table.

41| Point your mouse to the menu bar and click on *Analysis* → *Check Raw Data files* to make sure that all the data the programs needed are properly accessible and formatted.

42| Click on *Analysis* → *Fetch Raw Data* to fetch the data.

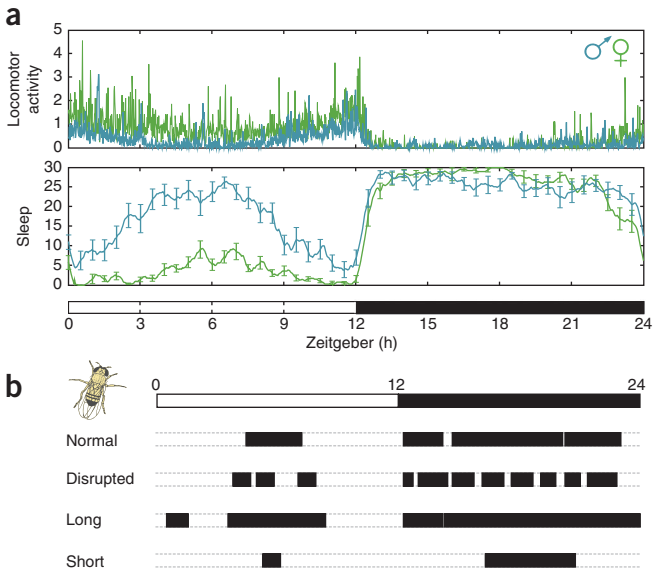

Figure 4 | Components of *Drosophila* sleep. (**a**) A hypnogram showing how locomotor activity calculated with the virtual beam-split method (top) translates into sleep (bottom). The graph shows average activity and sleep for actual male (blue) and female (green) flies over a 24-h period. Notice the difference in sleep during mid-day (the male siesta). Error bars in the sleep plot indicate s.d. (**b**) Graphic description of fly sleep over a 24-h period in a 12:12 light/dark cycle. Each black box represents a single sleep bout. Wild-type flies sleep primarily in the night. The fly labeled 'disrupted' has shortened bout lengths, with a similar total sleep time to wild-type flies. This phenotype can be distinguished from long- and short-sleeping flies that have differences in total amount of sleep.

43| Save the resulting file with extension '.dad'.

■ **PAUSE POINT** The resulting .dad file can be opened into the analysis window of pySolo at any time. Analysis can also be performed on a different machine, as it is now completely independent of the original raw data.

44| Send the acquired data to the analysis window by either clicking on *Analysis* → *Send Data to Analysis* or opening the analysis window from the *Windows* submenu of the menu bar and dragging and dropping the .dad file inside.

45| Click on the *Browser* tab to open the *Browser* panel.

46| Use the navigation tree on the left to select single genotypes, single days or single flies. Use the Control key on your keyboard to join together values from multiple selections.

47| When comparing different experimental groups, use the button marked with a blue plus sign on the bottom left hand side to add new item to the analysis.

48| Evaluate and compare values of the most important aspects of sleep (sleep duration, rebound to sleep deprivation, and so on; see **Fig. 4** and **Box 1** for other examples). The software will automatically look for dead flies, whose sleep pattern is beyond predetermined threshold values. By default, flies sleeping longer than 1,350 min a day are considered dead. If necessary, threshold levels can be modified in the *options* panel.

49| If necessary, export numerical data by clicking on the table to be exported with the right mouse button. Files will be saved in a spreadsheet-compatible format to allow external analysis or statistical comparison.

50| Similarly, export graphical data as images using the right mouse button.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 4**.

TABLE 4 | Troubleshooting table.

Step	Problem	Possible reason	Solution
7	Flies die or suffer after sedation	Flies are left too long on the narcotic pad	Be quicker when sorting flies or use pulses of CO_2 rather than continuous flow
8	Flies escape from the recording arena	Lid is not properly closed	Use a rubber band or tape to close the lid properly and make it adhere to the arena
8A(iii)	Flies die during transfer using CO_2 anesthesia	Flies touch the food while still unconscious and get stuck	Take special care not to push flies against the food
8B(vii)	Flies escape when transferring them to the arena by mouth pipette	Too many flies in the same vial	Use vials with fewer flies
		Vials are closed with hard pressed-paper stoppers	Replace stoppers with cotton wool
8B(vii)	Flies die during transfer by mouth pipette	Inlet hole of the mouth pipette is too small	Increase the diameter of the hole
13	The program crashes or does not start	The version of the operating system may not be supported	Try installing a supported and tested OS, such as Linux Ubuntu 10.10 or Microsoft Windows 7
	The program crashes when playing previews	The resolution is not supported by the camera	Try setting the camera resolution to 640 × 480
	The program crashes when using multiple cameras	Cameras are drawing too much current	Connect cameras to a USB hub with an independent power supply
16	Image is blurry	The camera objective is out of focus	Adjust focus manually and, if necessary, apply some glue to lock the lens in place
28A(iv)	Flies die during sleep manipulation	Sleep deprivation lasts too long	Reduce the duration of sleep deprivation
28B(iv)	Flies die during sleep manipulation	Concentration of the drug is too high	Reduce the concentration of the drug
	Flies do not eat the drug	Drug tastes unpleasant and flies will not eat it	Try changing the food mixture
	Sleep pattern is not very different between day and night	Too much light during the night	Make sure the incubator is properly sealed or, if necessary, use blackout foil to close residual openings
		Not enough light during the day	Make sure the lights are properly connected and will turn on during the day
	Flies die after few days of recording	Food dries out and flies have difficulty in eating	Make sure that humidity in the incubator is at desired levels
33	The arena bends under warm water	Temperature of the water is too high or the material has a high resin content	Use colder water or use a different material for printing

● TIMING

Steps 1 and 2, preparing the flies: 13 d (plan your crosses or expand your stocks in advance of the experiment so that enough flies will have hatched by the beginning of the experiment. For weak or sick stocks, it is wise to set up more than one vial.)
Steps 3–6, preparing the arena: 20–30 min (multiple arenas can be prepared at once: allow 1 more minute per each additional arena.)
Steps 7–9, loading the arenas with the flies: 20 min per arena (transferring awake flies in the arena by mouth pipette requires some experience and may be frustrating the first few times. Give yourself up to 1 h if you are new to this procedure and use some time to practice.)
Steps 10–12, preparing the software for data fetching: 30 min (needs to be done only once)
Steps 13–23, preparing the software for data recording: 1 h
Steps 24–29, recording activity data and interfering with sleep: ~1 week (see also **Fig. 2** for a better breakdown of experimental times during data recording.)
Steps 30–33, cleaning the arena: 10 min (preparing and cleaning the arena takes considerably less time than working with regular glass tubes (compare reference 14 with **Supplementary Video 3**).)

Steps 34 and 35, preparing the software for data analysis: 30 min (needs to be done only once)
Steps 36–50, analyzing the data: 0.5–2 h (data analysis can be performed at any time and from any computer on which pySolo is installed.)

ANTICIPATED RESULTS

Quality of image acquisition

Best results require optimal image acquisition conditions by the camera. A successful setting should result in an image whose quality is akin to the image in **Figure 3a**. Flies are detected with good contrast against the background, so that even wings and legs are recognizable despite the low resolution of the camera; no glare should be observable, or it should be limited to areas that are not included in the recording mask.

Internal controls

Duration of sleep and response to sleep deprivation will change considerably from line to line, even within wild-type stocks[17], whereas the patterns of activity throughout the day should remain roughly comparable among control stocks. A good setup should show a robust difference in activity between the relative day and relative night and an appreciable difference in the daily sleep patterns when comparing male to female flies. Peaks of activity should clearly appear at dusk and dawn. For these reasons, I recommend starting with pilot experiments to test the setup using wild-type (Canton S or Oregon R) male and female flies. **Figure 4a** shows an example of a good control experiment, in which male and female sleep patterns show a clear difference between night and day and in which the male siesta is clearly visible.

Note: Supplementary information is available via the HTML version of this article.

ACKNOWLEDGMENTS I am deeply indebted to A. Keene for his help with a first version of this manuscript. I would like to thank the open source community for creating and sharing valuable software. The work described here was partly funded by a Royal Society Research grant (RG110197) to G.F.G.

COMPETING FINANCIAL INTERESTS The author declares no competing financial interests.

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