Video tracking and analysis of sleep in *Drosophila melanogaster*

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30 **ABSTRACT**

In the past decade, *Drosophila* has emerged as an ideal model organism to study the genetic components of sleep, its regulation and functions. In fruitflies, sleep can be conveniently estimated by measuring the locomotor activity of the animals using techniques and instruments adapted from the field of circadian behaviour. However, proper analysis of sleep requires degrees of spatial and
35 temporal resolution higher than is needed by circadian scientists, and 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) created and developed in my laboratory. I focus on computer assisted video-tracking as instrument to monitor flies activity; I explain how to prepare the necessary equipment and how to plan a sleep analysis that covers the basic aspects of
40 sleep, closing with data analysis. A typical experiment for sleep analysis requires 5-7 days to perform.

INTRODUCTION

Overview

45 *Drosophila 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
50 back to 1956[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 demonstrated that the prolonged inactivity observed in *Drosophila* fully satisfies the five behavioural characteristics that define a sleep-like status[2]: (i) consolidated circadian periods of immobility, (ii) a species-specific posture and/or
55 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 need in *Drosophila*, as it does in vertebrates[7,8]. Conservation of sleep from humans to flies extends beyond behaviour to pharmacology (e.g.: caffeine and amphetamines promote wakefulness[3,4], whereas sedatives like
60 hydroxyzine promote sleep[4]) and flies, like vertebrates, show distinct electrophysiological correlates of wakefulness and sleep in the brain neuronal activity[9]. Together, these findings demonstrate an evolutionary conservation between *Drosophila* and human sleep at the behavioural and molecular levels, making flies an ideal system to study the mysterious functions of sleep and all its connections to pathological conditions in humans.

65 Because sleep is associated with sustained physical inactivity, the easiest way to record sleep in flies is to measure their locomotor behaviour. Traditionally, this has been done using tools already employed to measure circadian rhythms, namely detecting when a single fly breaks an infrared light beam transecting the midline of a small recording chamber[10] (**Fig. 1a**). Conceptually, this system dates back to the 1970s when Seymour Benzer adopted it in his mutagenesis screen for flies with

70 defective circadian rhythms[11] 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 since detecting movements only when the flies are crossing the midline has been reported to strongly overestimate sleep in some conditions[13]. Here

75 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 behaviour is an exciting emerging field in *Drosophila* neuroscience and several systems for computer assisted monitoring of sophisticated behaviours are currently available (**Supplementary Table 1**). In this protocol I concentrate on a system developed by our laboratory that was expressly

80 designed to be used for sleep detection and analysis, and carries specific features that other software with different aims do not provide. pySolo[14] is a software for data analysis characterised by an easy to use, powerful interface; its current alternative would be to analyse and plot data using elaborate spreadsheets or scripts, such as Microsoft Excel macros and Mathworks MatLab scripts (**Supplementary Table 1**). pySolo-Video translates video-detected motion into activity data: it is

85 integrated with pySolo, 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 summarised in **Table 1**. The main advantages of video tracking over infrared beam-split monitors are (i) a more faithful read out of

90 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

95 chamber and movements can be detected only if the fly is walking across (**Fig. 1a**). When the fly wanders on one side of the chamber, no movement is detected and sleep may be overestimated (Zimmerman et al[13] and our unpublished data). To overcome this drawback, new monitors were recently commercialized that detect activity using three, nine and up to 16 infrared beams per tube (LAM and MB5, TriKinetics Inc). Data using these new high resolution monitors are not yet

100   available in the scientific literature but it is easy to predict how an increase in spatial resolution will result in a more faithful read out. Video tracking, however, still offers a much higher level of resolution (an arena of 110 by 65 mm imaged at 640x480 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

105   cost around $3000, while the infrared split-beam alternative may range between $15000 and $100000, depending on the desired spatial resolution. 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 Inc.) were engineered to be ready

110   to use. The only assembly required is the connection of the recording monitors to the computer, which can be successfully done in 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

115   ability to host and record flies in an environment different from the traditional glass *cuvette*. Due 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 arena that are more convenient to use – like the ones described in this protocol - or suitable to complex experiments.

120   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, to unequivocally resolve the identity of single flies over prolonged times is still an open problem of computer vision and this kind of experiment should either be treated as "population readings" (rather than "individuals readings") or else be performed by

125   marking flies beforehand, using paints of different colours. Finally, video tracking offers an added value to the kind of data recorded: by analysing 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.

130   **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 the animals throughout the recording and provide them with the necessary nutrients.

135 Recording typically lasts 5-7 days and requires little or no human intervention. Flies are first monitored in a controlled environment for two or three consecutive days, to score their baseline sleep needs; 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 analysed at any time and from any computer.

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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 locomotor rest from sleep is to measure arousal threshold. When flies are asleep, their arousal threshold increases and it is therefore possible to
145 reliably infer if an animal 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 five minutes of immobility[16] and for this reason sleep in flies is commonly defined as "a period of inactivity lasting 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
150 more careful reading of activity will result in a more faithful estimate of sleep (**Fig. 3, panels e,f.** See **Box 1** for a glossary providing definitions for 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
155 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 Inc.) 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
160 the computer used to analyse 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 analyse movements; and software to analyse data (**Fig. 1b**). Virtually any video camera that can be
165 connected to a computer will work with pySolo-Video given that there is no need for high refresh rate 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 normally commercialized for video conferencing (*webcams,* see MATERIALS*)*: for less than $5 they offer enough image resolution (640x480 pixels), more than sufficient refresh rate (~10-20 frames per second) and the

170   ability to record under infrared illumination. A single camera can record over 30 flies simultaneously from a distance of about 20cm. **Figure 3a** offers a representative snapshot of a video collected using a similar camera.

The recording arena. In experiments measuring circadian rhythms, flies were traditionally housed
175   in small glass cuvettes, containing fly food on one end and cotton wool on the other (**Fig. 1a** and **Supplementary Figure 1b**). However, video analysis offers added freedoms in the choice of recording arena[17]. We use and recommend custom designed plates obtained through 3D printing techniques (**Fig. 1b** and **Supplementary Figure 1b,c**) because they provide more consistency between samples, are inexpensive and, most importantly, their preparation requires less time
180   (minutes versus hours or days). Custom recording arenas can be virtually modelled using 3D modelling 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 multi-well plastic containers normally adopted for cell cultures (see EQUIPMENT).

185   **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 affecting the quality of the image acquisition, 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
190   the LEDs in the camera and place an independent infrared LED strip underneath the arena, illuminating the flies from below: this will minimise 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 provide controlled intensity of light without sensibly raising the
195   temperature in the recording chamber (see MATERIALS).

Planning the experiment. A typical experiment may be composed of three successive phases (**Fig. 2**): baseline sleep (2-3 days), Fig 2a; sleep deprivation (6-24hours), Fig 2b; and recovery sleep (2-3 days), Fig 2c. During baseline sleep flies are left undisturbed and each individual animal is
200   monitored to set their baseline sleep need. On the third or fourth day, flies may undergo sleep deprivation using a protocol of choice (see 'Interfering with sleep', below). 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.

205 **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, like 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 ways to perform sleep deprivation in *Drosophila*: pharmacological;

210 mechanical; social; and starvation-induced. **Table 2** provides a summary of strengths and weaknesses of each of them but in the text procedural details are only provided 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 (reviewed in **Table 3**). Sleep or sleep loss can be

215 induced by feeding flies with food containing a sleep-altering compound. It is important to keep in mind that drugs may have side effects which extend beyond sleep control and 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.

220 - **Mechanical disruption of sleep.** It is possible to obtain robust sleep deprivation through mechanical stimulation of flies: for instance, tapping the vials where the flies are housed whenever they stop moving[3], spinning the flies upside down to interfere with their geotactic instincts[6,18], or placing flies on a laboratory orbital rotator[19]. Manual sleep deprivation is efficient but incompatible with high throughput experimental conditions; automatic sleep

225 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 naïve experimental fly is suddenly disturbed by another introduced into the arena. Socially-

230 induced sleep suppression may have greater ecological relevance than mechanically induced sleep suppression, but will work only when using male-male coupling (reference 7 and unpublished data, G.F.G.); other kinds of interactions may still induce changes in sleep behaviour that may be interesting to study[8]. Locomotor activity during social interaction must be measured using video tracking techniques; however, completely automatic tracking

235 of multiple flies interacting in an open arena for prolonged times is still difficult for computers and may require significant corrections by a human observer[20,21].

- **Starvation-induced sleep suppression.** This behaviour 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

240   fly food to agar, where they attain their water needs with no nutritional benefit, results in robust suppression of sleep[17,23,24].

Controls. Experimental groups should be carefully matched to reduce any influence of uncontrolled factors, such as the genetic background of the animal. A good description of this problem and how

245   to address it were 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 significantly 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 critical to compare flies of the same sex, age and with similar previous experience. Genotype should also be considered and

250   comparisons within *Drosophila* stocks with dissimilar genetic background should always be avoided. When comparing flies expressing transgenes under 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 animals and their sibling controls, distinguishable by eye colour or, *post hoc,* by PCR genotyping.

255   Sample size should vary as a function of statistical power, that is the difference in the mean values between experimental and control group 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 greatly even within sibling flies. Therefore, sample size should be empirically determined based on the experimental conditions and may vary

260   from as little 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 run in a dedicated environment, preferably in dedicated incubators.

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MATERIALS
REAGENTS

⚐ Fly food: The Bloomington *Drosophila* Stock Centre provides methods and recipes for cooking different kinds of fly media (http://flystocks.bio.indiana.edu/Fly_Work/media-

270   recipes/media-recipes.htm).

⚐ Organic cotton wool (Genesee Scientific, cat. no. 52-101).

EQUIPMENT

275   ⚲ Vials and plugs for raising flies (plastic flat bottom tubes 75 x 23.5 mm, Regina industries, cat. no. P1014L, Regina industries, cat. no. 49-102)

⚲ 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
280   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 1.8GHz processor, 2GB ram, 250Gb disk). A more powerful system will handle many more cameras (e.g.: HP Compaq 8200, Intel i7 2.8GHz processor, 4Gb ram, 500Gb Disk). Number of USB ports is not critical as they can be easily expanded using USB hubs (see below). The computer
285   should be connected to the network for data accessibility.

⚲ Recording and analysis software (http://www.pysolo.net)

⚲ One or more video cameras with USB connection **CRITICAL** The camera must be able to see under infrared (IR) light conditions. Many cameras carry an IR filter that would have to be forcefully removed. Cameras that provide in-built IR-LEDs do not have such a filter and
290   are therefore preferred (we routinely use http://www.ebay.com for inexpensive (<$5) cameras with integrated IR LEDs such as 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 (LEDLighting.co.uk, cat. no. 154798)
295   ⚲ Infrared LED lights (LEDlightsworld.com, 850nm Tri-Chip LED). Both visible and infrared LED strips are sold by 5m reel but can be conveniently cut to fragments of 5cm and wired directly to a power supply.

⚲ A digital mains timer socket (Amazon.co.uk, cat. no. B000TBGWIO)

⚲ Adhesive magnetic strips (RS components, cat. no. 297-9093)
300   ⚲ Food grade plastic container (Lock & Lock Storage 1.6L Medium Container)

⚲ One or more recording arenas to host flies. A purposely designed arena can be custom made using 3D printing (see EQUIPMENT SETUP)

⚲ PETG Clear copolyester sheet (RS Components, cat. no. 334-6444)

⚲ Multi-well cell culture plates (BD Falcon, cat. no. 353503) or glass tubes (Trikinetics,
305   Waltham, MA). 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 conditions of light, temperature and humidity. An artificial light-dark (LD) regimen of 12:12 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 to 5 days after setting up the vial.

Preparing food for the arenas Place a vial with fresh food in the microwave oven and heat for 10 seconds or until melted. Stir food with a pipette to remove clumps and heat few more seconds. For experiments using drug-induced sleep suppression, substitute fly food with a 1% 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 instruction 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 like the one shown in **Supplementary Fig. 1b** is around $20. STL file ready to print is provided as supplementary data and can be open and visualised using the free software meshlab (http://meshlab.sourceforge.net). Many third parties services offer remote 3D printing for comparable prices (e.g.: http://www.shapeways.com). Whether printed in house or by third parties, we recommend stereolithography, as the final product will be smoother and easy to clean under running tap water.

Prepare the lids for the recording arena Cut the copolyester sheet to fit as a sliding cover for the arena (63mm x 100mm, **Fig. 1b** and **Supplementary Figure 1b**). Cut a larger (>80mm x 100 mm) portion of the copolyester to be used as a transferring lid. The transferring lid should be wider than the normal lid and have two holes of about 1mm 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

345   container (i.e.: 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 (5cm) of visible LED lights and place it inside the container to illuminate the flies during

350   subjective day. Regulate timing of light transitions using a digital timer connected to the power supply. See **Figure 1c** for a schematic representation 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

355   humidity (i.e.: ideally an incubator or, if not available, an undisturbed cabinet in the fly room).

PROCEDURE

Preparing the experimental animals **TIMING** 13 days at 25° C

1| Approximately two weeks prior to the planned start of the experiment, prepare one or more

360   vials with the lines to be analysed and compared.

2| Allow flies to mate and lay eggs for 3 to 5 days then remove parents and wait for progeny to develop to imago.

Preparing activity arena **TIMING** 20-30 minute

365   3| Open the arena by sliding the transparent lid and fit the separation walls around the food chamber to avoid spilling.

CRITICAL STEP See **Supplementary Video 3** for a step-by-step description of how to prepare and load an arena.

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

370   5| Using a syringe, transfer melted food to the inner chamber of the arena and then wait for it to solidify (about 10 minutes 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.

375   **PAUSE POINT** Multiple arena can be prepared at once and then stored a 4-10°C for a few days.

Loading the activity arenas TIMING 20 minutes

7| Collect flies 1 or 2 days 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

380   flies are of the same age. If flies must be sorted to separate different genotypes, you may need to use CO_2 anaesthesia 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 using females, make sure to collect virgins; mated female flies will lay eggs

385   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 days old), transfer them to the arena. Flies can

390   be transferred to the arena using two systems: transferring sedated animals (using CO_2 or cold) (option A) or transferring the awake animals using a mouth-pipette (option B). **Supplementary Video 3** contains a detailed explanation of both procedures.

     TROUBLESHOOTING

     (A) Transferring flies using CO_2 sedation

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

       (ii) Using a small paintbrush, transfer each flys from the pad to a single groove in the arena

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

     TROUBLESHOOTING

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

400   (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 animal into the tip of the pipette

405   (iv)Transfer the animal 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 from 8B(ii) until all the required flies have been transferred.

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

410   **TROUBLESHOOTING**

CRITICAL STEP When comparing experimental and control animals, it is always preferable to host both in separate grooves in the same arena, mixing 16 control flies to 16 experimental animals, either randomly or placing 16 flies on each side of the arena. This will reduce the operational variability.

415 Remember to keep a record of fly genotypes and location in the arena

9| Place the arena with flies in the recording box and place the box in the incubator. **CRITICAL STEP** Let the animals 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
420 does have an effect on sleep amounts and regulation so allow one or two 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 minutes

CRITICAL STEP The steps from 10 to 13 are configuration steps, required only once at the
425 beginning of the procedure or whenever the location where they 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| Using your favourite text editor open the configuration file *copyfiles.cfg* and change the
430 relevant parameters. An explanation of each variable is offered in the **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 sake of data safety.

435 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 hour

440 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 option panel to customize the total number of available webcams and monitors, as well as the path where rawdata will be saved (same as *inputpath* in step 11).

14| Restart the program to reload the new settings.

445 **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.**TROUBLESHOOTING**

450 16| Move to the *live view* panel in the program interface.

17| Using the mouse, 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 holding down the left button of your mouse, or click on four arbitrary corners to draw a polygonal irregular area. Click on the

455 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 flies activity as you draw to mask (**Fig 3a**).

CRITICAL STEP Each mask should cover only one groove or channel. Pay special care in

460 drawing accurate borders. If you make any mistake, select the mask by clicking inside it and delete it 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 to save the data (see **Fig. 3b-d** for an explanation of the

465 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 TIMING 1 week

470 24| Start the pySolo-Video-Acquire component.

25| Click on Browse and locate the configuration file you saved on step 22. Hit Open.

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

27| Hit Start to begin tracking

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Interfering with sleep

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: option (A) Mechanical disturbance and option (B)

480 Pharmacological modulation.

TROUBLESHOOTING

(A) Mechanically induced sleep deprivation TIMING 3-24 hours

(i) Record baseline sleep for 48-72 hours

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

(iii)   Switch the orbital rotator on.

CRITICAL STEP High rotation speed, especially if performed for long stretches of time (>3hr) may be damaging and kill the flies. Optimal speed settings may vary depending on the

490   orbital shaker and should be empirically calibrated. Start at very low speed and go higher if necessary. A sleep deprivation of six hours is normally sufficient to induce noticeable effects. Sleep deprivations that extend beyond the 24 hours may result in death. Sick stocks may die even earlier[19]

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

495   incubator.

TROUBLESHOOTING

(B) Pharmacologically induced sleep deprivation TIMING 12-48 hours

Pharmacological sleep deprivation can be achieved by manually transferring flies from an arena with control food to another with drug-enriched food.

500   (i)   Prepare two arena: one filled with control food and one with laced food (either 1% 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 hours.

(iii)   At the beginning of the manipulation time, use a mouth-pipette to move flies from the

505   arena with regular food to the one with laced food. Place the arena back in the recording chamber.

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

510   **CAUTION** Sleep is an evolutionary conserved phenomenon and some drugs may have neurological effects on humans as on flies. Handle with care.

TROUBLESHOOTING

515

29| Measure rebound following sleep deprivation for at least 48 hours.

Cleaning the arena and discarding the flies TIMING 10 minutes

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

520   31| Fill a container with warm water and place the arena inside. Open the lid while 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 in deionised laboratory water and let dry.

33| Optionally, the arena can be washed using 70% ethanol or, depending on the material
525   adopted for printing, placed in a dish washer or autoclaved.

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

TROUBLESHOOTING

530   **Preparing the pySolo software for data analysis TIMING 30 minutes**

34| **Supplementary Video 6 and supplementary manual** provide a detailed description of how to configure and use pySolo for data analysis. Start 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
535   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 (same as *outputpath* from step 11); the extension of the files containing the daily data (*txt* by default); the format of the input data (see also **Fig. 3B-d**). pySolo must have
540   access to the folder where the daily are located to work

Analysing data using pySolo TIMING 30 minutes-2 hours
545   36| In the database window of the program, load your experimental database file or create a new one.

37| Hit 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
550   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 analyse 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

555   sure all the data the programs need are properly accessible and formatted

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

       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 later time. Analysis can also be performed on a different machine since it is now completely

560   independent by the original raw data.

       44| Send the acquire data to the analysis window by either clicking on *Analysis > Send Data to Analysis* or opening the analysis window from *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

565   46| Use the navigation tree on the left to select single genotypes, single days or single animals. 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

570   to sleep deprivation etc. See **Fig. 4** and the **Glossary** 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 1350 minutes a day are considered dead. If necessary, thresholds 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

575   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

580   Troubleshooting advice is provided in **Table 4.**

TIMING

Steps 1-2, preparing the animals: 13 days. 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

585   or sick stocks it is wise to setup more than one vial

Steps 3-6, preparing the arena: 20-30 minutes. Multiple arena can be prepared at once: add 1 more minute per each additional arena

Steps 7-9, loading the arena with the animals: 20 minutes per arena. Transferring awake flies in the arena using a mouth-pipette requires some experience and may be frustrating the first times. Give

590   yourself up to one hour if you are new to this procedure and use some time to practice

Steps 10-12, preparing the software for data fetching: 30 minutes (needs to be done only once).

Steps 13-23, preparing the software for data recording: 30 minutes.

Steps 24-28, recording activity data: about 1 week. See also **Fig. 2** for a better break down of experimental times during data recording.

595   Steps 29-32, cleaning the arena: less than one hour. Preparing and cleaning the arena takes considerably less time than working with regular glass tubes (compare reference 14 to **Supplementary Movie 3)**

Steps 33-34, preparing the software for data analysis: 30 minutes (needs to be done only once).

Steps 35-49, analysing the data: 0.5 – 2 hours. Data analysis can be performed at any time and from

600   any computer on which pySolo is installed.

ANTICIPATED RESULTS

Quality of image acquisition:

Best results require optimal conditions of image acquisition by the camera. A successful setting

605   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 should be limited to areas that are not included in the recording mask.

610   **Internal controls:**

Duration of sleep and response to sleep deprivation will change considerably from line to line, even within wild type stocks[17] whereas pattern 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 pattern when

615   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. Take **Figure 4a** as an example of a good control experiment in which male and female sleep show a clear difference between night and day and where the male *siesta* is clearly visible.

620

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625 **Competing financial interests**

The author declares that he has no competing financial interests.

Figures legend.

Figure 1: Principles of locomotor detection

630   **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.

635   **B.** *Arena for video recording.* Arenas of different shapes and complexities can be produced using 3D printing technology. The cartoon depicted in this figure shows an arena hosting up to 32 flies, 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 animals through

640   holes in a transferring lid (blue circles in the figure). A STL file ready to be used for 3D printing is provided as Supplementary Material (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 panel B. The arena is secured to the inner side of the lid through a paired couple of adhesive magnetic strips. A sheet of clear plastic slides onto the top of

645   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 resist to shaking and manipulations.

650   **Figure 2: Experimental workflow**

   The three main phases of the sleep assay. 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 four to seven days. At the end of the recoding, flies are discarded, the arena cleaned for next use and data analysed (**C**). The steps listed under each drawing refer to the ones described in the

655   experimental procedure.

   Figure 3: Custom devices for sleep detection and interference

   A. *Snapshot of pySolo Video in action.* Nocturnal view of flies in an arena, as seen by the webcam. Animals are in total darkness and infrared illumination comes from underneath the arena through

660   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. Motion of the animal can be recorded in three different formats. **B.** emulation of the infrared beam split. The position of the animal is calculated at every video frame (about 15-25 times a second) but motion is recorded only if the fly is trespassing the transversal midline of the chamber (red line in panels B and A). 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 one second. This latter format is to be considered as the raw data from which the other two can be derived. Additional custom analysis may provide also information on the activity of the animal, including the position held at any single time, speed and directions.

E. *Schematics of how sleep is estimated and processed.* Locomotor activity of the fly is plotted as function of time (leftmost, grey 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 (rightmost bar). In this example, the fly experiences three sleep bouts (black arrows) and two episodes of simple inactivity (blue arrow).

F. *Real activity vs. virtual split-beam.* Activity (upper panel) and sleep (lower panel) of a single fly recorded for 24 hours and plotted calculating the actual distance of the flies as shown in panel **c** (light blue) and the virtual beam-split as explained in panel **b** (pink). Both methods show clearly the daily peaks of activity (two, in this case) which would be used for circadian analysis. However, only by analysing distance can the real activity and sleep be properly estimated and beam-split can overestimate sleep amount by more than 100% (see also reference 13).

Figure 4: Components of Drosophila sleep

A. A hypnogram showing how locomotor activity calculated with the virtual beam split method (upper panel) translates into sleep (lower panel).

The graph shows activity and sleep data for actual male (blue) and female (green) flies over a 24 hour period. Notice the difference in sleep during mid-day (the male *siesta*).

B. Graphic description of fly sleep over a 24 hour 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 labelled '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.

700

Supplementary Figure 1: Photographs of the equipment.

A. The recording monitor.

B. A printed arena. The transparent lid is secured using a rubber band. The middle chamber is filled

705   with food.

C. A3D printed arena compared to a glass tube traditionally used for infrared beam-split analysis. The size of the walking chamber is almost identical.

Supplementary Video 1: Installing pySolo and pySolo video repositories in Ubuntu Linux

710   A brief screencast guiding through the steps necessary to install the pySolo suite in Ubuntu linux adding custom repositories. The repositories will take automatic care of future updates.

Supplementary Video 2: How to build a recording monitor

This video shows what are the the basic components of a recording monitor and what are the

715   criteria to follow to build one of your own

Supplementary Video 3: Preparing the arena for recording

How to prepare an arena for recording: from transferring the food to placing the flies.

720   **Supplementary Video 4: Configuring the *datafetcher* script**

Configuring the datafetcher for automatic fetching and formatting of data. Can be used for pySolo Video or for the original TriKinetics Software.

Supplementary Video 5: An introduction to pySolo Video

725   A brief introduction showing how to configure and use pySolo Video

Supplementary Video 6: Using pySolo for data analysis

A detailed guide with complete instruction on how to use pySolo for data analysis.

730   **Supplementary Table 1: Specialised software for computer assisted behavioural analysis**

Supplementary Table 2: Comparing costs of video tracking and infrared beam-split

735

Supplementary Data

The zip file contains an electronic drawing of a complete arena, as shown in **Supplementary figure 1b-c**. The file, provided here in STL format, was created using the free software Blender (http://www.blender.org) and can be opened and viewed using also the free STL viewer meshlab

740   (http://meshlab.sourceforge.net)

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

| | Split IR beam | | Video tracking |
	Single beam	Multiple beams	
Spatial resolution	Low	medium to high	High
Movement resolution	Binary (crossed / did not cross)	Up to 3mm	Can detect up to sub-millimetrical 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[*]
Cost of a single monitor	$500 - $800	$800 - $3200	$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

745   [*]. 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, also when estimating total costs.

Table 2 | Comparing methods of sleep modulation

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

Table 3 | Drugs used to manipulate sleep

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

750

CPT: 8-Cyclopentyll-1,3-dimethlxanthin

DPMX: 3,7-Dimethyl-1–2-propynylxanthine

W: Increases wake time

S: Increases sleep time.

755

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.
8A	Flies die during transfer using CO_2 anaesthesia	Flies touch the food while still unconscious and get stuck	Pay attention not to push flies against the food
8B	Flies escape when transferring them to the arena using 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	Flies die during transfer using mouth pipette	Inlet hole of the mouth pipette is too small	Increase diameter of the hole
8	Flies escape from the recording arena	Lid is not properly closed	Use a rubber band or scotch tape to close properly the lid and make it adhere to the arena
10	Program crashes or does not start	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
	Program crashes when playing preview	Resolution not supported by the camera	Try setting camera resolution to 640x480
	Program crashes when using multiple cameras	Cameras are drawing too much current	Connect cameras to an USB hub with an independent power supplier
15	Image is blurry	Camera objective is out of focus	Adjust focus manually and, if necessary, apply some glue to lock the lens in place
28	Flies die during sleep manipulation	Sleep deprivation lasting too long	Reduce the duration of sleep deprivation
28B	Flies die during sleep manipulation	Concentration of drug is too high	Reduce the concentration of 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 difficult eating	Make sure that humidity in the incubator is at desired levels
33	Arena bends under warm water	Temperature of the water is too high or material has high resin content	Use colder water or use a different material for printing

Box 1 | GLOSSARY

Arousal stimulus: a sensory stimulus that will induce motion in an otherwise immobile fly. 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 at least five consecutive minutes. Its duration is measured in minutes.

Sleep latency: time passing between light off and the start of the first sleep episode at night. It is used as a read out 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 hours 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 48h 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.
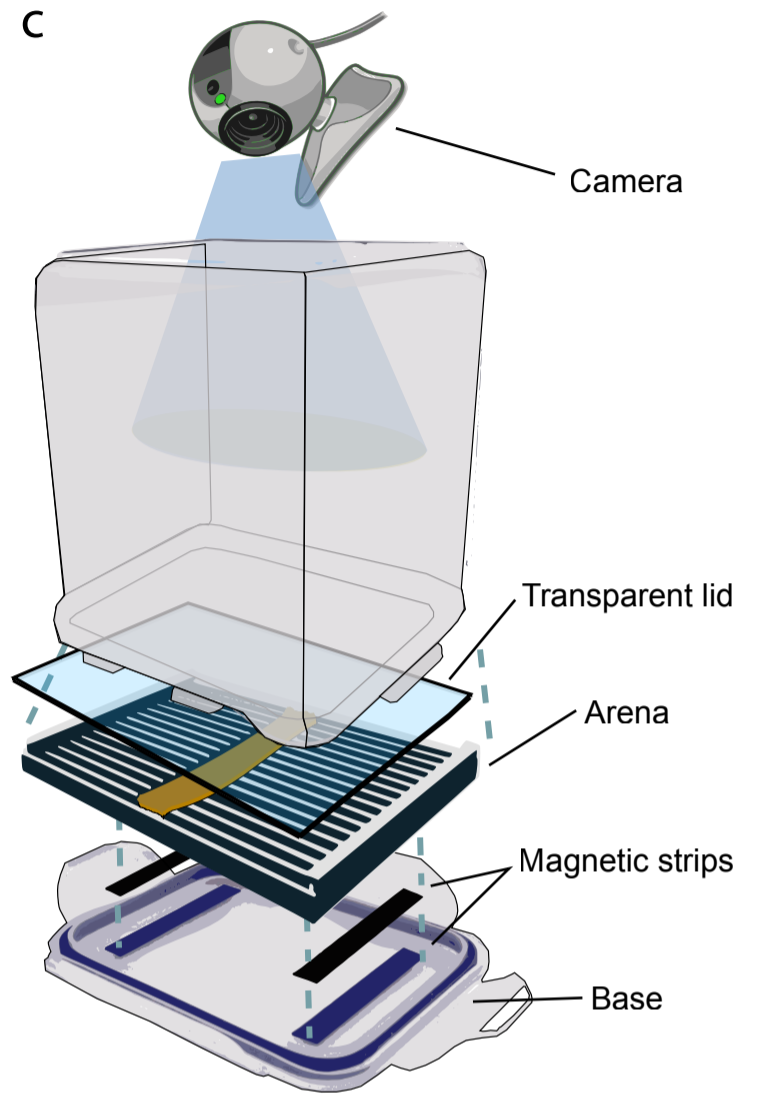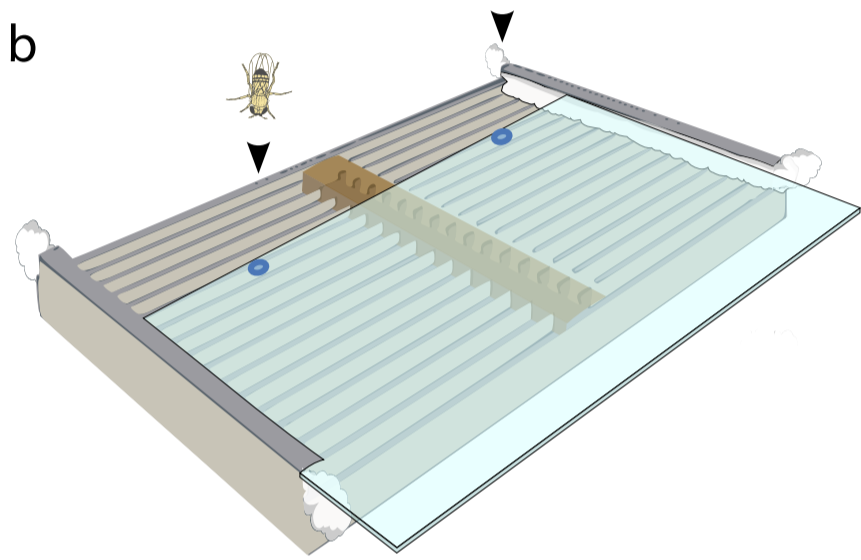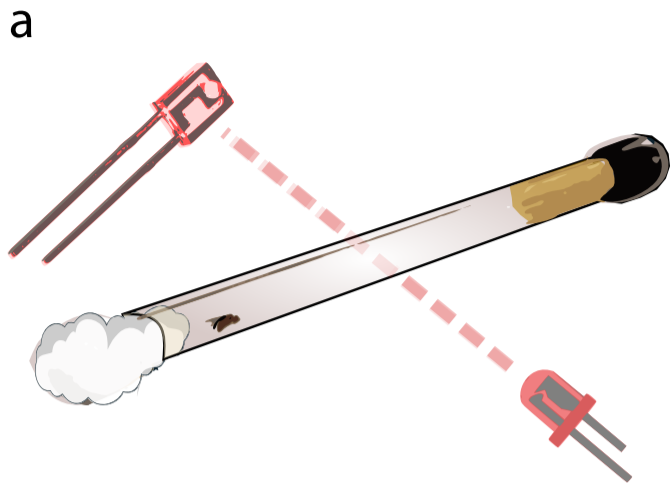
Activity index (AI): a relative measure of locomotor health. AI is calculated dividing the activity count by the total time spent awake in a 24 hours period. This is a way to normalize activity among flies with different total sleep duration.
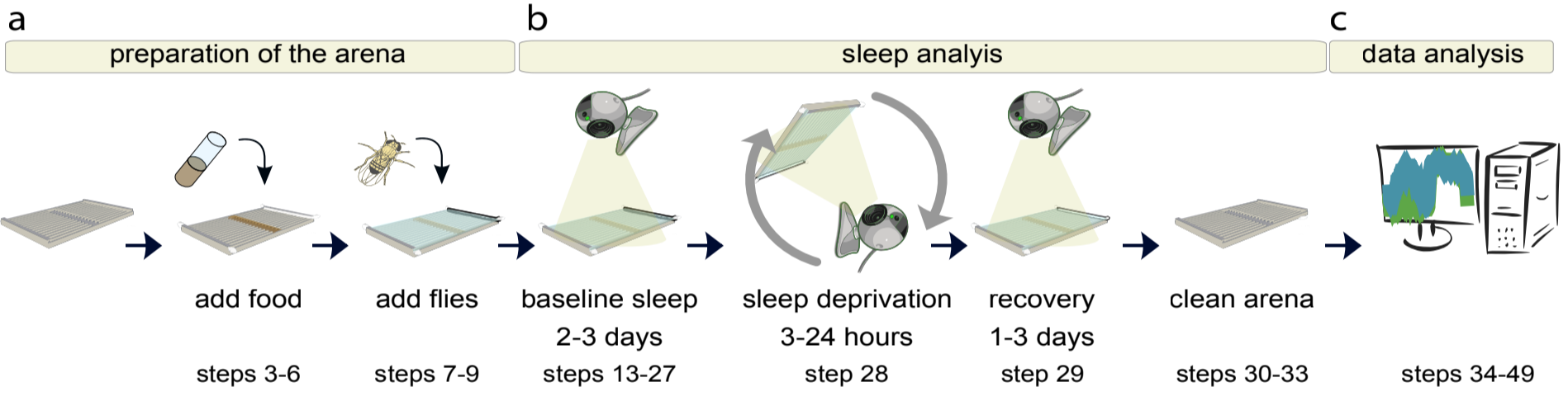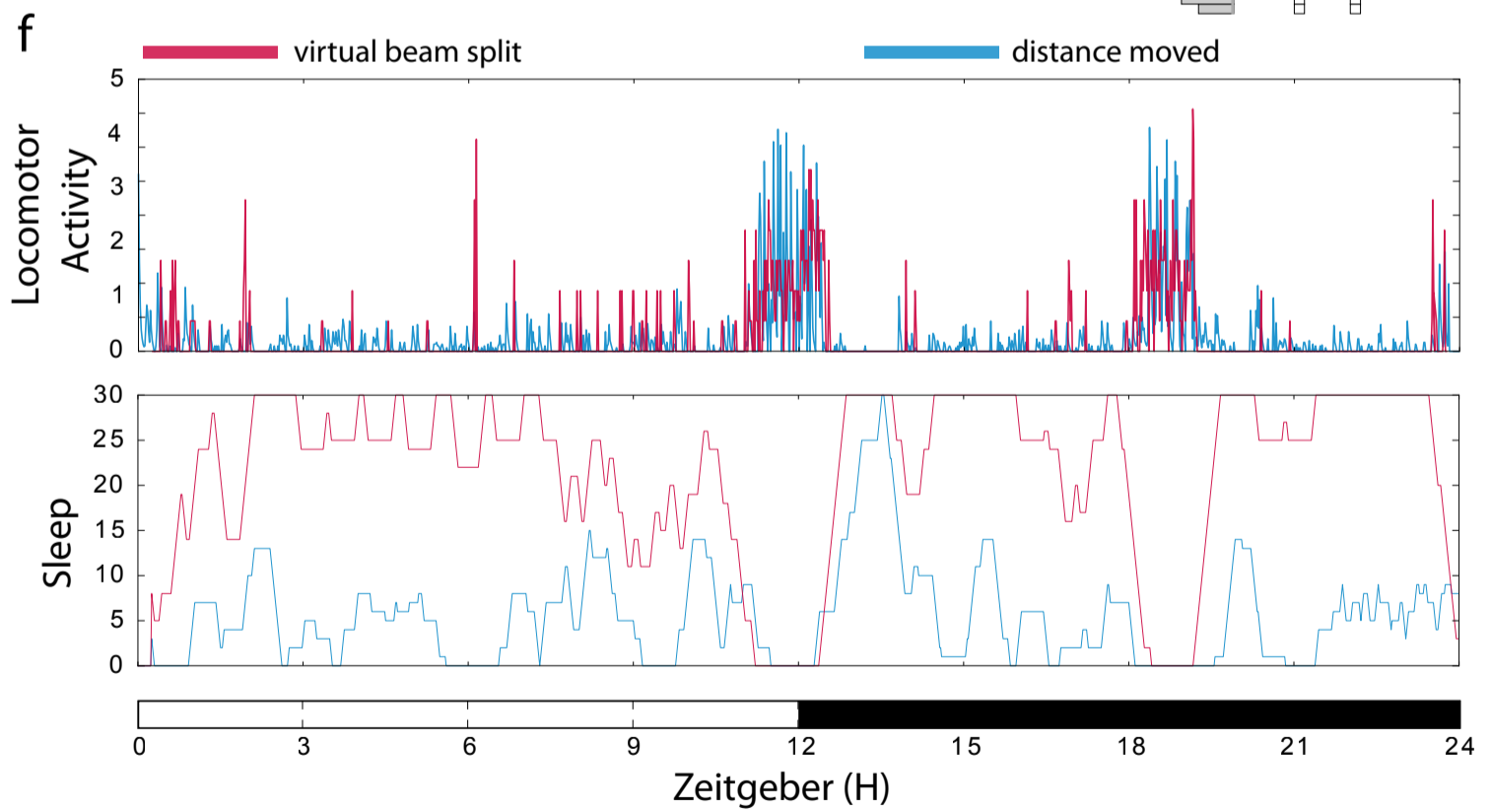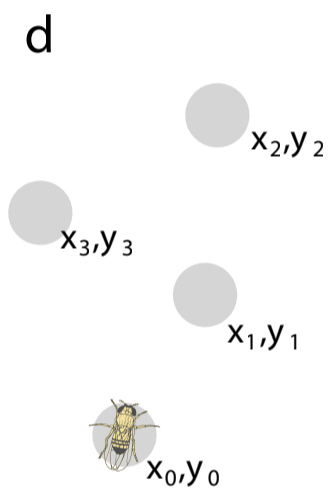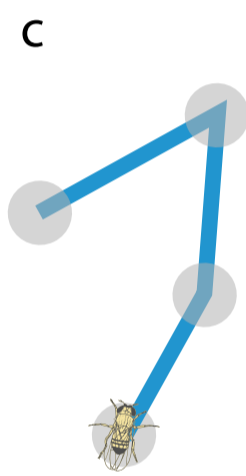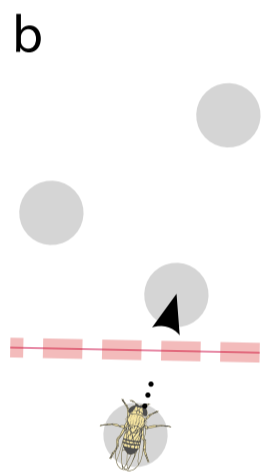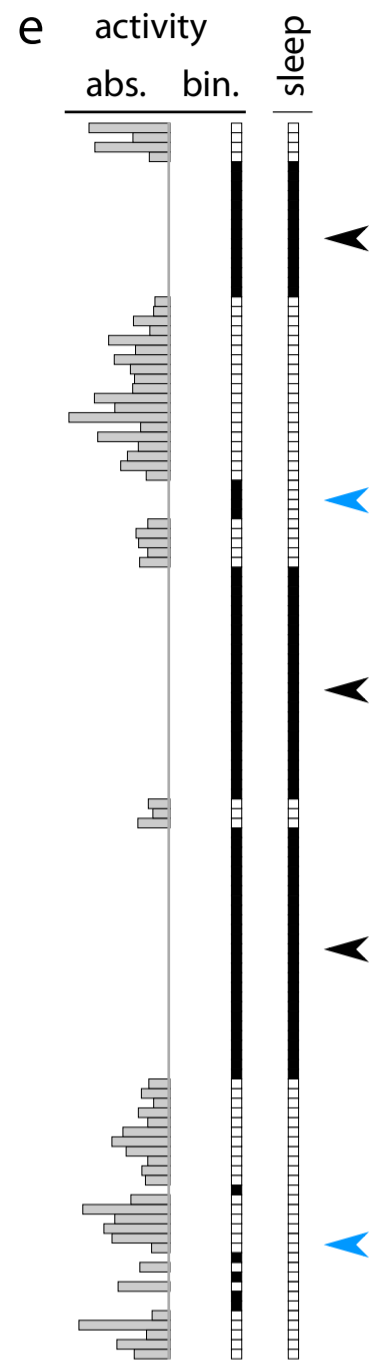
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a

b

c

Camera

Transparent lid

Arena

Magnetic strips

Base

a   preparation of the arena

add food   add flies

steps 3-6   steps 7-9

b   sleep analyis

baseline sleep   sleep deprivation   recovery   clean arena
2-3 days   3-24 hours   1-3 days

steps 13-27   step 28   step 29   steps 30-33

c   data analysis

steps 34-49

a

b

c

d x_2,y_2 x_3,y_3 x_1,y_1 x_0,y_0

e activity
abs. bin. sleep

f

virtual beam split   distance moved

Locomotor Activity

5
4
3
2
1
0

Sleep

30
25
20
15
10
5
0

0   3   6   9   12   15   18   21   24

Zeitgeber (H)

a

Locomotor Activity

Sleep

Zeitgeber (H)

b

normal

disrupted

long

short