

Topic Introduction

Activity Monitoring for Analysis of Sleep in *Drosophila melanogaster*

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Sleep is important for survival, and the need for sleep is conserved across species. In the past two decades, the fruit fly *Drosophila melanogaster* has emerged as a promising system in which to study the genetic, neural, and physiological bases of sleep. Through significant advances in our understanding of the regulation of sleep in flies, the field is poised to address several open questions about sleep, such as how the need for sleep is encoded, how molecular regulators of sleep are situated within brain networks, and what the functions of sleep are. Here, we describe key findings, open questions, and commonly used methods that have been used to inform existing theories and develop new ways of thinking about the function, regulation, and adaptability of sleep behavior.

EXAMINATION OF SLEEP IN *DROSOPHILA MELANOGASTER*

Sleep is a highly conserved neurobehavioral state that is critical for survival. However, many aspects of sleep are still mysterious—in particular, (1) the differences between the sleep state and wakefulness, (2) the mechanisms that regulate sleep, and (3) the purpose(s) of sleep. To explore these aspects of sleep, studies are needed in a range of organisms. The fruit fly *Drosophila melanogaster* was first established as a model system in which to study sleep more than two decades ago. Flies exhibit many key features indicative of a sleep state: They become immobile during specific time periods based on circadian (daily) time, have a preferred rest location, adopt a specific posture, are less sensitive to sensory stimuli during these rest phases, and show rebound sleep after rest deprivation (Hendricks et al. 2000a; Shaw et al. 2000). This indicates that the resting state is under homeostatic regulation, meaning that there is a set point for how much sleep flies need, and that the drive to rest builds up over extended periods of wakefulness. Furthermore, this period of inactivity is associated with lower-frequency oscillations in brain activity as compared to wake and arousal (Nitz et al. 2002; van Swinderen and Greenspan 2003; van Swinderen et al. 2004; van Alphen et al. 2013). *Drosophila* can also be used to study how sleep patterns are modified by other contextual factors in the environment, such as hunger, social interactions, lighting, and temperature states (Keene et al. 2010; Seidner et al. 2015; Chen et al. 2017; Geissmann et al. 2017; Machado et al. 2017; Beckwith and French 2019). As an example, sleep loss associated with starvation and courtship does not induce excessive “rebound” sleep afterward as expected by homeostatic drive alone, suggesting multiple interacting pathways for the control of sleep (Hendricks et al. 2000a; Geissmann et al. 2017; Brown et al. 2020). Thus, it is

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From the *Drosophila* Neurobiology collection, edited by Bing Zhang, Ellie Heckscher, Alex C. Keene, and Scott Waddell.

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Advanced Online Article. Cite this introduction as *Cold Spring Harb Protoc*; doi:10.1101/pdb.top108095

important to develop a holistic view of how and why animals switch between wake and sleep. As has been reviewed elsewhere (Sehgal and Mignot 2011; Allada et al. 2017; Cirelli 2009; Donlea 2017; Tomita et al. 2017), it has become clear that there are broad similarities between the genetic/molecular mechanisms that control sleep in mammals and in fruit flies.

The extensive genetic toolkit available to identify, manipulate, and characterize gene expression and neuronal function in *Drosophila* has significantly advanced our understanding of the mechanisms underlying sleep regulation. Multiple sleep- and wake-promoting neurons have been identified in the fly brain and are located within the mushroom body, central complex, pars intercerebralis, circadian clock network, and other distributed clusters (Joiner et al. 2006; Pitman et al. 2006; Parisky et al. 2008; Shang et al. 2008; Sheeba et al. 2008; Crocker et al. 2010; Aso et al. 2014; Donlea et al. 2014; Kunst et al. 2014; Haynes et al. 2015; Guo et al. 2016; Liu et al. 2016; Sitaraman et al. 2015a,b; Tomita et al. 2021). Recent reviews (Artiushin and Sehgal 2017; Ly et al. 2018; Shafer and Keene 2021) can be consulted for a more exhaustive discussion of these anatomical regions, network connectivity, and molecular mechanisms. Not surprisingly, these findings suggest that sleep regulation requires a complex interaction between multiple brain regions, which was recently reviewed by Shafer and Keene (2021). Since most of these regions have been resolved in an electron microscopy (EM) data set, there is tremendous potential in obtaining the first complete map of the sleep regulatory network in any organism (Scheffer et al. 2020). Interestingly, in recent years, research in *Drosophila* has shown that nonneuronal cells, specifically astrocytes, ensheathing glia, and glial cells of the blood–brain barrier, also regulate sleep parameters (Artiushin et al. 2018; Stahl et al. 2018; Vanderheyden et al. 2018; Davla et al. 2020; Blum et al. 2021; Titos et al. 2023). In addition to the brain, neurons of the ventral nerve cord (VNC), the fruit fly equivalent of the spinal cord, and some gut enteroendocrine cells have also been shown to regulate sleep, and function by signaling to identified sleep circuits in the brain (Titos et al. 2023). Long-term physiological recordings and measurements of sleep intensity/responsiveness in sleeping flies, combined with the role of specific neurons during phases of sleep, suggest that sleep is not a singular state and that stages of sleep can be observed and manipulated in flies (van Alphen et al. 2013, 2021; Faville et al. 2015; Tainton-Heap et al. 2021). Given the similarity between sleep behavior in vertebrates and flies, and the tools available in *Drosophila*, the *Drosophila* sleep field is poised to ask several important questions going forward:

- (1) How do sleep-regulating neurons sense sleep need?
- (2) How do various sensory inputs signal to sleep regulatory circuits?
- (3) How do sleep- and wake-promoting neurons interact within neural circuits?
- (4) How do sleep and circadian neurons interact?
- (5) How do sleep output neurons regulate behavior?
- (6) How do animals balance sleep with other behaviors like courtship, foraging, etc.?
- (7) How do cellular sleep networks intersect with specific gene functions?

To pursue these questions effectively, researchers will need to accurately assess sleep and circadian rhythms. Rest and activity in *Drosophila* are most commonly measured using high-throughput activity-monitoring systems. Here we describe many of the available options for activity monitoring in terms of both hardware and software that allow for high-throughput measurements of rest, activity, and positional preference. We also compare these methods with high-resolution, multiday video recordings as an alternative approach to study sleep behavior (Garbe et al. 2015; Guo et al. 2016).

BEAM BREAKS FOR SLEEP MEASUREMENT IN *DROSOPHILA*

Tracking activity as a way to estimate sleep has been used in several species during the last 50 years. For example, wrist actigraphy has been used in humans, and wheel-running and cage crosses have been used as metrics in rodents (Ibuka and Kawamura 1975; Fisher et al. 2012; Marino et al. 2013). These

techniques avoid the need for surgeries and attachment of electrodes and simplify the data into a single parameter that is simple to analyze. In flies, activity measurements were originally performed in the 1970s as a way to determine flies' circadian rhythms (Konopka and Benzer 1971).

Beam-Break Hardware

Devices to monitor fruit fly activity were developed by TriKinetics, Inc., in collaboration with the laboratories of Michael Rosbash and Jeffrey C. Hall at Brandeis University, and originally consisted of a small plank of wood (these devices are also called "boards") with eight small, clear tubes attached to them. Beams of infrared light passed across the center of each tube and any time a fruit fly placed in the tube crossed the center of its tube, an activity count was recorded (Rosbash 2021). These devices were termed *Drosophila* activity monitors or DAMs and used for multiday measurement of activity. In the late 1990s, this technique was used to estimate sleep (Hendricks et al. 2000a; Shaw et al. 2000). A threshold of 5 min of inactivity (0 beam crosses) was established as a criterion for sleep, with a sleep bout lasting until the next beam cross. The 5-min sleep threshold was chosen because it yielded data that fit well with accepted criteria for sleep, such as (1) altered body position, (2) preferred sleeping location, (3) heightened stimulation threshold for arousal, (4) homeostatic regulation, and (5) circadian regulation (Hendricks et al. 2000a,b). For example, the original studies observed that individual flies only assumed a new crouching posture after a handful of minutes of rest and that longer bouts of rest tended to occur in specific locations of the tube, near but not directly next to their food. The sleep measured in these studies was also organized according to a 24-h circadian rhythm and was observed to increase homeostatically following sleep deprivation (Hendricks et al. 2000a; Shaw et al. 2000). A few years later, local field potential (LFP) recordings from fruit fly brains revealed that electrical activity patterns changed in the brain during rest, but these changes took ~5 min to occur after rest behavior began (Nitz et al. 2002).

Modern DAMs

The original reports described above kick-started the study of sleep in *Drosophila*. Currently, standard DAMs consist of a horizontal, double-rowed arrangement of 32 individual-sized fly tubes, each with its own infrared beam. These have the advantage that lighting from above can provide nearly uniform light intensity across all of the tubes. In addition, simultaneous video recordings can be made of the flies. However, these horizontal monitors are large and block light from reaching monitors that are positioned below them. Vertical DAM2s also contain 32 individual-sized fly tubes, with four rows of eight tubes stacked on top of each other. Activity is measured by two infrared beams that cross each other at right angles at the same central location in the tube. These DAM2s take up much less space than the horizontal DAMs but have the drawback that light shone from above will have a different intensity at the top row than it will at the bottom row. In addition, flies that are in the plane of the monitor itself will be somewhat shielded from direct illumination from above. Nonetheless, DAM2s are currently the most commonly used monitor for *Drosophila* sleep studies.

Additional monitors have also been developed for other specific purposes. For example, DAM5Hs consist of the same horizontal arrangement used by the original DAMs but have 15 independent infrared beams crossing each tube. DAM5Ms are similar, but array their 32 tubes in one long row and contain four infrared beams per tube instead of 15. Multibeam activity monitors (MB5s) house 16 tubes within a box-like chassis that provides 17 beams per tube. MB5s use slightly longer tubes (80 mm) than the standard 65-mm length and can record from tubes ranging from the standard width of 5 mm up to 10 mm. The main advantage of DAM5H, DAM5M, and MB5 monitor types is that they allow for detection of each fly's location as well as its activity. This can be very useful for studies of location preference in combination with activity monitoring. They also allow greater stringency in sleep measurements, because any single beam break can be set to reset a sleep bout (whereas in the original DAMs and DAM2s, a fly must cross the center of the tube to register a beam break).

DAMs for Monitoring Multiple Flies

Locomotor activity monitors (LAMs) and *Drosophila* population monitors (DPMs) are systems that can monitor multiple flies simultaneously. LAMs allow for monitoring of rhythms and sleep in animals of different sizes, offering three different sizes of tubes. These are available with either a central ring of infrared beams or three such rings. DPMs can fit a standard vial containing up to 50 flies at a time. DPMs have three rings of infrared beams that encircle each tube, allowing for activity counts to be measured from the entire population at once. Although LAMs and DPMs can both be used to monitor populations of small fly species, individual fly activity among a population cannot be tracked using these systems, making it difficult to know whether any individual fly has reached the standard sleep criterion of 5 min of inactivity. However, both systems can be used to assess the role of social interaction on activity patterns. The commercialization of infrared-based beam break hardware and ease of installation and use by TriKinetics, Inc., have made these systems accessible and widely adopted for behavioral assays in *Drosophila* and other insects.

Beam-Break Software

Several software-based analysis tools have been developed to analyze beam-break data. One option is called ClockLab, available from ActiMetrics, which sells both hardware for studies of circadian rhythms and software for data analysis and was developed by David Ferster at Northwestern University (<https://actimetrics.com/products/clocklab/>). However, this is a fairly expensive option, and the software does not explicitly measure sleep parameters, only metrics dealing with rhythmicity. An alternative called Faas was developed by Michel Boudinot and François Rouyer at Paris-Saclay Institute of Neuroscience (<https://neuropsi.cnrs.fr/en/departments/cnn/group-leader-francois-rouyer/>). Faas is a freely downloadable 64-bit application that requires no other expensive software to function. However, it works exclusively on Macintosh computers, and the supported version requires MacOS 10.14 and above. Another program called ActogramJ, developed by Benjamin Schmid, Charlotte Helfrich-Förster, and Taishi Yoshii, functions as an ImageJ plug-in (Schmid et al. 2011). RhythmicAlly (Abhilash and Sheeba 2019) is a newer option from the laboratory of Vasu Sheeba that is based in the software R and the ShinyR-DAM package developed by the laboratory of Jay Hirsh (Cichewicz and Hirsh 2018). RhythmicAlly allows for analysis of both circadian and ultradian rhythms and works on most operating systems but requires a fair amount of setup and integration of other software packages. Like ClockLab, Faas, ActogramJ, and RhythmicAlly are very useful for circadian analysis but are not designed to examine sleep parameters.

There are several software packages for use with flies that examine sleep specifically. The first is pySolo, originally generated by Giorgio Gilestro in the laboratory of Chiara Cirelli (Gilestro and Cirelli 2009). This free software package based in Python can run on multiple operating systems but can be challenging to set up and run effectively for new users. Another option is Rethomics, generated by Quentin Geissmann in the laboratory of Giorgio Gilestro (Geissmann et al. 2019), which is also free and is based in R. Rethomics allows for the analysis of some sleep metrics, although it is still primarily focused on circadian analysis and requires substantial manual code entry during setup. Recently, a package called Rtivity was developed that uses Shiny and Rethomics packages in R to enable customizable analysis of activity data from a variety of species (Silva et al. 2022). Rtivity improves on ease of use compared with earlier analysis programs and can analyze several metrics about activity and circadian rhythms, as well as sleep parameters such as total sleep time, sleep bout duration, and latency to first sleep episode after lights off. A very recently published MATLAB-based analysis package called PHASE was developed jointly in the laboratories of Maria Fernandez and Orie Shafer (Persons et al. 2022). This program is multifaceted but most notably is helpful for identifying the phase during a circadian cycle when an animal's activity peak occurs, allowing for quantification of the timing of those peaks and the assessment of behavioral anticipation of environmental stimuli.

Another option that combines circadian and sleep analysis is the Sleep and Circadian Analysis MATLAB Program (SCAMP), generated by Chris Vecsey as a member of the laboratory of Leslie Griffith and originally reported by Donelson et al. (2012). SCAMP has a few downsides, in that it

requires some preprocessing of raw data using the DAMFileScan software available freely from the Trikinetics website and requires users to have access to MATLAB software. Nonetheless, SCAMP also has many strengths—it requires no coding, is based entirely on easy-to-use interfaces, generates both graphical and tabular outputs, and can generate data on seven circadian and 21 sleep parameters, the latter of which can be assessed over a variety of bin lengths throughout the day. These sleep parameters uniquely include probabilistic measures of the likelihood of transitions between sleep and wake states, as reported by Timothy Wiggin in the laboratory of Leslie Griffith (Wiggin et al. 2020). SCAMP has been updated and expanded repeatedly, and is actively maintained and supported. The most recent version (SCAMP_v4; see Protocol: Analysis of Sleep and Circadian Rhythms from *Drosophila* Activity-Monitoring Data using SCAMP [Vecsey et al. 2024b]) offers multiple new features, including improved circadian graphical output and the ability to graph comparisons of sleep parameters across days. This can be useful for examining how any type of manipulation affects sleep compared with a baseline period. For example, if neurons are conditionally activated, SCAMP can easily graph changes in sleep amount during the activation day compared with a baseline day [see Protocol: Neural Stimulation During *Drosophila* Activity Monitor (DAM)-Based Studies of Sleep and Circadian Rhythms in *Drosophila melanogaster* (Vecsey et al. 2024a) and Protocol: Analysis of Sleep and Circadian Rhythms from *Drosophila* Activity-Monitoring Data using SCAMP (Vecsey et al. 2024b)]. Importantly, SCAMP_v4 can also now analyze data from an updated DAM containing four separate infrared beams along the length of the tube (DAM5M). Having multiple beams allows for finer analysis of fly movement and position, which can be used for multiple purposes, including determining flies' preference for different foods placed at either end of the tube (see Protocol: Analysis of Positional Preference in *Drosophila* Using Multibeam Activity Monitors [Porter et al. 2024]).

AN ALTERNATIVE METHOD FOR SLEEP MEASUREMENT IN *DROSOPHILA*: VIDEO-BASED TRACKING

Although this review and associated protocols focus on activity monitoring based on beam breaks, it is worth noting that many laboratories also use video-based tracking to study sleep. In fact, high-resolution video was used in the original study identifying rest as a sleep-like state in flies, allowing the researchers to show that flies have a preferred sleeping location and posture (Hendricks et al. 2000a). More recent reports have specifically compared video and beam-break estimates of sleep time and structure (bout number and duration) (Zimmerman et al. 2008; Donelson et al. 2012; Garbe et al. 2015; Guo et al. 2016; Geissmann et al. 2017, 2019). Video-based systems compare consecutive frames and set a threshold for the number of pixels that have to change between frames in order to characterize the fly as having moved. This change is expressed as a percentage of fly body length (FBL). This technique has the large advantage of capturing much smaller movements than can be detected with a single beam in the center of a tube. However, direct comparisons suggest that beam-break and video-based monitoring yield measurements of sleep that are perhaps surprisingly similar. For example, video monitoring using a 100% FBL threshold found no significant differences in sleep compared with beam-break data. Only when the FBL was dropped to 50% was the estimated time asleep significantly reduced (Donelson et al. 2012), indicating that one must consider movements of less than the length of the fly as full arousals from sleep in order for video and beam-break analysis to diverge. It is worth noting that many users of video tracking software report that there is a high error rate that requires user input to proofread (Donelson et al. 2012; Garbe et al. 2015). Compared to beam-break analysis, video analysis is relatively lower throughput, requires much greater data storage, and is much more challenging to set up and calibrate across experiments but does have several important advantages—it allows researchers to use a variety of different-shaped arenas (although they still need to be flat in order to maintain focus, and a uniform size of the fly); it provides flexibility in the criteria for sleep; it captures detailed information about position preference; if multifly tracking is being used, it allows for social interactions and sleep to be analyzed concurrently; and in particular it provides an opportunity to analyze the ultrastructure of sleep patterns in more detail than is possible using beam-break

analysis. For example, studies using high-resolution video have suggested that flies experience multiple stages of sleep, including a stage that consists of small movements such as proboscis extensions, which would have not been detected by beam-break analysis (van Alphen et al. 2013, 2021). Hence, the ability to detect and record real-time activity via automated tracking has the potential to reveal new insights into sleep structure and function. Currently, as both video and beam-break measurements are made with a single fly in the area, it is unclear how sleep behavior is modified in groups. Although tracking and behavioral annotation have been established for group fly behaviors occurring in shorter timescales (minutes to a few hours) (Branson et al. 2009; Kabra et al. 2013), the ability to track flies over days in groups without mixing identities has been challenging. Both beam-break and video analysis have utility for studying sleep. Beam-break studies are advantageous for large-scale screening and are typically sufficiently accurate to detect differences in sleep patterns between experimental groups. However, video analysis allows researchers to examine behavior at a higher resolution, enabling a more thorough assessment of sleep characteristics.

OPTOGENETIC APPROACHES TO STUDYING SLEEP IN *DROSOPHILA*

To determine the roles of specific populations of cells in dictating sleep patterns, it is imperative for researchers to be able to manipulate neurons (and nonneuronal cells) to observe the effects on behavior. Optogenetics, thermogenetics, and chemogenetics compose a cluster of techniques that permit activation or inhibition of specific neurons—or, in some cases, specific intracellular pathways—at specific times of an experimenter's choosing, in response to light, heat, and chemicals, respectively (Bernstein et al. 2012; Becnel et al. 2013). The primary differences between these approaches are the modality of the environmental stimulus used to stimulate the sensor, the speed with which the sensor can be activated/deactivated, and whether the sensor is capable of activation or inhibition (Table 1).

In optogenetic and thermogenetic approaches, flies are genetically modified so that cells of interest express a protein sensor, either for light or temperature, allowing those cells to be stimulated electrically by “remote control.” Cell populations can be targeted using binary expression systems such as the GAL4/UAS system or more recently developed alternatives such as LexA/LexAop, QF2/QUAS, etc. (Brand and Perrimon 1993; Lai and Lee 2006; Potter et al. 2010).

Optogenetic approaches to study sleep and arousal have been fairly well established in mammals (Tyree and de Lecea 2017), but a growing number of studies have applied optogenetic methods to the examination of sleep mechanisms in *Drosophila* (see the Table in Supplemental Document 1, available online at <https://doi.org/10.6084/m9.figshare.24230701.v1>). One of the most straightforward uses of optogenetics to study sleep has been to activate neurons of interest using targeted genetic drivers and observe effects on sleep/waking behavior. Optogenetics, however, can be used in varied creative ways in *Drosophila* to explore circuit-, cellular-, and molecular-level mechanisms of sleep regulation.

Benefits of Optogenetic Approaches

Optogenetic stimulation has several benefits for studying the roles of different neural populations in regulating sleep:

1. When studying sleep, it is critical to allow the animal to continue to engage in its normal behavior with as little disturbance as possible. By triggering neural activation remotely, optogenetic activation allows flies to be physically undisturbed during the experiment. Red-light sensors in particular are beneficial because red light can reach the brain easily across the fly cuticle (Klapoetke et al. 2014), allowing for neural activation in intact, freely moving flies. Red light is also less disruptive to natural sleep and circadian rhythms than higher-intensity green or blue light (Helfrich-Förster 2020).
2. Optogenetics has rapid on/off kinetics; this is where optogenetics really “shines” in comparison with other activation techniques. Because optogenetic stimulation with light can be applied over a time-

TABLE 1. Comparison of techniques for conditional neural manipulation

Technique	Modality of activation	Timescale	Existing options for neural inhibition?	Examples of sensors	Function	Reference(s)
Optogenetics	Light	Milliseconds to hours	Yes	Channelrhodopsin (ChR2)	Blue-light activation	Developed by Schroll et al. (2006)
				ChR2.XXL	Blue-light activation	Developed by Dawydow et al. (2014)
				CsChrimson	Red-light activation (strong)	Developed by Klapoetke et al. (2014); used by Juneau et al. (2019), Pimentel et al. (2016), e.g.
				ReaChR	Red-light activation	Developed by Inagaki et al. (2014)
				Gtacr1	Green-light inhibition	Developed by Mohammad et al. (2017); used by Guo et al. (2018), e.g.
Thermogenetics	Temperature	Minutes to days	Yes	Halorhodopsin (eNPHR)	Green/amber-light inhibition	Developed by Inada et al. (2011)
				dTRPA1	Warmth activation (>27°C)	Developed by Hamada et al. (2008); used by Blum et al. (2021), Donlea et al. (2011); Sitaraman et al. (2015a)
				rTRPM8	Cold activation (<18°C)	Developed by Peabody et al. (2009)
Chemogenetics	Chemical (in food)	Hours to days	Yes	Temperature-sensitive Shibire (Shi ^{ts1})	Warmth inhibition (>29°C)	Developed by Kitamoto (2002); used by Pitman et al. (2006)
				hm3Dq	CNO activation (G _q)	Developed and used by Becnel et al. (2013)
				hm4Di	CNO inhibition (G _i)	Developed and used by Becnel et al. (2013)

In the References column, “Developed by” refers specifically to development of the tool for use in the fruit fly *Drosophila melanogaster*. “Used by” refers specifically to studies that used the tool in *Drosophila* to examine sleep and/or circadian rhythms.

scale that ranges down to milliseconds, researchers have been able to examine the effects of even momentary neuronal activation that can be timed to specific points across the day. For example, Juneau et al. (2019) used optogenetics to determine that even a brief (3-sec) period of activation of short neuropeptide (sNPF) neurons was able to induce a long-lasting period of sleep, and that the timing of the increase in sleep relative to the period of activation varied depending on the time of day of stimulation. Often, individual action potentials can be time-locked to each short pulse of light. Some studies have shown that optogenetic pulsing regimes can induce exactly the desired firing patterns in the targeted neurons, allowing for an examination of how particular firing patterns, beyond simply overall firing rates, in specific neurons influence sleep/wake behavior (Klapoetke et al. 2014). For example, the rapid kinetics of optogenetic activation have been leveraged to induce differing patterns of neuronal activity that are normally observed during daytime versus nighttime (Tabuchi et al. 2018). Interestingly, activating a specific cluster of clock neurons (DN1p) at night using a daytime pattern switched flies into a daytime pattern of rest/activity, indicating that these firing patterns were sufficiently instructive to drive sleep/wake behavior.

- Many optogenetic sensors, with varying properties, are available, allowing researchers to select the best option for their specific application. Among activators, there are blue-light sensors ChR2 (Boyden et al. 2005) and ChR2.XXL (Dawydow et al. 2014) and red-light sensors ReaChR (Lin et al. 2013), CsChrimson, and ChrimsonR (Klapoetke et al. 2014). Among inhibitors, there are green-light sensors eNpHR3.0 (Gradinaru et al. 2010) and GtACR1 (Govorunova et al. 2015). Most of these exist with UAS, LexAop, and QUAS control systems, and for many sensors there are versions

available with different numbers of UAS sequence repeats, allowing for variable expression levels of the sensor and therefore different effector strengths (Brand et al. 1994; Lai and Lee 2006; Potter et al. 2010).

4. Sensors can often be used in tandem with each other or with an ever-growing and freely available collection of *Drosophila* genetic tools. For example, because red-light activation of CsChrimson will not activate ChR2 or GtACR1, they can be used together with one set of neurons being activated by CsChrimson and a different set of neurons being activated or inhibited by a different sensor. However, it is important to note that blue light can activate CsChrimson if it is fairly bright, complicating these tandem sensor studies. Red light will also not interfere with infrared imaging of fly movement, allowing optogenetic stimulation to be performed while also tracking fly locomotion and other behaviors. Similarly, red light will not disrupt many types of live-cell fluorescent imaging, such as GCaMP for calcium (Nakai et al. 2001; Ohkura et al. 2005; Tian et al. 2009; Akerboom et al. 2012, 2013; Chen et al. 2013), EPAC-cAMPs for cyclic AMP (Shafer et al. 2008), and several sensors for voltage recently reviewed by Panzera and Hoppa (2019). This allows for researchers to carry out functional imaging studies while also activating neurons optogenetically. However, a consideration is that the light source for imaging can activate the optogenetic sensor, so these studies must be designed carefully with appropriate controls.
5. Several other tools, such as the temperature-sensitive GAL80 repressor (GAL80^{ts}) and the steroid-inducible GeneSwitch system (Osterwalder et al. 2001; McGuire et al. 2004), can be used to conditionally refine and manipulate the spatial and temporal expression of optogenetic (and thermogenetic) sensors. Optogenetic stimulation has also been used in a closed-loop feedback system, where optogenetic activation of a specific cell type (e.g., helicon cells within the central complex) was triggered only when flies reached a 3-min threshold of inactivity, allowing researchers to show a role for those cells in wake promotion (Donlea et al. 2018).
6. Optogenetic (and thermogenetic) activation of a neuronal population can also be paired with other manipulations to look for interactions. For example, optogenetic sleep promotion has been combined with presentation of arousal stimuli to show that sNPF neuron activation reduces but does not eliminate arousability (Juneau et al. 2019). Another example is the application of the sleep-promoting GABA agonist gaboxadol during optogenetic activation of sleep-promoting dorsal fan-shaped body (dFSB) neurons within the central complex (Donlea et al. 2011), which revealed that dFSB-mediated changes in local field potentials in the fly brain could be prevented by gaboxadol (Yap et al. 2017).
7. Optogenetic approaches can also be used to determine the functional connectivity of neurons within sleep regulatory circuits. One population of neurons can be activated optogenetically, while other cells are recorded from electrically or assessed using fluorescent calcium sensors. In one particular study, brief optogenetic activation of dopaminergic neurons using red light resulted in electrical silencing in dFSB neurons that correlated with an increase in wakefulness (Pimentel et al. 2016). This experiment showed that dopaminergic neurons functionally inhibit dFSB neurons, and that this effect is likely a mechanism of shifting flies from sleepy to wakeful states.

Limitations of Optogenetic Approaches

Like all scientific techniques, the use of optogenetics to study sleep has limitations that researchers should be aware of:

1. Optogenetic activation requires the application of light, which has the potential to disrupt sleep and rhythms. In mammalian studies, light can be delivered via fiber-optic cables surgically directed to specific desired locations in the brain (Fiala et al. 2010). In studies in *Drosophila*, this issue can be lessened by using sensors such as CsChrimson that can respond to light in the red range, which has less of an effect on sleep and rhythms than shorter wavelengths of visible light. However, typical “red”-light-emitting diodes (LEDs) with a peak at 630 nm are still visible to *Drosophila* and can

disrupt sleep, especially when applied at times of day when flies are already sleeping (Juneau et al. 2019).

2. Optogenetic activation requires feeding the flies the cofactor all-*trans*-retinal (ATR), adding another variable that must be controlled. Controls can be used that omit ATR, so that genetics are identical in experimental and control flies, and additional controls can be used that have ATR but do not express the red-light sensor. It can take a few days of feeding before the optogenetic sensor is fully activatable, so ATR feeding must be started at least 3–4 d in advance of experimentation. However, ATR can bleach in the presence of light, and once the optogenetic sensor is supplied with ATR, ambient light in the environment can cause unwanted activation of the optogenetic sensor. Therefore, once ATR is provided, flies need to be housed either in complete darkness or under very dim light (just bright enough to allow for circadian entrainment).
3. Optogenetic sensors can be “leaky,” meaning that they may activate the neurons where they are expressed even in the absence of light. We and others (N Stavropoulos, pers. comm.) have noticed this phenomenon in particular with the commonly used 20xUAS-CsChrimson construct (Klapoetke et al. 2014), although other sensors may also have similar effects. Whether or not this is an issue appears to depend on the particular driver line that is being used. In our experience, these effects seem to be more pronounced in male than in female flies and seem to build up over successive days as animals age (Juneau et al. 2019). If leakiness seems to be an issue in a given experiment, researchers could switch to using CsChrimson lines that have fewer UAS repeats (5× and 10×); other variants of Chrimson, such as ChrimsonR and Chrimson-TdTomato; or different sensors entirely, such as ReachR (Inagaki et al. 2014).
4. Optogenetic sensors may not be able to maintain neuronal spiking during continuous light exposure, although they can cause persistent levels of depolarization. This is one area where temperature-based thermogenetic activation can be advantageous, since adaptation seems to be much less of an issue when using dTRPA1 to activate neurons (Pulver et al. 2009). This issue can be avoided by using pulsed stimulation instead of constant light application, which has been shown to be able to induce repeated pulse-locked spiking (Tabuchi et al. 2018).

Methodological Considerations

Based on the benefits and limitations mentioned above, studies using optogenetics to examine sleep have varied the following notable aspects of their methodology (see the Table in Supplemental Document 1, available online at <https://doi.org/10.6084/m9.figshare.24230701.v1>):

1. **ATR cofactor treatment:** ATR must minimally be present at the time of stimulation with light for optogenetic sensors to absorb and respond to light. However, most studies expose flies to ATR for some period beforehand, ranging from a few days to the entire life of the animal. Researchers also use various concentrations of ATR, ranging from 0.4 to 2 mM. We have observed that maximal responsiveness can be achieved by feeding flies ATR for at least 3–4 d before experimentation.
2. **Lighting conditions during development of experimental flies:** The rearing conditions of flies depend on the presence or absence of ATR. If flies are raised in the absence of ATR, they can be entrained in standard light/dark (LD) conditions during development. In this situation, once experimental adult flies have been collected, they should be transferred onto ATR in total/constant dark (DD) conditions for at least 3–4 d before running sleep experiments. Conversely, if flies are raised on ATR food during development, they should not be exposed to bright light, as this will cause ATR to bleach and become ineffective. Therefore, studies in which ATR is administered throughout development typically involve flies raised in constant darkness. In our experience, adult flies’ circadian cycles during activity-monitoring experiments do not drift apart while being raised in constant darkness as one might expect. However, we have observed that circadian phase can be affected by when flies are exposed to light during loading into sleep monitors. Thus, it is important to load flies from all groups at the same time of day.

3. *Background lighting conditions during experiment:* Many studies maintain flies in constant darkness during the data collection portion of the sleep experiment until the moment of optogenetic stimulation with the appropriate color light (usually red). This method avoids unwanted activation of the optogenetic sensor but has the disadvantage that flies will be free-running in constant darkness, so any optogenetic experiment will have to be performed in that context. However, some studies have used a 12-h/12-h light/dark schedule throughout the experiment using relatively dim white or blue light. It is worth noting that this has sometimes caused background changes in sleep in the experimental animals, presumably due to low-level activation of the optogenetic sensor by the daily light being provided (Guo et al. 2018). Whether this is problematic in a given experiment will likely depend on which sensor is being used, which cells are being targeted, and the brightness and spectrum of the daily light being provided.
4. *Optogenetic light source characteristics:* Many different types of light sources have been used for optogenetic sleep experiments. Most studies have used red-emitting LEDs, typically with a peak emission of 615–630 nm. Depending on whether the goal is to illuminate a large number of DAMs filled with flies, a single fly at a time, or something in between, researchers have used 12-in square LED grids or single ultrabright LEDs, which optionally can be fitted with lenses to focus the light on the subject. Green LEDs with peak emission in the range of 540 nm have been used to activate the inhibitory sensor GtACR1 (Mohammad et al. 2017), and blue LEDs with peak emission at 458 nm have been used to activate the adenylate cyclase-activating sensor EPAC (Shafer et al. 2008).
5. *Timing and intensity of optogenetic stimulation:* Optogenetic stimulation can be applied as a single light pulse or repeated pulses. In the single-pulse strategy, pulses have ranged from 0.5 sec up to 24 h. In the pulsing strategy, many different paradigms have been used, but most stimulation frequencies have ranged from 1 to 20 Hz, with pulses typically 3–5 msec in duration. These pulsing stimuli have often been applied over long periods of time, on the order of 12–24 h. In addition to timing, the brightness of light stimulation affects how intensely the targeted neurons will be activated/silenced. Most studies have estimated that the brightness of their light stimulation was between 0.08 and 0.28 mW/mm², although flies will experience some variability in the light intensity when activating large populations of animals based on the geometry of their locations relative to the light source.

THERMOGENETIC APPROACHES TO STUDYING SLEEP IN *DROSOPHILA*

Thermogenetic tools that use temperature changes to activate or inhibit neurons have been used extensively in *Drosophila* neurobiology for the last two decades (Kitamoto 2002; Hamada et al. 2008). TRP channels are a class of evolutionarily conserved cation channels that transduce chemical and temperature inputs by altering membrane potential or intracellular calcium (Ca²⁺) concentration (Samanta et al. 2018). Two temperature-sensitive Trps have been developed as thermogenetic tools and are used in flies to study behavior: *Drosophila* TRPA1 (dTRPA1) and rat TRPM8 (Hamada et al. 2008; Peabody et al. 2009). Our associated protocol describes thermogenetic stimulation via dTRPA1 during activity monitoring [see Protocol: Neural Stimulation During *Drosophila* Activity Monitor (DAM)-Based Studies of Sleep and Circadian Rhythms in *Drosophila melanogaster* (Vecsey et al. 2024a)]. Temperature-dependent inactivation for high-throughput screening can be implemented using a different tool called Shibire (Shi^{ts1}). Shi^{ts1} codes for a temperature-sensitive mutant form of the *Drosophila* dynamin ortholog, which blocks vesicle endocytosis and limits the release of neurotransmitters when the temperature is raised above 29°C (Kitamoto 2002). Thus, Shi^{ts1} is a neuronal inhibitor rather than an activator like dTRPA1. Like dTRPA1, Shi^{ts1} has also been used for high-throughput screening for sleep phenotypes (Pitman et al. 2006).

Benefits of Thermogenetic Approaches

Thermogenetic stimulation has several benefits for studying the roles of different neural populations in regulating sleep:

1. In the context of studying the neural basis of behavior, dTRPA1 and Shi^{ts1} have proven to be extremely useful, as they can be expressed in single neurons or subsets of neurons to activate them within the preferred temperature range of 25°C–29°C (Kitamoto 2002; Hamada et al. 2008; Pulver et al. 2009).
2. dTRPA1 shows minimal adaptation in flies, meaning that it will continue to activate neurons as long as the temperature continues to be warm enough to open the channel, making it an excellent tool of choice for long-term activation ranging from hours to up to days in intact animals (Pulver et al. 2009). Most sleep studies using dTRPA1 are performed by simply shifting the temperature from a relatively cool baseline of 21°C–25°C to ~27°C–31°C, depending on the strength of the promoter or how much stimulation produces an observable phenotype.
3. Neither dTRPA1 nor Shi^{ts1} requires a cofactor, and temperature changes can be implemented in standard incubators, making them ideal for high-throughput screening. In fact, screening of various driver lines (GAL4 and split-GAL4) with dTRPA1 and Shi^{ts1} has revealed key regions of the brain involved in sleep and wakefulness, including but not limited to the mushroom body, central complex, clock neurons, pars intercerebralis, and aminergic and peptidergic clusters (Pitman et al. 2006; Parisky et al. 2008; Donlea et al. 2011; Sitaraman et al. 2015a; Liu et al. 2016). Identification of these regions has clarified how they interact and produce behavioral output relevant to sleep and wakefulness.

Limitations of Thermogenetic Approaches

1. Although the ease of implementation and minimal inactivation make dTRPA1 an excellent tool of choice for neural stimulation experiments, especially long-term ones, it must be noted that even small changes in temperature can significantly alter sleep behavior in wild-type flies independently of any targeted thermogenetic activation (Donlea et al. 2011; Sitaraman et al. 2015a; Liu et al. 2016; Alpert et al. 2022). For example, when wild-type flies experience a relatively small temperature shift from 25°C to 29°C, daytime sleep increases by almost 1 h and nighttime sleep decreases by over 2 h in both males and females (Parisky et al. 2016). Sleep structure in these flies is also altered, such that daytime sleep becomes more consolidated (increased bout duration) and nighttime sleep is fragmented (decreased bout duration). Thus, it is important to include controls to account for natural effects of temperature on sleep when interpreting results from dTRPA1 activation studies.
2. Thermogenetic activation experiments are often performed over a long period of time, and there is a lack of data on whether these extended neuronal activation states can equate with actual physiological patterns that occur during sleep and wakefulness.
3. It is worth noting that activation and inhibition tools may not always produce opposite phenotypes, given their different mechanisms of action. For example, dTRPA1 and Shi^{ts1} may have different on/off kinetics and work in different temperature ranges. Additionally, if a neuron is inactive at baseline, then Shi^{ts1} in that neuron might not have a noticeable behavioral change, whereas dTRPA1-mediated activation of the same neuron would.

Methodological Considerations

As with optogenetics, there are key aspects of experimental methodology that should be considered when using thermogenetic approaches to study sleep:

1. **Temperature to use for each sensor:** Although initial studies indicated that both dTRPA1 and Shi^{ts1} can be induced at ~29°C (Kitamoto 2002; Hamada et al. 2008; Pulver et al. 2009), behavioral studies have shown that Shi^{ts1}-mediated inhibition often requires higher temperatures of 31°C–32°C (Pitman et al. 2006; Gonzalez-Bellido et al. 2009). Because small changes in temperature itself can modify behavioral phenotypes, it is critical to test multiple genotypic controls at these temperatures to disassociate neuronal manipulation-based effects from temperature-mediated changes alone.

2. *Length of temperature elevation:* The length of temperature elevation for dTRPA1 and Shi^{ts1} manipulations varies based on the scientific question. For example, sleep is increased in flies expressing dTRPA1 in dFSB neurons when transferred to 31°C for 6 h (ZT0–ZT6) (Donlea et al. 2011). Flies are naturally awake during these time periods, and increases in sleep are therefore more detectable. Other dTRPA1-based excitation experiments have used a milder temperature of 27°C–29°C for longer periods ranging between 1 and 2 d (Liu et al. 2012, 2016; Aso et al. 2014; Haynes et al. 2015; Cavanaugh et al. 2016). In most cases, sleep is monitored in light–dark cycles, although some studies have used dTRPA1 activation in constant dark conditions (Ueno et al. 2012). Similarly, inhibition studies using Shi^{ts1} have involved transferring flies to 29°C–31°C for 6–24 h, and changes in sleep because of these manipulations are often used in identifying neurons that promote sleep or wake either during activation (Pitman et al. 2006) or during the postactivation period (Seidner et al. 2015).

FUTURE DIRECTIONS FOR NEURONAL ACTIVATION STUDIES OF SLEEP

Although researchers have already been quite creative about how they have made use of optogenetic and thermogenetic approaches to learn more about sleep, there are plenty of unexplored avenues, some of which we propose here.

1. *Optogenetic/thermogenetic cellular activation + molecular neuroscience:* Although optogenetics and thermogenetics have been paired extensively with behavioral assessment and, to a lesser degree, with electrophysiological analysis, so far it has been rare for optogenetic neural activation to be paired with molecular studies. For example, activation of a specific neural population could be followed by RNA sequencing or microarray analysis to determine how gene expression is affected by this activation. This could be done globally to compare, for example, the gene expression patterns during naturalistic sleep versus sleep induced by specific neural activation. Alternatively, the molecular analysis could be focused on specific populations; for example, by using the TRAP system, in which the RNAs that are actively being translated are immunoprecipitated and quantified (Huang et al. 2013). This could allow researchers to determine how bursting versus rhythmic firing patterns (induced optogenetically at any desired time of day) influence gene expression patterns either in the same activated neurons or in putative target neurons. Recently, a calcium-based sensor that activates the transcription of any desired genetic product was developed (TRIC) (Gao et al. 2015). Originally, this sensor was used as a way to monitor intracellular calcium levels through the calcium-induced expression of a fluorescent marker. However, other genes could be induced instead. For example, optogenetic or thermogenetic activation could raise calcium levels inside specific neurons, causing them to express a gene that then alters the function of those same neurons.
2. *Combinatorial activation of different neural populations:* Thus far, studies have activated one set of neurons using either optogenetics or thermogenetics but have not combined these approaches to activate or inactivate other neurons simultaneously. As the ability to manipulate distinct subsets of neurons has improved with the development of new drivers within the GAL4, split-GAL4, LexA, and Q systems, thermogenetic and optogenetic tools could be used simultaneously to activate (using dTRPA1, Chr2, etc.) and inhibit (using Shi^{ts1}, Kir2.1, etc.) distinct subsets of neurons to understand the information flow between clusters of sleep-regulating neurons. This already impressive genetic toolbox (for reviews, see Olsen and Wilson 2008; Venken et al. 2011) keeps expanding, offering researchers increased flexibility to perform combinatorial neuronal manipulations.
3. *Activation at specific times of day:* The fact that optogenetic activation can be performed with brief pulses of neuronal activation lends itself to studies using discrete activation at specific times of day. However, with the exception of a few studies (Juneau et al. 2019; Huang et al. 2021), most experiments thus far have used prolonged light exposures and/or have not explicitly compared

the behavioral/physiological effects of activating sleep-regulating neurons at different times of day. Thus, this is an area of research that should be expanded in the future. This ability could also be used to manipulate circadian rhythms. Researchers could perform repeated activation pulses with different circadian periods to assess the ability of rhythmic neuronal activity in specific populations to influence daily rhythms.

In conclusion, activity monitoring provides a powerful approach to measure sleep, arousal, and circadian rhythms in *Drosophila*, an organism that continues to lead the way in the discovery of broadly conserved mechanisms of sleep regulation and function. Optogenetic and thermogenetic manipulation have already been used to discover how specific sets of neuronal (and nonneuronal) cells contribute to sleep and circadian regulation and connect to each other, and there are many exciting avenues through which these studies can expand in the future.

ACKNOWLEDGMENTS

We gratefully acknowledge the following sources of funding: National Institutes of Health (NIH) Academic Research Enrichment Awards (AREA) grants 1R15NS101692-01A1 and 2R15NS101692-02A1 (to C.G.V.), College of Science Start-Up Funds (CSUEB) (to D.S.), NIH AREA grants 7R15GM125073-02 and 2R15GM125073-03 (to D.S.), and National Science Foundation Division of Integrative Organismal Systems (IOS) grant 2042873 (to D.S.).

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Cold Spring Harb Protoc; doi: 10.1101/pdb.top108095; published online February 9, 2024

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