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## CHAPTER 11

# Monitoring Sleep and Arousal in Zebrafish

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### Abstract

Zebrafish has emerged in the past 5 years as a model for the study of sleep and wake behaviors. Experimental evidence has shown that periods of behavioral quiescence in zebrafish larvae and adults are sleep-like states, as these rest bouts are regulated by the circadian cycle, are associated with decreases in arousal, and are increased following rest deprivation. Furthermore, zebrafish share with mammals a hypocretin/orexin system that promotes wakefulness, and drugs that alter mammalian sleep have similar effects on zebrafish rest. In this chapter, we review the zebrafish sleep literature and describe a long-term, high-throughput monitoring system for observing sleep and wake behaviors in larval zebrafish.

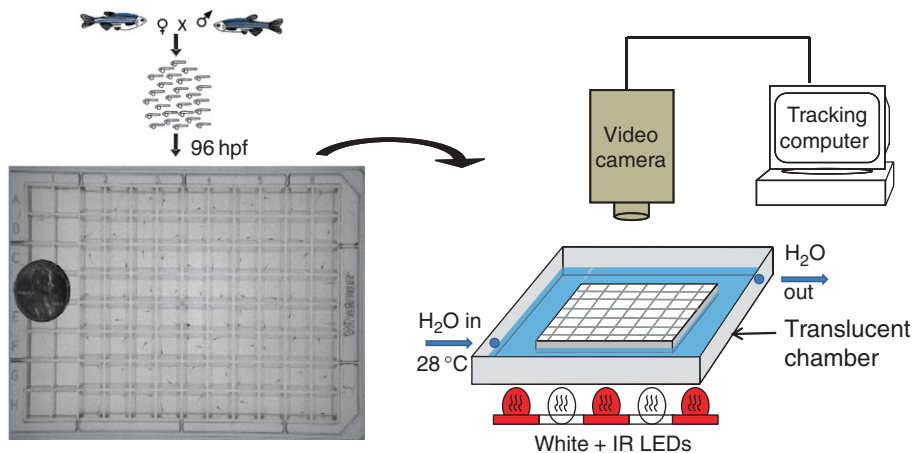
## I. Introduction

Sleep is essential, time consuming, and conserved across the animal kingdom, yet it remains one of the major mysteries of biology. What is the function of sleep, and how is it regulated by genes and neurons? Since the discovery of characteristic electroencephalographic (EEG) signatures for states of sleep and waking in the late 1930s (Davis *et al.*, 1937), mammalian model systems have dominated sleep research. However, behavioral observations over the past decade have demonstrated that non-mammalian systems, including *Drosophila* (Hendricks *et al.*, 2000; Shaw *et al.*, 2000), *Caenorhabditis elegans* (Raizen *et al.*, 2008; Van Buskirk and Sternberg, 2007), and zebrafish (Prober *et al.*, 2006; Yokogawa *et al.*, 2007; Zhdanova *et al.*, 2001), have sleep-like states. These “simple” model systems allow researchers to bring large-scale genetics and *in vivo* imaging to bear on fundamental questions of sleep biology. Zebrafish is an attractive model because it combines the facile genetics of invertebrates with brains that are morphologically and molecularly analogous to mammals. In this chapter, we review the progress of sleep studies in zebrafish and discuss the high-throughput methods (Fig. 1) we have developed to study larval zebrafish sleep/wake behaviors.

## II. Behavior, Genetics, and Pharmacology of Zebrafish Sleep

### A. Behavior

Sleep is a period of reversible, inattentive behavioral quiescence that can be distinguished from quiet wakefulness using several behavioral criteria (Borbely and



**Fig. 1** High-throughput tracking of zebrafish locomotor behavior. Zebrafish larvae older than 96 hpf are placed into a 96-well plate filled with blue water. The plate is then placed into a translucent chamber that is illuminated from the bottom continuously by infrared (IR) light emitting diodes (LEDs) and from 9:00 AM to 11:00 PM with white LEDs. Water heated to 28°C is pumped continuously through (water goes in and out of the chamber) the plate chamber to maintain temperature and humidity. The plate is observed by a video camera connected to a computer with video tracking software. The chamber, lights, and camera are housed inside a box (not shown) to prevent extraneous light from interfering with the experiment. Modified from Fig. 4 in Prober *et al.*, 2006.

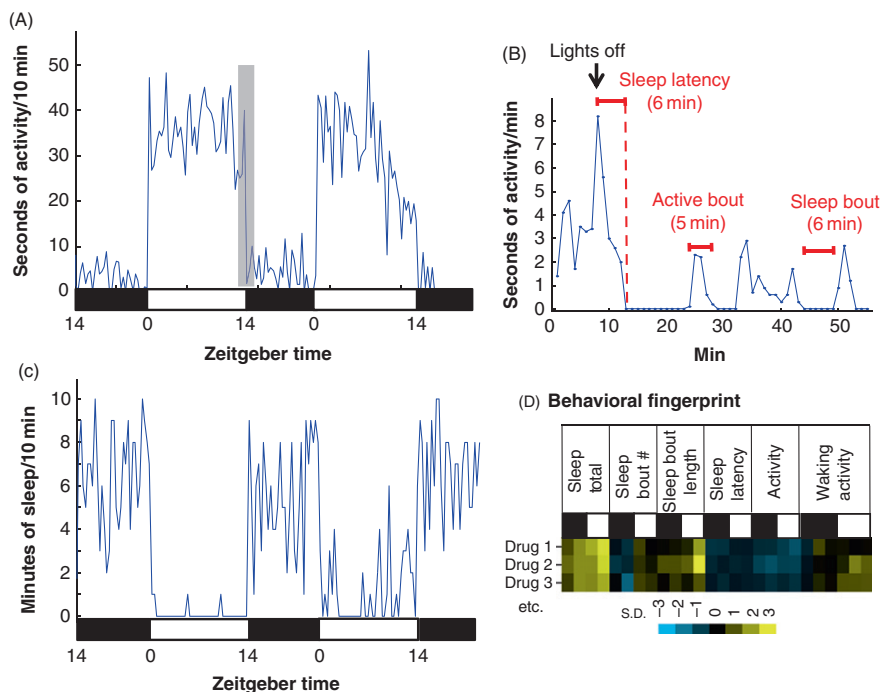
Tobler, 1996; Campbell and Tobler, 1984; Cirelli, 2009). Sleep-like rest periods are typically associated with a species-specific posture and location, and are regulated in a circadian (about 24 h) manner (Borbely and Tobler, 1996). Importantly, sleep is associated with an increased arousal threshold in response to stimuli, although strong stimuli can still wake the animal (thereby distinguishing sleep from coma or stupor). Finally, sleep is thought to be under homeostatic control, such that depriving an animal of sleep results in a subsequent increase in the duration or intensity of sleep, known as sleep rebound (Borbely and Tobler, 1996). Using automated tracking systems to observe individual animals for several days (see Section III), a variety of non-mammalian model systems, including fruit flies (Hendricks *et al.*, 2000; Shaw *et al.*, 2000), *C. elegans* (Raizen *et al.*, 2008; Van Buskirk and Sternberg, 2007), and both larval (Prober *et al.*, 2006; Zhdanova *et al.*, 2001) and adult zebrafish (Yokogawa *et al.*, 2007), have been shown to exhibit sleep-like states that meet these behavioral criteria.

### 1. Criterion 1—Quiescent Behavior Regulated by an Endogenous Circadian Rhythm

Zebrafish larvae first exhibit active spontaneous locomotor activity around 96 h post fertilization (hpf), shortly after inflation of the swim bladder (Hurd and Cahill, 2002; Prober *et al.*, 2006). When maintained on a 14 h:10 h light:dark cycle, these swim bouts occur maximally during the lights-on phase and are tightly synchronized with the light stimulus (Fig. 2A). If raised on a light:dark cycle and then transferred to constant dark conditions, the spontaneous locomotor activity of >96 hpf larvae and adults continues to cycle with a circadian rhythm of ~25–25.5 h, with the phase set by the prior entraining light:dark cycle (Hurd and Cahill, 2002; Hurd *et al.*, 1998). These observations demonstrate that zebrafish have an endogenously controlled circadian rhythm behavior that can be entrained by light. Consistent with the behavioral observations, rhythmic components of the molecular machinery that controls circadian rhythms in *Drosophila* and mammals also exhibit rhythmic expression with a light-entrainable circadian period in zebrafish. Such genes include the zebrafish orthologs of *Period1* (Dekens and Whitmore, 2008), *Period3* (Kaneke and Cahill, 2005; Pando *et al.*, 2001), *Clock* (Whitmore *et al.*, 1998), *Cryptochrome* (Kobayashi *et al.*, 2000), and the clock-binding partner *Bmal* (Cermakian *et al.*, 2000).

### 2. Criterion 2—Increased Arousal Threshold/Decreased Responsiveness during Quiescence

Throughout the 24 h light:dark cycle, zebrafish larvae exhibit bouts of quiescence that can last for several minutes or longer (Fig. 2B and C) and that occur maximally at night. During these quiescent periods, both zebrafish larvae and adults have an accompanying increase in arousal threshold, as measured by a decreased responsiveness of larvae to taps at night versus the day (Zhdanova *et al.*, 2001), an increase in response time to large changes in light intensity (Prober *et al.*, 2006), or a decreased responsiveness to electrical stimuli in adults (Yokogawa *et al.*, 2007). By correlating the length of a quiescent bout with the concomitant change in arousal state, sleep is defined as a quiescent bout lasting at least 1 min in larvae and at least 6 s in adults. This definition of a sleep-like state has served well in the analysis of molecules that regulate sleep and has been effective in uncovering conserved pathways (see Section II-B). The definition can now be refined by testing changes in arousal across multiple sensory modalities,



**Fig. 2** Zebrafish sleep/wake data. (A) The average activity of a single wild type larva is plotted per 10 min for two light:dark cycles starting at ~110 hpf. Activity occurs maximally during each day period. The gray area is expanded in (B). Zeitgeber time 0 = lights on; 14 = lights off. (B) The average activity of the same fish in (A) (gray area), expanded and replotted per 1 min. Examples of sleep latency, sleep bout, sleep bout length, active bout, and active bout length are indicated. (C) The behavior of the same fish shown in (A) and (B), plotted as minutes of sleep per 10 min. Sleep occurs maximally during each night period. (D) By normalizing each behavioral parameter to wild type controls, the data can be transformed into a behavioral fingerprint. Each square of the fingerprint represents the average relative value in standard deviations (black, higher than controls; white, lower than controls) for a single behavioral measurement. The black and white bars across the top represent night and day measurements, respectively. In this example, fingerprints are shown for three different drugs that increase sleep bout lengths, leading to increased total sleep.

experimental conditions, and behavioral situations, including a detailed analysis of changes in arousal states during different times of the day and night. For example, a recent report notes that larval respiration rate is reduced and arousal threshold elevated in nighttime rest compared to daytime rest (Zhdanova, 2006), indicating that these two quiescent states may not be equivalent. We also observe changes in average sleep bout length and in waking activity (see section III.C for details) during the day and night that may reflect underlying differences between these states (Table I).

### 3. Criterion 3—Sleep Rebound Following Sleep Deprivation (Homeostatic Regulation of Sleep)

Two studies have demonstrated an increase in total sleep amount in larval and adult zebrafish following nighttime sleep deprivation. Zhdanova and colleagues used a

**TABLE I**  
**Typical Sleep/Wake Measures Obtained from Wild Type Larvae (TLAB × TLAB Cross) in a 14h:10h Light:Dark Cycle**

	Mean	(± SD)	Median
TOTAL SLEEP (minutes)			
Day 6	189	138	135
Day 7	110	127	60
Night 6	252	125	240
Night 7	219	118	202
# SLEEP BOUTS			
Day 6	29	21	24
Day 7	37	34	26
Night 6	58	23	57
Night 7	64	20	63
SLEEP LENGTH (minutes)			
Day 6	7.7	5.4	6.6
Day 7	2.8	1.9	2.3
Night 6	4.5	2.8	3.9
Night 7	3.5	2.4	3.1
SLEEP LATENCY (minutes)			
Day 6	24.8	70.6	4
Day 7	59.2	147.0	7
Night 6	21.2	28.0	13
Night 7	19.6	27.0	14
AVERAGE ACTIVITY (seconds/minute)			
Day 6	4.04	1.88	3.90
Day 7	4.18	1.95	4.00
Night 6	0.85	0.46	0.78
Night 7	0.85	0.42	0.79
WAKING ACTIVITY (seconds/minute)			
Day 6	5.01	1.67	4.80
Day 7	4.58	1.88	4.30
Night 6	1.29	0.50	1.21
Night 7	1.21	0.41	1.19

$n = 321$ ; 14 h Day, 10 h Night

Each parameter ( $\pm$ SD) was calculated from the behavioral data of 321 wild type larvae. Day 6 starts at 120 hpf, and night 6 starts at 134 hpf. Day 7 starts at 144 hpf, and night 7 starts at 158 hpf. Sleep latency at night is calculated as the time from lights out to the first sleep bout; sleep latency during the day is calculated as the time from lights on to the first sleep bout.

mechanical shaker to deprive larvae of rest during the day and the night and found that night time deprivation decreased subsequent larval locomotor activity more than day time deprivation (Zhdanova *et al.*, 2001). Furthermore, following nighttime sleep deprivation, the larvae had an increased arousal threshold response to a tap stimulus. Together, these data suggest that nighttime deprivation causes a sleep rebound in both the amount and the depth of sleep. However, the decrease in general locomotion may indicate that the mechanical deprivation had caused some harm or stress. A long-term behavioral assessment demonstrating a return to baseline activity levels would help

clarify this point. Furthermore, it is unclear whether the arousal threshold changes occurred specifically during times of sleep-like states (see Section II-A, criterion 2). Large changes in arousal states during waking activity may also be an indicator of poor health. In another study, adult zebrafish were deprived of rest for 6 h with either electric shocks or light (Yokogawa *et al.*, 2007). Shock-induced deprivation at night resulted only in a modest rebound in total sleep relative to yoked controls; changes in arousal threshold following deprivation were not examined. Curiously, light-induced deprivation resulted in no observed sleep rebound, which may reflect a strong masking effect of light or the effect of light on the circadian rhythm in zebrafish.

Although these results are encouraging, much more work needs to be done to firmly establish that rest is under homeostatic control in zebrafish. In particular, it remains to be demonstrated that either the depth or duration of sleep rebound increases with increasing amounts of sleep deprivation. Also underexplored are the behavioral consequences of short- and long-term sleep deprivation, including the time course of the return to normal sleep patterns and altered performance in behavioral tasks (see Zhdanova, 2006). Finally, rebound sleep following short-term exposure to arousing drugs or genetic manipulations that dramatically reduce total sleep (see Section II-B) should also be investigated, as these treatments may represent less invasive and more reproducible methods for sleep deprivation.

## B. Genetics and Pharmacology

Genetic and pharmacological experiments in zebrafish indicate that mechanisms that regulate mammalian sleep are conserved in zebrafish. Of these, the most studied is the hypocretin/orexin (Hcrt) system, which has been shown to increase wakefulness in mammals (Sakurai, 2007). Hcrt peptide is produced by a population of hypothalamic neurons that project throughout the brain, particularly to other known wake-promoting centers (Peyron *et al.*, 1998). Deficiency in Hcrt signaling can be caused by loss-of-function mutations in the peptide (Chemelli *et al.*, 1999) or its receptors (Lin *et al.*, 1999; Willie *et al.*, 2003) or by a selective loss of Hcrt-producing neurons (Hara *et al.*, 2001; Peyron *et al.*, 2000; Thannickal *et al.*, 2000). In mammals, loss of Hcrt signaling leads to narcolepsy, a disease characterized by excessive daytime sleepiness, unstable sleep/wake states, and sudden loss of muscle tone during waking. Larval and adult zebrafish express their single *hcart* ortholog in a small number of hypothalamic neurons that project to putative wake-promoting centers of the brain and down the spinal cord, regions that also express the single *hcart* receptor (Appelbaum *et al.*, 2009; Faraco *et al.*, 2006; Kaslin *et al.*, 2004; Prober *et al.*, 2006). As expected for a wake-promoting peptide, overexpression of *hcart* in larval zebrafish leads to increased wakefulness at the expense of rest (Prober *et al.*, 2006). Furthermore, *in vivo* observation of Hcrt neural activity using the bioluminescent reporter GFP-Aequorin reveals that they are maximally active during episodes of spontaneous locomotor activity and inactive during rest (Naumann *et al.*, 2010), consistent with results obtained in mammals (Lee *et al.*, 2005; Mileykovskiy *et al.*, 2005). Finally, adult zebrafish with mutations in the Hcrt receptor exhibit sleep fragmentation (Yokogawa *et al.*, 2007), another hallmark of

narcolepsy (Mochizuki *et al.*, 2004; Overeem *et al.*, 2001). Taken together, these data confirm that at least some aspects of Hcrt's regulatory role in sleep/wake behavior are preserved in zebrafish.

There are some discrepancies between the Hcrt data obtained in larvae and adults that should be noted. Paradoxically, Hcrt receptor mutant adults were reported to have a small decrease in sleep at night and to be slightly hyperactive at night, although the latter effect was only significant compared to unrelated non-mutagenized wild type animals (Yokogawa *et al.*, 2007). These effects were not seen in Hcrt receptor mutant larvae under constant dim light conditions (Appelbaum *et al.*, 2009). In addition, some behavioral effects (e.g., hypoactivity and increased sleep in constant dark) appear only in Hcrt receptor heterozygotes (Yokogawa *et al.*, 2007). The reasons for these discrepancies are unclear but might include background mutations or the small number of adults that were analyzed. In addition, injection of a very high dose (280–2800 pmol/g body weight) of Hcrt peptide into adult zebrafish brains led to a slight decrease in activity (Yokogawa *et al.*, 2007). This observation contrasts with the long-term and strong increase in wakefulness following Hcrt overexpression in zebrafish larvae (Prober *et al.*, 2006) and the arousing effects of moderate levels (2.8–28 pmol/g body weight) of Hcrt peptide injected into adult goldfish (Nakamachi *et al.*, 2006). Additional genetic studies and more extensive behavioral analyses will be needed to resolve these discrepancies. Currently, the preponderance of evidence indicates that Hcrt has arousing effects in teleosts, and that loss of Hcrt receptor in adults leads to fragmented sleep/wake states, as in mammals.

Other evidence for the conservation of sleep-regulatory mechanisms in zebrafish comes from pharmacological studies. Tests of known sedative and hypnotic compounds, including the hormone melatonin (Zhdanova *et al.*, 2001), the GABA receptor agonists baclofen, phenobarbital, and diazepam (Renier *et al.*, 2007; Zhdanova *et al.*, 2001), and the alpha-2 adrenergic receptor agonist clonidine (Ruuskanen *et al.*, 2005), demonstrated that these compounds dose-dependently decrease locomotor activity and increase rest in zebrafish larvae 2–3 h following drug treatment. We have confirmed and extended these results through an unbiased screen of the long-term (3 days) effects of nearly 4000 small molecules on larval zebrafish sleep/wake behaviors. We found that many modulators of neurotransmitter systems, including the noradrenaline, serotonin, dopamine, GABA, glutamate, histamine, adenosine, and melatonin systems, induce similar sleep/wake phenotypes in zebrafish as observed in mammals (Rihel *et al.*, 2010).

Overall, the behavioral, genetic, and pharmacological evidence indicates that a sleep-like state exists in both larval and adult zebrafish and that this state is regulated by mechanisms that are conserved among vertebrates. Now that the conceptual groundwork for studying sleep in zebrafish has been established, future screens and experiments can begin to dissect novel mechanisms of sleep/wake regulation in earnest. Indeed, the pharmacological screen has already identified several pathways, including the ether-a-go-go related gene (ERG) potassium channel, verapamil-sensitive L-type calcium channels, and the immunomodulatory nuclear factor of activated T cells (NFAT) and nuclear factor kappa B (NF- $\kappa$ B) pathways, as targets for future zebrafish sleep/wake studies (Rihel *et al.*, 2010).

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### III. Methods for Monitoring Sleep/Wake Behavior in Zebrafish

#### A. Methodological Considerations—from *Drosophila* to *Danio*

Two major methods have been used to track locomotor behavior of animals, either by counting the number of times an animal breaks an infrared beam or by direct analysis of movements captured by video. Pioneering work on circadian rhythms (Konopka and Benzer, 1971) and sleep (Hendricks *et al.*, 2000; Shaw *et al.*, 2000) in *Drosophila* predominantly used the infrared beam break method, which measures when the fly crosses an infrared beam in the center of a tube. Some work on zebrafish (Hurd *et al.*, 1998) and goldfish (Azpeleta *et al.*, 2010; Iigo and Tabata, 1996; Vera *et al.*, 2007) also used this method to assess locomotion. Although useful for low time-resolution analysis of circadian rhythms, the beam break method suffers from blind spots, where an animal may move without crossing the beam. Indeed, a direct comparison of results from infrared beam breaks to direct video recording suggests that the beam break method can overestimate total sleep in flies by 10–90% (Zimmerman *et al.*, 2008). More fine-scale measures of sleep structure, including sleep latency, sleep bout number, and sleep bout length (see Section III-C for descriptions of these measures), can be even more dramatically overestimated (Zimmerman *et al.*, 2008). In addition, to obtain an accurate assessment of these important sleep parameters, individual animals must be tracked unambiguously throughout the experiment. Because sleep bouts occur non-simultaneously among individuals, methods that average activity across a population of animals lack details of sleep architecture. While methods that allow for the simultaneous and unambiguous tracking of animals within the same arena are under development (Branson *et al.*, 2009; Grover *et al.*, 2009; Kato *et al.*, 2004; Straw *et al.*, 2010), most available methods require animals to be individually housed.

There are a growing number of methods for automated detection of zebrafish locomotion using video-based analysis. These include commercially available zebrafish tracking systems from Noldus Information Technology (<http://www.noldus.com>) and Viewpoint Life Sciences, Inc. (<http://www.vplsi.com>) as well as custom algorithms designed for the analysis of short-term responses to stimuli captured by a high-speed camera (e.g., Burgess and Granato, 2007; Fontaine *et al.*, 2008). To simultaneously observe hundreds of animals over several days with minimal user input, we use a relatively simple frame-by-frame background subtraction method within an analysis suite from Viewpoint Life Sciences. We find that counting pixel changes per frame at a low frame rate (15 frames per second) gives a reliable readout of the timing and duration of each larva's locomotor activity and rest for days or weeks of continuous behavioral recording.

#### B. Experimental Design and Setup

Because sleep behavior can be strongly influenced by prior environmental conditions and experiences (Ganguly-Fitzgerald *et al.*, 2006 and personal observation), the conditions for raising larvae prior to behavioral testing must be rigorously maintained. Following fertilization, embryos and larvae are raised from a single cell on a 14 h:10 h



light:dark (LD) cycle at 28°C in petri dishes with conventional embryo water at a density of no more than 50 larvae per 100 mm dish. If the effect of a mutation or a transgene (e.g., heat shock driving *Hcrt* overexpression; Prober *et al.*, 2006) on behavior is being tested, all comparisons are ideally done within the same clutch or batch, raised in the same petri dish, and not pre-sorted by genotype. Each day, the dishes are cleared of any sick larvae, water levels are readjusted as necessary, and the chorions are removed post hatching.

Between 96 and 110 hpf, single larvae are placed into each well of a flat-bottom, square-well 96-well plate (650 µl well volume, Whatman) filled with embryo blue water. For the best optical properties, each well is filled so that the meniscus is flat and nearly flush with the top of the well. To test the long-term behavioral effects of small molecules, drugs may be added directly to the wells at this time by pipetting compound dissolved in DMSO. Usually the desired final concentration of drug in each well is between 100 nM and 1 mM, and the final DMSO concentration should not exceed 1% (above this level, DMSO can have behavioral consequences).

The 96-well plate is then placed into the zebrafish tracking setup (custom modified from Viewpoint Life Sciences; see Fig. 1). Inside a box, the plate chamber is illuminated continuously with an infrared LED panel and from 9:00 AM to 11:00 PM with white LEDs. The plate is monitored by a video camera (Dinion one-third inch Monochrome Bosch camera) fitted with a fixed-angle megapixel lens (50 mm focal length; Computar) and a filter that transmits infrared light. To slightly humidify the box and maintain a constant temperature, distilled water heated to 28°C is continuously pumped through the plate chamber. Embryo water is added daily to each well to maintain high water levels. Although the larvae need not be fed until the 7th day of development, paramecia can also be added to each well daily (they will not be detected by the software). By adding paramecia to the wells, we have monitored larvae in the same 96-well plate continuously from day 4 to day 14 of development without a noticeable decline in health (JR, unpublished data). Older animals can be monitored in plates with larger well sizes (e.g., 6-, 12-, and 24-well plates).

### C. Monitoring and Analysis of Sleep/Wake Behaviors

To collect the movement data from each larva in the 96-well plate, we use Viewpoint Life Sciences Videotrack software running in the quantization mode. In this software package, a detection threshold is set to distinguish the dark fish from the white background (in our setup, the threshold is 40, although this value depends on the infrared lighting used). For each camera frame, any pixels darker than this threshold that change are detected as a movement and stored in a raw data file as pixels changed per frame for each larva. These data are further processed by setting a threshold value for the number of pixels that must change to constitute larval movement instead of random pixel noise (the “freeze” threshold; for our experimental setups, a cutoff of 4 pixels). The data are then converted into total seconds spent moving per minute for each larva by summing the total time of pixel changes that exceed the threshold. A sample 56 h activity trace from a single fish is plotted in Fig. 2A.

From the activity data of each larva, we use custom-designed Matlab code to extract multiple additional parameters that measure the amount and structure of sleep for each day and night (Fig. 2B and Table I).

### 1. Total Sleep

We define sleep in larval zebrafish as a continuous period of inactivity that lasts at least 1 min, because 1 min of inactivity at night is associated with increased response latencies to changes in light intensity (Prober *et al.*, 2006). We measure the total sleep for each day and night period and plot the average sleep per 10 min to generate a sleep time course (Fig. 2C). Based on recent wild type data from our lab ( $n = 321$  animals, TLAB  $\times$  TLAB cross), zebrafish larvae sleep at night on average 235 min each 10 h night (23.5 min/h), but individual larvae can vary considerably from this value (Table I).

### 2. Sleep Bout Number and Sleep Bout Length

A sleep bout is defined as a continuous period of inactivity lasting 1 min or longer (see Fig. 2B). Because each larva has numerous sleep bouts that are interrupted by brief awakenings, plotting the sleep bout length distribution for each larva can also be useful. At night, larvae have on average 61 sleep bouts, with each bout lasting an average of 4 min (Table I).

### 3. Sleep Latency

Sleep latency is defined as the amount of time from the start of each day and night period until the first sleep bout (see Fig. 2B). Following lights out, wild type (TLAB  $\times$  TLAB cross) larvae have sleep latencies at night averaging about 20 min (Table I).

### 4. Average Activity per Waking Minute

Changes in a larva's locomotor behavior could be due to perturbations in muscle control and coordination, altered stress or arousal state, or other general health deficits. For example, an unhealthy larva with increased sleep may also move considerably less during active swimming. By measuring the average activity only during bouts of waking activity, we can assess whether the overall health and swimming ability of the fish have been compromised. Average activity per waking minute is calculated for each day and night period by summing the total activity and dividing by the number of active minutes (total active minutes = total time - total sleep time). This measure can also be used to determine whether an experimental perturbation causes a larva to be hyperactive when awake. In our experimental apparatus, wild type larvae have an average waking activity of 4.8 s per minute during the day and 1.3 s per minute at night (Table I).

These measures of sleep structure are biologically important, providing information about the initiation, maintenance, and timing of sleep. Given that these parameters can

be selectively perturbed by pharmacological agents (Rihel *et al.*, 2010), they are likely controlled by at least partially independent regulatory mechanisms. These parameters can be presented as a multi-dimensional “behavioral fingerprint” to facilitate comparisons among multiple genotypes, experimental manipulations, or small molecules. Each measurement is normalized to matched controls and then combined to create a vector that accounts for all of the parameters (see Fig. 2D; Rihel *et al.*, 2010). Clustering algorithms and principal component analyses can be used to organize large datasets by phenotype and to uncover small molecules or genotypes that have similar effects across multiple zebrafish sleep/wake behavioral parameters.

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## IV. Conclusion

Only a decade old, the study of sleep in non-mammalian systems is still in its infancy. While early zebrafish sleep studies have focused on establishing the existence of behavioral sleep regulated by conserved mechanisms, the challenge ahead is to use the zebrafish sleep model to uncover heretofore unsuspected aspects of the neuronal and genetic control of sleep/wake regulation. Recent studies that potentially link Hcr1 signaling to pineal gland regulation (Appelbaum *et al.*, 2009) and that uncover novel small molecule regulators of sleep/wake states (Rihel *et al.*, 2010) may represent two such discoveries.

A major advantage of the zebrafish system is the ability to efficiently perform genetic and pharmacological screens in a cost-, space-, and labor-effective manner. With this in mind, we have described an automated high-throughput method for observing long-term sleep/wake behavior in larval zebrafish. This methodology is highly flexible and can easily be adapted to other behaviors, for example to observe larval responses to temperature, vibration, noxious chemicals, and changes in light intensity (Emran *et al.*, 2007, 2008, 2010; Prober *et al.*, 2008). In principle, the behavioral space of future screens could be expanded by testing these and other behavioral modalities in conjunction with long-term sleep/wake behavioral monitoring, incorporating all the data into a single multi-dimensional behavioral fingerprint. Such screens could not only uncover novel mutants that affect specific behaviors but also identify correlated sets of behaviors that are regulated by similar underlying mechanisms. With new techniques to directly observe neural activity in behaving zebrafish, including neuroluminescence, whole-brain calcium imaging, and optogenetic techniques to manipulate the activity of neurons with light, future studies will also begin to directly elucidate the activities and functions of neural circuits that underlie sleep/wake behaviors.

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