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Desynchronizing the sleep–wake cycle from circadian timing to assess their separate contributions to physiology and behaviour and to estimate intrinsic circadian period

Wei Wang[1,2]✉, Robin K. Yuan[1,2], Jude F. Mitchell[3], Kirsi-Marja Zitting[1,2], Melissa A. St. Hilaire[1,2], James K. Wyatt[4], Frank A. J. L. Scheer[1,2,5], Kenneth P. Wright Jr[6], Emery N. Brown[7,8,9,10,11], Joseph M. Ronda[1,2], Elizabeth B. Klerman[1,2,10,12], Jeanne F. Duffy[1,2], Derk-Jan Dijk[13,14] and Charles A. Czeisler[1,2]

Circadian clocks drive cyclic variations in many aspects of physiology, but some daily variations are evoked by periodic changes in the environment or sleep–wake state and associated behaviors, such as changes in posture, light levels, fasting or eating, rest or activity and social interactions; thus, it is often important to quantify the relative contributions of these factors. Yet, circadian rhythms and these evoked effects cannot be separated under typical 24-h day conditions, because circadian phase and the length of time awake or asleep co-vary. Nathaniel Kleitman's forced desynchrony (FD) protocol was designed to assess endogenous circadian rhythmicity and to separate circadian from evoked components of daily rhythms in multiple parameters. Under FD protocol conditions, light intensity is kept low to minimize its impact on the circadian pacemaker, and participants have sleep–wake state and associated behaviors scheduled to an imposed non-24-h cycle. The period of this imposed cycle, T, is chosen so that the circadian pacemaker cannot entrain to it and therefore continues to oscillate at its intrinsic period (τ, ~24.15 h), ensuring circadian components are separated from evoked components of daily rhythms. Here we provide detailed instructions and troubleshooting techniques on how to design, implement and analyze the data from an FD protocol. We provide two procedures: one with general guidance for designing an FD study and another with more precise instructions for replicating one of our previous FD studies. We discuss estimating circadian parameters and quantifying the separate contributions of circadian rhythmicity and the sleep–wake cycle, including statistical analysis procedures and an R package for conducting the non-orthogonal spectral analysis method that enables an accurate estimation of period, amplitude and phase.

Introduction

In 1778, Hunter[1] reported that body temperature in humans during wakefulness was ~0.8 °C warmer than it was during sleep. In 1866, Ogle and Holmes[2] charted the diurnal rhythm of body temperature more precisely and observed that shielding the body from exposure to morning light, delaying food intake and minimizing activity did not prevent the early morning rise in body temperature, suggesting that it resulted from an endogenous source rather than as a result of waking, changing posture and becoming active in the morning. Then, in 1904, Benedict[3] documented the persistence of a nadir

[1]Division of Sleep and Circadian Disorders, Departments of Medicine and Neurology, Brigham and Women's Hospital, Boston, MA, USA. [2]Division of Sleep Medicine and Department of Medicine, Harvard Medical School, Boston, MA, USA. [3]Department of Brain and Cognitive Sciences, University of Rochester, Rochester, NY, USA. [4]Department of Psychiatry and Behavioral Sciences, Rush University Medical Center, Chicago, IL, USA. [5]Broad Institute, Cambridge, MA, USA. [6]Sleep and Chronobiology Laboratory, Department of Integrative Physiology, University of Colorado Boulder, Boulder, CO, USA. [7]Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, Boston, MA, USA. [8]Department of Brain and Cognitive Science, Massachusetts Institute of Technology, Cambridge, MA, USA. [9]Institute for Medical Engineering and Sciences, Massachusetts Institute of Technology, Cambridge, MA, USA. [10]Picower Institute for Learning and Memory, Massachusetts Institute of Technology, Cambridge, MA, USA. [11]Institute for Data Systems and Society, Massachusetts Institute of Technology, Cambridge, MA, USA. [12]Department of Neurology, Massachusetts General Hospital, Boston, MA, USA. [13]Surrey Sleep Research Centre, Faculty of Health and Medical Sciences, University of Surrey, Guildford, UK. [14]UK Dementia Research Institute Care Research and Technology Centre, Imperial College London and the University of Surrey, Guildford, UK. ✉e-mail: wwang4@bwh.harvard.edu

in body temperature during wakefulness at night, even after years of permanent night work, suggesting that the diurnal body temperature rhythm was (at least partially) resistant to inversion. Yet, a year later, Gibson[4] documented inversion of the body temperature rhythm within a couple of weeks after a trip from New Haven, Connecticut, United States to Manila, Philippines, suggesting that exposure to periodic environmental factors could have a more immediate and profound effect on the body temperature rhythm than exposure to 8 years of night shift work.

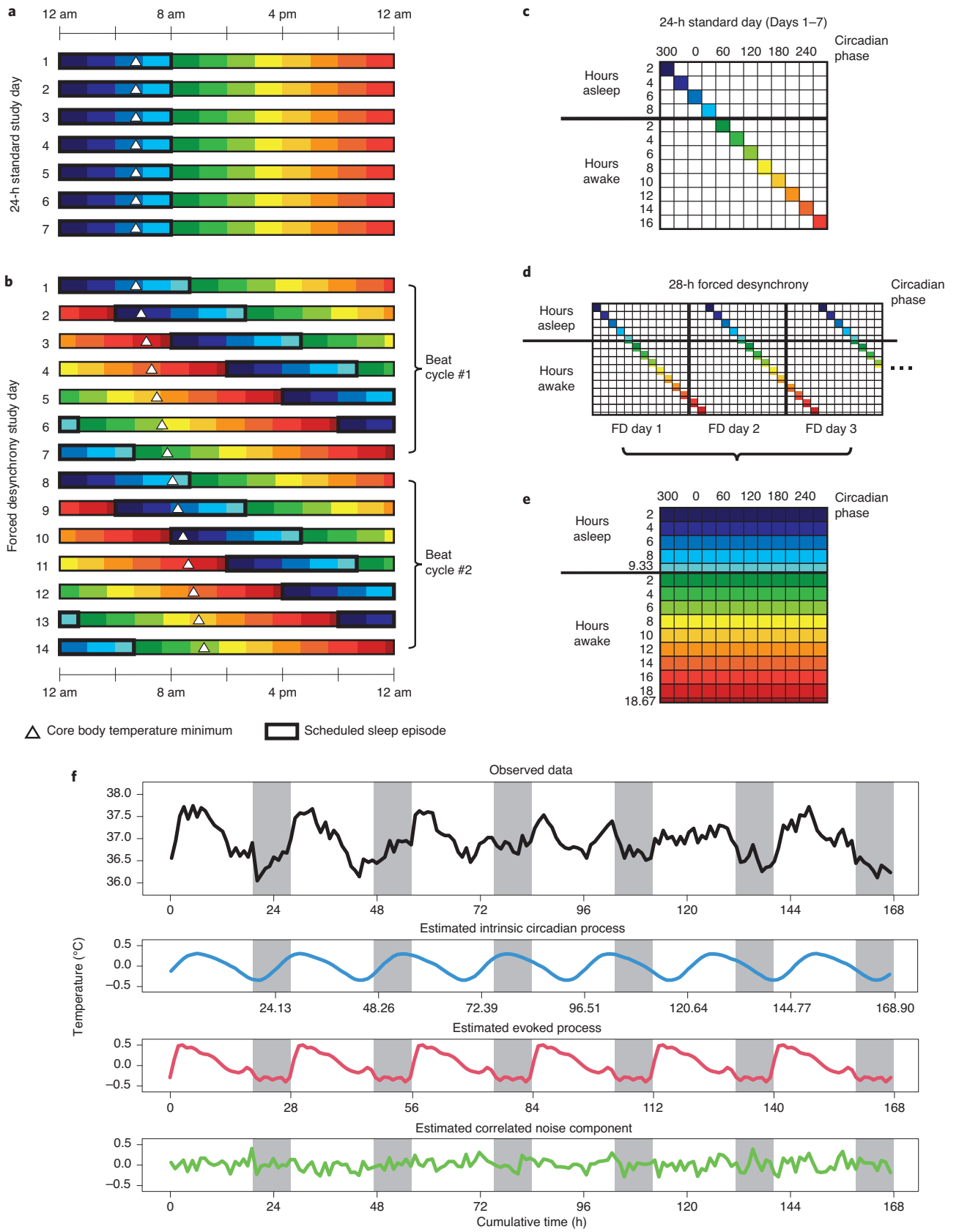
Committed to resolving this controversy, in 1938 University of Chicago Professor Nathaniel Kleitman and his student Bruce Richardson[5] descended 42.7 m underground to live for ~32 d in Mammoth Cave in Kentucky. In this cave with constant environmental conditions (constant ambient temperature of 12.2 °C, constant ambient silence and no exposure to natural light), Kleitman scheduled their sleep–wake, rest–activity, fasting–eating and dark–light times to a non-24-h cycle, with an imposed period, *T*, of 28 hours (T-cycle of 28 h), maintaining wakefulness and activity in artificial light for 19 h and scheduling 9 h in bed in absolute darkness for sleep each cycle. Despite there being only six of these 28-h cycles each week, Kleitman recorded seven endogenously generated cycles of body temperature per week in himself (though not in his student, who appeared to adapt to the 28-h routine and showed only six cycles of body temperature per week). This was the first attempt to investigate to what extent near-24-h (circadian) rhythmicity persisted when sleep and wakefulness were scheduled to non-circadian (28-h) cycles while living in an environment free of 24-h environmental and behavioral cyclicity. This Kleitman forced desynchrony (FD) protocol was later deployed by Aschoff and Wever[6,7] in an underground bunker in Germany, and by Mills, Minors and Waterhouse[8,9] in an isolation unit in England to separate endogenous circadian and evoked components of human daily physiology.

A 'free-running'[10] protocol has also been used for assessing the period of human circadian rhythms. This protocol allowed the study participants to choose freely when to go to sleep and when to awaken, and when to turn ambient lighting off and on. We now know that the free-running protocol biases period estimates because of both the phase-dependent impact of light exposure on the circadian system and the underlying circadian rhythm of sleep–wake propensity (the ability to remain asleep or awake, or the readiness to transition from wakefulness to sleep and vice versa)[11,12]. Specifically, when people are allowed to choose their own sleep times, most remain awake longer than usual, exposing themselves to light in the late evening and early night, which is a time when their circadian system shifts later in response to light (Fig. 2). Those later bedtimes are usually then accompanied by later wake times, which in turn shields the circadian system from morning light that would stabilize internal rhythms or shift them earlier. Thus, the pattern of self-selected sleep and wake results in a dark–light exposure pattern that systematically resets human circadian rhythms ~1 h later each cycle, resulting in an observed period of ~25 h[12,13].

In addition, sleep–wake and associated behaviors occur at only a subset of circadian phases (i.e., bedtimes occur much more frequently at some circadian phases than others, as do wake times). In long-term (>8 weeks) studies of individuals living in an environment free of time cues, nearly all participants eventually undergo what has been called 'spontaneous internal desynchronization'[14–16], in which their self-selected sleep–wake times uncouple from the timing of their body temperature and other endogenous circadian rhythms. In such cases, the sleep–wake cycle follows an extremely variable cycle length from day to day, often much shorter or longer than 24 h, whereas the endogenous circadian rhythms of many variables—core body temperature (CBT), cortisol and melatonin secretion and urinary volume and urinary potassium excretion—maintain a more stable cycle length that is close to 24 h.

Development of the protocol

To address problems inherent in the free-running protocol for assessing the circadian period, we developed a modified version of Kleitman's FD protocol. In our original adaptation, we maintained a 1:2 rest:activity ratio and scheduled participants to live on longer (28-h) or shorter (20-h) behavioral cycles. The choice of a 20- or 28-h imposed T-cycle was made to minimize the influence of the imposed T-cycle on the underlying near-24-h circadian rhythms, because the circadian pacemaker cannot entrain to these T-cycles or harmonics of these T-cycles when dim ambient light is used[12,17]. By scheduling participants to sleep and wake either 4 h later each cycle (T-cycle = 28-h FD) or 4 h earlier each cycle (T-cycle = 20-h FD), darkness and light, sleep and wakefulness and rest and activity are distributed across the entire ~24-h circadian cycle rather than occurring at a limited portion of the cycle, as occurs during entrained conditions (Fig. 1). We and other research groups have used other


a
12 am
8 am
4 pm
12 am
24-h standard study day
b
Forced desynchrony study day
Beat cycle #1
Beat cycle #2
Core body temperature minimum
Scheduled sleep episode
c
24-h standard day (Days 1–7)
Circadian phase
Hours asleep
Hours awake
d
28-h forced desynchrony
FD day 1
FD day 2
FD day 3
e
f
Observed data
Estimated intrinsic circadian process
Estimated evoked process
Estimated correlated noise component
Temperature (°C)
Cumulative time (h)

◀ **Fig. 1 | Example schedules and body temperature rhythms for different experimental protocols.** For **a**–**e**, the different colors of each square indicate the number of hours since scheduled sleep onset or wake onset, and the open triangles indicate the time of the CBT minimum (an established marker of circadian phase). **a**, A raster plot of a week of entrained 24-h days. Each sleep and wake episode occurs at the same clock time and has the same relationship to the underlying circadian phase of CBT, with the nadir occurring in the latter part of the scheduled sleep time. **b**, A raster plot of 2 weeks on a 28-h FD schedule. Sleep episodes are scheduled 4 h later each 'day' and over the course of a week cover the entire 24-h cycle, which is referred as one 'beat cycle'. Because the underlying circadian system cannot entrain to this imposed T-cycle, the CBT moves at its intrinsic ~24.2-h period, depicted by the white triangles moving slightly later each day. **c**, The colored blocks show that sleep and wake episodes occur only at the same limited portion of the ~24-h circadian cycle during the entire entrainment for the standard entrained 24-h day schedule in panel **a**. **d**, The colored blocks show that sleep and wake episodes occur at different portions of the ~24-h circadian cycle during the first 3 d of the FD schedule in panel **b**. This pattern continues beyond these 3 d throughout the 14 d of the FD schedule. **e**, The colored blocks show that sleep and wake episodes are distributed evenly across the entire circadian cycle when the 14 d of 28-h FD schedules are combined. **f**, An example of observed CBT data with a decomposed estimated intrinsic circadian process (estimated period =24.13 h), evoked process and serially correlated noise over time from seven 24-h days (168 h) of a 28-h FD protocol. The CBT data were standardized by subtracting the overall mean value of 36.93 °C. The shaded areas indicate the scheduled sleep time during the FD schedule. The *x*-axis scale for the circadian process is different from the two estimates.

T-cycles, many of which are much shorter than 20 h (from 20 min to 11 h[18–24]) and others that are longer than 28 h (from 30 to 42.85 h[25–28]).

Our research group first used the FD protocol in a series of studies designed to assess the period of the central circadian pacemaker, located in the mammalian suprachiasmatic nucleus, and to test whether it is shorter in healthy older adults than in healthy young adults[29]. We found no significant difference by age. We also found that the average period derived from healthy young adults studied in a 20-h T-cycle FD protocol did not differ significantly from the period derived from healthy young adults studied in a 28-h T-cycle FD protocol[29,30].

We then adapted the FD protocol to (i) assess the intrinsic period of the human circadian oscillator[29–31]; (ii) estimate the impact on physiology and behavior of circadian rhythmicity, acute evoked effects of periodic behaviors (e.g., posture, food intake, activity, sleep and wakefulness), chronic effects of changes in homeostatic sleep pressure and interactions of these factors[27,28,32–51]; and (iii) estimate the impact of countermeasures (such as melatonin or caffeine) for sleep disruption and/or impairment of wakefulness and circadian phase misalignment[52,53].

Validation of FD protocols

In the initial series of studies, we collected CBT throughout the FD protocols and collected hourly plasma melatonin and cortisol samples either throughout the FD protocols or at several intervals during the FD protocols, because these three physiologic rhythms were known to be strongly influenced by the central circadian pacemaker[29,54]. We observed that the intrinsic periods of the endogenous circadian components of the CBT, melatonin and cortisol rhythms collected in FD conditions were highly correlated within individuals[33,55,56], confirming that all three rhythms were probably driven by the same central circadian pacemaker. In those ~1-month-long studies, we also compared the intrinsic period estimates derived from FD data with those derived by extrapolating between the phases measured on the initial and final constant routine (CR) or constant posture protocols[29,56,57].

The CR protocol is used to assess endogenous circadian phase and involves having the participant remain in constant environmental (ambient light and temperature) and behavioral conditions (posture, activity level, sleep–wake state and feeding) for ≥24 h[58,59]. The month-long FD protocol allows the circadian system to oscillate at its intrinsic period, and thus the phases measured in the initial and final CRs (conducted immediately before and after the FD schedule, respectively) are at different clock hours; calculations using phases from these two CRs therefore allow for an independent assessment of circadian period, although in the absence of intermediary phase estimates, there are often several solutions that can be fit to two such points (shorter or longer than 24 h). The CR data are discussed more thoroughly below.

We found that the estimates from both methods were highly correlated in the 2- to 4-week-long FD protocols, suggesting that under the conditions used in those studies, the FD protocol accurately estimates intrinsic circadian period[29,57,60]. The resulting average intrinsic circadian period estimate of 24.15 h[30] derived from the FD protocol was also validated in a series of 1- to 2-month entrainment studies[60], which revealed that under conditions of a weak photic synchronizer—very-low-intensity light (~1.5 lux in the angle of gaze) during scheduled wakefulness and darkness during scheduled sleep—80% of participants' melatonin rhythms entrained to a 24.0-h T-cycle, whereas none of the participants' melatonin rhythms entrained to a 24.6-h T-cycle, demonstrating that the functional period of the central circadian pacemaker(s) driving the circadian melatonin rhythm is much closer

to 24 than to 25 h. In addition, in concordance with entrainment theory, interindividual variation in FD protocol–estimated circadian period correlates strongly with the phase relationship between the melatonin rhythm and the sleep–wake and associated dark–light cycle[30,61–63].

As a test of whether the length of the imposed T-cycle (20 h versus 28 h) has an impact on the period estimates derived from FD data, we studied several participants more than once by using different FD protocol T-cycles[29], and the results demonstrated that the length of the imposed T-cycle did not have a measurable impact on the assessed circadian period within individuals.

A second use of the FD protocol is the quantification of the contribution of endogenous circadian rhythmicity and that of the sleep–wake cycle to outcome measures of interest. Spontaneous desynchrony data and mathematical models had both suggested that sleep–wake regulation is governed by two oscillatory processes[64–66]. More specifically, on the basis of inferences from studies of sleep–wake propensity, human performance and risk of error or accident[28,67,68], it had been hypothesized that sleep–wake propensity and sleep structure are co-determined by circadian rhythmicity and sleep homeostasis—a hypothesis that has recently been substantiated by measurable changes in cortical and subcortical responsiveness as measured by functional magnetic resonance imaging, which vary with duration of sleep–wakefulness and/or circadian phase, with the nature of the interaction varying among cortical and subcortical brain regions[69]. Sleep homeostasis refers to the process that regulates the balance between sleep and waking such that effects of wakefulness on brain function and metabolism can be reversed during sleep[65,66]. The circadian rhythm was modeled as a self-sustained oscillator, while the sleep homeostat was modeled as a relaxation oscillator driven by the sleep–wake cycle. The FD protocol lends itself to estimating the effects of these two processes because, in contrast to the entrained condition, in the FD condition many phase relationships between the circadian oscillator and the sleep homeostat are observable (Fig. 1). Specifically, during the FD protocol, sleep is scheduled to occur across the circadian cycle, while at the same time the duration of scheduled wakefulness preceding scheduled sleep remains approximately the same; an unconfounded estimate of the circadian sleep–wake propensity rhythm can thereby be obtained.

The outcome measures of our initial interest were sleep propensity, sleep structure and waking performance. The FD protocol revealed that the circadian drive for wakefulness is strongest just before the circadian phase of habitual sleep onset (wake maintenance zone[67]), and the circadian drive for sleep is strongest at and just after the nadir of CBT (sleep maintenance zone[20,53,67,70–72]). Then, we used the FD protocol to obtain estimations of the effects of time awake (duration of time since the scheduled wake episode began) and time asleep (duration of time since the scheduled sleep episode began) on sleep and performance outcome measures separately from circadian phase[73]. Finally, because in FD studies, sleep–wake episodes are scheduled across the circadian cycle and many combinations of time awake, time asleep and circadian phase occur, the interaction between circadian phase and length of time awake or asleep can be estimated[25,26,73]. For example, the negative effects of time awake and chronic sleep restriction on neurobehavioral performance are much stronger near the sleep maintenance zone (close to the CBT minimum) than during the wake maintenance zone[28] (Fig. 3). These analyses, which allow us to test the contributions of the circadian and sleep homeostatic processes and their interaction on many outcome measures, demonstrated that the interactions are non-linear, such that the circadian effect is much greater at some durations of time awake (or asleep) than at others. For example, the circadian amplitude of the wake-promoting rhythm after being asleep for 7 h is very different than that after being asleep for 2 h[67].

These analyses of FD protocol data changed the view of how circadian clocks regulate behavior. For example, rather than acting as an alarm clock that sends one strong signal to wake people up in the morning, it is now recognized that the circadian pacemaker generates a circadian wake-promoting signal that becomes progressively stronger during the biological day with a maximum just before bedtime[67]. This circadian wake-promoting signal opposes the progressively increasing homeostatic drive for sleep that builds up during waking and thereby helps to consolidate wakefulness. Indeed, in the absence of this suprachiasmatic nucleus signal, the wake episode is no longer consolidated but fragmented[74,75]. The FD protocol data also imply that in the early morning hours, the circadian pacemaker actively promotes sleep and rapid eye movement sleep in particular, thereby contributing to consolidation of the nocturnal sleep episode[32].

Applications of the method

The FD protocol can be used for a wide range of applications. To date, it has been used to assess intrinsic circadian period in different populations, the relative contribution of sleep–wake and the

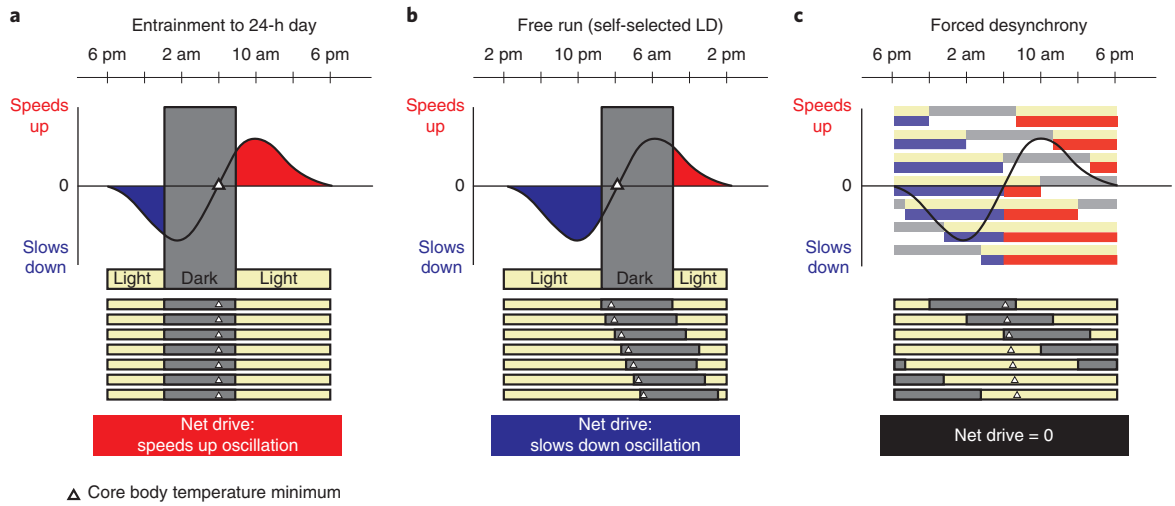


Fig. 2 | Schematic of wake-sleep and corresponding light–dark (LD) cycle effects of the FD protocol compared to the free-run protocol and to standard (entrainment) conditions. In all panels, time of day is across the horizontal axis. Horizontal bars indicate the timing of the LD cycle each day, with yellow representing light and gray representing darkness or sleep. White triangles indicate the timing of the circadian phase of the CBT minimum each day. The wave represents the phase-response curve to light, with blue shading indicating times at which light exposure slows down or shifts the clock later, red shading indicating times at which light exposure speeds up or shifts the clock earlier and gray shading indicating times with no light exposure. In the upper part of the panels, the overall drive onto the circadian system from daily exposure to light and darkness is illustrated, whereas in the lower part of the panels, change in LD cycle across protocol days is illustrated. **a**, Standard conditions in which an individual is entrained to a 24-h day. Because of the location of light exposure relative to the CBT phase, the overall net drive to a circadian system with an intrinsic longer-than-24-h circadian period will speed up the oscillation slightly each day, thereby allowing the intrinsic longer-than-24-h circadian system to remain entrained to the 24-h environment. **b**, Free-run conditions in which an individual is allowed to self-select sleep (dark) and wake (light) timing. In free-run conditions, individuals tend to go to bed later than under entrainment, choosing bedtimes close to the CBT minimum. Because of the phase-dependent influence of light exposure on the circadian system, this shift in the timing of sleep (darkness) leads to more exposure to light at times of day that slow down or shift the clock later and less exposure to light at times of day that speed up or shift the clock earlier. The overall net drive to the circadian system slows down the oscillation each day, making the observed period appear longer than its intrinsic period (the lower part of the panel). **c**, FD protocol in which an individual is scheduled to a sleep (dark) and wake (light) schedule that is several hours shorter or longer than 24 h. Light exposure is distributed nearly evenly with respect to the underlying phase-response curve to light, with light exposure at times that speed up the circadian system counter-balancing light exposure at times that slow down the circadian system. This produces an overall net drive of zero to the circadian system.

circadian system to multiple areas of physiology and neurobehavioral performance, the impact on performance of acute and chronic sleep loss on circadian sleep–wake interactions and the effects of interventions (e.g., medication) at different circadian phases of administration.

The 2- to 4-week FD protocols have revealed that the average intrinsic circadian period differs between men and women[30], with shorter periods in women; does not differ between healthy young and healthy older individuals[30,33]; might be longer in blind than in sighted individuals[31,76]; might be longer in circadian rhythm sleep disorder free-running–type patients[77]; is comparable or slightly longer in adolescents than in adults depending on the comparison group[78,79]; and is reproducible when assessed in the same individual months apart[29,80]. An abbreviated 5-h FD protocol in a dark–light cycle with 10–30 lux of light during wakefulness, which was designed to assess circadian period in only 3 d, has suggested that the average circadian period may differ between African-Americans (mean ± s.d. = 24.06 ± 0.19 h) and European-Americans (24.31 ± 0.24 h)[24] and that a sex difference in circadian period may be observed only in European-Americans (24.24 ± 0.23 h for women versus 24.41 ± 0.20 h for men) and not in African-Americans[81–83]. However, as noted below, that very abbreviated 3-d FD protocol (T-cycle = 5 h) has not been validated against the 2- to 4-week FD protocol described herein and appears to have greater variability and produces a substantially longer intrinsic period estimate (24.33 ± 0.23 h) than that derived from the 157 participants (24.15 ± 0.20 h) studied on the 2- to 4-week FD protocol (T-cycle = 20, 28 or 42.85 h). Nonetheless, the intriguing findings from that abbreviated FD protocol merit further investigation and replication by using the longer FD protocol in dimmer light to rule out the possibility that genetic differences in photic sensitivity may underlie the differences in apparent period observed in those studies.

The FD protocol has been used to assess endogenous and exogenous components in the circadian variation of CBT in humans[84], the effect of light on phase shifting[26], difference in period between in vitro measurements and plasma melatonin[85,86], the impact of circadian rhythmicity on sleep inertia[87–90], the timing of melatonin onset in men and women[91], the relationship between circadian

phase and body temperature on performance[38] and the aftereffects of entrainment to non-24-hour T-cycles on circadian period[56]. The relative contribution of the circadian system and sleep or wakefulness varies by metric, age and sex; this has been explored for sleep structure, activity, electroencephalography (EEG) oscillations during sleep and wakefulness, vigilance, cognition and mood during wakefulness[18,21,25,27,28,32–52,89,91–100].

The FD protocol has also been used to examine sleep loss and circadian disruption of metabolic, cardiovascular and pulmonary function, oral microbiota, bone outcomes, and disease risk factors[101–110]; to assess the effects of circadian rhythmicity and mistimed sleep on gene expression in whole blood[111]; and to examine genetic variants associated with intrinsic circadian period[112,113]. It also has been used to test the impact of sleep-promoting and wake-promoting compounds when they are given at different circadian phases[40,52,53].

In its current or modified form[114], the FD protocol could also be used to assess whether there are underlying circadian abnormalities in patients with circadian rhythm sleep–wake disorders, and how the circadian and sleep–wake process may influence symptoms in patients with mood disorders, epilepsy, dementia or neurodegeneration, as well as the pharmacokinetics of administered treatments[53,115].

The FD protocol with a number of T-cycles sufficient to complete at least one or two beat cycles (Fig. 1a) could also be used as a gold standard in validation of novel methods (e.g., digital biomarkers or multivariate -omics markers) to assess circadian rhythmicity. Novel biomarkers for circadian timing that will not require elaborate protocols or frequent assessment of blood samples or other physiological measures will greatly facilitate the implementation of circadian medicine at scale. However, any novel biomarker for intrinsic circadian period, phase or amplitude will need to be robust enough to maintain accuracy in the context of mistimed sleep and other masking factors and will need to be validated in the patient population being studied. Robustness and accuracy of these novel biomarkers can be assessed by using the FD protocol[59,116].

Variants of the FD protocol in which the T-cycle is very short (60 or 90 min) have also been used[18,23,117,118]. These ultra-short T-cycle FD protocols, when continued for sufficient duration, allow assessment of the circadian variation of outcomes of interest and may even permit estimation of circadian period, although validation of these ultra-short FD protocols against the much longer FD protocols described herein has yet to be undertaken for assessment of intrinsic circadian period. Furthermore, FD protocols with a short T-cycle (e.g., 3 h) are not useful for separating the sleep homeostatic from circadian contributions to outcome measures, because the sleep and wake durations are too short.

Comparison with other methods

The traditional protocol for demonstrating endogenous circadian rhythmicity and assessing circadian period in humans was the 'free-run' protocol[10], which—as described above—entails dark–light/rest–activity/sleep–wake timing selected by the study participant each day. Whereas the free-run protocol aims to remove all external periodic influences on the circadian timing system, it fails to remove the periodic influence of self-selected exposure to light and darkness generated by the participants' behavior. Because ethical and practical considerations would not generally allow humans to be studied in constant darkness, participants in free-run studies were typically allowed to turn lights on and off in synchrony with their self-selected sleep–wake times (or in other cases, some lights were kept on continuously). However, even in protocols in which the lights were on continuously, the participants' eyes were still exposed to a self-selected dark–light cycle because the participants closed their eyelids while asleep and opened them while awake.

When the free-running sleep–wake cycle remains in synchrony with the timing of endogenous circadian rhythmicity, the free-running protocol not only does not allow for accurate assessment of intrinsic circadian period, it also does not allow separation of circadian and sleep–wake–dependent contributions to sleep, performance or other functions because only a very limited range of sleep–wake and circadian phase combinations occur. In cases in which the free-running segment of the protocol continued for long enough that the sleep–wake cycle became desynchronized from endogenous circadian rhythmicity (spontaneous internal desynchrony), period estimates may be closer to that of the intrinsic period. However, spontaneous desynchrony does not always occur in free-run protocols, and if it does, it often takes 2–3 months to emerge[119], and it may not last long enough for accurate quantification of intrinsic circadian period[120,121]. Furthermore, although sleep–wake and circadian contributions to outcome measures can be assessed, the sleep and wake

durations vary widely and cluster more at some circadian phases than others, because these behaviors are distributed non-uniformly across circadian phases.

The CR protocol[58] has been used to assess the endogenous circadian component of physiological and behavioral measures by removing rhythmic influences such as the sleep–wake, dark–light and rest–activity cycles, and for those rhythmic influences that cannot be eliminated, such as food intake, by distributing them uniformly over the circadian cycle. The traditional CR involves sleep deprivation and thereby introduces a potentially confounding factor, particularly for performance outcomes because the time course of performance observed across a CR protocol reflects both rhythmic circadian and approximately linear sleep deprivation–related influences. Note that while circadian period can be estimated by extrapolating between two CRs that are multiple beat cycles of FD apart[56], to the best of our knowledge, this method has not been validated when a short FD segment is used (and therefore the interval between two CRs is only a few days). It has been shown that the 95% confidence intervals (CIs) of CBT phase collected during a CR are ~2 h[122]. If two CRs were only a few days apart—as is the case in studies with an FD protocol lasting only a few days—then inaccuracies in the phase estimates, which have a CI of 2 h, may lead to a misestimate of circadian period. This, combined with the wide 95% CI of the phase estimates, could thus lead to an estimate of intrinsic period with a very wide 95% CI as well. The CR protocol itself is too short to accurately assess circadian period. These are two reasons why the CR protocol is not optimal for assessing circadian period.

If there are many days of FD between the two CRs, then the accuracy of the period estimate increases. The accuracy of period estimates from CR protocols can be further improved by circadian phase markers that yield phase estimates with a narrower 95% CI, such as melatonin. However, this approach of estimating the period from two CRs makes the assumption that none of the properties of the circadian system change between the first and second CRs. Moreover, the slope of the intervening oscillation is unknown (shorter or longer than 24 h). These drawbacks are obviated in the FD protocol, because the data are continuously being observed. In addition, the acute sleep deprivation inherent in the CR protocols, which undermines the ability to assess circadian versus homeostatic contributions to regulation of sleep and performance, is avoided in the FD protocols, although some chronic partial sleep loss often occurs when using a 1:2 sleep/wake ratio because of the difficulties sleeping during the biological daytime (e.g., during phases when the circadian system is promoting wakefulness in humans).

Protocols for in vitro assessment of human circadian period based on luminometry of cultured peripheral fibroblasts in which the expression of firefly luciferase is driven by the promoter of a circadian gene (e.g., *Bmal1*) have been developed. Correlations between intrinsic periods derived from this in vitro method and those derived from the FD protocol are poor[85].

Limitations

The greatest limitations to the FD protocol are the duration and the requirement for a specialized inpatient research unit. These are burdensome for the participant and expensive for the investigator. An additional limitation is that because of the circadian rhythm of sleep propensity, the ability to sleep for the entire scheduled duration varies, resulting in a non-uniform distribution of sleep and wakefulness across the FD protocol, even though scheduled sleep and wakefulness are tightly controlled and evenly distributed. Nonetheless, for some experimental and clinical questions, the FD protocol remains the best protocol for collecting data with minimal confounders. One well-conducted FD protocol therefore may overall be less expensive than multiple less-well-controlled protocols.

Expertise needed to implement the protocol

These studies require multiple trained personnel because 24-h monitoring of the participant for the full duration of the study is necessary, for both safety and data integrity.

Materials

Participants

- Medical and psychological screening should be conducted to confirm that participants meet study inclusion criteria, including being in good health, being able and willing to comply with the conditions of the study and being unlikely to suffer harm as a result of taking part in the study. **! CAUTION** The study protocol should be approved by an institutional review board. Informed consent should be obtained following institutional and national guidelines.

- Women and minorities should be included in the study to ensure that the research findings can be generalizable to the entire population.
- Participants should not be using recreational drugs (including nicotine) and should have only moderate or no use of caffeine or alcohol before the inpatient portion of the protocol.
- Unless studying a clinical population, participants should be free from any acute, chronic or debilitating medical conditions.

Equipment

Facilities, equipment and staff

- Laboratory unit containing:
  - A private study room for each participant, free of time cues (no windows, clocks, timers on equipment or access to live television, radio, internet, telephone or text) and with 24-h monitoring by staff
  - A control room adjacent to the study room(s) with equipment for real-time monitoring of CBT, polysomnography (PSG) and performance/alertness testing plus audio and video of the study room for safety monitoring
  - Trained staff and equipment (centrifuge, freezers, ice machine and pipettes) for processing and temporary storage of samples
  - Equipment for physiological monitoring, including blood pressure, electrocardiogram and PSG, that is time-synched with other facility computers
  - Research nurses and/or trained technicians educated in the Standard Operating Procedures of the laboratory and certified in human subjects research
  - Registered PSG technicians for scoring the PSG recordings by using the American Academy of Sleep Medicine or other standard criteria
  - A researcher on call and available by phone within 5 min at all times during the study to help technical staff troubleshoot. This project leader is responsible for monitoring data integrity and meeting with study participant(s) and technical staff regularly during study execution.
- Controlled room lighting: approximately <4 lux in the angle of gaze during scheduled wakefulness evenly distributed across the study room and 0 lux during scheduled sleep episodes to minimize any circadian phase-shifting effects of light[123] or melatonin suppression. Equipment to measure room light levels at the start of each experiment and at regular intervals during the experiment.
- Temperature-monitoring system: continuous rectal temperature–recording system with real-time display of temperature (to detect transient data loss due to probe slips) in the control room.
- Indwelling intravenous (i.v.) catheter for frequent (every 30–60 min) blood sampling. When blood samples are not drawn, a heparinized saline solution should be infused through the sampling system at a slow rate to keep the i.v. catheter patent. Blood samples are processed for later assay of hormones or other features of interest (metabolites, proteins and RNA).
- Actigraphy device. A wrist-worn, research-grade actigraphy device is used to monitor the participant's rest–activity (and depending on the device, also light exposure) data for ≥1 week immediately before the laboratory study to verify their adherence (in terms of both timing and duration) to the assigned sleep schedule.

Hardware and software

- Computers and software capable of running higher-fidelity simulations of real-type performance tasks and systems that assess the cognitive components common to many work tasks (visual reaction time, cognitive throughout and short-term memory). For example, a psychomotor vigilance task (PVT) program runs on Windows/XP/7 and Windows 10 (Version 21H2) and is compatible with data collected with the AMI PVT-192 PVT monitor[124]. The hardware consists of a standard desktop computer with a cathode-ray tube (CRT) or light-emitting diode (LED) display, PS/2 trackball and keyboard. A custom-made, two-button response box is connected to the system via the computer's parallel printer port. The response box is attached to one of two status lines on the port. The software polls/samples the port for changes in the status line at an average sampling rate of 150 times/ms. The timing of each trial is synchronized to the beginning of the display monitor's refresh cycle of the first of the scrolling numbers (0000). This technique ensures the millisecond accuracy of the task. Each participant performed 10-min sessions spaced at predetermined intervals. Each session consisted of 50–100 stimulus–response events. The stimulus consists of a four-digit millisecond counter that appears in the display, and the response consists of a left or right button press, depending on the

configuration, which is based on the participant's handedness. The time difference between the stimulus presentation and the response constitutes the participant's reaction time (RT). At the end of each PVT testing session, RT data are automatically saved to a participant-specific directory in a text-based file format. Summary statistics, such as the mean RT or the number of lapses (RTs > 500 ms) per session, are also generated and saved.

- Computer or pencil-and-paper systems for other data collection, such as subjective assessments of mood, hunger or pain, that include exact timing of collection.
- Data-collection system that stores timing of events (light on/off, bedtime and wake time; time of blood and saliva samples, performance testing and PSG recordings) in addition to the scheduled time of these events.
- NOSA software package (available at https://github.com/wwang4bwh/FD-NOSA) to estimate period, amplitude and phase of CBT or melatonin.
- Computers and software to process and analyze the specific data being collected.

Procedure 1: the standard procedure

Experimental design

1 *Determine the T-cycle length*. T-cycle lengths that we have used include 11, 20, 27, 28 and 42.85 h[30].
▲ **CRITICAL STEP** A T-cycle length outside the range of entrainment[12,125] of the intrinsic circadian system and preferably not a factor of 24 h (e.g., not 3, 4, 6 or 8 h) should be chosen to accurately assess the intrinsic period. This ensures that under the FD conditions, dark–light and behavioral patterns (such as rest or activity, posture, fasting or eating and social interactions) that follow the scheduled sleep–wake cycle will be far enough away from the intrinsic circadian rhythm that has a period close to 24 h (mean ± s.d. = 24.15 ± 0.20 h)[30] that the circadian system cannot entrain to it.

2 *Determine the FD duration*. The duration of the FD protocol needs to be long enough to cover at least one full beat cycle (we recommend two beat cycles). The length of the beat cycle is equal to the least (smallest) common multiple of the T-cycle and 24 h (due to the nearly 24-h circadian cycle). For example, for a 28-h T-cycle FD, the length of one beat cycle is the least common multiple of 24 and 28, which is 168 h (seven 24-h days or one calendar week). Simulation studies with artificial datasets show that the difference between the estimated and the intrinsic periods decreases asymptotically as FD duration increases[12]. Theoretical studies show that the standard deviation of the period estimate is proportional to one over the FD duration to the 3/2 power[29,126]. Hence, the desired precision for the period estimate of the circadian process can also be used as an experimental design criterion to aid in the choice of the FD duration.
▲ **CRITICAL STEP** The duration of the FD needs to be long enough to cover at least one beat cycle. For greatest accuracy of the intrinsic period estimate, we recommend at least two beat cycles. An additional advantage of studying more than one beat cycle is a more fine-grained combination of sleep–wake and circadian phases.

3 *Determine the sleep/wake ratio and associated dark/light ratio*. One option is to match the typical ratio of dark/light exposure from a 24-h day—a 1:2 ratio of darkness/light (8 h of darkness or scheduled sleep and 16 h of light or scheduled wakefulness). Thus, a 28-h FD schedule would consist of 9.33 h of darkness or scheduled sleep and 18.67 h of light or scheduled wakefulness. Most of the FD studies that we have carried out have used this 1:2 sleep/wake ratio (see Supplementary Table 1). One factor to consider is whether the dark/light ratio will result in some chronic sleep loss across the study. For studies designed to investigate the effects of chronic sleep loss and its interaction with circadian rhythmicity, the ratio of sleep/wake can be increased. For example, we and others have conducted FD protocols with a larger 1:3.3 ratio, which is the equivalent of 5.6 h of sleep or darkness and 18.4 h of wakefulness or light per 24 h[28,49,104], or a 1:5 ratio, which is the equivalent of 4 h of sleep or darkness per 24 h[127]. Alternatively, the duration of sleep or darkness can also be extended to compensate for times when participants are sleeping at adverse circadian phases and will therefore have more wakefulness within those sleep episodes; a smaller ratio will minimize cumulative sleep loss by allowing more scheduled time for sleep, although the sleep may still be disrupted. We have used a 1:1.4 ratio[50].

4 *Determine the light level during wakefulness*. The light levels used in the protocol must be carefully considered and controlled to minimize effects of light on the circadian system or on any of the outcome measures (such as melatonin). On the basis of model simulations and our own observations, we recommend using the lowest feasible light intensity during scheduled wakefulness episodes (<15 lux maximum in the room at ~1.83 m (72 inches) in the direction of the light fixtures

(equivalent to approximately <4 lux in the angle of gaze looking toward the walls)) for a more accurate period assessment[12].

▲ CRITICAL STEP Although light is distributed evenly across the circadian cycle over the course of the entire experiment, brighter light levels will shift the circadian rhythms a greater amount from day to day, because of the larger amplitude of the phase response curve to brighter light. This creates a 'wobble' (also known as relative coordination) in the data, and the magnitude of the day-to-day wobble increases as the light level increases. With bright enough light and a short FD duration, this wobble will reduce the accuracy of the circadian period assessment significantly[12].

5 *Choose a biological marker and determine the sampling frequency*. Melatonin and CBT data are the most commonly used biomarker/physiological variables for determining intrinsic circadian period and phase.

▲ CRITICAL STEP Frequent sampling throughout the FD protocol is necessary to assess whether phase progresses uniformly; we recommend at least hourly measurements for melatonin and more frequent samples for temperature (at least every 10 min).

▲ CRITICAL STEP Melatonin secretion is suppressed by light exposure, so all blood or saliva samples that are collected for melatonin assay must be conducted in dim light (<15 lux).

▲ CRITICAL STEP For temperature collection, we recommend body temperature measurement from a rectal thermistor to obtain true core temperature assessment and collect continuous data. If a rectal sensor is used, we also recommend online real-time monitoring because there is a possibility that the sensor may become displaced as the participant moves about.

6 *Determine the sleep–wake activities*. All scheduled wake episodes across an FD protocol should be as similar as possible in terms of the types and pattern of activities. We recommend that participants rigorously follow a set schedule in which major events such as meals, showers and other daily tasks occur at the same time relative to scheduled wake time each wake episode.

▲ CRITICAL STEP Unless the intensity, duration and timing of exercise is strictly controlled[103] both within and between participants, we recommend minimizing exercise so that the level of physical activity is low throughout the FD protocol. This is because of evidence that exercise may induce phase shifts in a phase-dependent manner[128].

▲ CRITICAL STEP Participants should not be permitted to lie down or sleep during scheduled wake episodes; similarly, during scheduled sleep episodes the participants should be kept lying in bed without light exposure. Bedpans or urinals can be used as needed during scheduled sleep episodes to minimize posture changes (which might affect measurements of circadian phase) and the need to turn on lights.

7 *Determine outcome measures and frequency of assessment*. The appropriate metrics for other outcome measures of interest (sleep stages, neurobehavioral functions such as PVT reaction time and lapses, subjective alertness on a visual analog scale, mood, Karolinska sleepiness scale and tests of cognitive throughput such as addition calculation and digit symbol substitution tasks)[25,33,53,129,130] should be selected. The frequency and feasibility of repeated assessment, potential practice effects, learning curves, interference between assessments of outcome measures, participant burden and participant boredom should all be considered.

Data collection

8 *Recruit and screen participants*. Determine the required characteristics of the participants. For healthy individuals, we recommend a thorough medical (physical examination, medical history, electrocardiogram, blood count, urinalysis and comprehensive metabolic panel) and psychological screening.

! CAUTION Institutional review board approval must be obtained before the recruitment of participants. Informed consent must be obtained from all participants before beginning study procedures, including screening.

▲ CRITICAL STEP We recommend that researchers do not allow caffeine, tobacco or alcohol consumption by participants for ≥2 weeks before inpatient procedures if they will not be allowed during the inpatient study. This will reduce any withdrawal effects[131]. We recommend that participants are scheduled to at least 10 h of in bed or sleep time for at least 3 weeks prior to the protocol. This should be objectively verified (e.g., using actigraphy). This is to minimize variation within a participant and reduce variation across participants of any sleep loss prior to the protocol. We recommend that participants do not work rotating or night shifts within 3 months prior to the protocol and do not cross more than one time zone within 1 month prior to the protocol. This is to increase stability of circadian phase across participants.

9 *Admit the participant to the laboratory*. For each participant, complete the following steps:
- Orient the participant to the study environment and procedures.
- Collect and send a urine sample for a toxicology screen if researchers choose to do this as part of making sure that the participant is not taking any recreational drugs, alcohol or nicotine.
- Collect and send a urine sample for a pregnancy test (female participants only).
- To maintain a time-free environment, remove any devices that display time or have internet connections, such as watches, clocks, cell phones, tablets, computers or radios from the participant.
- To avoid possible contamination of study measurements such as saliva samples and PSG signals, remove food, mouthwash or lotions from the participant.
- Give the participant a CBT sensor and instruct them in insertion, anchoring and 'sensor-checking' procedures.
- Record light levels.

▲CRITICAL STEP The laboratory must be free of time cues. No windows, clocks or timers on equipment can be present, and no access to live television, radio, internet, telephone or text messaging can be permitted.

10 Run the study for the determined length of time with the determined parameters, collecting data as specified in the experimental design.

11 *Discharge and debrief the participant(s)*. At the conclusion of the study, a staff member should complete the follow tasks:
- Ask the participant for their feedback on their inpatient experience and answer any general questions that they might have.
- Complete a final health assessment of the participant.
- Review discharge instructions with the participant to ensure their safety after leaving the study, including how to get back on their usual sleep schedule and how to return to vigorous physical activity after being sedentary in the laboratory for days or weeks.

Data pre-processing

12 *Examine circadian marker data and exclude values out of physiological ranges*. If CBT was chosen as the circadian marker, exclude readings outside the physiological range (e.g., from instances where the sensor was removed for shower or toilet use). In addition, as an optional step, exclude readings within the physiological range that appear to be due to sensor slippage. If melatonin was chosen as the circadian marker, first have the collected blood samples assayed for melatonin by using commercially available methods (radioimmunoassay or ELISA)[132–135] and then exclude results outside of the detectable limit of the assay.

13 *Examine outcome measure data and exclude invalid measurements*. For physiological and metabolic measurements such as glucose or hormones, exclude values outside of physiological ranges. For neurobehavioral performance measures, exclude tests that were interrupted or incomplete and tests for which it appears that the participant did not follow the study instructions. In cases in which a performance test was taken late, determine whether it might be too close to the next scheduled test battery (in which case, consider keeping the late test results and discarding the following test results).

14 *Decide how to statistically treat missing data*. We do not impute missing neurobehavioral performance and mood data but rather apply models (such as mixed-effects models) that can deal with missing data[136].

▲CRITICAL STEP Interpolation methods can be used to impute missing physiological and metabolic data when the missing rate is ≤20%. Interpolation methods must account for the rhythmic nature of the data, and therefore the length of time between samples must be considered (e.g., do not interpolate between values 8 h apart).

Statistical modeling and analyses

15 *Estimate circadian period and phase using non-orthogonal spectral analysis (NOSA)*. The NOSA algorithm is a harmonic regression model with correlated noise. The model provides a decomposition of time series of physiological measurements into estimates of its circadian, evoked sleep–wake cycle and correlated noise components (Fig. 1f). The circadian component of CBT or melatonin consists of an oscillation with a period of ~24 h and its harmonics (12, 8 or 6 h). The evoked sleep–wake component captures the T-cycle influence on the data resulting from the

imposed FD protocol and its harmonics (14, 9.3 or 7 h for a 28-h T-cycle). The serially correlated noise component captures perturbations of the physiological time series due to events such as physical activity or showers that persist for some time after the perturbation itself.

A statistical model for the NOSA paradigm for a time series of data (CBT or melatonin) measurements $y_1, \ldots, y_n$ can be defined as

$$y_t = \mu + \sum_{k=1}^{2} A_k \cos\left(\frac{2k\pi t}{\tau_c}\right) + B_k \sin\left(\frac{2k\pi t}{\tau_c}\right) + \sum_{j=1}^{J} C_j \cos\left(\frac{2j\pi t}{\tau_{\mathrm{FD}}}\right) + D_j \sin\left(\frac{2j\pi t}{\tau_{\mathrm{FD}}}\right) + v_t$$

where μ is the mean, $\sum_{k=1}^{2} A_k \cos\left(\frac{2k\pi t}{\tau_c}\right) + B_k \sin\left(\frac{2k\pi t}{\tau_c}\right)$ is the circadian signal with period τ_c, $\sum_{j=1}^{J} C_j \cos\left(\frac{2j\pi t}{\tau_{\mathrm{FD}}}\right) + D_j \sin\left(\frac{2j\pi t}{\tau_{\mathrm{FD}}}\right)$ is the evoked sleep–wake signal with period τ_{FD} and v_t is the serially correlated noise component defined as

$$v_t = \exp(-\alpha\Delta) v_{t-1} + \varepsilon_t$$

where $\exp(-\alpha\Delta)$is the serial correlation coefficient, α is the approximate time constant of the correlated perturbations, Δ is the data sampling interval and ε_t is independent Gaussian noise with mean 0 and variance σ_ε^2. Two harmonics are usually used to describe the circadian component on the basis of empirical observations that the model accurately captures the non-sinusoidal shape in the CBT and melatonin oscillations[122,126,137]. More than two harmonics may be necessary to capture the amplitude of the evoked sleep–wake component; this is determined empirically. For the evoked component, the period τ_{FD} is set to the period of the T-cycle in the FD protocol. Scheduled and unscheduled activities such as showers can cause time-dependent, correlated fluctuations in CBT on a much shorter time scale than either circadian or evoked components. These data features are captured by v_t. The model is fit to the data by maximum likelihood[126]. A key feature of the NOSA algorithm is the formula derived from the likelihood analysis to estimate the uncertainty (standard deviation) in the period of the circadian signal[126], which is

$$\sigma_{\tau_c} = \left[\frac{6\tau_c^4 \sigma_\varepsilon^2 \Delta}{\pi^2 T^3 \sum_{k=1}^{2} k^2 (A_k^2 + B_k^2)\left[1 - 2\exp(-\alpha\Delta)\cos\left(\frac{2\pi k}{\tau_c}\right) + \exp(-2\alpha\Delta)\right]}\right]^{\frac{1}{2}},$$

where $T = n\Delta$ is the duration of the FD protocol[29,122,126]. If precision of the circadian period estimate is an experimental design criterion, then this equation can be used to guide the choice of FD protocol duration. Circadian phase can be computed from the estimated circadian signal by using this algorithm[122,137]. An R package is available (https://github.com/wwang4bwh/FD-NOSA) to apply the NOSA method for period estimation with the specification of τ_{FD} and k.

16 *Assign time awake, circadian phase and beat cycle for each physiological or behavioral measurement at a specific time t*. Assign a specific combination of a time awake (e.g., 4.3 h after scheduled wake), a circadian phase (with respect to the individual's intrinsic circadian period and phase at the start of the FD protocol, expressed in degrees from 0 to 359 defined as

$$\text{phase} = \frac{\mathrm{mod}(t - t_0, \hat{\tau}_c)}{\hat{\tau}_c} \times 360$$

where t_0 is the estimated time of the minimum for CBT data or maximum for melatonin data and $\hat{\tau}_c$ is the estimated period) and a beat cycle within the FD protocol duration. Data are typically binned with respect to time since scheduled wake or sleep onset and circadian phase, with the size of the bin depending on the frequency of data collection. For example, CBT data are typically collected multiple times per hour, and performance tests are typically collected every 2 h, so CBT bins might be 1 or 2 h wide, whereas performance bins might be 2 or 4 h wide.

17 Use longitudinal mixed-effects statistical modeling[136] to study the relationship between outcomes and length of time since scheduled wake or sleep onset (sleep–wake homeostasis), circadian phase and beat cycle of the protocol and their interactions. Participants can be treated as random effects to model interindividual differences and/or correlation from the repeated measures within the same individual.

▲CRITICAL STEP Longitudinal mixed-effects statistical modeling is more powerful and appropriate for these data than repeated *t* tests at the different time or circadian phase bins because the data are derived from multiple observations in the same individual and therefore are not independent.

Procedure 2: the real-life case study procedure

Data collection ● Timing ~3 weeks

▲CRITICAL These steps describe an experiment separating the influences of chronic sleep loss, many consecutive hours awake and circadian timing on human performance by using the ~3-week (three beat cycles), 42.85-h FD (1:3.3 sleep/wake ratio) protocol described in this research[28]. We have written the following steps to allow researchers to exactly repeat this study if they choose. Our understanding is that researchers would modify the precise parameters depending on their research questions by using the decisions outlined in Procedure 1.

1 Screen the participant for the study by history, physical exam, laboratory evaluations of urine and blood, electrocardiogram, psychological screening and clinical polysomnographic testing.

2 Maintain the participant on a consistent sleep–wake schedule with 10 h in bed at home for 3 weeks to ensure that the participant is well rested and does not have levels of sleep restriction different from other participants before entering the inpatient portion of the study. Confirm this with sleep logs and actigraphy and by checking calls to a time-stamped voicemail system during the 3-week pre-study period and again at admission.

▲CRITICAL STEP Participants not compliant with this schedule do not continue into the inpatient portion of the study.

3 Admit the participant to the Intensive Physiological Monitoring unit and orient them to the laboratory environment. All events including sleep–wake timing, blood and other sample collection and performance testing are based on each participant's at-home sleep–wake schedule. While in the laboratory, the project leader or other researcher visits the participant daily and reviews all data collected during each wake episode.

4 During the baseline segment at the beginning of the inpatient portion of the protocol, the participant's daily activities include three 24-h T-cycles, with 14 h of scheduled wakefulness and 10 h of scheduled sleep (a 1.4:1 ratio). This segment is to ensure that all participants are well rested and adapted to the laboratory environment. During the FD segment, the participant's schedule includes twelve 42.85-h T-cycles, with 32.85 h of scheduled wakefulness and 10 h of scheduled sleep (a 3.3:1 ratio). During the recovery segment, schedule the participant to ten 24-h T-cycles with 10 h of scheduled sleep, timed by using CBT period and phase measurements to be at the same relative circadian phase as during the baseline segment.

5 Implement 1:1 monitoring by technicians while the participant is awake during the FD segment of the study to ensure that the sleep restriction does not lead to any significant episodes of sleep during scheduled wakefulness. Score the PSG from the wake episodes within 2 d and provide feedback to the research technicians about any sleep that occurs during scheduled wake time.

6 Have participants wear a rectal thermistor continuously during the 42.85-h T-cycle FD protocol. Collect CBT readings once per minute and record them in real time.

? TROUBLESHOOTING

7 Collect samples of blood for melatonin measurement at hourly intervals during the last two baseline days, during the entire FD segment and during the first two recovery days after the FD protocol. Use a sample collection system that allows collection of samples during scheduled sleep without disturbing the participant, such as an i.v. catheter in a forearm vein connected to a 12-foot i.v. line and sampling apparatus. Follow local infection control guidelines for frequency of replacement of the i.v. tubing and sampling apparatus and replacement of the i.v. catheter.

? TROUBLESHOOTING

8 Administer multiple tests of neurobehavioral function for sleep inertia testing at 1, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 150 min after scheduled wake time and then once every 2 h during the remainder of the wake episode. We use a visual PVT (not for sleep inertia testing owing to time constraints), a test of sustained attention widely used in sleep research, followed by a subjective alertness scale.

9 Apply surface electrodes to the participant's face and scalp for recording of nightly sleep and daily wake PSG. Apply scalp electrodes for multi-channel EEG recordings according to the international standard 10-20 system of electrode placement and use locations for EEG and other sites recommended by the American Academy of Sleep Medicine. Record and score sleep PSG, including electro-oculogram, electromyogram and EEG according to the technique of Rechtschaffen and Kales[138]. Waking EEG is recorded during wakefulness to confirm that there is no inadvertent sleep and is also collected during the working memory task to assess blink artefacts and slow eye movements (SEMs)[139] and changes in theta and alpha activity that are associated with changes in sleep homeostasis[140,141] and working memory[141,142].

Data preprocessing ● Timing ~4 weeks per three beat cycles

10 Examine, clean and process the collected CBT (A), blood sample (B), neurobehavioral performance and mood (C) and PSG (D) data by following the appropriate option.
   (A) **CBT**
      (i) Exclude readings lower than physiological range.
      (ii) Exclude CBT readings from instances in which the CBT sensor had been removed for shower or toilet use.
      (iii) Exclude CBT readings in which the sensor had slipped.
   (B) **Blood samples**
      (i) Send EDTA plasma samples to a commercial company to assay for melatonin.
      (ii) Exclude results outside of the detectable limit of the assay.
   (C) **Neurobehavioral performance and mood**
      (i) Remove incomplete or interrupted test results.
      (ii) For tests within a few minutes of each other (e.g., if a test were delayed), use data from the first test.
   (D) **PSG data**
      (i) Score for sleep stages.
      (ii) Perform spectral analyses after removing EEG artefacts.

Data analyses ● Timing 4 weeks per three beat cycles

11 Analyze CBT and melatonin data by using NOSA as described in the general procedure for circadian period, amplitude and phase.
   ? TROUBLESHOOTING
12 Assign the circadian phase. Using the NOSA period and phase estimates, assign each outcome measurement from the study (e.g., PVT reaction time, PVT lapses, sleep stage of each 30-s PSG epoch and blood sample melatonin level) a circadian phase, with the fitted CBT minimum from NOSA set equal to phase 0°.
13 Assign the elapsed time awake or asleep. Assign each outcome measurement (e.g., PVT reaction time, PVT lapse, sleep stage of each 30-s PSG epoch and blood sample melatonin level) to a specific time relative to the start of the scheduled wake episode or sleep episode (such as 2 h since scheduled wakefulness or 4 h into the scheduled sleep episode).
14 Bin the outcome measurement data (PVT reaction time, PVT lapse, sleep stage of each 30-s PSG epoch and blood sample melatonin level) in 60° (4-h) circadian phase bins, with the first bin centered at 0°. Also bin the outcome measurement data in 4-h time awake or time asleep bins relative to the start of scheduled wakefulness or scheduled sleep.
15 Use linear or generalized linear mixed models to quantify the relationships between the outcome measurements and circadian phase, time awake and week within the FD duration. Include the participant as a random effect to model interindividual variability. Linear or generalized linear mixed models also appropriately control for correlation of performance and other measurements within an individual.

Troubleshooting

Troubleshooting advice can be found in Table 1.

Table 1 | Troubleshooting table for Procedure 2

Step	Problem	Possible reason	Solution
6	Temperature readings consistently lower than physiological range or missing despite thermistor being inserted	Sensor may be broken, disconnected or displaced	Check that the sensor is functioning and that all connections are secure. Ask the participant to check the placement of the sensor. If the sensor is broken, issue a replacement
	Temperature readings are higher than the expected range	Temperature may briefly spike after hot showers, bowel movements, strenuous exercise, etc. Sustained elevated temperature may indicate a fever	Brief temperature spikes are not problematic for NOSA. Sustained elevated temperature indicative of a fever may require medical examination of the participant and disempanelment for safety reasons

Table continued

Table 1 (continued)

Step	Problem	Possible reason	Solution
7	Melatonin values remain low (<2 pg/ml)[144] for the entire duration of the sampling segment	Light exposure was too high, and melatonin secretion was suppressed	Keep light exposure to a minimum (<15 lux) during melatonin sampling segments
11	One participant's melatonin values are outliers compared to others	Natural variability exists in melatonin levels among participants. Age, sex and individual differences may all influence melatonin secretion within an individual	As long as values are within the detectable limit for the assay, this should not affect the NOSA period and phase estimate
	Some melatonin samples were missed	Missing because of technical difficulties	NOSA can tolerate intermittent missing data. If >20% of the data are missing, the results might not be reliable. We recommend collecting both saliva and blood so that the saliva melatonin profile may be used if blood sampling difficulties occur

Timing

Procedure 2
Steps 1–9, data collection: ~4.5 weeks per participant for the entire study, 3 weeks for the FD segment
Step 10, data preprocessing: ~4 weeks per three beat cycles per participant
Steps 11–15, data analyses: 4 weeks per three beat cycles

Anticipated results

Using the real-life case study procedure of this protocol[28], we found that 10-h sleep opportunities were sufficient to dissipate an acute homeostatic process in the PVT task to baseline levels at the start of each wake episode even during the third beat period, but a separate chronic sleep homeostatic process led to accelerated PVT task performance deterioration over consecutive hours awake. Figure 3 shows the PVT mean reaction time at different combinations of circadian phase, time since wakefulness and week of sleep restriction under chronic sleep restriction and control conditions. The significant four-way interaction of circadian phase, length of time awake, week on the FD protocol and group on PVT mean reaction time suggests that within each week of the chronic sleep loss condition, circadian influence increased with longer consecutive hours awake. Across weeks, the cumulative effect of chronic sleep loss was more evident (red path) during circadian misalignment (late circadian night). Data from another 42.85-h FD protocol with a 1:2 scheduled sleep/wake ratio were included as a control to examine the effect of chronic sleep loss.

Other examples of outcome measures assessed by using the FD protocol

FD protocols used to estimate period

Example 1. FD protocols carried out between 1991 and 2011 were used to examine sex differences in circadian period by comparing data from 157 participants (52 women, 105 men; age (mean ± s.d.): 34.4 ± 17.7 years, range: 18–74 years)[30]. Intrinsic circadian periods derived from CBT were estimated by using NOSA and are shown in Fig. 4a. Circadian period estimates were significantly different between men and women (mean ± s.d. = 24.09 ± 0.20 h for women versus 24.19 ± 0.20 h for men, $F_{1,155} = 8.54$, $P < 0.01$). In fact, although an average of about one-quarter of participants exhibited intrinsic circadian periods that were shorter than 24 h, significantly more women than men had periods shorter than 24 h (34.6% versus 14.3%; Fisher's exact test, $P = 0.0061$)[30]. An abbreviated 5-h FD protocol also observed a sex difference in circadian period in European-Americans (24.24 ± 0.23 h for women versus 24.41 ± 0.20 h for men).

Example 2. A 73-d study that included two FD segments with a 1:2 ratio of scheduled sleep/wakefulness was used to test the hypothesis that the period of the circadian pacemaker in humans would be modified after entrainment to a non-24-h T-cycle. Specifically, the intrinsic circadian period would be lengthened after entrainment to a longer-than-24-h T-cycle, the 24.65-h Martian sol (Martian day length), as compared to a shorter-than-24-h T-cycle, the 23.5-h sleep/wake cycle that was often

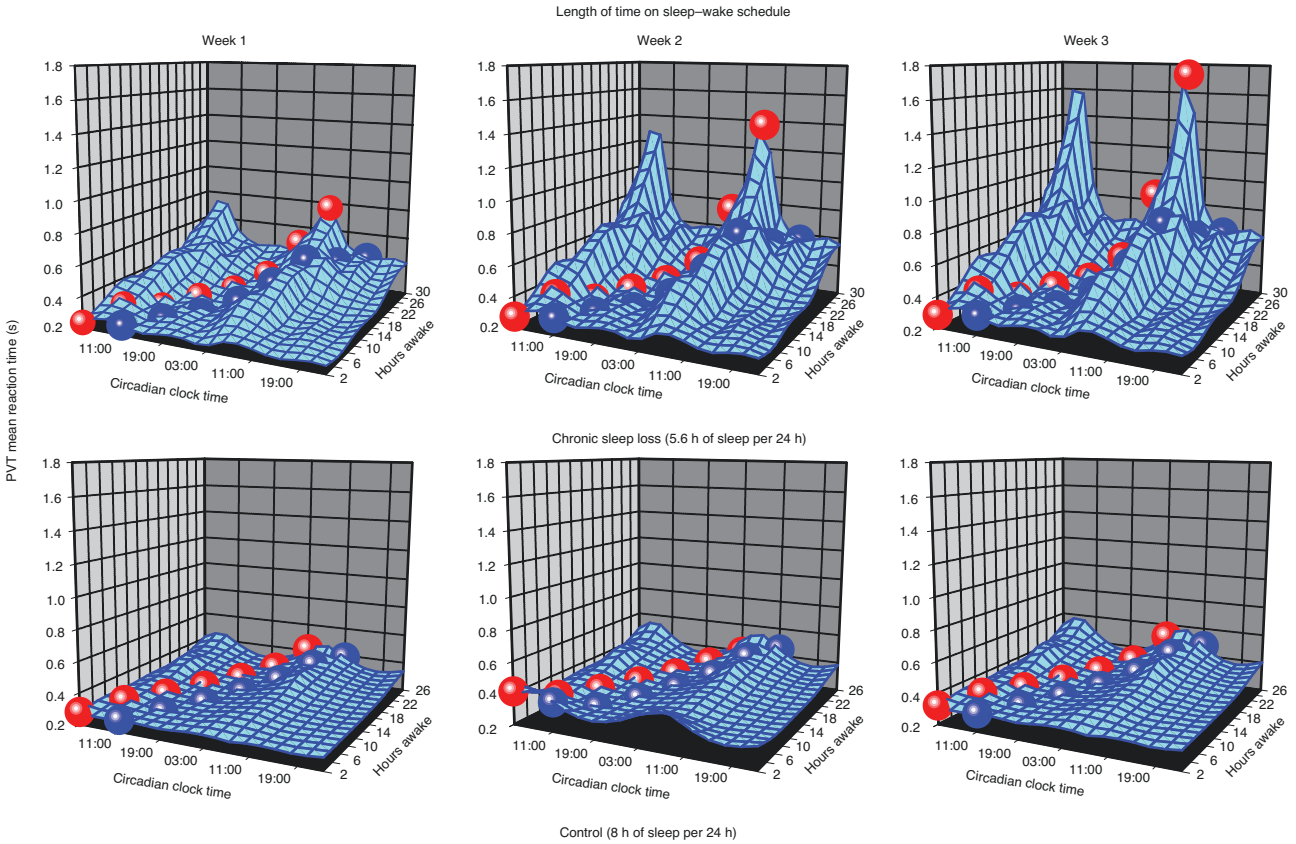


Fig. 3 | Circadian and homeostatic regulation of performance during a 42.85-h FD protocol. Mixed-effects statistical model predictions of PVT mean RT data double-plotted at each combination of circadian phase (in 24-h clock time) and length of time awake, within each week (or beat cycle) of the protocol. The blue dots show a projected path of the combination of consecutive hours awake and corresponding melatonin circadian time when individuals awaken at their habitual circadian time, whereas the red dots show the projected path when they awaken 2 h before the melatonin peak. Within each week of the chronic sleep loss protocol, PVT mean RT increased with longer consecutive hours awake. Across the 3 weeks, there was also a disproportionate deterioration of performance during the late circadian night (1 am to 9 am circadian clock time). Figure adapted with permission from ref. [28], AAAS.

experienced by astronauts in the Space Shuttle[56]. Seven participants lived for 2 weeks on a 23.5-h T-cycle with moderately bright light to enhance entrainment before their circadian period was estimated from data collected during a two-beat cycle 28-h FD segment (Fig. 4b), and for two weeks on a 24.65-h T-cycle with moderately bright light to enhance entrainment before their circadian period was estimated during a second two-beat cycle 28-h FD segment (Fig. 4c), with the order of imposed 23.5-h and 24.65-h T-cycle segments randomized. The composite estimates of the intrinsic circadian period—as computed by averaging the intrinsic period estimate from plasma melatonin, plasma cortisol and CBT data—were significantly shorter after entrainment to the 23.5-h T-cycle than they were after entrainment to the 24.65-h T-cycle (Fig. 4e), demonstrating that the human circadian pacemaker shows plasticity.

FD protocol used to study the effect of circadian pacemaker, the sleep–wake cycle and their interaction on sleep measures

Example 3. A 28-h T-cycle FD protocol with a 1:2 ratio of sleep/wakefulness was used to analyze the contribution of the circadian pacemaker and time since start of scheduled sleep as well as their interaction in the regulation of the internal structure of sleep[35]. Data from 11 young participants and 13 older participants were grouped into 4-h circadian phase bins and 1-h time bins since the start of sleep episode (Fig. 5). Older participants had much lower sleep efficiency when sleep was scheduled at adverse circadian phases, whereas young participants were able to maintain a higher sleep efficiency at most circadian phases.

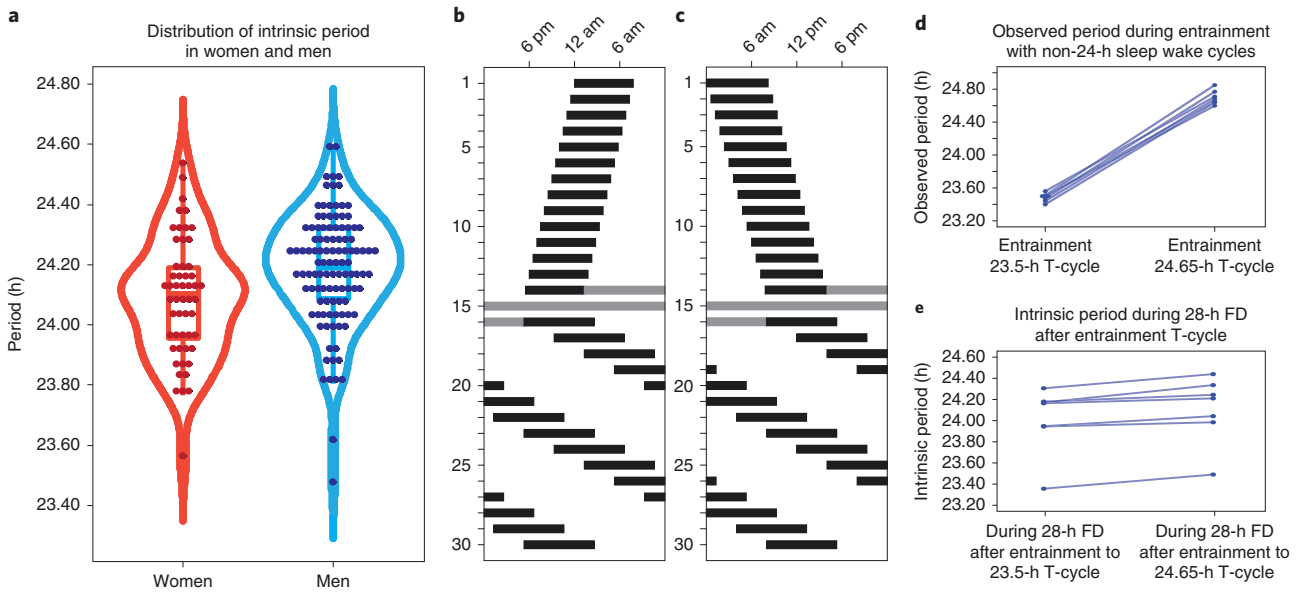


Fig. 4 | Circadian period estimation using FD protocols. a, Period estimate for women and men studied in different FD protocols. Intrinsic circadian period estimates were significantly different between men ($n = 110$, mean ± s.d. = 24.19 ± 0.19 h) and women ($n = 53$, mean ± s.d. = 24.09 ± 0.20 h) ($F_{1,161} = 9.48$, $P = 0.0024$). **b**, Raster plot of a 23.5-h entrainment T-cycle followed by a 28-h FD protocol. **c**, Raster plot of a 24.65-h entrainment T-cycle followed by a 28-h FD protocol. In the raster plots, the horizontal axis represents clock time within each 24-h day, and the vertical axis represents 24-h days across the FD protocol. **d**, Observed individual period estimates from melatonin data during a 23.5-h entrainment T-cycle and during a 24.65-h entrainment T-cycle. These results suggest that exposure to a model-based lighting regimen can entrain a human circadian pacemaker. **e**, Intrinsic period estimates from composite data during a 28-h FD protocol after two entrainment segments of different T-cycles. Intrinsic circadian period was significantly shorter after the 23.5-h T-cycle than after the 24.65-h T-cycle (mean ± s.d. of the difference = −5.8 ± 2.9 min, $P = 0.002$), as estimated during 2-week 28-h FD protocols in dim light conditions. This after-effect of entrainment reveals the plasticity of the circadian period. Data from ref. [56].

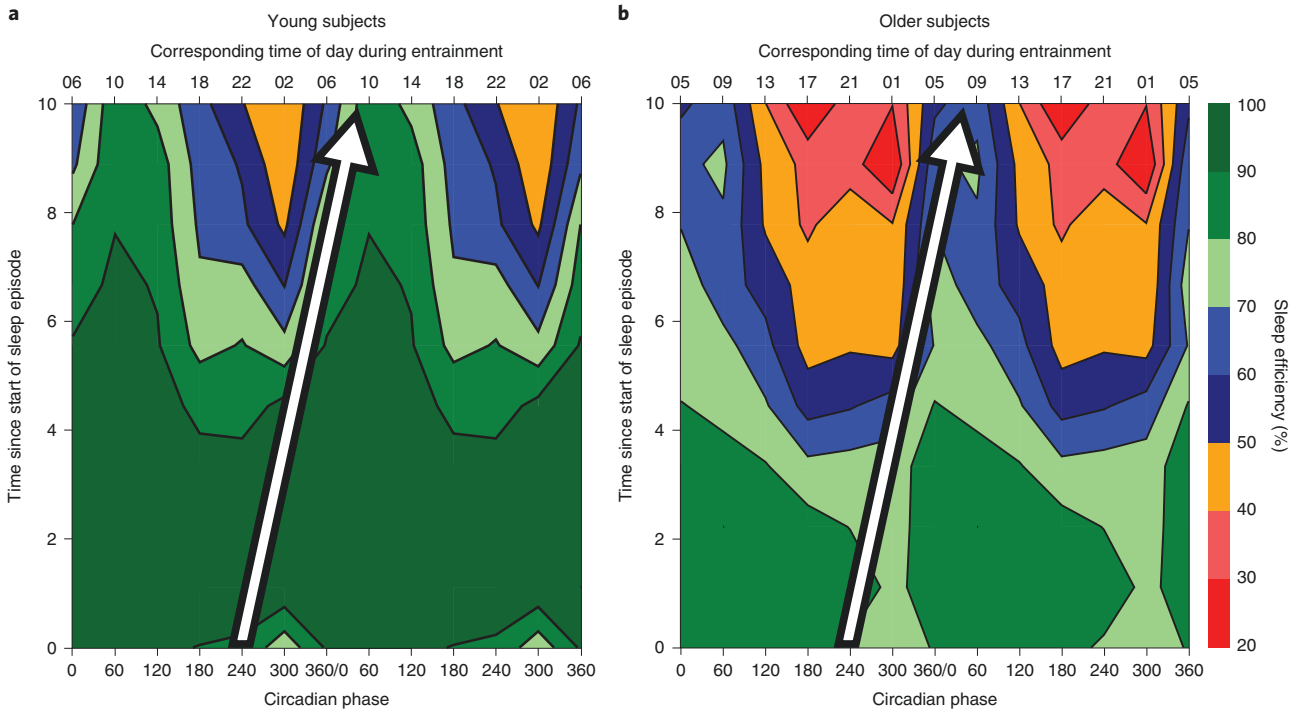


Fig. 5 | Sleep efficiency of young participants and older participants during a 28-h T-cycle FD protocol. Mean sleep efficiency data during sleep episodes of 11 young participants (**a**) and 13 older participants (**b**) were grouped into 60° (4-h) CBT circadian phase bins and 1-h time since start of sleep episode bins. The white arrows show the 'path' of sleep efficiencies from the beginning to the end of a sleep episode during circadian phases of habitual sleep timing under entrained conditions. Sleep efficiencies for young participants are higher than those for older participants. Data from ref. [35].

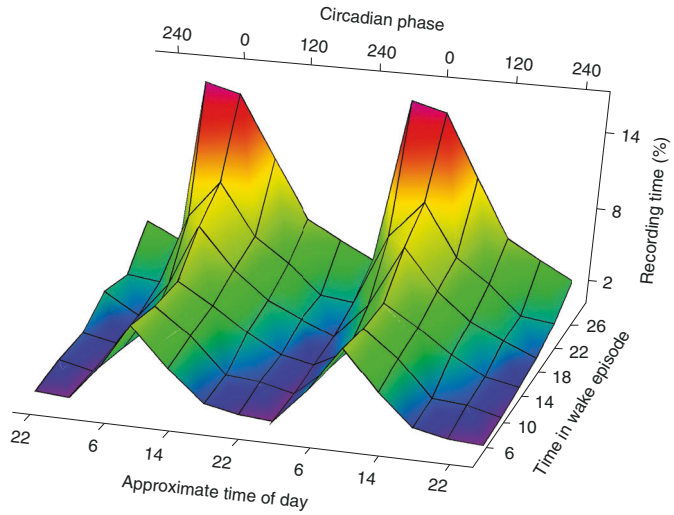


Fig. 6 | Percentage of recording time with SEMs during wakefulness under a 42.85-h FD protocol. Data of fitted percentage of recording time with SEMs from 7 men are double-plotted with circadian phase (4-h) bins and elapsed time awake (4-h) bins. SEMs increased over consecutive hours awake and were dependent on circadian phase, with the highest value occurring close to 0° and 26 h after wakefulness (in red). Data from ref. [27].

FD protocol used to study the effect of the circadian pacemaker, the sleep–wake cycle and their interaction on SEMs

Example 4. A 14-d 42.85-h T-cycle FD protocol with a 1:2 ratio of sleep/wakefulness was used to separate the contribution of circadian rhythmicity and elapsed time awake on EEG activity and SEMs during wakefulness[27]. The EEG signals during the 4-min eyes-open segment of the Karolinska drowsiness test[143] were visually inspected for SEMs. 2-s epochs containing SEMs were recorded, and the percentage of recording time containing SEMs was calculated. Data from 7 men were grouped into 4-h melatonin circadian phase bins and 4-h length of time awake bins (Fig. 6). The fitted percentage of recording time with SEMs increased over consecutive hours awake and was dependent on circadian phase, with the highest value occurring close to 0°.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All original datasets used in the preparation of this paper contain confidential or identifiable human data and are not publicly available due to human subject research regulations. De-identified sample datasets are available upon reasonable request and are subject to internal review and approval. Execution of a materials transfer agreement is required by our institution for transfer of data.

Code availability

SAS codes for data analyses and R codes for the NOSA program can be found at https://github.com/wwang4bwh/FD-NOSA.

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Author contributions

C.A.C. developed the concept and designed the FD protocol as implemented in our laboratory. J.M.R. designed and built the data-collection hardware and software, the protocol-scheduling software and the data-management system used in our laboratory. E.N.B. designed the statistical model, analysis and computational framework for the NOSA procedure. E.N.B., J.F.M., D.-J.D., and C.A.C. contributed to the implementation of the computational framework for the NOSA procedure. C.A.C., D.-J.D., E.B.K., and M.A.S.H. carried out simulations to determine the impact of light levels on period estimates from FD protocols and free runs. D.-J.D. developed the analyses framework for the separation of circadian and sleep–wake contributions to sleep and performance. R.K.Y., K.-M.Z., J.K.W., F.A.J.L.S., K.P.W., E.B.K., J.F.D. and D.-J.D. carried out FD studies and analyses. W.W. re-analyzed FD data presented here and wrote and updated the NOSA program in R for sharing. W.W., R.K.Y. and K.-M.Z. created figures for the manuscript. All authors contributed to drafting, writing and/or editing the manuscript.

Competing interests

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Chemical Solutions LLC, Amtrak, Casper Sleep Inc., C&J Energy Services, Catapult Energy Services Group, Covenant Testing Technologies, the Dallas Police Association, Enterprise Rent-A-Car, Espinal Trucking/Eagle Transport Group/Steel Warehouse Inc., FedEx, Greyhound Lines Inc./Motor Coach Industries/FirstGroup America, PAR Electrical Contractors Inc., Product & Logistics Services LLC/Schlumberger Technology Corp/Gelco Fleet Trust, Puckett Emergency Medical Services LLC, Puget Sound Pilots, Union Pacific Railroad, United Parcel Service and Vanda Pharmaceuticals Inc.; serves as the incumbent of an endowed professorship provided to Harvard University by Cephalon, Inc.; and receives royalties from McGraw Hill and from Philips Respironics for the Actiwatch-2 and Actiwatch Spectrum devices. C.A.C.'s interests were reviewed and are managed by the BWH and MGB in accordance with their conflict of interest policies. D.-J.D. was a paid consultant to F. Hoffmann-La Roche Ltd., Pfizer Inc., Eli Lilly and Company, Novo Nordisk A/S and Ono Pharma UK Ltd. and has received research support from Janssen Research & Development LLC, Eli Lilly and Company and GW Pharma. M.A.S.H. has provided paid limited consulting for The MathWorks, Inc. K.P.W. reports research support/donated materials: DuPont Nutrition & Biosciences, Grain Processing Corporation, and Friesland Campina Innovation Centre and being a consultant to and/or receiving personal fees from Circadian Therapeutics, Inc., Circadian Biotherapies, Inc., Philips, Inc, and U.S. Army Medical Research and Materiel Command - Walter Reed Army Institute of Research, outside the submitted work.

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Correspondence and requests for materials should be addressed to Wei Wang.

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