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Development and Characterization of a Tissue-Specific Reporter Line for Monitoring Circadian Clock Transcriptional Activity

Lilyan Mather

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ABSTRACT

Daily rhythms in physiology and behavior are produced by a circadian system consisting of a central clock located in the brain and peripheral clocks located in various peripheral tissues. Circadian clocks track time of day through the presence of a molecular clock that functions as a transcriptional-translational feedback loop. In the nucleus, the transcription factors CLOCK (CLK) and CYCLE (CYC) activate transcription of clock genes period (per) and timeless (tim). PER and TIM proteins slowly accumulate in the cytoplasm before entering the nucleus, where they inhibit CLK and CYC, and therefore repress their own transcription. Degradation of nuclear PER and TIM permits the next 24-hour cycle to begin. These molecular clocks must be coordinated across tissues, but the manner through which central and peripheral clocks communicate with one another to achieve this coordination is not well understood. We reasoned that the ability to track clock gene expression in specific tissues of living flies would facilitate an investigation into the relationship between different clock-containing tissues. Previous efforts to accomplish this in Drosophila have relied on reporter constructs in which regulatory elements of several different clock genes have been used to dictate expression of a luciferase reporter enzyme, the activity of which can be monitored using a luminometer. Although these reporter lines have been instrumental in our understanding of the circadian system, they generally lack cell specificity, making it difficult to compare molecular clock oscillations between different tissues. Here we report the generation of several novel lines of flies which allow for GAL4-
inducible expression of a luciferase reporter construct for clock gene transcriptional activity. We have expressed these reporter constructs selectively in neurons, the fat body, and ubiquitously, and show that molecular clock oscillations can persist in the fat body and in all tissues for several days in the absence of environmental cues. Additionally, we confirm previous reports that bioluminescence rhythms are generally more robust under 12hr:12hr light:dark conditions compared to constant darkness.
INTRODUCTION

Most organisms have a circadian system that regulates the timing of physiological and behavioral processes such as sleeping, waking, body temperature, feeding and digestion. These behaviors exhibit oscillations with a period of ~24 hours, and as a result, are called circadian rhythms (circa meaning “around”, diem meaning “day”). The first evidence of an endogenous circadian timing mechanism was documented when Jean Jacques d’Ortous de Mairan noticed the leaves of a plant rising and falling each day at predictable times. Mairan observed similar plant leaf movements even in the absence of light cues, providing evidence the plant was able to anticipate environmental changes and not just react to them (Klarsfeld 2013). The concept of an endogenous time keeping system was explored in humans in the mid-1900s when physiologist Nathaniel Kleitman subjected himself and another subject to a dark cave with a self-imposed 28-hour lighting cycle. After a month of observation, Kleitman reported he and the other subject exhibited oscillations in body temperature that repeated about every 24 hours despite the longer environmental cycles (Wolf-Meyer, 2013).

Illustrated by de Mairan and Kleitman, one critical property of circadian rhythms is that they persist in the absence of external time cues from the environment. In the lab, this is commonly studied by subjecting organisms to constant darkness (DD) conditions. The resulting rhythms are termed “free-running” because they are produced in the absence of light cues. In 1954, Colin Pittendrigh observed the fruit fly, *Drosophila melanogaster*, under DD conditions at
16°C and found the emergence of adults from their pupal cases, called eclosion, to have a free-running period of 24.5 hours (Pittendrigh, 1954). In mammals, free-running rhythms measured in DD tend to oscillate with a period greater than 24 hours in diurnal mammals, and less than 24 hours in nocturnal mammals (Aschoff, 1981).

The circadian system must also be able to receive inputs from the environment and adapt accordingly. When exposed to naturally occurring light:dark (LD) conditions, humans have a circadian rhythm with a period of 24 hours. Even though our bodies have an endogenous rhythm that is slightly longer than 24 hours, our biological clock synchronizes with the environment each day producing physiological processes with 24-hour oscillations. This process of biological clocks synchronizing to the environment is not specific to humans, though the period of free running rhythms may differ between species.

**Molecular Clock**

After identification of an endogenous time-keeping system, researchers began to investigate how this process occurs at the molecular level. Much of early work investigating the molecular clock utilized *Drosophila* as a model system. *Drosophila* exhibit robust circadian behaviors such as eclosion rates and locomotor activity. Rhythmic eclosion is characterized by high rates of eclosion during the morning that slow down into the afternoon and night. Locomotor activity is characterized by increased activity in the morning and evening, with decreased activity midday in between the morning and evening activity peaks (Hamblen-Coyle et al., 1992).

The first clock gene, *period* (*per*), was discovered by Ronald Konopka and Seymour Benzer by mutagenesis screens for eclosion behavior outside of a 24 hour period (Konopka and Benzer, 1971). They successfully identified three mutants, which they called *per*°,
*per*\(^S\), *per*\(^L\), that had arrhythmic, short, and long periods, respectively. All three mutations were traced back to a single gene, *per*, on the X chromosome. Researchers subsequently used RNase protection assays to measure *per* RNA levels from the head of *Drosophila* across the day, and found that *per* RNA levels oscillate with an approximately 24-hr rhythm in both LD and DD conditions (Hardin et al., 1990). Using immunohistochemistry, the PER protein was also found to exhibit circadian oscillations (Zerr et al., 1990). The oscillations of PER protein in *per*\(^S\) mutants had a shorter period, mimicking the *per*\(^S\) behavioral rhythms and implicating the *per* gene as a direct regulator of molecular clock oscillation speed.

A second clock gene, *timeless* (*tim*), was also identified through genetic screens and was found to produce arrhythmic eclosion and locomotor activity (Sehgal et al., 1994). Evidence suggested the *tim* mutation altered *per* RNA cycling, suggesting an interaction between the two genes. The relationship between *per* and *tim* was further elucidated when Vosshall et al. discovered the nuclear localization of PER is blocked by the mutant *tim* (Vosshall et al., 1994). Around the same time, the first mammalian circadian clock gene, *Clock* (*Clk*), was discovered and cloned (Vitaterna et al., 1994). Vitaterna et al. were the first to perform forward genetic screening in mice by injecting N-ethyl-N-nitrosourea into male mice to induce mutagenesis, mating the male mice to untreated females, and assessing circadian behavioral activity of offspring. They identified a heterozygous *Clk* mutant mouse that exhibited a lengthened period, and after further genetic crossing, observed an absence of sustained behavioral rhythmicity in homozygous *Clk* mutants. A homolog of the mouse clock gene was discovered through an independent forward genetic screen in *Drosophila*. *Drosophila Clk* mutants presented abolished locomotor activity rhythms and severely disrupted *per* and *tim* RNA cycling (Allada et al., 1998). That same year came the identification of a fourth clock gene, *cycle* (*cyc*),
the protein product of which was hypothesized to dimerize with CLK and bind the promoters of per and tim to regulate transcription (Rutila et al., 1998). Once characterized and mapped, the Drosophila cyc gene was found to be a homolog of the mammalian bmal1 gene. The discovery of these four major clock genes led to the proposal that the molecular clock consisted of a transcriptional-translational feedback loop, with clk and cyc acting as positive transcriptional regulators of the negative elements, per and tim, which would then feed back to inhibit their own transcription. This proposal received support through studies by Darlington et al. and Gekakis et al., which formally demonstrated the transcriptional activity of the CLOCK and BMAL1 genes on per transcription (Darlington et al., 1998; Gekakis et al., 1998).

In summary, per, tim, clk, and cyc interact through a transcriptional-translational feedback loop (Figure 1). During the morning, CLK and CYC dimerize and activate transcription of core clock genes per and tim in the nucleus by binding to a 6 bp promoter sequence, termed the E-box. PER and TIM proteins are translated, accumulate and dimerize in the cytoplasm during the evening, and eventually move to the nucleus and inhibit CLK and CYC, therefore repressing their own transcription (Allada and Chung, 2010). For the negative feedback loop to produce circadian cycling of clock genes, there must be a delay between the production of PER and TIM and their inhibitory functions on their own transcription. The kinase doubletime (dbt) and other kinases are essential to produce this delay by regulating the degradation and nuclear entry of the PER/TIM dimer via phosphorylation (Price et al., 1998). The genes involved in this mechanism are conserved in mammals, including humans (Stanewsky et al., 2003).
Figure 1. The molecular mechanism of the circadian clock. CLK and CYC form a heterodimer and bind to a specific E-box that drives transcription of *per* and *tim*. Translated proteins PER and TIM are then phosphorylated by kinase phosphorylation (e.g., DBT), which regulates protein levels and nuclear localization. PER and TIM accumulation causes their dimerization and entry into the nucleus, inhibiting the function of CLK/CYC. Photoreceptor CRY is activated by light and causes degradation of TIM, resetting and entraining this mechanism to environmental cues.

**Molecular Mechanisms of Entrainment**

To be beneficial, circadian clocks must entrain to external lighting patterns and adjust to seasonal changes in day length and temperature to ensure physiological processes occur at appropriate times. In flies, light exposure influences the molecular clock feedback loop by promoting degradation of TIM, which resets and synchronizes the phase of the clock. In a subset of core clock neurons, the blue light photoreceptor, *cryptochrome* (*cry*), mediates the circadian clock’s ability to entrain to external conditions. Upon light exposure, CRY binds to TIM and facilitates degradation by directing TIM to the proteosome. When TIM is absent, CLK and CYC activate *per* and *tim* transcription, therefore, resetting the clock mechanism (Figure 1).

In addition to its participation in the photoreceptive pathway, *cry* also plays a role in the molecular clock feedback mechanism in peripheral tissues. In flies without a functional *cry* gene,
molecular oscillations in PER/TIM are abolished in tissues such as the antennae, photoreceptor cells in the compound eyes, and Malpighian tubules (Ivanchenko et al., 2001; Krishnan et al., 2001; Stanewsky et al., 1998).

Central Clock

Once the clock genes were discovered, researchers used antibodies and in situ hybridization to localize clock-containing tissues. In Drosophila, clock gene expression was first identified in the brain, where ~150 clock neurons, collectively called the central clock, were found necessary to drive behavioral circadian rhythms. In mammals, central clock function is subserved by neurons in the suprachiasmatic nucleus of the brain (Ralph et al., 1990). Surprisingly, in addition to the brain, clock genes were found expressed throughout the body (Plautz et al., 1997).

In Drosophila, the central clock neurons are subdivided into groups based on their neuroanatomy: small ventral lateral neurons (s-LNvs), large ventral lateral neurons (l-LNvs), dorsal lateral neurons (LNds), lateral posterior neurons (LPNs), and three groups of dorsal neurons (DN1s, DN2s, and DN3s) (Nitabach and Taghert, 2008). The clock neurons are functionally heterogeneous between and within the subgroups, however all contribute to robust circadian behavioral rhythms. The different functions of clock neurons have been studied by observing fly locomotor activity, which has a predictable circadian pattern across the day. The s-LNvs and l-LNvs are identified by their expression of pigment dispersing factor (PDF) (Helfrich-Förster, 1995), a neuropeptide important for maintaining synchronization of molecular rhythms within the central clock network (Lin et al., 2004). Flies carrying a mutant version of the pdf gene and flies with ablated pdf neurons exhibit progressively arrhythmic rest:activity rhythms when subjected to DD conditions, likely due to a necessary role of PDF peptide in synchronizing
the molecular cycling and activity of the different groups of central clock cells (Renn et al., 1999). Under 12:12 hr LD conditions, pdf mutant flies report a lack of increased activity in anticipation of the morning “lights on” transition, which is normally present in wild type fly rhythms (Renn et al., 1999). The LNds have been implicated in production of the evening activity peak (Grima et al., 2004), and a subset of LNds have been found to contribute to rhythmic expression of metabolic genes via neuropeptide F signaling (Erion et al., 2016).

**Peripheral Clocks**

Like the central clock, peripheral clocks contain the molecular clock machinery necessary for keeping time. Peripheral clocks were first suggested in flies through experiments that utilized a per gene reporter in which per was fused to a bioluminescence gene. Fly body segments were cultured and analyzed for bioluminescence, indicative of per gene activity. Plautz et al. observed rhythmic bioluminescence from the head, thorax, abdomen, antennae, proboscis, legs, wings, and testes, demonstrating molecular clocks are widespread throughout the body (Plautz et al., 1997). In mammals, peripheral clocks are found in peripheral tissues such as the liver, pancreas, skeletal muscle, intestine, and adipose tissue (Albrecht and Eichele, 2003).

Different peripheral clocks in *Drosophila* have been described as having different levels of dependence on the central brain clock (Ito and Tomioka, 2016). Early studies by Hege et al. provided evidence the Malpighian tubules, functional analogs of the human kidneys, can be entrained directly by light and do not need input from the central clock (Hege et al., 1997). The presence of cry expression in peripheral clocks makes them directly light sensitive, and therefore less dependent on the brain for entrainment. The oenocytes, which regulate pheromone production, are an example of a peripheral tissue whose clock is regulated by the central clock. When PDF signaling is interrupted, the molecular clock of oenocytes is still intact, however,
PDF signaling from the central clock regulates the phase of oenocytes’ clock (Krupp et al., 2013). The dependency of the molecular clock of the prothoracic gland on the central clock is more complex, with the central clock regulating per expression but not tim expression (Morioka et al., 2012). Limiting food availability to a 6 hour interval per day has been shown to drive rhythmic expression of genes in the fat body, a tissue responsible for lipid storage and metabolism (Xu et al., 2011). This finding demonstrates the fat body can be synchronized to the environment via signaling outside of the central clock.

To better understand the relationship between the central clock and peripheral clocks, the circadian clock in separate tissues can be measured and compared. One way to quantify circadian rhythmicity is to track the activity of the per gene, whose cyclical expression is crucial for sustaining circadian rhythms. Per mRNA and protein levels cycle through time, generating a molecular rhythm, which is necessary for sustaining circadian behavioral rhythms. Drosophila with null mutations in per have arrhythmic behavioral patterns, and missense mutations in per produce behavioral rhythms whose cycles are either longer or shorter than 24 hours (Sehgal et al., 1994) (Konopka and Benzer, 1971). Both findings provide evidence that a functional per gene is necessary for production of rhythmic behavior. Therefore, monitoring per expression levels in various tissues provides a way to assess presence and oscillatory speed of a functioning circadian clock.

**Luciferase Reporters**

The ability to track clock gene activity depends on experimental methods to monitor gene or protein expression over time. Immunohistochemistry and in-situ hybridization are commonly used to quantify per activity in specific tissues, however, these methods require lethal tissue extraction, resulting in the inability to record data from the same fly through time. For a standard
temporal gene activity study in *Drosophila*, at least 80 flies are required for 8 measurements taken every 3 hours (10 flies/timepoint) (Schubert et al., 2020).

*Luciferase* gene reporter assays provide means to measure transcriptional activity of a gene in a living organism over days or even weeks. *Luciferase* is an enzyme that catalyzes the oxidation of D(-)-luciferin in the presence of ATP and O2 to produce oxyluciferin and light, in the form of a photon at ~560nm (Deluca and McElroy, 1978). A promoter sequence of a gene of interest can be placed upstream of the *luciferase* coding region, which puts *luciferase* expression under direct control of the gene’s regulatory elements. Thus, the amount of *luciferase* expression, measured by light emission, indicates the expression of the gene (Figure 2).

*Luciferase* reporters are a valuable tool for measuring expression levels of circadian genes. In 1992, Millar et al. utilized a luciferase reporter to monitor transcriptional activity of the *Cab2* circadian gene in the plant *Arabidopsis thaliana*, and observed circadian oscillations of luminescence that mimicked previously reported gene expression (Millar et al., 1992). Before it was widely accepted that prokaryotes exhibit circadian behavior, the use of a luciferase reporter provided strong evidence that the cyanobacteria gene, *psbAI*, is expressed in a circadian fashion that persists in constant conditions and is modulated by externally imposed lighting conditions (Kondo et al., 1993). Temporal gene activity analysis is possible due to luciferase enzyme kinetics and turnover. While the luciferase protein is quite stable, its enzymatic activity diminishes after a few catalytic cycles, allowing temporal resolution of rhythmic light emission (McClung, 2006).
Figure 2. Schematic representation of a luciferase reporter assay. A promoter, or piece of a promoter, of a gene of interest is placed upstream of the luciferase gene. When endogenous transcription factors activate the gene’s promoter, the luciferase enzyme will be transcribed and translated. The luciferase enzyme, when in the presence of luciferin, produces light, which indicates promoter activity.

**Per-luciferase reporters**

The first attempt to monitor expression levels of core clock genes in *Drosophila* using a luciferase reporter was presented by Brandes et al. in 1996. In this study, flies with a *per-luciferase* transgene made of a 4kb fragment of the *per* promoter (including nucleotides -3200 to +42) fused to the *luciferase* gene exhibited cycling luminescence with a period of about 24 hours in LD and DD conditions (Brandes et al., 1996). Using RNA protection assays, they observed a lag in luciferase RNA cycling compared to *per* RNA cycling. They also detected an overall decrease in luciferase signals over the six days of data collection, but this was reported as an artifact of the luciferase assay, as replenishment with fresh food and substrate restored bioluminescence levels. The next year Stanewsky et al. experimented with two different *per-luciferase* constructs. They found a reporter consisting of a sequence of the *per* promoter (-4200...
to +32) fused to luciferase cDNA was not sufficient to report endogenous per RNA cycling. However, a second reporter consisting of a sequence encompassing the per promoter and two-thirds of the PER protein (-4200 to +5627), produced bioluminescence signals similar to endogenous per RNA cycling (Stanewsky et al., 1997). In LD conditions, flies carrying the second reporter exhibited >99% rhythmicity with periods ranging from 22-26 hours. Stanewsky et al. observed a decrease in bioluminescence signal similar to Brandes et al., however, after adjusting for this artifact, found that luciferase from flies carrying either of the per-luciferase reporters dampened, or decreased in amplitude, the further into DD conditions. Subsequently, percent rhythmicity was much lower in DD than LD conditions.

Smaller sequences of the per promoter have been assessed for their ability to confer rhythmic luciferase cycling. Darlington et al. discovered the 18 bp E-box sequence can drive rhythmic luciferase signals that mimic endogenous per mRNA rhythms in LD and DD (Darlington et al., 2000). Flies carrying this transgene reported periods ranging from 22-27 hours and exhibited higher percent rhythmicity in LD compared to DD conditions. While these approaches successfully reported per expression in vivo, they did not do so in a tissue specific manner. This means all clock cells, central and peripheral, that normally express per, expressed the per-luciferase transgenes. The Brandes et al. study observed bioluminescence emitting mainly from the head (Brandes et al., 1996), and Stanewsky et al. reported bioluminescence emitting mainly from the abdomen (Stanewsky et al., 1997). Per-luciferase reporters can be used to monitor tissue-specific gene activity by dissecting fly body parts and observing bioluminescence in vitro. Dissection of individual body parts (e.g. head thorax abdomen) or individual tissues (e.g. antennae, leg, wing, eyes, prothoracic gland) have shown rhythmic per activity in LD and DD conditions (Plautz et al., 1997) (Emery et al., 1997). With recent
technological advances, this method has been used to observe bioluminescence of individual clock neurons (Schubert et al., 2020)(Versteven et al., 2020). However, despite these advances, an in vivo, tissue specific approach is needed to allow us to distinguish activity in central clock cells from peripheral tissues in living flies. This method will enable further research into the relationships between the central clock and peripheral tissues, and ultimately shed light on how the circadian system is organized.

**Research Aims**

The first goal of this study was to produce transgenic *Drosophila* to measure tissue-specific clock gene expression *in vivo* via a luciferase gene reporter. We created a modified *per-luciferase* reporter inducible in selective tissues by FLP recombinase. The second goal of this study was to use the luciferase reporter to observe CLK/CYC activity in distinct tissues, including the fat body and the brain, under differing lighting conditions. Tissue-specific GAL4 drivers were combined with a UAS-driven FLP recombinase to measure reporter activity in living *per-luciferase* flies over one week in DD and 12:12 LD conditions.
METHODS

Fly lines

The following fly lines were used: iso31 (isogenic w^{1118}) (Ryder et al., 2004), per^{91} (Konopka and Benzer, 1971), to-GAL4 (FBti0202314) and tub-GAL4 (RRID:BDSC_5138) provided by Amita Sehgal, UAS-FLP (RRID:BDSC_77141) provided by Michael Rosbash, otd-nls:FLP (Asahina et al., 2014) provided by David Anderson.

Generation of FLP-inducible circadian clock reporter lines

To monitor Drosophila circadian clock transcriptional activity in vivo, we produced two transgenes comprised of E-box sequences from the per promoter followed by the luciferase gene. The two constructs contain either a 69 bp sequence of the per promoter (P69E-f-luc) or three repeats of an 18 bp sequence of the per promoter (P18E-f-luc). These sequences contain a core E-box of the per gene and both have been shown to drive circadian cycling (Darlington et al., 2000; Hao et al., 1997).

Generation of the P69E-f-luc and P18E-f-luc plasmids

Both reporters were constructed from an existing plasmid (cre-f-luc) from the Yin Lab (Tanenhaus et al., 2012). Cre-f-luc contains three binding sites for dCREB2 followed by a transcription initiation site and ATG translation initiation codon. A cassette containing FRT sites flanking the mCherry gene with a translational stop codon is situated downstream. The luciferase coding region without its normal ATG start codon follows. The P69E-f-luc and P18E-f-luc constructs were created by replacing the three dCREB2 binding sites of the cre-f-luc plasmid.
with either a 69 bp fragment of the per promoter (-563 to -494 bp upstream of the transcriptional
start site, containing a consensus E-box sequence) (Hao et al., 1997), or 3 concatenated repeats
of an 18 bp per E-box element (Darlington et al., 2000) (Figure 3). We synthesized 69 bp and
3x18 bp sequences (Integrated DNA Technologies) with BamHI and NotI compatible ends (see
Table 1 for oligonucleotide sequences) and ligated these into BamHI and NotI-digested cre-f-luc
plasmids. The resulting P69E-f-luc and P18E-f-luc plasmids were verified by Sanger sequencing
(GeneWhiz) (see Table 2 for sequencing primers).

Creation of P69E-f-luc and P18E-f-luc transgenic flies

Prior to injection into embryos, we cloned the P69E-f-luc and P18E-f-luc fragments into
the pattB cloning vector, which contains the miniwhite gene for phenotypic marking and attB
sites that allow for phiC31 site-directed integration into the Drosophila genome. We digested
P69E-f-luc with XhoI and ligated the resultant DNA fragment into the XhoI site in the multiple
cloning region of pattB. P18E-f-luc was inserted between the NotI and XhoI sites in pattB
(Figure 3). The resulting target plasmids were confirmed by Sanger sequencing (see Table 2 for
sequencing primers). After sequencing, the plasmids were sent to BestGene Inc for phiC31-
mediated integration into attP sites located on the second chromosome (y[1] w[*];
P{y[+t7.7]=CaryIP}su(Hw)attP5) and third chromosome (y1 w1118; PBac{y+~attP-
9A}VK00005).
Figure 3. Creation of P69E-f-luc and P18E-f-luc plasmids. The cre-f-luc plasmid was digested with NotI and BamHI to remove the CREB2 binding sequences, which were replaced with either a 69 bp fragment of the per promoter (Hao et al., 1997), or 3 concatenated repeats of an 18 bp per E-box element (Darlington et al., 2000). The resulting PE-f-luc plasmid was digested with XhoI and the PE-f-luc fragments were ligated to a XhoI-digested pattB plasmid.

Table 1. Oligonucleotide constructs for PE-f-luc plasmids

<table>
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<tr>
<th>Plasmid</th>
<th>Oligonucleotide Sequence</th>
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<tr>
<td>P69E-f-luc</td>
<td>5'-GGCCGCATCTCGAGAAACCGTAGGCAGTGAAAAGCAGGCGGCATTG-3'</td>
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<td>CACGTGGAAGCTGGAGACATTTGCCCAGCAAATCCGCCATTTGGCTCGGAATTTGCC-3'</td>
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<td>P18E-f-luc</td>
<td>5'-GGCCGCATCTCGAGAAACCGTAGGCAGTGAAAAGCAGGCGGCATTG-3'</td>
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<td></td>
<td>TGGCGGAACATTTGCAGGCGCTCACTGAGCCGCCGCTACTG-3'</td>
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Table 2. Sanger sequencing primers

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<tr>
<td></td>
<td>PE-f-luc(puc)-R</td>
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**Luciferase assay**

Flies were reared on standard cornmeal-molasses food and entrained to 12:12 LD conditions at 25°C for at least 3 days before 4-10 day old males were loaded into a white 96-well microtiter plate (Berthold) containing 5% sucrose, 2% agar, and 10 mM luciferin (Perkin-Elmer). Bioluminescence was detected with a Centro-SX-3 luminometer at 25°C for 7 days starting at the beginning of the dark period and continuing in DD or 12:12 LD. Bioluminescence in relative light units (RLU) was measured for 10 seconds from each well every 30 minutes.

**Statistical Analysis**

To compare RLU across genotypes, mean RLU was determined for six consecutive days (excluding the first 12 hr) per fly, and these individual means were averaged across all flies of a given genotype. To normalize and detrend bioluminescence data, all data were passed through a 72 hr low pass Butterworth filter, and then each point was divided by the resulting trendline. During LD experiments, the lighting conditions imposed an artificial rhythm due to the plate absorbing light that is detectable by the luminometer. To account for this, we averaged bioluminescence traces of wells without flies to create a baseline, and then subtracted this baseline from experimental data. Chi-square periodogram analysis, performed in ClockLab, was
used to calculate rhythm strength (power) and period of bioluminescence readings. Rhythm power was calculated as the amplitude of the periodogram line at the dominant period minus the chi-square significance line (at significance of p < 0.01). Flies that died during the course of luciferase monitoring were identified via visual inspection of luminometer plates and removed from subsequent analysis. Because rhythm strength cannot be negative, flies with a calculated power < 0 were assigned a power of 0. A fly was considered rhythmic if analysis returned a period estimate between 16 and 32 hr and a rhythm power > 10. Welch’s t-test and Mann-Whitney test were used to compare rhythm power and period, respectfully. To compare bioluminescence across genotypes, Browne-Forsythe and Welch ANOVA and Dunnett’s T3 post hoc tests were performed.
RESULTS

Aim 1: Development of tissue specific reporter lines for in vivo monitoring of circadian clock transcriptional activity in Drosophila

Existing circadian gene reporter lines faithfully report circadian transcriptional activity (Brandes et al., 1996; Darlington et al., 2000; Stanewsky et al., 1997); however, they lack spatial resolution, since luciferase is present in all clock cells throughout the body. To confer cell specificity, we therefore sought to create reporter strains in which per promoter-driven luciferase expression could be induced in a cell specific manner. To that end, we produced two constructs that contain either a 69 bp sequence (Hao et al., 1997) or three repeats of an 18 bp sequence (Darlington et al., 2000) of the per promoter upstream of FLP-inducible luciferase (see Methods for promoter sequences). Both promoter sequences include the core E-box where transcription factors CYC and CLK bind. A transcription initiation site and ATG translation initiation codon are situated downstream of the per promoter, followed by a cassette containing FRT sites flanking the mCherry gene (which encodes for a monomeric red fluorescent protein) and two tandem translational stop codons. Downstream of the mCherry-STOP is the luciferase coding region without its normal ATG start codon (Figure 4A(1)). We used phiC31-mediated integration to insert these constructs onto either the second or third chromosome, resulting in four transgenic fly lines, which we call P69E-f-luc(II), P69E-f-luc(III), P18E-f-luc(II), and P18E-f-luc(III). In these lines, an absence of FLP should lead to translation termination at the
stop codon, and therefore no luciferase protein should be produced. In the presence of FLP, the mCherry-STOP is flipped out at the FRT sites, the remaining DNA is ligated together, and luciferase translation should occur. The presence or absence of FLP can be mediated by the GAL4/UAS system. Tissue-specific GAL4 expression drives the expression of UAS-FLP, which catalyzes site-specific recombination at the FRT sites, activating the reporter (Figure 4A(2)). FLP expression can also be mediated via a transgene consisting of a tissue-specific promoter that directly activates FLP expression (Figure 4A(3)).

Before utilizing the PE-f-luc reporters to monitor circadian transcriptional activity in various tissues, it was first important to investigate leakiness of the reporters, or potential bioluminescence produced by flies even in the absence of FLP. The average bioluminescence emitted by wildtype iso31 flies, which lack the luciferase gene, over the course of six days in DD conditions was ~17 RLU/reading. Importantly, there was no significant difference in bioluminescence levels between either of the second chromosome reporters and wildtype flies. In comparison, both third chromosome insertions had bioluminescence levels very slightly above background levels (Figure 4B). The negligible difference in RLU between wildtype flies and flies carrying a reporter suggest that there is minimal leakiness associated with the PE-f-luc reporter lines.

To test if FLP can induce luciferase expression, we first compared bioluminescence levels of flies carrying the PE-f-luc construct alone to those that also contain a transgene consisting of FLP directly activated by a neuronal promoter (otd-nls:FLP). The latter flies should allow CLK/CYC-driven luciferase expression to be restricted to nervous system cells, while the former should theoretically have no luciferase expression due to the lack of FLP-mediated removal of the translational stop codons. Consistent with this, we observed robust FLP-mediated
induction of luciferase expression in all four PE-f-luc reporters (Figure 4C). We noted that the bioluminescence signals varied depending on which chromosome held the PE-f-luc transgene, with flies carrying PE-f-luc on the third chromosome reporting higher RLU than flies carrying PE-f-luc on the second chromosome (Figure 4C). In addition, P18E-f-luc lines had marginally higher bioluminescence signals compared to P69E-f-luc reporters on the same chromosome (Figure 4C).

Different tissue specific promoters driving FLP should produce varying RLU intensities corresponding to the number of cells containing the specific promoter. We tested the ability of the PE-f-luc lines to report bioluminescence of different tissue-specific promoters by comparing bioluminescence levels of P18E-f-luc(II) flies with luciferase expression induced in neurons (otd-nls:FLP), in the fat body (to-GAL4 + UAS-FLP), or ubiquitously (tubulin-GAL4 + UAS-FLP). The tubulin driver produced the largest average RLU (Figure 4D), which is expected as luciferase expression in these flies is enabled in all cells. Additionally, average bioluminescence levels from flies with luciferase expression restricted to the fat body were larger than levels from flies with luciferase expression restricted to neurons (Figure 4D). These data indicate the PE-f-luc constructs, in conjunction with direct FLP driver or GAL4 driver, report luciferase expression in a cell specific manner.
Aim 2: Monitoring clock gene transcriptional activity in neurons, fat body, and ubiquitously

*PE-f-luc reporters exhibit rhythmic luciferase activity*
The 18 bp and 69 bp *per* promoter elements used in our PE-f-luc reporters have previously been demonstrated to confer rhythmic transcriptional activity when used to drive ubiquitously expressed reporter constructs (Darlington et al., 2000; Hao et al., 1997). To test whether this rhythmicity is retained in our FLP-inducible system, we conducted bioluminescence monitoring over extended time periods in flies in which our PE-f-luc reporter lines were crossed to otd-nls:FLP flies, which should restrict luciferase expression to neurons. In preliminary experiments, we found that reporters on the same chromosome, regardless of promoter sequence, performed comparably (data not shown), therefore, we report data from one second chromosome (P18E-f-luc(II)) and one third chromosome reporter (P69E-f-luc(III)).

In initial studies, we assayed for the presence of bioluminescence rhythms under DD conditions to assess endogenous clock mechanisms in the absence of light entrainment. We first compared bioluminescence levels of flies carrying the PE-f-luc construct alone to those that also contain otd-nls:FLP. In this case, we expect most luciferase signal to derive from photoreceptor cells, which are known to contain functional circadian clocks and vastly outnumber central clock neurons in the brain (Zerr et al., 1990). The average bioluminescence from control flies with no FLP (+>P18E-f-luc(II)) reports consistently low signal with no oscillations (Figure 5A-B). In flies containing the neuronally driven FLP and P18E-f-luc(II) (otdFLP>P18E-f-luc(II)), luciferase expression is enabled in neurons and dictated by the *per* E-box sequence, which is activated by transcription factors CYC and CYC. In DD, mean bioluminescence from otdFLP>P18E-f-luc(II) flies showed strong oscillations for the first few days, but decreased in amplitude, or dampened, as time proceeded in DD (Figure 5A-B). The rhythm power of individual otdFLP>P18E-f-luc(II) flies was variable, ranging from 0 to 91.73, with an average of 19.17 (Figure 5E-F, I). Notably, we found that many individual flies exhibited progressive
dampening of bioluminescence oscillations over multiple days in DD (Figure 5E); however, some retained cycling throughout the six-day recording period (Figure 5F).

Previous *per* gene luciferase reporters have shown increased levels of rhythmicity in LD conditions compared to DD conditions (Darlington et al., 2000). To test if this is also true in our inducible reporters, we monitored *otdFLP>P18E-f-luc(II)* flies in 12:12 LD for six days. In LD conditions, the mean bioluminescence trace of *otdFLP>P18E-f-luc(II)* flies showed persistent oscillations throughout the six days of data collection (Figure 5C-D). Surprisingly, however, there was no detectable difference in the average rhythm power of flies in DD and LD (Figure 5G-I). This could indicate that the apparent dampening observed under DD conditions may result from a lack of synchronization of clock gene oscillations between individual flies, which each express slightly different free-running periods. This possibility is supported by the fact that the average rhythm period of *otdFLP>P18E-f-luc(II)* in DD and LD conditions differed, with flies reporting an average period of 22.8 and 23.8, respectfully, with more individual period variability in DD (Figure 5J). A period of ~24 hr indicates the flies have successfully entrained to the LD schedule.
Figure 5. Neuronally-restricted P18E-f-luc(II) reporter exhibits rhythmic luciferase activity in DD and LD. Bioluminescence recordings of otd-FLP>P18E-f-luc(II) (black) and +>P18E-f-luc(II) controls (purple). (A-D) Group average bioluminescence recordings of flies expressing luciferase driven by a neuronal promoter. (A-B) Raw and normalized data from otdFLP>P18E-f-luc(II) flies (n=50) and control flies (n=20) in DD. (C-D) Raw and normalized data from otdFLP>P18E-f-luc(II) flies (n=37) in LD. (E-H) Normalized bioluminescence recordings of individual flies. For DD graphs, black bars indicate subjective night and grey bars represent subjective day. For LD graphs, black bars indicate lights off and yellow bars indicate lights on. Individual luminescence recordings are representative of flies that exhibited rhythm powers close to the group mean (top) and in the upper 10th percentile (bottom); rhythm powers are listed for each recording (I-J) Quantification of rhythm power and period of flies in DD and LD. Each circle corresponds to the strength of single fly’s normalized bioluminescence rhythm or period. Mean ± 95% CI are shown. The dashed line indicates the rhythmicity cut-off at 10. *p<0.05, Welch’s t-test.
We also tested flies carrying the P69E-f-luc(III) transgene with the neuronal driver in DD and LD. Similar to P18-f-luc(II), we observed that group mean bioluminescence rhythms rapidly dampened across the six days of recording in DD, but maintained strong oscillations throughout the duration of LD experiments (Figure 6A-D). However, in comparison to our second chromosome reporter, we found that of otdFLP>P69E-f-luc(III) flies had fairly weak DD rhythms. Thus, only ~22% qualified as rhythmic, and mean power was only 5.0±0.96, below the threshold for rhythmicity. Also as expected, overall luciferase signal was substantially higher in of otdFLP>P69E-f-luc(III) flies compared to otd-FLP>P18E-f-luc(II) flies (compare Figure 5A and 6A). We noted a substantial increase in mean power for this line under LD conditions, with most of these flies maintaining rhythmic oscillations throughout the duration of recordings (Figure 6I). Thus, for this line, the presence of environmental light cues appears necessary for continued and coherent clock gene expression rhythms in neurons. Although there was no difference in rhythm period of flies in DD and LD conditions, the periods of flies in DD were variable and flies in LD had an average period of 24.1, both indicating successful entrainment to the LD conditions (Figure 6J). Moreover, percent rhythmicity more than doubled for otdFLP>P69E-f-luc(III) flies in LD conditions (see Table 3).

The data from both reporters suggest core clock genes in neurons are transcriptionally active in a circadian fashion in constant and light-dark conditions, however, in LD the bioluminescence rhythms are generally more robust; either because light cues are necessary for the continued rhythmic oscillation of clock gene expression within individual neurons or for the synchronization of molecular clocks across different neurons.
Figure 6. P69E-f-luc(III) reporter exhibits increased rhythmic luciferase activity in LD. Bioluminescence recordings of otd-FLP>P69E-f-luc(III) (black) and +>P69E-f-luc(III) controls (purple). (A-D) Group average bioluminescence recordings of flies expressing luciferase driven by a neuronal promoter. (A-B) Raw and normalized data from otdFLP>P69E-f-luc(III) flies (n=40) and control flies (n=4) in DD. (C-D) Raw and normalized data from otdFLP>P69E-f-luc(III) flies in LD (n=37). (E-H) Normalized bioluminescence recordings of individual otdFLP>P69E-f-luc(III) flies. For DD graphs, black bars indicate subjective night and grey bars represent subjective day. For LD graphs, black bars indicate lights off and yellow bars indicate lights on. (I-J) Quantitation of rhythm power and period of otdFLP>P69E-f-luc(II) flies in DD and LD. Each circle corresponds to the strength of single fly’s normalized bioluminescence rhythm or period. Mean ± 95% CI are shown. The dashed line indicates the rhythmicity cut-off at 10. ***p<0.001, Welch’s t-test.
Rhythmic bioluminescence of PE-f-luc reporters depends on a functional molecular clock

If the PE-f-luc lines faithfully report endogenous clock gene transcriptional activity, then bioluminescence rhythms should be dependent on a functional circadian clock and flies without a functional clock should exhibit arrhythmic bioluminescence signals. We therefore compared luciferase signals in otdFLP>P18E-f-luc(II) and otdFLP>P69E-f-luc(III) flies in the presence and absence of the per\textsuperscript{01} mutation, which eliminates molecular clock function. We observed a loss of group mean bioluminescence rhythms in per\textsuperscript{01} mutant flies, as compared to flies with an intact per gene (Figures 7 and 8). For P18E-f-luc(II) flies, we found that mean rhythm strength was significantly lower in per\textsuperscript{01} flies as compared to non-mutant flies, both in DD (Figure 7F) and LD (Figure 7H). Importantly, the vast majority of individual per\textsuperscript{01} flies exhibited arrhythmic bioluminescence signals, while most non-mutant flies showed significant rhythmicity (Figure 7). Of note, in contrast to the results shown in Figure 5, we did find evidence in these experiments for increased rhythm strength in control otd-FLP>P18E-f-luc(II) lines in LD as compared to DD (compare Figure 7F and 5H). Additionally, the average RLU levels of per\textsuperscript{01} flies was increased compared to flies without per\textsuperscript{01} (Figure 7E and G). This is expected, as flies without PER are unable to repress CLK/CYC-mediated transcription such that CLK/CYC activity should be constitutively high.

We observed similar results with the P69E-f-luc(III) line (Figure 8). Consistent with our previous findings, otdFLP>P69E-f-luc(III) flies had relatively weak rhythms in DD conditions (Figure 8A-B), dampening out after a few rhythmic cycles, compared to persistent and robust cycling in LD (Figure 8C-D). In contrast, per\textsuperscript{01} flies were predominantly arrhythmic under both lighting conditions (Figure 8F and H). In addition, as was the case for the P18E-f-luc(II) line, mean RLU was significantly higher in per\textsuperscript{01} mutants (Figure 8E and G). The lack of rhythmicity
and increased RLU in $per^{01}$ mutants indicate the reporters are dependent on a functioning circadian clock and effectively report transcriptional activity of circadian clock genes.

Figure 7. Rhythmicity reported by P18E-f-luc(II) is dependent on a functional clock. (A-B) Raw and normalized group average bioluminescence recordings comparing $per^{01}$ mutants (n=42; red) and non-mutants (n=49; black) in DD. (C-D) Raw and normalized group average bioluminescence recordings comparing $per^{01}$ mutants (n=23; red) and non-mutants (n=23; black) in LD. For LD graphs, black bars indicate lights off and yellow bars indicate lights on. (E-H) Quantification of mean RLU and rhythm power in DD and LD. Each circle corresponds to a single fly. Mean ± 95% CI are shown. The dashed line indicates the rhythmicity cut-off at 10. ****p<0.0001, ***p<0.001, Welch’s t-test.
Figure 8. Rhythmicity reported by P69E-f-luc(III) is dependent on a functional clock. (A-B) Raw and normalized group average bioluminescence recordings comparing per\textsuperscript{\alpha} mutants (n=20; red) and non-mutants (n=23; black) in DD. (C-D) Raw and normalized group average bioluminescence recordings comparing per\textsuperscript{\alpha} mutants (n=22; red) and non-mutants (n=21; black) in LD. For DD graphs, black bars indicate subjective night and grey bars represent subjective day. For LD graphs, black bars indicate lights off and yellow bars indicate lights on. (E-H) Quantification of mean RLU and rhythm power. Each circle corresponds to a single fly. Mean ± 95% CI are shown. The dashed line indicates the rhythmicity cut-off at 10. ***p<0.0001, Welch’s t-test.

Ubiquitous expression of PE-f-luc reporters mimics results obtained with traditional non-inducible per-luciferase constructs

Thus far, we have monitored luciferase activity in flies in which reporter expression was restricted to neurons. To better compare to traditional non-inducible per-luciferase reporters, we next used a ubiquitously expressed tubulin-GAL4 line to drive UAS-FLP expression in all cells. In DD, flies carrying P18E-f-luc(II) and a UAS-driven FLP recombinase (FLP>P18E-f-luc(II))
exhibited detectable, but low luciferase levels (~80 RLU), indicating there is some leaky expression of the UAS-FLP in the absence of a GAL4 driver (Figure 9A and K). We attribute this leakiness to the UAS-FLP transgene because flies carrying only the PE-f-luc construct (→P18E-f-luc(II)) have even lower expression levels (~20 RLU) that are indistinguishable from flies lacking PE-f-luc (Figures 1 and 5A,K). Flies containing P18E-f-luc(II), tub-GAL4, and UAS-FLP (tubFLP>P18E-f-luc(II)) had significantly higher luciferase expression, with average RLU of ~1800 over the six days of recording (Figure 9A and K), indicating substantial reporter induction in the presence of both the GAL4 and UAS constructs.

*Per* E-box luciferase reporters have previously been used to record luciferase activity from all clock cells of living flies (Darlington et al., 2000). These studies found that bioluminescence oscillations dampen, or decrease in amplitude, in DD. This dampening was seen in individual flies and average traces, ruling out asynchrony of potentially rhythmic individual flies as a cause of dampening of the average traces. As tub-GAL4 is a ubiquitous driver, tubFLP>P18E-f-luc(II) flies can be compared to the results of these studies. Our experiments also revealed dampening at the group mean level, through visual inspection, demonstrates that weak oscillations are maintained through day six (Figure 9A-B). As was the case for neuronally-restricted PE-f-luc expression, we observed several flies that retained strong oscillations throughout the recording period (Figure 9H). Average rhythm power, calculated by analyzing each individual fly’s bioluminescence trace, indicates a strength of 25.8 ± 3.45 (Figure 9L), which is similar to otdFLP>P18E-f-luc(II) flies with luciferase expression driven in the neurons (Figure 5I). Darlington et al. also reported an increase in percent rhythmicity for flies in LD compared to DD. Our results are consistent with this finding, as ubiquitous luciferase expression reported by P18E-f-luc(II) had an average rhythm power of 58.66 ± 4.58 in LD (a
significant increase as compared to DD conditions) and restriction of individual period to values close to 24 hours (Figure 9D-F and I-M). Finally, we found that ~73% and 95% of individual flies exhibited rhythmic luciferase signals in DD and LD, respectively, which are both slightly higher than percent rhythmicity observed by Darlington et al. (see Table 3).

We also tested P69E-f-luc(III) with the ubiquitous GAL4 driver in DD and LD conditions. As seen with P18E-f-luc(II), there was some leaky expression of the UAS-FLP (Figure 10A,C and D,F). In DD, we observed clear rhythmicity at the group mean level throughout the entire recording period (Figure 10A-B), which differed from P18E-f-luc(II) (Figure 9A-B). Expectedly, strong rhythmicity was also evident at the individual level in DD conditions (Figure 10G-H). In LD conditions, the group mean bioluminescence and individual traces exhibited robust rhythmicity (Figure 10D-F, I-J), although there was no detectable difference in average rhythm strength in the different lighting conditions (Figure 10L).

Consistent with our previous reporter data, there was a significant difference in rhythm period in DD (average period = 23.3 ± 0.1 hr) and LD (average period = 24.0 ± 0.1 hr) conditions (Figure 10M), indicating successful entrainment.
Figure 9. Rhythmic luciferase expression from a ubiquitous driver: P18E-f-luc(II).

(A-C) Raw and normalized group mean bioluminescence signals of tubFLP>P18E-f-luc(II) flies (black; n=64), FLP>P18E-f-luc(II) controls (blue; n=21) and +P18E-f-luc(II) controls (purple; n=4) in DD. (D-F) Raw and normalized group mean data for tubFLP>P18E-f-luc(II) (black; n=56) (black; n=64) and FLP>P18E-f-luc(II) controls (blue; n=21) in LD. (G-J) Normalized data from individual flies in DD or LD. For DD graphs, black bars indicate subjective night and grey bars represent subjective day. For LD graphs, black bars indicate lights off and yellow bars indicate lights on. (K) Quantitation of mean RLU under DD conditions. Different letters indicate p<.0001, Brown-Forsythe and Welch ANOVA, Dunnett’s T3 post hoc. (L-M) Rhythm power and period. Each circle corresponds to a single fly. Mean ± 95% CI are shown. The dashed line indicates the rhythmicity cut-off at 10. ****p<0.0001, Welch’s t-test.
Figure 10. Rhythmic luciferase expression from a ubiquitous driver: P69E-f-luc(III).
Bioluminescence recordings of tubFLP>P69-f-luc(III) flies (black), FLP>P69-f-luc(III) (blue) and +>P69-f-luc(III) (purple). (A-C) Raw and normalized group mean data in DD. Black n=44. Blue n=38. Purple n=8. (D-F) Raw and normalized group mean data in LD. Black n=43. Blue n=39. (G-J) Normalized data from individual flies in DD or LD. For DD graphs, black bars indicate subjective night and grey bars represent subjective day. For LD graphs, black bars indicate lights off and yellow bars indicate lights on. (K) Quantitation of mean RLU under DD conditions. Different letters indicate p<.0001, Brown-Forsythe and Welch ANOVA, Dunnett’s T3 post hoc. (L-M) rhythm power and period. Each circle corresponds to a single fly. Mean ± 95% CI are shown. The dashed line indicates the rhythmicity cut-off at 10. ****p<0.0001, Mann-Whitney test.
Fat body clocks retain rhythmicity in both DD and LD conditions

We next sought to monitor clock gene transcriptional activity in the fat body. It has been previously reported that *per* expression in the abdomen of *Drosophila* quickly dampens in DD via a luciferase reporter (Stanewsky et al., 1997) and by RNA extraction (Xu et al., 2011). We again used the GAL4-UAS system to induce PE-f-luc expression, but in this case used a to-GAL4 driver, which is selectively expressed in the fat body (Dauwalder et al., 2002), to drive FLP, resulting in expression of the PE-f-luc reporter in the fat body. We monitored these offspring in a luminometer for six days in either DD or 12:12 LD conditions. When observing average bioluminescence readings, we detected weak luciferase oscillations for toFLP>P18E-f-luc(II) in DD (Figure 11A-B) that increased in rhythm strength when monitored in LD conditions (Figure 11D-E). We found similar results when analyzing rhythm power based on individual flies, with flies in LD reporting increased average rhythm power than in DD (Figure 11L). Importantly, even in DD conditions, we observed some individual flies that had persistent bioluminescence rhythms throughout day six (Figure 11H).

We also performed this same experiment with P69E-f-luc(III). As was the case with ubiquitous expression, group mean bioluminescence cycling from toFLP>P69E-f-luc(III) flies in DD was more robust than toFLP>P18E-f-luc(II) (compare Figure 11B and Figure 12B). There was no detectable difference in rhythm strength of toFLP>P69E-f-luc(III) flies in LD compared to DD (Figure 12D-E, L), which differed from toFLP>P18E-f-luc(II). However, in both toFLP>P18E-f-luc(II) and toFLP>P69E-f-luc(III) flies, the percent rhythmicity increased when monitored under LD conditions, going from 30.6% to 65.7% and 51.4% to 76.5%, respectively (see Table 3). Furthermore, rhythm periods were more variable for both lines in DD as compared to LD, and mean period length in LD was closer to 24 hours, indicating entrainment
(Figure 11M and 12M). Our experiments suggest that molecular clock oscillations persist in fat
body tissue for several days under DD and LD conditions, with rhythm strength increasing in the
presence of externally imposed light signals.
Figure 11. Rhythmic luciferase activity in the fat body: P18E-f-luc(II).

(A-C) Raw and normalized group mean bioluminescence signals for otd-FLP>P18E-f-luc(II) flies (black; n=36), FLP>P18E-f-luc(II) controls (blue; n=8) and +>P18E-f-luc(II) controls (purple; n=4) in DD. (D-F) Raw and normalized group mean bioluminescence signals for otd-FLP>P18E-f-luc(II) flies (black; n=35) and FLP>P18E-f-luc(II) controls (blue; n=8) in LD. (G-J) Normalized data from individual flies in DD or LD. For DD graphs, black bars indicate subjective night and grey bars represent subjective day. For LD graphs, black bars indicate lights off and yellow bars indicate lights on. (K) Quantitation of mean RLU under DD conditions. Different letters indicate p<.0001, Brown-Forsythe and Welch ANOVA, Dunnett’s T3 post hoc. (L-M) Rhythm power and period. Each circle corresponds to a single fly. Mean ± 95% CI are shown. The dashed line indicates the rhythmicity cut-off at 10. ***p<0.0001, Welch’s t-test.
Figure 12. Rhythmic luciferase activity in the fat body: P69E-f-luc(III).

(A-C) Raw and normalized group mean bioluminescence signals for otd-FLP>P69E-f-luc(III) flies (black; n=35), FLP>P69E-f-luc(III) controls (blue; n=8) and +P69E-f-luc(III) controls (purple; n=4) in DD. (D-F) Raw and normalized group mean bioluminescence signals for otd-FLP>P69E-f-luc(III) flies (black; n=34) and FLP>P69E-f-luc(III) controls (blue; n=10) in LD. (G-J) Normalized data from individual flies in DD or LD. For DD graphs, black bars indicate subjective night and grey bars represent subjective day. For LD graphs, black bars indicate lights off and yellow bars indicate lights on. (K) Quantitation of mean RLU under DD conditions. Different letters indicate p<.0001, Brown-Forsythe and Welch ANOVA, Dunnett’s T3 post hoc. (L-M) Rhythm power and period. Each circle corresponds to a single fly. Mean ± 95% CI are shown. The dashed line indicates the rhythmicity cut-off at 10. ****p<0.0001, Mann-Whitney.
Table 3. Quantitative analysis of all PE-f-luc lines expressed in various tissues under DD and LD conditions.

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<td>% Rhythmic</td>
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<td>41</td>
<td>29.33 ± 3.46</td>
<td>80.5</td>
<td>23.3 ± 0.1</td>
<td>43</td>
</tr>
<tr>
<td>toFLP&gt;P69E-f-luc(III)</td>
<td>35</td>
<td>17.15 ± 3.01</td>
<td>51.4</td>
<td>23.3 ± 0.2</td>
<td>34</td>
</tr>
</tbody>
</table>
DISCUSSION

In Aim 1, we have utilized the FLP/FRT and GAL4/UAS systems to make spatially restricted luciferase reporters of CLK/CYC activity at the E-box sequence present in the *per* gene promoter. The reporters, containing either a 69 bp sequence or three repeats of an 18 bp sequence of the *per* promoter, exhibit minimal leakiness, produce luciferase protein only in the presence of FLP, and report increasing strength of bioluminescence signal as the number of cells containing the luciferase reporter increases.

As noted, our reporter lines exhibited virtually no bioluminescence signal in the absence of FLP. This lack of leakiness suggests the translational stop codons present in the constructs can strongly suppress run-through translation. Though we did note slightly increased luciferase signal in third chromosome reporter lines compared is iso31 controls, the values differed by only a few RLU, which is insignificant compared to induced signal recorded in the presence of FLP. Overall, we conclude that our reporters are ideal for *in vivo* monitoring in a tissue-specific manner.

During transformation of fly embryos, phiC31 integration was used to insert the transgene at known attP sites in the fly genome, indicating the luciferase transgene should be located at the same position for reporters on the same chromosome. Consistent with this observation, reporters on the same chromosome displayed similar features, like bioluminescence intensity. P69E-f-luc(II) and P18E-f-luc(II) flies reported average bioluminescence levels ~150-250 RLU and P69E-f-luc(III) and P18E-f-luc(III) flies reported average bioluminescence levels
~1000-2000 RLU when driven by a neuronal promoter. This large difference in signal intensity between second and third chromosome reporters suggests there are significant insertional effects. It is possible that DNA sequences surrounding the second chromosome insertions act to suppress expression. Despite over similarities, we did also record slightly higher luciferase signal in P18E-f-luc flies compared to P69E-f-luc flies. This could be due to the fact that the P18E-f-luc construct contains 3 concatemerized E-boxes, compared to only a single E-box in the P69E-f-luc construct.

Our data comparing P18E-f-luc(II) flies with luciferase expression driven in different tissue types are consistent with the hypothesis that larger cell populations should report more luciferase activity, measured by larger RLU levels. This suggests the reporters can be used to measure CLK/CYC activity in any tissue for which a GAL4 driver or direct flippase driver exists. The cell specificity of our experiments relies on previous characterization of GAL4 lines because bioluminescence was recorded from whole flies. Cell culture experiments could be used to isolate specific cell populations from PE-f-luc flies and verify luciferase is being expressed in the expected cells.

In Aim 2, we utilized two reporters, P18E-f-luc(II) and P69E-f-luc(III), to monitor luciferase expression in neurons, the fat body, and all clock cells in DD and LD conditions. Results from this aim further validated the reporters and brought us closer to fully understanding the extent to which various clock cell populations maintain rhythmic cycling under constant environmental conditions, as well as contributing to our understanding of how central clock and peripheral clocks coordinate.
Our results suggest the P18E-f-luc(II) and P69E-f-luc(III) reporters are dependent on a functioning circadian clock. The loss of rhythmicity in per⁰¹ mutants was an essential finding to verify the lines are reporting activity of clock genes as intended. This conclusion could be further verified by monitoring reporter activity of flies without a functional cyc gene. In cyc mutants we would expect bioluminescence oscillations to cease and RLU to be constantly low, as clock gene transcription would not be activated in the absence of CYC.

Comparing clock gene transcriptional activity from P18E-f-luc(II) across all tested tissues in DD, bioluminescence rhythm strength was comparable in neurons and all clock cells (mean rhythm strength of ~20-25), and lower in the fat body (mean rhythm strength of ~7). For P69E-f-luc(III), bioluminescence rhythm strength was highest when expressed ubiquitously as compared to when specifically expressed in the fat body or neurons. This was somewhat unexpected, as we hypothesized rhythm strength would be stronger in specific tissues compared to all tissues. Clock gene activity has been shown to dampen when measured from all cells, presumably because the clocks of different peripheral tissues run at slightly different period lengths, resulting in loss of synchronization between clocks.

In particular, our experiments shed light on the function of the peripheral clock in the fat body. Previous experiments indicate cyclical per expression in the fat body dampens out by day six of constant conditions (Xu et al., 2011)(Fulgham et al., 2021), however, it is important to note that these experiments were done on combined tissue from multiple individual flies. For example, to quantify core clock gene expression, Xu et al. utilized quantitative PCR, which requires pooling of flies for each time point. Thus, it is impossible to determine whether the dampening is due to a lack of molecular clock function or synchronization within individual
flies, or the because flies have different endogenous periods, so they gradually become desynchronized despite the fact that individual flies may retain rhythmicity. Our luciferase reporter method for measuring core clock gene activity allowed for the first time for analysis of rhythmic clock-mediated transcription within the fat body of individual flies. Notably, our experiments suggest that molecular clock oscillations can persist in fat body tissue for several days under DD conditions, at least in a subset of flies. However, for individual bioluminescence traces that dampened in DD, we could not distinguish whether fat body cells within an individual fly become desynchronized or if molecular clock oscillations within individual cells truly dampened in DD.

Comparing reporter activity in DD and LD conditions resulted in a similar result across reporters and tissues: bioluminescence rhythms were stronger and more robust in LD compared to DD. This was expected, as peripheral clocks are able to synchronize to light schedules because light can penetrate the fly cuticle and peripheral tissues contain the blue-light photoreceptor CRY (Emery et al., 2000). The function of CRY in peripheral tissues was confirmed first by Stanewsky et al. when they observed loss or weakening of core clock gene oscillations in cry<sup>b</sup> mutants in LD conditions (Stanewsky et al., 1998).

There are several areas of circadian research that could benefit from the use of our luciferase reporters. It is suggested the fat body clock requires input from the central clock in constant darkness to maintain rhythmic clock gene oscillations (Erion et al., 2016). Erion et al. utilized RNA extraction to measure per mRNA levels on the second day of constant darkness and found ablation of the central clock resulted in arrhythmic per mRNA oscillation, while flies with an intact central clock exhibited wildtype per mRNA rhythms. Our PE-f-luc reporters could
be used further investigate the dependency of the fat body on the central clock by monitoring bioluminescence of toFLP>PE-f-luc flies that have an ablated central clock. Additionally, the fat body clock has been described as having varying effects on feeding behavior. Xu et al. provided evidence that disruption of the fat body clock increased food consumption (Xu et al., 2008). Contrastingly, Fulgham et al. found disruption of the fat body clock produced no effects on feeding behavior (Fulgham et al., 2021). Our PE-f-luc reporters could help verify the status of peripheral clocks in studies that aim to disrupt the clock and observe downstream effects.
BIBLIOGRAPHY


VITA

Lilyan Mather was born and raised in Sandusky, Ohio, until the age of ten when her and her family moved to Commerce, Georgia. She grew up alongside her older brother, Baron, and older sister, Morgan. Before attending Loyola University Chicago, she attended the University of Georgia where she earned a Bachelor of Science in Biology in 2018.

During her time at Loyola University Chicago, Mather conducted her thesis research in the Cavanaugh Lab, served as a teaching assistant for Introduction to Neuroscience and General Genetics, and was elected President of the Biology Graduate Student Association in her second year. While researching in the Cavanaugh Lab, she grew to appreciate the fruit fly *Drosophila melanogaster* as a model organism and the intricacies of their circadian rhythms. Additionally, she presented her thesis research at the Midwest Drosophila Conference.

Mather is continuing her education at the University of North Carolina Eshelman School of Pharmacy where she is on track to earn a Ph.D. in Pharmaceutical Sciences with a specialty in Medicinal Chemistry and Chemical Biology.