Supplementary Methods

Animal Housing

Standard mouse cages measuring 7 1⁄2" x 11 1⁄2" x 5" (Ancare, Bellmore, NY) with 30 g TekFresh bedding were used for all experiments in the LumiCycle In Vivo units, with a few simple modifications. To minimize obstruction in the field of view of the PMTs, cage lids were modified by removing the divider that normally contains chow. Chow (Teklad 2014) was available in a stainless-steel hopper (Ancare) side-mounted inside the cage. An aluminum bar measuring 4" x 1" x 5/8" was placed under the hopper to keep mice from nesting beneath. Reduced height square water bottles (Ancare) with #6 stoppers allowed the cages to be moved easily in and out of the unit. For delivery of a substrate in drinking water, 50 mL conical tubes were used instead of water bottles, also with # 6 stoppers. To ensure the availability of the solution through the stopper, the angle of the tubes was kept constant by an extension spring clipped to the cage lid (See Supplementary Figure 2). In addition to measuring bioluminescence, the In Vivo system was also used to record locomotor activity and, in some cases, drinking bouts. Locomotor activity was recorded via a wireless node with a PIR (motion) sensor positioned over the cage. Each node sends data by radio to a receiver which connects to a computer, and to the Clocklab Wireless data acquisition program. Drinking bouts were measured using a sensitive circuit to detect each contact between the animal and a custom sipper tube; counts were also transmitted via a wireless node. We recorded general locomotor activity by a passive infrared motion sensor using Clocklab software (RRID:SCR_014309). Files for motion sensor counts from each animal are included in our data files posted on the open science framework site.

Possible sources of background noise include the type of bedding and the composition of the diet. To rule out these possible contributors in our system we tested an empty cage as well as ones bedded with Aspen bedding, white crinkled paper, and two amounts of TekFresh (7099, Envigo). The lesser amount of TekFresh was closest to the level of the empty cage and all subsequent experiments used that amount. To avoid abdominal autofluorescence as reported (Inoue et al. 2008) we used a global, alfalfa-free diet (Teklad 2014, Envigo). We did a further comparison between that and a purified diet (AIN-93M, Envigo) and found no significant difference between the two in our system.

Pump Preparation

Alzet® subcutaneous osmotic minipumps (Durect, Cupertino, CA), calibrated to deliver for either 7 or 14 days were used. On the day prior to implantation, pumps were filled with substrate which had been sterile-filtered using 1 mL Luer Slip syringes (Grainger Industrial Supply, Springfield, MA) and Millipore syringe filters (Thermo Fisher Scientific, Waltham, MA). 0.5cc BD Insulin Syringes with no residual volume (MWI Veterinary Supply, Boise, ID) were used to fill the pumps. To measure the final volume in the filled pump, each pump, flow moderator, and packaging was weighed before and after filling. Weights were recorded to determine initial filled volume and to

compare with the residual volume at the end of treatment, to measure the actual delivered dose. Pumps were then primed for up to 24 hours in 1.5 mL sterile saline in 15 mL conical tubes, at 37° C.

Surgery for Pump Implantation

One hour prior to surgery animals were weighed and injected subcutaneously with buprenorphine (0.05 mg/kg) and either ketoprofen (5 mg/kg) or meloxicam (2 mg/kg) as analgesics. At the beginning of surgery animals were placed in an induction chamber with 3% isoflurane, then transferred to a nose cone. Body temperature was maintained with a heated gel pack placed under the animal throughout surgery. Eyes were protected with veterinary ophthalmic ointment and anesthesia was maintained at 2.5% to 3%.

Animals were shaved with an OsterFinisher clipper (Osterpro.com) on the back, from shoulder blades to pelvic bones, and around both sides and the complete abdomen (See Supplementary Figure 3). After washes with Betadine and 70% EtOH and using sterile surgical technique, a ~10 mm skin incision with a #15 scalpel was made below the shoulder blade, perpendicular to the spine. A 20 mm subcutaneous pocket was created using a blunt hemostat and flushed with 0.5 mL sterile saline. Pumps were removed from the priming solution and inserted into the pocket. Incision was closed with 3-4 absorbable sutures (Ethicon #023434, MWI Veterinary Supply, Boise ID) and treated with a splash block (Lidocaine 0.2%, Bupivicaine 0.05%) and antibacterial ointment (Vetropolycin, MWI Veterinary Supply).

During recovery animals were housed individually in large standard mouse cages (10 1⁄2" x 19" x 6 1⁄8", fitted with Micro Filter Top™, Ancare) with TekFresh bedding. Cages were placed on top of heated discs (SnuggleSafe.com) overnight and animals monitored for recovery and freedom of movement. Besides normal chow ad libitum and water, soft food and apple slices were placed in dishes on the cage floor. After three days recovery in 12:12 LD, animals were moved into smaller standard cages and placed into individual LumiCycle In Vivo units in constant darkness, just prior to the time of lights off in 12:12 LD. Data were recorded for 7 days in DD, and animals were checked once a day at randomized times using an infrared viewer. As well as bioluminescence, locomotor activity was recorded throughout the experiment with a passive infrared motion sensor using ClockLab (Actimetrics). After completing the pump experiments, animals were euthanized and tissue samples were collected for confirmation of genotype.

Surgery for Pump Removal

For those experiments in which an animal was to be tested with a second pump after recovery, a pump removal protocol was followed. Animals were briefly anesthetized with 3% isoflurane in an induction chamber, to allow for subsequent subcutaneous injections of buprenorphine and ketamine or meloxicam as previously described, without restraint. Animals were returned to their home cages for at least 30 minutes before beginning surgery. An additional brief induction

followed by transfer to a nose cone preceded the pump removal and body heat was again maintained with a gelpack. Any remaining sutures were removed and an incision was made in the original site or parallel to it, depending on the degree of healing present. The pump was removed, the subcutaneous pocket flushed with 0.5 mL saline and incision closed as for pump insertion. Postoperative care was the same as for pump insertion.

Dose determination

Immediately following a pump removal, any remaining substrate was removed from the pump using a 0.5 cc BD Insulin Syringe. Actual dose delivered was calculated by the following formula, as provided by the manufacturer Alzet:

Mean Pumping Rate x Infusion Duration (in hours) = Volume Infused.

Actual Fill Volume – Volume Infused = Residual Volume

During the hours of priming before surgical implantation in the animals, the rate per hour was calculated at half the mean pumping rate. From the time of implant until removal the rate was calculated at the normal pumping rate.

Data analysis

For consistency, the la12 filter was applied to 15-min binned time series for all DWT analyses. To check the robustness of these choices, we compared to other choices of filter length and binning size, with consistent results. The 15-min bin size was chosen to yield a DWT scale D6 spanning 16-32 h, which includes all periods that could be considered circadian. We did not examine ultradian scales of the DWT in these analyses.

The data used for the MetaCycle analysis were 3-day windows beginning on the second day of recording with 90-min median binning but no other processing.

Because the spacing of peaks can be variable, methods assuming stationarity will not be as reliable in this context as the wavelet transform. Hence we did not apply those methods. The DWT circadian component captures the rhythms well and provides a reliable peak time estimate

Thermal stability of luciferins

D-luciferin sodium salt was purchased from GoldBio. CycLuc1 was synthesized as previously described (Reddy et al. 2010). The luciferin substrates were dissolved in PBS to a final concentration of 100 mM (D-luciferin) and 5 mM (CycLuc1), then incubated at 4 °C or 37 °C for the indicated time (0-14 days). Aliquots were removed for LC-MS and bioluminescence imaging comparisons.

FVB/NJ mice were purchased from Jackson Laboratories (Bar Harbor, ME). Intravenous injection of AAV9-CMV-WTluc2 was performed as previously described (Mofford et al. 2015). Bioluminescence assays were performed on a Xenogen IVIS-100 system in the UMass Medical

School Small Animal Imaging facility. All luciferin aliquots were sterile-filtered through a 0.22 µm syringe filter (Millex-GV) prior to injection. Each mouse was weighed to determine substrate dosing and anesthetized using 2.5% isoflurane in 1 L/min oxygen. Each luciferin substrate was injected i.p. at a dose of 4 µL/g mouse and mice were imaged ventrally 12 minutes after injection. Each mouse served as its own control, and imaging for different conditions were performed on different days. Data acquisition and analysis were performed with Living Image® software. Data were plotted and analyzed with GraphPad Prism 7, and reported as the total flux (p/s) for the whole mouse. All these experiments were conducted in accordance with the Institutional Animal Care and Use Committee of The University of Massachusetts Medical School (docket #A-2474-14).

LC-MS analyses were performed on an Agilent Technologies 6130 quadrupole LC-MS connected to an Agilent diode array detector. Chromatography was conducted on an Agilent InfinityLab Poroshell 120 EC-C18 column (4.6 x 50 mm, 2.7 micron particle size), with a mobile phase of water/acetonitrile with 0.1% formic acid. Samples were prepared by dilution of an aliquot of the 100 mM and 5 mM PBS stocks with Milli-Q water to 500 µM D-luciferin and 250 µM CycLuc1, respectively. Of this solution, 20 µL was injected onto the C18 column, and a gradient of H_2O and acetonitrile (+0.1% formic acid) was used as the mobile phase.

Time (min)	Water (0.1% Formic acid)	Acetonitrile (0.1% Formic acid)	Flow rate ml/min	Pressure (bar)
2	100	0	1.0	600
10	0	100	1.0	600
12	100	0	1.0	600
14	100	0	1.0	600

Materials

Name	**Company**	**Catalog Number**
LumiCycle In Vivo	Actimetrics	https://actimetrics.com/products/lumicycle/lumicycle-in-vivo/
D-luciferin Potassium Salt	GoldBio	Catalog # LUCK-1G
CycLuc1	gift of Dr. Steve Miller	
CycLuc1	MedChemExpress (MCE)	HY-111653
Alzet osmotic minipumps 7	Durect	Model 1007D Cat # 0000290

day		
Alzet osmotic minipumps 14 day	Durect	Model 1002 Cat #0004317
Luer slip syringes	Grainger Industrial Supply	Item # 19G334 Mfr. Model # 4010.200V0
Millipore syringe filters	Thermo Fisher Scientific	CAT # SLGV004SL
0.5cc BD Insulin Syringes	MWI Veterinary Supply	MWI SKU: 19545
Vetropolycin Antibiotic Ointment	MWI Veterinary Supply	MWI SKU: 001479
Oster Finisher clipper	OsterPro.com	Oster® Finisher® Trimmer (Model 59)
Ethicon Absorbable sutures	MWI Veterinary Supply	Cat # 023434
Recovery cage	Ancare	N40
Recovery cage heat discs	SnuggleSafe	Microwave heat pad
Micro Filter Top	Ancare	N40 Micro Filter Top
Recording cage	Ancare	N10
Reduced Height Square water bottle	Ancare	Reduced Height Square Bottle
Food hopper	Ancare	Mouse Feeder
Carson OPMOD DNV viewer	www.opticsplanet.com	99-NV-OPMOD-DNV-DN-300
Pulsar Edge Night Vision Goggles	www.nightgearstore.com	PU-75095