Recording of diurnal gene expression in peripheral organs of mice using the RT-Biolumicorder

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Running head: Recording of gene expression in peripheral organs *in vivo*.

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Abstract

There is high interest in investigating the daily dynamics of gene expression in mammalian organs, for example in liver. Such studies help to elucidate how and with what kinetics peripheral clocks integrate circadian signals from the suprachiasmatic nucleus, which harbors the circadian master pacemaker, with other systemic and environmental cues, such as those associated with feeding and hormones. Organ sampling around the clock, followed by the analysis of RNA and/or proteins, is the most commonly used procedure in assessing rhythmic gene expression. However, this method requires large cohorts of animals and is only applicable to behaviorally rhythmic animals whose phases are known. Real-time recording of gene expression rhythms using luciferase reporters has emerged as a powerful method to acquire continuous, high-resolution datasets from freely moving individual mice. Here, we share our experience and protocols with this technique, using the RT-Biolumicorder setup.

1. Introduction

Circadian clocks are pervasive in most light-sensitive organisms and serve to synchronize a variety of cellular, physiological and behavioral processes with the 24h solar day. Organisms possessing such biological timekeepers can actively anticipate daily recurring events, such as light-dark cycles, temperature rhythms, and nutrient availability, and thereby adapt their

physiology in a proactive manner. Molecularly, the mammalian clock relies on negative feedback loops in transcription that generate oscillations in gene expression. It is composed of about a dozen core clock genes whose daily oscillations drive the cyclic expression of hundreds to thousands of clock-controlled genes (that drive clock output) (see [1] for a comprehensive review of the mammalian circadian system). Anatomically, the circadian system is organized as a hierarchical network of self-sustained cell-autonomous clocks. These are capable of measuring time autonomously in the absence of external timing cues. However, they also integrate a variety of signaling cues and, in some cases, interact with the clocks of neighboring cells (coupling). At the top of the hierarchy, the master pacemaker in the suprachiasmatic nucleus (SCN) receives photic inputs from the retina. This allows the SCN to track external time and to relay internal time to the clocks found throughout virtually all peripheral organs. The synchronization of peripheral clocks by the SCN occurs via several, partially redundant, direct and indirect phase entrainment signals. While feeding-fasting rhythms, depending on rest-activity cycles, are the dominant synchronization cues for many if not most organs, rhythmic hormones and body temperature oscillations also participate in the phase adjustments of peripheral clocks [2–6]. In the liver, the best studied peripheral organ in the field, several studies have shown that feeding rhythms can even control the rhythmic expression of certain genes independently of local liver clocks [7, 8]. These findings reflect the necessity of this metabolically highly active organ to operate in synchrony with nutrient availability and to temporally coordinate the energy demands of the organism accordingly.

Continuous recording of oscillations in gene expression from peripheral cell types is readily achieved with *in vitro* cultured cells. Thus, many protocols are available for circadian reporter assays using cell lines and primary cells. These include a recent methods article in this journal for the real-time recording of bioluminescence rhythms in human primary cells expressing a firefly luciferase reporter gene [9]. Moreover, many peripheral tissues display circadian oscillations for several cycles when cultured as organ explants [10]. Such *in vitro* and *ex vivo* approaches are instrumental in studying the clock in isolation, but fail to recapitulate the complex interactions

65 occurring *in vivo*. Moreover, explant and culture conditions likely inflict artefactual signals onto
66 the clock, such as those elicited by medium and temperature changes.

67 Rhythmic gene expression studies in animals have mostly relied on the analysis of tissues
68 collected around-the-clock. Yet, even at a dense sampling rate (e.g. every 2 or 4 hours) the
69 temporal resolution can be unsatisfactory for some purposes [11]. Moreover, the resulting data
70 are non-continuous, providing a sequence of static snap-shots of circadian states from
71 independent animals. This renders it virtually impossible to study kinetic properties or cause-
72 consequence relationships of the intrinsically dynamic clockwork. Moreover, biochemical time
73 series are not feasible with behaviourally arrhythmic animals whose phase is not known. Another
74 drawback is the need for large cohorts of animals. Real-time recording of peripheral circadian
75 rhythms in intact animals can solve these issues. Initial approaches using the commercially
76 available In Vivo Imaging System (IVIS) still relies on snap-shots taken at different time points
77 from anesthetized mice that express rhythmically regulated luciferase reporter genes (*Per2::Luc*
78 or *Bmal1-ELuc)* [12, 13]. The general anesthesia and the limited time resolution are major
79 shortcomings of this system.

80 The RT-Biolumicorder (Figure 1A-B), a setup first described in 2013, allows for the long-term
81 monitoring of bioluminescence in real time at a high temporal resolution in freely moving mice
82 [6]. It is particularly suited for the recording of bioluminescence rhythms generated by large
83 organs, such as the liver, or entire animals (see also Note 1). Circadian reporter genes can be
84 delivered either genetically (e.g. *Per2::Luc* [14]) or through adenoviral vectors. Here, we describe
85 a protocol that we have established for RT-Biolumicorder experiments. It starts with the choice
86 and administration of reporter genes and covers the animal operation procedures. It ends with
87 considerations about the RT-Biolumicorder strategy and the description of settings used for
88 recording and data analysis.

89 ## 2. Materials

2.1 Mice and reporter gene

Mice carrying a genetically encoded reporter allele, for example the circadian *Per2::Luc* knock-in (ki) reporter, can be used for RT-Biolumicorder studies [14]. Alternatively, the reporter can be introduced using an adenoviral vector. If none of the available reporter gene vectors (see Note 1) is suitable for the envisioned experiment, it is possible to custom-design, clone and prepare alternative adenoviral reporter constructs. Adenoviral vectors injected into the tail vein of animals predominantly accumulate in the liver [15], a well characterized process often applied for gene therapy [16]. In our experiments we use adult male mice at the age of 10 - 14 weeks, but female animals or animals of different ages may be used as well, depending on the addressed research question.

2.2 Adenovirus vector production and purification

Adenovirus (Ad) genome transfection

1. Restriction enzyme for Ad vector genome linearization
2. Polyethylenimine (PEI) solution: 7.5 mM linear 22 kDa PEI, pH 7.0 (adjust pH with NaOH). Sterile filtrate. Store at 4°C.
3. Adenovirus producer cells: E1-complementing cell line for production of first generation ΔE1 Ad vectors, e.g. HEK293 (ATCC, CRL-1573)

Adenovirus harvest, rescue and amplification

1. Adenovirus buffer: 50 mM HEPES, 150 mM NaCl, pH 7.8 (adjust pH with NaOH). Sterile filtrate. Prepare fresh, protect from light and store at 4°C.
2. Culture plates, 6 cm and 15 cm diameter
3. Cell scrapers
4. Conical centrifugation tubes
5. Liquid nitrogen
6. Water bath at 37°C

Adenovirus purification

1. Liquid nitrogen
2. Water bath at 37°C
3. Adenovirus buffer (Ad-buffer): 50 mM HEPES, 150 mM NaCl, pH 7.8; sterile filtrate. Prepare fresh, protect from light and store at 4°C.
4. CsCl step gradient buffer: CsCl=1.27 g/cm^3 in Ad buffer, pH 7.8; sterile filtrate. Prepare fresh, protect from light and store at 4°C.
5. CsCl step gradient buffer: CsCl: 1.41 g/cm^3 in Ad buffer, pH 7.8; sterile filtrate. Prepare fresh, protect from light and store at 4°C.
6. Glycerol (autoclaved)
7. 200 ml centrifugation tubes
8. 13.2 ml ultracentrifugation tubes, e.g. 13.2 ml UltraClear, Beckman Coulter
9. Ultracentrifuge with swing-out rotor, e.g. Beckman SW41
10. Syringes and needles
11. PD-10 size exclusion chromatography column (GE Healthcare)

Adenovirus titration by measurement of OD260

1. Sodium dodecyl sulfate (SDS) solution: 10% SDS in autoclaved ddH2O
2. Blank sample: Adenovirus buffer, 10% glycerol

2.3 Adenovirus administration

If a viral vector is the approach of choice, an additional experimental step is required for its delivery. Our method of choice is tail vein injection. The materials required for tail vein injections of virus are:

1. Adenoviral reporter (stored at -80° C or below)
2. Restrainer for tail injection (commercially available or homemade). We use "The Mouse Tail Illuminator" from Braintree Scientific, Inc (Braintree, MA, USA) that restrains the mouse while at the same time warming up and illuminating the tail.
3. 70% ethanol

4. Heating box or a heating lamp for preheating the animals prior to the injection (recommended)
5. Single-use 1 ml plastic syringe and 30G needles; alternatively, insulin syringes or others can be used.

2.4 Pump implantation

Luciferin can be administered both via drinking water (see Note 2) or with the use of a micro-osmotic pump for constant supply. When delivery by pump is preferred, it must first be filled with luciferin, activated at 37°C, followed by the subcutaneous, dorsal implantation. The required material is:

1. Mini-osmotic pumps. Different pumps are commercially available (ALZET, https://www.alzet.com), differing in size/reservoir volume, flow rate, and duration of delivery. The choice will be mainly dictated by the experimental design and the age/weight of the animal. We use the 1007D ALZET pump with a reservoir volume of 100 µl and a diffusion rate of 0,5 µl/h, which has a duration of about one week with the *Per2::Luc* ki mouse. With viral vectors we use the 2001 ALZET pump with a reservoir volume of 200 µl and a diffusion rate of 1 µl/h, lasting approximately 7 days. For experiments in which the feeding schedule is modified during recording (recording of clock resetting kinetics), the use of pumps with longer duration is recommended (i.e., models with 200 µl reservoir).
2. Blue or teal coloured flow moderators (ALZET) (see Note 4)
3. D-Luciferin sodium salt, lyophilized powder - dissolved 90 mg/ml in Phosphate Buffered Saline (PBS, pH 7.4) For luciferin supply via drinking water, D-luciferin is diluted in tap water with 0.8 mM NaOH to a final concentration of 1.56 mg/ml.
4. Single-use 1 ml plastic syringe for filling the pump
5. Needle for filling the pump (filling tube provided by the pump supplier, or any sterile needle with appropriate diameter)

6. Sterile 0.9% NaCl for pump activation (at 37°C)
7. Shaver or epilation cream (see Note 3), unless genetically hairless animals - such as the immunocompetent SKH1 mouse strain - are used [6].

2.5 Animal care and room disinfection

1. 70% ethanol, deconex or other disinfectant for disinfecting the surfaces
2. Bepanthen® Plus creme and Povidone-iodine ointment for disinfection and care of the site of invasive procedure (sutures after pump implantation)
3. Anesthetics (isoflurane, ketamine/xylazine or others, according to the approved animal experimentation authorisation)
4. Painkillers (water soluble paracetamol, carprofen or others, according to the approved animal experimentation authorisation)
5. Topical ophthalmic ointment for eye protection during anesthesia
6. Heating pad for keeping animal temperature stable during anesthesia
7. Sterile scissors, hemostatic forceps, straight and curved forceps and surgical suture (all tools that are used for invasive procedures, such as in pump implantation, must be meticulously cleaned and sterilized)
8. Appropriate scoresheets for the monitoring of animal well-being according to the approved animal experimentation authorisation (see Supplemental attachment).

All animal procedures must ensure animal welfare and follow the regulations of your research institution and local legislation for animal experimentation. In most countries a detailed form describing the procedures must be submitted for approval to local authorities prior to starting experiments.

2.5 Recording

Hardware:

1. RT-Biolumicorder (Lesa-Technology), including air pumps and power supply, feeders and water bottles for each of the RT-Biolumicorder setups (see Notes 5, 6,7 & 8)
2. A computer with the relevant software (Biolumicounter, Lesa-Technology) (Notes 6 & 9)

Consumables:

1. Polyethylene-coated paper that allows infrared transmission in the 7-14 μm range (used to cover the infrared sensor at the center of the recording cage; Lesa-Technology)
2. Highly absorbent bedding (Lesa-Technology)
3. Sodium hydrogen carbonate ($NaHCO_3$) to apply to the bedding for neutralization of urine smell during recording (optional)
4. Chow (or any other) diet pellets with a diameter of approximately 12 mm, which are loaded into the feeder of the RT-Biolumicorder (see Note 9).

3. Methods

3.1 Choice of reporter gene

Since the experimental readout relies on bioluminescence signals, the first decision to take concerns the selection of the appropriate firefly luciferase reporter and the mode of its delivery. The two main possibilities are, (i) to use a mouse strain genetically carrying a suitable reporter allele, or (ii) to use a reporter gene delivered through viral transduction. Both methods need preparation (weeks/months) before the start of the actual recording experiments, e.g. to breed the reporter allele into the desired mouse strain, or to clone and prepare the adenoviral vectors (Figure 1C). Please see Note 1 for a detailed account of advantages and weaknesses of the two approaches.

3.2 Adenoviral preparation

Adenovirus is a non-enveloped virus harbouring a double-stranded DNA genome of 36-40 kb. Human adenoviruses, such as adenovirus type 5 (hAd5, Ad5), have been extensively studied and their cell entry and replication processes have been characterized in great detail. The virus genome can easily be cloned into a plasmid or bacmid backbone, and the virus is rendered replication-defective by removal of the early adenoviral gene region E1 from its genome. Plasmid/bacmid systems based on human Ad5, which allow for the insertion of heterologous transgene expression cassettes into the replication-defective vector genomes, are commercially available. Due to its high genome stability, its ability to transduce a wide variety of proliferating and post-mitotic cells, and the possibility to produce infectious virions to high titers with relative ease, replication-deficient Ad5 can serve as an attractive tool for transient gene transfer in various animal models. The amplification of Ad5-based vectors requires producer cells which complement the lack of the early viral gene region E1 in trans. HEK293 cells are the most commonly used cell line that constitutively expresses Ad5 E1 and that is thus able to support the production of replication-deficient adenoviral vectors after transient transfection of ΔE1 vector genomes. Although the efficiency with which vector particles are produced from transfected genomes is poor, the number of produced virions is still sufficient to re-infect fresh HEK-293 cells. Prior transfection, Ad5ΔE1 genomes have to be released from the respective bacmid/plasmid backbone by using a suitable restriction enzyme.

For vector production and purification, the following steps should be performed:

1. Seed HEK-293 cells in 24-well plates with 2x10E5 cells/well and transfect the next day with 500 ng/well of the E1-deleted, linearized vector genome using Polyethylenimine (PEI).
2. A full cytopathic-effect (CPE), reflecting successful vector amplification, should be visible 7-12 days after PEI transfection. A full CPE is characterized by a morphological shift from an adherent to globular appearance and aciniform cell detachment. At this time point, most vector particles are retained intracellularly. We advise to perform multiple

transfections in parallel since the efficiency of virus vector rescue from plasmid/bacmid can be low.

3. Release cells from the dishes by scraping and harvest by centrifugation for 10 min at 400 x g. Next, the pellet is resuspended in 1 ml Ad-buffer and subjected to three freeze/thaw cycles, using liquid nitrogen for freezing and a water bath at 37°C for thawing.
4. The lysate, now containing rescued Ad vector particles, can be used for reinfection of 1x10E6 cells seeded the day before in a 6-cm dish. Now, cells should exhibit a full CPE 48-72 h post-infection.

This serial amplification procedure should be repeated as described by Kratzer and Kreppel (17) up to a final reinfection of 1-2 x 10E8 cells (10 to 15 15-cm dishes). To obtain highly purified Ad vector particles, cells from final reinfection are harvested, lysed and subjected to two consecutive discontinuous CsCl gradients. It is very important to perform the purification with two subsequent discontinuous gradients in order to remove impurities which might interfere with vector performance in vivo.

5. Harvest cells from final reinfection into 200 ml centrifugation tubes and pellet as described above.
6. After centrifugation, resuspend the pellet in 3 ml Ad-buffer and rescue vector particles by three freezing/thawing cycles.
7. Subsequently, load the lysate onto a discontinuous CsCl density gradient (lower phase: 3 ml of ϱCsCl: 1.41 g/cm³; upper phase: 5 ml of ϱCsCl: 1.27 g/cm³) and centrifuge for 2 h at 176.000 x g and 4°C using an ultracentrifuge.
8. After centrifugation, the white-blue vector band is visible at the border of the two different CsCl solutions. Collect the vector band by puncturing the ultracentrifugation tube using a syringe.
9. Dilute aspirated Ad vector particles with Ad-buffer to a final volume of 3 ml, load onto the second discontinuous CsCl density gradient and centrifuge as described above.

10. Collect the vector band with a syringe as described above and dilute with Ad-buffer to a final volume of 2.5 ml.
11. To remove CsCl, disposable PD-10 columns are equilibrated with Ad-buffer (5 x 5 ml) before being loaded with Ad vector particle dilution.
12. The virus is eluted with a volume of 3 ml Ad-buffer and subsequently supplemented with glycerol to a final concentration of 10%.
13. Suitably sized aliquots (50 – 200 µl) can be stored at -80°C.

To maintain the integrity and infectivity of Ad vector preparations, the vectors should always be stored at -80°C in the presence of 10% glycerol. Note that repeated freezing/thawing cycles can significantly reduce vector infectivity. Therefore, it is recommended to store appropriately sized aliquots.

To assess the quality of purified Ad vectors, the physical and infectious titers, and the purity of the preparation are evaluated.

Physical titer

Physical titers are quantified by either quantitative PCR or the determination of the optical density at 260 nm.

1. For measuring OD260, mix 20 µl purified vector solution with 79 µl deionized water and 1 µl of 10% sodium dodecyl sulfate (SDS) and incubate for 10 min at 56°C for capsid denaturation.
2. Physical particle titers can be calculated from measured OD260, with one OD260 unit corresponding to 1.1 x 10E9 vector particles per µl (18, 19).

Infectious titer

1. To determine the infectious titer, infect 2x105 HEK-293 cells with a serial dilution (1:10,000 - 1:10) of purified vector and incubate at 37°C, 5% CO_2.
2. After 48 h cells are visually screened for a full CPE.

Since the ratio of infectious to non-infectious particles usually varies between 1:10 and 1:30 [20] a full CPE 48 h post-infection represents a multiplicity of infection (MOI) of 300 and can therefore be used to calculate the infectious titer.

Purity of vector preparation

Purity of Adenovirus vector preparation is verified by polyacrylamide gel electrophoresis (SDS-PAGE).

1. According to physical particle titers, incubate 1-2 x 10E10 vector particles for 10 min at 75°C for denaturation.
2. Load on a 8% SDS-PAGE gel.
3. After electrophoresis, develop the gel by silver-staining [21]. This highly sensitive staining procedure allows the detection of even weak protein bands and contaminant traces (i.e. non-viral proteins).

3.3 Tail vein injection

Tail vein injection of viral vectors is a rapid procedure and allows for a quick recovery of the animal; please also refer to Figure 2A-B for a schematic drawing relating to this procedure.

1. Recommended: warm up the animals prior to the intervention. We place the mice in thin-walled plastic cages on a heated metal surface at 37°C for 1-2 hours before the injections. Similarly, the restrainer for tail vein injection and 70% ethanol for disinfecting the tail should be pre-warmed to 37°C.
2. Thaw the adenoviruses on ice or at room temperature for the time period of an experiment that involves injecting several animals. Avoid multiple freezing/thawing cycles of viral preparations.
3. Anesthetize the mouse by inhalation (constant supply of 2% isoflurane) or injectable anesthesia (80/12.5 mg/kg ketamine/xylazine intraperitoneally, which results in ~50 min anesthesia). Tail vein injection in non-anesthetized animals is also possible, if allowed by the local animal experimentation legislation. In our lab we have been using anesthesia by

inhalation (2% isoflurane) as it allows for quicker recovery and is considered more animal-friendly (see Note 11).

4. Once the mouse is restrained apply topical ophthalmic ointment and disinfect the tail.
5. Draw the virus with the syringe (be sure to avoid any air/bubbles), place the needle on the syringe and remove any residual bubbles.
6. With the applied heat from the restrainer, the veins of the animal become visible; the mouse tail has two caudal veins laterally to the artery (Figure 2B). Locate one of them, place the animal on its side, and insert the needle into the vein, almost parallel to the tail (the angle should be no greater than 15 degrees to the vein). In most cases, we have found injections within the middle third of the tail most convenient (closer to the tail base, the vein is larger; closer to the tip, it can be more visible). A sign for correct placement in the vein is entry of a small amount of blood into the syringe, either spontaneously or after slowly drawing back the syringe.
7. Once proper localisation of the needle is ensured by this strategy, slowly inject the virus (ca. 5 seconds for the 100 µl). In case of resistance during injection, slightly readjust the needle or try with the other vein to avoid further irritation on the same side of the tail.
8. At the end of the injection carefully remove the needle and apply slight pressure to the puncture with a clean piece of cotton until the bleeding has stopped (blood at the end of the injection is a good indication of a successful injection).
9. Monitor the animal until the anesthesia has passed. About 24-48 hours after viral injection, the animal can proceed to pump implantation. We would like to point out that several videos showing correct tail vein injection practice are also available on YouTube.

3.4 Luciferin solution preparation, pump filling & activation

There are two ways of luciferin administration during recording, either via drinking water or via constant diffusion using a mini-osmotic pump. There are several pros and cons to both approaches.

For *in vivo* imaging, luciferin can be administered via drinking water, as it is not significantly degraded in the digestive tract [22]. Luciferin supply in drinking water has no toxic side-effects. This method is more animal-friendly and shortens the overall protocol. Even though this route of administration has been used in circadian studies using the RT-Biolumicorder before [6], other observations indicate that daily changes in liquid uptake (drinking) by the mice can lead to rhythmic fluctuations in *in vivo* luciferin availability (see Note 2) [23]. However, the drinking rhythms do not markedly confound the results when high-amplitude circadian bioluminescence rhythms are recorded [5].

By contrast, mini-osmotic pumps provide constant diffusion of luciferin for several days. Pumps furthermore allow to choose the appropriate diffusion rate and duration according to the study design. Lastly, mice tolerate the implanted pumps well, especially the smaller models with a 100 µl reservoir volume. However, the pump implantation requires an extra round of anesthesia and painkiller administration and it prolongs the overall protocol for at least two more days. In addition there is a possibility that daily locomotor activity might influence the diffusion rate of the luciferin via the pump and thus create low-amplitude activity-related rhythms [23]. Animal experimentation approval is required prior to the pump implantation procedure.

Pump filling and activation are performed under sterile conditions, according to the following protocol:

1. Dissolve the D-Luciferin sodium salt at 90 mg/ml in sterile PBS, pass it through a 0.45 µm filter and aliquot it. Dissolved luciferin is stored at -20°C. Luciferin is light sensitive, so keep the aliquot tubes wrapped in foil.
2. Filling the pump with luciferin solution is performed using a single-use 1 ml syringe, either with the needle provided together with the pump, or with a needle of your choice. If not using the needle provided with the pumps, be sure to use a needle that has a blunt tip in order to avoid damaging the inner wall of the pump.

3. Draw the luciferin without the needle to avoid any small bubbles, place the needle and remove excess air.
4. Place the needle carefully inside the opening of the pump and slowly inject the luciferin until the pump is completely full. Carefully, release the needle and place the flow moderator, always ensuring that there are no air bubbles (see Note 12). Air bubbles can potentially block the pump and affect its function.
5. Place the pump in a 15 ml tube containing ~5 ml sterile NaCl 0.9% covered with foil and leave for activation at 37°C. The pump activation duration varies depending on the pump type (we typically activate in the range of a few minutes to a few hours).

3.5 Pump implantation

Before the pump implantation, the animals are anesthetized. In addition, some of the dorsal fur coat is shaved or removed with epilation cream (see Note 3), in particular around the area of interest for recording, notably the liver (Figure 2A). Depending on the mouse strain, the dark fur can strongly reduce the signal intensity of emitted bioluminescence. For this procedure, injectable anesthetics are preferred, in order to shave the mice more easily and quickly. However, inhalational anesthetics are tolerated better by some mice strains and in general allow quicker recovery (see Note 10). All surfaces and surgical instruments must be disinfected, and animal body temperature must constantly remain at ~37°C by the use of a heating pad.

1. Place the anesthetized and shaved mouse on the head pad, apply ophthalmic ointment and inject painkiller (if an injectable painkiller is used). We inject 4-5 mg/kg of Carprofen subcutaneously at the beginning of the pump implantation procedure. Check the animal reflexes to evaluate the status of anesthesia.
2. Using sterile forceps and surgical scissors, cut a small incision into the skin of the right upper back of the animal. The incision must be long enough to slip in the pump (pumps

with 200 µl of reservoir require larger incisions compared to those with 100 µl of reservoir).

3. With a pair of straight forceps, carefully lift the skin and insert a pair of hemostatic forceps. Opening and closing the hemostatic forceps inside the incision will create a "pocket" in the skin that will accommodate the pump.
4. Remove the pump from the NaCl solution, slowly take out the hemostatic forceps, and insert the pump with the flow moderator facing downwards.
5. Once the whole pump is inserted under the skin (be careful not to implant the pump intraperitoneally), apply iodine, suture the wound using surgical suture, and administer Bepanthen® Plus creme at the back of the mouse.
6. Place the anesthetized animal in a heat-controlled environment (a dedicated recovery cage or, alternatively, a heating lamp) and monitor until it fully recovers from anesthesia.

3.6 Monitoring of animal welfare

The wellbeing of animals must be evaluated all along the experiment, especially after the invasive procedures, i.e. following the tail vein injection (if applicable) and the pump implantation (if applicable). The researchers should look for signs of discomfort or pain, such as altered grooming behavior, posture, or locomotor dysfunction. Body weight must also be monitored. Painkillers and antibiotics can be administered if required. In the case of severe signs of pain or infection the animal has to be sacrificed. We have created two dedicated score-sheets, according to the Swiss animal experimentation legislation. A carefully processed datasheet helps in the evaluation of animal health and in making the choice of the most appropriate action (medication, sacrifice) (see Supplemental attachment).

3.7 Recording

Figure 1A shows a schematic representation of the RT-Biolumicorder that was developed at the University of Geneva [6] and is now commercially available from Lesa-Technology. One

single-housed mouse can be recorded in each RT-Biolumicorder setup at a time. The RT-Biolumicorder consists of a cylindrical cage with photon-reflecting walls, equipped with a photomultiplier tube (PMT) centrally at the top of the cage, a food container (right side) and a water container (left side). In addition, the device contains a large reflecting cone on top of the cage (external cone) that projects photons to the photomultiplier tube and a small reflecting cone in the center of the cage floor (central cone) that projects photons to the reflecting walls. An infrared sensor built into the small cone records the locomotor activity of the mouse.

1. Use highly absorbent bedding in order to reduce the background light in the recording cage. The bedding must cover the entire surface at the cage bottom.
2. Apply sodium hydrogen carbonate on the bedding to neutralize the urine smell that can otherwise accumulate during the recording period (a small amount ~20 g equally dispersed on the bedding; optional).
3. Cover the small central cone at the center of the recording cage with special polyethylene-coated paper.
4. Lastly, after the bedding and the central cone, place the round metal grid on the cage floor.
5. Provide a small amount of bedding on top of the grid, as nesting material for the animals. It is important to not provide too much bedding material, as this might interfere with signal detection (in particular during the sleep phase).
6. Carefully fill the food and water containers. The food pellets must have a specific size in order to fit in the feeding tube (pellets of maximum 12 mm diameter). Take special care to ensure that there is no leakage from the water containers. We recommend manually tracking food and water consumption over the course of the experiment as a means to ensure that there are no issues with access to food/water and/or with animal wellbeing. It is recommended not to open the recording cages during the experiment (see Note 10).

7. Place the animals in the recording cages and turn on the power supply of the RT-Biolumicorders (multiple RT-Biolumicorders can be attached to one supply) and the air pumps for each recording cage (see Notes 5 & 8).
8. Constant air pressure in the cages is very important during the recording, as the air exchange is required for oxygen supply and removal of urine odors (see Note 8).
9. Using the ''Biolumicounter'' software, select the desired recording parameters (timing of lights-on, lights-off and food access) for each of the recording cages and initiate. During the recording period it is possible to change recording parameters and to evaluate photon levels and animal activity in real-time.

3.8 Recording parameters: habituation period, feeding schedules, skeleton photoperiods

Depending on the experimental design and the research questions, various parameters during the recording period are adjusted. An initial habituation period with 12h lights-on and 12h light-off (in phase with the previous timing experienced by the animal) of approximately 24-48 hours is recommended before releasing the animals into constant darkness or other experimental conditions (see Note 12).

After the first 24h of the habituation period, the experimenter may potentially open the cages (during lights-on phase) and visually check the animals, which can inform on whether they have become accustomed to the new environment (in particular to the feeder/water supply). Prolonged habituation periods may be considered according to the scientific questions of the experiment. However, it is important to keep in mind that there is no recording during the lights-on period and that mini-osmotic pumps have a limited delivery duration (by contrast, the duration of adenoviral vector expression has rarely been a limiting factor in our experiments, as it persists for several weeks).

After habituation, animals are typically released into constant darkness, if data collection under free-running conditions is desired. Alternatively, a skeleton photoperiod is applied, if the experiment requires measurements under phase-entrained conditions. In our hands, a

simple skeleton photoperiod consisting of two 30 min light pulses at the beginning and end of the ''light'' phase (ZT0-ZT0.5 and ZT11-ZT11.5) is sufficient to ensure a stable phase-entrainment, while causing minimal disruptions to the recordings (that are, of course, suspended during illumination).

Through the programmable feeder, food availability can be set as desired. Typical settings used in many studies in the chronobiology literature are *ad libitum* feeding or time-restricted feeding. The activity cycles, entrained by skeletal photoperiods through the synchronisation of the SCN, can be deduced from locomotor activity records. High and low mobility periods correspond to subjective nights and days, respectively, for nocturnal animals like mice (see Note 7).

3.9 Termination of experiments

1. At the end of the recording period, stop the recording and open the RT-Biolumicorder devices.
2. Evaluate the health status of the animal (body weight, grooming) and proceed with sacrificing the mice and, if applicable, collecting their tissues.
3. Total food consumption may also be evaluated at the end of the experiment.
4. Mini-osmotic pumps are designed for single usage, so discard them at the end of the recording (depending on the local biological waste regulation, removal of the pumps from the animal body and separate disposal might be required).

3.10 Data analysis

Data are obtained as numeric values of photon number and activity levels per minute in text files (*.txt*). Time, light and feeder information are also included in the data files. Data can be analyzed using the computational program of choice. In the past, we have used the commercially available software Igor Plus (Wavemetrics) [6]. In addition, we have recently programmed a software that

enables easy visualisation and analysis of RT-Biolumicorder recording data, both for individual mice and by experimental groups (see Note 8) .

We have developed a custom-made application that runs under the MATLAB environment (The Mathworks, Inc). The application, that we named Osiris, allows the user to easily load, visualize and analyse the *.txt* format data generated by the RT-Biolumicorder. Briefly, the signals obtained for locomotor activity and the bioluminescence data from an individual animal can be displayed as a smoothing average across the recording period (the raw data are displayed on demand, Figure 3A-B). Tracks for food access and illumination are available for display as well. Osiris also allows selecting different smoothing parameters (in particular moving average window size) for the rather spiky bioluminescence raw data.

In addition, with Osiris, the user can create a database that includes multiple animals within one or more groups. Within a group, it uses the information provided by the user to align the data across the individual animals and to visualize their signals (mean and dispersion, with options: standard error of the mean, standard deviation, 95% confidence interval) for the selected animals in each group (Figure 4A). The database of multiple animals can be saved in *.mat* format, and the output images in both *.tiff* and *.eps* formats.

Finally, for the current (first) version of Osiris, relevant (albeit still basic) rhythmicity analyses on the bioluminescence and activity signals can be executed. Thus, it is possible to collapse an animal's multi-day recording into a mean cycle across the 24 hour period (Figure 4B). Measures of peak-trough amplitude, peak phase (ZT values) and the steepness/rate of change from through to peak values in the 24 hour period of the mean cycle can be exported in *.csv* format, and graphics outputs in both .tiff and .eps formats. In summary, all these features aim at making analyses and conclusions easier and more user friendly. Moreover, they greatly improve traceability and objectivity of the analyses. The Osiris application is publicly available at https://github.com/aosorioforero/Osiris.git.

4. Notes

1. Different considerations come into play when deciding on which reporter gene to use, and whether to rely on adenoviral delivery or a genetically encoded allele. A genetically encoded reporter allele has multiple advantages, such as the high reproducibility of its expression that occurs from a defined genetic locus and that is not limited in time. As no viral injection is required, the experimental procedure is shorter and the setup considerably simpler and with less stress for the animal. By contrast, the genetic alleles will typically be expressed across tissues and cell types, which can have implications for the interpretability of the signal that is recorded from the whole animal. In published work [6] and additional experiments in our laboratories, the genetic reporter allele that has been successfully used is the *Per2::Luc* allele (official nomenclature: *Per2*^{tm1Jt}) developed by the Takahashi laboratory [14]. It is a knock-in of the coding sequence (cds) of firefly luciferase into the endogenous *Per2* locus, in frame with the *Per2* cds (lacking its termination codon) such that a fusion protein PER2-Luciferase is produced. This fusion protein fully replaces the function of PER2 within the circadian clockwork circuitry. Hence, the allele is more than a simple reporter of circadian transcriptional oscillations, but luciferase activity quantifies the abundance of the core clock protein Period 2. We have used the *Per2::Luc* allele in RT-Biolumicorder experiments in both heterozygous (*Per2::Luc* / +) and homozygous settings (*Per2::Luc* / *Per2::Luc*). The former already gives sufficiently robust signals for most purposes. Finally, it has been observed that the *Per2::Luc* reporter reacts relatively sensitively to changes in feeding rhythm [6], making it a suitable tool to study phase readjustment kinetics mediated by food-derived signals. Beyond the popular *Per2::Luc* allele, a number of other circadian luciferase alleles are available. Yet testing their performance and properties in RT-Biolumicorder experiments is, to the best of our knowledge, still pending. Thus, *mPer1-Luc* transgenic mice have been constructed independently by different labs. In these mice the luciferase expression is driven by mouse *Per1* promoter/5' UTR elements (e.g. *Tg(Per1-luc)025Jt* [24] or *Tg(Per1-luc)Chron* [25]). We expect these alleles to be suitable real-time reporters of *Per1* expression rhythms in RT-Biolumicorder experiments. Mice carrying the *Bmal1-ELuc* transgene [26] express

548 enhanced green-emitting luciferase (ELuc) from the 5′-flanking region of the *Bmal1* promoter
549 and would be suitable in RT-Biolumicorder experiments to track the rhythmic transcriptional
550 activity at the *Bmal1* promoter.
551 Nevertheless, the number of available rhythmically expressed genetic luciferase alleles
552 remains rather limited (and crossing them into a specific mouse model/genetic background
553 can be time-consuming). Transduction with viral reporters is an alternative way of delivery.
554 Moreover, this method allows testing reporter variants, as well as the co-delivery of
555 additional genes. In our experiments, we use E1/E3-deleted replication-incompetent first-
556 generation adenovirus vectors based on human adenovirus serotype 5 (plasmid pCV100, a
557 variant of pGS66 [27] with an additional deletion of Ad5 nt 28133-30818) purified to high titres
558 (>10E9 infectious particles/µl) that are safe (replication-incompetent; no wild-type virus
559 contamination; depending on your local biosafety regulations, they can be considered non-
560 biohazardous) and do not elicit a severe immune response in the mice. Upon tail vein
561 injection, adenoviral vectors transduce primarily liver cells. We typically inject 10E11
562 infectious particles through the tail vein, which results in robustly detectable signals for
563 several weeks. Two reporter constructs have been particularly useful in our hands. The *Rev-*
564 *erbα-Luc* reporter (recapitulating the rhythmic transcription at the *Rev-erbα*/*Nr1d1* locus) is
565 fast-reacting to changes in feeding rhythm, whereas the *Bmal1-Luc* reporter (*Luciferase* cloned
566 with *Bmal1*/*Arntl* promoter and 5′ UTR sequences) has slower readjustment kinetics to food-
567 derived signals and may thus be more dependent on SCN signaling cues [6]. Moreover, the
568 *Bmal1-Luc* construct also contains an expression cassette for Cre recombinase (driven from
569 hCMV promoter) [28], which allows the recombination of floxed alleles and recording of
570 transcriptional rhythms from the same cells. Other reporter constructs are in the making in
571 our group, and possibly other laboratories as well, which should extend the repertoire of
572 useful reporter viruses in the future.
573 In principle, every organ/tissue emitting sufficient amounts of photons would be suitable for
574 RT-Biolumicorder recording experiments. We anticipate that lungs, heart and skeletal

muscles, kidney, pancreas, whole brain, stomach, parts of the gut and skin would fall into this category of organs/tissues. Transgenic mice, whose genomes carry luciferase reporter genes with flox-stop signals, could probably be engineered for all of these tissues. In such animals, the tissue-specific expression of the reporter could be achieved by expressing a Cre recombinase transgene from cell type-specific transcriptional regulatory elements, such as locus control regions, enhancers, or promoters.

2. When administering luciferin via drinking water, it is important to exclude the possibility that drinking rhythms interfere with the rhythm of the reporter of interest. In this case it is recommended to measure temporal drinking profiles during the recording in order to control for potential bias due to rhythmic drinking of the animals. A way of doing so is by using an ultrasonic detector to record water consumption throughout the experiment (prototype at Lesa-Technology). A recent study detected rhythmic expression of an otherwise constitutively expressed reporter when luciferin was administered via drinking water and the observed rhythm resembled the expected rhythm of the drinking behaviour [23]. We speculate that environmental conditions of the experimental room, such as the humidity levels, or the reporter in use (genetic, viral, expression levels) could potentially determine whether bioluminescence levels are affected by the drinking behaviour. We would like to emphasize, however, that the bioluminescence cycles engendered by drinking rhythms are of much lower amplitude than bioluminescence cycles driven by circadian reporter genes. Indeed, Sinturel *et al.* [5] and Martin-Burgos *et al*. [29] have recently shown that drinking rhythms do not markedly confound the results obtained with circadian luciferase reporter genes.
3. By shaving/depilation of a dorsal patch of the fur coat (corresponding to the position of the liver), it is possible to limit detected signal mostly to that emitted from hepatic tissue. Nevertheless, possible contributions from other organs must be kept in mind.
4. We use blue flow moderators from ALZET, which are recommended for bioluminescence imaging applications. These were specifically developed to avoid background luminescence

caused by the standard (white) flow moderators, which may interfere with the real signal from the reporter gene.

5. Hardware problems: It is important to check that all parts of the equipment function properly before initiating experiments. Potential problems with hardware can include: power supply and computer problems, infrared sensor problems (often accidentally blocked by bedding), the indicator lights at the RT-Biolumicorder panel (for activity, PMT, shutter) might be out of order or burnt (and need replacement), or the shutter might be blocked.
6. Light settings: when setting the lights-on and lights-off timing in each of the RT-Biolumicorders, always remove all pre-existing settings and set up the desired new settings. Often the software has a particular default setting assigned when starting the program and it can cause confusion if not removed.
7. Feeder opening/closing: When placing an animal for recording, always check that the feeder is placed in the correct position and the animal can access the food. Especially in cases when altered feeding schedules are applied, it is very important to evaluate the proper opening and closure of the feeder. During cleaning of the feeders between experiments it has happened to us that the position got slightly altered, which resulted in the mechanics of food access not closing properly. This will have repercussions on the obtained data and their interpretation. Of note, we found that *Bmal1* knockout mice require a longer period of adaptation before recording can be started.
8. Air pump control: It is important to daily check the proper function of the air pump by evaluating the pressure at the recording cage (there is an air pressure indicator outside of each RT-Biolumicorder setup). Air pumps can be out of function over time or accidentally detach from the power supply, causing severe problems to the recorded mouse and eventually its death.
9. Data files and storage: it is recommended to securely save the data on an external disk at multiple time points during the recording period. Potential problems of the computer or the software might lead to loss of data. Moreover, it is important that there are no empty spaces

at the end of the *.txt* file to be properly loaded by Osiris. This can happen in a small percentage of files created by the RT-Biolumicorder, in which case the empty spaces should be manually modified before loading for the current version. No further modifications of the files are needed.

10. Food/water consumption: When refilling the feeding tube, remove the metal weight, place the food pellets and then put the metal weight back. Always use a specific type of food granule that fits into the food supplier of the RT-Biolumicorder (as mentioned above we use a diet consisting of 12 mm pellets). Avoid using too small/broken pellets as they might block the tube and restrict food supply. The feeder cover should be well closed in order to avoid any potential light introduction from the external environment to the recording cage. When evaluating the water consumption, the floater/indicator might be stuck and show no changes in the water levels in the tube.

    When filling up the water supplier always ensure that there is no leakage. To do so, assemble carefully all the parts of the supplier, fill in the water and let it stand for a few minutes or hours. If there are no drops coming out of the bottle, it can be placed in the recording cage. When placed in the cage, proper position and water access must be evaluated (water needs to be able to drip out when the animals touch it with their tongues. Check manually that the water is freely coming out of the reservoir and there is no blockage by air bubbles). Make sure that the indicator can freely move and is floating on the water, before concluding that the mouse is not drinking any water.

11. Anesthesia troubleshooting: In our experiments we have occasionally faced problems with mice recovering poorly from injectable anesthesia with ketamine/xylazine after mini-osmotic pump implantation. Please also note that some circadian knockout mice are particularly sensitive to xenobiotics, as detoxification by the liver is under clock control. We have thus changed to anesthesia by inhalation using isoflurane. Briefly, animals are placed in a plexiglass chamber, anesthetized and quickly shaved with an electric razor. We perform the pump implantation in a laminar hood using a homemade nose mask (which can be easily

constructed using a small plastic funnel or the conical base of a 50 ml tube) for isoflurane administration that adapts to the animal head. Mice recover much more quickly and with no signs of pain or discomfort.

12. While filling the mini-osmotic pump, let the luciferin slightly overflow and create a small liquid bubble at the opening of the pump. Place the metal flange of the flow moderator at the overflowed luciferin and gently press the moderator to close the pump. This avoids the introduction of any air bubbles in the internal part of the pump reservoir. A tiny amount of luciferin is expected to come out of the flow moderator cap while closing the pump. This is an indication that there are no air bubbles blocking the flow.
13. Food/water supply habituation: We have observed that mice relatively frequently fail to become habituated to the food and water supply in the recording cage, even with a prolonged habituation under 12h light/12h dark conditions. This may critically confound the obtained data, in addition to the undesired effects on animal health. In such cases it is recommended to add, early in the experimental period, during the lights-on phase a pad of water gel or food gel. This will ensure enough food/water supply during the recording without disturbing the experiment (naturally, this intervention is only compatible with *ad libitum* feeding experiments). At the beginning of the experiment, when placing the food and water suppliers, ensure their proper installation for unperturbed access.

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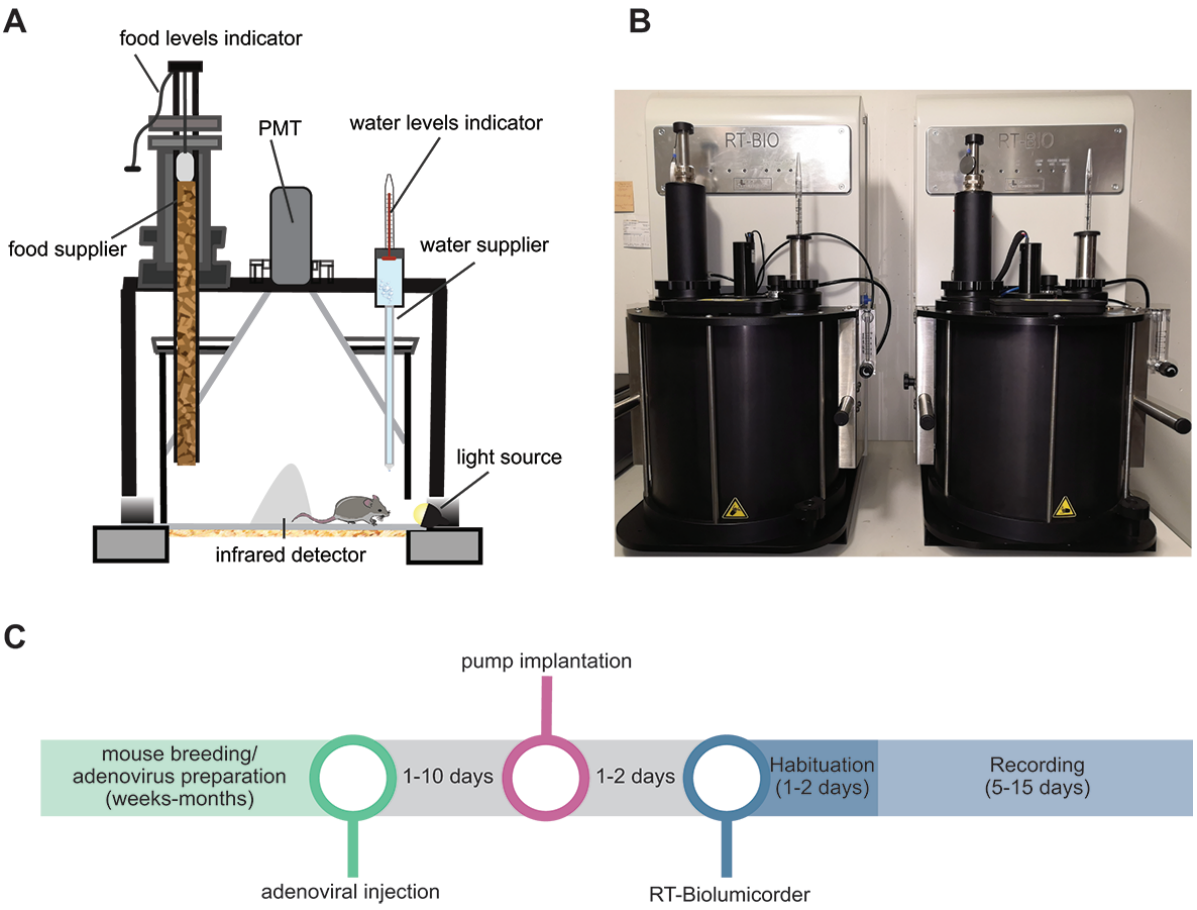
Figures and Figure Legends

Figure 1. View of the RT-Biolumicorder setup.



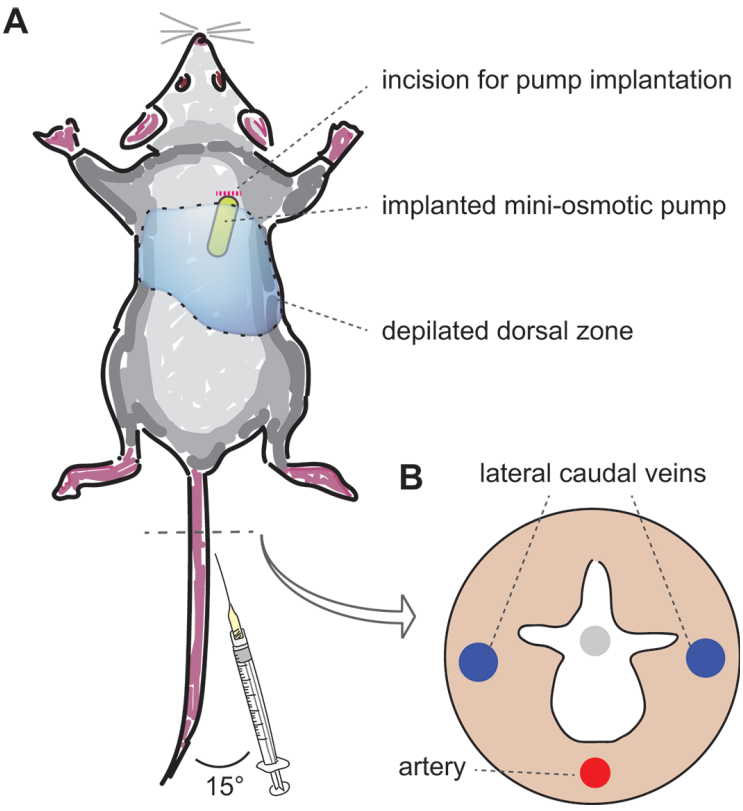
A. Schematic representation of the RT-Biolumicorder (Lesa-Technology) that allows for simultaneous real-time recording of bioluminescence and locomotor activity in freely moving mice. The RT-Biolumicorder consists of a cylindrical cage with reflective walls equipped with a photomultiplier tube (PMT) that is centrally placed above the recording cage and records bioluminescence levels. An infrared detector that records locomotor activity is built into a small cone in the middle of the cage floor. Water and food suppliers with their corresponding consumption indicators are depicted.

B. A picture of two RT-Biolumicorders at the animal facility of our department. The medium-size black cylinder at the left side of each machine is the food supplier and the smaller metallic

792 cylinder at the right is the water supplier with the water level indicator. Each RT-Biolumicorder
793 has a control panel (silver metal panel labelled "RT-BIO"), which indicates: (a) activity, (b) food
794 access off, (c) light on, (d) shutter on, (e) PMT on.

795 **C.** Overview of the typical experimental workflow. After mouse breeding (to introduce
796 luciferase reporter allele) or adenoviral reporter preparation, the whole experiment typically
797 takes 2-3 weeks. After adenoviral injection, micro-osmotic pump implantation should occur
798 within the following 1-10 days. Because the availability of luciferin limits the length of the
799 recording, the animals should then be transferred rapidly (1-2 days) to the RT-Biolumicorder.
800 After short habituation (1-2 days), the actual recording takes place for approximately 5-15 days
801 (i.e. until signal becomes too weak due to exhaustion of luciferin reservoir). Note that when the
802 reporter gene is genetically encoded (e.g. *Per2::Luc* mice), the adenoviral injection is omitted. This
803 makes the protocol significantly shorter and less stressful for the animal. Likewise, if luciferin is
804 delivered through the drinking water, no operation for the micro-osmotic pump is required, thus
805 eliminating the main invasive operational procedure. While in principle this would represent the
806 ideal protocol, we would like to point out that delivery via drinking water may not be possible
807 when low-amplitude rhythms are recorded (see Note 2).
808

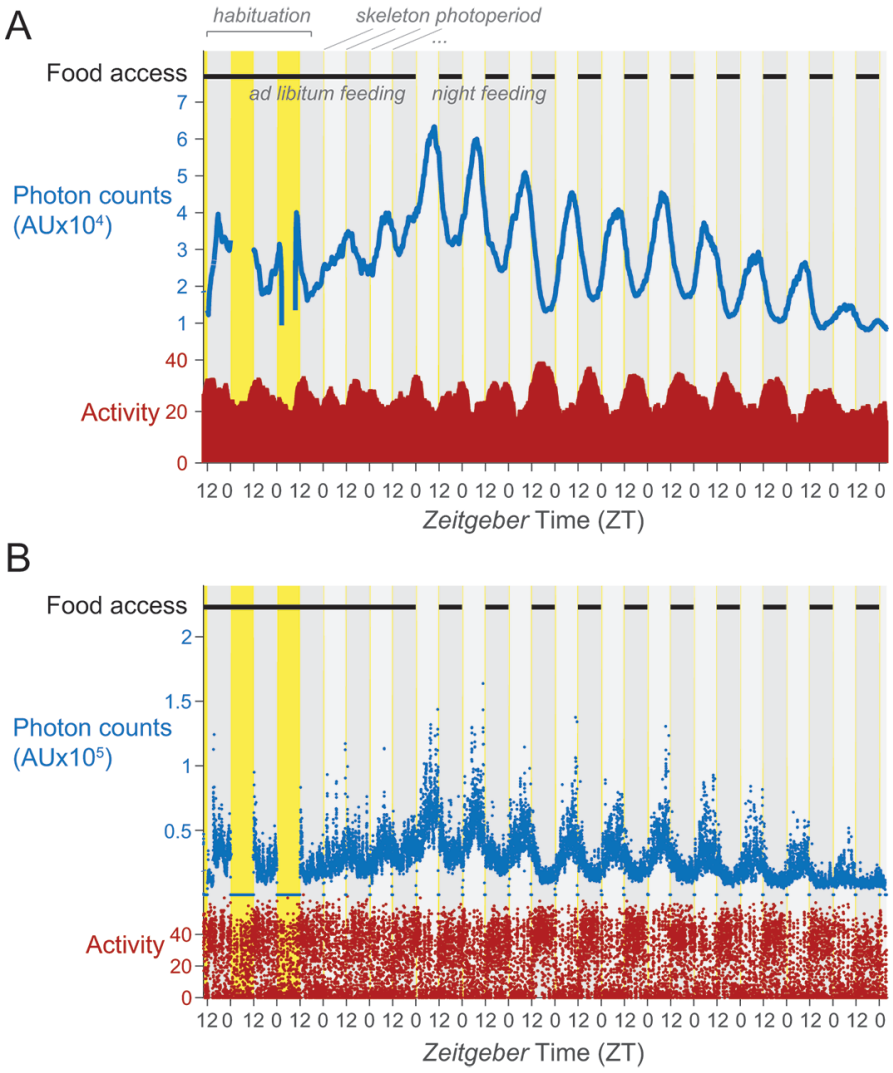
Figure 2. Schematic representation of main sites of animal handling.



A. Dorsal view of an experimental animal. Blue shading depicts the dorsal zone that is depilated/shaved. The site of the incision made in the skin between the scapulae is shown as a red dotted line. Using a hemostat, a small pocket is formed by spreading the subcutaneous connective tissues apart, and the pump is inserted (depicted in yellow). The skin incision is then closed with sutures, and the area is disinfected. Adenoviral delivery (prior to the pump implantation) occurs through tail vein injection that is best performed at a very shallow angle (<15°).

B. Diagram of a sectional view of a mouse tail, with the two lateral caudal veins and the ventral artery. With the applied heat from the restrainer, the veins become more visible. After locating one of the veins, the animal is placed in a lateral position, the needle is inserted and the virus is carefully injected. Typically, we have had high-quality outcomes with injection sites located in the middle third of the tail. The vertebrae is shown in white.

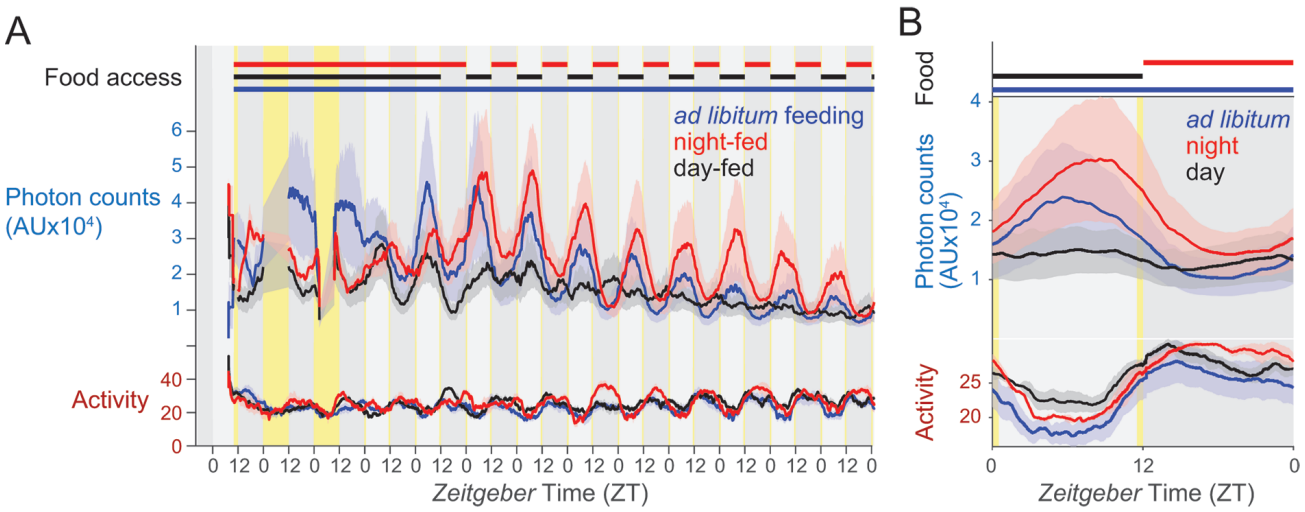
Figure 3. The Osiris software allows for straightforward display of RT-Biolumicorder data.



A. Example of bioluminescence (blue) and activity (red) dynamics of a single animal, after detrending and smoothening (with a 5 min sliding window). Lights on, subjective days and subjective nights are depicted by yellow, light grey and dark grey shadings, respectively. Food access is indicated at the top of the graph.

B. Corresponding raw data for tracks shown in (A), with photon counts in blue and activity in red. Data are from a C57BL/6 mouse that was injected with an adenoviral reporter, expressing luciferase (with the 5′ UTR of the mouse *Rpl30* gene) from the human *Pgk1* promoter (to be reported elsewhere).

Figure 4. Data analysis for groups of animals and for rhythmicity parameters using the Osiris software.



A. Luminescence and activity dynamics for three groups of animals with different timing of food access, *ad libitum* (blue, N=4), night-fed (red, N=3) and day-fed (black, N=4). The shaded areas represent the standard error of mean (SEM) of the data. The same reporter as in Figure 3 was used.

B. Comparison of the mean cycle across the 24 hour period between three groups of animals with different timing of food access shown in (A). Yellow, light grey and dark grey backgrounds represent the skeleton photoperiod, subjective day, and subjective night, respectively.