Video Article

Monitoring Cell-autonomous Circadian Clock Rhythms of Gene Expression Using Luciferase Bioluminescence Reporters

Chidambaram Ramanathan, Sanjoy K. Khan, Nimish D. Kathale, Haiyan Xu, Andrew C. Liu

Department of Biological Sciences, The University of Memphis

Correspondence to: Andrew C. Liu at acliu@memphis.edu

URL: <http://www.jove.com/video/4234/>

DOI: 10.3791/4234

Keywords: Genetics, Issue 67, Molecular Biology, Cellular Biology, Chemical Biology, Circadian clock, firefly luciferase, real-time bioluminescence technology, cell-autonomous model, lentiviral vector, RNA interference (RNAi), high-throughput screening (HTS)

Date Published: 9/27/2012

Citation: Ramanathan, C., Khan, S.K., Kathale, N.D., Xu, H., Liu, A.C. Monitoring Cell-autonomous Circadian Clock Rhythms of Gene Expression Using Luciferase Bioluminescence Reporters. *J. Vis. Exp.* (67), e4234 10.3791/4234, DOI : 10.3791/4234 (2012).

Abstract

In mammals, many aspects of behavior and physiology such as sleep-wake cycles and liver metabolism are regulated by endogenous circadian clocks (reviewed^{1,2}). The circadian time-keeping system is a hierarchical multi-oscillator network, with the central clock located in the suprachiasmatic nucleus (SCN) synchronizing and coordinating extra-SCN and peripheral clocks elsewhere^{1,2}. Individual cells are the functional units for generation and maintenance of circadian rhythms^{3,4}, and these oscillators of different tissue types in the organism share a remarkably similar biochemical negative feedback mechanism. However, due to interactions at the neuronal network level in the SCN and through rhythmic, systemic cues at the organismal level, circadian rhythms at the organismal level are not necessarily cell-autonomous⁵⁻⁷. Compared to traditional studies of locomotor activity *in vivo* and SCN explants *ex vivo*, cell-based *in vitro* assays allow for discovery of cell-autonomous circadian defects^{5,8}. Strategically, cell-based models are more experimentally tractable for phenotypic characterization and rapid discovery of basic clock mechanisms^{5,8-13}.

Because circadian rhythms are dynamic, longitudinal measurements with high temporal resolution are needed to assess clock function. In recent years, real-time bioluminescence recording using firefly *luciferase* as a reporter has become a common technique for studying circadian rhythms in mammals^{14,15}, as it allows for examination of the persistence and dynamics of molecular rhythms. To monitor cell-autonomous circadian rhythms of gene expression, luciferase reporters can be introduced into cells via transient transfection^{13,16,17} or stable transduction^{5,10,18,19}. Here we describe a stable transduction protocol using lentivirus-mediated gene delivery. The lentiviral vector system is superior to traditional methods such as transient transfection and germline transmission because of its efficiency and versatility: it permits efficient delivery and stable integration into the host genome of both dividing and non-dividing cells²⁰. Once a reporter cell line is established, the dynamics of clock function can be examined through bioluminescence recording. We first describe the generation of P(*Per2*)-dLuc reporter lines, and then present data from this and other circadian reporters. In these assays, 3T3 mouse fibroblasts and U2OS human osteosarcoma cells are used as cellular models. We also discuss various ways of using these clock models in circadian studies. Methods described here can be applied to a great variety of cell types to study the cellular and molecular basis of circadian clocks, and may prove useful in tackling problems in other biological systems.

Video Link

The video component of this article can be found at <http://www.jove.com/video/4234/>

Protocol

1. Construction of Lentiviral Luciferase Reporters

A mammalian circadian reporter construct usually contains an expression cassette in which a circadian promoter is fused with the *luciferase* gene. Both ligation- and recombination-based strategies are commonly used for DNA cloning. As an example, here we describe a recombination-based Gateway cloning method for generating a P(*Per2*)-dLuc lentiviral reporter, in which the destabilized *luciferase* (dLuc) is under control of the mouse *Per2* promoter.

1. Cloning of *Per2* promoter. Use PCR to amplify the *Per2* promoter DNA fragment of 526 bp, upstream of the transcription start site from a mouse *Per2* BAC clone⁹⁻¹³, using a forward primer (5'-CTCGAGCGGATTACCGAGGCTGGTCACG TC-3') and a reverse primer (5'-CTCGAGTCCCTTGCTCGGCCCGTCAC TTGG-3'), and clone into pENTR5'-TOPO vector (Invitrogen) to generate pENTR5'-P(*Per2*).
2. Cloning of dLuc. The dLuc contains the firefly *luciferase* gene and a C-terminal PEST sequence for rapid protein degradation as previously described²¹. Use PCR to amplify the dLuc DNA fragment, and clone into pENTR/D-TOPO vector (Invitrogen) to generate pENTR/D-dLuc.
3. Construction of reporter vector. Mix the two pENTR plasmids, pENTR5'-P(*Per2*) and pENTR/D-dLuc, with the lentiviral destination vector pLV7-Bsd (*Bsd*, blasticidin resistance gene), and perform the recombination reaction using Clonase to generate a pLV7-Bsd-P(*Per2*)-

dLuc reporter (**Figure 1**). pLV7-*Bsd* is a modified version (made in our lab) of pLenti6/R4R2/V5-DEST (Invitrogen) in which the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) sequences²² were inserted immediately downstream of the expression cassette to enhance gene expression.

2. Production of Lentiviral Particles

1. Seed 293T cells (day 1)

1. Grow human embryonic kidney (HEK) 293T cells to 90-100% confluence in regular DMEM supplemented with 10% FBS and 1x Penicillin-Streptomycin-Glutamine (PSG) on 10 cm culture dishes. (Rapidly growing cells with low passage number are critical for efficient transfection.)
2. Prior to seeding the cells for transfection, coat 6-well culture plates by adding 1 ml of 0.001% poly-L-lysine in PBS to each well and incubate at room temperature for 20 min. Aspirate the solution and rinse once with 1x PBS before use.
3. Dissociate 293T cells with trypsin and seed 0.75×10^6 cells onto each well of the pre-coated plates with 2 ml regular DMEM. Swirl the plates thoroughly to obtain an even distribution of cells in each well. Grow the cells in the incubator at 37 °C overnight.

2. Transient transfection via CaPO_4 /DNA precipitation (day 2)

1. Observe the seeded cells from day 1. Cell should reach confluence of 80-90%.
2. Prepare plasmid transfection mix in a 1.5 ml microcentrifuge tube by adding 2 µg of a lentiviral reporter plasmid DNA (e.g., pLV7-P(*Per2*)-*dLuc*; Liu lab) and the 3 packaging vectors (1.3 µg Gag/Pol, 0.5 µg Rev, and 0.7 µg VSVG; Invitrogen). As a control for both transfection and subsequent infection, we usually include an additional well in transfection for a lentiviral GFP expression vector, pLV156-CMV-EGFP (**Figure 1A**), harboring enhanced green fluorescent protein (EGFP) under the control of the CMV promoter as described previously²⁰.
3. Add 100 µl of 0.25 M CaCl_2 (diluted with DNase/RNase-free ddH₂O from 2.5 M stock) to the plasmid mix in step 2 and mix thoroughly. Then add 100 µl of 2x BBS solution (50 mM BES, 280 mM NaCl, 1.5 mM Na_2HPO_4 , pH 6.95) and mix gently but thoroughly. Incubate the DNA mix at room temperature for 15 min.
4. While waiting, aspirate medium from 293T cells and change to 2 ml fresh medium. Return the plate to the incubator for at least 10 min to equilibrate medium pH before transfection.
5. Add transfection mix from step 3 to 293T cells drop by drop. Swirl the plate gently and observe particle formation under a microscope. Incubate at 5% CO_2 , 37 °C overnight. (Fine particle formation of CaPO_4 /DNA precipitate is critical for efficient transfection.)

3. Harvest viral particles (days 3-4)

1. About 16 hours post-transfection (day 3) by which time cells should reach 100% confluence, aspirate medium from cells and replace with 2 ml fresh regular DMEM. Incubate at 37 °C overnight.
2. On day 4, assess transfection efficiency by observing EGFP expression in transfection control cells (Transfection efficiency of 90-100% with high EGFP expression is a reliable predictor of a good viral prep.)
3. Collect the medium containing secreted, infectious viral particles. Centrifuge at $>2,000 \times g$ for 5 min to remove residual 293T cells and collect the virus-containing supernatant. Alternatively, the medium may be cleared with a 0.45 µm membrane filter. The viral particles are ready for use in infection.

3. Infection of 3T3 Cells

1. Seed 3T3 cells (day 3)

Split and seed appropriate number (~12,000) of 3T3 cells on a 12-well plate to obtain 20-30% confluence by next day. Incubate at 37 °C overnight.

2. Infect 3T3 cells (day 4)

1. Observe the seeded cells. Confluence of 20-30% (less than 50%) is desired for infection.
2. Add polybrene to a final concentration of 5 µg/ml to the collected medium containing viral particles. Mix well by pipetting.
3. Aspirate medium from 3T3 cells, and add 1 ml of the above viral mixture per well. Incubate at 37 °C overnight. (Polybrene is used to enhance infection efficiency, but is not absolutely required. As it may be toxic to some cells, prior testing is recommended.)

3. Select infected cells (day 5 and onward)

1. Twenty-four hours post-infection, aspirate medium containing virus and polybrene from infected cells, wash once with 1x PBS, and change to fresh medium. Incubate at 37 °C overnight for recovery and growth.
2. When confluent (usually 1-2 days later), split the cells and incubate at 37 °C overnight.
3. The following day, aspirate medium from cells (<50% confluence is desired) and replace with fresh medium containing 10 µg/ml Blasticidin to select for stably transduced cells. (Blasticidin kill-curve needs to be empirically determined for a particular cell line.)
4. Change to fresh medium containing Blasticidin every 2-3 days for continuous selection of antibiotic-resistant cells expressing clock reporters (generally 4-6 days total).

4. Bioluminescence Recording of Reporter Cells

1. Seed reporter cells

Propagate Blasticidin-resistant reporter cells and split onto 35-mm culture dishes. Incubate at 37 °C until confluent. We usually prepare ≥3 dishes for each reporter cell line under each condition for circadian phenotyping.

2. Synchronization and change to recording medium

1. Aspirate medium from confluent reporter cells, wash once with PBS, and replace with DMEM containing 10 μM forskolin (or 200 nM dexamethasone). Incubate at 37 °C for 1 hour to synchronize the cells. (Alternatively, cells can be synchronized by temperature cycles²³ or serum shock²⁴.)
2. While waiting, prepare recording medium for 3T3 cells as follows: 1x DMEM (HyClone) containing 10% FBS, 1x Pen/Strep/Gln, 1 μM forskolin, 1 mM luciferin, 25 mM HEPES, pH 7.4. Serum and forskolin concentration may be determined empirically. For very dim cells, phenol red-free medium may be used.
3. At the end of forskolin treatment, aspirate medium and replace with freshly made recording medium.

3. Bioluminescence recording of reporter cells

1. Following medium change, cover culture dishes with 40 mm sterile coverslips and seal in place with vacuum grease to prevent evaporation.
2. Load the dishes onto the LumiCycle luminometer, which is kept inside an incubator set at 36 °C without H₂O or CO₂.
3. Start real-time bioluminescence recording. We usually record rhythms for 1 week, followed by medium change and continuous recording for a second week (see Savelyev *et al.* for details)²⁵. (For recording of 96-well plates, Synergy SL2 was used as the recording device; see Discussion 1.1 for detail.)

5. Data Analysis and Presentation

Reporter cells facilitate high-resolution quantitative luminescence recording, critical for determining phenotypic effects on circadian clock function. To obtain circadian parameters including phase, period length, rhythm amplitude, and damping rate, we use the LumiCycle Analysis program (Actimetrics) to analyze bioluminescence data^{5,14}. Briefly, raw data are baseline fitted first, and baseline-subtracted data are fitted to a sine wave, from which the parameters are determined. For samples that show persistent rhythms, goodness-of-fit of >90% is usually achieved. Due to high transient bioluminescence upon medium change, we usually exclude the first cycle of data from analysis.

For data presentation, we usually plot raw data (bioluminescence, counts/sec) against time (days). When necessary, baseline-subtracted data can be plotted to compare amplitude and phase.

6. Representative Results

1. Phase-specific circadian reporters

The circadian clock is based on a biochemical negative feedback mechanism¹. The core feedback loop consists of transcriptional activators BMAL1 and CLOCK, and repressors PERs and CRYs, which act on the circadian E/E'-box enhancer elements to produce rhythmic gene expression (with morning phase, *e.g.*, *Rev-erba*). The core loop regulates and integrates at least two other circadian cis-elements, the DBP/E4BP4 binding element (D-box; for day phase, *e.g.*, *Per3*) and the ROR/REV-ERB binding element (RRE; for night phase, *e.g.*, *Bmal1*)¹⁷. Combinatorial regulation by multiple circadian elements can generate novel intermediate phases. For example, *Cry1* transcription is mediated by all three circadian elements (*i.e.*, E/E'-box and D-box elements in the promoter and RREs in the first intron of the *Cry1* gene), giving rise to the distinct *Cry1* evening-time phase¹³.

Based on these mechanisms of gene regulation, we generated four different reporter constructs: P(*Per2*)-dLuc and P(*Cry1*)-dLuc reporters containing both E/E'-box and D-box elements in the regulatory region^{17,26,27}; P(*Cry1*)-Intron-dLuc representing combinatorial regulation by all three elements (*i.e.*, E/E'-box, D-box, and RRE)^{13,17}; and P(*Bmal1*)-dLuc regulated exclusively by RRE^{9,17,19,21}. We introduced these reporters into 3T3 cells to produce the anticipated distinct phases of reporter expression (Figure 2).

2. Gene knockdown via RNAi and pharmacologically active compounds

When transfection efficiency is high, synthetic siRNA can be transiently transfected into cells to knock down gene expression. When transfection is technically difficult, an shRNA expression vector can be stably transduced into cells via lentiviral infection, so that shRNA produced by the cell is processed to siRNA for gene knockdown (KD). Here we present KD effects of *Cry1* and *Cry2* genes using siRNA in U2OS cells (Figure 3A) and shRNA in 3T3 cells using a pLL3.7 Gateway expression vector⁹ (Figure 3B). In addition to 3T3 cells, the U2OS model has become another preeminent cellular clock model largely because it meets the key requirements for high-throughput screening of commercially available human siRNA libraries (*e.g.*, human origin, capable of generating robust circadian rhythms, validated function of all known clock genes, and amenable to highly efficient transfection and quantitative luminescence recording). In both cell types, RNAi-mediated KD resulted in clock phenotypes consistent with previous mouse knockout (KO) and cellular KD studies^{5,10,11,28}. For example, *Cry1* KD shortens period length and reduces rhythm persistence, whereas *Cry2* KD lengthens period. In addition, selected small molecules can be used to pharmacologically target and perturb protein function (Figure 3C).

Figure 1. The lentivirus-mediated gene delivery system. (A) Schematic diagram of two lentiviral P(*Per2*)-dLuc reporter vectors and a CMV-EGFP construct. Only the region for integration into the host cell genome is shown. In both reporter constructs, the transcription of dLuc is under direct control of the *Per2* promoter. In the pLV7-Bsd-P(*Per2*)-dLuc vector (recombination-based cloning), a coexpressed Blasticidin resistance gene (Bsd) facilitates selection of infected cells. In the pLV156-P(*Per2*)-dLuc vector (ligation-based cloning), EGFP translation is mediated by an internal ribosome entry site (IRES) downstream of dLuc, allowing for visual observation and FACS sorting of infected cells. In addition, an SV40 promoter/terminator (P/T) is used as an insulator (see discussion 1.3). In the CMV-EGFP control vector, EGFP expression is under control of a strong CMV promoter. (B) Fluorescent images of transfected and infected GFP-expressing cells. Typically, we achieve high efficiency both in transient transfection of 293T cells and in lentiviral infection of cell lines of our interest, as indicated by GFP expression in these cells. [Click here to view larger figure.](#)

Figure 2. Phase-specific expression of bioluminescence reporters in 3T3 cells. The lentiviral reporter vectors used in this experiment are pLV7-Bsd-P(*Per2*)-dLuc, P(*Cry1*)-dLuc, P(*Cry1*)-Intron-dLuc, and P(*Bmal1*)-dLuc. Each reporter exhibits a distinct phase of oscillation, as indicated by the arrows. While the *Per2* and *Cry1* promoters drive peak bioluminescence at morning-day phases and the *Bmal1* promoter at night phase, combinatorial regulation by the P(*Cry1*)-Intron harboring E-box, D-box, and RRE elements confers evening phase of peak bioluminescence. [Click here to view larger figure.](#)

Figure 3. Genetic and pharmacological perturbation of circadian bioluminescence rhythms in reporter cells. (A) Effects of *Cry1* and *Cry2* knockdown by siRNAs on cellular rhythms of U2OS reporter cells. The LumiCycle luminometer was used for bioluminescence recording of cells in 35 mm dishes. Figure is adapted from Reference #10, with permission from Elsevier (2009). (B) Effects of *Cry2* knockdown by shRNAs on cellular rhythms of 3T3 reporter cells. A pLL3.7 Gateway vector containing a U6-shRNA cassette was used for *Cry2* gene knockdown. shRNA2 has a better knockdown efficiency than shRNA1 as determined by Western blot analysis (data not shown). A Synergy luminometer was used for bioluminescence recording of cells in a 96-well plate. The settings for recording are as follows: incubator temperature, 33 °C; integration time, 15 sec; interval time, 30 min. (C) Effects of small molecule inhibitors on cellular rhythms of U2OS reporter cells. Chir99021 and Roscovitine are inhibitors directed against GSK-3 and CDK, respectively. The ViewLux system (Chir99021 assay) and a Tecan luminometer (Roscovitine assay) were used for bioluminescence recordings of cells in 384-well plates. Figure is adapted from Reference #19 (Copyright 2008 National Academy of Sciences, U.S.A.). [Click here to view larger figure.](#)

Discussion

1. Modifications to Current Protocol

1.1 Recording devices and throughput considerations

Because of its commercial availability, the LumiCycle (Actimetrics) has become the most commonly used automated luminometer device for real-time recording^{4,5,9,19,29-31}. The LumiCycle employs photomultiplier tubes (PMTs) as light detectors, which provide extremely high sensitivity and low noise¹⁴, and therefore is particularly suitable for data acquisition of extremely dim luciferase-based bioluminescence. Other similar PMT-based devices (e.g., Kronos, Atto Co.; and POLARstar Optima, BMG labtech Inc.) have also been used in many studies^{32,33}.

The LumiCycle assay using 35-mm dishes can be adapted to 96-, 384- or even 1,152-well plate formats for high-throughput screening (HTS) experiments. HTS assay development mainly focuses on the following two aspects: 1) better reporter cells with brighter luciferase and/or higher reporter expression (see below), and 2) highly sensitive recording devices that accommodate multi-well plates. We and others have used several microplate readers such as Synergy SL2 (BioTek), Infinite M200 (Tecan)¹⁹, TopCount (PerkinElmer)²⁸, and EnVision (Perkin Elmer)³⁴. In our lab, both the LumiCycle and Synergy devices are placed in a temperature-controlled and light-tight incubator. Alternatively, if resources permit, the recording units can be placed in a temperature-controlled room. For HTS, critical settings such as integration/exposure time and interval between time points must be determined empirically for a given reporter and recording device.

The LumiCycle assay does not provide spatial information at single cell resolution. Yamaguchi *et al.*³⁵ and Welsh *et al.*^{4,5} pioneered real-time bioluminescence imaging by integrating a specially designed microscope with a highly sensitive, low-noise CCD camera¹⁵ to achieve single cell resolution. In recent years, more sensitive imaging systems have become commercially available, including LV200 (Olympus) and CellGraph (AB3000-B, Atto) with an ultrasensitive cooled and back-illuminated electron multiplying CCD and multicolor filters^{36,37}. Imaging has also been used in more sophisticated HTS experiments, e.g., a ViewLux CCD imager (Perkin-Elmer) combined with the GNF automated robotic system (GNF Systems). This system enabled large-scale screens for novel genes and small molecules that affect clock function^{10,12,19}.

1.2 Luciferase reporters

Luciferases were first used in real-time luminescence recording of circadian gene expression in the early 1990s in plants and cyanobacteria, and have been commonly used in the mammalian system since 2000^{29,35,38-40} (also see reviews^{14,15}). Luciferase reporters are generally less toxic and more sensitive than fluorescent reporters such as GFP, due to much lower background⁴¹. The most commonly used bioluminescent reporter is firefly (*Photinus pyralis*) luciferase (*Luc+*) constructed in the pGL3 vector series (Promega), in which the coding region of the native *Luc* was modified for optimized transcription and translation. The combination of firefly luciferase and its highly stable and cell-permeable substrate, D-luciferin, is ideal for long-term recording. *dLuc* is a modified version of *Luc+* with a PEST sequence fused at its C-terminus to allow for rapid protein degradation. A further improved version, *Luc2*, is available in the pGL4 vector series (Promega) with higher and less anomalous expression. However, we did not observe a significant difference in performance between *Luc+* and *Luc2* in transiently transfected 3T3 and U2OS cells (data not shown). In a recent report, Brazilian click beetle luciferase (*ELuc*) was shown to exhibit a much brighter signal than *Luc+*, suitable for single cell imaging⁴².

Many recent studies have taken advantage of the mPER2::LUC fusion knock-in reporter mouse^{4,5,9,19,25,29-31,43}. This reporter system allows circadian phenotyping of cells and tissue explants including the SCN. In our previous studies, we crossed this reporter mouse line with many of the behaviorally characterized clock gene knockouts and examined the dynamics of bioluminescence rhythms in SCN, liver and lung explants cultured *ex vivo*, and in dissociated SCN neurons and fibroblasts cultured *in vitro*^{4,5,9,31}. These studies allowed us to gain important insights into the molecular details of clock operation at the cell and organismal levels.

1.3 Gene delivery methods

Vector-based clock reporters provide great versatility, as they can be introduced into various cell types via transient transfection^{11,13,16,17} or lentiviral transduction^{5,9,18}. Although cumbersome and less reproducible, transiently transfected cells can also be grown in the continuous presence of antibiotics to generate stable cell lines. Lentiviral vectors are still preferred because of their stable integration into the cell's genome, as well as greater efficiency and versatility. Besides the pLV7 vector presented above in which infected cells can be selected with antibiotics, we have used other vectors. For example, the pLV156-P(*Per2*)-*dLuc* vector contains a P(*Per2*)-*dLuc*-IRES-EGFP expression cassette in which EGFP translation is mediated by an internal ribosome entry site (IRES) downstream of *dLuc*, allowing for visual observation and fluorescence-activated cell sorting (FACS) of cells with integrated vectors⁵ (**Figure 1**) (see below). To shield the reporter from integration site effects, a constitutive promoter and terminator (P/T) can be introduced as an insulator or decoy immediately upstream of the P(*Per2*)-*dLuc* reporter expression cassette (for example, bottom construct in **Figure 1A**), as reported by Brown *et al.*¹⁸ and Liu *et al.*⁵

Previous studies established the groundwork for exploration of tissue-specific genetic perturbation both *in vivo* and/or *ex vivo*⁴⁴⁻⁴⁸. For example, viral particles can be injected into the SCN region followed by SCN slice preparation. As an alternative to this *in vivo* followed by *ex vivo* assay, SCN slices can be dissected first and cultured *ex vivo*, followed by incubation with high-titer virus. However, due to low infection efficiency of relatively thick tissue slices, a highly consistent *ex vivo* protocol remains to be developed.

1.4 Choice of cell lines and recording conditions

Much of what we know about the biochemistry and cell biology of the clock mechanism is based on three cellular models: 3T3 mouse fibroblasts^{16,17}, U2OS human osteosarcoma cells^{10,11,19,28}, and mouse fibroblasts derived from mice^{9,13,34}. The lentiviral vector system permits efficient delivery and stable integration into the host genome of both dividing and non-dividing mammalian cells, and therefore is not limited to certain cell types as in transient transfection. Given the roles of circadian clocks in the regulation of a wide range of cellular and physiological responses, future studies will certainly extend to a wide variety of cell types, either commercially available cell lines or primary cells derived from mice. These cell-autonomous clock studies will help uncover tissue-ubiquitous, as well as tissue-specific, properties of circadian clocks.

It is worth noting that generating reporter cells may require empirical testing of different reporters (e.g., various vector and promoter types), and sometimes further optimization. Not all cell types have cell-autonomous rhythms. To improve cell health and persistence of cellular rhythms, optimization of recording medium is often necessary. For example, as an alternative to the 3T3 recording medium, the recording medium used for U2OS cells is as follows: 1x DMEM (Invitrogen), 2% B-27, 1 μ M forskolin, 1 mM luciferin, 14.5 mM NaHCO₃, 10 mM HEPES (pH 7.2). 1 mM luciferin is usually sufficient, but a final concentration (0.1-1 mM) may be determined for each cell/reporter type. Forskolin may not be necessary and its concentration may be empirically determined for each cell type.

1.5 Transfection efficiency in lentiviral prep

High transfection efficiency of 293T cells is critical for a good viral prep. Major considerations include health of 293T cells and choice of a transfection reagent. 293T cells can be very finicky and require careful handling. Thus, cell growth rate and morphology must be carefully monitored. For consistency, we recommend that serum be tested first and the same tested batch used thereafter for maintaining the cells. We always test new 2x BBS and CaCl₂ stock solutions and optimize working concentration of CaCl₂ around 0.25 M. Cells of high passage number and/or displaying slow growth rate should not be used. 293T cells are readily transfectable with a number of transfection reagents. In addition to CaPO₄, we also achieved excellent transfection efficiency with Fugene 6 (Roche or Promega) or Lipofectamine-2000 (Invitrogen). These lipid-based transfection (lipofection) reagents are more expensive, but give better transfection consistency than CaPO₄. For large-scale lentiviral preps, we recommend using CaPO₄ for transfection because of lower cost.

1.6 Generation of clonal cell lines

Un-concentrated crude viral particles are of relatively low titer (10⁶ viral particles/ml), and reporter expression in transduced cells is low. Although this is sufficient for most applications such as LumiCycle recording, brighter reporters are often needed, e.g. in high-throughput assays using a less sensitive recording device. Reporter expression can be increased by using higher titer viral preps. To do this, we recommend using

larger culture vessels such as 15 cm dishes for transfection, and collecting media twice at day 4 and day 5. For 10⁷ viral particles/ml, perform filtration using centrifugal filter devices (Millipore). For 100x or more concentration ($\geq 10^8$ viral particles/ml), perform ultracentrifugation as previously described²⁰. It is noted that, while viral titer may affect luminescence signal amplitude, it does not alter key circadian parameters such as period length^{5,18}. If a homogenous cell population is desired, clonal cell lines may be obtained by FACS-based single cell sorting in 96-well plates. However, it is imperative that these clonal cells be carefully examined; cell morphology should be indistinguishable from parental cells, and clock properties such as period length and amplitude should be representative of the infected cell population.

2. Application of Cell-autonomous Reporter Clock Models

Unlike tissue or animal models, cell-based models are amenable to genetic and pharmacologic perturbations, and when necessary, also use in HTS formats. Perturbation of gene function can be achieved by over-expression or RNAi-mediated knockdown. Selective small molecules can be used to interfere with protein function. Here we briefly discuss some uses of cell-autonomous clock models in circadian studies.

2.1 Study of core clock mechanisms

Cell-autonomous clock models have greatly facilitated mechanistic studies. Hogenesch and colleagues used the 3T3 model to show that feedback repression by CRYs is required for clock function¹⁶, and the U2OS model to probe the system-level properties of clock function¹¹. We employed the *Cry1*^{-/-}:*Cry2*^{-/-} fibroblast model derived from mice to show that delayed feedback repression is necessary for clock function¹³, and the *Bmal1*^{-/-} fibroblast model to show that *Rev-erba* and β play redundant roles in regulating RRE-mediated rhythmic gene expression, and that *Bmal1* rhythm is not critically required for core clock function⁹. Furthermore, chemical screening in reporter cells allowed identification and/or clarification of the functions of GSK-3 β (period shortening when perturbed)¹⁹, CK1 σ and ϵ (period lengthening when perturbed)⁴⁹, and CK1 α ¹². As discussed below, these models facilitate identification of additional clock components. Strategically, these cellular models are more tractable for rapid discovery of basic mechanisms, which then provide new entry points for *in vivo* validation and exploration.

2.2 Identification of clock factors

In addition to the core feedback loop, the clock mechanism also integrates diverse signaling and regulatory factors. For identification of additional clock components and modifiers, cell-autonomous clock models are advantageous over genetic screening in mice due to the inherent lethality, pleiotropic effects, and developmental genetic compensation associated with mutant animal models⁵⁰. Cell-based screens, in combination with functional genomics approaches, have been effectively carried out using high-throughput recording systems to screen genome-wide siRNA and shRNA libraries for identification of novel clock factors^{10,28}. Similarly, one can employ chemical biological approaches and screen for diverse small molecules to study their effects on clock function^{12,19,34,49}. Although major clock genes and their functions have been identified, these screens have identified additional components or modifiers that are involved in regulation or modulation of the clock. Many of these modifiers represent particular modalities of integrating signal transduction of synchronous or asynchronous cues (clock inputs).

2.3 Study of clock outputs

Although virtually all cells *in vivo* have circadian clocks, cells cultured *in vitro* do not necessarily display overt circadian rhythms. In this context, the reporter approach provides a useful tool to test whether the clock mechanism exists in a particular cell type. Presence of cell-autonomous clock mechanisms warrant studies of clock outputs in these cells, as well as in tissues *in vivo*, including the transcriptome by microarray or RNA sequencing^{51,52}, transcriptional regulatory networks, the proteome, and the metabolome. These studies examine genome-wide RNA and protein expression patterns and thus would complement cell-based luminescence reporter assays that do not necessarily reflect the endogenous expression.

3. Significance of Cell-autonomous Clock Models

Coordination of circadian function within the SCN and across different cell and tissue types is a critical aspect of normal physiology³⁰. The central SCN and peripheral clocks are all essential parts of the overall circadian organization. The SCN receives light input directly from the retina to entrain to the light/dark cycle, and serves as a coordinator to synchronize peripheral clocks through both neuronal and hormonal connections. In addition to internal synchronization, the molecular clockwork within peripheral tissues and cells also orchestrates a myriad of transcriptional output networks, which ultimately govern overt circadian rhythms in physiology and behavior.

Thus, both systemic and local signals regulate circadian physiology, and there is a need to uncover circadian genes directly regulated by cell-autonomous clocks vs. those indirectly regulated by systemic signals emanating from the SCN. The role of peripheral clocks can be studied in cell-autonomous clock models, in which systemic influences are removed. In the case of the liver, tissue-specific regulatory networks generate rhythms in local physiology^{51,53}. By comparing *in vitro* and *in vivo* circadian rhythms, we will be able to discriminate between cell-autonomous and systemic cue-driven clock functions. Indeed, recent studies have begun to reveal a significant role for the liver clock in hepatic gene expression^{6,51,52}. Future research in the field warrants development of various tissue- and cell type-specific clock models representing different physiological outputs.

Previous studies of the biochemistry and cell biology of the clock mechanism have largely relied on a limited number of cell and tissue types, including particularly 3T3, U2OS, mouse fibroblasts, and liver. An implicit assumption in most circadian studies is that the clock works the same way in all cell and tissue types, and gene function determined in one cell or tissue type is generally considered to apply universally in all cells⁷.

However, by establishing and characterizing more cell-autonomous clock models, future studies are expected to uncover tissue specific clock properties underlying local circadian biology.

Disclosures

No conflicts of interest declared.

Acknowledgements

This work was supported in part by the National Science Foundation (IOS-0920417) (ACL).

References

1. Reppert, S.M. & Weaver, D.R. Coordination of circadian timing in mammals. *Nature*. **418**, 935-941 (2002).
2. Hastings, M.H., Reddy, A.B., & Maywood, E.S. A clockwork web: circadian timing in brain and periphery, in health and disease. *Nat. Rev. Neurosci.* **4**, 649-661 (2003).
3. Nagoshi, E., *et al.* Circadian gene expression in individual fibroblasts: cell-autonomous and self-sustained oscillators pass time to daughter cells. *Cell*. **119**, 693-705 (2004).
4. Welsh, D.K., *et al.* Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. *Curr. Biol.* **14**, 2289-2295 (2004).
5. Liu, A.C., *et al.* Intercellular coupling confers robustness against mutations in the SCN circadian clock network. *Cell*. **129**, 605-616 (2007).
6. Kornmann, B., *et al.* System-driven and oscillator-dependent circadian transcription in mice with a conditionally active liver clock. *PLoS Biol.* **5**, e34 (2007).
7. Hogenesch, J.B. & Herzog, E.D. Intracellular and intercellular processes determine robustness of the circadian clock. *FEBS Lett.* **585**, 1427-1434 (2011).
8. DeBruyne, J.P., Weaver, D.R., & Reppert, S.M. Peripheral circadian oscillators require CLOCK. *Curr. Biol.* **17**, R538-539 (2007).
9. Liu, A.C., *et al.* Redundant function of REV-ERB α and β and non-essential role for Bmal1 cycling in transcriptional regulation of intracellular circadian rhythms. *PLoS Genet.* **4**, e1000023 (2008).
10. Zhang, E.E., *et al.* A genome-wide RNAi screen for modifiers of the circadian clock in human cells. *Cell*. **139**, 199-210 (2009).
11. Baggs, J.E., *et al.* Network features of the mammalian circadian clock. *PLoS Biol.* **7**, e52 (2009).
12. Hirota, T., *et al.* High-throughput chemical screen identifies a novel potent modulator of cellular circadian rhythms and reveals CK1 α as a clock regulatory kinase. *PLoS Biol.* **8**, e1000559 (2010).
13. Ukai-Tadenuma, M., *et al.* Delay in feedback repression by cryptochrome 1 is required for circadian clock function. *Cell*. **144**, 268-281 (2011).
14. Yamazaki, S. & Takahashi, J.S. Real-time luminescence reporting of circadian gene expression in mammals. *Methods Enzymol.* **393**, 288-301 (2005).
15. Welsh, D.K., Imaizumi, T., & Kay, S.A. Real-time reporting of circadian-regulated gene expression by luciferase imaging in plants and mammalian cells. *Methods Enzymol.* **393**, 269-288 (2005).
16. Sato, T.K., *et al.* Feedback repression is required for mammalian circadian clock function. *Nat. Genet.* **38**, 312-319 (2006).
17. Ueda, H.R., *et al.* System-level identification of transcriptional circuits underlying mammalian circadian clocks. *Nat. Genet.* **37**, 187-192 (2005).
18. Brown, S.A., *et al.* The period length of fibroblast circadian gene expression varies widely among human individuals. *PLoS Biol.* **3**, e338 (2005).
19. Hirota, T., *et al.* A chemical biology approach reveals period shortening of the mammalian circadian clock by specific inhibition of GSK-3 β . *Proc. Natl. Acad. Sci. U.S.A.* **105**, 20746-20751 (2008).
20. Tiscornia, G., Singer, O., & Verma, I.M. Production and purification of lentiviral vectors. *Nat. Protoc.* **1**, 241-245 (2006).
21. Ueda, H.R., *et al.* A transcription factor response element for gene expression during circadian night. *Nature*. **418**, 534-539 (2002).
22. Zufferey, R., Donello, J.E., Trono, D., & Hope, T.J. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J. Virol.* **73**, 2886-2892 (1999).
23. Buhr, E.D., Yoo, S.H., & Takahashi, J.S. Temperature as a universal resetting cue for mammalian circadian oscillators. *Science*. **330**, 379-385 (2010).
24. Balsalobre, A., Damiola, F., & Schibler, U. A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell*. **93**, 929-937 (1998).
25. Savelyev, S.A., Larsson, K.C., Johansson, A., & Lundkvist, G.B.S. Slice Preparation, Organotypic Tissue Culturing and Luciferase Recording of Clock Gene Activity in the Suprachiasmatic Nucleus. *J. Vis. Exp.* (48), e2439, DOI: 10.3791/2439 (2011).
26. Akashi, M., Ichise, T., Mamme, T., & Takumi, T. Molecular mechanism of cell-autonomous circadian gene expression of Period2, a crucial regulator of the mammalian circadian clock. *Mol. Biol. Cell*. **17**, 555-565 (2006).
27. Ohno, T., Onishi, Y., & Ishida, N. A novel E4BP4 element drives circadian expression of mPeriod2. *Nucleic Acids Res.* **35**, 648-655 (2007).
28. Maier, B., *et al.* A large-scale functional RNAi screen reveals a role for CK2 in the mammalian circadian clock. *Genes Dev.* **23**, 708-718 (2009).
29. Yoo, S.H., *et al.* PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 5339-5346 (2004).
30. Liu, A.C., Lewis, W.G., & Kay, S.A. Mammalian circadian signaling networks and therapeutic targets. *Nat. Chem. Biol.* **3**, 630-639 (2007).
31. Ko, C.H., *et al.* Emergence of noise-induced oscillations in the central circadian pacemaker. *PLoS Biol.* **8**, e1000513 (2010).
32. Izumo, M., Johnson, C.H., & Yamazaki, S. Circadian gene expression in mammalian fibroblasts revealed by real-time luminescence reporting: temperature compensation and damping. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 16089-16094 (2003).
33. Izumo, M., Sato, T.R., Straume, M., & Johnson, C.H. Quantitative analyses of circadian gene expression in mammalian cell cultures. *PLoS Comput. Biol.* **2**, e136 (2006).

34. Chen, Z., *et al.* Identification of diverse modulators of central and peripheral circadian clocks by high-throughput chemical screening. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 101-106 (2011).
35. Yamaguchi, S., *et al.* Synchronization of cellular clocks in the suprachiasmatic nucleus. *Science*. **302**, 1408-1412 (2003).
36. Akashi, M., Hayasaka, N., Yamazaki, S., & Node, K. Mitogen-activated protein kinase is a functional component of the autonomous circadian system in the suprachiasmatic nucleus. *J. Neurosci.* **28**, 4619-4623 (2008).
37. Hoshino, H., Nakajima, Y., & Ohmiya, Y. Luciferase-YFP fusion tag with enhanced emission for single-cell luminescence imaging. *Nat. Methods*. **4**, 637-639 (2007).
38. Asai, M., *et al.* Visualization of mPer1 transcription *in vitro*: NMDA induces a rapid phase shift of mPer1 gene in cultured SCN. *Curr. Biol.* **11**, 1524-1527 (2001).
39. Wilsbacher, L.D., *et al.* Photic and circadian expression of luciferase in mPeriod1-luc transgenic mice *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 489-494 (2002).
40. Yamazaki, S., *et al.* Resetting central and peripheral circadian oscillators in transgenic rats. *Science*. **288**, 682-685 (2000).
41. Welsh, D.K., & Noguchi, T. Cellular bioluminescence imaging. In: *Imaging: A Laboratory Manual*, Yuste, R., Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 369-385 (2011).
42. Nakajima, Y., *et al.* Enhanced beetle luciferase for high-resolution bioluminescence imaging. *PLoS One*. **5**, e10011 (2010).
43. Guilding, C., *et al.* A riot of rhythms: neuronal and glial circadian oscillators in the mediobasal hypothalamus. *Mol. Brain*. **2**, 28 (2009).
44. O'Neill, J.S., *et al.* cAMP-dependent signaling as a core component of the mammalian circadian pacemaker. *Science*. **320**, 949-953 (2008).
45. Fuller, P.M., Lu, J., & Saper, C.B. Differential rescue of light- and food-entrainable circadian rhythms. *Science*. **320**, 1074-1077 (2008).
46. Mukherjee, S., *et al.* Knockdown of Clock in the ventral tegmental area through RNA interference results in a mixed state of mania and depression-like behavior. *Biol. Psychiatry*. **68** (6), 503-511 (2010).
47. Saijo, K., *et al.* A Nurr1/CoREST pathway in microglia and astrocytes protects dopaminergic neurons from inflammation-induced death. *Cell*. **137**, 47-59 (2009).
48. Elias, G.M., *et al.* Synapse-specific and developmentally regulated targeting of AMPA receptors by a family of MAGUK scaffolding proteins. *Neuron*. **52**, 307-320 (2006).
49. Isojima, Y., *et al.* CKepsilon/delta-dependent phosphorylation is a temperature-insensitive, period-determining process in the mammalian circadian clock. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 15744-15749 (2009).
50. Bucan, M. & Abel, T. The mouse: genetics meets behaviour. *Nat. Rev. Genet.* **3**, 114-123 (2002).
51. Hughes, M.E., *et al.* Harmonics of circadian gene transcription in mammals. *PLoS Genet* **5**, e1000442 (2009).
52. Atwood, A., *et al.* Cell-autonomous circadian clock of hepatocytes drives rhythms in transcription and polyamine synthesis. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 18560-18565 (2011).
53. Panda, S., *et al.* Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell*. **109**, 307-320 (2002).