In vivo bioluminescence analyses of circadian rhythms in *Arabidopsis thaliana* using a microplate luminometer

Masaaki Okada[1] and Paloma Mas[1,2,3]

[1]Centre for Research in Agricultural Genomics (CRAG), CSIC-IRTA-UAB-UB, Campus UAB, Bellaterra, 08193 Barcelona, Spain.

[2]Consejo Superior de Investigaciones Científicas (CSIC), 08028 Barcelona, Spain.

[3]Correspondence to: paloma.mas@cragenomica.es

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Monitoring bioluminescence rhythms in Arabidopsis

Abstract

Our understanding of the circadian clock function in plants has been markedly assisted by studies with the model species *Arabidopsis thaliana*. Molecular and genetics approaches have delivered a comprehensive view of the transcriptional regulatory

networks underlying the *Arabidopsis* circadian system. The use of the luciferase as a reporter allowed the precise *in vivo* determination of circadian periods, phases and amplitudes of clock promoter activities with unprecedented temporal resolution. An increasing repertoire of fine-tuned luciferases together with additional applications such as translational fusions or bioluminescence molecular complementation assays have considerably expanded our view of circadian protein expression and activity, far beyond transcriptional regulation. Further applications have focused on the *in vivo* simultaneous examination of rhythms in different parts of the plant. The use of intact versus excised plant organs has also provided a glimpse on both the organ-specific and autonomy of the clocks and the importance of long distance communication for circadian function. This chapter provides a basic protocol for *in vivo* high-throughput monitoring of circadian rhythms in *Arabidopsis* seedlings using bioluminescent reporters and a microplate luminometer.

Key Words: Circadian rhythms, luciferase, promoter activity, gene expression, *Arabidopsis thaliana*, bioluminescence, reporter assays, microplate luminometer.

1. Introduction

Circadian rhythms are orchestrated by a timing mechanism or circadian clock that is present in nearly all organisms examined to date. The circadian function ensures that the organism's biology is in sync with the external environmental conditions *(1)*. Due to their sessile nature, the perception and adjustment to these environmental conditions is particularly important for plants *(2)*. Over the years, the components and mechanisms of the plant circadian clock have been well studied in the model system *Arabidopsis thaliana* *(3)*. Research has been lately considerably expanded to crops, opening interesting opportunities for improving plant productivity and resilience to environmental insults *(4)*.

As in some other organisms, circadian rhythms in *Arabidopsis* rely on the rhythmic expression of clock genes, which form transcription-translation reciprocal regulations at the core of the central oscillator *(3)*. A number of core clock components, whose expression oscillates in a circadian manner and with specific phases during the day-night cycle has been characterized *(5-7)*. Analyses of circadian rhythms of core gene expression have uncovered fundamental properties of the *Arabidopsis* circadian system including its

robustness and flexibility under different environmental conditions *(8-10)*. One crucial step for our understanding of the circadian system in plants was the fine time-resolved elucidation of the circadian waveforms of the main oscillating genes. Gene expression analyses by time courses of RNA abundance requires sampling of plants and the subsequent RNA extraction at the different time points. Although this procedure is amenable and produces reliable results, it is destructive and the time-series data are necessarily obtained from different samples.

In vivo long-term analyses using reporter genes provide an advantage for circadian studies. The approach is based on the use of a reporter gene fused to a promoter, gene or protein of interest that is exogenously introduced into cells, and literally "reports" its expression through the signal readout without the need of sample extraction. In plant cells, chlorophyll and carotenoids in plastids, and lignin and other phenolic compounds in cell walls cause auto-fluorescence, which often interferes and complicates the detection of the fluorescent reporters *(11)*. In addition, fluorescent reporters that require excitation by light impose restrictions on the experimental conditions and might affect the circadian outputs

as circadian systems are sensitive to light. *In vivo* bioluminescence-based assays, on the other hand, rely on the chemical reaction between the enzyme luciferase and a luminogenic substrate. Thus, monitoring bioluminescence does not require excitation light and provides a sensitive and reliable way for long-term monitoring of circadian rhythms.

Luciferase genes cloned from bacteria, beetles, deep sea shrimp and Renilla are generally used as bioluminescent reporters. Each one of these luciferases has specific properties related to the size of the enzyme, the luminogenic substrate, wavelength and intensity of luminescence, half-life, etc. High-resolution bioluminescence assays have been achieved by both optimizing reporter genes and by improving the sensitivity of the detectors for luminescence. Native luciferases have been optimized for bioluminescent assays by genetic modifications. Adjusting the codon usage increased luciferase expression up to several hundred-fold in some instances *(12)*. Monitoring luminescence using photo-multiplier tubes (PMTs) or the highly sensitive electron-multiplying charge-coupled-device (EM-CCD) camera enables to minimize the duration of the photon count or

exposure time, thus reducing the time in which samples are under darkness. The microplate luminometers use PMTs and can be easily controlled by appropriate software that precisely program the measurements. In this way, microplate luminometers can automatically and simultaneously record bioluminescence rhythms within appropriate time intervals. This high-throughput monitoring system for bioluminescence is suitable for screening clock-related mutants or chemical compounds affecting circadian rhythms in *Arabidopsis (13)*.

In this chapter, we describe how to monitor *in vivo* circadian rhythms in *Arabidopsis* seedlings using bioluminescent reporters and a microplate luminometer. The protocol has been successfully used to follow circadian rhythms under a variety of light and temperature conditions, and will likely be useful for researchers not only with an interest in chronobiology but in many other areas of plant biology.

2. Materials

2.1 Equipment

1. Orbital incubator

2. Electroporation cuvette (Bio-Rad)

3. Bio-Rad Gene Pulser (Bio-Rad)

4. 50 mL sterile Falcon tube (FALCON)

5. Disposable plastic bag

6. 1.5 mL microcentrifuge tubes (Eppendorf)

7. Tube rotator

8. Sterile filter paper

9. Surgical paper tape (3M)

10. Aluminum foil

11. Laminar flow cabinet

12. 96-well Microplate: white, sterile with lid (Berthold Technologies)

13. Cover film (Ratiolab)

14. Tweezers

15. Hypodermic needle (BD)

16. Luminometer LB-960 (Berthold Technologies)

17. Software Microwin (Mikrotek Laborsysteme)

18. BioDare2 software

19. Environmentally-controlled chamber (Inkoa Sistemas)

2.2 Reagents

1. YEB media: beef extract 5 g/L, yeast extract 1 g/L, peptone 5 g/L, sucrose 5 g/L, $MgCl_2$ 0.5 g/L

2. 1 mM HEPES, pH 7.0

3. 10% glycerol

4. Luria-Bertani (LB) plate: yeast extract 5 g/L, bacto-tryptone 10 g/L, NaCl 10 g/L, agar 15 g/L

5. Floral dip solution: 5% sucrose solution containing 0.03% of Silwet L-77 (Lehle Seeds).

6. Ethanol

7. Sterilization solution: 70% ethanol (v/v), 0.1% Triton X-

100 (v/v)

8. Murashige and Skoog (MS) medium

9. Luciferin solution: 1.4 mM D-Luciferin acid form

(Biothema) in 2.6 mM 2-(N-morpholino)ethanesulfonic acid (MES)

pH 5.8. Filter the solution under sterile conditions.

2.3 Bacteria   *Agrobacterium tumefaciens* GV2260

2.4 Plants   Transgenic *Arabidopsis thaliana* lines expressing a

bioluminescent reporter *(14)*.

3. Methods

3.1 Luciferase vectors and bioluminescent reporter constructs

1. Choose the appropriate luciferase vector for your studies and generate transcriptional

or translational fusions of the promoters, genes or proteins of interest fused to the chosen

luciferase.

2. Use standard molecular cloning techniques for the generation of the constructs *(15)*.

A number of bioluminescent reporters with different luciferases, such as the Firefly luciferase, the Renilla luciferase, or the NanoLUC luciferase, are currently available (Promega). Each one of these luciferases has specific properties related to the size of the enzyme, the luminogenic substrate, wavelength and intensity of luminescence, half-life, etc. (please see Table 1). Choosing the appropriate luciferase depends on the particular analysis to be performed. In general, instability (shorter half-life) of the luciferase activity is preferred for monitoring promoter activities fused to the reporter, as the luminescence reflects the actual dynamics of the promoter (not interfered by a stable reporter). Recent studies have reported that NanoLUC has longer half-life compared to firefly luciferase *(16)*. The NanoLUC can be a useful tool for tracking protein accumulation and dynamics in plants (see Note 1, 2).

The *luc+* gene has been modified to improve its function as a genetic reporter. These modifications include the removal of the peroxisomal translocation sequence, resulting in the transport of luciferase to the cytoplasm and the removal of glycosylation sites. Together these changes produce a several fold increase in the light signal *(17)*. The cryptic regulatory sequences in the *luc* gene that may adversely affect transcription, resulting in

anomalous expression of the reporter, were removed without changing the amino acid

sequence to create the *luc2* gene. The generated *luc2* gene displays higher expression than

the *luc+* gene. In addition, sequences resembling splice sites, poly(A) addition sequences,

Kozak sequences (translation start sites for mammalian cells), *E. coli* promoters or *E. coli*

ribosome-binding sites were removed wherever possible. This process has led to a

reduced number of cryptic regulatory sequences and therefore a reduced risk of

anomalous transcription. A similar process was performed using *Renilla* luciferase to

produce the *hRluc* gene. (Promega website: "Reporter Genes and their Applications").

3.2 Transformation of *Arabidopsis thaliana*

Bioluminescent reporter constructs can be used to transform *Arabidopsis* plants to

generate stable transgenic lines following the methods described in this section. Please

see Note 3 for alternative analyses with transient expression.

3.2.1 Transformation of Agrobacterium

This section is adapted from **ref.** 18.

1. Inoculate 10 mL of YEB media with the *Agrobacterium* strain GV2260 (rifampicin, kanamycin resistant). Incubate overnight at 28 ºC in an orbital incubator.

2. Use the overnight culture to inoculate 300 mL of YEB and incubate in an orbital incubator at 28 ºC until an optical density at 600 nm (OD_{600}) of 0.5 is reached. Chill bacteria on ice and harvest by centrifugation. Wash bacteria with 10 mL of 1 mM HEPES, pH 7.0, three times and once with 10% glycerol, and finally suspend them in 3 mL 10% glycerol. Snap-freeze in 200 μL aliquots (competent *Agrobacterium*) and store at -80 ºC.

3. Transform competent *Agrobacterium* by using the electroporation method with the constructs generated in *3.1*. Thaw competent cells on ice and add approximately 200 ng of vector. Put them in a 0.2-cm electroporation cuvette and place it in the Bio-Rad Gene Pulser. Apply a single electric pulse of 2.5 kV initial voltage using the 25 μF capacitor. After the pulse add 500 μL YEB media into the cuvette and pipet gently. Transfer to a microcentrifuge tube and incubate for 1 h at 28 ºC.

4. Spread aliquots on selective LB plates and incubate for 48 h at 28 ºC.

3.2.2 Agrobacterium-mediated transformation of Arabidopsis thaliana by the floral dip

method

This section is adapted from **ref.** 19.

1. Prepare Arabidopsis plants, which are flowering. Optimal plants have many immature flower clusters and only few fertilized siliques. As an option, first bolts can be clipped to encourage proliferation of many secondary bolts.

2. Select an *Agrobacterium* colony from selective LB plates generated in *3.2.1*, and resuspend bacteria in 10 μL H_2O. Spread half of the volume immediately on a selective YEB plate, and incubate at 28 ºC for 2 – 3 days. Use the other half to verify the presence of transformed DNA construct by PCR analysis.

3. Collect the densely grown bacteria from the plate by scraping, and resuspend them in 30 mL YEB in a sterile Falcon tube. OD_{600} should be about 2.0.

4. Prepare 120 mL of floral dip solution per transformation, and pour solution into a disposable plastic bag and add the bacteria.

5. Dip the inflorescence of the plants into the *Agrobacterium* solution for 10 seconds under gentle agitation. A film of liquid coating the plants should be observed. The bacteria are distributed to all plant parts by gently pressing the outside of the bag.

6. To maintain high humidity, cover dipped plants for 16 to 24 hours. Avoid exposure to excessive sunlight, otherwise the temperature inside can get high.

7. Water and grow the plants as normal, tying up loose bolts. Stop watering as seeds become mature.

8. Harvest dry seeds.

9. Select for transformants using appropriate antibiotics or herbicides. Transformation of luciferase constructs can be confirmed by measurements of bioluminescence. Homozygous lines containing a single copy of the reporter gene should be selected.

3.2 Seed sterilization, seedling growth and entrainment

1. For sterilization, seeds are placed in 1.5 mL microcentrifuge tubes and surface sterilized by soaking them in sterilization solution for 10 min on a rotator, followed by 2 washes with 70% ethanol and 1 wash with 100% ethanol (Figure 1) (Note 4).

2. Seeds are then dried on a sterile filter paper in a laminar flow cabinet for 1 h and sown on plates containing MS medium (3% sucrose, 0.8% Agar, see Note 5).

3. Plates are sealed with surgical paper tapes and covered with aluminum foil.

4. After 2-3 days of stratification at 4ºC in the dark, plates are then transferred to an environmentally-controlled plant growth chamber.

5. Seedlings are grown for about 7-12 days in the chamber at the appropriate entraining (synchronizing) cycles, such as light-dark cycles and/or high-low temperature cycles, before time-course bioluminescence assay.

3.3 Preparation of 96-well plates

All procedures should be done in a laminar flow cabinet.

1. Use 160 µL of agar (or liquid) MS medium and add 40 µL of a luciferin solution into each well of the 96-well plate.

2. The 7-12 day-old seedlings expressing the luciferase and grown on the MS agar medium as specified above should be then removed from the plates one by one using sterile tweezers and placed into the wells of the 96-well plates (one seedling per well). Each seedling should be picked carefully to avoid damaging the shoots, hypocotyls or breaking the roots. The seedling should be placed in the centre of the well. Bioluminescence rhythms of different organs can be also examined *(10, 20, 21)*.

Luminescence from distinct parts of seedlings (e.g. shoots and roots) can be also measured simultaneously *(10, 21)*. For this method, one side of the walls of the 96-wells should be slightly serrated to allow communication between two adjacent wells. Seedlings are then horizontally positioned such that the shoot can be placed in one well and the roots in the contiguous well *(10, 21)*.

3. Cover the plate tightly with a cover film. Make 4-6 small holes on the seal at each well with a sterile hypodermic needle.

3.4 Setting the luminometer and chamber conditions

Install a microplate luminometer (e.g. LB-960, Berthold Technologies) into a growth chamber, in which conditions of illumination and temperature can be programmed (see Note 6). Set the chamber conditions under which the measurement should be done, i.e. continuous light, light/dark cycles, high/low temperature cycles, etc.

3.5 Setting measurement conditions

Set the measurement conditions using the software Microwin (Mikrotek Laborsysteme).

To that end, click on settings and:

1. Select the wells of the plate to be measured (normally the 96 wells).

2. Click on "measurements" and select 5 seconds per well.

3. Select the option "unload" to let the plate out of the luminometer after every time point. Introduce the seconds (time interval) between two measurements in "use delay time".

4. Set the number of repeats, e.g. 168 repeats for the 1-week measurements with 1-hr intervals or 84 repeats for 1-week measurements every two hours.

5. Introduce the name of your experiment including the relevant information to be easily identified. Start measurements.

3.6 Data Analyses

The circadian analysis of luminescence data can be achieved by using the BioDare2 software *(22)*. BioDare2 is a repository for circadian, biological data, providing a platform for data sharing and rhythmic analysis. You can estimate periodic functions, such as period lengths, phases and amplitudes, of your luminescence data by uploading it to

the BioDare2 website.

3.6.1 Upload data sets

1. After measurements, export bioluminescence data as an excel file. The excel file should be with one of the columns holding the measurement time and the other columns recording measured values.

2. To analyze data with BioDare2, go to the New Experiment page on the BioDare2 website and create an experiment with descriptions. (registration is required the first time).

3. To upload the data file, choose Excel Table as File Format, and select data layout, such as data in rows or columns, depending on your file layout.

4. After selecting Time unit, Time offset, and information about rows and columns of the data file, timeseries can be imported.

3.6.2 Analysis of data

After importing timeseries, individual bioluminescence rhythms are shown, and those are ready to be analyzed. Choose periodic functions, which you need to analyze, and set conditions, such as Data window, Analysis method, etc. Results of analyses can be shown

on the webpage, and also can be downloaded to your local disk.

3.6.3 Further information about using BioDare2

To ensure that the data are in the correct format for BioDare2, you should follow the

instruction described in the Documentation page on the BioDare2 website. Furthermore,

descriptions about analyses performed in BioDare2 are available in the page.

4. Notes

1.   Several possible luciferases isolated from different species are available for

studies in plants *(23)*. These luciferases emit bioluminescence with different

wavelengths (colors), which can be separated with appropriate interference

filters. Thus, using different bioluminescent reporters, multiple gene expression

levels are simultaneously monitored.

2.   Clock-gene promoter activities in specific tissues can be monitored by the

tissue-specific luciferase assay (TSLA) developed by Endo et al. (2014) *(24)*.

In TSLA, the carboxy- and amino-terminal fragments of firefly luciferase are

driven by tissue-specific and clock promoters, respectively. Fragmented N- and

C-terminal luciferase can be also used for bioluminescence complementation

assays in which physical interactions between genes of interest in the circadian

system can be assayed *(25)*.

3. Transient expression of the bioluminescent reporter can be also assayed by

    particle bombardment with detached leaves or polyethylene glycol (PEG)-

    mediated transfection of protoplasts *(26, 27)*. The transient methods allow to

    analyze circadian rhythms with available *Arabidopsis* mutant/over-expression

    lines without generating stable reporter lines. Also, these methods are useful for

    plants/crops, in which the generation of stable transgenic lines is very difficult

    or not possible *(28, 29)*. By using the particle bombardment method, circadian

    rhythms in individual cells on leaves can be analyzed, and rhythms can be

    genetically modified with effector constructs, such as RNAi, CRISPR/Cas9,

    over-expression. By PEG-mediated transfection with protoplasts, cell

    autonomy on circadian rhythms can be assessed by transient expression of

amiRNAs targeting clock genes.

4. Bleach solution can be used for sterilizing the seeds. To dilute bleach, add 100 mL of bleach to 100 mL of distilled water. Add 50 μL of Tween 20 detergent to the bleach solution. In a laminar flow hood, add 500 μL of the bleach solution to the microcentrifuge tube containing seeds. Shake the tube for 10 min with a platform shaker to keep the seeds suspended. Remove the bleach solution from the microcentrifuge tube using a pipette or an aspirator fitted with a pipette tip on the end. Add 500 μL of sterile distilled water to the tube. Close the tube and invert to mix. Allow seeds to settle to the bottom of the tube. Once seeds have settled to the bottom of the tube carefully remove the bleach solution by pipetting. Repeat this rinsing process 6 times. Add 1 mL of autoclaved distilled water to the tube to suspend the seeds *(30)*.

5. The properties of circadian rhythms, such as robustness and/or period length, are affected by the presence of exogenous sucrose *(8)*.

6.   To observe spatiotemporal patterns of clock-genes expression, the microscope

or the highly sensitive EM-CCD camera can be also used *(31, 32)*. Controlling

devices by an imaging software, such as HOKAWO (Hamamatsu photonics),

enables us to capture a series of bioluminescence images with appropriate

intervals automatically *(33)*.

Relevant web links:

Promega website: "Reporter Vectors"

https://www.promega.es/en/resources/vector-sequences/reporter-vectors/

Promega website: "Reporter Genes and their Applications"

https://www.promega.es/en/resources/guides/cell-biology/bioluminescent-reporters/

BioDare2 website

https://biodare2.ed.ac.uk

BioDare2 website: "TimeSeries data and formats"

https://biodare2.ed.ac.uk/documents/timeseries-data

Acknowledgements

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Figure Caption

Figure 1. Schematic drawing depicting the main steps for the *in vivo* bioluminescence analyses of circadian rhythms in *Arabidopsis thaliana* seedlings using a microplate luminometer.

Table Caption

Table 1. List of common luciferases used for circadian studies in plants *(34)*

Table

Table 1.

Luciferase	Substrate	Size (kDa)	Wavelength (nm)	Vector series*
Firefly	D-luciferin	61	560	pGL3 (*luc+*) pGL4 (*luc2*)
NanoLUC	Furimazine	19	460	pNL
Renilla	Coelenterazine	36	480	pGL4 (*hRluc*)

*Available from Promega.