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High throughput and quantitative approaches for measuring circadian rhythms in cyanobacteria using bioluminescence

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Abstract

The temporal measurement of a bioluminescent reporter has proven to be one of the most powerful tools for characterizing circadian rhythms in the cyanobacterium *Synechococcus elongatus*. Primarily, two approaches have been used to automate this process: (1) detection of cell culture bioluminescence in 96-well plates by a photomultiplier tube-based plate-cycling luminometer (TopCount Microplate Scintillation and Luminescence Counter, Perkin Elmer) and (2) detection of individual colony bioluminescence by iteratively rotating a Petri dish under a cooled CCD camera using a computer-controlled turntable. Each approach has distinct advantages. The TopCount provides a more quantitative measurement of bioluminescence, enabling the direct comparison of clock output levels among strains. The computer-controlled turntable approach has a shorter set-up time and greater throughput, making it a more powerful phenotypic screening tool. While the latter approach is extremely useful, only a few labs have been able to build such an apparatus because of technical hurdles involved in coordinating and controlling both the camera and the turntable, and in processing the resulting images. This protocol provides instructions on how to construct, use, and process data from a computer-controlled turntable to measure the temporal changes in bioluminescence of individual cyanobacterial colonies. Furthermore, we describe how to prepare samples for use with the TopCount to minimize experimental noise, and generate meaningful quantitative measurements of clock output levels for advanced analysis.

Keywords

luciferase; *Synechococcus elongatus*; Kai proteins; single colony bioluminescence; temporal automated measurement

Theory

In vivo bioluminescence measurements have been invaluable in the determination of circadian oscillations in many organisms, and especially in the cyanobacterium *Synechococcus elongatus*, for which no other visible circadian phenotype is evident (Mackey, Golden & Ditty, 2011). One of the most successful approaches to identify the

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genetic components that determine a complex phenotype in any organism, has been to systematically mutate the genome by targeted or random mutations, and screen for phenotypic variants (Brenner, 1974; Mayer, Ruiz, Berleth, Miseéra & Juürgens, 1991; Nolan, Kapfhamer & Bucan, 1997). This method initially was used to identify many of the core genes involved in the *S. elongatus* clock (Kondo, Tsinoremas, Golden, Johnson, Kutsuna & Ishiura, 1994), and currently is being used to elucidate subtler features of the circadian gene network. Unfortunately, the only commercially available machine to monitor the temporal expression of bioluminescence in cyanobacteria is limited in the scale of mutants that it can assay, requires clones to be inoculated individually, and has a long set-up time (Mackey, Ditty, Clerico & Golden, 2007). The lab of T. Kondo (Nagoya University) has shown that bioluminescence from individual *S. elongatus* colonies on Petri dishes can be reliably measured over time (Kondo & Ishiura, 1994). Kondo and colleagues built a computer-controlled turntable that iteratively rotates Petri dishes under a CCD camera for imaging, and significantly increased the throughput of cyanobacterial mutant screening (Kondo et al., 1994). Construction of a similar apparatus by other labs has been limited because of technical hurdles involved in coordinating and controlling both the camera and the turntable, and in processing the resulting images. Applications developed for the software packages Matlab and R have simplified these challenges. Here, we describe how to construct and operate a computer-controlled turntable and how to analyze a temporal image series of bioluminescent cyanobacterial colonies. This system is reliable and can accurately characterize the periods of approximately 300 colonies per plate, or about 2700 colonies per run of 5 days, enabling the high-throughput screening for mutant colonies that have altered circadian phenotypes. This protocol can easily be adapted to other applications that require temporal image acquisition and processing.

While quantifiable differences in period, phase and amplitude can be measured from individual colonies using the turntable described above, additional information is contained in the magnitude of bioluminescence, which can only be extracted from larger cell cultures that are carefully prepared. For example, the magnitude of bioluminescence can provide information directly related to whether the oscillator output is activating or repressing transcription (Paddock, Boyd, Adin & Golden, 2013). Here, we also report on methods and internal tests to establish quantitatively informative bioluminescence measurements. Although this quantification can be done using either the computer-controlled turntable or a TopCount, we have found that using a TopCount is easier to control for culture density. Therefore, the protocol presented here is for use with a TopCount. General considerations for the measurement of bioluminescence have been described previously in detail (Mackey et al., 2007). Our focus will be on strain treatment and criteria for validation of quantitative bioluminescent measurements in cyanobacterial systems.

1 Build a computer-controlled turntable

The primary elements are: a sensitive cooled CCD camera that can detect bioluminescence from colonies, a precise stepper motor that can be externally controlled, a secure base to minimize movements during operation, a light source that uniformly illuminates the turntable's surface, and efficient light shielding of the Petri dish during image acquisition.

Here we present our design (Fig. 1), but as long as the above elements are satisfied, a machine that differs in some aspects should function correctly.

1.1 Materials

1. Sherline P/N 8700 CNC Rotary Table (<http://www.sherline.com/8700.htm>)
2. Computer with parallel port that can run 32 bit Matlab
3. Large camera/ copy stand
4. Pixis 1024B CCD Camera (Princeton Instruments)
5. 25mm F0.95 Lens (Navitar)
6. 1-1/31" thick by 23-3/4" diameter edge-glued pine round (Home Depot)
7. 5" Black PVC Female Adapter (screw type)
8. 5" Black PVC Male Adapter (screw type)
9. Aluminum Base Plate (Fig. 2C)
10. Aluminum Light Shield (Fig. 2B)
11. Black butcher paper
12. Black paint
13. Two 4" diameter Aluminum Disc Spacers (Fig. 2C)
14. Black electrical tape
15. Adhesive-backed black felt at least 8.5" by 12" by 1/4"
16. 4" to 5" rubber drain coupling
17. 8-pin Mini-Din Male MAC to DB25 Male Hayes-Compatible Model Cable (Cables to Go Part 02966)
18. Fluorescent light and stand at least 24" wide

1.2 Programs

1. Matlab (Mathworks)
2. Data Acquisition Toolbox for Matlab
3. PVCAM (Photometrics)
4. Micro Manager (Edelstein, Amodaj, Hoover, Vale & Stuurman, 2010)

1.3 Protocol

1. Download scripts and installation files—We have written several scripts to control the rotary motor and cooled CCD camera with Matlab and to analyze the resulting images in R. These files along with additional useful software installation instructions can be downloaded from "http://golden.ucsd.edu/turn_table.html" and will be referred to throughout this protocol.

2. Cooled CCD Camera—We use a Princeton Instruments Pixis 1024B CCD camera with a 25mm F0.95 lens (Navitar Part DO-2595). This lens allows an entire Petri dish to be imaged sharply at 6 inches. A short focal length is preferable as it decreases the length of the Light Shielding Assembly required to eliminate external light during bioluminescence detection. The CCD camera is both cooled and back-illuminated, features that reduce noise during the long exposure times necessary to detect bioluminescence. The camera should be attached to an optical post or a large copy stand that positions it at least 2 feet above the base. The base of the stand should be sufficiently large that the turntable assembly can be attached to it while the edge of the turntable is directly under the camera (Fig. 1B).

3. Controlling the camera—Two additional programs are required to control the Pixis 1024B on a Windows machine with Matlab: PVCAM and Micro Manager. PVCAM is required to install camera drivers, and Micro Manager can set camera properties and acquire images (Edelstein et al., 2010). We provide specific instructions on how to install and use these programs in the file Camera_setup.txt, which is included in the files downloaded in the section **Download scripts and installation files**. We recommend getting your camera working prior to final assembly of the turntable. This preparation will allow you to adjust camera focus during construction. Simple snapshots can be taken through the Micro Manager GUI interface.

4. Turntable surface—We could not find a prefabricated Table Top that could hold Petri dishes, so we had to have one machined. Twelve Petri dish sized holders were cut into a 1-1/31" thick by 23-3/4" diameter edge-glued pine round (Home Depot) as shown in Fig. 2A. Dimensions in this figure are given in cm rather than inches to be consistent with the units of a standard Petri dish. There are several considerations to keep in mind when making this part. (1) The holders should be tight around the plates to reduce translational movements during table rotation, which affect image analysis. (2) The centers the holders need to be evenly spaced around the circumference of the platform. (3) There needs to be adequate space between plates, to ensure that only 1 plate is visualized at a time. (4) Holders should not be close enough to the edge to prevent the incursion of external light during imaging. (5) Bolt holes need to be cut into the Table Top to connect the Table Top to the Rotary Table described below. After fabrication, cover the Table Top with black butcher paper to reduce light noise during imaging and to reduce friction between the Table Top and the Light Shielding Assembly. To further reduce light noise, you can paint the plate holders black.

5. Rotary Table—We use the Sherline P/N 8700 CNC Rotary Table and Motion Controller (Fig. 2C). This is a stepper-motor-based rotary table that can be externally controlled by Matlab as described below.

6. Attaching the Table Top to Rotary Table and Copy Stand—To attach the Rotary Table to the wooden Table Top, we had a 4" diameter Aluminum Disc Spacer fabricated that had 8 bolt holes in it: four of which were unthreaded and used to attach the Aluminum Disc Spacer to the Rotary Table and four threaded holes used to attach the Table Top to the Aluminum Disc Spacer. The stepper motor on the Rotary Table drops below the base of the assembly, preventing the Rotary Table from sitting flat on a uniform surface. We attached a

second 4" diameter Aluminum Disc Spacer to the base of the Rotary Table to raise the stepper motor. To this assembly we also attached a fabricated 5" x 7" Aluminum Base Plate with 4 oblong screw holes cut into each corner, increasing the overall stability of the turntable (Fig. 2C).

7. Light Shielding Assembly—One of the most difficult aspects of building this turntable is properly shielding the Petri dish from ambient light during imaging. Our approach was to attach a 5" black PVC pipe to the camera. We used a threaded connector pipe, so that the height of the shielding could be adjusted. The PVC pipe was attached to the camera with a 4" to 5" Rubber Drain Coupling. The seal between the PVC pipe and the table did not provide sufficient light shielding for imaging. To address this shortcoming, we fabricated an 8.5" by 12" by 1/4" Aluminum Light Shield with a Petri dish sized hole cut into the middle of it (thanks to Carl H. Johnson at Vanderbilt University, for this suggestion). To the base of this Aluminum Light Shield, we attached a 1/4" thick piece of felt, which both prevented light from entering the imaging chamber, and allowed the table to slide smoothly under the plate. The top of the Aluminum Light Shield had a 3/16" deep circular groove that the PVC pipe could fit in (Fig. 2B,D). This part covers three Petri dish holders on the Table Top, and therefore reduces the number of plates that can be assayed to 9. The large footprint of the Aluminum Light Shield was necessary for us to get sufficient light shielding during imaging. The entire Light Shielding Assembly was wrapped in a heavy black curtain to further reduce light noise. If any aspects of the Light Shielding Assembly moves during table rotation, it can be stabilized using a ring stand.

8. Lighting System—We use a Jump Start 2 Foot Fluorescent Grow Light System (Hydrofarm) to illuminate the Table Top (Fig. 1), but any lighting system that provides strong uniform illumination across the table surface is acceptable. A ring-shaped light may be superior, but we have not tested one.

9. Programming the Rotary Table—To program the Motion Controller: (1) Plug it into the Rotary Table and turn it on. (2) Push the Mode button until the display says Division Mode and press Enter. (3) Enter the number of divisions that you want; it will be 12 if you use the Table Top described above. (4) Push the Next button and the table will rotate 30° clockwise. Each time you hit Next the table will rotate another 30°. Instead of hitting Next, you can also rotate the table by sending an electric TTL pulse into the Interface port that is located on the back of the controller. This pulse can be sent by Matlab as described below. To initially align your table, hit the Stop / Jog button to enter Jog mode, and then use push either "1" or "3" on the number pad, to move the table left or right respectively.

2 Use a computer-controlled turntable

2.1 Programs

1. Matlab (Mathworks)
2. Data Acquisition Toolbox for Matlab
3. Matlab script RTinit.m

4. Matlab script RTturn.m
5. Matlab script RTfull.m
6. Matlab script RTexp.m
7. PVCAM (Photometrics)
8. Micro Manager (Edelstein et al., 2010)

2.2 Protocol

1. Cyanobacterial strains—Two different luciferase reporters have been used in *S. elongatus*: the bacterial *luxAB* operon and the firefly *luc* gene (Kondo, Strayer, Kulkarni, Taylor, Ishiura, Golden, et al., 1993; Andersson, Tsinoremas, Shelton, Lebedeva, Yarrow, Min, et al., 2000). Although both work well, the substrate for the bacterial reporter can be synthesized within *S. elongatus* by expressing the *luxCDE* operon, whereas bioluminescence from Luc is dependent upon the addition of D-Luciferin. Moreover, the absolute signal strengths are higher with Lux. To ensure continuous bioluminescence over the course of the experiment without substrate reapplication, and to achieve the highest sensitivity of detection, we suggest using a strain that contains the bacterial Lux reporter.

2. Preparing plates—*S. elongatus* strains containing the reporter and the genes necessary for substrate synthesis are plated on Petri dishes containing the BG-11 solid medium previously described (Mackey et al., 2007), and grown until colonies are 1 mm in diameter. Plates are then entrained for two 12:12 light / dark cycles before testing. The raised upper edge on the top of many Petri dish lids can scatter external light across the plate, obscuring the bioluminescent signal. To mitigate these effects, we wrap the edge of the Petri dish in black electrical tape. To allow for air exchange on the plate, it is necessary to cut ventilation slits into the tape with a razor blade between the Petri dish base and lid. Place the plates in the holders on the turntable. The plates should be snug in the holders, so they do not rotate during the course of the experiment. If the plates can easily rotate, they can be stabilized by sliding a small piece of hard plastic, or part of a metal twist tie, between the plate edge and the holder.

3. Initializing the Rotary Table for use with Matlab—As previously mentioned, the Motion Controller can be used as a programmable interface between Matlab and the Rotary Table. For this to work, you will need to install the Data Acquisition Toolbox for Matlab, which can control the parallel port on the computer. Using an 8-pin Mini-Din Male MAC to DB25 Male Hayes-Compatible Model Cable (Cables to Go Part 02966), connect the 25-pin parallel port on the back of the computer to the 8-pin interface port on the back of the Motion Controller **while it is turned OFF**. Initialize the parallel port for use with the RTinit.m script in Matlab. After initialization, turn the Motion Controller on, choose Division mode, and pick 12 divisions as described in **Programming the Rotary Table** above. The RTturn.m script sends a single TTL pulse to the interface and triggers the Motion Controller to turn one division. RTfull.m turns the table 12 divisions, resulting in one full rotation.

4. Controlling the CCD camera with Matlab—The CCD camera can be controlled using Matlab and the Micro Manager Matlab library. We provide the Matlab script `micro2.m`, which takes a single picture with a 3 minute exposure. Exposure length within this script can be modified by changing the value in the function `core.setExposure`.

5. Running a time-course experiment—To run a full experiment, use the Matlab script `RTexp.m`. This script uses `RTturn.m` and `micro2.m` to turn the table and take pictures respectively. Parameters within this script can be modified to adjust the number of time points taken and the duration between time points. The script is currently set to take pictures once every 2 hours for 10 days. Five days worth of data is a sufficient sample to get reliable period predictions using the programs described below. To adjust the interval at which pictures are taken, adjust the value in the pause function at the end of the script, which is currently set to 5242 seconds. Images are saved as tiff files and named according to plate number and time point (*i.e.* `plate_1_001.tiff`). An image of a plate is shown in Fig. 3A.

3 Analyzing data from turntable

3.1 Programs

1. R (CRAN)
2. R package `EBImage`
3. R package `biOps`
4. R package `Rwave`
5. R package `waveclock`
6. R script `RCFinder.R`
7. R script `WC.R`
8. R script `EBI2biOps.R`
9. `ImageJ` (Rasband, 1997)

3.2 Protocol

1. Install R libraries—Our R scripts for image analysis and period quantification are dependent upon several R packages: `EBImage`, `biOps`, `Rwave` and `waveclock`. Instructions on how to install these packages and links to packages are given in the `R_Package_Install.txt` file included with those downloaded in **Download scripts and installation files**. These scripts were tested and work with R version 3.0.1. We used the following versions of each of the other packages: `EBImage` version 4.2 (Pau, Fuchs, Sklyar, Boutros & Huber, 2010), `biOps` version 0.2.2, `Rwave` version 2.2, and `waveclock` version 1.04 (Price, Baggs, Curtis, FitzGerald & Hogenesch, 2008).

2. Process plates—Move all plate images and the `RCFinder.R`, `wc.R` and `EBI2biOps.R` files into a new directory for processing. `RCFinder.R` is the main plate processing script that identifies individual colonies on a plate, calculates the intensity of each colony for each time point, and determines the period of its bioluminescence. To do this, it sums all images for a

given plate and generates a mask of colony objects. An example mask is shown in Fig. 3A. This mask is applied to all images, and the pixel intensity within each colony object is calculated. The period of each colony is then determined using waveclock (Price et al., 2008). Bioluminescence data from individual colonies are shown in Fig. 3B. Three image files are generated for each plate in the results sub-directory. The first is plateX_numbered.tiff which shows the mask and object ID numbers for all identified colony objects. The second is plateX_rc.tiff which is the same as the first image, except it shows only the colony ID numbers for those colonies that have a rhythmic circadian phenotype. These colonies are also circled in red (center image in Fig. 3A). The third is plateX_per.tiff which is the same as the second image, except it reports the period of the colony instead of the object ID number. To overlay any of these masks with a raw image of a plate, like in Fig. 3A, we use the Overlay function in ImageJ (Rasband, 1997). Finally, the period of each colony and all bioluminescence data is reported in the file all_rc.xls, also in the results sub-directory.

4 Steps to extract reliable quantitative information from bioluminescence levels

The method described above is extremely useful for identifying mutants that have altered circadian properties, but the signal from individual colonies is low, and more quantitative data can be attained from a greater number of cells. A stronger signal can be acquired using the computer-controlled turntable by streaking colonies into larger patches, but to get comparable measurements of bioluminescence levels between strains, you need to start with liquid cultures that have the same cell density. Here, we present a strategy to achieve meaningful bioluminescent measurements with the TopCount Microplate Reader, which is better suited to handle a large number of liquid cultures, enabling the direct comparison of clock output levels between strains. Corrections for sample size are necessary and can be made easily in bacterial cultures. Additional concerns about interpreting bioluminescence levels have arisen because bioluminescence is a function of not only the level of luciferase, but also its substrate and metabolites such as ATP or FMNH₂, depending on the species source of the enzyme. Therefore, comparison of quantitative values for bioluminescence in cyanobacterial studies has rarely been emphasized. These concerns can be addressed by carefully controlling cell counts, growth rates, and luciferase substrate levels. Errors in any of these variables will result in changes to the measured level of bioluminescence that do not necessarily reflect the genotype of the mutant strains. This protocol describes how to minimize these errors through careful sample preparation.

4.1 Equipment

1. Laminar flow hood with ultraviolet (UV) light
2. Packard TopCount Microplate Scintillation and Luminescence Counter (PerkinElmer Life Sciences, Boston, MA)
3. Black 96-well microtiter plates and clear plastic lids (ThermoLabsystems, Franklin, MA)
4. Packard Topseal (Perkin Elmer Life Sciences)

5. Clear 96-well plates (ThermoLabsystems, Franklin, MA)

4.2 Programs

1. Excel (Microsoft)
2. BRASS: Biological Rhythms Analysis Software System (Millar Lab)

4.3 Protocol

1. Strain growth (5 – 7 days)—Start cultures from colonies on an agar plate following transformation or recovery. Pick five colonies using a sterile toothpick from each transformation and patch (spread inoculum with toothpick) onto a new plate that includes the appropriate antibiotics. Two to three of these that pass growth criteria described below will be used as biological replicates to ensure that the data are best representative of the genotype, and are not subject to spontaneous secondary mutations. More details for media preparation, how to setup TopCount runs, and how to handle strains are described in (Mackey et al., 2007).

- 1A** After several days, when colony color develops, pick cells and start 5 ml BG11 liquid cultures. Grow for 2 – 3 days until color is developed.
- 1B** Measure absorbance of cultures at 750 nm (optical density, OD₇₅₀). Dilute cells into new BG11 medium with appropriate antibiotics to about 10⁸ cells/ml, which has an OD₇₅₀ ~ 0.2 (Beckman Coulter DU 640B Spectrophotometer).

TIP: Note that OD measurements for cultures can be different for different instruments because the OD is measuring a scattering from the culture. Thus, the measured value will depend on the details of the detection system, in particular, the cross section of the scattering that the detector captures. Calibration of the OD₇₅₀ with cell count may be necessary for accurate density measurements. However, comparisons can be made between cultures at standardized readings even if absolute cell counts are not known. As a general rule, work with samples in the range of OD₇₅₀ 0.1–0.5.

- 1C** Grow for 2 days, monitoring OD₇₅₀ to make sure cells are healthy and that the growth rates, which will vary depending on light penetration, are the same for all of the cultures. Discard cultures that do not meet this criterion. If comparing mutants that have distinctly different growth rates, it may not be possible to make quantitative comparisons among strains. If a clone of a strain that is usually impaired in growth relative to wild type (WT) unexpectedly grows robustly, this improved growth is evidence of a suppressor mutation and should be considered with caution.
- 1D** Differences in growth rates will be evident as changes in the slope of the bioluminescence data with time. If there are differences, then the best window for quantitative comparison should be at the early time points (days 1, 2, and 3) when the cell densities are still similar.
- 1E** Aim for final density of 2 x 10⁸ cells/ml (OD₇₅₀ = 0.3 with our instrument).

TIP: It is best to use cultures that begin the experiment with similar growth histories. We grow precultures that have been recently diluted and grown for 2–3 days under standard conditions to use as the inocula for the samples that will go onto the TopCount. Avoid comparing samples from cultures that are significantly different in culture density, as those cultures would have been growing under different conditions such as lower overall light intensity, possible limitation of some nutrients, and differences in entry into stationary phase.

TIP: Avoid a high cell culture density as it can result in significant shading and an effective lower light intensity and slower growth. The reduction in light penetration into a cyanobacterial culture as density increases is dramatic; remember that these cells actively absorb photons for a living! The center of a stationary phase 100 ml culture in a 250 ml flask is essentially dark, regardless of the intensity of the lights in your chamber. All cultures should be in a similar state of growth at the start of the bioluminescence experiment.

2. TopCount Microplate Preparation (1–2 days)—The TopCount measures bioluminescence from the top of up to 8 X 96-well black Microplates that carry samples. Clear plates that allow light penetration through the plate stacker are also present. The general considerations for using a TopCount to measure bioluminescence from cyanobacteria are presented elsewhere (Mackey et al., 2007). To set up sample plates for measurement, liquid cyanobacterial cultures are pipetted onto pads of BG11 solid medium in each well as described below. Using solid medium in the well places the entire sample near the top of the well, where its bioluminescence can be readily detected; liquid cultures that distribute and scatter the emission are not suitable.

- 2A** Prepare BG11 solid medium as described in (Mackey et al., 2007). Equilibrate to ~60°C before making 96-well plate for the TopCount. Add antibiotics (if appropriate for the strains), the anti-fungal benomyl to 10 µg/ml, and filter-sterilized antioxidant Na₂SO₃ to 1 mM.

TIP: Because the cells will divide only once or twice per experiment, it is not necessary to maintain all antibiotics during the course of the experiment. Previous measurements of growth rates during the culture preparation would reveal any significant differences between WT and mutants' growth rates. Consider that even if there were 0.1% of the culture contaminated with a revertant that had a growth rate that is twice that of the parental strain, the level of background would increase to between 0.2 and 0.4% by the end of the experiment, a level that is insignificant compared to other uncertainties. Maintain antibiotic selection for the most critical genetic elements in the strains; experience with your strains and thoughtful planning are key.

- 2B** Use a multichannel pipette to fill each well of a black 96-well microtiter plate with 280 µl melted BG11 solid media. Let dry for 30 – 60 minutes.

Tip: White opaque plates produce background signals and are not suitable.

- 2C** Measure the OD₇₅₀ of each sample immediately prior to use, and dilute the more dense cultures with sterile BG11 liquid medium so that all cultures have the same cell density. Try to maintain cell densities 2×10^8 cells/ml (OD₇₅₀ = 0.3 with our instrument) for sufficient signal intensity.
- 2D** Test aliquots of each culture for possible contamination during Microplate set-up. Add 10 μ l on a rich medium plate such as an OMNI plate (Mackey et al., 2007) and incubate in the dark overnight. Most contaminating bacteria can utilize the carbon sources whereas *S. elongatus* cannot.
- 2E** Inoculation: Ideally, 30 μ l of each culture is tested in at least 10 wells on the plate, requiring a 300 μ l sample. If you use the Luc reporter instead of Lux, add 10 μ l of 100 mM firefly D-Luciferin to each 300 μ l aliquot prior to plating.
- TIP: It is best to prepare a slightly larger sample of inoculum than needed in case of accidental sample loss; scale D-Luciferin appropriately.
- 2F** Distribute the samples into wells in a pattern across the plate to span the row from positions 2 to 11 (see tip below), as there is a gradient of light intensity across the plate if an external light source is used. Averaging the wells (provided they remain alive and healthy) provides a first-order assessment of the circadian phenotypes of the strains.
- TIP: Arrange strains such that you have a positive and negative control on the same plate with the strains to be tested.
- TIP: Avoid using the wells at the perimeter of the plates for samples as they are most subject to drying out during a 5–7 day run. Cell death can be detected by a loss in the bioluminescence over time; any wells that exhibit cell death should be discarded from the analysis, else they will lead to a systematic decrease in the bioluminescence levels which is not accounted for in statistical error treatments.
- TIP: When using the TopCount to directly compare the circadian periods of different strains, the pattern of sample distribution must be considered so that only samples equidistant from the edges of the plate nearest the light source are compared. Cyanobacteria follow “Aschoffs Rule” and exhibit slight variations in period as a function of incident light intensity. This consideration is covered more fully elsewhere (Mackey et al., 2007).
- 2G** Seal the plate with a TopSeal cover. Using a 16-gauge sterile needle, poke a hole in the plastic seal above each well, being careful not to touch the samples. The hole allows gas exchange throughout the TopCount run.

3. Run TopCount and analyze data

- 3A** For circadian measurements, entrain the TopCount sample plates with a 12-h dark incubation. For experiments that include mutants that are sensitive to light/dark cycles, such as *rpa-* strains, keep the light intensity during light periods relatively low (50 μ E m⁻² s⁻¹) and limit to one dark pulse.

TIP: Administer dark incubations during local solar night. You should assume that unentrained cultures already have their clocks generally set to local time, and a major change in day/night cycle would add an unintentional phase shift to the experiment.

- 3B** Load plates onto the TopCount instrument as described in (Holtman, Chen, Sandoval, Gonzales, Nalty, Thomas, et al., 2005).
- 3C** After the run is complete analyze data using BRASS (Biological Rhythms Analysis Software System, <http://millar.bio.ed.ac.uk/PEBrown/BRASS/BrassPage.htm>; A. J. Millar laboratory, University of Edinburgh, Scotland, United Kingdom). Check that each well remained healthy over the course of the experiment, and exclude samples that showed a dramatic decline in bioluminescence signal during the run.
- 3D** Average the data from usable wells of the same strain (ideally all 10 wells). Normalize data to the peak signal amplitude of the WT strain at some time early in the run, such as at or near the 36-h time point. We found this time frame to yield the lowest variance among experiments, avoiding variations at the light/dark transition very early in the run and changes due to growth or drying after several cycles. Plot the averages of the time-dependent bioluminescence with standard deviations of the mean (SEM). We present data for WT and arrhythmic mutants to show reproducibility in measurements (Fig. 5) (Paddock et al., 2013).

TIP: Bioluminescence magnitudes vary from run to run. Thus, comparison of absolute bioluminescence values among different runs or even different plates in a single run is subject to systematic errors. However, relative values among plates and runs, normalized in the manner presented here, are very reproducible when cell numbers and growth conditions are standardized.

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Figure 1. Fully constructed turntable

(A) Photograph of assembled computer-controlled turntable. (B) Cross-sectional schematic of assembled computer-controlled turntable.

Figure 2. Turntable components

(A) Schematic of turntable surface (referred to in text as Table Top). (B) Schematic of Aluminum Light Shield. (C) Photograph of Rotary Table attached to Aluminum Disc Spacers, Aluminum Base Plate and Copy Stand base. (D) Photograph of Aluminum Light Shield on turntable surface. The PVC pipe is moved from its final location to show the hole in the center of the Aluminum Light Shield.

Figure 3. Example data from time course experiment

(A) The image on the left is a raw image of a plate with *luxAB-luxCDE* expressing cyanobacteria. The image in the center is the mask generated by the RCFinder.R script. Each white spot represents an identified colony. Those spots that are numbered and circled in red were identified as rhythmic. The number is displaced down and to the right of the spot. The right image is an overlay of the first two images to show which colonies on the plate are rhythmic. (B) Bioluminescence data for five rhythmic colonies found in (A). Colony intensity is a measure of the average pixel intensity for a colony object and varies between 0 and 1 (arbitrary units).

Bioluminescence flowchart:

Figure 4.
Flow chart for quantitative bioluminescence sample preparation.

Figure 5. Example data from time course experiment

Default high bioluminescence level for class 1 promoter P_{kaiBC} measured from both the *luc* (A) and *lux* constructs (B). Time-dependence of bioluminescence from the WT (black squares) and mutants that carry disruptions of KaiC (blue diamonds) or KaiABC (purple circles) following a 1-d and 2-d entrainment period for (A) and (B) respectively. The bioluminescence using *luc* was measured with the TopCount (Mackey et al., 2007), whereas the bioluminescence using *lux* was measured on the turntable as described above. Averages of the replicates and the standard error of the means for the bioluminescence values are indicated. Bioluminescence was converted to counts per pixel in (B) for easier comparison with the TopCount (Paddock et al., 2013). Each genetic background showed the same general behavior with both reporter systems, even though the luciferase systems and the detection systems are distinct. Thus, the constitutively high levels of bioluminescence observed in the knockouts strains are attributed to the genetic lesions and are not a consequence of detection methods or luciferase reporter constructions. This figure was adapted from *Proc. Natl. Acad. Sci. U S A.* 110(40):E3849–E3857; M.L. Paddock, J.S. Boyd, D.M. Adin, and S.S. Golden; *The active output state of the Synechococcus Kai circadian oscillator*; Copyright (2013), with permission from the National Academy of Sciences.