TopCount Topics

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Automated Recording of Luciferase-Reported Gene Transcription in Living Seedlings and Fruit Flies

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Abstract

Firefly luciferase is an ideal *in vivo* reporter for temporally regulated events due to its short biological half-life, a phenotype that can be assayed non-invasively, its versatility in a number of systems, and the outstanding sensitivity of its assay using single photon counting technology. The utility of this reporter gene is increased by the ability to monitor its expression automatically in the Packard TopCount Microplate Scintillation and Luminescence Counter. We have applied this novel combination of technologies to address circadianly regulated gene transcription in *Arabidopsis* (a mustard plant) and *Drosophila* (the common fruit fly). The nondestructive, repeated-measures approach to circadian studies has proven useful in applications ranging from assays of large populations for their circadian phenotype to revealing previously undetectable features of temporally regulated gene expression.

Introduction

Gene transcription can be monitored in many ways. Reporter genes such as β-galactosidase or biochemical assays such as Northern blots have proven their usefulness in certain circumstances. However, the stability of many reporters and the destructive nature of biochemical assays limit their practicality for monitoring temporally regulated gene expression. This has been especially evident in the study of circadian gene expression. For instance, much preliminary molecular analysis of the first clock gene to be cloned, the *period* (*per*) gene of *Drosophila,*[1] was accomplished through the RNase protection assay (RPA), which involves homogenizing the sample, extracting RNA from the homogenate and hybridizing an RNA probe to the message of interest. Although this assay does allow the monitoring of rhythmic transcription, it is extremely labor-intensive, somewhat noisy and prone to error, and necessitates the destruction of the sample. Furthermore, while these assays may reveal the average molecular characteristics of a population, individual rhythms potentially out of sync with a population cannot be detected.

To address these problems, our group has adopted the firefly luciferase enzyme as a reporter of circadian activity in *Arabidopsis* and *Drosophila.* Transgenic *Arabidopsis* were produced that contain luciferase coding sequences fused to the promoter of an endogenous clock-controlled gene, providing an artificial circadian phenotype of bioluminescence.[2,3] Similarly, transgenic *Drosophila* were produced expressing luciferase under control of *per* regulatory sequences.[4] In both cases, production of luciferase and the resulting bioluminescence reflect transcription of these clock-related genes. Luciferase is a useful reporter for circadian studies as it has a relatively short half-life of activity (2-4 hours[4]) and can be assayed non-invasively and repeatedly in the same individual. Moreover, we have automated the assay of bioluminescence in these organisms through the use of the TopCount multidetector microplate scintillation counter. The sensitivity of the detectors (rivaled only by dedicated luminometers) allows meaningful data to be extracted from extremely low-light samples. This automated, non-invasive monitoring of the molecular clock represents the state of the art in circadian biology.

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Materials and Methods

Bioluminescence Monitoring of Transgenic *Arabidopsis* Seedlings

Transgenic *Arabidopsis* seedlings were germinated on agar media. Approximately three days post-germination, individual seedlings rooted in plugs of agar were transplanted into the wells of black 96-well microplates (Dynatech) previously partially filled with media (100 µL/well) and luciferin solution (2.5 mM, 20 µL/well). Plates were sealed with TopSeal-A adhesive film (Packard) and one small hole was made in each well to allow for gas exchange.

Light was supplied to samples in the TopCount external plate stackers by red LED lamps (Quantum Devices, Inc.) positioned on either side. To allow illumination of the samples, stackers were modified by removing side and rear panels and clear microplates were intercalated between the opaque sample plates. Seedlings were in continuous light (LL) except for one brief period during each cycle when the sample was taken into the counting chamber. This type of interrupted LL regime does not significantly affect free running circadian rhythms.[3] Two wells were counted simultaneously for six seconds; seven plates (672 seedlings) were assayed with a cycle time of 1.15 hours.

Bioluminescence Monitoring of Transgenic *Drosophila*

Flies were assayed in black 96-well microplates. Plates were prepared by first filling each well half full (200 µl) of 1.5% agar, then adding 100 µl of fly food fortified with luciferin to a final concentration of 1 mM. Flies were anesthetized with CO_2 and placed individually into wells. Plates were sealed and ventilated as described above. For light/dark (LD) cycle experiments, light was supplied by fluorescent light bulbs and the modified stackers and spacer plates were used. Two wells were counted simultaneously for 17 seconds, one plate was counted in approximately 14 minutes, and three plates (288 flies) cycled every hour. (Editor's note: The TopCount used in these studies measures two wells simultaneously. For higher throughput, options are available for measuring luminescence in six or 12 wells simultaneously.)

Results and Discussion

Bioluminescence Record of *Arabidopsis*

After completion of counting, data was compressed, copied using the TopCount floppy disk drive, and transferred to laboratory microcomputers for analysis. The data was processed and plotted using Microsoft® Excel® and the characteristics of the bioluminescence rhythm, including period and phase, were subsequently described using custom software.

Figure 1 shows the bioluminescence record over time of two individual seedlings of different genotype as recorded by the TopCount. The "wild type" trace is of the original transgenic line, while "*toc1*" denotes a mutant line that exhibits a shortened but otherwise normal bioluminescence rhythm.[5] These data show robust cycling of bioluminescence throughout the entire six days of the experiment. Various aspects of the rhythm, such as period, phase, and waveform, are evident and as expected for these two lines. While the sensitivity of the TopCount assay allows for very short count times, minimum bioluminescence counts of the samples (in the troughs of the rhythms) are still at least an order of magnitude above background (data not shown).

Seedlings selected for further study can be transplanted from microplates to soil, allowing collection of material from the selfsame plant for molecular analysis as well as propagation of the line.

Rhythmicity in *Drosophila*

As seen in Figure 2, the per^{+}*; per-luc* transgenic flies clearly express luciferase rhythmically, while the per^{01} transgenics (per^{01} is a *per* null mutation that abolishes the clock[6]) do not. Moreover, these experiments allow molecular circadian rhythms in *individual* flies to be monitored at the molecular level for the first time (inset). The rhythms persisted whether the animals were monitored in alternating periods of 12 hours light/12 hours darkness (inset) or constant darkness (main figure), showing that the rhythm is not dependent on external cues. Finally, the relative lack of noise and high time resolution have revealed a second peak of *per* transcription occurring just after the main peak. This peak has not been seen in previous RPA experiments, presumably due to experimental noise and/or the coarse time resolution common to these labor-intensive assays.

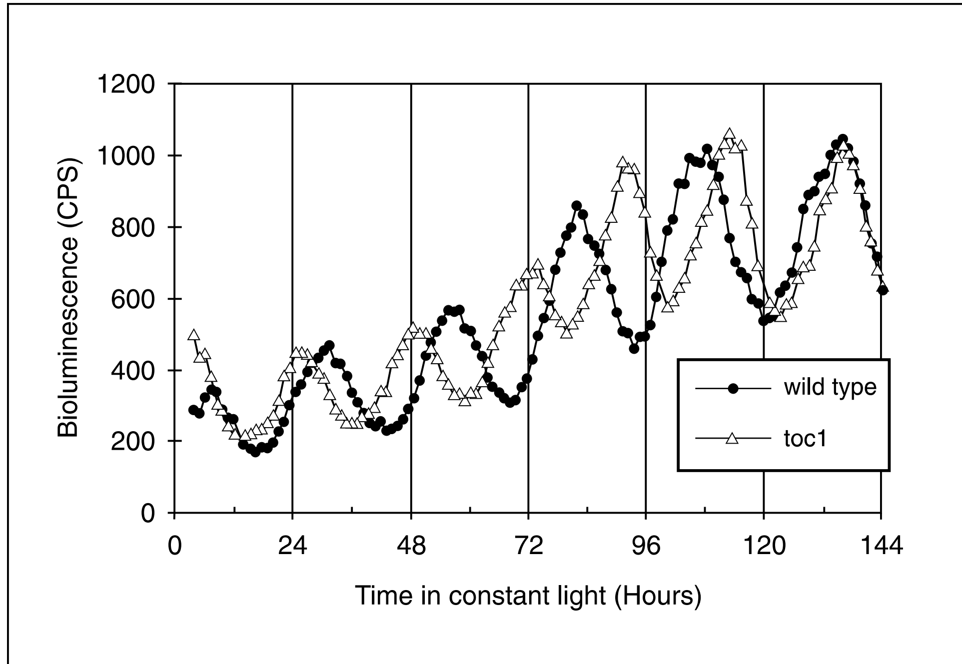

Figure 1.
Bioluminescence levels over time of transgenic *Arabidopsis* seedlings. Wild-type and *toc1* seedlings containing the *luc* reporter transgene were automatically counted over the indicated time course in the Packard TopCount.

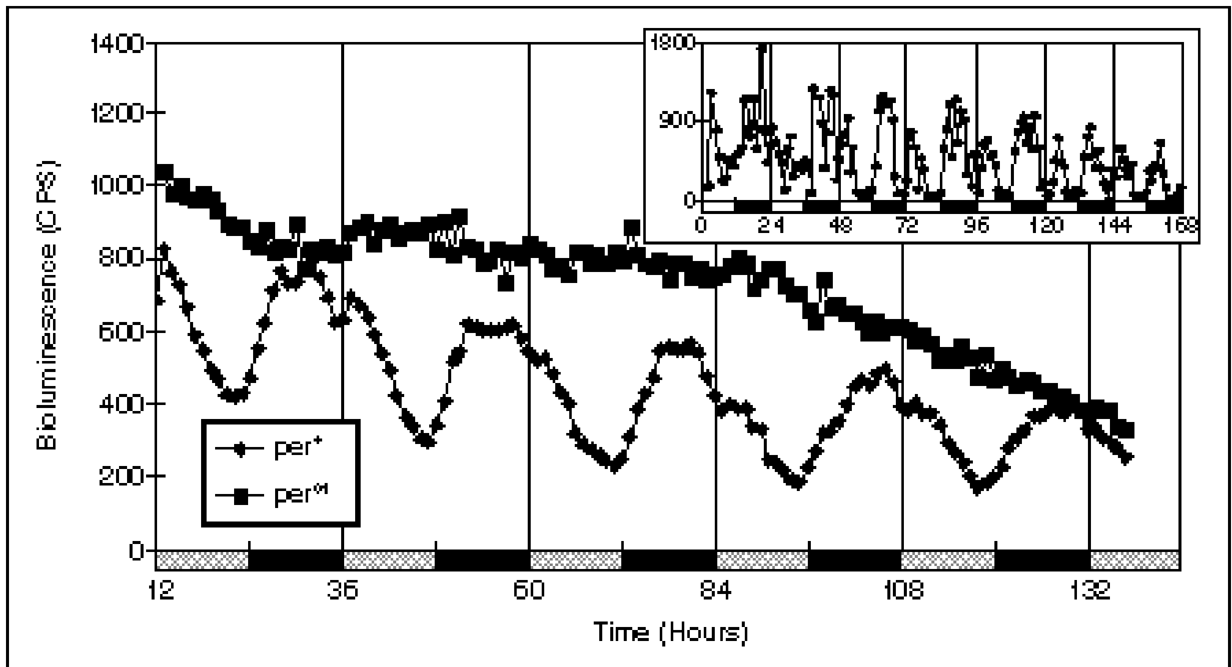

Figure 2.
Transgenic *per-luc Drosophila* are rhythmic in LD and DD. The main figure shows the average luminescence levels for a population recorded in constant darkness. Note the secondary peak of transcription just after subjective dawn. The inset shows the rhythmic bioluminescence of an individual transgenic fly in LD conditions. The second peak is again apparent. Open bars indicate light conditions, filled bars indicate dark conditions, and gray bars indicate subjective light conditions.

The significance of these findings is great, both in general technological and circadian biology terms. Using RNase protection assays to generate the same amount of data that we get from three full plates of flies monitored hourly for one week would require over 40,000 separate RPA reactions and the sacrifice of more than 2.5 *million* flies over several years; the data would still hide individual rhythms. With bioluminescence and the TopCount, that data can be obtained with less than two hours of hands-on work.

Biologically, the observation that *per* promoter-driven luminescence levels in a per^{01} background are consistently at or above the peak levels in a per^{+} background supports a feedback inhibition model for circadian regulation, which predicts (since PER protein inhibits its own transcription[7]) that the *per* promoter would be fully active in the absence of PER protein. This was not seen with RNase protection assays.[8] Finally, our discovery of a second peak of *per* transcription may help explain one of the mysteries of the circadian clock — how is a molecular cycle "stretched" to fill approximately one day? We hypothesize that this second peak could represent the major delay that lengthens the clock's free-running molecular cycle to a circadian pace. Further work to elucidate the importance of this peak may have profound impact in *Drosophila* and possibly general circadian biology.

Conclusion

Bioluminescence monitoring in the TopCount opens the door to high-throughput *in vivo* molecular analysis of gene transcription. We have successfully applied this system to the analysis of circadian regulation in organisms as diverse as plants and insects. This system should be equally useful for other temporal studies, such as developmental regulation of gene transcription, and for many other organisms and cell culture systems. The technological ease of automated bioluminescent recording in the TopCount combined with the utility of luciferase as a temporally sensitive reporter will help address a multitude of biological questions.

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