

# Video Imaging of Regulated Firefly Luciferase Activity in Transgenic Plants and *Drosophila*

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*Luciferase as a genetic reporter combined with low-light imaging systems offers a means to study dynamic fluctuations in transcriptional activity. In addition, these methods provide a non-invasive way to study these changes as well as allow the development of genetic screens based on subtle differences in transcriptional activity.*

## Introduction

Reporter gene technologies, particularly the bacterial beta-glucuronidase (GUS) system, have been central to many recent advances in plant molecular biology. Both the GUS enzyme and its reaction products are stable, making it difficult to use to assay dynamic changes in gene expression in intact plants. The same is true for the *lacZ* reporter gene used in many *Drosophila* gene expression studies. The firefly luciferase gene (*luc*) provides a more versatile reporter, as luciferase activity is relatively unstable *in vivo*. Reductions in luciferase mRNA abundance are therefore reflected by reduced luciferase activity over several hours.

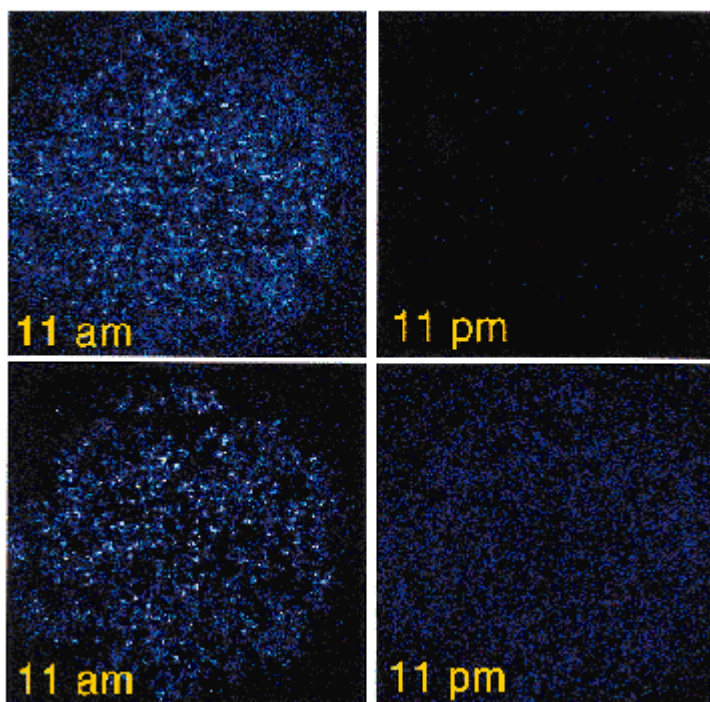
The luciferase enzyme emits green light, concomitant with the ATP- and O<sub>2</sub>-dependent oxidation of beetle luciferin. Cells transformed with luciferase will luminesce when supplied with luciferin because ATP and O<sub>2</sub> are supplied by endogenous pools. The low intensity of light produced may be measured by low-light video imaging using appropriate optics; single-cell resolution is achievable under a microscope. Alternatively, large numbers of intact seedlings may be examined using a wide-angle lens. In this report, we illustrate the power of the bioluminescent marker system as a quantitative, real-time assay of regulated gene expression *in vivo*, when luciferase-generated bioluminescence is measured by ultra low-light imaging equipment.

## Assays of *cab2* Gene transcription on transgenic plants

*Cab2* is one of a family of nuclear genes that encodes the chlorophyll **a/b**-binding proteins of the photosynthetic light-harvesting complexes. A number of cellular signaling pathways regulate *cab* genes,

and our laboratory has focused on the regulation of *cab* gene transcription by light and circadian clock rhythms. Because we measure gene expression at high time density, sometimes for several days, we need much more reliable methods for monitoring cyclic gene regulation than RNA-based assays. Furthermore, we wished to create novel phenotypes for genetic screens based upon subtle changes in gene transcription under a given condition. To accomplish both of these goals, we used firefly luciferase since it offers the advantages of relatively unstable *in vivo* activity and the ability to measure activity non-invasively by photo counting imaging.

We used several luciferase gene fusions to study the regulated expression of the *cab2* gene of *Arabidopsis thaliana* (1-4). We fused a 323bp fragment of the 5' upstream region (-322/+1) of the *Arabidopsis cab2* gene (*cab2::luc*) and studied transgenic *Arabidopsis* plants bearing this construct using a Hamamatsu VIM camera and an Argus-50 photon-counting image processor. This imaging method stores 1 pixel of unit intensity for each photoelectric event detected. The image in [Figure 1](#) shows that the population of plants exhibits a dramatic fluctuation in bioluminescence which accurately reflects the rhythmic changes that occur in *cab2::luc* transcription due to regulation by the circadian clock. The image also demonstrates two important pieces of information. First, transcription can be quantitated by the digital measurement of light output. Second, transcription in individual seedlings can be monitored and compared within a complex population.

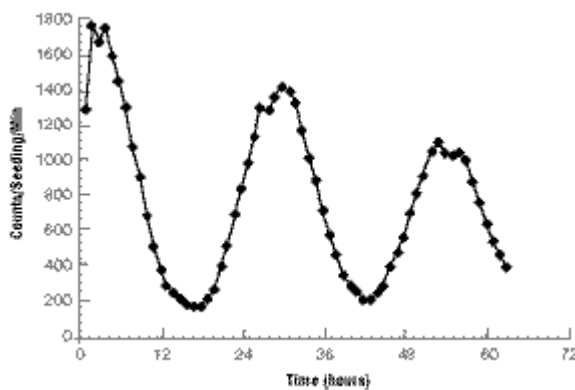


**Figure 1. Imaging of transgenic *Arabidopsis* seedlings containing a *cab2::luc* gene fusion.**

Approximately 500 transgenic *Arabidopsis* seedlings were sown onto agar growth medium on a 15cm petri dish, germinated and grown for seven days in 12 hours light/12 hours dark cycles. The seedlings were then sprayed with 5mM d-luciferin in 0.01% Triton® X-100 three times at 6-hour intervals prior to imaging. This "pre-spray" treatment removes a bulk pool of luciferase activity that accumulates prior to the first luciferin treatment. The seedlings were then sprayed with 1mM luciferin/0.01% Triton® X-100 at the indicated times. At each timepoint, bioluminescence was collected for 25 minutes using a Hamamatsu intensified VIM camera (I-CCD) and Argus-50 photon counting images processor in centroid processing mode. A Nikon 50mm f 1/.2 lens was used on the camera. The top panels show the bioluminescence emitted at 11 a.m. and 11 p.m. in a

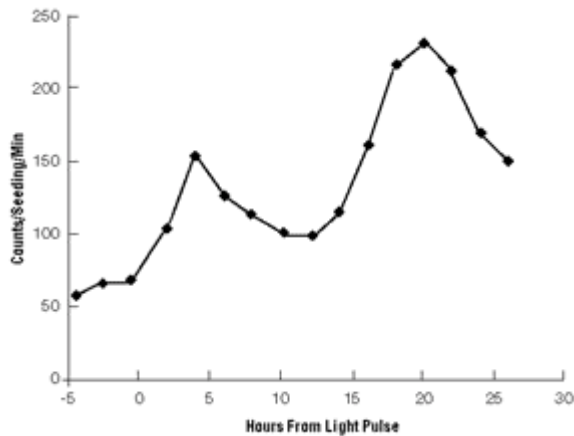
light/dark cycle, whereas the bottom two panels show light emitted at the same time, but after placing the plants in constant light for three days.

**Figure 2** provides a good example of quantifying *cab2::luc* transcription from the analysis of images taken at several time points. In this experiment, the same *cab2::luc* construct from **Figure 1** was used and the circadian regulation was studied in transgenic tobacco seedlings. The images demonstrate that the -322/+1 *cab2* DNA fragment confers a robust circadian rhythm in bioluminescence that is easily monitored for several days. An equivalent analysis based on RNA extraction and protection assays would have required a much greater amount of tissue and is far more laborious. We have taken advantage of the ease of the luciferase assay to conduct a detailed analysis of the regulatory elements present in the *cab2* promoter that are responsible for circadian-regulated transcription. Furthermore, we have used luciferase activity as a novel phenotype that has enabled us to identify mutants in the *Arabidopsis* circadian clock (6).



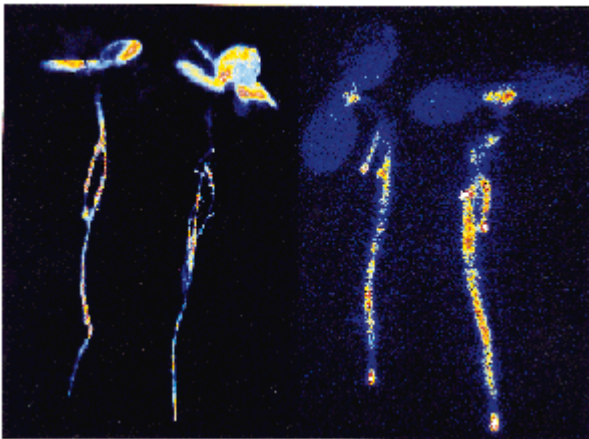
**Figure 2. Circadian-regulated transcription in *cab2::luc* transgenic tobacco seedlings.** A plate of 30 transgenic tobacco seedlings containing the *cab2::luc* fusion were grown as described in **Figure 1**. The plate was placed in constant light, and images were taken every hour following luciferin spraying as described in **Figure 1**, except that the images were recorded for 10 minutes. Photon counts were measured using the Hamamatsu Argus-50 image processor. The photon counts were then measured for each timepoint and divided by the number of seedlings expressing luciferase.

The light regulation of the *cab2* gene is mediated principally by the phytochrome photoreceptor system (7), which is responsive to red light (R). The activating effects of brief R treatments are largely abrogated by a subsequent irradiation with far-red light (FR), giving phytochrome-mediated responses a characteristic reversibility. **Figure 3** presents the use of luciferase to report these more rapid changes in transcription, using transgenic tobacco seedlings bearing a -199/+1 *cab::luc* fusion. This *cab::luc* fusion transcript accumulates in patterns that are very similar to those reported for native *cab* genes: brief R treatment induces *cab::luc* mRNA accumulation in a cyclic pattern peaking at 2-4 hours and again at approximately 20 hours after the R treatment. In **Figure 3**, peaks of bioluminescence occur at 4 hours and 21 hours after the R induction. The images also show that the luminescence was restricted to the cotyledons of the seedlings, accurately reflecting the tissue-specificity of *cab* gene expression (see below). We have observed no significant damage to seedlings following up to 6 days of imaging.



**Figure 3. Phytochrome-induced *cab2::luc* expression in etiolated transgenic tobacco.** A plate of 30 transgenic tobacco seedlings carrying the -199/+1 *cab::luc* construct were germinated and grown in complete darkness for seven days, resulting in a very low basal level of *cab* transcription. At time 0, the plants were exposed to red light for 2 minutes to activate the phytochrome photoreceptors. Plants were imaged as described in [Figure 2](#)

In addition to monitoring temporal regulation of gene transcription, luciferase is suitable for studies of spatial regulation. [Figure 4](#) shows how spatial regulation can be recorded using appropriate optics on two different imaging systems. The left-hand panel of Figure 4 shows an image taken with a Photometrics liquid nitrogen-cooled CCD (versus the intensified Hamamatsu CCD; see below). The images in Figure 4 are transgenic tobacco seedlings expressing *luc* fused to a -199/-33 *cab2* fragment that is attached to a heterologous promoter (-90/+8) derived from the cauliflower mosaic virus 35S promoter (35S promoter). This heterologous promoter drives reporter gene expression in roots as is evident from the bioluminescence pattern exhibited by these seedlings.



**Figure 4. Firefly luciferase reports spatial regulation of transcription.** The left hand panel shows two seedlings bearing a -198/-33 *cab2::35S::luc* fusion which were imaged using a Nikon 105mm f2.8 lens attached to a Photometrics liquid nitrogen-cooled CCD camera. The image was processed using PMIS software and Adobe Photoshop. The right panel shows two seedlings containing a -322/-74 *cab2::35S::luc* fusion. These images were acquired using a Nikon 105mm f/2.8 lens attached to the Hamamatsu VIM camera for 5 minutes.

We have been able to combine both temporal and spatial measurements in our studies of *cab*

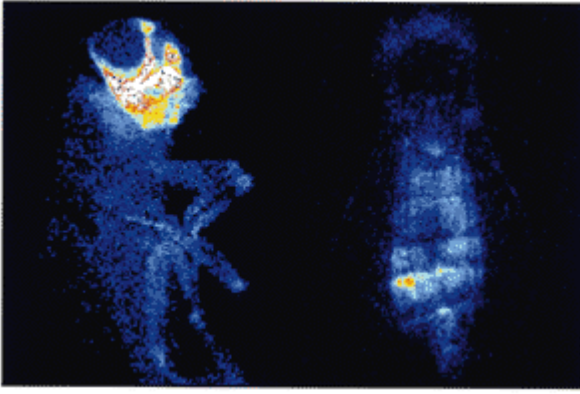
transcription. The image of seedling bioluminescence shown in the right panel of [Figure 4](#) was acquired using the Hamamatsu I-CCD VIM camera. The two seedlings contain a construct composed of the -322/-74 region of *cab2* fused to -90/+8 TATA-containing region of the 35S promoter to control luciferase expression. In this construct, weaker expression driven by the -322/-74 region is detectable in the cotyledons, but the expression clearly seen in the roots is derived from the 35S promoter -90 region. We have been able to demonstrate that the luminescence observed in the leaves cycles in a circadian manner, whereas luminescence in the roots does not oscillate. This suggests that *cab2* clock and phytochrome regulatory elements do not function in roots. The ability to measure spatial-temporal components of transcription in living tissues provides a powerful tool for studying the underlying molecular events that regulate development.

## Measuring transcription in living *Drosophila*

Many obvious applications combining luciferase expression with imaging technology exist in a wide range of organisms. Besides plants, we are particularly interested in studying clock-regulated transcription in *Drosophila* in which the circadian clock has been subjected to detailed genetic analysis (8). Ultimately the goal of using luciferase in *Drosophila* is to develop the tools necessary for measuring neuronal gene transcription and behavior simultaneously, thereby providing molecular correlates for the control of complex brain function.

To initiate these studies, we used transgenic fly lines bearing luciferase fused to the heat shock hsp70 promoter (*hsp70::luc*) for pilot experiments. [Figure 5](#) shows two images of living flies expressing *hsp70::luc*, which directs strong bioluminescence throughout the animal. During these pilot experiments, we discovered two successful methods for luciferin applications: simply spraying anesthetized animals with luciferin prior to imaging or including luciferin at a concentration of 1mM in the upper layer of the animal's food. It usually requires 2-4 hours for the adult fly to acquire sufficient luciferin by ingestion for imaging. As shown in [Figure 5](#), it is quite simple to measure *hsp70::luc* luminescence in a living, anesthetized animal. We recently expanded this analysis to several different promoters, including that of the *period* gene (C. Brandes, R. Stanewsky, C. Jameson, J. Plautz, J.H and S.K, unpublished data).

Several factors may affect the results of the bioluminescence pattern in *Drosophila*. Pigmentation can be present in different body parts that occludes the green light emitted by firefly luciferase activity. [Figure 5](#) demonstrates this point, where no luminescence is observed from the flies' eyes. However, when *hsp70::luc* transgene is crossed into genetic backgrounds containing the yellow-white mutation, which removes pigment from the eye and greatly reduces pigment in the body, strong bioluminescence is seen from the eyes (data not shown). We therefore recommend that the use of firefly luciferase as an *in vivo* reporter gene in *Drosophila* will be maximally effective in strains carrying mutations that reduce pigmentation. As with plants, we have not observed any deleterious effect of exposing flies for long periods to luciferin.



**Figure 5. Hsp70::luc transcription in living *Drosophila*.** Adult *Drosophila* bearing an hsp70::luc fusion were heat shocked in a dry vial at 40°C for 40 minutes, followed by a 30-minute recovery period at room temperature. The animals were then anesthetized with ether, sprayed with 5mM luciferin and imaged for 10 minutes using a 10X objective on a Nikon upright microscope attached to the Hamamatsu VIM camera.

### Choice of low-light imaging systems

One major decision facing researchers who wish to purchase an imaging system to monitor luciferase activity is the choice of slow-scan cooled CCD versus an intensified CCD such as the Hamamatsu system (see [Figure 4](#)). The cooled CCD camera systems (cryogenic cooling is required for most applications) will often give higher spatially resolved images than the intensified CCD systems because the resolution is degraded in the microchannel plate of the intensified camera. In our current experience, however, there is a crossover point in the light intensity detected by extremely weak signals. We urge researchers to assess several different camera systems using their own material before making a camera purchase as the choice of a particular system will be governed by many user-specific parameters.

### Summary

Luciferase fusions, combined with a low-light imaging system, have a unique potential to report both temporal and spatial regulation of gene expression in intact multicellular organisms. Many current imaging systems, such as those employed in these studies, are supplied as turn-key packages, including all components necessary to collect and analyze low-light data. Using appropriate luciferase fusion constructs, this technology should be readily applicable to any gene expression system that is transparent to green light and accessible to exogenous luciferin substrate. Moreover, the expression pattern of the individual organisms can be resolved in a large population, such that genetic screens may isolate mutants in any gene that regulates the expression of the luciferase transgene using phenotypes of aberrant luminescence.

### References

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## Ordering Information

Product	Size	Cat.#
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Beetle Luciferin, Potassium Salt	5mg	E1601
	50mg	E1602
	250mg	E1603
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pSP- <i>luc</i> + Vector	20µg	E1781
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pSP- <i>luc</i> +NF Fusion Vector	20µg	E4471
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