Kinetic Analysis and Modeling of Firefly Luciferase as a Quantitative Reporter Gene in Live Mammalian Cells

Jolene M. Ignowski, David V. Schaffer

Department of Chemical Engineering and The Helen Wills Neuroscience Institute, MC 1462, University of California, Berkeley, Berkeley, California; 94720-1462; telephone: 510-643-5963; fax: 510-642-4778; e-mail: schaffer@cchem.berkeley.edu

Received 2 September 2003; accepted 6 January 2004

Published online 10 May 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/bit.20059

Abstract: Firefly luciferase has proven to be a highly sensitive and quantitative reporter gene for studying gene delivery and regulation, and its recent use in live cells and organisms promises to further expand its utility. However, the intracellular behavior and properties of the enzyme are not well characterized. Specifically, information on the intracellular kinetics and stability of luciferase activity is necessary for real-time luminescence counts from live cells to be quantitatively meaningful. Here, we report a dynamic analysis of luciferase activity in the context of living mammalian cells. We have determined the relative light units measured in living cells to be proportional to that found in cell lysate. We have also calculated the K_m of luciferase in living cells to be $\approx$ 1 m*M*, a value much higher than the 10 μM found for pure enzyme in vitro. In addition, a 2-hour half-life of luciferase activity in live cells was measured in real time. Finally, we have modeled luciferase activity in live cells for the purposes of understanding and translating the luciferase signal into a more effective metric of gene expression and cell behavior. © 2004 Wiley Periodicals, Inc.

Keywords: firefly; luciferase; reporter gene; enzyme; kinetic; model

INTRODUCTION

Reporter genes, genes that encode proteins whose presence is readily detected and quantified, have significantly advanced a number of efforts in biology and biotechnology, including studies of gene regulation, gene delivery, and signal transduction (Chalfie et al., 1994; Gould and Subramani, 1988; Naylor, 1999). The reporter genes used initially, such as chloramphenicol acetyltransferase and β-galactosidase, have gradually yielded to more sensitive, nonradioactive reporters based on fluorescence and luminescence. These include fluorescent proteins, such as the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, and luciferases, including firefly luciferase (Fluc) from *Photinus pyralis* (Tsien, 1998; Wilson and Hastings, 1998). Green fluorescent protein has the advantages that its intrinsic fluorescence is readily visualized, and that it is nonenzymatic and thus does not require a substrate; however, considerable concentrations of this fluor ($\approx$ 1 μM) must be present inside the cell to detect a signal over the background noise (Cubitt et al., 1995; Niswender et al., 1995). Furthermore, it is highly stable intracellularly, with a half-life of over 24 hours. While this stability enhances its sensitivity by yielding higher concentrations, is poses a significant disadvantage for dynamic studies of short time scale gene expression events, and GFP variants with lower half-lives have a correspondingly lower sensitivity (Li et al., 1998).

Firefly luciferase catalyzes the reaction of D-luciferin with O_2 to produce light in the presence of Mg^{2+} and ATP, as shown below:

$$Luciferin + O_2 + ATP \xrightarrow{Luciferase, Mg^{2+}} Oxyluciferin + hv + AMP + PP_i + CO_2$$

Luciferases have the advantage of a very low background noise, thus decreasing the number of molecules needed for a detectable signal. This attribute is particularly useful in studies of promoters with low or transient activity. The relatively short half-life reported for luciferase can also serve a practical purpose when the enzyme is utilized to study the dynamics of gene expression (Thompson et al., 1991). Because of these advantageous properties, luciferases have been employed in a wide variety of studies including gene delivery (Taniyama et al., 2002), growth factor regulation of gene expression (Harrison et al., 1999), and gene silencing (Paddison et al., 2002). Real-time imaging of luciferase expression in cell culture and in live rodents is also developing into a standard technique for noninvasive localization and quantification of gene expression (Greer III and Szalay, 2002; Nunez et al., 1998; Ray et al., 2002; Rutter et al., 1995; White et al., 1995; Wu et al., 2001). Furthermore, such real-time imaging may be well suited when the quantification of gene expression in single live cells may be necessary (White et al., 1995). These include phenomena

Correspondence to: D. Schaffer

Contract grant sponsors: ONR Young Investigator Award; American Parkinson's Disease Association Pilot Grant; University of California Dissertation-Year Fellowship

where there is significant variability within a cell population, such as for stochastic events where uniform conditions and stimuli can ultimately lead to highly divergent cell responses and behavior, a hallmark of stem cells for example (Gage, 2000).

Although purified Fluc has been extensively studied since the 1940s, relatively little is known about its activity in the context of a living cell. This information is important for understanding the quantitative relationship between gene expression, protein concentration, and light output, the signal that is experimentally measured. We have therefore investigated the linearity, half-life, and enzyme kinetics of luciferase bioluminescence in live human embryonic kidney (HEK 293) cells. We have also created a model of the luciferase-luciferin reaction in the context of living mammalian cells based on our own experiments and values found in literature. This systematic investigation of the enzyme provides new and important information for the quantification and interpretation of luciferase signals within live cells.

MATERIALS AND METHODS

Materials

Iscove's Modified Dulbecco Medium (IMDM), L-15 Medium, and penicillin-streptomycin were obtained from Invitrogen (LaJolla/San Diego, CA). BioWhittaker produced the fetal bovine serum (FBS). D-luciferin, free-acid and potassium salt, were obtained from Regis Technologies and Promega Corp. (Madison, WI), respectively with Quantilum luciferase also from Promega. All other reagents were acquired from Sigma-Aldrich Chemicals Co. (St. Louis, MO), unless otherwise noted.

Plasmid Construction

The firefly luciferase gene (*Fluc*) gene was amplified from the plasmid MARSHA luc (a kind gift of F. Gage, the Salk Institute for Biological Studies, La Jolla, CA) using the polymerase chain reaction (PCR). The primers used (5′-AGGGGCCGCCTCGGCCGTCGACGGTAT CGATAAG-3′ and 5′-GTGGCGGCCGCTCTAGAACTAGTG-3′) contain the restriction enzymes sites of *Sfi* I and *Not* I for ready insertion into several vectors. The *Fluc* gene was then inserted into the MoMLV retroviral vector pCLPCX under the control of the human CMV IE (immediate early) promoter to create pCLPC Fluc. *Fluc* was also inserted into the MoMLV retroviral vector pCLPIT under the control of the tetracycline-off gene regulation system (Gossen and Bujard, 1992). DNA manipulation and purification was performed using standard molecular biology techniques (Ausubel et al., 1999).

Cell Culture

Human embryonic kidney 293T cells were maintained at 37°C and 5% CO_2 in IMDM with 10% FBS and 100 units/mL penicillin and 0.1 mg/mL streptomycin. Cells were counted using a hemacytometer.

Viral Packaging and Cell Line Production

The retroviral vectors pCLPC Fluc and pCLPIT Fluc were packaged by transient transfection of the plasmid of interest into a 10 cm dish of 70% confluent 293T human embryonic kidney cells (ATCC) with two helper plasmids, CMV gag-pol and CMV VSVG, which encode the retroviral Gag-Pol proteins and vesicular stomatitis virus envelope glycoprotein necessary for viral production. Cells were allowed to produce virus for 48 hours before harvesting. The viral-containing cell media was then centrifuged and filtered to clarify cell debris. The virus was then purified via ultracentrifugation, and resuspended in 100 μL of phosphate buffered saline. Purified virus was stored at −20°C.

The 293T CLPC Fluc and 293T CLPIT Fluc cell lines were produced by infecting ≈ 10^6 naïve 293T cells with 1 μL purified retrovirus in the presence of 8 μg/mL polybrene. Stably infected cells were selected by culturing cells in the presence of 1 μg/mL puromycin.

Luciferase Assays

Luciferase assays were performed on cell lysate as follows. Media was aspirated from the cells, and the plate was rinsed with phosphate-buffered saline (PBS). The cells were lysed with 200 μL of a cell lysis buffer (25 m*M* Tris-phosphate, pH 7.8, 2 m*M* dithiothreitol, 2 m*M* 1,2-diaminocyclohexane-*N*, *N*, *N*, -tetraacetic acid, 10% glycerol, and 1% Trition X-100), scraped from the plate, and placed in a 1.5 mL Eppendorf tube. The samples were then frozen at − 80°C. Prior to assay, samples were thawed and briefly centrifuged to remove cell debris. The luciferase assay buffer was prepared in semi-darkness (20 m*M* Tricine, 1.07 m*M* magnesium carbonate pentahydrate, 2.67 m*M* magnesium sulfate, 0.1 m*M* EDTA, 0.5 m*M* ATP sodium salt, 0.27 m*M* Coenzyme A sodium salt, 33.3 m*M* dithiothreitol, and 0.47 m*M* D-luciferin potassium salt). The luminometer (Turner Designs TD 20/20) was blanked with 100 μL of the luciferase assay buffer in a 12 × 75 mm polypropylene tube. The sensitivity was adjusted as necessary to ensure light levels remained within the instrument's detection limits. To assay the samples, 10 μL of the cell lysate were added to 100 μL of the luciferase assay buffer, swirled briefly, and placed in the luminometer. The signal was integrated for 30 seconds with a 2 second delay and was reported in Relative Light Units (RLU).

Live Cell Assays

The 35-mm plates were typically seeded with 10^5 cells in 1 mL of IMDM or L-15 media 24 hours prior to the assay. Various amounts of a 1 m*M* D-luciferin stock in PBS or water were added to cells just prior to placing the plates in the luminometer. The luminometer was set to collect data

every 0.2 s. To create a temperature-controlled environment inside the sample chamber, the luminometer was outfitted with a light-tight water reservoir. A rubber stopper was inserted into the neck of a black, 12-inch helium quality balloon and placed into the adapter opening on the lid of the luminometer sample chamber. Tubing was connected to the holes of the stopper to serve as a water inlet and outlet for continuous circulation through the reservoir from a water bath via a small pump. The temperature inside the chamber, monitored by a thermocouple, was controlled by adjusting the temperature of the water bath and allowing the system to reach equilibrium.

Mathematical Modeling

The Fluc model was formulated and analyzed using BERKELEY MADONNA software (www.berkeleymadonna.com).

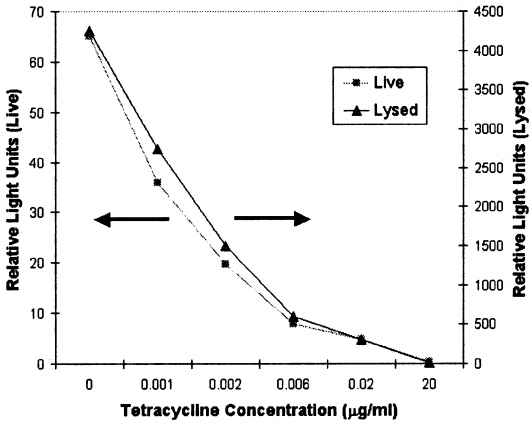
RESULTS AND DISCUSSION

CLPIT Fluc

It has long been recognized that bioluminescence signal is linearly related to luciferase concentration for purified enzyme or cell lysate. However, while a number of studies of luciferase activity in live cells in culture or in animals have been reported in literature (Greer III and Szalay, 2002), to our knowledge it has never been determined how light output from inside a cell is quantitatively related to luciferase expression upon the extracellular addition of enzyme substrate to the cell culture media. To compare the signal from living cells vs. cell lysates, approximately 10^5 293T cells infected with a retroviral vector expressing firefly luciferase under the control of a tetracycline regulable promoter (CLPIT Fluc cells) were seeded per 35-mm plate. Various concentrations of tetracycline were added to the media, and the cells were incubated for 48 h. Half of the plates were individually lysed for luciferase assays, and luciferase expression was monitored in live cells on the other plates. To assay the live cells in the luminometer, 100 μM of D-luciferin in water was added to the media, and data were collected for 15 min.

In the tetracycline-off regulation system, increasing the tetracycline concentration reduces the level of luciferase expression by inhibiting the promoter induction (Gossen and Bujard, 1992). As shown in Figure 1, the luminescence emitted by luciferase activity in lysed and living cells at different tetracycline concentrations are directly proportional. These data demonstrate the signals obtained from living cells, while $\approx$ 70 times weaker, exhibit the same linear relationship found in luciferase assays performed on cell lysates.

While luciferases are highly sensitive reporter genes, this reduced signal in live cells poses a potential challenge for certain cases where it may be difficult to monitor the light signal produced by these enzymes. To compensate, strong promoters, an integrated signal from larger cell populations (as in rodent tissue), highly sensitive equipment, or a combination of the above may be necessary. Indeed, most of the single cell studies thus far have used high copy numbers of plasmids coupled with strong promoters that are then imaged by photon-counting digital cameras (White et al., 1995).



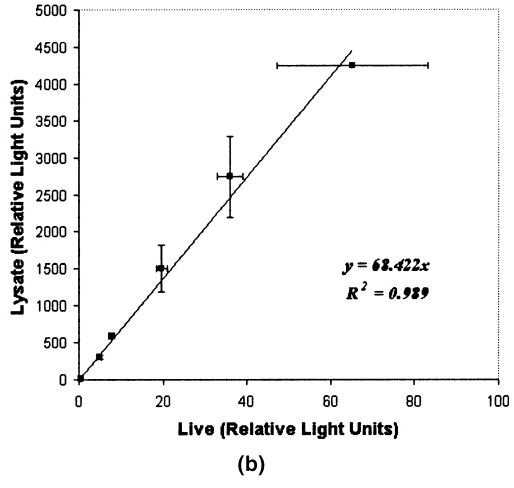


Figure 1. (a) A comparison of the RLU signal between live and lysed 293T cells expressing luciferase from the retroviral vector CLPIT Fluc. (b) There is a clear linear relationship between the live and lysed cell signals, but with a 70-fold lower value in the intact cells. Concentrations of tetracycline in μg/mL are (1−r): 20, 0.02, 0.006, 0.002, 0.001, and 0. Forty-eight hours prior to the experiment, 10^5 cells were plated in 35-mm dishes with 2 mL of media. Triplicate readings were taken at ambient temperature, and the error bars represent 1 standard deviation from the average.

Kinetics

The decreased sensitivity of luciferase when monitoring live cells is not surprising, as the live cells have a variety of factors that may contribute to a reduced signal. Since we have determined that luciferase signals in live vs. lysed cells are proportional, we next measured the enzyme kinetic constants in each context. To determine the $K_{M(luciferin)}$ for living cells at 37°C, we heated the sample chamber of the luminometer without compromising the low thermal

noise of the PMT detector, as described in the Materials and Methods section. Approximately 10^5 293T CLPC Fluc cells were plated in a 35-mm dish 24 h prior to the measurements, D-luciferin in PBS was added to the media at various concentrations. The luminometer readings were recorded for 15 min and averaged. The concentrations of the cofactors magnesium ($\approx$ 2 m*M*) and ATP ($\approx$ 1 m*M*) inside a living cell are saturating relative to the $K_{M(ATP)}$ of luciferase (25–250 μ*M*), and the reaction rate can therefore be considered zeroeth order with respect to ATP (Eu and Andrade, 2001; Fukuda et al., 1983; Lee et al., 1970; Millart et al., 1995).

To calculate the Michaelis constant, $K_{M(luciferin)}$, the initial enzymatic reaction velocity, v_{zero} (RLU/second) was recorded as the average of triplicate sample readings (Fig. 2a). From a double reciprocal plot, the $K_{M(luciferin)}$ was found to be 1.55 m*M* with a 95% confidence interval of ± 0.35 m*M*. This

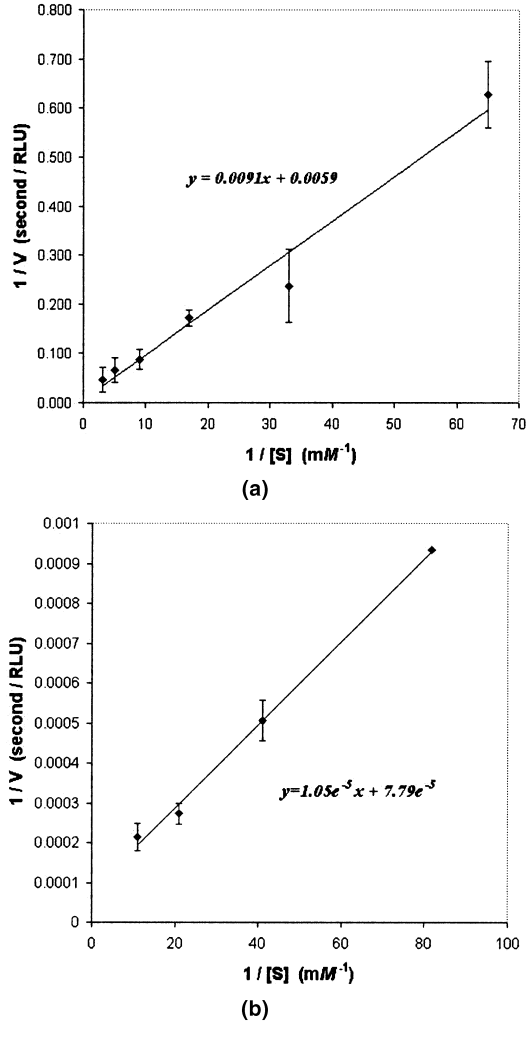


Figure 2. (a) A double reciprocal plot of luciferase bioluminescence in living 293T CLPC Fluc cells at various luciferin concentrations. Measurements were taken from cells in 35-mm plates at 37°C, and the readings were averaged over a 15-min time course. (b) A double reciprocal plot of 293T CLPC Fluc lysate. Triplicate readings were averaged in this case over initial 10-s time course. Error bars represent 1 standard deviation from the average.

is considerably higher than the 0.2–10 μ*M* reported previously for the enzyme in vitro (Eu and Andrade, 2001), and also higher than evidence presented by Vitvitsky et al. (1992), who found the K_M for luciferin within whole erythrocytes to be between 4 and 21.5 μ*M*.

These discrepancies have two potential explanations. The conditions or components inside the 293T cells could by several mechanisms effectively reduce the binding affinity of the luciferase for the luciferin. For example, it has been proposed that pyrophosphate can slightly inhibit luciferase activity (Gandelman et al., 1994). Alternatively, the concentration of the luciferin inside the cell could be lower than in the surrounding media as suggested by Gandelman et al. (1994). To test this possibility and to find the K_M inside the cells, the enzyme kinetics were monitored in cell lysate with disrupted cellular membranes. 293T CLPC Fluc cells were harvested from 10-cm plates and homogenized by shearing with an 18G needle to liberate the enzyme. Increasing amounts of D-luciferin were added to 25 μL of the crude cell lysate, and the luminescence was measured (Fig. 2b). The K_M for the enzyme in lysate was found to be 134 μ*M* with a 95% confidence interval of ± 22 μ*M*. This value, lower than that obtained for live cells but still higher than that reported for pure enzyme or erythrocytes, suggests that there are additional factors inside the cells that may contribute to the increased Michaelis constant not simply a luciferin gradient. Luciferin should readily cross the cell membrane considering its relatively small size and amphipathic properties, and the gradient observed by Gandelman et al. (1994) may result from the high luciferase concentrations inside their cells and their measurement techniques. The K_M for live cells may be higher than that for cell lysate due to the localization of the enzyme inside the cells unable to access the free luciferin in the surrounding media.

Luciferase Decay

To further characterize the intracellular properties of luciferase, we measured the half-life of the enzyme in living cells, in real time. Live cells at 37°C were incubated with 0.1 mg/mL of cycloheximide to halt protein synthesis (Bennett et al., 1965). A separate experiment in which we added cycloheximide to cell lysate demonstrated that this compound has no direct effect on luciferase activity, that is, it inhibits protein synthesis only and not enzyme activity (data not shown). The data presented in Figure 3 indicate the enzyme decays following first-order kinetics with a half-life of approximately 2 h. This result is faster than the value of 3 h found by Thompson et al. (1991) for the thermal decay of luciferase in cell lysate, a discrepancy that may be due to differences in measurements between live cells and lysates.

Fluc Model

We developed a model of luciferase expression and activity in living cells at 37°C to synthesize the results of the above experiments and dynamically track the activity of the

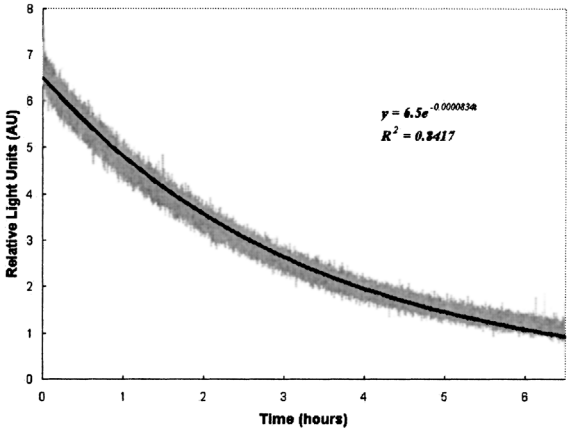


Figure 3. Thermal decay of Fluc in living 293T cells. 0.1 m*M* D-luciferin was added to cells several hours prior to start of the experiment, and 0.1 mg/mL cycloheximide and added at t = 0. Data are the average of two runs.

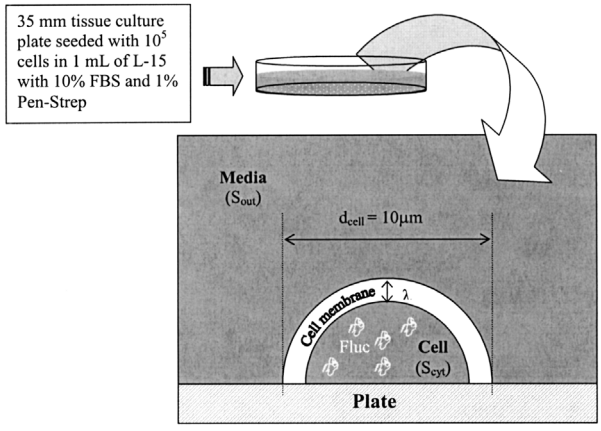


Figure 4. Schematic representation of model structure and parameters. Luciferin at concentration S_{out} from the culture media transports across the cell membrane of thickness λ to establish an intracellular concentration S_{cyt}. The intracellular substrate then reacts with cytosolic luciferase to release light.

luciferase–luciferin reaction to relate the amount of light produced (in Relative Light Units) to luciferase levels in real time. The model is based on a set of differential equations that describe the changes of luciferin concentration in the media and inside an individual cell, the changes in the amount of enzyme inside an individual cell, and the light produced by a population of cells. Figure 4 shows a schematic representation of the model. The model equations make the following assumptions:

- Mass transfer of luciferin across the cell membrane occurs by simple diffusion.
- The reaction follows Michaelis-Menten kinetics (Michaelis and Menten, 1913).
- No luciferin is present within the cells at $t = 0$.
- For surface area calculations, the cell geometry is idealized as a hemisphere.

The equations are shown below:

$$\frac{dS_{out}}{dt} = -\frac{D_{mem}A}{\lambda}(S_{out} - S_{cyt})N/V_{media} - k_{dluc}S_{out} \quad (1)$$

$$\frac{dS_{cyt}}{dt} = \frac{D_{mem}A}{\lambda}\frac{(S_{out} - S_{cyt})}{V_{cell}} - k_{dluc}S_{cyt} - \frac{k_{cat}ES_{cyt}}{K_m + S_{cyt}} \quad (2)$$

$$\frac{dE}{dt} = k_s - k_dE \quad (3)$$

$$\frac{dL}{dt} = v = \frac{k_{cat}ES_{cyt}}{K_m + S_{cyt}}N * RLU \quad (4)$$

$$\frac{dN}{dt} = k_cN \quad (5)$$

Equation (1) describes the change in the concentration of luciferin in the media (S_{out}). The first term within this equation is the diffusion of the luciferin into the cells, and the second term is the natural degradation of the luciferin. The second equation [Eq. (2)] for the concentration of luciferin inside an individual cell (S_{cyt}) is essentially the same as for S_{out}, but it is based on a single cell and includes a term for the reaction of luciferin to oxyluciferin. Equation (3) describes the formation and degradation of luciferase (E) inside a cell. Equation (4) provides the light (L) produced in terms of Relative Light Units per second, where v is the velocity of the light production reaction for the population of cells. The parameter *RLU* is the conversion factor that translates the moles of luciferin reacted into the RLU measurement by the instrument. The final equation [Eq. (5)] describes the growth of the cells during the course of the experiment.

All of the parameters in these equations were either experimentally measured in Figures 1–3 or taken from literature, and they are summarized in Table 1. For example, the initial level of luciferase in the cells was determined using a standard curve based on purified recombinant luciferase from Promega (data not shown). In addition, this recombinant, standard luciferase was also utilized to calculate the conversion between moles of luciferin reacted and the instrument's RLU measurements. The luciferin decay constant at 37°C was calculated from experimental data (data not shown). Furthermore, we based the mass transport of luciferin into the cells on values given by Stein (1986).

Using the values in Table 1, we were able to successfully reproduce the experimental data (Fig. 5A and 5B) through the model. The first comparison (Fig. 5A) was the thermal decay of luciferase (determined above) by setting the synthesis rate of luciferase (k_s) and the cell growth (k_c) to zero (Baxter and Stanners, 1978; Brooks, 1977). It is interesting to note that the model indicates the luciferin transport across the cell membrane is essentially instantaneous (data not shown).

The second comparison was the long-term monitoring of the CLPC Fluc cells at 37°C in the absence of cycloheximide as a control (Fig. 5B). From the model, we calculated the

Table I. List of parameters used in Fluc model with relevant references listed.

Parameter	Definition	Values	Units	Reference
Initial S_{out}	Initial concentration of luciferin in the media	0.0001	mol/L	100 μ*M* luciferin added to media
Initial S_{cyt}	Initial concentration of luciferin in an individual cell	0	mol/L	Assume no luciferin in cell
Initial E	Initial amount of luciferase in an individual cell	1.44 e -19	mol/cell	From luciferase calibration (Experiment)
D_{mem}	Diffusion coefficient across a cell membrane	1.3 e -7	dm^2/s	From Stein (1986)
A	Area of a cell	1.57 e -8	dm^2/cell	Calculated area from hemisphere assuming diameter = 10 μm
λ	Thickness of cell membrane	4 e -8	dm	From Stein (1986)
N	Number of cells	(1-4) e 5	–	Number of cells
V_{cell}	Volume of a cell	1e -12	L	Calculated volume from hemisphere assuming diameter = 10 μm
V_{media}	Volume of media	0.001	L	1 mL of media on cells
k_c	Growth rate of cells	1.93 e -5	s^{-1}	10 h doubling time (Experiment)
k_s	Synthesis rate for luciferase	1.19952 e -23	mol/(cell·s)	Assume steady state for CMV promoter (i.e., equals $k_d \cdot E$)
k_{dluc}	Decay constant for luciferin	4.83 e -6	s^{-1}	From Luciferin half-life determination (Experiment)
k_d	Decay constant for luciferase	8.33 e -5	s^{-1}	From Luciferace half-life determination (Experiment)
k_{cat}	Catalytic rate constant	0.04	mol oxyluciferin/ (mol luciferase·s)	From Brovko et al.
K_M	Michaelis constant for luciferin	0.0001	mol/L	From Michaelis constant determination (Experiment)
RLU	Conversion factor	1.4775 e 16	RLU·s/ (mol oxyluciferin)	From RLU conversion (Experiment)

luciferin consumption to be approximately 0.5 n*M*/s. If we assume one ATP is consumed per luciferin, the 1 m*M* ATP concentration in the cell is not limiting, and luciferase consumption of ATP would fortunately not be expected to perturb cellular metabolism. Furthermore, we observe a gradual increase in the value of the signal due to cell proliferation, and this time scale for the increase exactly matched the modeled 10-h doubling time of the cells.

For a model to be useful, it must not only match the data used to obtain parameter values for the model, but it must be able to predict new results. To test the ability of the model to predict new experimental data, we performed an experiment in which cycloheximide was initially added to cells, then removed to allow luciferase synthesis to recover. Cycloheximide (10 μg/mL) was added to the cells for 100 min, and the media was then removed and replaced with fresh media containing D-luciferin (0.1 m*M*). The cycloheximide would then gradually diffuse out of the cells across a concentration gradient proportional to its intracellular concentration, leading to a predicted exponential decrease in intracellular cycloheximide effects on protein synthesis. Therefore, within the model the synthesis rate was allowed to recover by the equation:

$$k_s = k_{so}(1 - e^{-bt})$$

where k_{so} is the steady-state value of k_s, and b is the recovery rate constant. Christopher et al. (1971) experimentally measured that protein synthesis returns to normal levels 8 h after removal of cycloheximide, and we therefore set 8 h equal to three exponential time constants, where $k_s = 0.95k_{so}$. Furthermore, Brooks (1977) showed that cycloheximide blocks cell proliferation even at low levels (i.e., 33–100 ng/mL). Keeping cell growth at zero, our model predicts the initial exponential decay and the eventual recovery of the luciferase upon cycloheximide removal. The deviation toward the end of the time window is probably due to the reinitiation of cell growth, and for the first 12 h, the model prediction fits the experimental data very well.

To our knowledge, this is the first model of luciferase in living mammalian cells, and it will significantly aid the interpretation of the RLU signals generated by luciferase intracellularly. We anticipate that the model parameters may be largely independent of a particular cellular and experimental context, expanding the model's general applicability. For example, the model indicates that the cell membrane does not represent a major transport barrier for luciferin (a result also supported by the rapid rise in luminescence upon addition of luciferin to the media), and cell geometry should therefore not significantly affect model applicability. The intracellular luciferase decay is dependent upon chaperones, which may vary modestly between cell types (Michels et al., 1995; Robinson et al., 1996). The Michaelis constant, which is very different between intracellular and free enzyme, may be dependent upon numerous factors such as intracellular enzyme inhi-

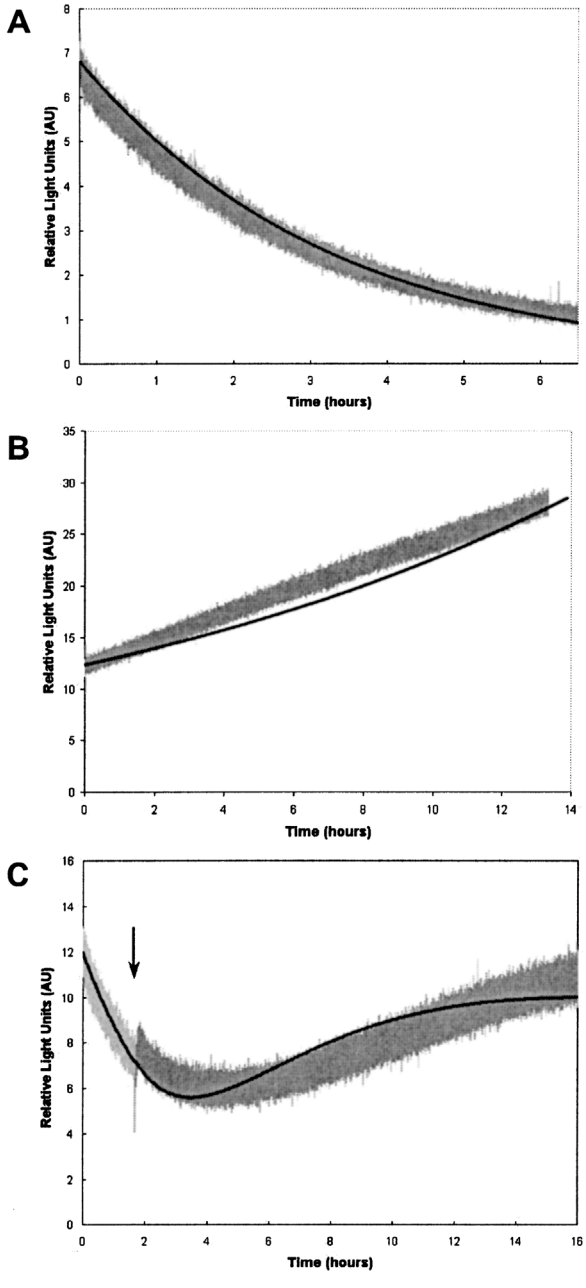


Figure 5. Comparison of model with experimental data. (A) Thermal decay of luciferase in living cells. Model parameters: N = 1.6e5 cells, k_s = 0, k_c = 0. (B) Long-term monitoring of luciferase in living cells. Model parameters: N = 2.9e5 cells, k_s = 1.19952e-23 moles/(cell · seconds), k_c = 1.93e-5 s^{-1}. (C) Cycloheximide challenge and rescue. The arrow represents the time point at which the medium was changed to remove the cycloheximide. Model parameters: N = 2.8e5 cells, b = 8.88646e-5 s^{-1}, k_c = 0. For all graphs, lines are model (black) and experiment (gray).

bitors, such as pyrophosphate (Gandelman et al., 1994), or components that bind and sequester luciferin. This constant may therefore be worthwhile quantifying in other cell types, and future work will include measuring luciferase properties in other systems such as neural stem cells (Lai et al., 2003). The conversion factor between luciferin consumption and RLU measurement is dependent upon the instrument and should be measured for each experimental system; however, this measurement is not necessary to yield data on the dynamics of relative levels of luciferase expression. The overall model structure, as well as the values of many parameters, are independent of cell type, and the model will therefore have general utility for numerous systems. In particular, it will be useful for converting light output to the rate of luciferase expression, and therefore the level of promoter activity for both promoter activation measurements in signal transduction and gene regulation studies, as well as gene therapy and delivery experiments.

CONCLUSIONS

Firefly luciferase has been used as a reporter gene for a broad variety of applications, including the analysis of promoter activity (Ciana et al., 2003), gene therapy vector efficacy (de Roos et al., 1997; Iyer et al., 2002; Rogers et al., 1997), and dynamic imaging of cell signaling and gene expression (Rutter et al., 1995). The imaging and quantification of luciferase expression in live cells is particularly promising because it allows for nondestructive, real-time analysis of gene expression. However, it is interesting that although this promising real-time analysis of luciferase expression has been conducted numerous times not only in cell culture, but also in living animals (see Greer III and Szalay, 2002, for a review), to our knowledge there has not yet been a systematic and quantitative study of the properties of luciferase enzymatic activity in the context of a living cell. This information is necessary to draw accurate correlations between the measured luminescence signal and the level of intracellular luciferase expression. Regardless of the application, or whether measurements are conducted in cell populations, single cells, or in animals, quantitative information on the behavior of luciferase in the context of living cells is necessary to convert the quantity measured, relative light units or photon counts per time, into the relevant quantity, the relative enzyme activity within the cell. The quantitative data available for luciferase kinetics and activity are from in vitro experiments conducted with purified protein or cell lysate, and alone they do not provide sufficient information for understanding the behavior of the enzyme inside a living cell. We have collected intracellular luciferase activity data to build a predictive model of firefly luciferase's activity in the context of a mammalian cell, and this information will be useful for the real-time monitoring of gene expression and regulation.

We would like to thank Jeannes Tirtadidjaja, Sameera Thakore, and Khoi Thai for technical assistance. This work was partially funded by an ONR Young Investigator Award (to DS), an American Parkinson's Disease Association Pilot Grant, and a University of California Dissertation-Year Fellowship (to JI).

References

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K, editors. 1999. Short protocols in molecular biology: A

compendium of methods from current protocols in molecular biology. New York: Wiley.
Baxter GC, Stanners CP. 1978. The effect of protein degradation on cellular growth characteristics. J Cell Physiol 96:139–145.
Bennett LL, Ward VL, Brockman RW. 1965. Inhibition of protein synthesis in vitro by cycloheximide and related glutarimide antibiotics. Biochim Biophys Acta 103:478–485.
Brooks RF. 1977. Continuous protein synthesis is required to maintain the probability of entry into S phase. Cell 12:311–317.
Brovko LY, Gandelman OA, Polenova TE, Ugarova NN. 1994. Kinetics of bioluminescence in the firefly luciferin-luciferase system. Biochemistry (Moscow) 59:195–201.
Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. 1994. Green fluorescent protein as a marker for gene expression. Science 263:802–804.
Christopher CW, Ishikawa K, Amos H. 1971. Anomalous stimulation of protein synthesis: Evidence for translational control in primary cells. Cell Tissue Kinet 4:75–92.
Ciana P, Raviscioni M, Mussi P, Vegeto E, Que I, Parker MG, Lowik C, Maggi A. 2003. In vivo imaging of transcriptionally active estrogen receptors. Nat Med 9:82–86.
Cubitt AB, Heim R, Adams SR, Boyd AE, Gross LA, Tsien RY. 1995. Understanding, improving and using green fluorescent proteins. Trends Biochem Sci 20:448–455.
de Roos WK, Fallaux FJ, Marinelli AW, Lazaris-Karatzas A, von Geusau AB, van der Eb MM, Cramer SJ, Terpstra OT, Hoeben RC. 1997. Isolated-organ perfusion for local gene delivery: Efficient adenovirus-mediated gene transfer into the liver. Gene Ther 4:55–62.
Eu J, Andrade J. 2001. Properties of firefly luciferase immobilized through a biotin carboxyl carrier protein domain. Luminescence 16:57–63.
Fukuda J, Fujita Y, Ohsawa K. 1983. ATP content in isolated mammalian nerve cells assayed by a modified luciferin-luciferase method. J Neurosci Methods 8:295–302.
Gage FH. 2000. Mammalian neural stem cells. Science 287:1433–1438.
Gandelman O, Allue I, Bowers K, Cobbold P. 1994. Cytoplasmic factors that affect the intensity and stability of bioluminescence from firefly luciferase in living mammalian cells. J Biolumin Chemilumin 9: 363–371.
Gossen M, Bujard H. 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA 89:5547–5551.
Gould SJ, Subramani S. 1988. Firefly luciferase as a tool in molecular and cell biology. Anal Biochem 175:5–13.
Greer III LF, Szalay AA. 2002. Imaging of light emission from the expression of luciferases in living cells and organisms: A review. Luminescence 17:43–74.
Harrison PT, Dalziel RG, Ditchfield NA, Quinn JP. 1999. Neuronal-specific and nerve growth factor-inducible expression directed by the preprotachykinin-A promoter delivered by an adeno-associated virus vector. Neuroscience 94:997–1003.
Iyer M, Berenji M, Templeton NS, Gambhir SS. 2002. Noninvasive imaging of cationic lipid-mediated delivery of optical and PET reporter genes in living mice. Mol Ther 6:555–562.
Lai K, Kaspar BK, Gage FH, Schaffer DV. 2003. Sonic hedgehog regulates adult neural progenitor proliferation in vitro and in vivo. Nat Neurosci 6:21–27.
Lee RT, Denburg JL, McElroy WD. 1970. Substrate-binding properties of firefly luciferase: II. ATP-binding site. Arch Biochem Biophys 141:38–52.
Li X, Zhao X, Fang Y, Jiang X, Duong T, Fan C, Huang C, Kain S. 1998. Generation of destabilized green fluorescent protein as a transcription reporter. J Biol Chem 273:34970–34975.
Michaelis L, Menten ML. 1913. Die Kinetik der Invertinwirkung. Biochem Z 49:333–369.
Mickels AA, Nguyen VT, Konings AW, Kampinga HH, Bensaude O. 1995. Thermostability of a nuclear-targeted luciferase expressed in mammalian cells: Destabilizing influence of the intranuclear microenvironment. Eur J Biochem 234:382–389.
Millart H, Durlach V, Durlach J. 1995. Red blood cell magnesium concentrations: analytical problems and significance. Magnesium Res 8:65–76.
Naylor LH. 1999. Reporter gene technology: The future looks bright. Biochem Pharmacol 58:749–757.
Niswender KD, Blackman SM, Rohde L, Magnuson MA, Piston DW. 1995. Quantitative imaging of green fluorescent protein in cultured cells: Comparison of microscopic techniques, use in fusion proteins and detection limits. J Microsc 180:109–116.
Nunez L, Faught WJ, Frawley LS. 1998. Episodic gonadotropin-releasing hormone gene expression revealed by dynamic monitoring of luciferase reporter activity in single, living neurons. Proc Natl Acad Sci USA 95:9648–9653.
Paddison PJ, Caudy AA, Hannon GJ. 2002. Stable suppression of gene expression by RNAi in mammalian cells. Proc Natl Acad Sci USA 99:1443–1448.
Ray P, Pimenta H, Paulmurugan R, Berger F, Phelps ME, Iyer M, Gambhir SS. 2002. Noninvasive quantitative imaging of protein-protein interactions in living subjects. Proc Natl Acad Sci USA 99:3105–3110.
Robinson AS, Bockhaus SA, Voegler AC, Wittrup KD. 1996. Reduction of BiP levels decreases heterologous protein secretion in *Saccharomyces cerevisiae.* J Biol Chem 271:10017–10022.
Rogers BE, Douglas JT, Ahlem C, Buchsbaum DJ, Frincke J, Curiel DT. 1997. Use of a novel cross-linking method to modify adenovirus tropism. Gene Ther 4:1387–1392.
Rutter GA, White MR, Tavare JM. 1995. Involvement of MAP kinase in insulin signaling revealed by non- invasive imaging of luciferase gene expression in single living cells. Curr Biol 5:890–899.
Stein WD. 1986. Transport and diffusion across cell membranes. Orlando, FL: Academic Press. 685 p.
Taniyama Y, Tachibana K, Hiraoka K, Aoki M, Yamamoto S, Matsumoto K, Nakamura T, Ogihara T, Kaneda Y, Morishita R. 2002. Development of safe and efficient novel nonviral gene transfer using ultrasound: enhancement of transfection efficiency of naked plasmid DNA in skeletal muscle. Gene Ther 9:372–380.
Thompson JF, Hayes LS, Lloyd DB. 1991. Modulation of firefly luciferase stability and impact on studies of gene regulation. Gene 103:171–177.
Tsien RY. 1998. The green fluorescent protein. Annu Rev Biochem 67:509–544.
Vitvitsky VM, Ataullakhanov FA, Sinauridze EI. 1992. ATP monitoring in human red blood cells with luciferase introduced intracellularly. Adv Exp Med Biol 326:149–156.
White MR, Masuko M, Amet L, Elliot G, Braddock M, Kingsman AJ, Kingsman SM. 1995. Real-time analysis of the transcriptional regulation of HIV and hCMV promoters in single mammalian cells. J Cell Sci 108:441–455.
Wilson T, Hastings JW. 1998. Bioluminescence. Annu Rev Cell Dev Biol 14:197–230.
Wu JC, Sundaresan G, Iyer M, Gambhir SS. 2001. Noninvasive optical imaging of firefly luciferase reporter gene expression in skeletal muscles of living mice. Mol Ther 4:297–306.