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Chapter 5

BIOLUMINESCENCE MICROSCOPY IN LIVE CELLS: CONSIDERATION OF EXPERIMENTAL FACTORS AND PRACTICAL RECOMMENDATIONS

Hirobumi Suzuki[1,*], Ryutaro Akiyoshi[1] and Katsunori Ogoh[1]

[1]Evaluation Technology Department 1, Olympus Corporation, Hachioji, Tokyo, Japan

ABSTRACT

Since the firefly luciferase gene was cloned, luciferase has been used as a reporter enzyme in luminometer-based bioluminescence assays to measure the activity of specific gene promoters *in vitro*. Recently, microscopy-based luciferase reporter assays of gene promoter activity have been performed in live cells. However, live-cell luciferase reporter assays have been quantitatively analyzed and interpreted using enzymatic kinetic properties of luciferase that were determined *in vitro*. Since *in vitro* buffers do not recapitulate the intracellular milieu, the kinetics of luciferase activity *in vitro* are also likely different from that in live cells, and the results of live-cell luciferase reporter assays should be interpreted

* Corresponding Author Email: hirobumi2_suzuki@ot.olympus.co.jp.

with caution. Herein, we discuss experimental factors that should be considered when imaging luciferase activity in live cells, including choosing among commercially available beetle luciferase enzymes based on their kinetic and bioluminescence properties.

Keywords: bioluminescence microscopy, beetle luciferase, gene promoter assay, live cells

1. INTRODUCTION

Bioluminescence is a special form of enzyme-catalyzed chemi-luminescence found in living organisms. In beetles, bioluminescence involves the conversion of luciferin (photon-emitting substrate) into adenylated luciferin (luciferyl-AMP as an enzyme-bound intermediate) by luciferase, which requires Mg^{2+}-ATP. Adenylated luciferin is then oxidized by molecular oxygen (O_2) to create oxyluciferin (another enzyme-bound intermediate), which exists in a high-energy state. A photon is emitted during the transition from the unstable high-energy state to the ground state [1].

Since the firefly luciferase gene was first cloned in the late 1980s, photon-counting luminometer methods have been used to quantify the *in vitro* activity of specific gene promoters that drive the expression of luciferase as a reporter enzyme [2-4]. More recently, gene promoter activity has been measured in live cells using luciferase-based bioluminescence microscopy, which is now possible with ultra-low-light imaging cameras such as liquid nitrogen-cooled charge-coupled device (CCD) cameras, photon-counting CCD cameras, or image-intensifying CCD cameras [5-13]. In addition, recent advances in bioluminescence imaging technology, such as with electron multiplying CCD (EM-CCD) cameras, provide higher sensitivity and image quality than previous ultra-low-light imaging cameras. In addition, short-focal-length imaging lenses [14-15] facilitate bioluminescence imaging of single cells, which has now been used in several research fields [16-33]. Live-cell bioluminescence imaging studies have revealed heterogeneous gene expression to the same stimulus among genetically identical individual cells.

The enzymatic activity of luciferase, which is indirectly measured as luminescence, is generally quantified by correlating light intensity to the chemical concentration of the reaction components (i.e., standard curve), which include ATP, luciferin, or luciferase. A standard curve is generated by varying only one of the reaction components. In live cells, luciferase activity is

quantified by measuring luminescence and using the following assumptions: the intracellular concentrations of reaction components (ATP, luciferin, O_2, and Mg^{2+}) are constant (except for luciferase), and the *in vitro* and *in vivo* kinetic properties of luciferase, such as its affinity for substrate ($1/K_M$), are similar or the same. However, the latter assumption is likely not true in all cases. For instance, Ignowski and Schaffer (2004) showed that the affinity of luciferase for luciferin was 100-fold lower in cells than *in vitro* [34]. This finding underscores the fact that although the *in vitro* kinetic properties of luciferase have been extensively characterized, its *in vivo* kinetic properties remain poorly characterized. Therefore, we discuss experimental factors to consider when imaging gene promoter activity in live cells and provide practical recommendations of parameter choices and ranges, including of commercially available beetle luciferase enzymes based on their kinetic and bioluminescence properties.

2. CONSIDERATION OF FACTORS THAT AFFECT BEETLE LUCIFERASE ASSAYS IN LIVE CELLS

2.1. Cell Permeability of Luciferin

We first examined the cell permeability of luciferin because luciferin, which is added to the cell culture medium, is accessible to transiently expressed luciferase only by its influx. Figure 1 shows the time course of luminescence intensity in HeLa cells, transiently expressing click beetle luciferase (pCBG99 control vector, Promega, Madison, WI, USA), that were cycled between Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) with or without 500 mM D-luciferin (Promega). HeLa cells were cultured on 35-mm glass-bottom dishes, and luminescence intensity was measured with a Kronos luminometer (AB-2500, Atto, Tokyo, Japan) at 1 min intervals for a total of 70 min with a photon integration time of 10 s at 35°C. After exchanging to medium containing luciferin, luminescence intensity increased to near-peak values within 1 min, and after exchanging to medium without luciferin, luminescence intensity decreased to the background level within 1 min. Thus, luciferin is highly cell permeable and can enter and exit cells within 1 min.

2.2. Luciferin Concentration in Cells

We estimated the intracellular and extracellular concentrations of luciferin by laser scanning confocal microscopy, which can be used to image the same confocal volume inside or outside of HeLa cells [35].

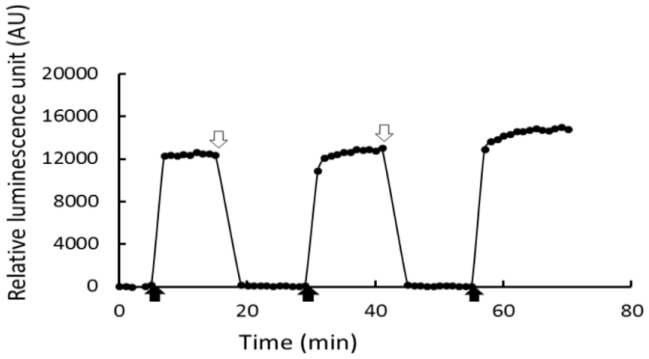

Figure 1. Time course of luminescence intensity in HeLa cells transiently expressing CBG99 click beetle luciferase and cycled between DMEM with or without 500 mM D-luciferin. Black and white arrows indicate exchange of medium to DMEM with or without 500 mM D-luciferin, respectively. Nearly all of the changes in bioluminescence associated with the influx and efflux of luciferin occur within 1 min.

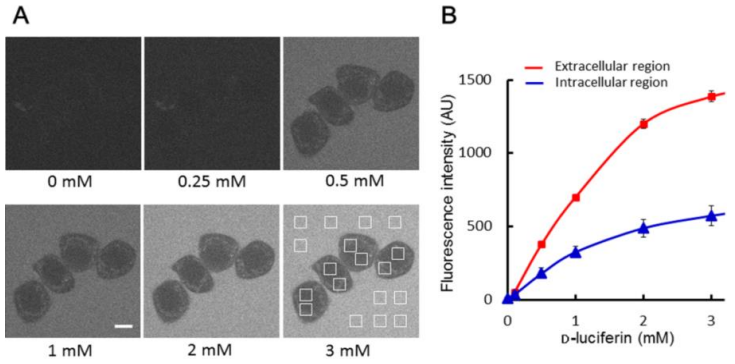

Figure 2. Estimation of intracellular luciferin concentration. A: Confocal fluorescence images of HeLa cells cultured in PBS containing 25 mM HEPES and D-luciferin ranging from 0 to 3 mM. Eight and ten equally sized square regions were drawn inside and outside of cells, respectively. Scale bar = 10 μm. B: Average luminescence intensities of regions inside and outside of cells (shown in panel A) were plotted relative to the luciferin concentration added to the culture medium. Data are shown as the mean ± SD. This figure was reproduced from ref. [35] in accordance with Wiley's open access terms and conditions.

Figure 2A shows confocal fluorescence images of HeLa cells cultured in phosphate-buffered saline (PBS) containing between 0 to 3 mM D-luciferin. The average fluorescence intensities of eight and ten equally sized square regions that were drawn inside and outside of cells, respectively, were plotted relative to the input luciferin concentration in the culture medium (Figure 2B). Although the quantum yield of luciferin fluorescence increases in alkaline conditions (pK_a = 8.25) [36], the intracellular and extracellular pH values are likely the same under the culture conditions tested; therefore, the quantum yield of luciferin fluorescence is likely the same inside and outside of cells. As shown in Figure 2B, the intracellular luciferin concentration was approximately half that of the extracellular concentration [35].

Expression of luciferin transporter proteins (ATP binding cassette transporter proteins, multidrug resistance proteins, organic anion transporter proteins) was reported to increase intracellular luminescence [37-39]. Thus, the extracellular-to-intracellular concentration gradient of luciferin is regulated not only by simple diffusion but also by transporter proteins.

2.3. Intracellular Luciferin Concentration Required for Live-Cell Luciferase Reporter Assays

We estimated the K_M value of Luc+ firefly luciferase (Promega) for luciferin by fitting data with a Michaelis-Menten model using the least squares method [35]. The K_M value of the luciferin-luciferase interaction *in vitro* is ~15.7 μM (Figure 3). Assuming that *in vitro* K_M values are similar to *in vivo* K_M values, an intracellular luciferin concentration of at least twice the *in vitro* K_M value of the luciferin-luciferase interaction (i.e., 32 μM luciferin in cells or 64 μM in the culture medium) is required for live-cell luciferase reporter assays. Therefore, under this assumption, D-luciferin concentrations higher than 100 μM in the culture medium should be sufficient for imaging luciferase activity in live cells [35].

However, Ignowski and Schaffer (2004) showed that the affinity of firefly luciferase for luciferin in HEK 293T cells was 100-fold lower (K_M = 1.6 mM) than that *in vitro* (K_M = 0.2–10 μM) [34, 40]. Patrick et al. (2014) also reported that the affinity of Luc2 luciferase for D-luciferin was 422 and 57 μM in HEK 293T cells and cell extracts, respectively [39]. This contrasts with the temperature-dependence of the affinity of luciferase for D-luciferin, which increases from 25 to 37°C (Table 1).

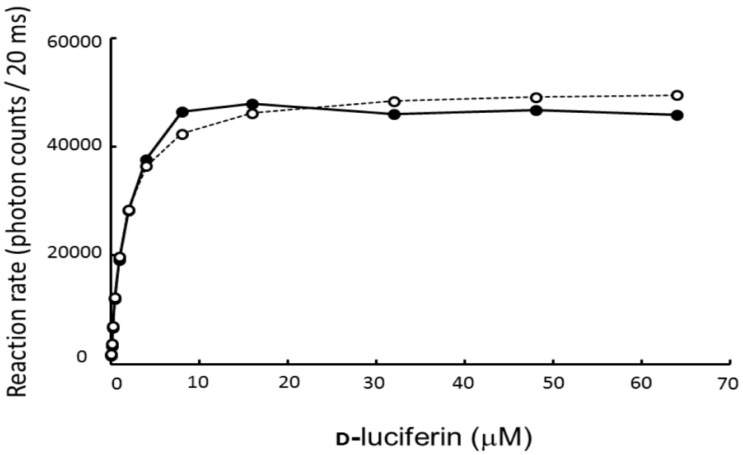

Figure 3. Determining K_M of luciferase for luciferin. The reaction rate (ordinate), which was estimated by the number of photon counts in 20 ms time gates, was plotted relative to the input luciferin concentration (abscissa). Closed and open circles show measured and expected values, respectively. Data are shown as the mean ± SD (N = 6).

In our studies, luminescence intensity increased in HeLa cells expressing Luc+ luciferase at luciferin concentrations ranging from 100 to 1000 μM in the culture medium (data not shown). This indicates that 100 μM luciferin, which is estimated to saturate luciferase enzymes in live cells based on the *in vitro* K_M value, is actually subsaturating. Therefore, for live-cell luciferase reporter assays, we add at least 1,000 μM of luciferin to the culture medium [41].

2.4. ATP Concentration in Cell

Traut (1994) reported that the intracellular ATP concentration is 2–3 mM [42]. K_M values of commercially available luciferases for ATP are 22–230 μM at 37°C *in vitro* (Table 1). The affinity of luciferase for ATP was estimated using similar methods as those described [35] to determine the affinity of luciferase for luciferin but with slight modifications: 50 mM Tris-HCl (pH 8.0) buffer containing 50 μg/mL of partially purified luciferase, 1 mM D-luciferin, 4 mM $MgSO_4$, and ATP (3–1556 μM) at 25°C and 37°C.

Table 1. A list of commercially available beetle luciferase reporter vectors for bioluminescence microscopy with their estimated kinetic properties

Luciferase	Provider	Origin (Species)	λmax (nm) *in vitro* at 25°C (upper line) or 37°C (lower line)			λmax (nm) in HeLa cell at 25°C (upper line) or 37°C (lower line)	Km (μM) for D-luciferin at 25°C (upper line) or 37°C (lower line)	Km (μM) for ATP at 25°C (upper line) or 37°C (lower line)
			pH 6	pH 7	pH 8		pH 8	pH 8
Luc2	Promega	Firefly (*Photinus pyralis*)	609 (25°C) 613 (37°C)	567 598	562 566	569 605	27.1 21.2	72.6 21.6
CBG99	Promega	Click beetle (*Pyrophorus plagiophalam*)	550 552	550 552	550 552	548 549	1.5 0.5	55.4 33.1
CBR	Promega	Click beetle (*Pyrophorus plagiophalam*)	617 618	617 618	617 618	618 618	34.3 28.8	45.0 26.6
ELuc	Toyobo	Click beetle (*Pyrearinus termitilluminans*)	540 544	540 544	541 544	542 542	20.6 12.2	279.6 230.3

The intracellular ATP concentration is likely sufficient for live-cell luciferase reporter assays but only under the assumption that the affinity of luciferase for ATP is similar *in vitro* and in cells. Therefore, it is important to measure the affinity of luciferase for ATP in cells directly.

2.5. Wavelength Spectra of Bioluminescence in Cells

With decreasing pH or increasing temperature, the peak emission wavelength and quantum yield of bioluminescence from *in vitro* purified firefly luciferase, but not from click beetle luciferase [45-46], is shifted to a longer wavelength and reduced, respectively [43-44]. The temperature-dependent shift in the peak emission wavelength has also been confirmed in live cells [35, 47]. It is important to consider environment-dependent shifts in the peak emission wavelength when using different combinations of luciferase enzymes as reporters in multiplexed assays.

Figure 4 shows wavelength spectra of bioluminescence in U2OS cell lines expressing Luc2 firefly luciferase (Figure 4A) or CBG99 click beetle luciferase (Figure 4B) [15]. U2OS cells were cultured in DMEM containing 10% FBS and incubated in a humidified 5% CO_2 incubator at 25°C or 37°C. The peak emission wavelength of luminescence in Luc2-expressing cells shifted from yellow (597 nm) at 25°C to orange (609 nm) at 37°C, and the peak intensity increased with temperature. In contrast, the peak emission wavelength of luminescence in CBG99-expressing cells (549 nm) did not shift with temperature, although the peak intensity also increased with temperature. Therefore, when using firefly luciferase for multiplexed imaging assays, the temperature of the experiment should be considered when choosing the appropriate set of optical filters.

3. COMMERCIALLY AVAILABLE BEETLE LUCIFERASE REPORTER VECTORS

Luciferase genes are commercially available as reporter vectors. For reference, we have provided a list of commercially available beetle luciferase reporter vectors, which are suitable for bioluminescence microscopy, and their estimated kinetic properties in Table 1. Luciferase genes were cloned from firefly (Luc2) or various click beetles (CBG99, CBR, and ELuc).

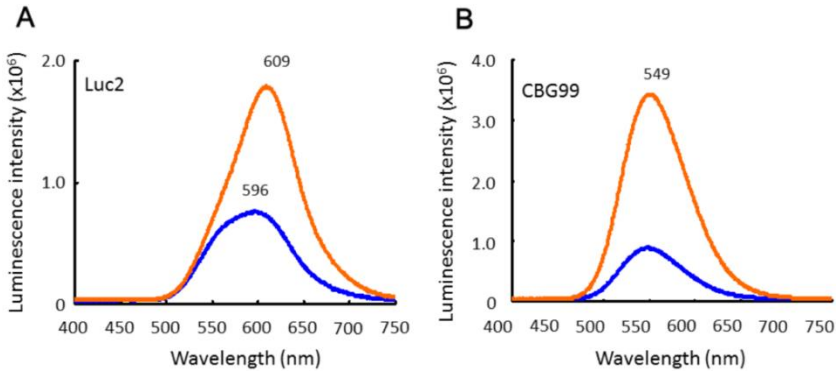

Figure 4. Wavelength spectra of bioluminescence in Luc2- and CBG99-expressing U2OS cell lines at 25°C and 37°C. U2OS cells were cultured in DMEM containing 10% FBS and incubated in a humidified 5% CO_2 incubator, and 1 mM D-luciferin was used as the substrate. Peak emission wavelength of luminescence in cells expressing Luc2 shifted from 596 nm at 25°C (blue line) to 609 nm at 37°C (orange line). Peak luminescence intensity in both Luc2- and CBG99-expressing cells increased with increasing temperature. This figure was partially reproduced from ref. [15] in accordance with Wiley's open access terms and conditions.

The peak emission wavelength of luminescence from firefly luciferase, but not from click beetle luciferase, is shifted to a longer wavelength at lower pH *in vitro* and at higher temperatures in cells. K_M values of luciferase for luciferin at 37°C ranged from 0.5 μM to 28.8 μM, and were slightly lower at 25°C. The K_M value of ELuc for ATP at 37°C is a log-order greater (230 μM) than that of other luciferases for ATP at the same temperature (22–33 μM).

CONCLUSION

Gene expression is a fundamental cellular process that underlies diverse cellular behaviors, including proliferation and differentiation. Bioluminescence assays using the luciferase enzyme have provided insight into gene promoter activity and its regulation *in vitro* and in cells. Bioluminescence imaging is a powerful new tool to concomitantly observe morphological changes and determine gene promoter activity at the single cell level. However, live-cell luciferase reporter assays are currently interpreted based on the assumption that *in vitro* and *in vivo* luciferase enzyme kinetics are similar or the same. Therefore, we have investigated experimental factors

that affect luciferase-based bioluminescence imaging in live cells, and recommended parameter ranges for live-cell luciferase reporter assays. Our studies underscore the importance of directly measuring luciferase enzyme kinetics in live cells.

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BIOGRAPHICAL SKETCH

Hirobumi Suzuki

Affiliation: Evaluation Technology Department 1, Olympus Corporation

Education: Doctor of Science in Biology, March 1997. Graduate School of Science, Tokyo Metropolitan University.

Research and Professional Experience: firefly biology, bioluminescence microscopy

Professional Appointments: Deputy General Manager

Honors: Zoological Science Award in 1989, the Zoological Society of Japan.

Publications Last 3 Years:

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