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Simultaneous Multicolor Luciferase Reporter Assays for Monitoring of Multiple Genes Expressions

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Abstract: Multicolor luciferase reporter assays are powerful tools for simultaneously monitoring the expression of several genes. Dual-color luciferase reporter assays were established in 2004 and have been used in broad range of application from basic researches to applications in clinical research, e.g., drug screening and alternative toxicological tests. The advantage of this method is that it can be used to simultaneously monitor sequential and complex changes in the expression of multiple genes from the single-cell level to the tissue level. In this review, I focus on the simultaneous analysis of multiple genes using multicolor beetle luciferases.

Keywords: Beetle luciferase, bioluminescence, multicolor luciferase assay, imaging luciferase assay, reporter assay.

INTRODUCTION

Bioluminescence is a form of "cold light" in living organisms, based on the chemical reaction; that is, the measured light activity depends on the amount of enzyme, substrate, and cofactor(s). Bioluminescence reporters, such as luciferase (enzyme) and luciferin (substrate), are widely used for the analysis of various biological functions, such as protein-protein interactions, and gene expression [1-3]. The structural relationship between luciferin and luciferase is restricted in each bioluminescence reaction. Additionally, bioluminescence can be reproduced *in vitro* using a chemically synthesized luciferin reporter and a genetically produced luciferase enzyme. The bioluminescence system comprising these two components produces a unique light source used widely in the field of biotechnology for visualizing biological functions. Thus, luciferases are good candidate reporter enzymes in cell-based assays. Advanced biotechnologies using multicolor luciferases permit the quantitative analysis of multiple genes from the single-cell level to the tissue level using a luminometer/charged-coupled device (CCD) camera equipped with optical filters. Bioluminescent protein techniques are sensitive approaches for understanding cell physiology, whereas fluorescence imaging that use fluorescent proteins (e.g., green fluorescent protein [GFP]) as probes have contributed to the understanding of cell physiology researches. Especially, bioluminescent probes are highly sensitive, display good linearity, and are easy to perform compared with other fluorescent probes. Beetle luciferases are particularly suitable as reporter enzyme because they produce stable color lights ranging from green to red under any condition; in contrast, the light produced by firefly luciferases can change depending on pH or temperature. In this review, I discuss the simultaneous analysis of multiple genes using multicolor beetle luciferases.

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FIREFLY AND BEETLE BIOLUMINESCENCE

Bioluminescence is a well-known phenomenon. In the presence of firefly and beetle luciferases as a catalytic enzyme and ATP and Mg^{2+} as cofactors, the bioluminescence reactions produc photons (Fig. **1**) [4-6]. In light-emitting reactions, the emission maxima of firefly D-luciferin-type bioluminescence are found at around 535-630 nm (Fig. **2A**, **B**). Beetle bioluminescence is also known to produce much higher quantum yield (QY) values than other types of luminescence [7, 8]. The QY value of native firefly D-luciferin varies from 0.15 to 0.60 depending on the luciferase enzyme; for example, the green-yellow-emitting luciferase of the American firefly (*Photinus pyralis*) has a QY value of 0.41. Moreover, the QY value of the firefly luciferase is correlated with the luminescence peak wavelength. The red-emitting luciferase of the Brazilian railroad worm (Phrixothrix hirtus), which has the reddish peak wavelength, has the lowest QY (0.15), whereas the green-emitting luciferase of the Brazilian click beetle (Pyrearinus termitilluminans), whose luminescence spectrum has the greenish peak of all reported luciferases, possesses the highest quantum yield (0.61) [9].

Interestingly, beetle bioluminescence, including that of fireflies, can be classed into two large groups according to bioluminescent spectral sensitivity to pH: 1) pH-sensitive luciferases (fireflies) that exhibit a red color shift with increasing temperature or decreasing pH [4, 5]; and 2) pH insensitive (railroad worm and click beetle) luciferases that are not affected by these changes in pH and temperature [9, 10]. Additionally, the spectra of pH-insensitive beetle luciferases are narrower than those of pH-sensitive firefly luciferases, although many color mutants have still been constructed. For example, Fig. (**2**) shows the corrected luminescence spectra for pH-insensitive (Fig. **2A**) and -sensitive luciferases (Fig.

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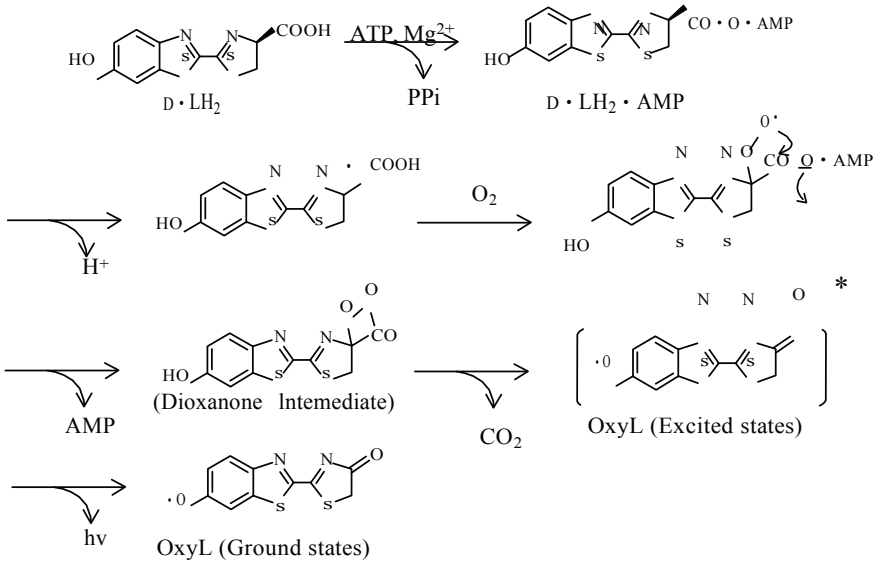

Fig. (1). Proposal mechanism of chemical reaction in firefly bioluminescence. Luciferin (D-LH_2) is converted into an adenylate (D-LH_2-AMP) in the presence of ATP, which is oxidized in the presence of oxygen, forming a peroxide intermediate by splitting of AMP. Decomposition of the intermediate *via* the dioxetanone intermediate is sufficiently energetic to produce the excited state of the oxyluciferin (OxyL) monomer or dianion, and then to produce the light from excited state to ground state.

2B). The spectra of the multicolor luciferase mutants are broader than those of pH-insensitive red and green light-emitting luciferases [9]. For the various beetle luciferases, the QY values and spectra of the bioluminescence reaction have been analyzed in order to explain their relationships.

The mechanisms mediating the different color emissions of beetle bioluminescence were proposed based on active site determined by the 3D-structure of the *Luciola cruciata* firefly luciferase [11]. For example, Hirano *et al*. investigated the spectroscopic properties of the phenolate anion of firefly luciferin and proposed that the excited luciferin as a light emitter was modulated by the polarity of the active-site environment of firefly luciferase and protonated basic moiety in the active site [12]. On the other hand, the emitter of firefly bioluminescence may relate to the lability of firefly oxyluciferin. Maltsev *et al*. explained that its lability is due to autodimerization of the coexisting enol and keto forms in a Mannich-type reaction based on NMR spectroscopy and X-ray crystallography data of a side product [13]. Nazivet *et al*. demonstrated that emitting light depends on the micro-environmental polarity at the phenolate/phenol of the benzothiazole fragment in oxyluciferin, and furthermore, denied that the color modulation of the emitting light depends on the size of the compact luciferase protein which is a cavity embedding the excited oxyluciferin molecule [14]. Nazivet *et al*. also demonstrated that based on a model of Cypridina oxyluciferin and coelenteramide, carbonyl group of firefly oxyluciferin or the different chemical environment of the dioxetanone is more rigid in the firefly bioluminescence

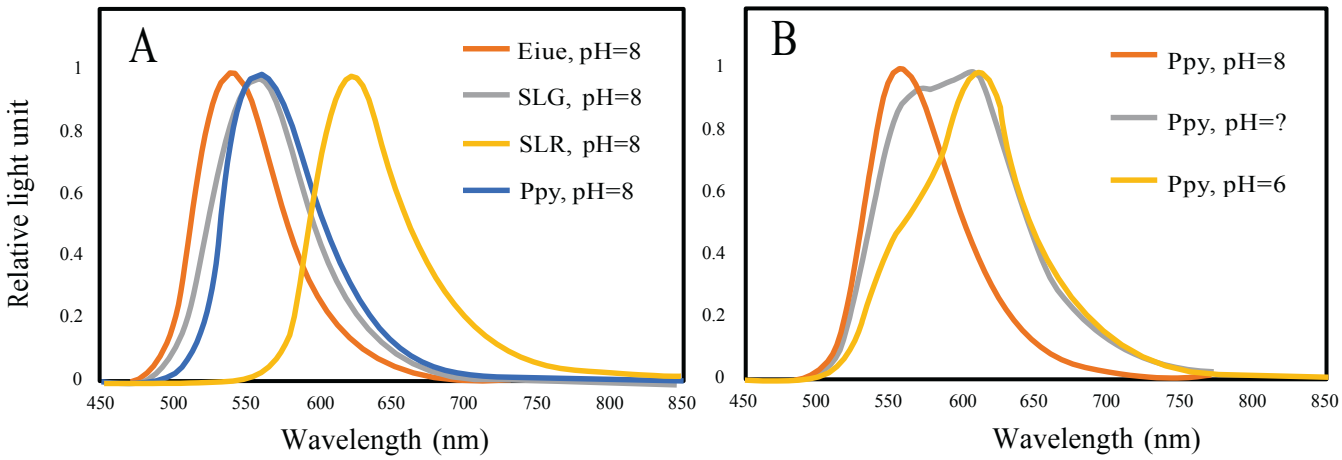

Fig. (2). Luminescence spectra of bioluminescence reactions for various beetle luciferases. Spectra were measured using PicaGene reagent at 24°C, corrected for spectral sensitivity of the sphere spectrometer, and normalized at each luminescence maximum [9]. Ppy; *Photinus pyralis* luciferase, ELuc, SLG, SLR (see Table **1**).

system [15]. However, the mechanisms mediating the pH sensitivity (or insensitivity) of the beetle luciferase enzyme remain unknown. In particular, the mechanism of pH insensitivity in the beetle luciferases is not clear because the 3D-structure of pH-insensitive luciferases has not been determined. Thus, improving our understanding of the mechanisms of beetle bioluminescence could facilitate the development of novel luciferase assays and biological applications of luciferases in a variety of research fields.

CELL-BASED MULTICOLOR LUCIFERASE REPORTER ASSAYS

Fig. (**3**) demonstrates a simple luciferase reporter assay. In general, the reporter plasmid containing the luciferase gene plus the target promoter region of interest is transfected into target cells, and luciferase-expressing cells are lysed after an appropriate period, e.g., 1–2 days. We can measure the amount of expressed luciferase as a light signal *in vitro*, and we can estimate the target promoter activity as a light intensity. Table **1** summarizes the characteristic properties of bioluminescence systems based on commercially available luciferases from beetles, sea pansy, copepods, and ostracods. Commercially available luciferins comprise only three types: firefly D-luciferin, coelenterazine and *Cypridina* luciferin, although other luciferins, including dinoflagellate [16] and *Latia* luciferins [17], have been identified. In light-emitting reactions, the emission maxima of firefly D-luciferin-type, coelenterazine-type, and *Cypridina* luciferin-type bioluminescence are found at around 535–630, 460–480, and 460 nm, respectively. The molecular weights of luciferases vary widely (20–62 kDa), and their molecular structures, which originate from phylogenetically distant systems, belong to different super families. *Cypridina* [18], *Gaussia* [19], and *Metridia* [20] luciferases (Cluc, GLuc, and MetLuc, respectively) are secreted enzymes. The luciferin-luciferase reaction is triggered by adding luciferin, although the bioluminescence of firefly, click beetle and railroad worm luciferases requires ATP and magnesium ions as cofactors.

Advances in the luciferase assay system have an additional luciferase as an internal control reporter (dual-reporter assay), thereby minimizing inherent experimental variability that can undermine experimental accuracy, such as differences in the number and viability of cells used and the efficiency of cell transfection and lysis. Thus, the first generation of dual-reporter assays is a combination of firefly luciferase and sea pansy *Renilla* luciferase using firefly D-luciferin-type and coelenterazine-type bioluminescence. Of the luciferases identified to date, the firefly luciferase from *P. pyralis* is the commonly used bioluminescent reporter in commercial vectors. Only the expression of one gene or one target event can be monitored at a time, although this luciferase has been extensively used to monitor cellular events in cell-based assays and *in vivo* imaging [21-23].

Gene expression events are both complex and sequential because of the elaborated regulatory pathways found in living cells. Many researchers have sought new reporter assay systems, focusing on the characteristics of multicolor beetle luciferases. Advanced luciferase technology, involving progressives in both the luciferase and the detection equipment, as well as newly cloned luciferase genes, have allowed us to simultaneously monitor the expressions of multiple genes when luciferases are used that induce different color emission spectra in the catalysis of a single D-luciferin substrate. The advantages of beetle luciferases producing multiple colors are as follows: (1) the colors are separable with an optical filter; (2) the number of substrates is minimal; (3) the temperature dependences are similar; and (4) the half-lives are similar (Fig. **4**). Thus, these mixed emission spectra are measured simultaneously. Each intensities can be quantified by splitting them with optical filter(s) [24].

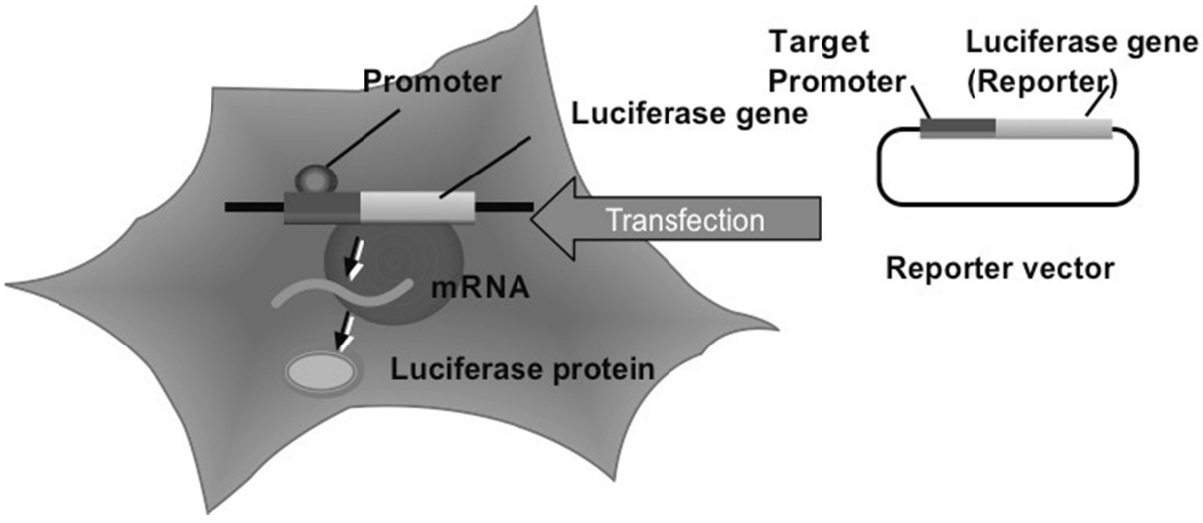

Fig. (3). Principle of a simple luciferase reporter assay. The reporter plasmid vector consists of the target promoter sequence and luciferase gene sequence. After transfection of the plasmid into target cells, the promoter region regulates the expression of luciferase gene in living cells. The expressed luciferase protein catalyzes a reaction with luciferin to produce light. In the transient transfection luciferase assay, luciferase-expressing cells are lysed for an appropriate period. The amount of expressed luciferase protein can be estimated from the light intensity which indicates the promoter activity in living cells. In this case, the promoter activity is normalized by cell numbers or cellular enzymatic activity.

Table 1. Summary of characteristic properties of commercialized bioluminescence system.

Organism	Gene Symbol	Luciferin	Mass (kDa)	λmax (nm)	Main Company
Non-Secreted					
Firefly	luc(+), luc2	firefly luciferin	61	562	Promega
Sea pansy	Rluc	coelenterazine	36	480	Promega
Click beetle (Jamaica)	CBGluc	firefly luciferin	60	537	Promega
Click beetle (Jamaica)	CBRluc	firefly luciferin	60	613	Promega
Click beetle (Brazil)	ELuc	firefly luciferin	61	638	TOYOBO
Railroad-worm	SLR	firefly luciferin	60	630	TOYOBO
Railroad-worm (Japan)	SLG	firefly luciferin	60	550	TOYOBO
Railroad-worm (Japan)	SLO	firefly luciferin	60	580	TOYOBO
Secreted					
Copepoda	GLuc	coelenterazine	20	480	NEB
Copepoda	MetLuc	coelenterazine	24	480	Clontech
Ostracod	Cluc	cypridinid luciferin	61	465	NEB

APPLICATION OF CELL-BASED MULTICOLOR LUCIFERASE REPORTER ASSAY

In the first example of dual-color luciferase assays in 2004, Kitayama *et al*. constructed a simple dual-reporter system monitored simultaneously two promoter activities in living cyanobacterial cells [24]. Two Phrixothrix railroad-worm luciferases [10] catalyzing the generation of different color emissions served as the dual reporters; each emissions was separated by interference filters to estimate the individual emission signals using photomultiplier tubes. Using this system, they clearly demonstrated the expression profiles between promoters in the same cells.

As a second example, Nakajima *et al*. developed a simultaneous monitoring system [25] in mammalian cell line using green light emitting luciferase (SLG, see Table **1**) and red light-emitting luciferase (SLR, see Table **1**) from the Phrixothrix railroad worm. The two spectral mixed emissions were divided using a > 600-nm long-pass filter, and the respective luciferase activities were calculated. Splitting the emissions with a long-pass filter is advantageous in that the emission loss is less than when interference filters are used. The linear response range of this system using cell extracts which are expressed the green and red light-emitting luciferases could be estimated to be more than two orders of magnitude.

Furthermore, Nakajima *et al*. established a tricolor reporter assay using SLG, orange-emitting luciferase (SLO, see Table **1**), and SLR [26]. Because all of these enzymes emit light with D-luciferin, their respective activities can be detected in a one-step reaction from a single sample. Using this method, they estimated that the linear response range of the system exceeds two orders of magnitude, although the low-threshold light intensities require one order of magnitude higher intensity than those estimated in the dual-color luciferase assay. Using this system, they introduced SLG as an internal control reporter and measured its activity separately from those of SLO for *Bmal1* and SLR for the part of *Bmal1* promoters for retinoic acid receptor-related orphan receptor α response element (RORE) (Fig. **5**). They demonstrated a simultaneous monitoring of the clock genes expressions *in vitro* and clarified the role of retinoic acid receptor-related orphan receptor α (RORα) in the transcriptional regulation of the clock genes *Bmal1* and RORE in mammalian cell.

Since 2005, many researchers have published papers describing the application of the dual-color luciferase assay. Branchini *et al*. developed a model system for the dual-color luciferase assay by combination of green light-(Val241Ile/Gly246Ala/Phe250Ser, λmax = 549 nm) and red (Ser284Thr, λmax = 615 nm) emitting firefly luciferase mutants. They divided the emissions using two band-pass filters and confirmed that the green light-emitting GST fusion protein could be measured over a 10,000-fold range from about 20 amol to 200 fmol when the amount (10 fmol) of red light-emitting GST fusion protein was kept constant in the crude cell extracts [27]. Moreover, based on the results, they generated thermostable firefly luciferase mutants and quantified both luciferase activities at 1.0 fmol in a mixture using the microplate luminometer format [28]. Using a similar approach, Michelini *et al*. simultaneously monitored the expression of two genes in cell extracts using two beetle luciferases (green light-emitting luciferase from *P. pyralis* [λmax = 560 nm] and red light-emitting luciferase from *Luciola italica* [λmax = 613 nm]), which emit light *via* reaction with firefly D-luciferin as the experimental reporter. The latter enzyme is a mutant of the wild-type form, displaying a red-shift emission and better thermostability [29].

Furthermore, in plant cells, Ogura *et al*. used reporter plasmids harboring the green light-emitting luciferase from the Jamaican click beetle *Pyrophorus plagiophthalamus* (CBG; λmax = 537 nm) connected with the chlorophyll *a/b* binding protein (*Cab*) promoter and the red light-emitting luciferase from *P. plagiophthalamus* (CBR; λmax = 613 nm) connected with the cauliflower mosaic virus promoter as a control. They co-injected these plasmids into plant cells

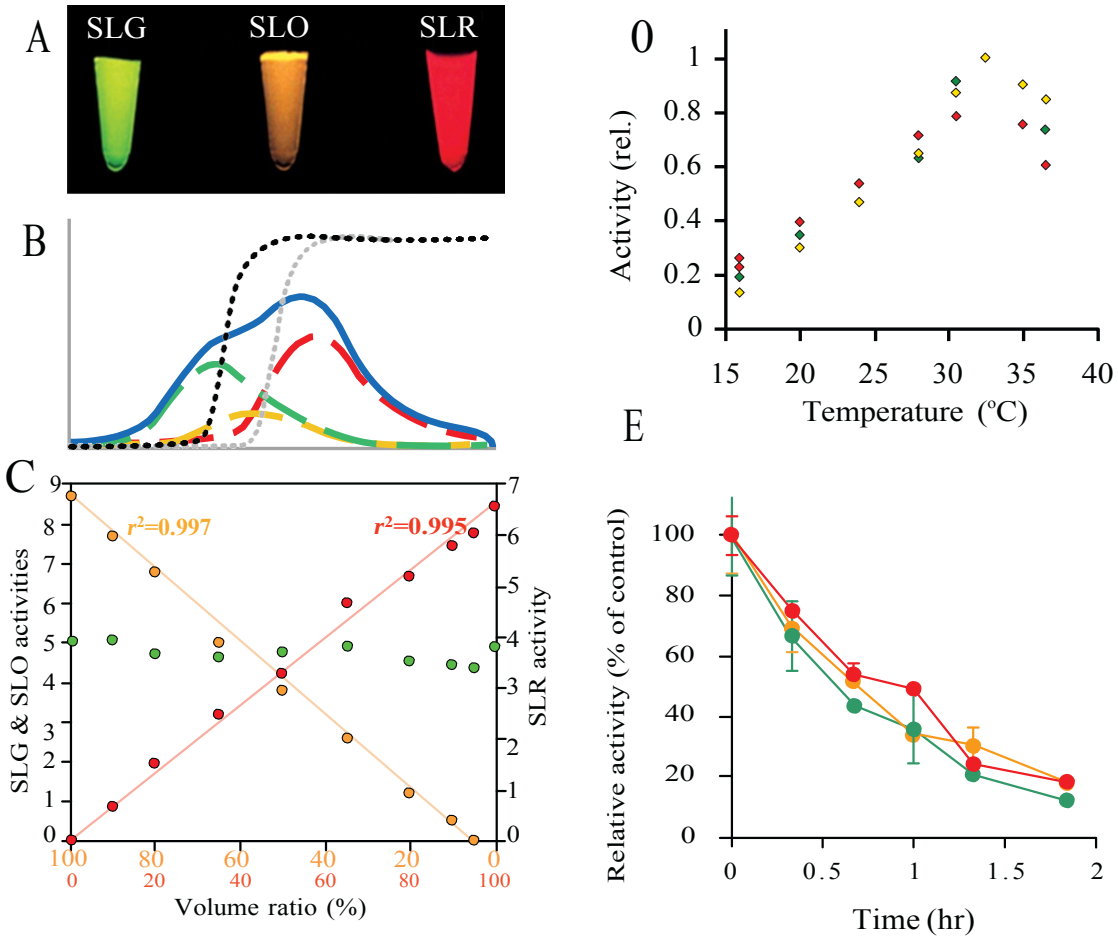

Fig. (4). Characteristic properties of SLG, SLO, and SLR. **A**; Bioluminescence color of SLG, SLO, and SLR. **B**; Bioluminescence spectra example (blue line) of mixture of SLG, SLO, and SLR and individual spectra of SLG (green dotted line), SLO (orange dotted line) and SLR (red dotted line) luciferases, and the transmission spectra of > 560 nm (O56, black dotted line) and > 600 nm (R60, gray dotted line) long-pass filters. **C**; Quantitative relationship among SLG (green circle), SLO (orange circles), and SLR (red circles) activities in a mixture of each proteins expressed in silkworm. Each samples were diluted with PicaGene Dual lysis buffer (Toyo B-net, JP) at the indicated volume ratio were mixed. The respective luciferase activities were measured with an AB2250 luminometer (ATTO, JP) after injecting PicaGene. RLU, relative light unit. **D**; Temperature dependence of SLG (green circle), SLO (orange circles), and SLR (red circles) activities of each proteins. Luminescence activities under different temperatures were measured as followings. 5 ∝L of each luciferases solution (0.1 ∝g/mL) at 4°C was mixed with 50 uL of Tripluc Luciferase Assay Reagent (TOYOBO, JP) that was pre-incubated at 16, 20, 24, and 28°C for 10 min. All the apparatus except for the luminometer at room temperature were also incubated at each temperatures in advance. **E**; Half-lives of PEST (rapid degradation sequence) sequence fused-SLG (green circle), -SLO (orange circles), and -SLR (red circles) activities. Functional half-life of PEST-fused SLG, SLO and SLR luciferases in NIH3T3 cells. Expression plasmids were independently transfected into NIH3T3 cells. Forty hours after transfection, the culture medium was replaced with DMEM supplemented with 10% FBS and 100 µM cycloheximide, and incubated for 30 min to block protein synthesis. After 30 min (time = 0), incubation was continued in the same medium. At the indicated periods, the cells were disrupted and measured their activities in Tripluc Luciferase Assay Reagent (TOYOBO, JP).

using the microprojectile bombardment system and demonstrated the simultaneous measurement of dual-color luciferase activities in extracts of spinach leaves [30]. Respective light intensities were measured simultaneously with two interference filters using a CCD camera and demonstrated the light-induced expression of the *Cab* promoter.

PRACTICAL APPLICATIONS OF CELL-BASED MULTICOLOR LUCIFERASE REPORTER ASSAYS

For the purposes of diagnostics, drug discovery and alternatives for animal models, the reliability and high-throughput features of reporter assays need to be improved. Assays must also save time and money, reduce the amount of sample needed, and facilitate the interpretation of data. The dual-color reporter assay is a simple method in which only one luciferin is used and the similar characteristic properties of luciferases are exploited; however, special equipment is required for measuring the different color emissions. The internal control reporter as a simultaneous dual-reporter assay can be optimized for inherent experimental reliability in order to improve experimental accuracy.

Indeed, to establish a practical drug screening, Davis *et al.* developed a high-throughput dual-color luciferase assay system using a 1,536-well plate format for screening small

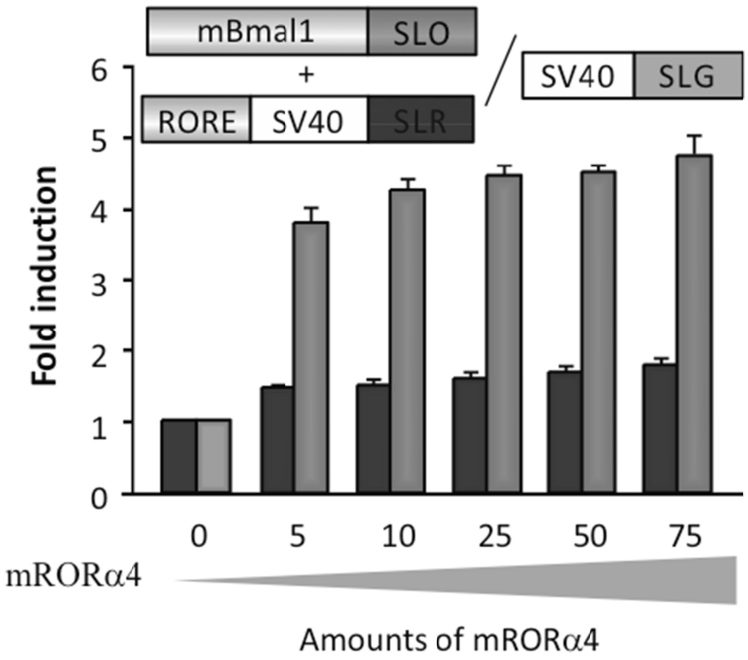

Fig. (5). Simultaneous monitoring of mRORα4-dose-dependent induction of RORE-mediated SLR gene (red bars) and *mBmal1* promoter mediated SLO gene (orange bars) transcription as a control of simultaneous expression of SV40 derived SLG gene. Reporter plasmids pRORE-SLR, Bp915-SLO and pSV40-SLG were co-transfected with or without indicated amounts of expression plasmids carry mRORa4 into NIH/3T3 cells. The amount of DNA added per well was kept constant by adding control plasmid. All values are shown as multiples. The diagram of the reporter plasmids shown location of elements. RORE, Rev-Erb/ROR response element in the *mBmal1* promoter; mBmal1 915, 915-bp fragment of the *mBmal1* promoter region; SV40, simian virus 40 promoter.

molecules that stabilize inhibitor of kappaB alpha (IκBα), a critical repressor of nuclear factor (NF-κB) [31]. They generated a dual luciferase cell line (OCI-Ly3), in which IκBα was fused to the CBG and CBR. Both luciferases were placed under an inducible promoter and integrated into the chromosomes. In screening of drug candidates, the IκBα-fused green light-emitting luciferase as an experimental reporter and the red light-emitting luciferase as an internal control reporter. By measuring their activities simultaneously in cell extracts with two band-pass filters using a CCD-based plate reader, they succeeded in screening and identifying known and unknown inhibitors of NFκB signaling from a collection of bioactive molecules.

As a toxicological test using a dual-color luciferase system, Takahashi *et al*. established a stable THP-1-derived interleukin (IL)-8 reporter cell line, THP-G8, which harbors SLO gene under IL-8 promoter and SLR gene under glyceraldehyde 3-phosphate dehydrogenase promoter as a control, respectively [32]. They evaluated the performance of this assay using values in at least two of three independent experiments as the criteria of a sensitizer. These experiments showed the test accuracies of 82% for the selected 22 chemicals and of 88% for the chemicals proposed by the European Center for the Validation of Alternative Methods. This assay is a candidate replacement for the animal tests of skin sensitization according to OECD Guidelines because of its accuracy, high-throughput performance, reliability and convenience.

On the other hand, the dual-color luciferase system is a powerful and simple technique that can be used to analysis the complex interactions of two genes, even at the whole-organism level. Noguchi *et al*. established a dual color transgenic mice and monitoring simultaneously expression of two genes at the tissue or whole-organism level [33]. They clearly monitored antiphasic oscillations of *Bmal1* and *Per2* genes names should be italicized, consistent with their endogenous mRNA profiles in the superchiasmatic nucleus and in peripheral tissues. The demonstrating results clearly show that the system allows the long-term, quantitative, and simultaneous monitoring of the expressions of the two genes.

CELL-BASED MULTICOLOR LUCIFERASE REPORTER IMAGING ASSAYS

Bioluminescence imaging at the single cell level using multicolor luciferases is a sensitive approach for understanding cellular physiology. This advance in luciferase technology has enabled quantitative and long-term visualization of cellular events at single-cell resolution using a luminescence microscope equipped with a highly sensitive cooled CCD camera [34]. However, it is possible to visualize single events only in living cells. Zhang *et al*. performed a real-time imaging of ATP release from a single cell using immobilized firefly luciferase and acquired time-lapse images of ATP release and diffusion from the cell surface, allowing determination of the actual ATP concentration at the surface of single living cells [35]. Hoshino *et al*. also developed a luciferase fused fluorescent protein composed of Rluc and EYFP (BAF-Y) for real-time single cell imaging [36]. They showed that BAF-Y exhibits the enhancement of Rluc emission intensity and appropriate subcellular distribution when fused to target-signal peptides, thus permitting the use of highly spatial and temporal resolution

microscopy. However, bioluminescence imaging using coelenterazine is unsuitable for long-term experiments because coelenterazine is degraded by auto-oxidation in living cells.

For the spatiotemporal resolution of bioluminescence long-term imaging at the single-cell level, beetle bioluminescence is advantageous because firefly D-luciferin is stable for auto-oxidation and degradation in living cells. Moreover, the background of beetle bioluminescence is lower than that of other systems, and a CCD camera can detect weak signals at the single-cell level. Nakajima *et al*. developed an enhanced green light-emitting beetle luciferase from *P. termitilluminans* (ELuc; see Table **1**), whose light signal intensity in mammalian cells is more than 10-fold greater than that of the firefly luciferase [37]. Although the current luciferase reporter is used for bioluminescence imaging, it is difficult to perform subcellular level imaging due to the inadequate signal intensity in these viable cells; indeed, higher bioluminescence intensity is required for subcellular imaging with a higher-magnification lens. They demonstrated the subcellular localization of ELuc with high resolution in mammalian cells.

In the first example of dual-color luciferase imaging in a single cell, Kwon *et al*. simultaneously visualized the expression patterns of two genes for several days in a single cell using ELuc and SLR luciferases [38]. They constructed a new dual-path optical luminescence imaging system. The light signal generated in a target cell is collimated by the objective lens and is divided by the dichroic mirror into green and red lights. Dual-color bioluminescence imaging clarified that the expression levels of genes encoding two circadian proteins (*mBmal1* and *mPer2*) oscillate in antiphase, with a circadian period of ~24 h in individual cells over 4 days. However, disorders such as 'blinking' and varying periodicities were observed several times at the single-cell level, although the bioluminescence monitoring data from the cell population showed robust and a stable circadian rhythm within the same timeframe.

Moreover, current researches in bioluminescence technique have made it possible to visualize the subcellular localization of color difference luciferases and monitor simultaneously the expression levels of two genes at subcellular resolution. Yasunaga *et al*. visualized simultaneously the subcellular localization of ELuc in the peroxisome and SLR in the nucleus in a single cell using a high-magnification objective lens with a 3-min exposure time using a combination of optical filters without binning [39]. They simultaneously quantified the kinetics of activation of NFκB using nuclear-targeted SLR and transcriptional changes in the internal control promoter using peroxisome-targeted ELuc at a single cell level and showed that the activation kinetics, including activation rate and amplitude (Fig. **6**).

FUTURE SCOPE

In the postgenomic era, we must clarify quantitatively and spatially the complex phenomena of biological systems in real time. Bioluminescence is a unique light source and

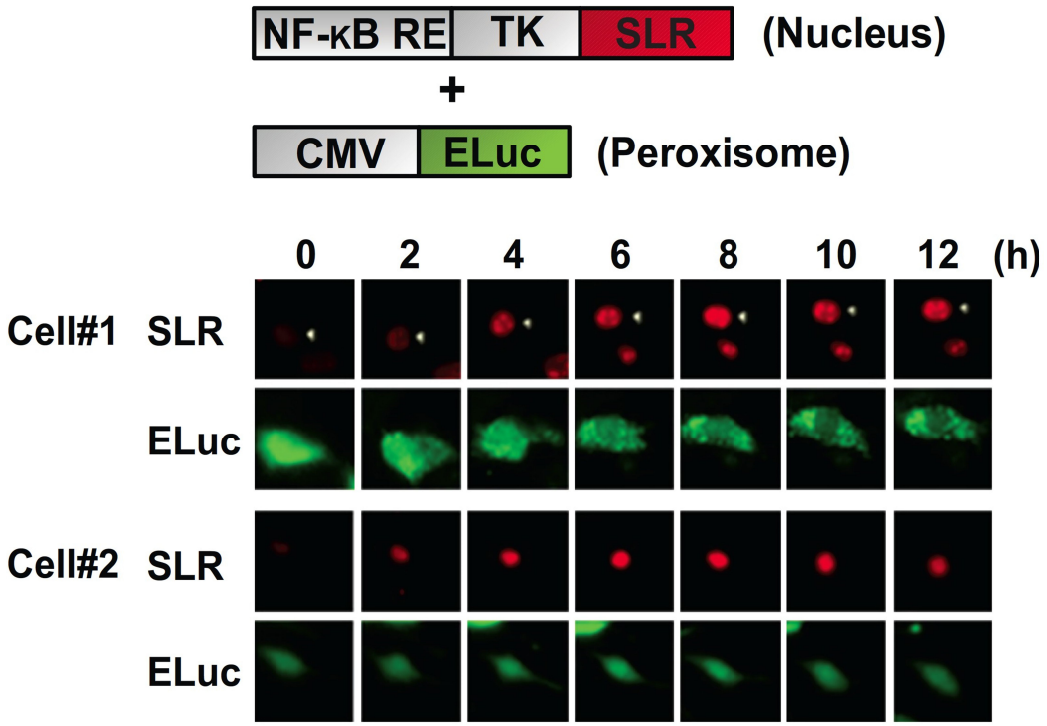

Fig. (6). Time-lapse dual-color bioluminescence imaging of tumor necrosis factor α (TNFα)-induced nuclear factor κB response element (NF-κB RE)-dependent transactivation using reporter plasmid pCMV-ELuc (peroxisome) and pNFκB-TK-SLR (nuclear) in NIH/3T3 cells. NIH/3T3 cells were transfected with pCMV-ELuc and pNFκB-TK-SLR plasmids. One day after the transfection, the culture medium was replaced with Dulbecco's modified Eagle's medium supplement with TNFα. CCD images were acquired using 3-min exposure time at 2 h intervals for 12 h with a x40 objective lens without binning. Representative serial luminescence images acquired using R62 (red, indicated as SLR) and BG39 (green, indicated ELuc) filters.

luciferase is a good candidate reporter enzyme in the field of bioresearch. The sensitivity and linearity of bioluminescence assays are superior to that of other reporter enzymes. The combination of beetle luciferases and firefly D-luciferin produce stable spectra from green to red under different buffer conditions, and their different color lights can be measured by equipment for measuring light intensity using color separation techniques. Thus, the use of different color beetle luciferases can provide new methods to analyze multiple genes both *in vitro, in cellulo* and *in vivo*. The multicolor luciferase assay is a powerful tool that will, in the near future, reveal the relationship between the core biological clock gene and cell cycle-related or metabolic-related genes, and the balance between the expressions of different genes in the immune system. However, it is a big limitation to establish the bioluminescent reporter cell, tissue, and animal. For instance, we need a hard effort to establish several genes expressed cell line. Furthermore, it is also too hard to keep the potential of reporter cell line for a long time. At the next stage, we have to develop new fusion technologies with bioluminescent reporter system for gene engineering or cell-tissue-animal engineering including artificial chromosome technology, genome editing system and etc.

CONFLICT OF INTEREST

The author confirms that this article content has no conflict of interest.

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