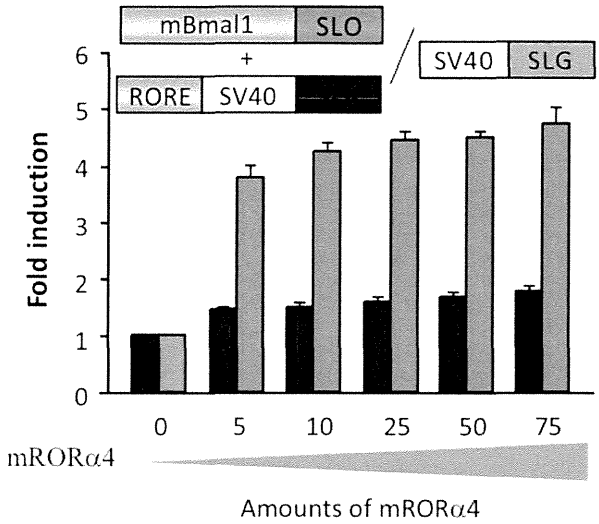

Fig. (5). Simultaneous monitoring of mRORα4-dose-dependent induction of RORE-mediated SLR gene (red bars) and *mBmal1* promoter mediated SLO gene (orange bars) transcription as a control of simultaneous expression of SV40 derived SLG gene. Reporter plasmids pRORE-SLR, Bp915-SLO and pSV40-SLG were co-transfected 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

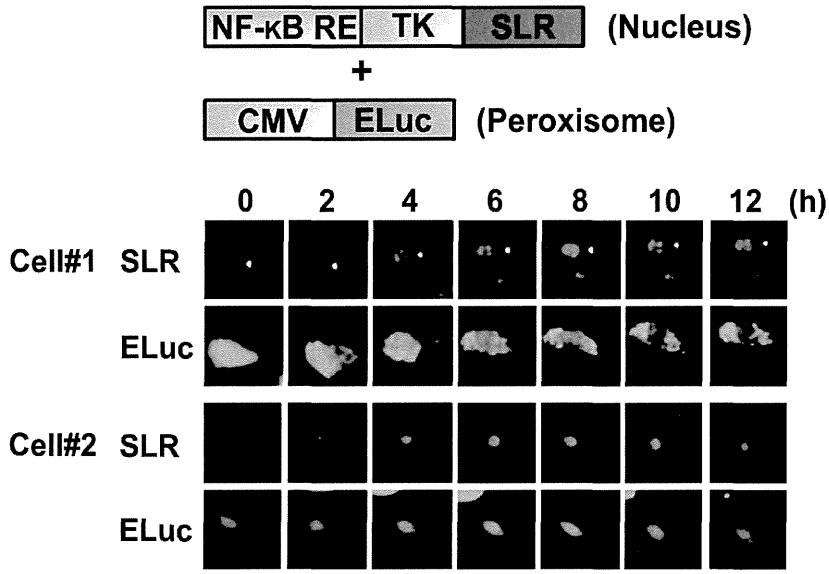

Fig. (6). Time-lapse dual-color bioluminescence imaging of tumor necrosis factor α (TNFα)-induced nuclear factor κB response element (NF-κB RE)-dependent transactivation using reporter plasmid pCMV-ELuc (peroxisome) and pNFκB-TK-SLR (nuclear) in NIH/3T3 cells. NIH/3T3 cells were transfected with pCMV-ELuc 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.

ACKNOWLEDGEMENTS

I wish to thank my colleagues who supported our research: Drs. Y. Nakajima and K. Niwa.

REFERENCES

[1] Bronstein, I.; Fortin, J.; Stanley, P.E.; Stewart, G.S.; Kricka, L.J. Chemiluminescent and bioluminescent reporter gene assays. *Anal. Biochem.*, **1994**, *219*, 169-181.

[2] Wilson, T.; Hastings, J.W. Bioluminescence. *Annu. Rev. Cell Dev. Biol.*, **1998**, *14*, 197-230.

[3] Greer, L.F.; Szalay A.A. Imaging of light emission from the expression of luciferases in living cells and organisms:a review. *Luminescence*, **2002**, *17*, 43-74.

[4] DeLuca, M. Firefly luciferase. *Adv. Enzymol.*, **1976**, *44*, 37-68.

[5] Wood, K.W. The chemical mechanism and evolutionary development of beetle bioluminescence. *Photochem. Photobiol.*, **1995**, *62*, 662-673.

[6] Fraga, H. Firefly luminescence: A historical perspective and recent developments. *Photochem. Photobiol. Sci.*, **2008**, *7*, 146-158.

[7] Seliger, H. H.; McElroy, W.D. Quantum yield in oxidation of firefly luciferin. *Biochem. Biophys. Res. Commun.*, **1959**, *1*, 21-24.

[8] Ando, Y.; Niwa, K.; Yamada, N.; Enomoto, T.; Irie, T.; Kubota, H.; Ohmiya, Y.; Akiyama, H. Firefly bioluminescence quantum yield and colour change by pH-sensitive green emission. *Nat. Photonics*, **2008**, *2*, 44-47.

[9] Niwa, K.; Ichino, Y.; Kumata, S.; Nakajima, Y.; Hiraishi, Y.; Kato, D.; Viviani V.R.; Ohmiya, Y. Quantum yields and kinetics of the Firefly bioluminescence reaction of beetle luciferases. *Photobiology & Photochemistry*, **2010**, *86*, 1046-1049.

[10] Viviani, V. R.; Bechara, E.J.; Ohmiya, Y. Cloning, sequence analysis, and expression of active *Phrixothrix* railroadworms luciferases: Relationship between bioluminescence spectra and primary structures. Biochemistry, **1999**, *38*, 8271-8279.

[11] Nakatsu, T.; Ichiyama, S.; Hiratake, J.; Saldanha, A.; Kobashi, N.; Sakata, K.; Kato, H. Structural basis for the spectral difference in luciferase bioluminescence. *Nature*, **2006**, *440*, 372-376.

[12] Hirano, T.; Hasumi, Y.; Ohtsuka, K.; Maki, S.; Niwa, H.; Yamaji, M.; Hashizume, D. Spectroscopic studies of the light-color modulation mechanism of firefly (beetle) bioluminescence. *J. Am. Chem. Soc.*, **2009**, *131*, 2385-2396.

[13] Maltsev, O.V.; Nath, N.K.; Naumov, P.; Hintermann, L. Why is firefly oxyluciferin a notoriously labile substance? *Angew. Chem. Int.*, **2014**, *53*, 847-850.

[14] Navizet, I.; Liu, Y.; Ferre, N.; Xiao, H.; Fang, W.; Lindh, R. Color-tuning mechanism of firefly investigated by multi-configurational perturbation method. *J. Am. Chem. Soc.*, **2010**, *132*, 706-712.

[15] Navizet, I.; Roca-Sanjuan, D.; Yue, L.; Liu, Y.; Ferre, N.; Lindh, R. Are the bio- and chemiluminescence states of the firefly oxyluciferin the same as the fluorescence state? *Photochem. Photobiol.*, **2013**, *89*, 319-325.

[16] Nakamura, H.; Kishi, Y.; Shimomura, O.; Morse, D.; Hastings, J. W. Structure of dinoflagellate luciferin and its enzymatic and nonenzymatic air-oxidation products. *J. Am. Chem. Soc.*, **1989**, *111*, 7607-7611.

[17] Shimomura, O.; Johnson S.H. The structure of *Latia* luciferin. *Biochemistry*, **1968**, *7*, 1734-1738.

[18] Nakajima, Y.; Kobayashi, K.; Yamagishi, K.; Enomoto, T.; Ohmiya, Y. cDNA cloning and characterization of a secreted luciferase from the luminous Japanese ostracod, *Cypridina noctiluca*. *Biosci. Biotechnol. Biochem.*, **2004**, *68*, 565-570.

[19] Verhaegent, M.; Christopoulos, T.K. Recombinant *Gaussia* luciferase. Overexpression, purification, and analytical application of a bioluminescent reporter for DNA hybridization. *Anal. Chem.*, **2002**, *74*, 4378-4385.

[20] Markova, S.V.; Golz, S.; Frank, L.A.; Kalthof, B.; Vysotski, E.S. Cloning and expression of cDNA for a luciferase from the marine copepod *Metridia longa*. A novel secreted bioluminescent reporter enzyme. *J. Biol. Chem.*, **2004**, *279*, 3212-3217.

[21] Roda, A.; Guardigi, M.; Michelini, E.; Mirasoli, M. Bioluminescence in analytical chemistry and *in vivo* imaging. *Trend Anal. Chem.*, **2009**, *28*, 307-322.

[22] Nakajima, Y.; Ohmiya, Y. Bioluminescence assays: multicolor luciferase assay, secreted luciferase assay and imaging luciferase assay. *Expert Opin. Drug Discov.*, **2010**, *5*, 835-849.

[23] Michelini, E.; Calabretta, M.M.; Calabria, D.; Mirasoli, M.; Roda, A. Exploiting *in vitro* and *in vivo* bioluminescence for the implementation of the three Rs principle (replacement, reduction, and refinement) in drug discovery. *Anal. Bioanal. Chem.*, **2014**, *406*, 5531-5539.

[24] Kitayama, Y.; Kondo, T.; Nakahira, Y.; Nishimura, H.; Ohmiya, Y.; Oyama, T. An *in vivo* dual-reporter system of cyanobacteria using two railroad-worm luciferases with different color emissions. *Plant Cell Physiol.*, **2004**, *45*, 109-113.

[25] Nakajima, Y.; Ikeda, M.; Kimura, T.; Honma, S.; Ohmiya, Y.; Honma, K. Role of orphan nuclear receptor ROR a in clock gene transcriptions demonstrated by a novel reporter assay system. *FEBS Lett.*, **2004**, *565*, 122-126.

[26] Nakajima, Y.; Kimura, T.; Sugata, K.; Enomoto, T.; Asakawa, T, Kubota H, Ikeda M, Ohmiya, Y. A multicolor luciferase assay system, one-step monitoring of multiple gene expressions with a single substrate. *Biotechniques*, **2005**, *38*, 891-894.

[27] Branchini, B.R.; Southworth, T.L.; Khattak, N.F.; Michelini, E.; Roda, A. Red- and green-emitting firefly luciferase mutants for bioluminescent reporter applications. *Anal. Biochem.*, **2005**, *345*, 140-148.

[28] Branchini, B.R.; Ablamsky, D.M.; Murtiashaw, M.H.; Uzasci, L.; Fraga, H.; Southworth, T.L. Thermostable red and green light-producing firefly luciferase mutants for bioluminescent reporter applications. *Anal. Biochem.*, **2007**, *361*, 253-262.

[29] Michelini, M.; Cevenini, L.; Mezzanotte, L.; Ablamsky, D.; Southworth, T.; Branchini, B.; Roda, A. Spectral-resolved gene technology for multiplexed bioluminescence and high-content screening. *Anal. Chem.*, **2008**, *80*, 260-267.

[30] Ogura, R.; Matsuo, N.; Wako, N.; Tanaka, T.; Ono, S.; Hiratsuka, K. Multi-color luciferase as reporters for monitoring transient gene expression in higher plants. *Plant Biotech.*, **2005**, *22*, 151-155.

[31] Davis, R.E.; Zhang, Y-Q.; Southall, N.; Staudt, L.M.; Austin, C.P.; Inglese, J.; Auld, DS. A cell-based assay for IκBα stabilization using a two-color dual luciferase-based sensor. *Assay Drug Dev. Technol.*, **2007**, *5*, 85-103.

[32] Takahashi, T.; Kimura, Y.; Saito, R.; Nakajima, Y.; Ohmiya, Y.; Yamasaki, K.; Aiba, S. An *in vitro* test to screen skin sensitizers

using a stable THP-1-derived IL-8 reporter cell line, THP-G8. *Toxicol. Sci.*, **2011**, *124*, 359-369.

[33] Noguchi, T.; Michihata, T.; Nakamura, W.; Takumi, T.; Shimizu, R.; Yamamoto, M.; Ikeda, M.; Ohmiya, Y.; Nakajima, Y. Dual-color luciferase mouse directly demonstrates coupled expression of two clock genes. *Biochemistry*, **2010**, 4i9, 8053-8061.

[34] Welsh, D.K.; Kay, S.A. Bioluminescence imaging in living organisms. *Curr. Opin. Biotechnol.*, **2005**, *16*, 73-78.

[35] Zhang, Y.; Phillips, G.J.; Li, Q.; Yeung, E.S. Imaging localized astrocyte ATP release with firefly luciferase beads attached to the cell surface. *Anal. Chem.*, **2008**, *80*, 9316-9325.

[36] Hoshino, H.; Nakajima, Y.; Ohmiya, Y. Single-cell imaging with fluorescent protein excited by luciferin-luciferase reaction. *Nature Methods*, **2007**, *8*, 637-639.

[37] Nakajima, Y.; Yamazaki, T.; Nishii, S.; Noguchi, T.; Hoshino, H.; Niwa, K.; Viviani, V.R.; Ohmiya, Y. Enhanced beetle luciferase for high-resolution bioluminescence imaging. *PLoS One*, **2010**, *5*(4), e10011.

[38] Kwon, H.J.; Enomoto, T.; Shimogawara, M.; Yasuda, K.; Nakajima, Y.; Ohmiya, Y. Bioluminescence imaging of dual gene expression at the single-cell level. *BioTechniques*, **2010**, *48*(6), 460-462.

[39] Yasunaga, M.; Nakajima, Y.; Ohmiya, Y. Dual-color bioluminescence imaging assay using green- and red-emitting beetle luciferases at subcellular resolution. *Anal. Bioanal. Chem.*, **2014**, *406*, 5735-5742.

Received: May 28, 2015 **Revised: July 28, 2015** **Accepted: September 15, 2015**