Benchmarks

Bioluminescence imaging of dual gene expression at the single-cell level

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BioTechniques 48:460–462 (June 2010) doi 10.2144/000113419

Keywords: bioluminescence; gene expression; multicolored luciferase; single-cell imaging

Bioluminescence imaging reveals the long-term dynamics of individual gene expression in a single cell. However, methods for simultaneously imaging multiple gene expression patterns have been unknown to date. Here, we constructed a dual-path optical luminescence imaging system using a two-color reporter system and could simultaneously track two gene expression patterns for several days in a single cell.

Beetle luciferases are used as reporter enzymes for monitoring gene expression in living cells because the amount of luciferase present is correlated with emitted light intensity in the presence of excess intracellular luciferin. This method is widely used in chronobiological and pharmacological research (1–3). When the luciferase gene containing a target promoter region is transfected into target cells using a plasmid and the transfected cells are cultured

in medium containing luciferin, the expression of luciferase can be estimated from bioluminescence for several days or even weeks using suitable equipment. Recently, Noguchi et al. developed a dual-color real-time reporter system in which the expression of two genes could be monitored simultaneously by splitting emissions from green- and red-emitting beetle luciferases using an optical filtered luminometer (4). This system is very

convenient for comparing the expression of target and control genes in living cell populations. However, although bioluminescence imaging of cells is a powerful tool (5–7), monitoring has been limited to single target genes. In this study, we constructed a dual-path luminescence imaging system to simultaneously monitor the expression of two genes in a single cell using a dual-color reporter system.

Our dual-path luminescence imaging system is based on a commercial single-cell luminescence imaging apparatus, the Cellgraph AB-3000B (8) (ATTO Corp., Tokyo, Japan) (see Supplementary Materials, Method 1). Figure 1A shows a schematic diagram of our final imaging apparatus. Luminescence generated in a target cell is collimated by the objective lens and is divided by the dichroic mirror DM1 and is divided by the dichroic mirror DM1 into green and red light. Green light, with a wavelength shorter than the cutoff wavelength of DM1, passes through, whereas red light, with a longer wavelength, is reflected. Light is then reflected by the mirrors M1 and M2 to the dichroic mirror DM2, which has the same optical property as DM1, so that green light passes through and the red is reflected. The optical axes of the mirror are parallel but the distance between them is half the width of the charge-coupled device (CCD) sensor, which has dark current of 0.001 e⁻/pixel/s and a readout noise of <1 e⁻ at 1MHz with electron multiplication. The green and red luminescence emissions are focused on different areas of the CCD by the imaging lens. Therefore, the single CCD obtains two images of the cell simultaneously. Figure 1B shows the bioluminescence spectra of green- and red-emitting luciferases expressed by the pEluc-test and pSLR-SV40 plasmid vectors (Toyobo, Osaka, Japan), respectively, and the transmission spectrum of the dichroic mirror.

Figure 1. Dual-path luminescence imaging apparatus. (A) Bioluminescent light generated in a target cell is collimated by the objective lens and is divided by the dichroic mirror DM1 into two color components: green and red. Green goes straight through while red is reflected by DM1 orthogonally. Each light path is reflected by the mirrors M2 and M1, respectively, to mirror DM2, which has the same optical properties as DM1. Green goes through DM2 and red is reflected. Their optical axes are parallel but the distance between them is half the width of the CCD sensor so that the green and red images are focused on different regions of the CCD by the imaging lens. Thus, a single CCD camera obtains two images of the cell at the same time. (B) Bioluminescence spectra of green (Eluc, green line) and red (SLR, red line) luciferases and the transmission spectrum (black line) of the dichroic mirror.

Figure 2. (A) The dual-color imaging of NIH3T3 cells cotransfected with the Bmal1-pSLR plasmid (red) and the Per2-pEluc plasmid (green), showing circadian rhythms of green and red luminescence, respectively. Each spot is on the separate cell. Scale bar, 20 μ m. (B) Representative circadian bioluminescence rhythms from individual NIH3T3 cells cotransfected with Bmal1-pSLR plasmid (red) and Per2-pEluc plasmid (green) for 3–4 days. Each graph represents real-time analysis data of quantitative bioluminescence intensity for the respective spot shown in Figure 2A. The graphs are each for one spot on one cell. Since cells often changed into long and narrow shapes during live imaging, a spot was defined as a circle small enough to fit well inside the cell. The spot location chosen was always adjusted to the position of highest bioluminescence intensity in the cell. The three spots shown in Figure 2A are all the same size, which was kept constant during live imaging.

For multicolor imaging, the green and red luminescence intensities (G and R) from the two luciferases in the living cell were calculated from the equation

$$\begin{pmatrix} F_1 \\ F_2 \end{pmatrix} = \begin{pmatrix} \kappa Rr & \kappa Gr \\ \kappa Rt & \kappa Gt \end{pmatrix} \begin{pmatrix} R \\ G \end{pmatrix}, \quad [\text{Eq. 1}]$$

where F_1 and F_2 are the relative light units (RLU) of light components that are either reflected by the dichroic mirror or pass through it, respectively; similarly, κRr and κRt are the RLU ratios of red luminescence that are reflected or pass through and κGr and κGt are the RLU ratios of green luminescence that are reflected or pass through. In this experiment, the respective κGr and κGt values were 0.235 and 0.765, and the κRr and κRt values were 0.97 and 0.03, respectively. Based on the above calculation, the green and red components could be reconstituted and visualized as a two-color image (Supplementary Materials, Method 2).

We visualized the expression of two circadian rhythm genes, *mBmal1* and *mPer2*, using our system. The vectors, plasmid Bmal1-pSLR containing the *mBmal1* 0.9-kb promoter (9) and plasmid Per2-pEluc containing the *mPer2* 1-kb promoter (10), were transfected into NIH3T3 cells maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin. Aliquots of 7.5×10^5 cells were plated in 35-mm dishes, cultured for 24 h, and then transfected with 1.6 μ g Bmal1-pSLR and 0.8 μ g Per2-pEluc plasmids using Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were

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treated with 0.1 μ M dexamethasone (Sigma-Aldrich, St. Louis, MO, USA) for 2 h to synchronize their circadian rhythms (4) and then the medium was replaced with 2 mL DMEM supplemented with 10% FBS, 10 mM HEPES buffer pH 7.2, 0.1 mM D-luciferin (Wako, Osaka, Japan), 100 U/mL penicillin, and 100 μ g/mL streptomycin. The cellular bioluminescence signals were recorded continuously with the dual-path luminescence imaging apparatus for 4 days. Detection of luminescence required 29-min exposures every 30 min. The electron multiplier gain of the CCD camera was set to 900. The resulting images were analyzed and processed using PC software (Cellgraph Viewer, ATTO; see Supplementary Materials, Method 2).

Dual-color bioluminescence imaging showed that the expression of *mBmal1* and *mPer2* oscillate in anti-phase, with a circadian period of $\sim$ 24 h in individual cells over 4 days (Supplementary Movies S1 and S2). 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 shows robust and stable circadian rhythm during the same time (Supplementary Figure S1). Figure 2A shows the representative real-time imaging data at each time point, in which each spot is on a separate cell. We analyzed the bioluminescence intensity of *mBmal1* and *mPer2* at single-cell level in selected cells. As shown in Figure 2B and Supplementary Figure S2, the expression of *mBmal1* and *mPer2* shows a circadian rhythm but sometimes also an unstable oscillation pattern, which may be primarily due to toxicity resulting from DNA transfection,

or may depend on cell conditions. Also, circadian rhythms in individual cells might not be synchronized perfectly even though the rhythmic pattern in a whole cell population is shown to be robust and stable.

This monitoring system revealed the dynamics of dual-gene expression in a single cell and illustrated the possibility of analyzing cell-cell interactions in the population. This system could be used for the detailed analysis of transcriptomes and promoteromes, and for screening new drugs or detecting harmful chemicals in a single cell.

Acknowledgments

The construction of equipment was supported by the Dynamic Biology Project (NEDO). K.Y. and Y.O. were supported by the Takeda Foundation. H.K. and K.Y. were also supported financially by the Matching Program for Innovations in Future Drug Discovery and Medical Care. We thank Masaaki Ikeda of Saitama Medical University for providing the *mBmal1* promoter.

Competing interests

The authors declare no competing interests.

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Received 12 January 2010; accepted 29 April 2010.

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