Absolute bioluminescence imaging at the single-cell level with a light signal at the Attowatt level

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Bioluminescence imaging (BLI) demonstrates cellular events as a light signal at the single-cell level using a highly sensitive, cooled CCD camera. However, BLI signals are relative values and thus, images taken on different days or using different equipment cannot be compared directly. We established a reference LED light source that was characteristic of the total flux and light distribution and calibrated the BLI system as an absolute light signal. This calibrated BLI system revealed that the average light signal of beetle luciferase was at an attowatt level per sec at the single cell level.

Bioluminescent reactions have been widely used as a convenient reporter system in gene expression analysis of living cells [1–3]. Light signal from luciferin and luciferase reactions within cell populations can be measured as an average value using a luminometer. Furthermore, recent advances in bioluminescence imaging (BLI) have facilitated the demonstration of cellular events as light signals, such as the dynamics of cellular organelles or changes in promoter activity, using highly sensitive, cooled CCD cameras [4–6]. However, BLI signals are relative values and images taken on different days, using different equipment or BLI probes cannot be directly compared. For instance, light intensities cannot be compared quantitively between blue-emitting BLI and yellow-emitting BLI, as the detecting efficiencies of detectors such as CCD depend on the wavelength of the emission light. Thus, we propose absolute BLI based on a reference LED light source to overcome this challenge.

For the calibration of the BLI system, we established the reference LED light source with a pulse-width modulation (PWM) control (Figure 1), which possessed a light-emitting aperture at an emission wavelength of 632 nm because of light-output stability over the long term. This source had a hole 250 μm in diameter, with total radiant flux (Rf) of 903 (±10%) pW. Total Rf (W) of the LED was evaluated by NMIJ/AIST (Tokyo, Japan) based on the absolute sphere method. The characteristics of light distribution of the source were measured by a previously described method [7]. Photon energy of the light source was found to be 3.14×10^{-19} J and the number of photons was 7.18×10^{8} per sec.

For BLI, we used the single-cell BLI system, Cellgraph AB-3000 (ATTO, Tokyo, Japan), to which a 20x objective lens Nikon Plan Fluor (NA 0.45) and 0.5x Nikon conversion lens, with a reference LED light source set at 226 pW/aperture, was attached (Tokyo, Japan). The CCD camera used was a frame transfer type iXon (Andor, Belfast, GB), with a pixel size of 16 μm, and the number of pixels was 512 × 512. Preamplifier gain was set at 1 and EM gain was set at 3500. Figure 1 illustrates the schematic of calibration for the BLI system and Table 1 summarizes the conversion process we followed to obtain the absolute value of the light signal from the reference LED light source. As the light distribution of the light source is $I(\theta) = \cos^{n}\theta$, the light distribution of the reference LED light source is $\cos^{1.33}\theta$. The light collection efficiency of the objective lens $\eta(\theta)$ is calculated as follows:

$$\eta(\theta) = 1 - \cos^{n+1}\theta = 1 - \cos^{2.33}\theta$$

The total number of photons (Tp) during the exposure time t (sec) is determined based on the accumulated light in the CCD camera and photon energy at 632 nm using the following equation:

$$Tp = \frac{Rf \times \eta(\theta) \times t}{E_{632}}$$

To determine the absolute sensitivity, we measured the reference LED light source five times at exposure times of 0.1 and 0.03 sec. The relative luminescence activity (RLU) of the reference LED light source was measured by CS Analyzer (ATTO) of the image analysis software. The average RLU values were 1.799×10^{8} and 9.393×10^{7} at 0.1 and 0.03 sec, respectively. Using the average RLU value, which corresponds to the total number of photons at an exposure time of 0.07 sec, the photon conversion

METHOD SUMMARY

We present a method for absolute bioluminescence imaging using a reference LED light source that was characteristic of the total flux and light distribution.

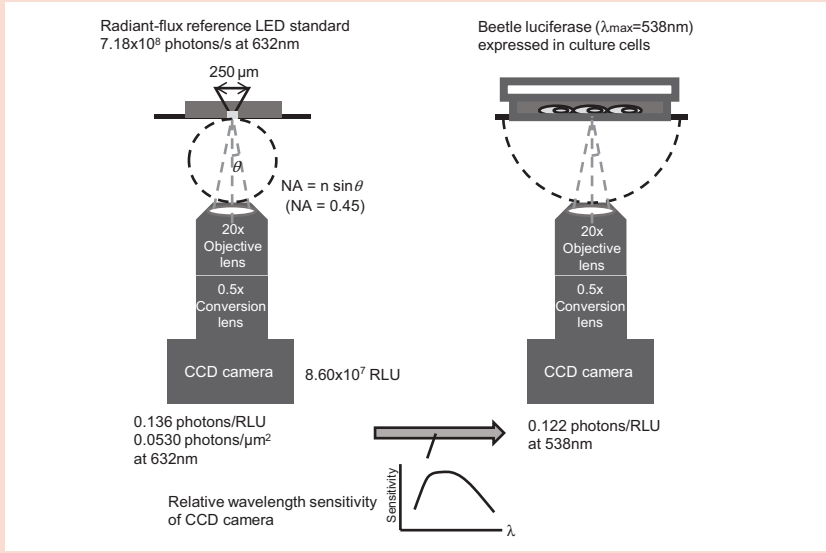

Figure 1. Illustration of the schematic of calibration for the bioluminescence imaging system based on the reference LED signal. The number of 7.18×10^8 photons/s at 632 nm in Radiant-flux reference LED standard was detected 8.60×10^7 RLU on CCD camera. The detail of the conversion process was summarized in Table 1.

coefficient (PC_{632}) was determined to be 0.136 photons/RLU and 0.0530 photons/µm² at 632 nm.

Next, we visualized beetle luciferase (ELuc), expressed in NIH3T3 cells controlled by a TK-promoter, which emitted green light (λmax = 538 nm) [8,9]. Aliquots of 7.5×10^5 NIH3T3 cells were plated in 35-mm dishes, cultured for 24 h, followed by transfection with 1.6 µg of TK-pELuc vector DNA in which TK promoter was inserted to pELuc vector (TOYOBO, Osaka, Japan), using Lipofectamine LTX (Invitrogen, CA, USA) per the manufacturer's protocol. 24 h after transfection, the medium was replaced with 2 ml of Dulbecco's modified Eagle's medium without phenol red supplemented with 10% fetal bovine serum, 10 mm HEPES buffer, 100 units/ml penicillin, 100 µg/ml streptomycin, and set to a pH of 7.2. For BLI, we cannot use pH indicators such as phenol red in the medium because the color indicators absorb light signals. Based on the relative wavelength sensitivity of λmax between 538 nm and 632 nm in the CCD camera (Supplemental Figure 1), we determined the photon conversion coefficient to be 0.122 photons/RLU and 0.0477 photons/µm² at 538 nm [7]. BLI of ELuc-expressing NIH3T3 cells were visualized by the BLI system having been subjected to exposure for 10 min following the addition of 0.2 mM firefly luciferin, which was measured under the same conditions as that of the reference LED light source.

Figure 2 and Supplemental Figure 2 show the BLI of ELuc-expressing NIH3T3 cells, in which the light signals were detected in the whole of the cell except the nucleus because ELuc without C-terminal SKL signal sequence was in the cytoplasm. Next, we normalized the BLI from the relative light signal (RLU/pixel) to the absolute light signal (photons/pixel and photons/µm²) based on the reference LED. As the signal of bioluminescent cells is radiated uniformly in all directions at the same intensity, we determined the light

Table 1. The conversion process to absolute CCD light signal from the reference LED light source.

	Calibrated wavelength (nm)	632	538	460	Uncertainty (%) k = 2
Standard light	Radiant-flux standard wavelength (nm)	632	538	460	10.0
	Total radiant flux (W)	9.03×10^{-10}			2.3
	Total radiant flux for imaging system calibration (W)	2.26×10^{-10}			
	Radiation pattern	$\cos^{1.33}\theta$			
	Photon energy (J)	3.14×10^{-19}	3.69×10^{-19}	4.32×10^{-19}	
	Number of photons/s	7.18×10^8			
Objective lens	NA	0.45			
	Collection efficiency	0.232			
Camera	CCD PreAmp Gain	1			
	EM-Gain	3500			
	CCD exposure time (s)	0.070			
	Pixel size (µm)	16			
	Relative wavelength sensitivity	1.00	1.11	0.96	2.0
	Average relative light intensity (RLUs) of 5 images	8.60×10^7			5.2
BLI system	Absolute sensitivity (photons/RLU)	0.136	0.122	0.141	11.7
	Optical magnification	10	10	10	
	Image size/pixel (µm)	1.60	1.60	1.60	
	Image area/pixel (µm²)	2.56	2.56	2.56	
	Absolute sensitivity (photons/µm²)	0.0530	0.0477	0.0522	

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collection efficiency $\eta_{BL}(\theta)$ at the convergence angle θ as follows:

$$\eta_{BL}(\theta) = \frac{1-\sqrt{1-\sin^2\theta}}{2} = \frac{1-\sqrt{1-NA^2}}{2}$$

Tp at the single cell was deduced from the measured light intensity (P_{cell}) of BLI as follows:

$$Tp = P_{Cell} / \eta_{BL}(\theta)$$

We selected several single cells in an obtained image and measured the total relative light signal in each region of interest. Based on the absolute sensitivity and the light collection efficiency, we determined the total absolute light signal ranged from 1100 to 2500 photons/s in a single cell. This was an extremely weak light intensity corresponding to ~600 aW (attowatt). For example, cell #1 was estimated to possess (1.17 ± 0.14) × 10^3 photons/s per cell, while cell #2 possessed (1.63 ± 0.19) × 10^3 photons/s per cell, and cell #3 possessed (1.82 ± 0.21) × 10^3 photons/s per cell (Figure 2). In the measurement on a different day, cell #1 was estimated to possess (1.35 ± 0.16) × 10^3 photons/s per cell, while cell #2 possessed (9.35 ± 1.09) × 10^2 photons/s per cell, and cell #3 possessed (2.23 ± 0.26) × 10^3 photons/s per cell (Supplemental Figure 2). These results suggested this system could allow the quantitation of individual cells and determine the variations in photons within the population. Moreover, given that the quantum yield for ELuc is 0.61 [10], the number of expressed luciferase protein molecules could be estimated to be approximately 1900–3000 in a single cell, assuming ELuc cannot turnover as an enzyme.

Furthermore, we visualized red-emitting beetle luciferase (SLR) expressed in an NIH3T3 cell, which resulted in the total absolute light signals being estimated to possess from (8.00 ± 0.94) × 10^3 photons/s cell to (1.18 ± 0.14) × 10^4 photons/s per cell (Supplemental Figure 3). We also estimated the total absolute light signals of NanoLuc luciferase stable expressed NIH3T3 cell from (3.30 ± 0.39) × 10^4 photons/s per cell to (4.58 ± 0.54) × 10^4 photons/s per cell (Supplemental Figure 4). These results indicated that we can evaluate the transformation efficiency of the reporter gene or the stability of the bioluminescent stable transformant cell. Although several examples for single-cell BLI as a relative light unit were reported [6,11,12], to our knowledge this is the first example of the absolute photon number being determined directly in a single-cell expressing luciferase. However, at present, absolute BLI could be limited to a mono-layer single-cell level. We could also expect the limitation of linearity range to be between about 200 and 3 × 10^7 photons/s per cell. Furthermore, it is not easy to estimate absolute BLI for color change, such as a BRET signal.

In conclusion, we established absolute BLI using a reference light source standardized by the absolute sphere method. The average light signal of the expressed beetle luciferase was at an attowatt level corresponding to a few thousand photons per sec in a single cell. Using this information, we can directly compare several images regardless of when they were taken, the equipment used, or BLI probe used.

Author contributions

TE and HK carried out the total experiments, HK, KM, MS, MY and HA established and confirmed the absolute BLI system, TE and YO wrote the manuscript, and YO planNed total experiments.

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Financial & competing interests disclosure

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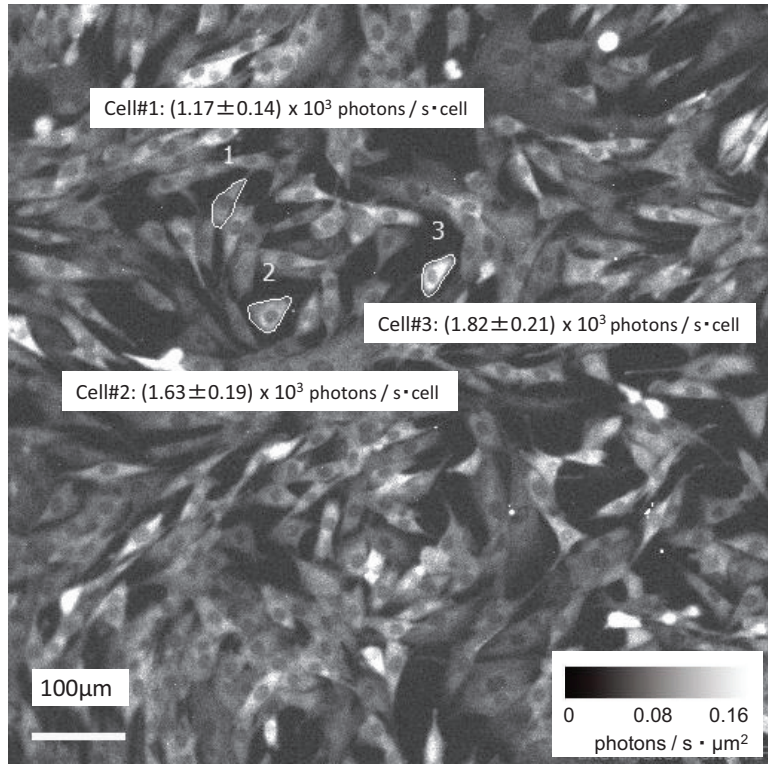

Figure 2. Absolute bioluminescence imaging of the luciferase expressed cells. NIH3T3 cells transfected with the TK-pELuc plasmid, cultured in Dulbecco's modified Eagle's medium without phenol red supplemented with 10% fetal bovine serum, 10 mM HEPES buffer, 100 units/ml penicillin, 100 μg/ml streptomycin and maintained at a pH of 7.2. BLI were visualized by the BLI system having been subjected to exposure for 10 min following the addition of 0.2 mM firefly luciferin, which was measured under the same conditions as that of the reference LED light source. The BLI of the selected cells were determined the photon number per μm^2 sec as a total absolute light signal.
BLI: Bioluminescence imaging.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Open access

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2018-0043

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