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Cloning and mutagenetic modification of the firefly luciferase gene and its use for bioluminescence microscopy of *engrailed* expression during *Drosophila* metamorphosis

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ABSTRACT

Bioluminescence microscopy is an area attracting considerable interest in the field of cell biology because it offers several advantages over fluorescence microscopy, including no requirement for excitation light and being phototoxicity free. This method requires brighter luciferase for imaging; however, suitable genetic resource material for this purpose is not available at present. To achieve brighter bioluminescence microscopy, we developed a new firefly luciferase. Using the brighter luciferase, a reporter strain of *Drosophila* Gal4-UAS (Upstream Activating Sequence) system was constructed. This system demonstrated the expression pattern of *engrailed*, which is a segment polarity gene, during *Drosophila* metamorphosis by bioluminescence microscopy, and revealed drastic spatiotemporal change in the *engrailed* expression pattern during head eversion in the early stage of pupation.

1. Introduction

Ever since the luciferase gene was cloned from the American firefly [1], luciferase has been used as a reporter enzyme for the promoter assay of gene expression [1–3] instead of conventional reporter enzymes such as β-galactosidase and chloramphenicol acetyltransferase, because of its greater sensitivity and operational simplicity. Luciferase has also been used as a luminescent tag for bioluminescence image analysis of tumor invasion and metastasis by whole-mouse *in vivo* imaging [4,5] instead of the fluorescent tag, green fluorescent protein (GFP), because of the absence of autofluorescence interference from mouse skin and hair. However, light intensity from mammalian cells expressing the luciferase gene is much weaker than that measured from cells expressing GFP. Therefore, high-sensitivity detectors such as a photo-multiplier tube (PMT) and an electron-multiplying charge-coupled device (EM-CCD) camera are used for the luminometric reporter and *in vivo* imaging assays.

Furthermore, bioluminescence imaging at the single-cell level by microscopy is gaining attention in the field of cell biology. Because bioluminescence microscopy requires no excitation light, it lacks the phototoxicity associated with fluorescence microscopy and permits the long-term, nonlethal observation of living cells, such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells. Therefore, bioluminescence microscopy is expected to be a powerful tool in cellular biology that complements fluorescence microscopy [6–10]. To carry out bioluminescence microscopy, a low-light imaging microscope using a short-focal length imaging lens system [11,12], and brighter luciferases were developed [13–17]. In particular, NanoLuc luciferase, derived from deep-sea shrimp, was 150 times brighter than firefly luciferase [14], and Nano-lantern and its derivatives attained bioluminescent sensing of Ca^{2+}, cAMP, and ATP with multicolor imaging obtained from the bioluminescence resonance energy transmittance (BRET) system [16,17]. Red-shifted firefly luciferase in the meanwhile accomplishes video-rate *in vivo* bioluminescence imaging from a neurons in striatum [18].

Brighter luciferase promotes bioluminescence microscopy at the single-cell level, and more genetic resource materials for luciferase are expected to facilitate bioluminescent sensor probe development. To obtain a variety of luciferase genetic resource materials, we cloned and modified firefly luciferase genes and applied them in the imaging of *Drosophila* metamorphosis. In *Drosophila*, the Gal4-UAS (Upstream Activating Sequence) system is widely used for tissue-specific and time-specific gene expression analyses by crossing Gal4 promoter strain with UAS-*luciferase* reporter strain. However, the reporter strain was not suitable for bioluminescence microscopy because conventional *luciferase* was employed for the luminometric assay. We constructed the

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UAS-*luciferase* reporter strain of *Drosophila* using our newly developed, brighter *luciferase*, and imaged *engrailed* expression pattern during metamorphosis; this is because a pupal body is immobile and hence suitable for multilayer imaging. Furthermore, morphological and gene expression data are fragmented and scarce at the pupal stage. The bright luciferase shortened exposure time and facilitated multilayer bioluminescence microscopy for all-focused imaging during *Drosophila* metamorphosis.

2. Materials and methods

2.1. Firefly

Five firefly species, namely *Pyrocoelia matsumurai kumejimensis, Pristolycus sagulatus*, *Luciola* sp., *Drilaster kumejimensis,* and *Stenocladius flavipennis*, were used in this study. They were stored at −80 °C until use. Taxonomic and collection data are described in Supplementary Table 1.

2.2. Cloning of the firefly luciferase gene

Lanterns obtained from three specimens (either larvae or adults) were crushed in an RNA extraction matrix D tube (MP-Biomedicals, Santa Ana, CA, USA) with 1 mL of TRlzol reagent (Invitrogen, Carlsbad, CA, USA) by Fast Prep FP100A (MP-Biomedicals) homogenizer. Total RNA was isolated from the homogenate by chloroform extraction and ethanol precipitation methods. To obtain the full-length *luciferase* cDNA from the total RNA, cDNA library construction and RNA ligase-mediated rapid amplification of 5′ and 3′ cDNA ends (5′-RACE and 3′-RACE) were performed with the GeneRacer Kit (Invitrogen).

To design 5′-RACE primers, amino acid sequences of firefly luciferase previously reported were compared using the sequence information analysis software DNASIS Pro (Hitachi Software Engineering, Tokyo, Japan). Supplementary Table 2 shows the 12 primers deduced from conserved amino acid sequence, L-I-K-Y-K-G-Y-Q-V, situated at around the 440th residue position by aligned sequence comparison among the 10 firefly species, namely *Lampyris noctiluca* (Genbank Accession No. CAA61668), *Luciola cruciata* (P13129), *Luciola lateralis* (Q01158), *Luciola mingrelica* (Q26304), *Hotaria parvula* (AAC37253), *Photinus pyralis* (BAF48390), *Photuris pennsylvanica* (Q27757), *Pyrocoelia miyako* (AAC37254), *Pyrocoelia rufa* (AAG45439), and *Rhagophthalmus ohbai* (BAF34360). This region corresponded to the 2nd to 10th amino acid positions of the C-terminal domain of luciferase [19] (Supplementary Fig. 1).

The 5′-ends of cDNA were amplified by PCR using the primers designed and cloned into the TA cloning vector, pGEM-T Easy Vector System I (Promega, Madison, WI, USA), and sequenced by the 3130xl Genetic Analyzer (Applied Biosystems, Foster, CA, USA) with the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Based on the read sequences, primer sets for the first and nested second PCRs for 3′-RACE and full-length cDNA of luciferase were designed at the 5′-untranslated region (UTR) (Supplementary Table 2), and the nested second PCR product of the full-length cDNA was cloned into the TA cloning vector and sequenced.

The luciferase-coding region of the full-length cDNA was inserted in-frame into *Bam* H1/*Eco* RI multicloning sites of pRSET-B (Invitrogen), and expressed in *Escherichia coli* strain JM109 (DE3) (Promega). Light-emitting colonies were picked up after spraying 1 mM D-luciferin (Promega) in 0.1 M citrate buffer, pH 5.0.

2.3. Protein purification and emission spectrum

The luciferase expressed in JM109 (DE3) was purified with a Ni-NTA agarose resin column (Qiagen, Venlo, Limburg, Netherlands). The emission spectrum was determined with a LumiFl-Spectrocapture AB-1850 (Atto, Tokyo, Japan) at room temperature (25 °C) in a solution of 0.1 M citric acid/0.1 M Na_2HPO_4 buffer (pH 6.0–8.0) containing 1 mM D-luciferin, 2 mM ATP, 4 mM $MgCl_2$, and 10 μg/mL of purified luciferase. Luc2 luciferase expressed by using pGL4-Control vector (Promega) was used as the control.

2.4. Kinetic constant of luciferase

The Michaelis–Menten constant (Km) values of luciferase for luciferin and ATP were determined with a luminometer, Luminescencer JNR II (Atto) [20]. Luminescence intensity was measured in 50 mM Tris-HCl (pH 8.0) containing 50 μg/mL of partially purified luciferase (enough for Km determination), 2 mM ATP, 4 mM $MgSO_4$, and luciferin (0.6–640 μM) for Km of luciferin, and in 50 mM Tris-HCl (pH 8.0) containing 50 μg/mL of purified luciferase, 1 mM luciferin, 4 mM $MgSO_4$, and ATP (5–1920 μM) for Km of ATP. The time course of light emission was measured for 10 s with 0.02 s gated time, and the reaction rate was determined by peak light intensity. The Km value was estimated by curve fitting against the Michaelis–Menten equation using the least squares method.

2.5. Mutagenesis

Based on the nucleotide sequence of the full-length cDNA, codon usage of *luciferase* was optimized for expression in mammalian or *Drosophila* cells, and the synthesized sequence including a Kozak sequence was inserted in-frame into *Bam* H1/*Eco* RI multicloning sites of pRSET-B. Then, site-directed mutations were introduced [21]. Light-emitting colonies of JM109 (DE3) were picked up after spraying 1 mM D-luciferin in 0.1 M citrate buffer, pH 5.0.

2.6. Comparison of luminescence intensity in live cells

The *luciferase* mutated in the pRSET vector was replaced with a co-expression vector, pF9A CMV hRluc-neo Flexi (Promega), into *Sgf* I/*Pme* I multicloning sites. The vector (1 μg) was transfected into HeLa cells (ECACC, Salisbury, UK) using a FuGene HD transfection reagent (Roche, Basel, Switzerland), and the cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) containing 10% foetal bovine serum (FBS) for overnight incubation. The luminescence intensity of the live cells expressing the target *luciferase* was determined by the Luminescencer with addition of 2 mM D-luciferin in the culture medium, and the intensity was normalized with the alternative luminescence of *Renilla* luciferase co-expressed in the cells as an internal control according to the Dual-Glo Luciferase Assay System (Promega) instruction manual.

2.7. Bioluminescence microscopy and emission spectrum of live cells

The mutated *luciferase* was inserted into *Bam* H1/*Eco* RI multicloning sites of pCDNA3.1 (Invitrogen), and the vector was transfected into HeLa cells by FuGene HD. The cells were cultured in DMEM containing 10% FBS overnight and subjected to bioluminescence microscopy by the addition of D-luciferin (1 mM) in the culture medium.

The bioluminescence image of the cells was captured by a luminescence microscope LV200 (Olympus, Tokyo, Japan) [11,12] equipped with UPLFLN60 × OI objective lens (NA 1.25, Olympus) and DP74 color CCD camera (Olympus) or ImagEM C9100-13 EM-CCD camera (Hamamatsu Photonics, Shizuoka, Japan).

The culture medium of HeLa cells expressing *luciferase* was replaced with Hank's Balanced Salt Solution (Invitrogen) containing 1 mM D-luciferin. The emission spectrum of the cells was determined with the LumiFl-Spectrocapture AB-1850 at 37 °C.

2.8. Bioluminescence microscopy of Drosophila pupa using Gal4-UASp system

2.8.1. Engrailed-Gal4 strain

Engrailed-Gal4 strain (yw; en-Gal4/Cyo) was provided by Prof. T. Aigaki, Tokyo Metropolitan University (strain stock No. TMU1118 in Tokyo Metropolitan University). The FlyBase ID of *engrailed* is FBgn0000577.

2.8.2. UASp-pmat luciferase system

Luciferase cloned and modified from *Pyrocoelia matsumurai kumejimensis* (Pmat) optimized for *Drosophila* cell expression was inserted into *Kpn* I and *Xba* I restriction sites in pUASp vector [22], and transgenic flies carrying the UASp-*Pmat* construct were generated using a phi C31-based integration system [8,23]. The UASp-*Pmat* construct DNA (400 μg/mL) was introduced into the 86Fb strain (*yw*; M{eGFP. vas-int. Dm}ZH-2A; M{RFP. attP'}ZH-86Fb) by microinjection using Inject Man NI2 (Eppendorf, Hamburg, Germany). After hatching, adult males were crossed with virgin *yw* strain females. The red-eyed male progeny were crossed with virgin NDB6 strain (*yw*; Sp/SM1; Pr Dr/TM6C, Sb Tb) females to stabilize the UASp-*Pmat* insertion.

2.8.3. Bioluminescence microscopy of Drosophila pupa

Homozygotes of *engrailed*-Gal4 and UASp-*Pmat* strains were crossed, and collect progenies (third insular lave) and feed yeast paste containing 3 mM D-luciferin for more than 6 h. Prepupa (white pupa) was placed on 35-mm glass-bottom dish, and bioluminescence images were captured by LV200 attached with ImagEM EM-CCD camera (EM gain 1200, exposure time 30 s), UPLFLN10×PH objective lens (NA 0.30, Olympus), motorized focus drive, HP1762 (Prior, Cambridge, UK), and Z-focus controller, ES10ZE (Prior). This system was controlled by CellSens Dimension software v. 1.9 (Olympus).

Fifty-one images were captured at steps of every 10 μm for 500-μm thickness, and all-focused images (transmitted light option) were generated by CellSens Dimension. For time-lapse imaging, the capture sequence was repeated at 30-min intervals for 93 h.

3. Results and discussion

3.1. Luciferase

The amino acid sequences of the modified luciferases deduced from cDNA sequences ranged from 544 to 555 residues (Table 1) and were aligned (Supplementary Fig. 1). Sfla luciferase from *Stenocladius flavipennis* was about 10 residues longer than the other luciferases at the N-terminal end. Peroxisome target sequences, SKL and CKL [24], were found at the C-terminal end in Pmat and MALuci2 from *Luciola* sp., respectively, but SKM in others, Dkum from *Drilaster kumejimensis*, Sfla, and Psag from *Pristolycus sagulatus*.

3.2. Spectral and kinetic properties in vitro and in the cell

Bioluminescence spectra at several pH *in vitro* for wild-type and mutated luciferases are described in Supplementary Figs. 2 and 3, respectively. The wild-type luciferases except MALuci2 (wt) were pH sensitivity in wavelength and luminescence intensity. With decreasing pH, wavelength increases, and luminescence intensity decreases. Maximum wavelength values of the wild-type luciferases at pH 8.0 are listed in Table 1. On the other hand, all the mutated luciferases were pH insensitive, and maxmum wavelength value at pH 8.0 of Pmat, Dkum, and MALuci2 were almost same as the wild type, while those of Sfla and Psag were shifted longer form wild type. The Km values of wild-type and mutated luciferases *in vitro* are listed in Table 1. As a reference, kinetic properties of Luc2 [25] are also listed.

It has been reported that amino acid residues at 243 (Ala) [26] and 534 (Arg) [27] were conserved in pH-insensitive luciferases, and these positions corresponded to 255 and 541 amino acid residues of aligned sequence (arrowhead Nos. 1 and 2), respectively, in Supplementary Fig. 1. Although the mutated luciferases are pH insensitive, the amino acid residue at 255 is Gly, and that at 541 is Ser for Pmat and Dkum, or Thr for MALuci2, or Gly for Psag, except for Sfla. Red-shifted mutations were previously introduced at S286 N [28,29], I288V or A [30], G326S [28], H433Y [27,28], and E457K [27], and these positions corresponded to 293, 295, 333, 440, 464 amino acid residues of aligned sequence (arrowhead Nos. 3 to 7), respectively. However, the matched residue was S333 in Psag only in the red-shifted luciferases of Sfla and Psag.

Bioluminescence spectra of cells expressing the luciferases at 37 °C are described in Fig. 1A. The values of maximum wavelength were the same at 25 °C (Table 1), but luminescence intensities at 25 °C were lower than those at 37 °C (data not shown).

3.3. Bioluminescence intensity in cells

Bioluminescence intensities of cells expressing the luciferases at 37 °C were compared with those of Luc2 (Fig. 1B). Pmat was 12 times brighter than Luc2, but the intensity was compensated to 1.2 times after normalization with internal control of *Renilla* luc. Dkum and MALuci2 were also 7.7 and 7.3 times brighter than Luc2, but the intensities were compensated to 5 and 2 times, respectively, by normalization with

Table 1
Kinetic properties of wild-type and modified luciferases in the present study.

Luciferase	Amino acid length	Mutation sites	λmax (*in vitro*)	λmax (in cell)	Km (luciferin μM)	Km (ATP μM)	DDBJ Accession No	BRC catalog No
Pmat (wt)	548	wild type	560	–	14.2	104	LC495923	
Pmat	548	E355R, V367A, I424L, K446Q	560	568	62.2	98.1	LC495924	RDB14359
Pmat (Dro)	548	E355R, V367A, I424L, K446Q	–	–	–	–	LC495925	
Dkum (wt)	547	wild type	557	–	60.1	127.3	LC495926	
Dkum	547	N2D, I424L	558	560	19.4	26.8	LC495927	RDB14360
MALuci2 (wt)	544	wild type	557	–	4.25	188	LC495928	
MALuci2	544	N2D	556	557	1.2	88.9	LC495929	RDB14363*
Sfla (wt)	555	wild type	559	–	16.6	21.5	LC495930	
Sfla	555	P2A, S293F, N387S, C400Y, I432V, V451I, A486D, S517C, K520R	609	612	6.6	9.0	LC495931	RDB14362
Psag (wt)	545	wild type	583	–	4.9	194	LC495932	
Psag	545	I148V, S283L, V401 M, F464I, G503R	605	609	221.7	281.7	LC495933	RDB14361
Luc2	550		562	605	27.1	72.6		

The position numbers of mutation sites are indicated as amino acid sequence position for the length of each luciferase. Maximum wavelength (λmax) *in vitro* (at pH 8.0) and in the cell was measured at 25 °C and 37 °C, respectively. These materials were deposited at RIKEN BioResource Research Center (Tsukuba, Japan), and the deposition numbers are also listed. However, it is to be noted that amino acid position at 2 of MALuci2 (RDB14363*) was not altered to N2D (N2N). –: not determined.

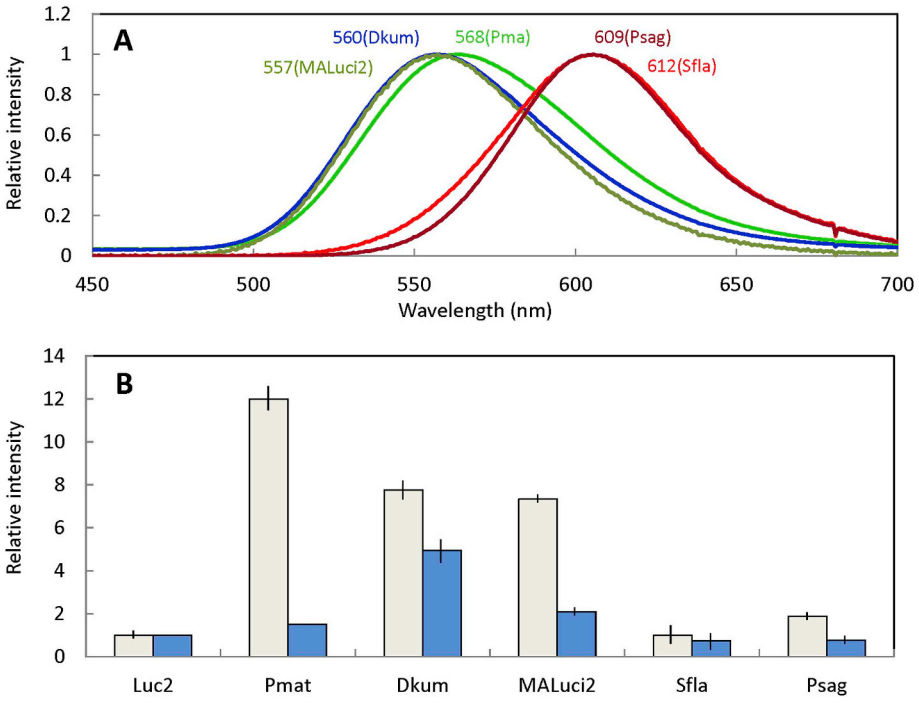

Fig. 1. Properties of luciferase expression in HeLa cells at 37 °C. Normalized bioluminescence spectrum and maximum wavelengths of the five luciferases (Pmat, Dkum, MALuci2, Sfla, and Psag) are described (A). Normalized bioluminescence intensities of the five luciferases (B). The left bar indicates normalized value by Luc2 intensity, and the right bar indicates normalized value by Luc2 intensity with internal control of *Renilla* luc intensity. Bar height: mean ± SD, n = 4.

Renilla luc. For red-shifted luciferases (Sfla and Psag), the intensities were almost the same as Luc2. As shown in the result, the comparison result among the two normalization methods was different, but we have no clear answer to this discrepancy.

3.4. Bioluminescence microscopy of live cells

Bioluminescence images of HeLa cells expressing the luciferase transiently are described in Fig. 2. By using a conventional color CCD camera (Fig. 2A), cell images could be captured within 30-s exposure time. The luminescence intensity of Dkum was stronger than that of Pmat with internal compensation of *Renilla* luc by luminometry. But the brightness of the images corresponded to the luminescence intensity with normalization of Luc2, and Pmat was the brightest (Figs. 1B and 2A). As bright image can actually reduce exposure time, we adopt *Pmat* for *Drosophila* pupa imaging. By using an EM-CCD camera, cell images could be captured within 500-ms exposure time.

3.5. Bioluminescence microscopy of Drosophila pupa

All-focused images of pupa are described sequentially in Fig. 3. The *engrailed* activity was observed at the posterior edge of each abdominal segment and posterior spiracle (ps) at the prepupal stage (Fig. 3A–E). The activity in the ps decreased gradually from 3 to 4.5 h APF (after puparium formation) (Fig. 3B–E) and disappeared at 5 h APF (Fig. 3 F). In the thorax, the *engrailed* activity was greater than that in the abdomen, and the spatiotemporal expression pattern changed drastically during head eversion in the early stage of pupation. Posterior and a pair of lateral edges of the thoracic segment 3 (T3) demonstrated the activity from 0 h to 8.5 h AFP (Fig. 3A–F). On the other hand, in the thoracic segment 2 (T2), a pair of lateral edges and central part demonstrated the activity from 0 to 3 h AFP (red pseudocolor sites in Fig. 3A-B), but the active area of the pair of the lateral edges moved to the inner part of the active areas of the T3 from 3 to 4 h AFP (Fig. 3B–D), and further moved to the posterior edge of T3 and fused in line from 4.5 to 5.5 h AFP (Fig. 3E-F). The active area of the central part disappeared in 3.5 h AFP (Fig. 3C). The whole area of the thoracic segment 1 (T1) demonstrated the activity from 0 to 2.5 h AFP (blue pseudocolor sites in Fig. 3 A), and the active area moved to T3 through T2 from 3 to 6 h AFP (Fig. 3B–F). The whole area of pseudo cephalon (psc) demonstrated the activity from 0 to 2.5 h AFP (yellow pseudocolor sites in Fig. 3 A), and the active area moved to T2 from 3 to 4 h AFP (Fig. 3B–D) and formed a pair of mushroom-like shape from 4.5 to 6 h AFP (Fig. 3E-F). The *engrailed* activity in the thorax disappeared once at 9 h AFP. In this stage, head eversion is completed. The *engrailed* reappeared at abdominal segments from 10 h AFP and gradually decreased (Fig. 3 G) and disappeared to 25 h AFP. On the contrary, it appeared at an area that is considered to be midgut in the abdomen at 25 h AFP (Fig. 3. H), and continued until eclosion. We have not confirmed uniform distribution and consumption rate of the substrate in

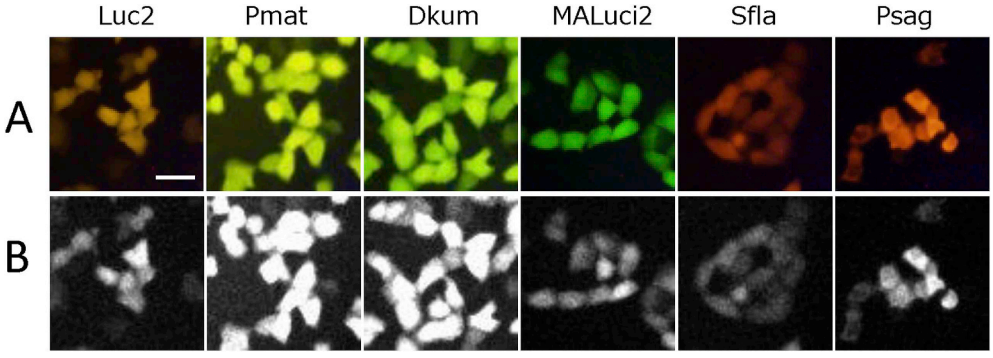

Fig. 2. Bioluminescence images of HeLa cells expressing the luciferase transiently at 37 °C. Cell images were captured by color CCD camera (DP74) with 30-s exposure time (A), and captured by EM-CCD camera (ImagEM C9100-13) with 500-ms exposure time (B). Images A and B are displayed with the same level of adjustment for each. Scale bars: 50 μm.

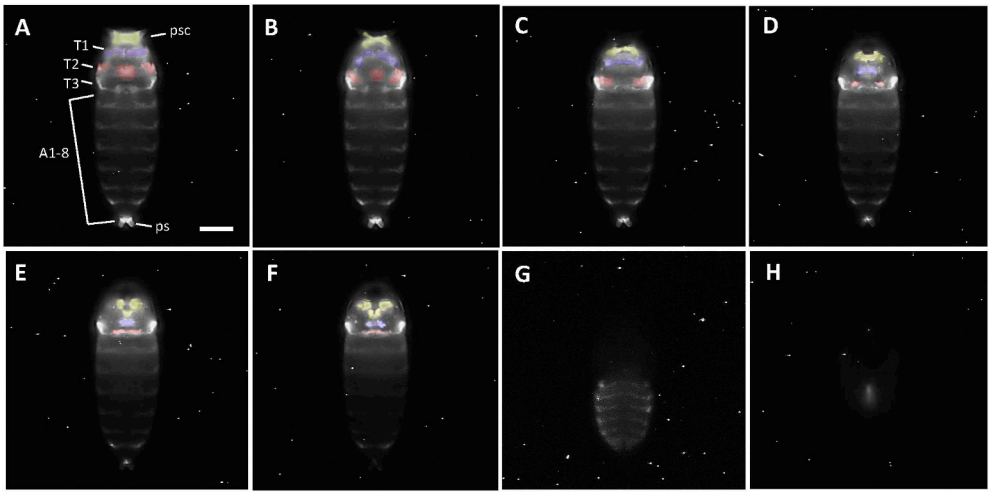

Fig. 3. Bioluminescence images of the *engrailed* expression pattern during *Drosophila* metamorphosis. Images are described sequentially in 2.5 h APF (A), 3.0 h APF (B), 3.5 h APF (C), 4 h APF (D), 4.5 h APF (E), 5 h APF (F), 11 h APF (G), and 50 h APF (H). T1: thoracic segment 1 (blue pseudocolor), T2: thoracic segment 2 (red pseudocolor), T3: thoracic segment 3, A1-8: abdominal segment 1–8, psc: pseudo cephalon (yellow pseudocolor), ps: posterior spiracle. Scale bars: 200 μm. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the embryo, and the luciferase used in this study contains no degradation sequences such as PEST sequence. The correlation between the luminescence intensity and promoter activity of the *engrailed* expression is not strictly guaranteed.

The *engrailed* expression pattern was studied intensively in the segmentation process of the embryo [31,32] and also in the nervous system and head formation process of larva and adult [33,34]. These analyses were conducted with whole-body *in situ* hybridization and immunochemistry methods. These methods provide endpoint data of one individual. To obtain time-series data, experiments using many individuals are required. On the other hand, bioluminescence microscopy of promoter activity provides spatiotemporal data from one individual, but spatial resolution is not superior to previous methods used. Time-lapse images of *engrailed* expression during *Drosophila* metamorphosis constitute the first data obtained in this study. However, the *engrailed* active area is mapped on segmental layers of prepupa (Fig. 3). Since pupation proceeds inside the prepupal case and creates a gap between the prepupal case and the head of the pupa inside, it is not appropriate to show the gene expression pattern data on the position of prepupal segments. Histological time-series data of pupa are fragmented and scarce. Once histological information is accumulated, such as in the adult *Drosophila* brain atlas, the gene expression patterns could be superimposed onto the atlas [35].

CRediT authorship contribution statement

Katsunori Ogoh: Data curation, Investigation, Methodology. **Ryutaro Akiyoshi:** Data curation, Investigation, Methodology. **Hirobumi Suzuki:** Project administration, Supervision, Writing - review & editing.

Declaration of competing interest

The authors K. Ogoh, R. Akiyoshi, and H. Suzuki are employees of Olympus Corporation (Tokyo, Japan) and are inventors on patent applications US9523082, JP6032861, JP6006070, JP5980608, JP5896624, and JP5860651 submitted by Olympus, Nimura Genetic Solutions (Tokyo, Japan) and Perak State Development Corporation (Perak, Malaysia) that covers the modified luciferase genes.

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Appendix A. Supplementary data

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