Chapter

Imaging Promoter Assay of Adenylyl Cyclase A Gene in *Dictyostelium discoideum* during Fruiting Body Formation by Dual-Color Bioluminescence Microscopy

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Abstract

Cyclic adenosine monophosphate (cAMP), which is derived from adenosine triphosphate through adenylyl cyclase A (acaA), acts as an intracellular secondary messenger and an extracellular chemotactic substance in important biological processes. In the social amoebae *Dictyostelium discoideum*, cAMP mediates cell aggregation, development, and differentiation to spore and stalk cells during fruiting body formation. The *acaA* gene is transcribed under the control of three different alternative promoters. This study aimed to develop a promoter assay for *acaA* in *D. discoideum* using bioluminescence microscopy. Here, we inserted green- and red-emitting luciferase genes into downstream of promoter regions 1 and 3, respectively. Promoter activities were visualized by bioluminescence microscopy. We confirmed the differential expression of *acaA* under the control of promoters 1 and 3 at the different stages of *D. discoideum* development. We also demonstrated the application of dual-color bioluminescence imaging in the development of an imaging promoter assay.

Keywords: *Dictyostelium discoideum*, adenylyl cyclase A promoter, dual-color luciferase, bioluminescence microscopy, imaging promoter assay, fruiting body formation

1. Introduction

Gene expression and regulation are essential processes in cellular proliferation and differentiation and are involved in morphogenesis and embryogenesis. The social amoebae *Dictyostelium discoideum* is known to have a simple life cycle, short generation time, and small genome size. Thus, it is a model organism that is often used in research on morphogenesis, especially the formation of fruiting bodies from amoeba cell aggregation. This cell aggregation is mediated by extracellular cyclic adenosine monophosphate (cAMP) [1]. Then, cAMP is secreted from the tip of the cell mound for prestalk and prespore cell migration [2]. High concentrations of extracellular cAMP are required for prestalk and prespore cell differentiation [3, 4] and spore

formation [5]. Thus, cAMP plays an important role in *Dictyostelium discoideum* development. Its synthesis is catalyzed by adenylyl cyclases A, B, and G, which are encoded by the genes *acaA*, *acrA*, and *acgA*, respectively [6, 7].

Adenylyl cyclase A is considered a development-specific enzyme [8, 9]. Galardi-Castilla et al. [10] characterized the promoter region of the *acaA* gene in *Dictyostelium discoideum* Ax4 cells by histochemistry using a *lacZ*/X-Gal staining system, β-galactosidase reporter system, quantitative RT-PCR, and *in situ* hybridization. The *acaA* gene is transcribed under the control of three different alternative promoters: promoter 1 (distal region), promoter 2 (intermediate region), and promoter 3 (proximal region). Promoter 1 is active during the cell aggregation stage, and promoters 2 and 3 are active in the mound, slug, and fruiting body stages [10].

Promoter assays using histochemical techniques are quite cumbersome, as the samples have to be fixed, stained, and observed over time sequentially for each promoter. For this purpose, many samples must be prepared. On the other hand, a promoter assay using bioluminescence microscopy can be used to obtain time-lapse image data from a single experiment using one sample. This method is often used in the study of clock genes [11–13] and developmental biology [14–16]. In this chapter, we applied bioluminescence microscopy and used two luciferases in the development of an *acaA* promoter assay that can monitor *acaA* promoters 1 and 3 simultaneously during *Dictyostelium discoideum* development, and compared the result with those of histochemistry and β-galactosidase reporter system [10]. We also demonstrated the advantages of this promoter assay and discussed the perspectives that need further consideration.

2. Materials and methods

2.1 Dictyostelium discoideum

Under the National BioResource Project (NBRP), the National Institute of Advanced Industrial Science and Technology (AIST) in Japan provided the *Dictyostelium discoideum* strain Ax2 (NBRP ID: S00001). The Ax2 cells were cultured in SM/5 medium on a 1.4% agar plate at 21°C with *Klebsiella aerogenes* bacterial cells (provided by Prof. H. Kuwayama, Tsukuba University, Japan) as feed.

2.2 Firefly luciferase gene

Green- and red-emitting luciferases were used for the dual-color bioluminescence promoter assay. The green-emitting luciferase gene *Luci sp1* was cloned from *Luciola* sp. collected in the Belum forest, State Park, Malaysia. *Luci sp1* was modified and optimized for mammalian cell expression. Variant 1 of *Luci sp1* [17] (DNA Data Bank of Japan [DDBJ] accession no. LC632706) was used for the green vector construction. The red-emitting luciferase gene *Psa* (wild-type) was cloned from *Pristolycus sagulatus* collected in Tokyo, Japan. *Psa* (Wildtype) was modified and optimized for mammalian cell expression as *Psa* [18] (DDBJ accession no. LC495933). This luciferase gene was used for the red vector construction and was also deposited in the RIKEN BioResource Research Center (BRC), Tsukuba, Japan (BRC catalog no. RDB14361).

2.3 Construction of *adenylyl cyclase A* reporter vector

The *Dictyostelium* extrachromosomal expression vectors pDM304 (NBRP ID: G90008) and pDM358 (NBRP ID: G90009) were provided by Tsukuba

University under the NBRP and were used in the construction of two *acaA* reporter vectors involving promoters 1 and 3. The actin 15 promoter (*Xho*I/*Bgl*II restriction sites) of the pDM358 and pDM304 vectors was replaced with promoters 1 and 3 of *acaA*, respectively. The promoter regions were amplified using PCR from *Dictyostelium discoideum* Ax2 genomic DNA using the primer sets for promoter 1 (5′-GCctcgagCTTGATGAGTGGCCCAAAACC-3′ and 5′-GCagatctATTTTTAAAGATCCAAGAATTCG-3′) and promoter 3 (5′-GCctcgagACCTCACTTCATAAATATATCTTTG-3′ and 5′-GCagatct TTTTTAATAATTTTTTAATATTATTAC-3′) [10]. Variant 1 of *Luci sp1* (green) and *Psa* (red) luciferase genes, including the *Dictyostelium* Kozak sequence (AAAA) before the start codon, was inserted into downstream of the promoter in the pDM358 and pDM304 vectors, respectively, *via* the *Spe*I/*Hind*III restriction sites. These vectors contained the actin 8 terminator.

2.4 Transformation and fruiting body formation

The Ax2 cells were co-transfected with the two constructed vectors by electroporation using a MicroPulser (Bio-Rad, California, USA) according to the protocol for *Dictyostelium*. The transformant cells were selected using hygromycin (50 mg/mL) and neomycin (10 mg/mL) in HL5 medium.

Millicell Cell Culture Insert PICM 0RG50 (Merck, Darmstadt, Germany) was placed onto a 35-mm glass bottom dish, and 1.2 mL of D buffer (saline solution for *Dictyostelium*) containing 3 mM D-luciferin potassium salt (Promega, Wisconsin, USA) was added to the basolateral side of the glass bottom dish. The transformant cells were seeded onto the inside of the cell culture insert above the membrane and cultured at room temperature (21°C), facilitating the development of the mound, slug, and fruiting body.

2.5 Bioluminescence microscopy

The bioluminescence images of the cells were captured using an LV200 bioluminescence microscope (Olympus, Tokyo, Japan) [19, 20] equipped with a UCPLFLN 20XPH objective lens (Olympus) and an ImagEM C9100-13 EM-CCD camera (Hamamatsu Photonics, Shizuoka, Japan). The activities of *acaA* promoters 1 and 3 were visualized using BA495-540GFP (Olympus) and 610ALP (Omega, New Jersey, USA) emission filters, respectively, at an exposure time of 30 s for 24 h at 90s intervals. In addition, bright-field images were captured at an exposure time of 200 ms using the same capture sequence as that of the bioluminescence images. The time course of luminescence intensity was analyzed using TiLIA, a time-lapse image analysis software [21].

2.6 Fluorescence microscopy

The autofluorescence images of the cells were captured using an IX83 inverted microscope (Olympus) equipped with a U-HGLGPS excitation light source (output level 100 with ND25 filter), a U-FGFP mirror unit, and a DP74 color CCD camera. A UCPFLN 10XPH objective lens was used for mound and slug observations, and a UCPFLN 4xPH objective lens was used for fruiting body observation. Exposure time was 500 ms for all experiments.

The irradiation power of the excitation light was measured at 480 nm using a PM100D optical power meter (Thorlabs, New Jersey, USA) with an S170C sensor probe for microscopy.

3. Results and discussion

Bright-field and bioluminescence images of the activities of promoters 1 and 3 are shown in **Figure 1**. According to the bright-field image, the amoeba cells began to aggregate after 10 h of seeding (**Figure 1A**) and formed the mound (**Figure 1D**), slug, and fruiting body (**Figure 1F**) after 16, 18, and 20 h, respectively. The image of the slug is not shown in **Figure 1**, since the moving slug disappeared from the field of view. In the case of **Figure 1**, the slug stage was extremely short and the fruiting body formation occurred immediately from the mound.

According to the bioluminescence image, promoter 1 activity was observed in single amoeba cells. It increased gradually (**Figure 1A–C**) and peaked at the mound

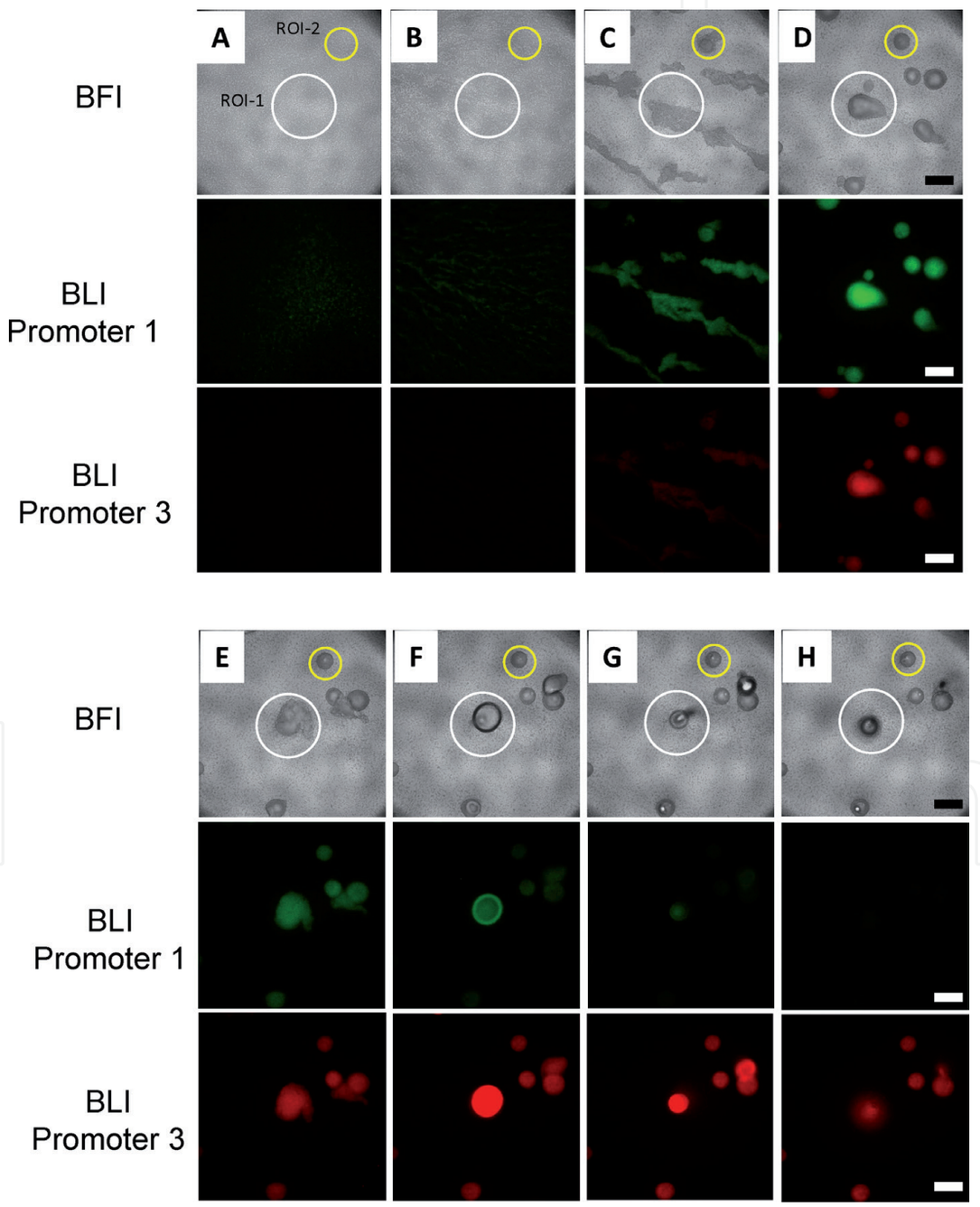

Figure 1.
Bright-field images (BFI) and bioluminescence images (BLI) reflecting the activities of promoters 1 and 3 at 10 h (A), 12 h (B), 14 h (C), 16 h (D), 18 h (E), 20 h (F), 22 h (G), and 24 h (H) after seeding ameba cells. Regions of interest 1 and 2 (ROI-1 and ROI-2) were assigned to cover the process from cell aggregation to fruiting body formation and are marked as circles on the BFI. Scale bar: 500 mm.

stage after 16 h (**Figure 1D**). Then, it decreased during fruiting body formation (**Figure 1E–G**) and eventually disappeared (**Figure 1H**). On the other hand, promoter 3 activity increased during the cell aggregation stage (**Figure 1C**), peaked during the fruiting body stage (**Figure 1F**), and then decreased (**Figure 1G** and **H**). Thereafter, the activity of the stalk cells disappeared. Histochemical detection of promoter activity using a *lacZ*/X-Gal staining system [10] showed that promoter 1 activity was detected in the cell aggregation and early mound stages, but was not detected after the slug stage. On the other hand, promoter 3 activity was detected in the early mound, slug, and spore stages. This activity was particularly strong in the upper cup of the spores. The two methods yielded almost the same results, but

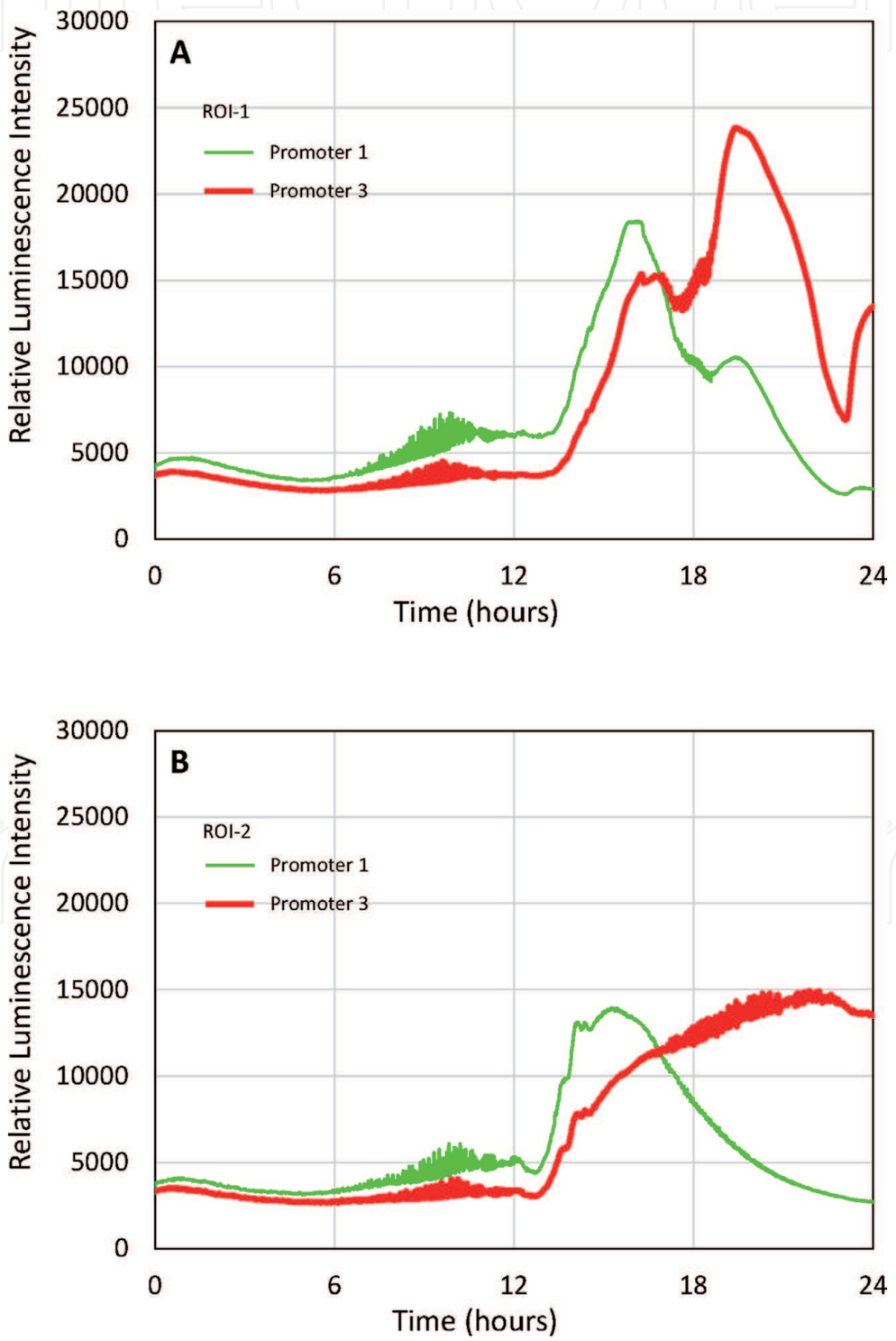

Figure 2.
Time course of luminescence intensity reflecting the activities of promoters 1 and 3 in ROI-1 (A) and ROI-2 (B).

promoter 3 activity was detected earlier by bioluminescence imaging than by histochemical detection at the cell aggregation stage after 14 h (**Figure 1C**). However, the resolution of the images obtained by histochemical detection was superior to that by bioluminescence imaging.

To show the time course of the promoter activities, two regions of interest (ROI) (ROI-1 and ROI-2) were assigned to cover the process from cell aggregation to fruiting body formation, as shown in **Figure 1**. **Figure 2** shows the time course of luminescence intensity reflecting the activities of promoters 1 and 3 in ROI-1 (**Figure 2A**) and ROI-2 (**Figure 2B**) using 961 time-lapse images captured at 90s intervals for 24 h. The intensity of promoter 1 activity increased after 13 h, peaked after 16 h, decreased, and disappeared after 24 h in ROI-1 and ROI-2. On the other hand, the intensity of promoter 3 activity increased to the same timing as that of promoter 1 after 13 h, but peaked after around 20–22 h. Then, the intensity decreased gradually in ROI-2, but rapidly decreased and recovered in ROI-1. Since the measurement of the intensities involves live imaging, the discrepancy may be caused by the movement of the spores in the ROI during fruiting body formation. The measurement of the time courses of the activities of promoters 1 and 3 by bioluminescence imaging and by a β-galactosidase reporter system [10] showed similar results.

The results of the promoter assay using bioluminescence microscopy were the same as those of the promoter assays using histochemistry and β-galactosidase, confirming the convenience of this imaging promoter assay for *Dictyostelium* studies. Moreover, the imaging promoter assay enabled the spatiotemporal information of promoter activity to be obtained sequentially in a single experiment. Based on the result, detailed analysis of promoter activity can be performed efficiently by histochemical or immunofluorescence microscopy. However, several experiments are required for each measurement in histochemical and β-galactosidase promoter assays. Multiple promoters can also be analyzed using multicolor luciferases, but the number of promoters evaluated is limited to the number of luciferases of different colors. Moreover, there are some concerns regarding the use of multicolor luciferases in bioluminescence microscopy as follows. **Figure 3** shows the normalized

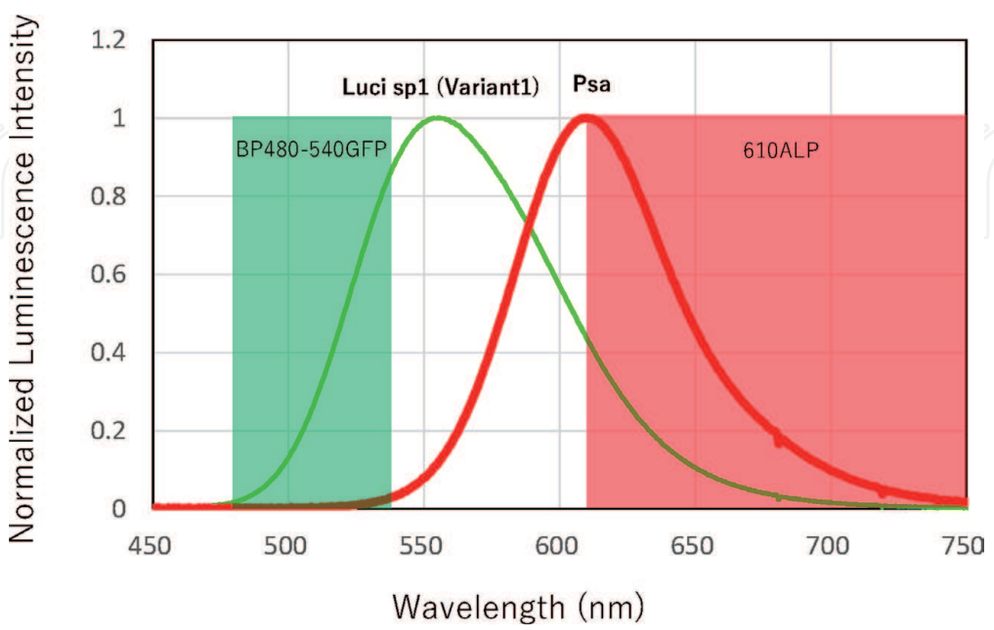

Figure 3.
Normalized luminescence spectra of luciferases (variant 1 of Luci sp1 and Psa) expressed in HeLa cells in the transparent range of the emission filters, BP480-540GFP and 610ALP.

luminescence spectra of the luciferases (variant 1 of *Luci sp1* and *Psa*) in the transparent range of the emission filter for each luciferase channel. The luciferases were expressed in HeLa cells [17, 18]. In the transparent range of the 610ALP filter, cross talk between the two spectra was observed between 610 and 700 nm. To prevent spectral cross talk, a spectral unmixing operation must be done, as is performed in fluorescence microscopy [22]. Nakajima et al. [23] demonstrated the unmixing of tri-colored bioluminescence for a luciferase promoter assay using one color to normalize the activity of two genes. In addition to the number of promoters to evaluate, we need one more color luciferase to normalize different promoter activities.

One of the advantages of bioluminescence microscopy is that it is not affected by autofluorescence background. **Figure 4** shows the bright-field and autofluorescence images of the mound, slug, and fruiting body stages of *Dictyostelium discoideum* development captured by fluorescence microscopy with a mirror unit for green fluorescent protein (GFP). Autofluorescence from the upper tip of the mound, the periphery of the slug body, and the spore and stalk of the fruiting body was observed. Therefore, the imaging conditions used GFP as a reporter require optimization of the excitation intensity, etc.

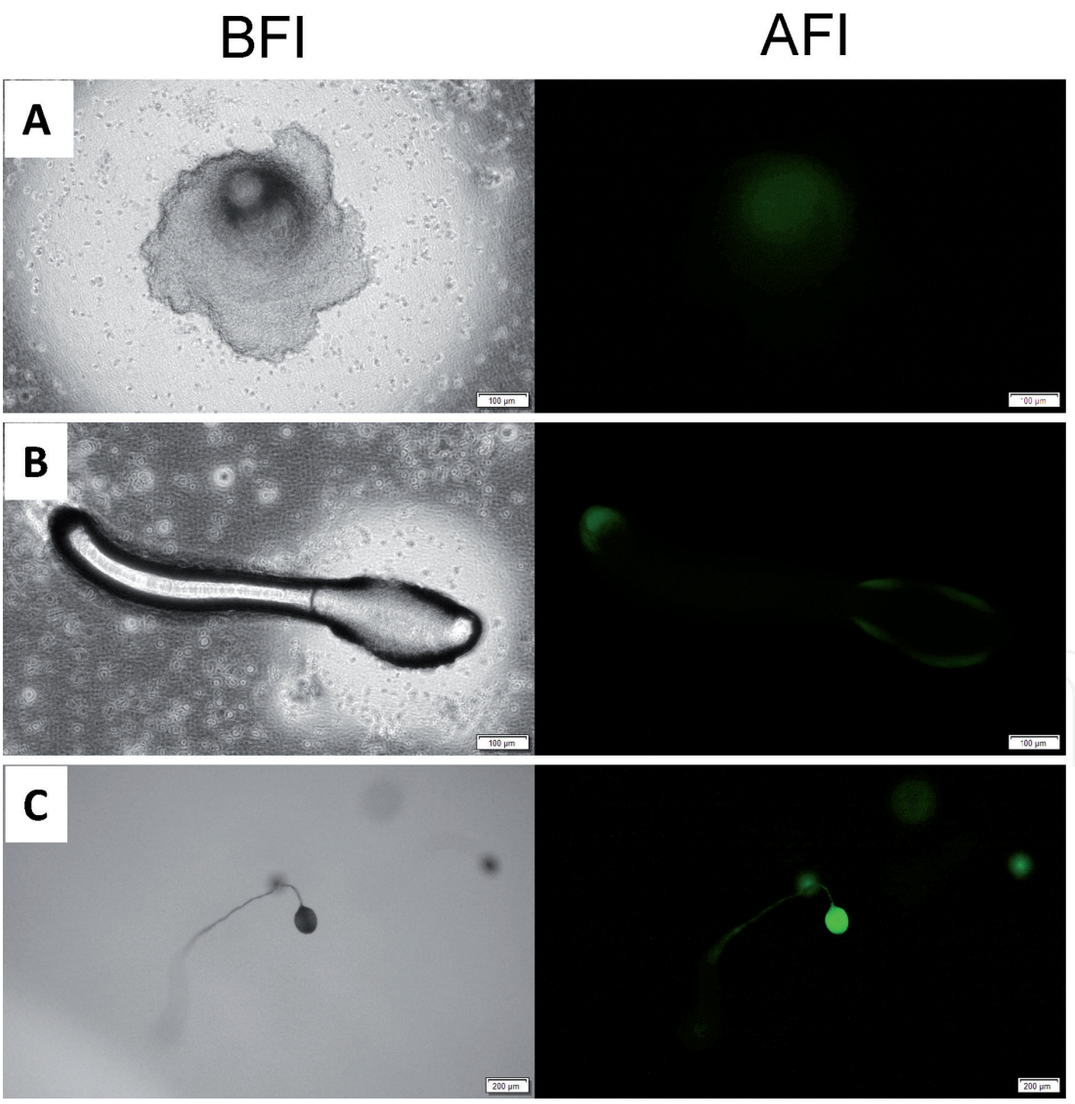

Figure 4.
Bright-field images (BFI) and autofluorescence images (AFI) of the mound (A), slug (B), and fruiting body (C) stages of Dictyostelium discoideum *development. Exposure time was 500 ms, and excitation light power was 0.8 mW for the mound and slug stages and 1.0 mW for the fruiting body stage. Scale bar: 100 mm for A and B and 200 mm for C.*

4. Conclusion

The imaging promoter assay of the *acaA* promoters 1 and 3 by bioluminescence microscopy and the histochemical and β-galactosidase promoter assays yielded similar results in the evaluation *acaA* promoter activity in the different stages of *Dictyostelium discoideum* development. Moreover, we found that obtaining an overall picture of promoter activity spatiotemporally during the entire developmental process is possible with the bioluminescence imaging promoter assay. However, spectral unmixing is required to effectively normalize the different promoter activities. We also found that the imaging promoter assay is not significantly affected by autofluorescence.

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Conflict of interest

The authors T. Hayashi, K. Ogoh, and H. Suzuki are employees of Olympus Corporation (Tokyo, Japan).

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