

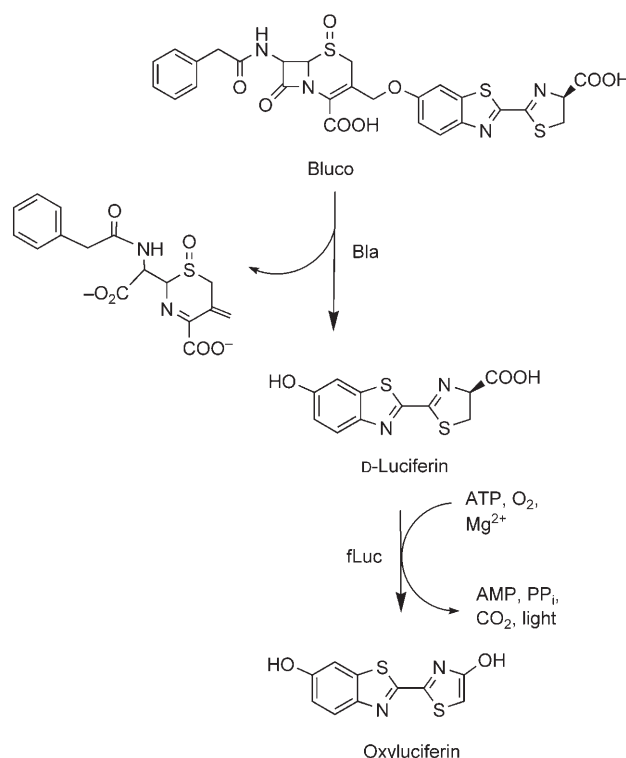
A Bioluminogenic Substrate for In Vivo Imaging of β -Lactamase Activity**

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β -Lactamases are an important family of bacterial enzymes for research in bacterial infection and biotechnology. Bacterial resistance to antibiotics is becoming a growing global health problem, and bacteria expressing β -lactamases are resistant to β -lactam antibiotics because β -lactamases efficiently hydrolyze β -lactam antibiotics such as penicillins and cephalosporins.^[1] Rapid and sensitive detection of β -lactamase activity in vitro and in vivo is thus clinically important for the identification of bacterial pathogens that are resistant to β -lactam antibiotics. A number of colorimetric and fluorometric substrates have been developed for the detection of β -lactamase in vitro and in living cells,^[2–7] but a substrate that can detect β -lactamase in living animals has not yet been reported. Highly sensitive imaging of bacteria will find many health and environmental applications.^[8–11] In contrast, β -lactamases have been developed as a powerful tool with a number of biotechnology applications. For example, TEM-1 β -lactamase (Bla), a small (29 kDa) and monomeric enzyme, is a sensitive reporter system for detecting and imaging biological processes and interactions in single living mammalian tissue culture cells. It has been applied for examining promoter/regulatory elements activity,^[12] imaging RNA splicing,^[13,14] monitoring viral infection,^[15] and detecting protein–protein interactions.^[16,17] The β -lactamase enzymatic activity has recently also been applied to control formation of supramolecular hydrogels.^[18] To extend the reporter utility of Bla to whole living animals is highly desirable but has been impeded by lack of a suitable substrate that can image the Bla activity in vivo. Herein, we report the first bioluminogenic substrate for Bla and demonstrate its application for in vivo imaging of the Bla activity.

Our detection strategy utilizes the bioluminescent enzyme firefly luciferase (fLuc) to image the Bla activity in vivo, because luciferase-based bioluminescence imaging offers great sensitivity for probing biology in small living animals^[19]

(Scheme 1). Firefly luciferase catalyzes the oxidation of its substrate D-luciferin in the presence of O_2 , ATP, and Mg^{2+} and



Scheme 1. The structure of Bluco and proposed two-step reaction for detection of Bla.

emits light, which can be imaged with a sensitive CCD camera. The 6-hydroxy group of D-luciferin is critical to its oxidation by fLuc and has been modified to design probes for detection of target enzyme activity, for example, β -galactosidase,^[20] monoamine oxidase,^[21] and caspases.^[22] We thus designed the bioluminogenic substrate by coupling this hydroxy group to the 3' position of the cephalosporin through an ether bond (Scheme 1). The resulting conjugate should be a poor substrate for fLuc but remain as a substrate for Bla. The opening of the β -lactam ring by Bla would trigger spontaneous fragmentation, leading to the cleavage of the ether bond at the 3' position and subsequent release of free D-luciferin that can now be oxidized by fLuc in a light-producing reaction. The Bla activity can thus be imaged from the light emission. To improve the stability of the conjugate, the sulfide group on the cephalosporin was oxidized to sulfoxide.^[6] The final structure was termed Bluco (beta-lactam and D-luciferin conjugate) and was efficiently prepared by a multiple-step

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organic synthesis (see the Supporting Information). The purity of the final substrate was validated by the HPLC analysis (see the Supporting Information).

Bluco displayed a maximal fluorescence emission at 440 nm, and, after the Bla treatment, the maximal emission red-shifted to 540 nm (Figure 1). This fluorogenic feature of

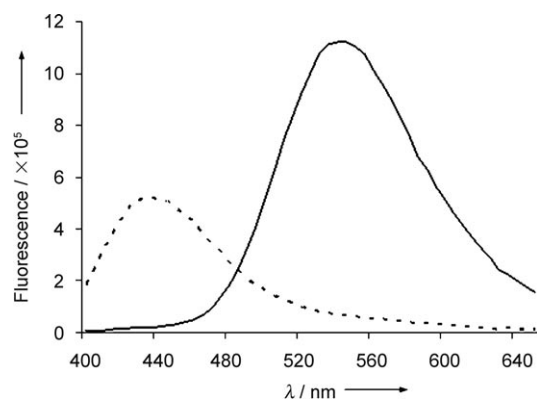


Figure 1. Fluorescence emission spectra of Bluco (1 μM) in 10 mM PBS before (dashed line) and after (solid line) Bla treatment ($\lambda_{\text{ex}} = 350 \text{ nm}$).

Bluco can be applied to measure its hydrolysis kinetics by Bla: $K_m = 90 \mu\text{M}$ and $k_{\text{cat}} = 1.0 \text{ s}^{-1}$ (see the Supporting Information). The small value of k_{cat} suggests that the leaving group D-luciferin is not as efficient as thiolphenol used in CCF2.^[5] Subsequent addition of fLuc to the Bla-treated Bluco solution produced strong light emissions, validating our design of the bioluminogenic substrate for Bla.

Since Bluco is a polar molecule containing two carboxylate groups, we first evaluated whether Bluco was cell-permeable and able to detect the Bla activity in intact cells. We added Bluco (62.5 μM in phosphate buffered saline (PBS)) to 1×10^5 intact COS7 (monkey kidney) cells cotransfected with Bla and fLuc (Bla + fLuc), and observed strong bioluminescence emission (Figure 2). In comparison, control COS7 cells transfected only with fLuc displayed similar fLuc activity but produced little light emission upon addition of Bluco (Figure 2). The contrast between the luminescence

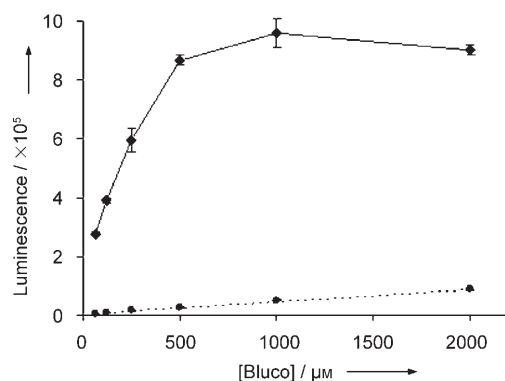


Figure 2. Bioluminescence emission of 1×10^5 Bla + fLuc (solid line) and fLuc (dashed line) COS7 cells after incubation for 10 min with various concentrations of Bluco in 10 mM PBS.

of Bla + fLuc and fLuc cells was more than 55-fold. As the concentration of Bluco increased, the bioluminescent emission from Bla + fLuc cells proportionally increased, but reached a maximum after the Bluco concentration was higher than 500 μM , which might reflect the saturation of the Bla activity (Figure 2). The bioluminescent emission from Bla + fLuc cells was also proportionally dependent on the cell numbers, and as few as 1500 intact cells were detected in the presence of 500 μM Bluco (Figure 3). These results demonstrate that Bluco is cell-permeable and able to detect the Bla activity in living mammalian cells.

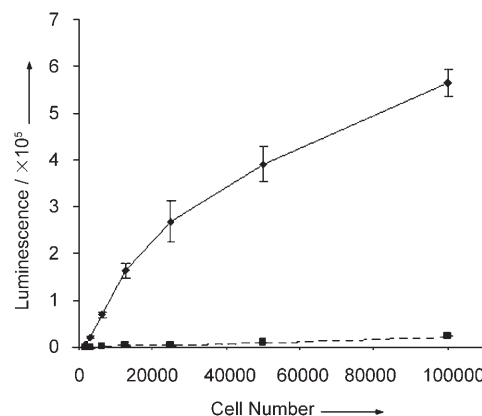


Figure 3. Dependence of luminescence emission on cell number. Serially diluted solutions of Bla + fLuc (solid line) and fLuc cells (dashed line) were exposed to Bluco (500 μM) in 10 mM PBS.

To test whether Bluco can work *in vivo*, we injected 1×10^6 COS7 cells transiently cotransfected with Bla and fLuc into the left rear thigh of a nude mouse. The same number of only fLuc transfected cells was injected into the right rear thigh as a control. Two hours after the implantation, we injected 78 μg of Bluco ($5 \mu\text{mol kg}^{-1}$) via the tail vein into each mouse and measured the bioluminescent signal. The left injection site exhibited a strong emission, which was about 15–25-fold higher than the emission from the right injection site (Figure 4). The emission peaked at about 30 min and lasted for approximately 30 min, then gradually decreased by 40% over 1 h. At the end of the Bluco measurement, D-luciferin was injected into the same mice for measuring the fLuc activity at both injection sites. Both sites showed similar bioluminescent emission (Figure 4), indicating that the fLuc activity was similar and that the observed contrast from the Bluco imaging was not due to different fLuc activity at each site. All these data demonstrate that Bluco can image the Bla activity *in vivo* in the presence of fLuc.

Finally, we applied Bluco to image the RNA-splicing reaction *in vivo*. We have previously reported a Bla-based RNA-splicing reporter for imaging *Tetrahymena* group I intron ribozyme-splicing activity in living cells (Figure 5a).^[13] This ribozyme reporter construct RzB was transiently transfected with COS7 cells that stably expressed fLuc (RzB + fLuc). An inactive mutant of RzB with a single mutation at the catalytic site (RzBm) was used as the control.^[13] The

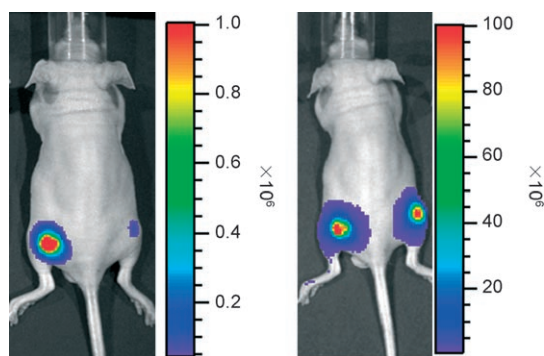


Figure 4. Imaging of Bla activity in living mice ($n=4$; n is number of mice). 1×10^6 COS7 cells were cotransfected with Bla and fLuc and injected into left rear thigh of a nude mouse, and the same number of cells transfected with fLuc only were injected at the right rear thigh. After implantation for 2 h, Bluco (78 μ g) was injected intravenously into each mouse for bioluminescence imaging of the Bla activity (left image), and then D-luciferin (0.14 mg) was injected to measure the fLuc activity at both sites (right image). Intensity unit: photons $s^{-1} cm^{-2} steradian^{-1}$.

cis splicing of RzB was confirmed by in vitro RT-PCR (reverse-transcription polymerase chain reaction) analysis (see the Supporting Information). The in vitro Bluco assay revealed that the Bla activity from transfected cells was 4 times higher than that of the control (Figure 5b). As shown previously,^[13] the Bla activity resulting from the RzB splicing was able to be imaged with CCF2/AM in single live cells under a fluorescence microscope (Figure 5c). The blue fluorescence indicated Bla activity from the splicing reaction, and the mutant construct (RzBm) did not emit any blue fluorescence but only green fluorescence from uncleaved CCF2.

We then injected these transfected COS7 cells (1×10^6) into nude mice at the left (transfected with RzB + fLuc) and right rear thighs (transfected RzBm + fLuc as the control). Two hours after the cell implantation, Bluco (0.3 mg) was injected intravenously into each mouse for bioluminescence imaging of the Bla activity (Figure 5d). The left injection site exhibited about twofold emission of that from the right injection site (see the Supporting Information). We verified the fLuc activity at both sites to be identical through separate D-luciferin injection and imaging. These data indicate that Bluco can image the Bla activity produced from the splicing of *Tetrahymena* ribozyme in vivo. This example also demonstrates that with the availability of Bluco and fluorogenic substrates for individual experimental settings, the Bla reporter assay can now be performed in vitro, in single living cells, and in whole living animals.

In conclusion, the work presented herein describes the design and evaluation of a new bioluminogenic substrate Bluco for imaging the Bla activity in vivo. Together with previously reported fluorogenic substrates,^[5–7] the bioluminogenic substrate reported herein completes the reporter enzyme Bla as a unified reporter for imaging in vitro, in single cultured living cells, and in whole living animals, and should enable many in vivo applications of the Bla reporter. Although this demonstration focused on mammalian cells,

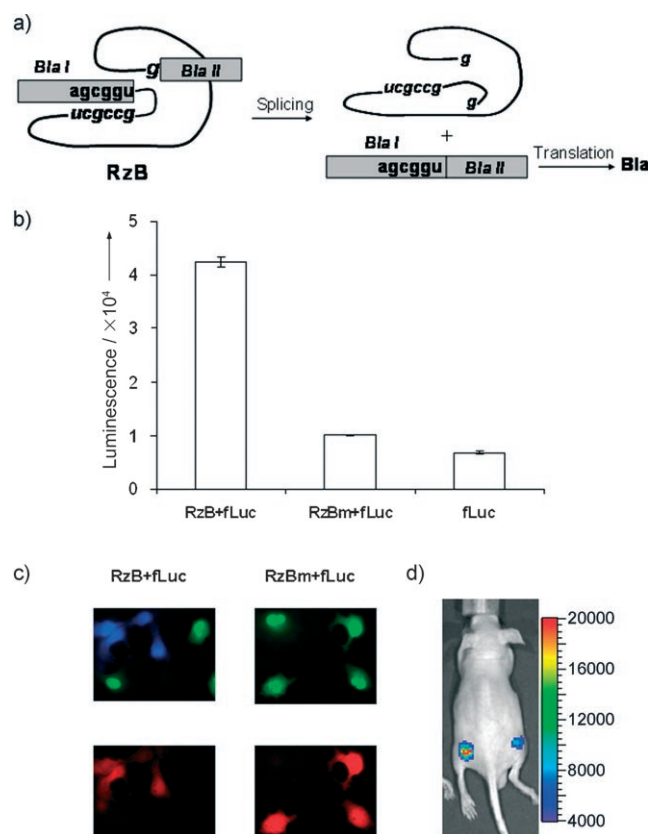


Figure 5. Detection and imaging of the *Tetrahymena* ribozyme-splicing activity in vitro, in single living cells, and in whole living mice with Bla as reporter. a) Schematic of the *Tetrahymena* ribozyme-splicing reporter RzB. b) Bluco assay of 1×10^5 intact COS7 cells transfected with indicated constructs (500 μ M Bluco; 30 min incubation). c) Imaging the ribozyme splicing in single living cells. COS7 cells were transfected with indicated constructs, and red fluorescent protein (dsRed) was used as the transfection marker. Cells were incubated with CCF2/AM for 1 h. Top panel images are an overlay of frames captured at 530 nm (green emission) and 460 nm (blue emission), and images in lower panel are dsRed emission at 605 nm with excitation at 560 nm. d) Representative image of mice ($n=4$) implanted with COS7 cells expressing RzB + fLuc (left rear thigh) and RzBm + fLuc (right rear thigh) and imaged with the similar procedure described in Figure 4.

Bluco may also be applied for in vivo imaging of β -lactam-resistant bacteria pathogens that express β -lactamase.

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- [1] K. Bush, R. B. Sykes in *Antimicrobial Drug Resistance* (Ed.: L. E. Bryan), Academic Press, New York, **1984**, pp. 1–31.
- [2] C. H. O'Callaghan, A. Morris, S. M. Kirby, A. H. Shingler, *Antimicrob. Agents Chemother.* **1972**, *1*, 283–288.
- [3] R. N. Jones, H. W. Wilson, W. J. Novick, Jr., *J. Clin. Microbiol.* **1982**, *15*, 677–683.
- [4] J. T. Moore, S. T. Davis, I. K. Dev, *Anal. Biochem.* **1997**, *247*, 203–209.

- [5] G. Zlokarnik, P. A. Negulescu, T. E. Knapp, L. Mere, N. Burren, L. Feng, M. Whitney, K. Roemer, R. Y. Tsien, *Science* **1998**, 279, 84–88.
- [6] W. Gao, B. Xing, R. Y. Tsien, J. Rao, *J. Am. Chem. Soc.* **2003**, 125, 11 146–11 147.
- [7] B. Xing, A. Khanamiryan, J. Rao, *J. Am. Chem. Soc.* **2005**, 127, 4158–4159.
- [8] C. P. Bleeker-Rovers, O. C. Boerman, H. J. Rennen, F. H. Corstens, W. J. Oyen, *Curr. Pharm. Des.* **2004**, 10, 2935–2950.
- [9] J. W. Hardy, K. P. Francis, M. DeBoer, P. Chu, K. Gibbs, C. H. Contag, *Science* **2004**, 303, 851–853.
- [10] X. Zhao, L. R. Hilliard, S. J. Mechery, Y. Wang, R. P. Bagwe, S. Jin, W. Tan, *Proc. Natl. Acad. Sci. USA* **2004**, 101, 15 027–15 032.
- [11] R. Edgar, M. McKinstry, J. Hwang, A. B. Oppenheim, R. A. Fekete, G. Giulian, C. Merrill, K. Nagashima, S. Adhya, *Proc. Natl. Acad. Sci. USA* **2006**, 103, 4841–4845.
- [12] M. Whitney, E. Rockenstein, G. Cantin, T. Knapp, G. Zlokarnik, P. Sanders, K. Durick, F. F. Craig, P. A. Negulescu, *Nat. Biotechnol.* **1998**, 16, 1329–1333.
- [13] S. Hasegawa, W. C. Jackson, R. Y. Tsien, J. Rao, *Proc. Natl. Acad. Sci. USA* **2003**, 100, 14 892–14 896.
- [14] S. Hasegawa, J. W. Choi, J. Rao, *J. Am. Chem. Soc.* **2004**, 126, 7158–7159.
- [15] M. Cavois, C. de Noronha, W. C. Greene, *Nat. Biotechnol.* **2002**, 20, 1151–1154.
- [16] A. Galarneau, M. Primeau, L.-E. Trudeau, S. W. Michnick, *Nat. Biotechnol.* **2002**, 20, 619–622.
- [17] J. M. Spotts, R. E. Dolmetsch, M. E. Greenberg, *Proc. Natl. Acad. Sci. USA* **2002**, 99, 15 142–15 147.
- [18] Z. Yang, P.-L. Ho, G. Liang, K. H. Chow, Q. Wang, Y. Cao, Z. Guo, B. Xu, *J. Am. Chem. Soc.* **2007**, 129, 266–267.
- [19] C. H. Contag, M. H. Bachmann, *Annu. Rev. Biomed. Eng.* **2002**, 4, 235–260.
- [20] T. S. Wehrman, G. von Degenfeld, P. O. Krutzik, G. P. Nolan, H. M. Blau, *Nat. Methods* **2006**, 3, 295–301.
- [21] W. H. Zhou, M. P. Valley, J. Shultz, E. M. Hawkins, L. Bernad, T. Good, D. Good, T. L. Riss, D. H. Klaubert, K. V. Wood, *J. Am. Chem. Soc.* **2006**, 128, 3122–3123.
- [22] K. Shah, C. H. Tung, X. O. Breakefield, R. Weissleder, *Mol. Ther.* **2005**, 11, 926–931.