

## Development of cell-impermeable coelenterazine derivatives†

Cite this: *Chem. Sci.*, 2013, **4**, 4395Eric Lindberg,<sup>a</sup> Shin Mizukami,<sup>ab</sup> Keiji Ibata,<sup>cd</sup> Takashi Fukano,<sup>d</sup> Atsushi Miyawaki<sup>de</sup> and Kazuya Kikuchi<sup>\*ab</sup>

We describe the development of the first cell-membrane impermeable coelenterazine derivative (CoelPhos). CoelPhos was constructed by the alkylation of coelenterazine with a linker containing a terminal anionic phosphonate moiety. The bioluminescence activity of CoelPhos with *Gaussia* luciferase (GLuc) showed a significantly higher activity in comparison with *Renilla* luciferase. In imaging studies with living cells, outer membrane bound GLuc was clearly imaged with CoelPhos. On the other hand no signal could be detected with intracellularly localized GLuc, demonstrating the impermeability of this novel coelenterate substrate derivative. CoelPhos has potential utility as a new bioluminogenic tool for the monitoring of dynamic fusion events at the cell-surface interface.

Received 16th July 2013  
Accepted 3rd September 2013

DOI: 10.1039/c3sc51985f

www.rsc.org/chemicalscience

## 1 Introduction

Bioluminescence imaging (BLI) is a valuable technique that is readily used in the life sciences. Bioluminescence is a natural phenomenon that involves the conversion of chemical energy into the emission of visible light. A luciferase enzyme catalyzes the chemical reaction between a luciferin substrate and molecular oxygen, generating a high energy species which, upon decaying, results in the emission of light. Firefly and *Renilla* luciferases are the most commonly used luciferases, with D-luciferin and coelenterazine as their respective bioluminogenic substrates. Firefly luciferase (FLuc) is usually the preferred choice due to its more favorable emission spectrum (560 nm). It is not suitable though, for the monitoring of extracellular events due to the requirement of intracellular co-factors such as ATP, which can activate purinergic ion-channel receptors. Coelenterate luciferases on the other hand do not require extra co-factors besides molecular oxygen. *Gaussia* luciferase (GLuc) is the brightest among all reported luciferases and displays a flash-type luminescence profile.<sup>1</sup> These

luciferases have mostly been used as reporters to monitor dynamic changes in gene expression and transcription.<sup>2</sup>

The monitoring of single exocytotic events in living cells has mostly employed methods such as total internal reflection fluorescence (TIRF), and two-photon laser scanning microscopy.<sup>3–6</sup> However, these methods are confined to monitoring a limited section of the cells and the use of fluorogenic dyes for accurate localization can be difficult due to diffusion. In addition, fluorescence methods require continuous irradiation, causing cell damage and photobleaching. Moreover due to their temporal resolution properties, confocal and two-photon laser scanning microscopies are more suitable when monitoring the membrane fusion of vesicles with slower kinetics.<sup>7</sup>

BLI can offer distinct advantages over fluorescence imaging in the monitoring of protein secretion and other exocytotic events in living cells. The visualization of luciferase secretion in living mammalian cells was first realized with *Cypridina* luciferase (CLuc) in CHO cells,<sup>8</sup> and in live mouse embryos to monitor the transcriptional activation of genes.<sup>9</sup> CLuc was also utilized for imaging neurotransmitter release.<sup>10</sup> Recently secretion of the brighter GLuc in PC12D cells was monitored in real-time.<sup>11</sup> However, in general BLI has suffered from poor resolution due to low luminescence intensity. Using an electron multiplying charge coupled device (EM-CCD) camera, video-rate BLI was employed in studying the secretory dynamics of MMP-2 with GLuc at much improved resolutions.<sup>12</sup> In another study, the same group also demonstrated that video-rate BLI could be used for the quantitative analysis of insulin oscillations in pancreatic MIN6  $\beta$  cells, which could have potential drug-screening applications.<sup>13</sup>

Recently, in an effort to monitor exocytosis in synaptic boutons *via* BLI, a mutant GLuc with enhanced luminescence output was fused to the part of a vesicle-associated membrane

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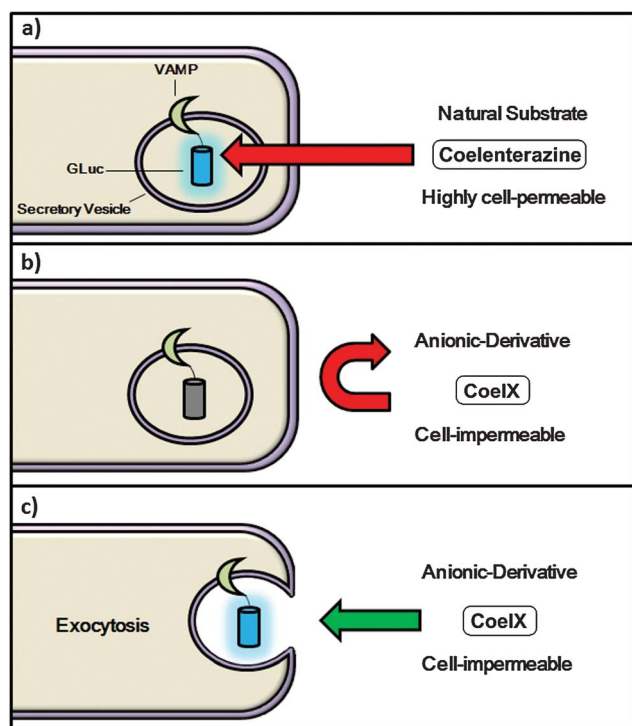
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† Electronic supplementary information (ESI) available: Details of synthesis and characterization of coelenterazine, 2-BnO-TEG-CTZ, 6-BnO-TEG-CTZ, and CoelPhos. In addition, details of generation of GLucM23 is included. See DOI: 10.1039/c3sc51985f

protein (VAMP) located within the interior of the synaptic vesicle (unpublished data). Hence, when the vesicle fuses with the cell membrane, undergoing exocytosis, the luciferase would react with its substrate coelenterazine, generating a bioluminescent response. However, a very poor signal-to-noise ratio was observed which was attributed to the high cell-membrane permeability of coelenterazine, *i.e.* coelenterazine could diffuse across the synaptic and vesicle membranes and react with the luciferase before the exocytotic event, giving rise to high background luminescence (Fig. 1a). Miesenbock *et al.* also observed a similar issue with *Cypridina* luciferin when imaging patterns of synaptic activity in hippocampal neuronal cells.<sup>10</sup> Therefore a modified coelenterazine substrate with decreased cell-membrane permeability is desired (Fig. 1b and c). However modifying the bioluminogenic substrate without having a negative impact on the bioluminescence activity is very difficult. This is especially true in the case of GLuc, which has very high substrate specificity. Hence, even the smallest modification of the coelenterazine substrate will result in a significant drop in bioluminescence activity.<sup>14–16</sup> Therefore it is not surprising that only one coelenterazine derivative (s-CTZ) with improved bioluminescence activity with GLuc has been reported.<sup>17</sup> The compound structure has yet to be published, however, the published data strongly implies that s-CTZ is highly cell-permeable. Hence we sought to design and synthesize a cell-membrane impermeable coelenterazine derivative with retained bioluminescence activity.



**Fig. 1** Monitoring membrane fusion events via BLI; concept (a) coelenterazine readily penetrates the cell-membrane, giving rise to background noise prior to fusion taking place; (b) coelenterazine derivative CoelX has a negative charge, making it highly cell-impermeable and unable to react with GLuc; (c) following fusion of the secretory vesicle with the cell-membrane CoelX can react with GLuc, generating a bioluminescence signal.

## 2 Results and discussion

### 2.1 Design and synthesis of 2-BnO-TEG-CTZ and 6-BnO-TEG-CTZ

When conceiving of a proper rational substrate design strategy, it is very useful to perform docking simulations of substrate derivatives before undertaking actual experimental work. Urano *et al.* constructed near-infrared-emitting firefly luciferins by initially performing docking simulations with luciferase from *Photinus pyralis*.<sup>18</sup> However, as there is no existing X-ray crystal structure of *Gaussia* luciferase (GLuc), and a lack of homology with other coelenterate luciferases, for which crystal-structures are available, the rational design and modification of coelenterazine remains a difficult task in terms of being able to predict the effect of any modification of the coelenterazine substrate on bioluminescence activity. Modification of the coelenterazine substrate has mostly been focused on increasing light output, red-shifting the emission spectrum of coelenterazine and increasing the stability of coelenterazine to decrease autoluminescence background noise.<sup>19–24</sup> As mentioned above, coelenterate luciferases share very little homology and hence any modification of coelenterazine that might have an advantageous impact on the luminescence output of one luciferase, might have the reverse effect on another. This is especially true for GLuc, which has been shown to have an extremely narrow substrate specificity.<sup>14–16</sup> In order to construct a cell-impermeable derivative of coelenterazine, we decided to attach a flexible linker with a terminal anionic phosphonate group. Initially we synthesized two coelenterazine derivatives with a highly flexible polyethylene glycol (PEG) linker with terminal benzyl-protecting groups installed at the 2 and 6 positions of coelenterazine (Fig. 2) to investigate the effect on the bioluminescence activity of GLuc.

We prepared **11** from tetraethylene glycol (TEG) by mono-benzylation with benzyl bromide, followed by bromination to **12** via the Apple reaction (Scheme S2†). Synthetic intermediates **6** and **10** were synthesized as previously described (Scheme S1†).<sup>25,26</sup> **13** was synthesized via standard alkylation of coelenteramine **6** with **12**. Following acid-catalyzed condensation reaction of **13** and the  $\alpha$ -ketoacetal **9** under reflux the desired compound 6-BnO-TEG-CTZ (**2**) was synthesized (Fig. 2). Synthesis of 2-BnO-TEG-CTZ (**3**) was accomplished by initial alkylation of deprotected  $\alpha$ -ketoacetal **10** with the TEG linker **12** to give **14**. Acid-catalyzed condensation reaction between **6** and **14** under reflux generated the desired product, 2-BnO-TEG-CTZ (**3**).

Next the luminescent properties of 2- and 6-BnO-TEG-CTZ were evaluated with GLuc in comparison with native coelenterazine. Both substrates 2-BnO-TEG-CTZ and 6-BnO-TEG-CTZ showed more than 200- and 950-fold reduction in total bioluminescence activity, respectively (Table 1). Interestingly, Urano *et al.* observed similarly poor bioluminescence activity when attaching flexible PEG linkers to aminoluciferin.<sup>18</sup> Installing rigid alkyl linkers also resulted in a low bioluminescence output. The poor observed activity was attributed to that these linkers may be linear and adopt conformations that hinder access of the luciferin substrate to the active site of the luciferase enzyme. Hence, it was concluded that long PEG



linkers conjugated near the luminophore are unsuitable. This conclusion also seems to be valid in the case of installing PEG linkers on coelenterazine as well.

## 2.2 Design and synthesis of CoelPhos

Due to the detrimental effect on bioluminescence activity of PEG-modified coelenterazines we decided to change to more rigid alkyl linkers. Although our initial results suggested that both 2- and 6-positions of coelenterazine were sensitive to modification, we decided to focus on modification of the 2-position. We synthesized a phosphonate alkyne, dimethyl-(prop-2-ynyloxy)methylphosphonate **16** in two steps (Scheme S3†). Alkylation of **10** with 1,3-dibromopropane resulted in the formation of **17**, which was converted into the propyl azide **18**. Copper(I)-catalyzed azide-alkyne Huisgen cycloaddition<sup>27</sup> gave the desired 1,2,3-triazole **19**. In the final step, acid-catalyzed condensation reaction of **6** and **19** was conducted, generating the desired product CoelPhos (Fig. 2) (see ESI† for further description and details).

Next, we evaluated the luminescent properties of CoelPhos with GLuc (Table 1) and *Renilla* luciferase (RLuc) in comparison with coelenterazine (Table S1†). CoelPhos yielded a much stronger luminescence with GLuc than RLuc and also a significant improvement in bioluminescence activity compared with both 2- and 6-BnO-TEG-CTZ, although CoelPhos still showed about a 30-fold reduction in luminescence intensity in comparison with coelenterazine. With the exception of s-CTZ,<sup>17</sup> the highest reported bioluminescence activities of any coelenterazine derivative with GLuc are those of MeO-CTZ (methylation of the 2-hydroxy group) (13.6%) and 3iso-CTZ (replacement of 2-hydroxy group with 3-isopropylbenzene), (14.3%).<sup>16</sup>

## 2.3 Bioluminescence imaging with CoelPhos

Recently GLuc mutants with improved luminescence output and stable luminescence profiles have been reported.<sup>28,29</sup> We utilized a recently developed mutant GLuc (GLucM23) with roughly 10-fold improvement in luminescence intensity in living cells, and a glow-type kinetic profile (see ESI†). We evaluated CoelPhos by measuring bioluminescence activity with a charge-coupled device (CCD) camera in HeLa cells expressing outer-membrane localized wild-type GLuc or GLucM23 (GLucM23<sub>Mem</sub>) to determine if the improved mutant GLuc had a negative or positive impact on the luminescence intensity of CoelPhos (Fig. 3). We determined the relative intensities of coelenterazine and CoelPhos by comparing the relative mKO

**Table 1** Relative and maximum luminescence of 2- and 6-BnO-TEG-CTZ, and CoelPhos

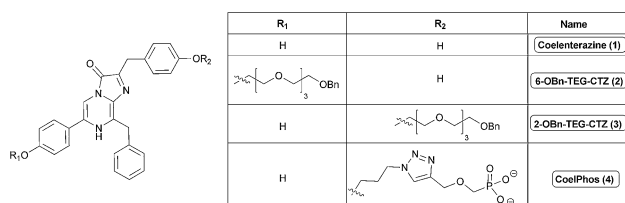
Compound	<i>Gaussia</i> luciferase	
	$I_{\text{Total}}^a$ (%)	$I_{\text{Max}}^b$ (%)
Coelenterazine ( <b>1</b> )	100	100
2-BnO-TEG-CTZ ( <b>2</b> )	0.45	0.17
6-BnO-TEG-CTZ ( <b>3</b> )	0.10	0.14
CoelPhos ( <b>4</b> )	3.23	2.70

<sup>a</sup>  $I_{\text{Total}}$ : total luminescence integrating for 60 s in 1 s intervals. <sup>b</sup>  $I_{\text{Max}}$ : maximum observed intensity at any 1 s interval.

fluorescence and luminescence intensities of 5 cells (Fig. 4). CoelPhos showed similar relative bioluminescence activity to the mutant GLucM23 in comparison with the wild-type GLuc relative to coelenterazine (~3–4%). Hence the GLuc mutant did not affect the bioluminescence activity of CoelPhos relative to coelenterazine. The contrast observed in HeLa cells expressing outer membrane bound GLuc was very poor in comparison with GLucM23. This is related to the flash-type luminescence of GLuc, together with the luminescence sensitivity of our imaging system. The stable glow-type luminescence of GLucM23 and its higher luminescence output makes it more suitable for BLI in living cells. This was especially true for our derivative CoelPhos which could be imaged with much improved signal-to-noise ratio with GLucM23<sub>Mem</sub> (Fig. 3k and l) over GLuc<sub>Mem</sub> (Fig. 3h and i).

Finally, we evaluated the relative cell-membrane permeability of our probe CoelPhos in comparison with coelenterazine. We transfected HeLa cells with an endoplasmic reticulum (ER)-localizing GLuc construct (GLuc<sub>ER</sub>). The construct also contained Venus fluorescent protein as a control for expression levels as well as confirmation of localization. We reasoned that whilst coelenterazine would give a strong signal due to its high cell-permeability, CoelPhos would not give any bioluminescence signal due to its inability to cross the cell-membrane (Fig. 5). When coelenterazine was added, strong bioluminescent signals were observed in HeLa cells expressing GLuc<sub>ER</sub>. On the other hand, when CoelPhos was added, no signal could be detected, even after extending the exposure time to 100 s (20-fold) (Fig. 6). The observed data seem to suggest that attachment of a terminal phosphonate moiety is enough to significantly decrease cell-membrane permeability of the coelenterate substrate.

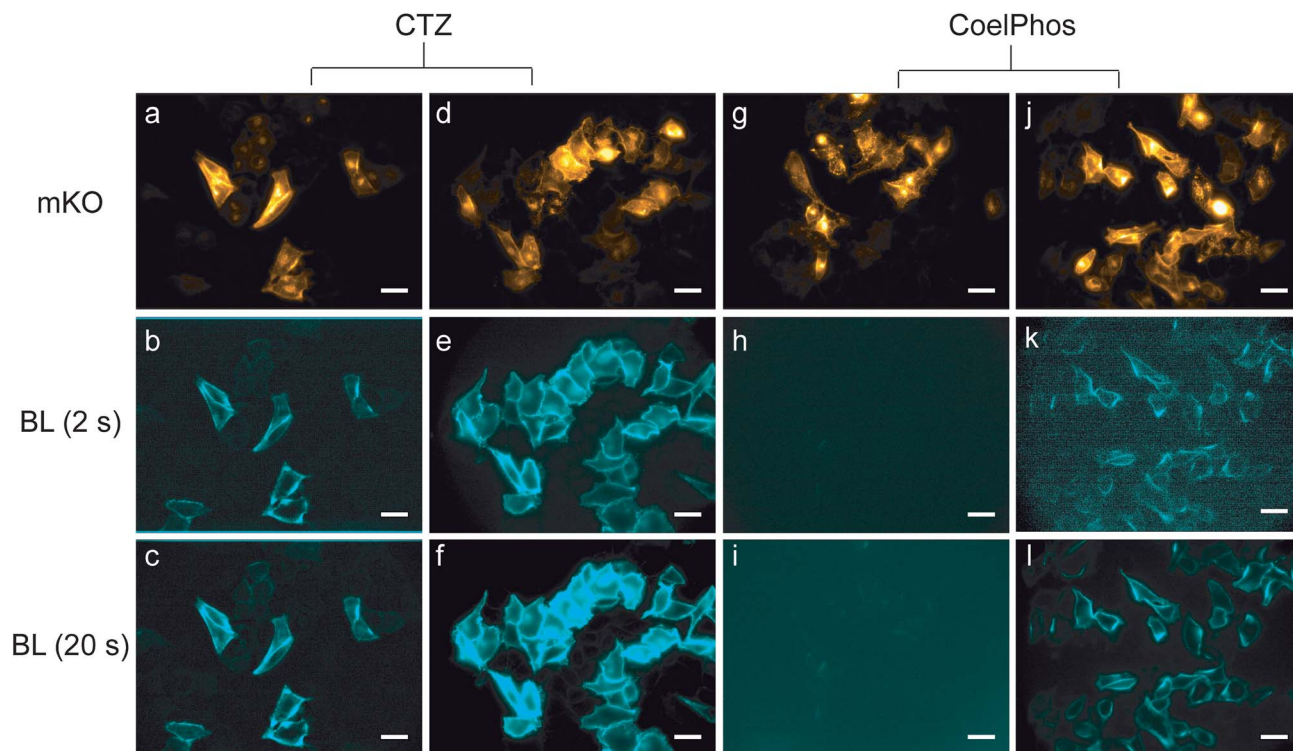
It has been shown that GLuc produces a 1000-fold higher BL signal than RLuc or FLuc in mammalian cells.<sup>1,30</sup> GLucM23 has roughly 10 times higher BL intensity over wild-type GLuc in mammalian cells, suggesting that this novel mutant GLuc could have a 10 000-fold higher BL signal over RLuc and FLuc in mammalian cells. Although there was an over 30-fold decrease in BL activity of CoelPhos with GLuc and GLucM23 in comparison with native coelenterazine, it seems reasonable to assume that CoelPhos together with GLucM23 would show a stronger BL intensity over RLuc with coelenterazine. Further investigation is required however, in order to be able to make a direct comparison.



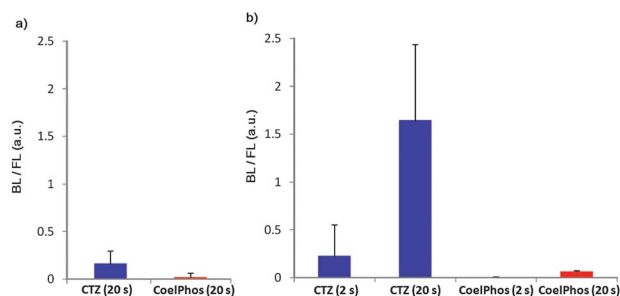
**Fig. 2** Structures of native coelenterazine and synthesized derivatives.







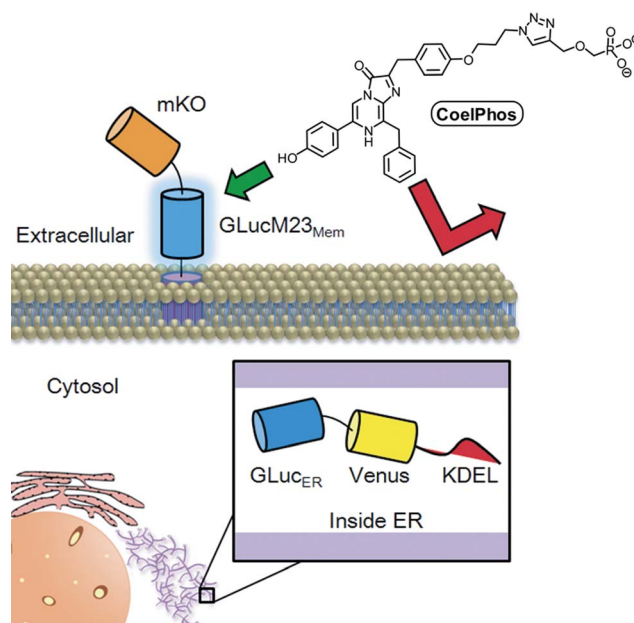
**Fig. 3** Bioluminescence imaging with coelenterazine (CTZ) and CoelPhos in HeLa cells expressing outer-membrane bound GLuc or GLucM23. (a, d, g and j) Fluorescence images of mKO ( $\lambda_{\text{ex}} = 548 \text{ nm}/\lambda_{\text{em}} = 559 \text{ nm}$ ; 200 ms) before addition of bioluminogenic substrate. CTZ with GLuc<sub>Mem</sub>, exposure time: 2 s (b) and 20 s (c); CTZ with GLucM23<sub>Mem</sub>, exposure time: 2 s (e) and 20 s (f); CoelPhos with GLuc<sub>Mem</sub>, exposure time: 2 s (h) and 20 s (i); CoelPhos with GLucM23<sub>Mem</sub>, exposure time: 2 s (k) and 20 s (l). Concentration of CTZ and CoelPhos: 22.7  $\mu\text{M}$ . Objective lens: 60 $\times$ . Scale bar: 40  $\mu\text{m}$ .



**Fig. 4** Relative activity of CoelPhos with GLuc and GLucM23 versus coelenterazine (CTZ) in HeLa cells. (a) CTZ and CoelPhos with GLuc; (b) CTZ and CoelPhos with GLucM23 ( $n = 5$  cells); signal was quantified using ImageJ (2 or 20 s) = exposure time.

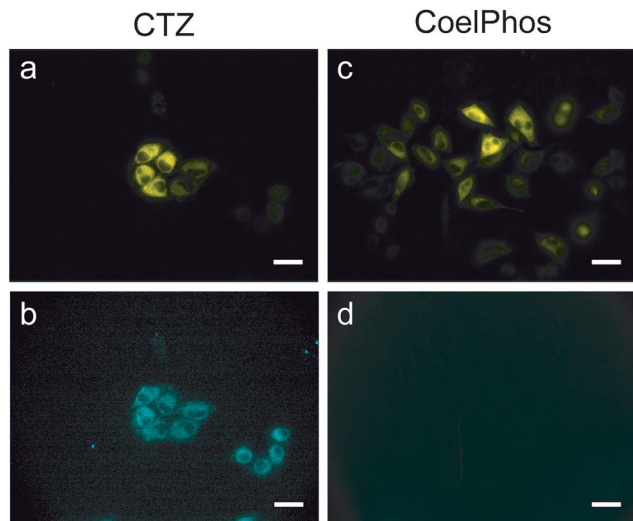
Not only does the substrate specificity of GLuc differ from other coelenterate luciferases such as *Renilla* and *Oplophorus* luciferase but GLuc has been shown to contain two catalytic domains; both with similarly narrow substrate specificities.<sup>30</sup> Significant differences are also observed in the kinetic properties. Unlike other marine luciferases (RLuc, CLuc) which respond to their respective coelenterate luciferin concentration in a linear non-cooperative manner, it was recently shown that GLuc operates in a cooperative manner, possibly *via* an allosteric mechanism.<sup>31</sup>

Previous studies utilizing bioluminescence imaging for monitoring of real-time protein secretion showed the



**Fig. 5** Illustration of the cell-permeability of CoelPhos. Because of the attached anionic phosphonate group CoelPhos does not penetrate the cell-membrane as readily as native coelenterazine. Therefore a bioluminescent signal will be observed with outer-membrane bound GLucM23<sub>Mem</sub> but not with intracellularly localized GLuc<sub>ER</sub>.





**Fig. 6** Fluorescence and bioluminescence images of HeLa cells expressing GLuc<sub>ER</sub> with coelenterazine (CTZ) and CoelPhos. (a) Venus FL; (b) CTZ (22.7  $\mu$ M); exposure time: 5 s; (c) Venus FL; (d) CoelPhos (22.7  $\mu$ M); exposure time: 100 s. Objective lens: 60 $\times$ . Scale bar: 40  $\mu$ m.

importance of the type of camera used. Exocytotic events of native GLuc in CHO-K1 cells were visualized in real time with a time resolution of 10 s. However, the resolution of the luminescence spots was low, which made it difficult to identify single exocytotic spots of luminescence due to the low luminescence sensitivity of the camera used.<sup>12</sup> In more recent studies the same group utilized a more powerful EM-CCD which resulted in a 60-fold increase in sensitivity, which allowed the real-time monitoring of localization and dynamics of proteins on the surfaces of cells with millisecond temporal resolution.<sup>13,14</sup> Notwithstanding the fact that the CCD camera employed in our imaging studies was inferior to cameras utilized in the above references in terms of quantum efficiency and signal-to-noise ratio, we were able to visualize CoelPhos with GLucM23<sub>Mem</sub> at a sampling rate of 2 s. Hence it is plausible to venture that utilization of EM-CCD cameras with improved sensitivity will allow bioluminescence imaging of CoelPhos with much improved temporal resolution and luminescence sensitivity.

### 3 Conclusions

In conclusion, we have developed a cell-membrane impermeable coelenterazine derivative, CoelPhos, as a potential tool for monitoring membrane fusion events in living cells. The derivative was constructed by alkylating the phenolic hydroxy group at the 2-position of coelenterazine with an alkyl linker containing a terminal phosphonate group. While displaying a 30-fold lower activity with GLuc compared with native coelenterazine, CoelPhos showed a high specificity for GLuc over RLuc with a 30-fold higher activity. By utilizing a new mutant GLuc, GLucM23, we were able to image membrane-localized GLucM23 despite the lower luminescence intensity of CoelPhos. We demonstrated that CoelPhos has decreased cell-membrane permeability in comparison with native coelenterazine. Our

probe, CoelPhos, has the potential to be used as a cell-impermeable bioluminescent tool for the monitoring of exocytotic events.

### Acknowledgements

This work was supported in part by the Cabinet Office, Government of Japan and through the Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program) from JSPS, by the Grant-in-Aid for Scientific Research from MEXT of Japan (nos 20675004, 22108519, 25620133, 25242072, and 24108724 for K.K.; 24685028, 24115513, and 24651259 for S.M.; 23700387 for K.I.), by the CREST from JST, by the Grant-in-Aid from MHLW of Japan, by the Naito Foundation, by the Takeda Science Foundation, by the Magnetic Health Science foundation, and by the Asahi Glass Foundation. The authors are also grateful for a Japanese government scholarship (Monbukagakusho) for E.L. to perform his PhD thesis work in the international frontier biotechnology program. We would like to thank Dr Yutaro Kumagai and Prof. Shizuo Akira for providing us with the plasmid for expressing *Renilla luciferase*.

### Notes and references

- 1 B. A. Tannous, D.-E. Kim, J. L. Fernandez, R. Weissleder and X. O. Breakefield, *Mol. Ther.*, 2005, **11**, 435–443.
- 2 T. Ozawa, H. Yoshimura and S. B. Kim, *Anal. Chem.*, 2013, **85**, 590–609.
- 3 I. Akopova, S. Tatur, M. Grygorczyk, R. Luchowski, I. Gryczynski, J. Boredjo and R. Grygorczyk, *Purinergic Signalling*, 2012, **8**, 59–70.
- 4 J. G. Burchfield, J. A. Lopez, K. Mele, P. Vallotton and W. E. Hughes, *Traffic*, 2010, **11**, 429–439.
- 5 N. Takahashi and H. Kasai, *Endocr. J.*, 2007, **54**, 337–346.
- 6 A. Oshima, T. Kojima, K. Dejima, Y. Hisa, H. Kasai and T. Nemoto, *Cell Calcium*, 2005, **37**, 349–357.
- 7 M. Matsuzaki, G. C. R. Ellis-Davies, T. Nemoto, Y. Miyashita, M. Iino and H. Kasai, *Nat. Neurosci.*, 2001, **4**, 1086–1092.
- 8 S. Inouye, Y. Ohmiya, Y. Toya and F. Tsuji, *Proc. Natl. Acad. Sci. U. S. A.*, 1992, **89**, 9584–9587.
- 9 E. M. Thompson, P. Adenot, F. Tsuji and J.-P. Renard, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, **92**, 1317–1321.
- 10 G. Miesenbock and J. E. Rothman, *Proc. Natl. Acad. Sci. U. S. A.*, 1997, **94**, 3402–3407.
- 11 T. Suzuki, S. Usuda, H. Ichinose and S. Inouye, *FEBS Lett.*, 2007, **581**, 4551–4556.
- 12 T. Suzuki, C. Kondo, T. Kanamori and S. Inouye, *Anal. Biochem.*, 2011, **415**, 182–189.
- 13 T. Suzuki, C. Kondo, T. Kanamori and S. Inouye, *PLoS One*, 2011, **6**, e25243.
- 14 S. Inouye and Y. Sahara, *Biochem. Biophys. Res. Commun.*, 2008, **365**, 96–101.
- 15 T. Kimura, K. Hiraoka, N. Kasahara and C. R. Logg, *J. Gene Med.*, 2010, **12**, 528–537.
- 16 S. Inouye, Y. Sahara-Miura, J.-I. Sato, R. Iimori, S. Yoshida and T. Hosoya, *Protein Expression Purif.*, 2013, **88**, 150–156.



- 17 D. Morse and B. A. Tannous, *Mol. Ther.*, 2012, **20**, 692–693.
- 18 R. Kojima, H. Takakura, T. Ozawa, Y. Tada, T. Nagano and Y. Urano, *Angew. Chem., Int. Ed.*, 2012, **51**, 1–6.
- 19 O. Shimomura, B. Musick and Y. Kishi, *Biochem. J.*, 1988, **251**, 405–410.
- 20 O. Shimomura, B. Musick and Y. Kishi, *Biochem. J.*, 1989, **261**, 913–920.
- 21 C. F. Qi, Y. Gomi, T. Hirano, M. Ohashi, Y. Ohmiya and F. Tsuji, *J. Chem. Soc., Perkin Trans. 1*, 1992, 1607–1611.
- 22 S. Inouye and O. Shimomura, *Biochem. Biophys. Res. Commun.*, 1997, **233**, 349–353.
- 23 C. Wu, H. Nakamura, A. Murai and O. Shimomura, *Tetrahedron Lett.*, 2001, **42**, 2997–3000.
- 24 M. P. Hall, J. Unch, B. F. Binkowski, M. P. Valley, B. L. Butler, M. G. Wood, P. Otto, K. Zimmerman, G. Vidugiris, T. Machleidt, M. B. Robers, H. A. Benink, C. T. Eggers, M. R. Slater, P. L. Meisenheimer, D. H. Klaubert, F. Fan, L. P. Encell and K. V. Wood, *ACS Chem. Biol.*, 2012, **7**, 1848–1857.
- 25 M. Adamczyk, D. D. Johnson, P. G. Mattingly, Y. Pan and R. E. Reddy, *Org. Prep. Proced. Int.*, 2001, **33**, 477–485.
- 26 M. Adamczyk, S. R. Akireddy, D. D. Johnson, P. G. Mattingly, Y. Pan and R. E. Reddy, *Tetrahedron*, 2003, **59**, 8129–8142.
- 27 V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2002, **41**, 2596–2599.
- 28 S. B. Kim, H. Suzuki, M. Sato and H. Tao, *Anal. Chem.*, 2011, **83**, 8732–8740.
- 29 M. H. Degeling, M. S. S. Bovenberg, G. K. Lewandrowski, M. C. de Gooijer, C. L. A. Vleggeert-Lankamp, M. Tannous, C. A. Maguire and B. A. Tannous, *Anal. Chem.*, 2013, **85**, 3006–3012.
- 30 S. Inouye and Y. Sahara, *Biochem. Biophys. Res. Commun.*, 2008, **365**, 96–101.
- 31 G. Tzertzinis, E. Schildkraut and I. Schildkraut, *PLoS One*, 2012, **7**, e40099.

