

# A Biocompatible “Split Luciferin” Reaction and Its Application for Non-Invasive Bioluminescent Imaging of Protease Activity in Living Animals

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## ABSTRACT

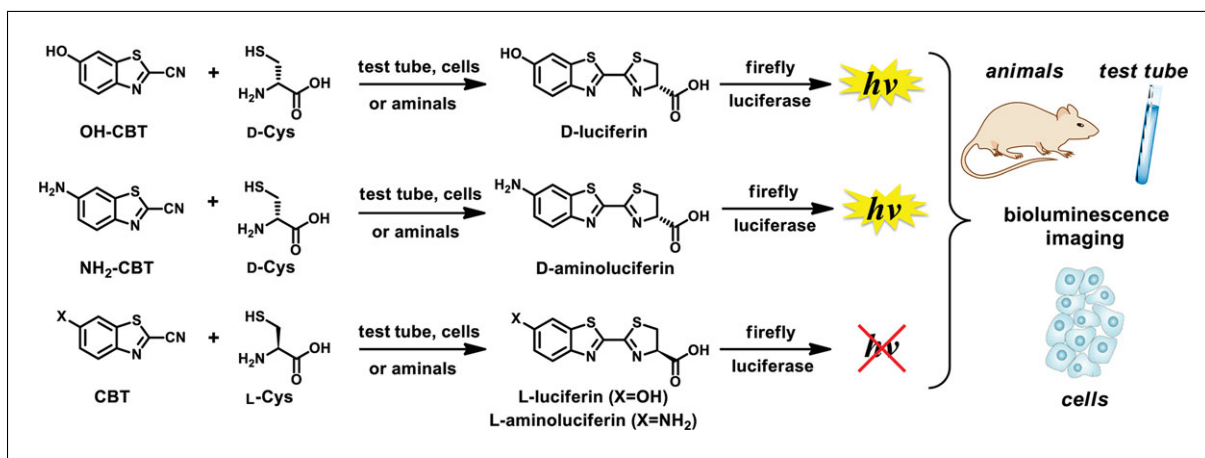
The great complexity of many human pathologies, such as cancer, diabetes, and neurodegenerative diseases, requires new tools for studies of biological processes on the whole organism level. The discovery of novel biocompatible reactions has tremendously advanced our understanding of basic biology; however, no efficient tools exist for real-time non-invasive imaging of many human proteases that play very important roles in multiple human disorders. We recently reported that the “split luciferin” biocompatible reaction represents a valuable tool for evaluation of protease activity directly in living animals using bioluminescence imaging (BLI). Since BLI is the most sensitive *in vivo* imaging modality known to date, this method can be widely applied for the evaluation of the activity of multiple proteases, as well as identification of their new peptide-specific substrates. In this unit, we describe several applications of this “split luciferin” reaction for quantification of protease activities in test tube assays and living animals. *Curr. Protoc. Chem. Biol.* 6:169-189 © 2014 by John Wiley & Sons, Inc.

Keywords: protease activity • biocompatible reaction • non-invasive • bioluminescent imaging • *in vivo*

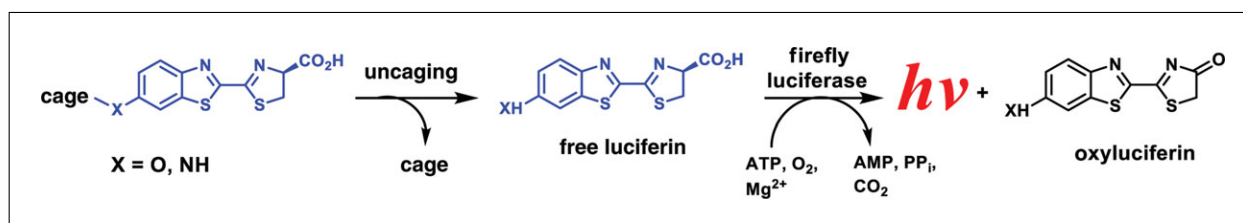
## INTRODUCTION

The discovery of biocompatible reactions had a significant impact on chemical biology and allowed studies of many biological processes directly in living systems. However, despite the fact that multiple biocompatible reactions have been developed in the past decade, very few work efficiently in complex living organisms. We previously reported that D-cysteine and 2-cyanobenzothiazole (CBT) can selectively react with each other *in vivo* to generate luciferin substrates for firefly luciferase (“split luciferin reaction,” Fig. 1) (Godinat et al., 2013).

Since the production of luciferin substrates can be visualized and quantified directly in genetically modified living animals by bioluminescence imaging (BLI), it has immediate application for studies of many biological processes. For example, this reaction is well suited for interrogation of targeted tissues using a “caged” luciferin approach. This



**Figure 1** “Split luciferin” reaction: Overall schematic of the split luciferin ligation reaction between D- or L-cysteine and hydroxy- or amino-cyanobenzothiazole derivatives (OH-CBT and NH<sub>2</sub>-CBT) in various biological environments. Adapted with permission from Godinat et al. (2013). Copyright 2014 American Chemical Society.

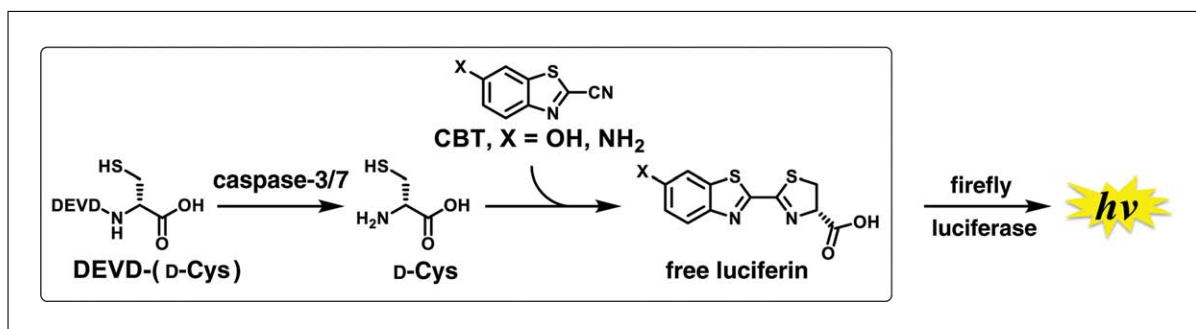


**Figure 2** Probing molecular signatures of target tissues through the use of caged luciferin substrates.

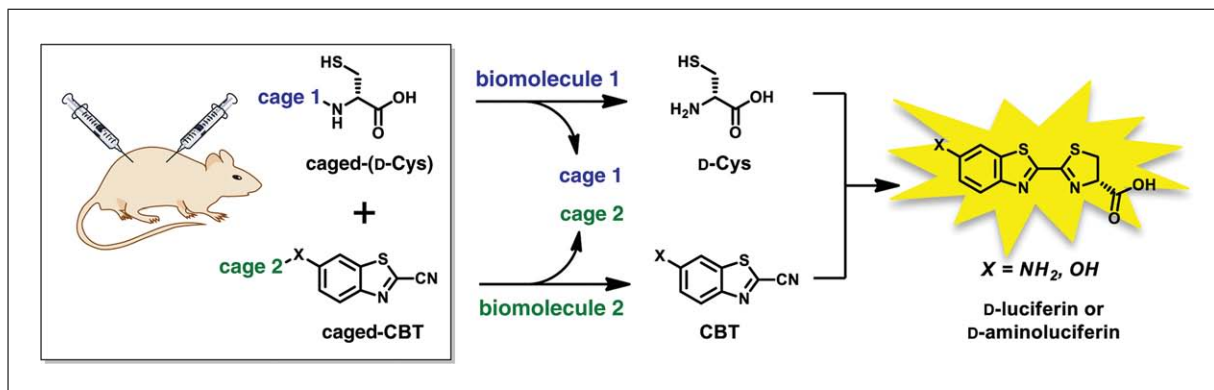
technique is based on the fact that luciferins caged on the phenolic oxygen or aryl nitrogen do not lead to light production (Wehrman et al., 2006; Cohen et al., 2010; van de Bittner et al., 2010; Henkin et al., 2012) (Fig. 2). Despite the fact that caged luciferin compounds have been previously generated to quantify activities of several biological processes (Shah et al., 2005; Goun et al., 2006; Wehrman et al., 2006; Cosby et al., 2007; Yao et al., 2007; Dragulescu-Andrasi et al., 2009; Biserni et al., 2010; Cohen et al., 2010; Hickson et al., 2010; van de Bittner et al., 2010; Scabini et al., 2011; Henkin et al., 2012), the synthesis of their scaffolds involves much more complex and low-yielding synthetic procedures, representing a major limitation of this technology (Massoud and Gambhir, 2003; McCaffrey et al., 2003; Liu et al., 2005; Shah et al., 2005; Wehrman et al., 2006; Yao et al., 2007; Dragulescu-Andrasi et al., 2009; Cohen et al., 2010; Prescher and Contag, 2010; Reddy et al., 2010; van de Bittner et al., 2010; Harwood et al., 2011; Conley et al., 2012; Henkin et al., 2012; Liang et al., 2012a,b; McCutcheon et al., 2012).

We previously reported application of this “split luciferin reaction” for the real-time and non-invasive imaging of apoptosis, associated with activation of caspase 3/7 (Godinat et al., 2013). Caspase-dependent release of free D-cysteine from the caspase 3/7-specific peptidic substrate Asp-Glu-Val-Asp-D-Cys [DEVD-(D-Cys)] allowed further reaction with 6-amino-2-cyanobenzothiazole (NH<sub>2</sub>-CBT) *in vivo* and subsequent formation of 6-amino-D-luciferin. Since this compound is known to be an excellent substrate for luciferase, its formation leads to the generation of light that is proportional to the activity of caspase 3/7. Importantly, this “split luciferin” strategy was found to be more sensitive when compared to the commercially available DEVD-aminoluciferin substrate (Godinat et al., 2013), in which the entire luciferin is caged with the same peptidic sequence. The underlying principle of the use of “split luciferin reaction” for protease imaging is depicted in Figure 3 using caspase 3/7 peptide-specific sequence (DEVD) as an example.

This result was particularly exciting because the “split luciferin” approach can now be applied for imaging and quantification of many other proteases that are known to cleave



**Figure 3** Imaging of caspase 3/7 activity using the “split luciferin” reaction. Modification on the D-cysteine moiety with DEVD sequence allows selective formation of D-luciferin upon caspase-3/7 cleavage and subsequent production of light, proportional to activity of caspase 3/7. Adapted with permission from Godinat et al. (2013). Copyright 2014 American Chemical Society.



**Figure 4** Overall representation of the dual imaging concept for luciferin ligation. Both luciferin ligation precursors could be caged as sensors for two different biomolecules. Only when both become uncaged, D-luciferin or D-aminoluciferin is formed as the result of split luciferin ligation reaction, allowing the production of light by luciferase enzyme. Reprinted with permission from Godinat et al. (2013). Copyright 2014, American Chemical Society.

at the end of specific amino acid sequences (Wilson et al., 2002; Cali et al., 2006; Zhou et al., 2006a,b; Yao et al., 2007), as well as identification of new protease-specific peptides associated with various human diseases. Immediate examples of mammalian proteases include dipeptidyl peptidase 4 (GP and VP), tryptase (PRNK), and various caspases such as caspase 2 (VDVAD), caspase 6 (VEID), caspase 8 (LETD), caspase 9 (LHTD), and caspase 12 (ATAD) (O’Brien et al., 2005; Cali et al., 2006; Geiger et al., 2006; Zhou et al., 2006a,b; Ren et al., 2009). In addition to mammalian proteases, this “split luciferin” methodology could also be used for studies of a wide variety of viral, parasite, and bacterial proteases that play very important roles in the replication and the spread of infectious diseases (Wilson et al., 2002). They include SARS protease (TSAVLQ), caspase-like (nLPnLD), and trypsin-like (LRR) proteases (Cali et al., 2006; Geiger et al., 2006; Zhou et al., 2006a,b).

Importantly, the synthesis of short peptide sequences with C-terminal D-cysteine can be easily performed with the help of automated peptide synthesis, which is a widely available and versatile technique (Merrifield, 1965; Merrifield and Stewart, 1965). On the contrary, the synthesis of caged luciferin substrates like DEVD-aminoluciferin represents a major synthetic challenge, which is reflected by a very high cost of commercially available probes. Moreover, the new approach suits itself perfectly for multiplex imaging based on the modular construction of bioluminogenic sensors, where either or both reaction partners (D-Cys and CBT) are caged to report on multiple biological events (Fig. 4).

Therefore, we believe that this protocol can be applied to evaluation of activity of multiple biologically important proteases that are known to cleave at the end of corresponding

specific amino acid sequences and allow sensitive imaging and quantification of their activities directly in animal models of disease.

Basic Protocol 1 describes the methodology for imaging and quantifying protease activity in a cell-free in vitro experimental setup. The procedure is detailed for caspase-3 imaging. Thrombin protease activity imaging is also described in the Alternate Protocol. Finally, Basic Protocol 2 describes in detail the use of “split luciferin” based bioluminescent probes for real time non-invasive caspase-3/7 imaging in luciferase-expressing mice.

*NOTE:* All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

## **BASIC PROTOCOL 1**

### **IN VITRO (CELL-FREE) BIOLUMINESCENCE IMAGING OF PROTEASE ACTIVITY USING “SPLIT LUCIFERIN” REACTION**

The following procedure describes in detail all the steps necessary for evaluation of activity of thrombin and caspase 3 proteases in a plate reader format. However, the same approach can be adapted to studies of activity of other proteases that are known to cleave their peptide-specific sequences on the C-terminal of the cleavage site.

The development of novel probes for evaluation of protease activities should start from the selection of protease-specific amino-acid sequence that can be selectively recognized and cleaved by a protease of interest (POI). When such sequence is selected, a D-cysteine (D-Cys) residue should be introduced at the C-terminal position using common and widely used automated or manual peptide synthesis techniques (Merrifield, 1965; Merrifield and Stewart, 1965). If evaluation of thrombin or caspase 3/7 proteases are intended, the exact protocol described below should be followed. To test the viability of this approach with the desired POI, the selected peptide containing the D-Cys on the C-term will be incubated with the purified POI. After addition of CBT and luciferase enzyme, light emission will be acquired.

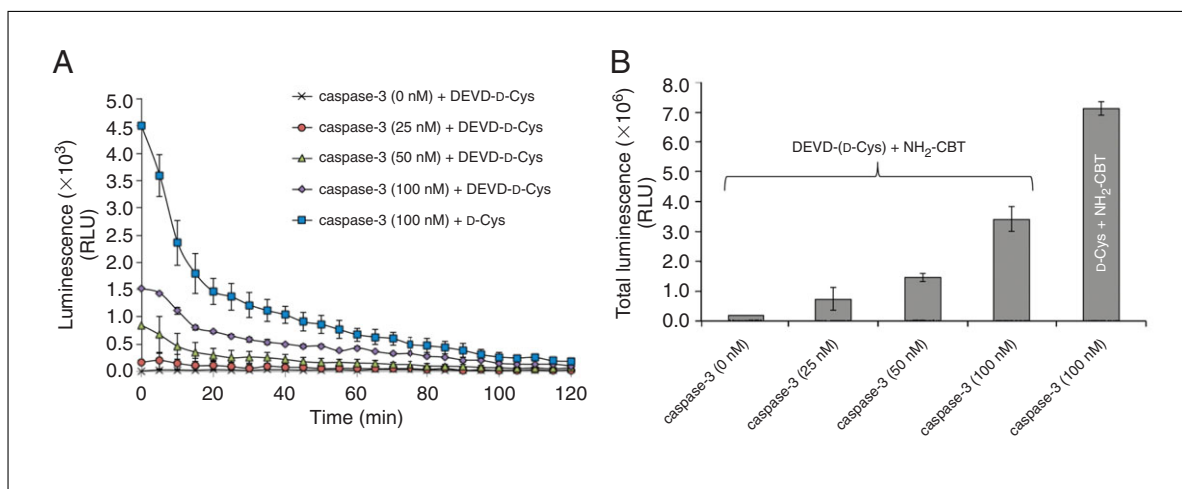
#### ***Example with caspase 3***

In the first step, activated caspase-3 enzyme is incubated with DEVD-(D-Cys) peptide. Once cleaved by the protease, the resulting D-Cys reacts with NH<sub>2</sub>-CBT leading to the formation of amino-D-luciferin and further light production, proportional to the activity of caspase 3 (Fig. 3). An example of the data that was acquired for quantification of caspase-3 activity in vitro using the split luciferin approach is depicted in Figure 5 (Godinat et al., 2013). In these experiments, the DEVD-(D-Cys) probe was incubated with increasing concentrations of caspase-3 followed by addition of NH<sub>2</sub>-CBT. The resulting light emission was acquired over 2 hr and plotted as a function of time (Fig. 5A). Quantification of the total light produced over this period of time was performed by integrating the area under the curves and plotting in the form of bar-graph (Fig. 5B).

Caspase-3 was purified and characterized following the reported procedure (Stennicke and Salvesen, 1999).

#### ***Materials***

- Caspase buffer (see recipe)
- 200 nM of purified caspase-3 solution (see recipe)
- 800 μM D-cysteine solution (see recipe)
- 800 μM DEVD-(D-Cys) peptide solution (see recipe)
- 400 μM 6-amino-2-cyanobenzothiazole (NH<sub>2</sub>-CBT; see recipe)
- 60 μg/ml Firefly luciferase enzyme solution (see recipe)



**Figure 5** Quantification of caspase-3 activity in test-tube assay using the “split luciferin approach.” **(A)** Bioluminescent signal as a function of time from DEVD-(D-Cys) peptide (200  $\mu\text{M}$ ) or D-cysteine (200  $\mu\text{M}$ ) after incubation with different concentrations of caspase-3 (25, 50, and 100 nM) over 3 hr at 37°C, followed by addition of NH<sub>2</sub>-CBT (400  $\mu\text{M}$  in MeOH), and incubation for additional 1 hr at 37°C. The signal acquisition was started immediately after addition of 5  $\mu\text{l}$  of the premade luciferase buffer (see recipe) and acquired for the duration of 2 hr using the IVIS Spectrum (Perkin Elmer). (Error bars are  $\pm$  SD for three measurements.) **(B)** Total light output collected over the period of 2 hr (performed by integrating the area under the curves in panel A and plotted in the form of a bar-graph). Adapted with permission from Godinat et al. (2013). Copyright 2014, American Chemical Society.

V-shaped 96-well plate (Vitaris, cat. no. 3897-COR)

37°C incubator

Black 96-well plate (BD Falcon, cat. no. 353219)

Bioluminescence plate reader *or* imager (Spectramax M5, Molecular Devices)

1. Prepare different solutions of caspase-3 in caspase buffer by diluting 200 nM caspase-3 stock solution as follows:
  - 0 nM: only caspase buffer
  - 50 nM: dilute 100  $\mu\text{l}$  of 200 nM caspase-3 stock solution to 400  $\mu\text{l}$  using caspase buffer
  - 100 nM: dilute 200  $\mu\text{l}$  of 200 nM caspase-3 stock solution to 400  $\mu\text{l}$  using caspase buffer
2. Add caspase-3 solutions (0, 50, 100 nM) in triplicate to a V-shaped 96-well plate with 50  $\mu\text{l}$  per well.
3. Add 200 nM caspase-3 stock solution into six wells of the same plate with 50  $\mu\text{l}$  per well.
4. Cover the plate with a lid to avoid evaporation and incubate for 15 min at 37°C in order to activate the caspase-3 enzyme.
5. Add 50  $\mu\text{l}$  of 800  $\mu\text{M}$  D-cysteine solution into three of the wells containing the 200 nM caspase-3 solution.
6. Add 50  $\mu\text{l}$  of 800  $\mu\text{M}$  DEVD-(D-Cys) solution into all of the other wells.
7. Cover the plate with a lid to avoid evaporation and incubate for 3 hr at 37°C.
8. Add 100  $\mu\text{l}$  of the 400  $\mu\text{M}$  NH<sub>2</sub>-CBT solution into each well. Mix the solutions by pipetting up and down several times.
9. Protect the plate from light and incubate for 1 hr at 37°C.



10. Prepare a second 96-well plate by adding 115  $\mu$ l of the 60  $\mu$ g/ml luciferase solution per well.

*The type of 96-well plate that should be used here is dependent on the bioluminescence imaging system used. If an imager, such as IVIS Spectrum (PerkinElmer), is used, a black 96-well plate is necessary. Please follow the recommendations of the plate reader/imager manufacturer.*

11. Immediately before reading bioluminescence emission, quickly transfer 5  $\mu$ l of the reaction solutions in the plate containing luciferase. Measure bioluminescence signal from the plate every 5 min for a duration of 2 hr.

*As soon as the incubated solutions are added to the luciferase buffer, light emission will be produced. As this process is very fast, it is important to add the luciferase buffer at the same time to all the wells and start data acquisition right after addition. Therefore, we suggest using multichannel pipets.*

*If necessary, the delay between measurements can be shortened to allow collection of more data points.*

*If the plate reader is equipped with a liquid handling system, a different procedure could be used. This would consist of adding 5  $\mu$ l of the caspase-3 containing solutions into a 96-well plate and adding 115  $\mu$ l luciferase buffer using the automated liquid handling system.*

#### **ALTERNATE PROTOCOL**

#### **IN VITRO (CELL-FREE) IMAGING OF THROMBIN PROTEASE ACTIVITY**

This alternate protocol focuses on the evaluation of thrombin protease activity using thrombin-specific Gly-Gly-Arg-(D-Cys) [GGR-(D-Cys)] peptide, which is designed to be cleaved by thrombin protease between Arg and D-Cys residues.

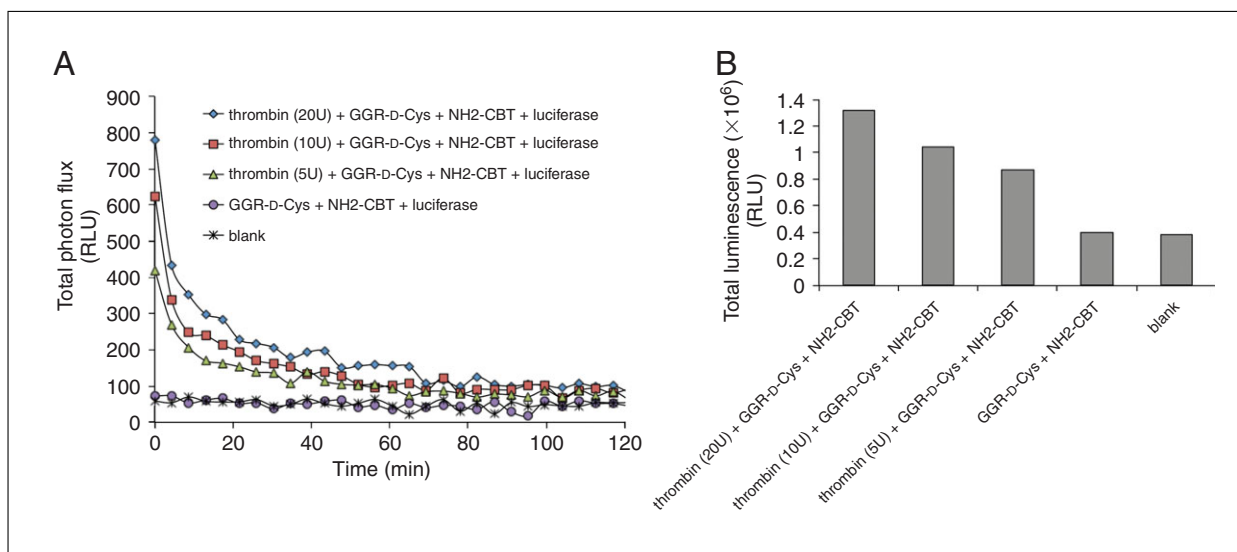
Thrombin enzyme is first incubated with GGR-(D-Cys) peptide. Upon enzymatic cleavage of peptidic sequence, D-Cys is liberated and reacts with NH<sub>2</sub>-CBT, resulting in the formation of D-aminoluciferin and further produces light that is proportional to the activity of thrombin enzyme.

Figure 6 is an example of the data acquired for this experiment (Fig. 6) (Godinat et al., 2013). In this study, GGR-(D-Cys) peptide was first incubated with increasing concentration of thrombin enzyme, followed by addition of NH<sub>2</sub>-CBT. Subsequent light emission was acquired over 2 hr (Fig. 6A). Integration of the area under each curve represent total photon flux produced over this time period and plotted in the form of bar-graphs in Figure 6B.

#### **Materials**

- Thrombin stock solution (see recipe)
- Thrombin buffer (see recipe)
- D-cysteine solution (see recipe)
- 500  $\mu$ M GGR-(D-Cys) peptide solutions (see recipe)
- 500  $\mu$ M NH<sub>2</sub>-CBT solution (see recipe)
- 60  $\mu$ g/ml Firefly luciferase enzyme solution (see recipe)
- V-shaped 96-well plate (Vitaris, cat. no. 3897-COR)
- 37°C incubator
- Black 96-well plate (BD Falcon, cat. no. 353219)
- Bioluminescence plate reader (Spectramax Gemini, Molecular Devices) *or* camera-like IVIS Spectrum (Perkin Elmer)

1. Prepare the different solutions of thrombin enzyme in thrombin buffer by diluting the 1000 U/ml thrombin stock solution as follows:



**Figure 6** Evaluation of thrombin activity using the “split luciferin approach”. **(A)** Bioluminescent signal as function of the time from GGR-(D-Cys) peptide incubated with thrombin protease at different concentrations (5, 10, and 20 U) over 3 hr at 37°C before addition of NH<sub>2</sub>-CBT and an additional incubation for another 1 hr at room temperature. Signal acquisition was started right after addition of 5  $\mu$ l of the premade luciferase buffer (see recipe) and continued for about 2 hr using the IVIS Spectrum (Perkin Elmer). The blank represents the signal from the luciferase buffer. **(B)** Total light output collected over the period of 2 hr obtained by integrating the area under the curves in panel A and plotted in the form of a bar-graph. Adapted with permission from Godinat et al. (2013). Copyright 2014, American Chemical Society.

0 U/ml: only thrombin buffer

250 U/ml: dilute 20  $\mu$ l of 1000 U/ml thrombin stock solution to 80  $\mu$ l using thrombin buffer (1:4 dilution)

500 U/ml: dilute 40  $\mu$ l of 1000 U/ml thrombin stock solution to 80  $\mu$ l using thrombin buffer (1:4 dilution)

2. Add thrombin solutions (0, 250, 500 U/ml) in triplicates into a V-shaped 96-well plate with 20  $\mu$ l per well.
3. Add 1000 U/ml thrombin stock solution into six wells of the same plate with 20  $\mu$ l per well.
4. Add 80  $\mu$ l of 500  $\mu$ M D-cysteine solution into the three wells containing the 250 U/ml thrombin stock solution
5. Add 80  $\mu$ l of 500  $\mu$ M solutions of GGR-(D-Cys) peptide solution to all the other wells.
6. Cover the plate with a lid to avoid evaporation and incubate for 3 hr at 37°C.
7. Add 100  $\mu$ l of 400  $\mu$ M NH<sub>2</sub>-CBT solution to all of the wells. Mix the solution by pipetting “up and down.”
8. Protect the plates from light and incubate for 1 hr at room temperature.
9. Prepare a second 96 well-plate by adding 115  $\mu$ l of the 60  $\mu$ g/ml luciferase solution per well.

*The type of 96-well plate used for experiments depends on the bioluminescence imaging system used. If a camera, such as IVIS Spectrum (PerkinElmer) is used, a black 96-well plate is recommended. Please follow the recommendations of the plate reader/imager manufacturer.*

10. Immediately before reading bioluminescence light output, quickly transfer 5  $\mu$ l of the reaction solutions into the plate containing luciferase.

*It is recommended to acquire bioluminescence signal every 5 min for 2 hr.*

*As soon as the sample solutions are added to the luciferase buffer, light emission will be produced. As this process is very fast, it is important to add the solution to all the wells at the same time and start imaging right after addition.*

*If necessary, the delay between measurement points can be shortened in order to have more data points.*

*If the plate reader is equipped with a liquid handling system, a different procedure could be used. This would consist of adding 5  $\mu$ l of the caspase-3-containing solutions into a 96-well plate and adding the 115  $\mu$ l luciferase solution using the liquid handling system.*

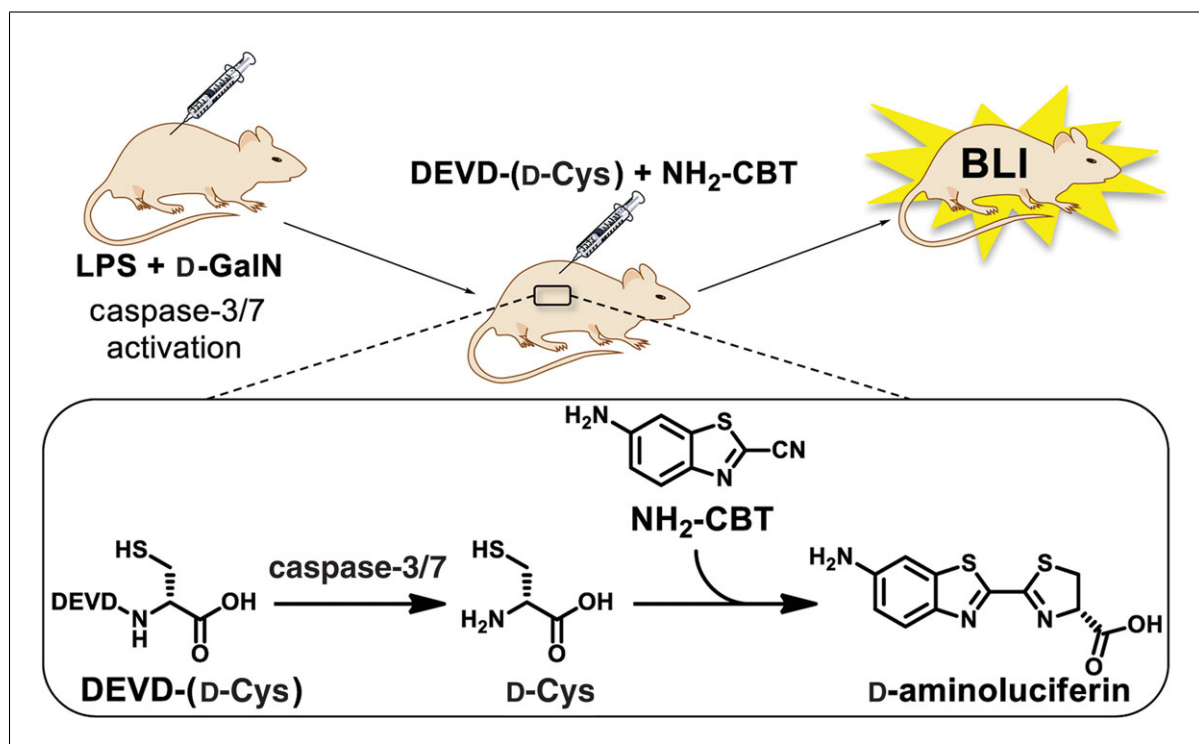
## BASIC PROTOCOL 2

### REAL-TIME NON-INVASIVE IMAGING OF CASPASE-3/7 ACTIVITIES IN TRANSGENIC REPORTER MICE (FVB-Luc+)

In this protocol, the procedure for evaluation of caspase-3/7 activity in living animals is described in details. It consists of two major parts: (1) induction of caspase-3/7 in live animals; (2) imaging steps using the “split luciferin” approach (Fig. 7). A commercial kit is now available for the second step based on this technology [“Caspase 3 and Caspase 7 mouse kit (z-DEVD-D-Cys)”, Intracel Medical].

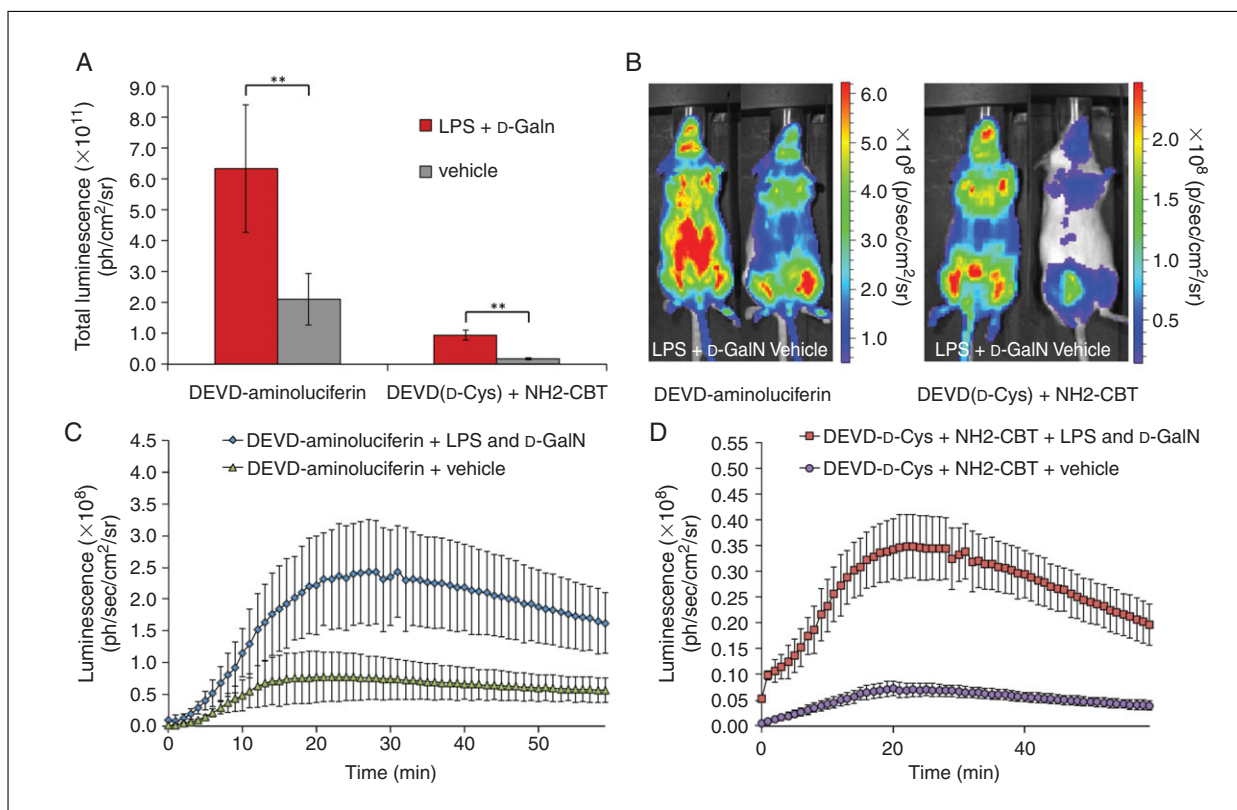
In this study, we used transgenic mice ubiquitously producing luciferase in every cell of their body under actin promoter (Cao et al., 2004). These and several other types of transgenic mice with luciferase expression in different organs are commercially available (from Taconic or The Jackson Laboratory).

Caspase 3/7 was induced in the liver of FVB-luc+ mice by injecting the animals with lipopolysaccharide (LPS) and D-galactosamine (D-GalN), following a procedure that was previously established for bioluminescence imaging of caspase-3/7 (Biserni et al., 2010). Six hours post-administration of LPS and D-GalN, animals were injected with



**Figure 7** Imaging of caspase-3 activity in live FVB+*luc* animals using the “split luciferin” approach. Adapted with permission from Godinat et al. (2013). Copyright 2014, American Chemical Society.





**Figure 8** Evaluation of caspase-3/7 activity using the “split luciferin” reaction in living transgenic reporter mice (FVB+luc). **(A)** Total luminescence over 1 hr from FVB+luc mice treated with either PBS (control group) or a combination of LPS (100  $\mu\text{g}/\text{kg}$  in 50  $\mu\text{l}$  of PBS) and D-GalN (267 mg/kg in 50  $\mu\text{l}$  of PBS). Six hr post-treatment, the animals received i.p. injections of either DEVD-aminoluciferin (34 mg/kg in 100  $\mu\text{l}$  of PBS) or a combination of DEVD-(D-Cys) peptide (22.6 mg/kg in 100  $\mu\text{l}$  of PBS) and NH<sub>2</sub>-CBT (6.8 mg/kg in 20  $\mu\text{l}$  of DMSO). Statistical analyses were performed with a two-tailed Student’s *t* test. **\*\*** $p < 0.01$  [ $n = 8$  for DEVD-aminoluciferin groups and  $n = 4$  for combination of DEVD-(D-Cys) and NH<sub>2</sub>-CBT reagents]. Error bars are  $\pm$  SD for eight and four measurements respectively. **(B)** Representative image of mice, 15 min post-injection of DEVD-aminoluciferin or a combination treatment with DEVD-(D-Cys) and NH<sub>2</sub>-CBT reagents. **(C)** Bioluminescent signal produced over 60 min following i.p. injection of DEVD-aminoluciferin in LPS/D-GalN ( $n = 8$ ) or vehicle ( $n = 8$ ) treated mice. **(D)** Luminescence emission over 60 min following i.p. injection of DEVD-(D-Cys) peptide and NH<sub>2</sub>-CBT in LPS/D-GalN ( $n = 4$ ) or vehicle ( $n = 4$ ) treated mice. Error bars are  $\pm$  SD for eight or four measurements. Adapted with permission from Godinat et al. (2013). Copyright 2014, American Chemical Society.

“split luciferin” substrates [DEVD-(D-Cys) and NH<sub>2</sub>-CBT, Intrace Medical] and the signal was acquired using IVIS Spectrum camera (Perkin Elmer) (Godinat et al., 2013). Commercially available DEVD-aminoluciferin compound was used as a positive control and for comparison. The data resulting from this experiment are shown on Figure 8.

Taking into account that the current protocol could be potentially used in a different type of mouse or animal disease models, we thought it appropriate to add a few important points. Expression of firefly luciferase is mandatory in order to obtain readout using this approach. The animals can express luciferase ubiquitously, as in our study, or only in certain organs or tissues (like xenografts models or spontaneous cancer models where tumor cells express luciferase). It is also recommended that mice selected for the experiment should preferably be of approximately the same sex, age, and weight.

The amount of light output depends on the amount/level of luciferase expression that becomes particularly important if tumor models are used. In order to account for the difference in levels of luciferase expression between animals, the experiment should start with quantification of “basal” level of light resulting from administration of D-luciferin. This data set is important and can be later used for “calibration” of resulting signal from protease activation to the basal level of luciferase expression in each mouse.

## Materials

3.1 mg/ml D-luciferin potassium salt (Intrace Medical) solution (see recipe)  
Phosphate-buffered saline (PBS; Life Technologies, cat. no. 20012-068), sterile  
FVB-Luc+ mice [FVB-Tg(CAG-luc,-GFP)L2G85Chco/J, The Jackson Laboratory; Cao et al., 2004]  
Isoflurane for anesthesia (Phoenix)  
50 µg/ml lipopolysaccharide (LPS) (from *Salmonella typhosa*, Sigma-Aldrich, cat. no. L7895) solution (see recipe)  
133.5 mg/ml D-(+)-galactosamine hydrochloride (D-GalN) solution (see recipe)  
5.65 mg/ml DEVD-(D-Cys) peptide solution (see recipe)  
8.5 mg/ml 6-amino-2-cyanobenzothiazole (NH<sub>2</sub>-CBT) solution (see recipe)  
Dimethyl sulfoxide (DMSO), sterile

0.5-ml insulin syringes (U-100 insulin syringe, 28-G × ½-in. needle; BD, cat. no. 329465)  
Clear, Plexiglas box  
Bioluminescence imaging system for small laboratory animals (IVIS Spectrum, PerkinElmer) or similar device  
37°C heating pad

**NOTE:** The following procedure is described for an average 25 g FVB-luc+ mouse. If the mice used have different weight, it is advised to adjust the concentrations of solutions in order to keep the doses constant.

### Perform D-luciferin injections

1. Prepare D-luciferin solution in order to inject 12.4 mg/kg of mouse body weight in 100 µl of sterile PBS.

*For a typical mouse weighing 25 g, this represents 0.31 mg of D-luciferin potassium salt in 100 µl of sterile PBS.*

2. Preload 0.5-ml insulin syringes with 100 µl of the luciferin solution.
3. Place the mice into a clear Plexiglas anesthesia box (3% isoflurane) that allows unimpeded visual monitoring of the animals.

*Depending on the imaging system used, as well as the legislation that applies in the country where experiments are performed, other anesthesia methods could be used*

4. Once the mice are fully anesthetized, inject them intraperitoneally (i.p.) with 100 µl of the corresponding D-luciferin solutions.
5. Place the mice on the heating pad (37°C) in the imaging chamber and keep them under anesthesia during the entire imaging period (it is advised to decrease the overall level of anesthesia to the equivalent of 1.5% isoflurane for imaging sessions over 1 hr).
6. Acquire bioluminescent signals for about 1 hr, recording measurements every 1 to 5 min.

*In order to minimize the time between D-luciferin injection and the beginning of data acquisition, it is advised to preload syringes with corresponding solutions prior to the anesthesia step. Once all the mice are anesthetized, they should be injected sequentially and placed in the imaging chamber right after.*

7. After imaging, turn off the isoflurane manifold to 0 and put the animals back into cages when awake.

*At least 48 hr of rest for the animals are allowed before continuing with the experiment.*

*The resting time between two imaging sessions might vary depending on the legislation that applies in the country where experiments are performed. It is also important to ensure that no residual signal is left from previous imaging with luciferin before starting step 10. Therefore, it is advised to perform quick imaging of mice before proceeding with the next imaging step.*

8. Prepare two experimental groups of animals defined as “treatment group” and “control group.” Label each group of mice according to the legislation that applies in the country where the experiment is performed (at least four mice per group is recommended).

### **Activate caspase-3**

9. Prepare 300  $\mu$ l of a sterile solution of lipopolysaccharides (LPS) in PBS at 50  $\mu$ g/ml.

*The total volume of the above LPS solution should be sufficient to treat all the mice of the “treatment group.” See step 15 for dosage.*

10. Prepare 300  $\mu$ l of a sterile solution of D-(+)-galactosamine hydrochloride (D-GalN) in PBS at 133.5 mg/ml.

*The total volume the above D-GalN solution should be sufficient to treat all the mice of the “treatment group.” See step 16 for dosage.*

11. Prepare 500  $\mu$ l of a sterile solution of PBS.
12. Preload 0.5-ml insulin syringes with 50  $\mu$ l of sterile LPS solution (one for each animal in the “treatment group”).
13. Preload four syringes with 50  $\mu$ l of sterile D-GalN solution (one for each animal in the “treatment group”).
14. Preload four syringes with 100  $\mu$ l of sterile PBS (one for each animal in the “control group”).
15. Inject the four mice from the “treatment group” intraperitoneally (i.p.) with 100  $\mu$ g/kg of LPS in 50  $\mu$ l sterile PBS (one for each animal in the “control group”).
16. Inject the same four mice from the “treatment group” i.p. with 267 mg/kg D-GalN in 50  $\mu$ l of sterile PBS.

*An average 25 g mouse should receive a total amount of 2.5  $\mu$ g LPS injected in 50  $\mu$ l of sterile PBS solution (50  $\mu$ g/ml).*

*A typical 25 g mouse should receive a total amount of 6.7 mg D-GalN in 50  $\mu$ l of sterile PBS solution (133.5 mg/ml).*

17. Inject the “control group” of mice i.p. with 100  $\mu$ l of sterile PBS.
18. Return all of the mice to their cages and wait 6 hr before proceeding the next step.

### **Image caspase-3/7 activity**

19. Prepare 500  $\mu$ l of DEVD-(D-Cys) peptide solution in sterile PBS at 5.65 mg/ml.
20. Prepare 500  $\mu$ l of NH<sub>2</sub>-CBT solution in sterile DMSO at 8.5 mg/ml.
21. Preload syringes with 100  $\mu$ l of sterile DEVD-(D-Cys) solution (one for each mouse)
22. Inject the animals from “treatment group” i.p. with 22.6 mg/kg dose of DEVD-(D-Cys) peptide solution in 100  $\mu$ l of PBS.

*An average 25 g mouse should receive a total amount of 0.565 mg of DEVD-(D-Cys) peptide in 100  $\mu$ l of sterile PBS (5.65 mg/ml solution of the peptide in sterile PBS).*

*A delay of 10 min is respected before proceeding to the next step.*

23. Place the animals from the “treatment group” into a clear Plexiglas anesthesia box (3% isoflurane) that allows unimpeded visual monitoring of the animals.
24. Preload syringes with 20  $\mu$ l of sterile NH<sub>2</sub>-CBT (one for each mouse in the “treatment” group).
25. After the mice are fully anesthetized, inject the mice i.p. with a 6.8 mg/kg dose of NH<sub>2</sub>-CBT solutions in 20  $\mu$ l in sterile DMSO.

*An average 25 g mouse should receive a total amount of 0.17 mg of NH<sub>2</sub>-CBT in 20  $\mu$ l sterile DMSO (8.5 mg/ml solution).*

26. Place the mice on the heating pad (37°C) in the imaging chamber and keep them under anesthesia during the entire imaging period (it is advised to decrease the overall level of anesthesia to equivalent of 1.5% isoflurane for imaging sessions over 1 hr).
27. Acquire bioluminescent signals for  $\sim$  1 hr, recording measurements every 1 to 5 min.
28. After the end of data acquisition, sacrifice the mice from “treatment group” according to the legislation that applies in the country where experiments are performed.
29. Repeat the experiment from steps 18 to 27 with the “control group.”
30. After the end of data acquisition, turn off the instruments and return the mice from the “control group” back to their cages (they normally do not have to be sacrificed due to non-invasive nature of the control experiments).
31. Perform data analysis.

*Data analysis is performed using Living Image In Vivo Software (PerkinElmer). Regions of interest (ROI) are defined around each animal. Mice tails are not taken into account in the ROI. This is performed for every time point and quantified values are plotted in function of time (Fig. 8C,D). To calculate the overall light emission per mouse during the complete imaging time, areas under the curves are calculated and plotted in a bar graph (see Fig. 8A).*

## REAGENTS AND SOLUTIONS

*Use Milli-Q purified water or equivalent in all recipes and protocol steps.*

### **6-Amino-2-cyanobenzothiazole (NH<sub>2</sub>-CBT) solution, 8 mg/ml**

Dissolve 8.5 mg of 6-amino-2-cyanobenzothiazole (NH<sub>2</sub>-CBT; Intrace Medical) in 1 ml of sterile dimethyl sulfoxide (DMSO)  
Prepare fresh

*The dosage of NH<sub>2</sub>-CBT is 6.8 mg/kg of mouse body weight and should be injected in approximately 20  $\mu$ l volume. For a typical mouse weighing 25 g, prepare a 8.5 mg/ml solution of NH<sub>2</sub>-CBT in sterile DMSO and inject 20  $\mu$ l of the solution.*

### **6-Amino-2-cyanobenzothiazole solution, 400 $\mu$ M**

Dissolve 1 mg of 6-amino-2-cyanobenzothiazole (mol. wt. 175.20 g/mol) in 1.427 ml of pure methanol. Once dissolved, take out 200  $\mu$ l of this solution and further dilute to 2000  $\mu$ l with methanol to obtain a 400  $\mu$ M solution.

*The solution should be protected from light and stored at  $-20^{\circ}$ C. A fresh solution should be prepared before every experiment.*

### **Caspase buffer**

100 mM HEPES, pH 7.4  
0.1% CHAPS  
1 mM EDTA

10 mM DTT  
1% sucrose  
Store up to 3 months at 4°C

***D-cysteine solution, 500  $\mu$ M***

Dissolve 1 mg of D-cysteine (mol. wt. 121.15 g/mol) in 16.508 ml of degassed thrombin buffer (see recipe)

*A fresh solution should be prepared before every experiment. If possible, before dissolving the D-cysteine, the thrombin buffer should be degassed by bubbling inert gas (e.g., nitrogen or argon) in the solution for 30 min.*

***D-cysteine solution, 800  $\mu$ M***

Dissolve 1 mg of D-cysteine (mol. wt. 121.15 g/mol) in 10.318 ml of degassed caspase buffer (see recipe)

*A fresh solution should be prepared before every experiment. If possible, before dissolving the D-cysteine, the caspase buffer should be degassed by bubbling inert gas (e.g., nitrogen or argon) through the solution for 30 min.*

***DEVD-(D-Cys) peptide solution, 5.65 mg/ml***

Dissolve 5.65 mg of DEVD-(D-Cys) peptide in 1 ml sterile phosphate-buffered saline (PBS)

Prepare fresh

*The dosage of DEVD-(D-Cys) peptide is 22.6 mg/kg of mouse body weight and should be injected in  $\sim$ 100  $\mu$ l. For a typical mouse weighing 25 g, prepare a 5.65 mg/ml solution of DEVD-(D-Cys) peptide in sterile PBS and inject 100  $\mu$ l of the solution.*

***DEVD-(D-Cys) peptide solution, 800  $\mu$ M***

Dissolve 0.5 mg of peptide in 1.08 ml caspase buffer (see recipe)

Prepare fresh

***D-(+)-galactosamine hydrochloride (D-GalN) solution, 133.5 mg/ml***

Dissolve 133.5 mg of D-(+)-galactosamine hydrochloride (D-GalN; Applichem GmbH, cat. no. A6859) in 1 ml sterile phosphate-buffered saline (PBS)

Prepare fresh

*The dosage of D-GalN is 267 mg/kg of mouse body weight, and should be injected in  $\sim$ 50  $\mu$ l volume. For a typical mouse weighing 25g, prepare a 133.5 mg/ml solution of D-GalN in sterile PBS and inject 50  $\mu$ l of the solution.*

***D-luciferin potassium salt, 3.1 mg/ml***

Dissolve 3.1 mg of D-luciferin potassium salt (mol. wt. 318.4 g/mol) in 1 ml sterile phosphate-buffered saline (PBS)

Store up to 1 month at  $-20^{\circ}\text{C}$  protected from light (repeated freeze-thaw is not recommended)

*The dosage of D-luciferin potassium salt is 12.4 mg/kg of mouse body weight, and the resulting solution should be injected in  $\sim$ 100  $\mu$ l. For a typical mouse weighing 25 g, prepare 3.1 mg/ml solution of D-luciferin in sterile PBS and inject 100  $\mu$ l of the solution.*

***Firefly luciferase enzyme, 60  $\mu$ g/ml***

Dissolve 1 mg of firefly luciferase (Sigma Aldrich) in 16.7 ml of luciferase buffer (see recipe).

Alternatively, a 2 mg/ml stock solution of Firefly luciferase can be prepared in PBS. 100  $\mu$ l of the stock solution will then be diluted up to 3.333 ml with luciferase buffer (see recipe) in order to obtain the desired 60  $\mu$ g/ml luciferase solution.

*continued*



*Freeze-thaw cycle should be avoided on the luciferase enzyme and, if possible, used batch of Luciferase that have the same number of freeze-thaw cycle.*

#### **GGR-(D-Cys) peptide solution, 500 $\mu$ M**

Dilute 0.5 mg of Gly-Gly-Arg-(D-Cys) peptide in 2.555 ml thrombin buffer (see recipe)  
Prepare fresh

#### **Lipopolysaccharide (LPS) solution, 50 $\mu$ g/ml**

Dissolve 50  $\mu$ g of LPS in 1 ml sterile phosphate-buffered saline (PBS)  
Store at  $-20^{\circ}\text{C}$

*Repeated freezing and thawing is not recommended. No indication is given by the provider as to how long the solutions can be stored at the temperature provided.*

*The dosage of LPS is 100  $\mu$ g/kg of mouse body weight and should be injected in  $\sim 50$   $\mu$ l volume. For a typical mouse weighing 25 g, prepare a 50  $\mu$ g/ml solution of LPS in sterile PBS and inject 50  $\mu$ l of the solution.*

#### **Luciferase buffer**

0.1 M Tris-Cl, pH 8  
2 mM ATP  
5 mM  $\text{MgSO}_4$

*0.1 M Tris-Cl supplemented with 5 mM  $\text{MgSO}_4$  can be stored up to several weeks at  $4^{\circ}\text{C}$  but fresh ATP should be supplemented just before performing the experiments. It is not recommended to store the luciferase buffer once ATP has been supplemented.*

#### **Purified caspase-3, 200 nM**

Caspase-3 can be purified and characterized following the reported procedure (Stennicke and Salvesen, 1999)  
A caspase-3 solution is prepared freshly by dissolving 0.2 nmol of purified caspase-3 in 1 ml of caspase-3 buffer (see recipe)

#### **Storage buffer**

50 mM sodium citrate, pH 6.5  
200 mM NaCl  
0.1% PEG-8000  
50% glycerol  
Store up to 2 weeks at  $4^{\circ}\text{C}$

#### **Thrombin buffer**

0.02 M Tris-Cl, pH 8.4  
0.15 M NaCl  
2.5 mM  $\text{CaCl}_2$   
Store up to 2 weeks at  $4^{\circ}\text{C}$

#### **Thrombin stock solution**

Thrombin enzyme (Sigma-Aldrich, cat. no. T1063-1KU) is used without additional purification steps  
1000 U/ml thrombin stock solution is prepared by diluting 1000 U of thrombin enzyme in 1 ml storage buffer (see recipe)

*Thrombin stock solution should be stored at  $-20^{\circ}\text{C}$  and multiple freeze/thaw cycles should be avoided.*

## COMMENTARY

### Background Information

In the past 10 years, several biocompatible “click” reactions have been developed and successfully applied for various study of biological processes (Sletten and Bertozzi, 2009). These reactions include copper-free cyclooctyne-type cycloaddition (Agard et al., 2006; Ning et al., 2008; Sletten et al., 2010; Jewett et al., 2010; Chang et al., 2010; Neves et al., 2011a,b), Staudinger ligation (Nilsson et al., 2000; Saxon and Bertozzi, 2000; Saxon et al., 2000; Presher et al., 2004; Lin et al., 2005; Dube et al., 2006; Chang et al., 2007; Hangauer and Bertozzi, 2008), and alkene-tetrazine reactions (Devaraj et al., 2008; Blackman et al., 2008; Budin et al., 2011; Devaraj et al., 2012; Lang et al., 2012; Liang et al., 2012a,b; Yang et al., 2012). However, most of these reactions only work in cell lysates or live cells, and the great complexity of human pathologies requires tools that allow studies of biochemical transformations on the level of the whole organism.

Since multiple animal models of various human pathologies have been successfully established, development of new biocompatible reactions applicable for studies of biological processes on the level of a live animal plays a crucial role in biology and medical research (Fedele et al., 2012; Laferla and Green, 2012; Langdon, 2012; Li et al., 2012; Peters et al., 2012; Rogers, 2012).

Only few biocompatible reactions have been shown to work efficiently in living animals, and one of them is known as Staudinger Ligation (Saxon and Bertozzi, 2000; Prescher et al., 2004; Neves et al., 2011a,b; van Berkel et al., 2011). Recently, [4 + 2] tetrazine/trans-cyclooctene cycloaddition has also been used in living mice, despite the fact that the tetrazine reagent was bound to a polymeric support to improve its pharmacokinetic properties of the reagent in vivo settings (Devaraj et al., 2012).

It has been previously reported that D-cysteine efficiently reacts with 6-hydroxy-2-cyanobenzothiazole (OH-CBT) in physiological solutions (Ren et al., 2009; Liang et al., 2010; Ye et al., 2011) and was first reported by White and co-workers in 1963 as the final step in the synthesis of D-luciferin (Fig. 1) (White et al., 1963). Recently, others have reported novel applications of this reaction for the selective labeling of proteins on N-terminal cysteines (Ren et al., 2009; Nguyen et al., 2011), as well as the controlled assembly of

polymers in physiological solutions and living cells (Liang et al., 2010; Ye et al., 2011). Remarkably, the rate of this reaction was found to be three orders of magnitude faster than Staudinger ligation (Ren et al., 2009; Yuana and Liang, 2014).

We have recently reported that this reaction can also occur directly in living animals and be efficiently used in combination with bioluminescence imaging (Godinat et al., 2013), which is currently the most sensitive imaging technique in living animals (McCaffrey et al., 2003; Massoud and Gambhir, 2003; Prescher and Contag, 2010). Indeed, in the past, BLI has been extensively used for tracking luciferase-expressing cells in living animals and visualization of transcriptional activation (Geiger et al., 2006). More recent applications of BLI include probing of molecular signatures of target tissues through the use of “caged” luciferin probes that are only uncaged by the activity of specific biological molecules. The underlying principle in the design of all these caged luciferin substrates is based on the fact that luciferins substituted on the phenolic oxygen or aryl nitrogen are not capable of light emission (Wehrman et al., 2006; Cohen et al., 2010; van de Bittner et al., 2010; Henkin et al., 2012). This approach was reported for the design of probes to sense enzymatic activities directly in vivo, such as those of beta-galactosidase (Wehrman et al., 2006) and caspases (Shah et al., 2005; Cosby et al., 2007; Biserni et al., 2010; Hickson et al., 2010; Scabini et al., 2011). Furin (Dragulescu-Andrasi et al., 2009), as well as beta-lactamases (Yao et al., 2007) were also sensed using a similar methodology. Previously, we successfully reported the use of this concept for real-time imaging and quantification of fatty acids uptake (Henkin et al., 2012), cell surface glycosylation (Cohen et al., 2010), and hydrogen peroxide fluxes (van de Bittner et al., 2010), as well as studies of efficiency of delivery, linker release, and biodistribution of cell-penetrating peptide conjugates (Goun et al., 2006). Recently, the use of new red-shifted luciferin derivatives and their corresponding luciferase enzymes for multi-color application were reported (Woodrooffe et al., 2008; Reddy et al., 2010; Harwood et al., 2011; Conley et al., 2012; McCutcheon et al., 2012).

In addition, to prove that the “split luciferin” reaction can occur directly in living mice to form luciferin, we used this new methodology to image caspase-3/7 activation

**Table 1** Troubleshooting Tips for Cell-Free and In Vivo Split Luciferin Experiments

Problem	Cause	Solution
<i>Troubleshooting cell-free experiments</i>		
Very low or non-existent signal	Inactive luciferase enzyme	Run a control experiment by incubating D-luciferin with luciferase enzyme. If no light emission is observed, luciferase might be degraded. Use another batch of luciferase enzyme
	Compromised luciferase buffer	Prepare a fresh luciferase buffer (with freshly added ATP) and control activity by incubating the luciferase enzyme with D-luciferin
	Exposure (integration) time is too short	Increase the exposure time on the bioluminescence reader/imager and repeat the experiment
	Concentration of the probes is too low	Increase the probe concentrations
	Delay between addition of the luciferase and the beginning of acquisition is too long	Decrease the delay between luciferase addition and acquisition
Low signal-to-background ratio (no increase in light emission when the probe is incubated with protease enzyme)	Inactive protease enzyme	Verify enzymatic activity of the protease using a commercial colorimetric or fluorescent kit (e.g., for caspase-3: Sigma-Aldrich: NAc-Asp-Glu-Val-Asp-pNA, ref A2559)
	Quality of protease-specific peptide is compromised	Verify that the peptide probe is pure and not oxidized or degraded. HPLC/MS can be used for analysis
	Low purity of peptide	Very high degree of purity is necessary since traces of free D-Cysteine can result in unspecific formation of D-luciferin and significantly increase background signal. Verify peptide purity using HPLC and MS. If stored solution was used, prepare a fresh one.
<i>Troubleshooting in vivo experiments</i>		
Very low or non-existent signal	Low luciferase expression in the selected animal model	Overall, light emission from protease-specific bioluminescent probe is expected to have a lower intensity than light resulted from D-luciferin injection. If light emission generated by D-luciferin is already low, increase dosage of protease-specific peptide and CBT
	Quality of reagent issue	Verify that the peptide probe is not oxidized or degraded using HPLC/MS analysis

*continued*

**Table 1** Troubleshooting Tips for Cell-Free and In Vivo Split Luciferin Experiments, *continued*

Problem	Cause	Solution
Low signal to background ratio (no difference in light emission between treatment and control groups)	Low level of protease activation	Increase drug/effector used to activate (or decrease) protease activity  Verify protease activation using commercial probe (if available) or use classical ex vivo methods
	Quality of protease-specific peptide is compromised	Verify that the peptide probe is pure and not oxidized or degraded. HPLC/MS can be used for analysis.
Low purity of peptide	Very high degree of purity is necessary as traces of free D-Cysteine can result in unspecific formation of D-luciferin that significantly contribute to background. Verify peptide purity using HPLC and MS analysis. If stored solution was used, prepare a fresh one.	

directly in living mice by “caging” the D-Cys component with the sequence of amino acids that can be specifically recognized by caspase 3/7 protease (Godinat et al., 2013). This novel technology opens up new ways for imaging many biological processes and can even be used for dual analyte detection, as reported recently for simultaneous imaging of caspase-8 and hydrogen peroxide (van de Bittner et al., 2013). Moreover, this novel technology could be applied for studies of many other proteases that are known to cleave at the end of peptidic sequences using sensitive bioluminescent imaging.

### Critical Parameters

#### *Multi-well plates*

Even though several types of 96-well or similar plates could be used in this experiment, it is essential that the chosen plates are compatible with bioluminescent imaging and have been validated with the equipment available. For example, we propose to first measure the background signal from the plate with PBS solution before starting the real measurements. Careful attention must be paid to those parameters in order to maximize assay sensitivity.

#### *Choice of animal model*

Many animal models can be used for this study. However, in order to obtain light emission, the animals should express luciferase either ubiquitously or in certain organs or tissues. For example, animals ubiquitously ex-

pressing luciferase under control of the beta-actin promoter, as well as mice expressing luciferase in certain organs, are now commercially available (The Jackson Laboratory). Alternatively, animals can be injected with luciferase-expressing cells subcutaneously, intraperitoneally, intravenously, by an intracardiac route, or orthotopically.

In order to quantify the signal from protease activation, it is important to determine the basic level of expression of the luciferase enzyme, as it will greatly influence the amount of light produced. It is particularly important when this experiment is performed in tumor models where the tumor size differs significantly from one animal to another. The basic level of luciferase expression should be measured before activation of protease step, and these data can be used for calibration to the level of protease expression. For generation of these data, please follow Basic Protocol 2 (steps 1 to 8).

In addition, several controls are essential for distinguishing background from protease-specific signal, such as negative control without treatment (see Basic Protocol 2, steps 17-27 and 30).

If the animal model used or the protease of interest are different from the ones described in the protocol, we recommend conducting an initial pilot trial on a smaller group of animals. This should help to reduce number of animals and help to optimize the imaging conditions.

### Reagent purity and storage

Probes used in assays have to be of the highest purity available to minimize contaminating side products. Purification of cysteine-labeled peptide is generally performed by HPLC. Luciferin and CBTs derivatives should be kept protected from direct light during experiment and storage. In order to maximize possible signal production, buffers have to be degassed when possible to avoid oxidation of cysteine and its derivatives. All the solutions that are to be injected in animals have to be sterile-filtered or sterile buffers have to be used for preparation of solutions.

### Acquisition of bioluminescence signal

For all the experiments (cell-free and in vivo), it is recommended to acquire a sequence of data over time for at least one hour by taking measurements every 1 to 10 min. One acquisition per minute is usually a good starting point. It is also important to do the same for measuring the signal from luciferin alone, as this signal changes several fold over time and therefore one-point measurement will certainly lead to significant mistakes in calculations.

### Caspase activation

Activation of caspase 3/7 in mice using LPS/D-GalN may result in pain and distress in animals. Therefore, this experiment should be performed in agreement with the legislation that applies in the country where the experiments are realized.

### Troubleshooting

Troubleshooting guides for “cell-free” and “live mice” protease activity imaging experiments are presented in Table 1.

### Anticipated Results

Detection of apoptosis has become an important factor in understanding tumor pathology and finding new antitumor treatment. This assay can be used to investigate the apoptotic profile of diseases and also to evaluate cytotoxicity of drugs in vitro and in vivo by measuring accurately cell death parameters.

Figures 5 and 6 demonstrate the examples of data expected from cell-free assays. Figure 8 represents an example of results that can be obtained from in vivo experiments.

The signal to background ratio obtained is the key parameter in these experiments. A significant difference in bioluminescent light emission between treated and untreated animals is expected for both types of assays.

### Time Considerations

Once all the necessary reagents are obtained, the described procedure for cell-free test tube assays can be performed within a day. In practice, a few more days should be considered if optimization process is necessary.

Experiments in live animals described in the paper typically take three days. However, if taking into account the initial pilot trial in small group of animals in order to optimize conditions, a week or two could be required.

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