Chapter 22

Imaging Proteolytic Activities in Mouse Models of Cancer

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Abstract

Proteases are “protein-cleaving” enzymes, which, in addition to their non-specific degrading function, also catalyze the highly specific and regulated process of proteolytic processing, thus regulating multiple biological functions. Alterations in proteolytic activity occur during pathological conditions such as cancer. One of the major deregulated classes of proteases in cancer is caspases, the proteolytic initiators and mediators of the apoptotic machinery. The ability to image apoptosis noninvasively in living cells and animal models of cancer can not only provide new insight into the biological basis of the disease but can also be used as a quantitative tool to screen and evaluate novel therapeutic strategies. Optical molecular imaging such as bioluminescence-based genetically engineered biosensors has been developed in our laboratory and exploited to study protease activity in animal models with a high signal to noise. Using the circularly permuted form of firefly luciferase, we have developed a reporter for Caspase 3/7, referred to as Caspase 3/7 GloSensor. Here, we discuss the use of the Caspase 3/7 GloSensor for imaging apoptotic activity in mouse xenografts and genetically engineered mouse models of cancer and present the potential of this powerful platform technology to image the proteolytic activity of numerous other proteases.

Key words Caspase 3/7, GloSensor, Firefly luciferase, Proteases, Mouse models, Cancer

1 Introduction

Proteases are enzymes that cleave the peptide bond between two amino acids and were originally identified as destructive enzymes essential for protein catabolism [1]. Subsequently, in addition to their non-specific degradative function, a role for proteases in catalyzing a substrate-specific proteolytic cleavage resulting in the production of new protein products has become appreciated [2]. There is hardly a signaling process in a living cell that is not associated with protease activity in one way or another. Besides regulating the localization and activity of proteins, proteases create new bioactive molecules as well as generate, transduce, and amplify cellular signals through modulation of protein-protein interactions [3]. Thus proteases regulate multiple biological processes including cell proliferation, differentiation and morphogenesis, DNA replication and transcription, tissue morphogenesis and remodeling,
autophagy, apoptosis, etc. [4–11]. Considering the multitude of cellular functions performed by proteases, it is no surprise that alterations in proteolytic systems occur during several pathological conditions such as cancer, neurodegenerative disorders, inflammation, and cardiovascular diseases.

Apoptosis or programmed cell death is a genetically regulated process to self-destruct unwanted and damaged cells [12]. Apoptosis can be activated extrinsically through the activation of cell surface death receptors like TNF-α or intrinsically through mitochondria-mediated release of cytochrome c. Irrespective of extrinsically or intrinsically activated apoptosis, the two pathways converge on activation of terminal, effector caspases, culminating in cell death [12]. Caspases are a family of cysteine-aspartic proteases that drive the process of apoptosis by cleaving essential proteins and thus dismantling the unwanted or damaged cell [13]. Caspases 3 and 7 are effector caspases that are activated in response to initiator caspases, caspases 8 and 9, which are activated in response to an extrinsic or intrinsic signal, respectively [13, 14]. Caspase 3/7 activation is used as a surrogate marker for apoptosis [15, 16]. Activation of the proteolytic activity of Caspase 3/7 has been used to design assays to interrogate the initiation of apoptosis and its deregulation in cancer [17, 18].

Bioluminescence is the emission of light by a chemiluminescent reaction involving light-generating enzymes called luciferases, which are a large family of enzymes that catalyze the oxidation of a substrate, luciferin, into oxyluciferin, with the concomitant production of light [19, 20]. At the cellular level, this light can be captured and detected using an extremely sensitive cooled charge-coupled device (CCD) camera or a photomultiplier. With the current advancement of instrumentation, bioluminescence imaging can be used to carry out real-time functional imaging of biological processes like protein-protein interactions, kinase activities, cellular trafficking, disease progression and response to therapy, etc. [21]. This is achieved by using a reporter gene that is genetically engineered and encodes the light-generating enzyme luciferase in a form such that its catalytic activity is modulated in context of a protein modifying activity (e.g., protease or a kinase). In the presence of a substrate, luciferase-expressing cells emit a blue to yellow-green light with an emission spectra peaking at a wavelength between 490 and 620 nm. This noninvasive imaging modality is extremely sensitive over a broad dynamic range with an exceptionally large signal-to-noise ratio. Presently, more than 30 luciferase-luciferin systems are known, but the most frequently used luciferase for in vivo molecular imaging is the ATP-dependent firefly (Photinus pyralis) luciferase [19]. The main advantage of using firefly luciferase is the high signal-to-noise ratio as 30% of the light produced by firefly luciferase has an emission spectrum above 600 nm, a region with the least amount of signal attenuation due to absorbing and
scattering properties of live mammalian tissue [19]. More recently, a smaller but very bright luciferase NanoLuc from deep sea shrimp (Oplophorus gracilirostris) has been successfully engineered [22]. NanoLuc uses a new substrate to produce light which is 100-fold brighter than that of firefly luciferase [23].

In addition to studies wherein the luciferase reporter technology is used to interrogate transcriptional activity of promoters, protein complementation assays are becoming a method of choice to detect the activity of an enzyme or protein in response to therapeutics. Conceptually, protein complementation technology involves reporter gene manipulation, wherein a monomeric reporter is split into two inactive components, which when brought together can reconstitute a functionally active molecule. Split-luciferase reporters where the luciferase has been divided into two halves (N-Luc and C-Luc) have been developed to study protein-protein interactions. These split-luciferase reporters are based on either the intermolecular or intramolecular complementation of the luciferase fragments to generate signal in response to cellular cues [24, 25]. In our laboratory we have utilized the split-luciferase technology to develop reporters for the activities of several kinases including AKT [26, 27], GSK3β [28], CK1α [28], and ATM [29] and more recently proteases like Caspase 3/7 [30].

Previously, we have reported the generation of a hybrid polypeptide that noninvasively reports on Caspase-3 activity in living cells and animals [31]. This reporter, called as ANLucBCLuc, comprised of a fusion of two small interacting peptides, peptide A and peptide B, with NLuc and CLuc fragments of luciferase on their N terminals, respectively, with an intervening Caspase-3 cleavage site (DEVD) between pepANLuc (ANLuc) and pepBCLuc (BCLuc) [31]. In response to apoptosis, activated Caspase 3 cleaves the engineered reporter, enabling dissociation of ANLuc from BCLuc and thus facilitating the high-affinity interaction between peptide A and peptide B. This interaction restores luciferase enzymatic activity by NLuc and CLuc complementation resulting in the generation of light in the presence of substrate, luciferin [31]. The reporter was found to be very useful in vivo but had limitations in high-throughput screens in vitro due to its significant background signal. Continuing our efforts to develop an effective and highly sensitive imaging reporter for Caspase 3/7 activity, we utilized recent advances in biosensor research including circularly permuted forms of firefly luciferase and thermostable Photinus pyralis luciferases [32–34]. In collaboration with Promega Corporation, we screened a library of randomly mutated thermally stable luciferase constructs permuted at residue 358 and found a reporter with >50-fold induction of bioluminescence activity following stimulation. The reporter has been further engineered such that the magnitude of the luminescence increase is directly proportional to the amount of analyte or protease activity present and has
been made commercially available as GloSensor™ luciferase [35]. This reporter has been used by several investigators to generate biosensors for the activity of various other proteases. Li et al. used the GloSensor technology to develop biosensors for the proteolytic activity of granzyme B/Caspase 8 and used it in combination with the Caspase 3/7 reporter to examine the distinct pattern of caspase activation cascade induced by granzyme B vs Fas [36]. Kilianski et al. utilized the GloSensor reporter to develop reporters for imaging the activity of papain-like protease and chymotrypsin-like protease [37]. These reporters were then used to identify potential inhibitors of Middle East respiratory syndrome coronavirus (MERS-CoV) [37]. These studies exemplify a wide applicability of this powerful technology to image the activity of many specific and regulated proteases.

In this chapter we discuss the generation of GloSensor Caspase 3/7, its validation in vitro in cell culture and in high-throughput screens and its application in imaging proteolytic activity in mouse models.

## 2 Materials

### 2.1 Vector Construction

1. pGloSensor plasmid pGlo-30F (Promega, WI).
2. Oligonucleotides corresponding to the amino sequence DEVD for Caspase 3/7, IETD for granzyme b/Caspase 8, etc.
3. Restriction enzymes BamHI and HindIII.
4. Expression vectors and packaging plasmids for generating lentiviral particles (optional).
5. High-fidelity polymerases (Pfu), dNTP, primers for cloning and sequencing, buffers, thermocycler, restriction endonuclease, DNA ligase, pipettes, filter tips, etc.
6. High-efficiency competent cells, antibiotic, bacterial growth media (LB, SOC), agar plates, plasmid DNA extraction kits, DNA gel purification and sequencing kits, etc.

### 2.2 Cell Culture

1. RPMI 1640.
2. Fetal bovine serum (FBS).
3. Trypsin-EDTA.
4. D54-MG and MDA-MB-231-1833 cells maintained in 10% FBS-RPMI 1640.
5. Antibiotic G418 for selection of stable clones.
6. FuGENE 6 for transfection.
7. Flat bottom 96-well culture plates.
8. 10 cm culture dishes.
9. Tissue culture supplies.
2.3 Cell Imaging

1. Translucent black- or white-walled 96-well clear bottom plates.
2. GloSensor c-AMP reagent at 40 mg/mL stock concentration in 1× PBS, stored in dark-colored vials at −80 °C.
3. Drugs for treating cells: TRAIL, CV3988, docetaxel, anti-Fas antibody, and pan-Caspase inhibitor Z-VAD-FMK.
4. DMSO for control.
5. Luminometer to image bioluminescence.
6. Liquid handling instrument, plate handling robot, and a cell culture incubator compatible with high-throughput instruments for high-throughput assays (optional).

2.4 Animal Imaging

1. Immunocompromised mice (athymic nude or NOD/SCID) for human tumor xenografts.
2. Calipers for tumor measurement. Calculate the tumor volume using the formula \[ V = \frac{W(2) \times L}{2}, \] where \( V \) is tumor volume, \( W \) is tumor width, and \( L \) is tumor length.
3. pCLEX vector with CAG and floxed GFP/stop cassette.
4. FVB/N females.
5. Tissue-specific Cre-recombinase mouse (CMV-Cre or p48-Cre).
6. Primers for CMV-Cre.
   Forward oIMR1084 5′-GCGGTCTGGGCAGTAAAAACTATC-3′.
   Reverse oIMR1085 5′-GTGAAACAGCATTGCTGTCACTTT-3′.
7. Primers for p48Cre.
   Forward p48 GT-Cre 5′-CATGCTTCATCGTCGGTCC-3′.
   Reverse p48 GT-Cre 5′-GATCATCAGCTACACCAGAG-3′.
8. Primers for reporter mouse.
   Forward 358 F 5′-AGTTTCAACAGCCAAATGG-3′.
   Reverse 358 R 5′-CCGGAATAGCTGCATAACGAGAT-3′.
9. Cerulein for inducing pancreatitis.
10. Luciferin (VivoGlo Luciferin) at 4 mg/mL in sterile 1× PBS as stock concentration. The stock can be stored in dark tubes at −20 or −80 °C.
11. 1 mL insulin syringes for intraperitoneal (i.p.) luciferin injection in mice.
12. IVIS imaging system with temperature-controlled platform and isoflurane anesthesia injection and controller system.
3 Methods

3.1 Construction of Biosensor Expression Plasmid

1. The GloSensor Caspase 3/7 biosensor is encoded by the pGLS-30F construct and is also commercially available (GLS Caspase 3/7, Promega). The GLS.DEVD version is based in a thermal stable variant of Photuris pennsylvanica luciferase that has been evolved for enhanced detection of Caspase 3/7 activity.

2. To construct plasmids for Caspase 3/7 or any other desired proteolytic activity, anneal and ligate the oligonucleotides corresponding to the amino sequence of the protease of interest, e.g., DEVD for Caspase 3/7, IETD for granzyme B/Caspase 8, RLKGG for papain-like protease, or VRLQS for chymotrypsin-like protease into the BamHI/HindIII sites of pGLC-30F plasmid (see Note 1).

3.2 Generation of Stable Transfectants Expressing Caspase 3/7 Reporter

1. Transfect Caspase 3/7 reporter into desired cell lines like D54-MG and MDA-MB-231/1833 using FuGENE reagent following manufacturer’s protocol (see Note 2).

2. Selection can be started after 48 h by placing the cells in selection media (400 μg/mL G418 in 10% FBS-RPMI 1640).

3. Single-cell clones can be selected by limited dilution method by plating the transfected cell population at a density of 0.3 cells/well using 96-well culture plates (see Note 3).

4. Once single-cell clones are selected, they can be tested for reporter expression by Western blotting with luciferase antibody and for bioluminescence. In response to apoptosis, Caspase 3/7 activation will lead to an increase in bioluminescence as compared to untreated control cells (Figs. 1 and 2). Clones with similar reporter expression and bioluminescence activity can be selected for further experiments.

5. Expand and freeze 3–4 best reporter expressing stable clones at low passage for future use.

6. Revive and maintain cells in 10 cm dishes in 10% FBS-RPMI 1640 with appropriate amount of G418 (see Note 4).

3.3 Cell-Based Bioluminescence Assay

1. It is important to carry out cell-based and in vivo bioluminescence assays using the reporter expressing stable cell lines.

2. Select the cell lines which represent appropriate cellular and biological context as desired.

3. Stable cell lines expressing Caspase 3/7 reporter are plated overnight in black-walled or white-walled, clear bottom 96-well plates for live cell assays at a density of 1–2 × 10⁴ cells/well 24 h prior to treatment.
4. Treat the cells with the drug of interest like 100–200 ng/mL TRAIL, 12.5 μM CV3988, 50 μM docetaxel, or 25–100 ng/mL anti-Fas antibody in fresh media.

5. For studies evaluating the specificity of the reporter, protease inhibitors such as the pan-Caspase inhibitor Z-VAD-FMK can be utilized. Cells should be preincubated with 20 μM Z-VAD-FMK or DMSO (vehicle control) 1 h prior to treatment.

6. Add 100 μg/mL GloSensor cAMP reagent to the assay media, and perform live cell imaging at desired time points.

7. If cells are to be imaged at multiple time points, cells can be incubated with 100 mL of CO₂-independent media containing 300 μg/mL of GloSensor cAMP reagent for 2 h prior to the beginning of the treatment. Photon counts can be acquired over time pre- and posttreatment using the EnVision Luminometer (see Notes 5–7).

8. Image the black-walled 96-well plates on the live cell imaging system (such as Xenogen IVIS) immediately after adding the
substrate at medium binning for 30–60 s. For a time course measurement, image every 3–10 min.

9. The white-walled plates can be read on the live cell plate reader (such as EnVision) right after addition of the substrate. Typically each well of the plate is read for 0.01–1.0 s. For a time course activity measurement, the images can be acquired with a delay of 15–60 min between each read. If using high throughput system, for each read the robot takes the plate out from the incubator, loads it on the reader where the plate is read, and transfers it back to the incubator until the next time point.

10. Quantify bioluminescence acquired on the IVIS imaging system by region-of-interest (ROI) analysis using Living Image software. The bioluminescence data from the live cell plate reader is automatically saved in quantitative form in tab-delimited file format.

11. Validate the bioluminescence measurements in parallel experiments by immunoblot analysis of the cleavage products. This can include cleavage of the reporter or cleavage of cellular substrates of the protease (e.g., PARP).

### 3.4 In Vivo Imaging of Caspase 3 Activity

1. For flank xenografts, expand low-passage D54-MG cells expressing Caspase 3/7. Trypsinize and resuspend in RPMI 1640 media at 40 × 10^6 cells/mL. Inject 50 μL of this suspension subcutaneously into each flank (2 × 10^6 cells) of NOD/SCID mice. Monitor tumor size by caliper measurements, and start the treatment once the tumor size reaches around 100 mm³.

2. For breast cancer bone metastasis model, implant MDA-MB-231/1833 cells stably expressing the Caspase 3/7 GloSensor into the tibia of SCID mice. Follow tumor growth by MRI, and start the treatment when tumor reaches size 5–15 mm³.

3. For in vivo bioluminescence imaging, anesthetize the mice using 2% isoflurane/air mixture, and inject with a single i.p. dose of 150 mg/kg VivoGlo Luciferin (see Note 8).

4. Acquire baseline bioluminescence measurements 6 h before starting the treatment.

5. Transfer mice to the bioluminescence instrument while maintaining under anesthesia. A maximum of five mice can be imaged at once isolated by a plastic separator. Acquire bioluminescence images using a 15–30 s acquisition time at medium sensitivity to begin with. Adjust the settings depending on the signal. To account for the variation in signal intensity between tumors and in order to be able to acquire bioluminescence at its peak signal, it is helpful to do sequential acquisition of 10–20 reads with a 1–2 min delay between the reads (see Note 9).
6. Remove mice from the imaging instrument, and monitor for complete recovery from anesthesia.

7. Treat the mice with appropriate inhibitors or activators, and again monitor bioluminescence over time (see Note 10).

8. Acquire images posttreatment at required time points. It is important to give a 4–6 h gap between consecutive imaging so that the bioluminescence signal from the previous substrate injection has dissipated.

9. Quantify imaging data by ROI analysis of bioluminescence produced by the tumor using units of photon flux (see Notes 11–14).

10. Fold induction of bioluminescence activation can be calculated by normalizing posttreatment values to pretreatment values of each individual animal.

1. Caspase 3/7 reporter can be conditionally activated in a tissue-specific manner in mice to visualize apoptosis in real time but noninvasively.

2. A transgenic construct for Caspase 3/7 reporter can be generated by cloning the Caspase 3/7 reporter in pCLEX vector with CAG and floxed GFP/stop cassette. Constitutively active CAG promoter drives the expression of green fluorescent protein EGFP to determine the expression of transgene in various tissues. The EGFP coding sequence is flanked by loxP sites and a polyA signal with a strong termination sequence preventing the transcription of the downstream-located bioluminescent reporter construct (Fig. 3).

3. The transgenic Caspase 3/7 reporter mice can be generated by pronuclear microinjection of the transgene containing the apoptosis reporter into fertilized eggs obtained from FVB/N females.

4. Intercross the transgene reporter mice with a tissue-specific Cre-recombinase mouse (CMV-Cre or p48-Cre). Upon Cre-mediated recombination driven by CMV or p48 Cre expression, the EGFP lox-stop-lox cassette will be excised thus allowing for transcription of the reporter in Cre-expressing cells (Fig. 3).

5. Genotyping can be done using the primers specified above (in Subheading 2.4) for CMV-Cre, p48Cre, and apoptosis reporter mice.

6. To evaluate reporter activity, treat the mice with an agent that initiates the activation of apoptosis. In mice wherein the reporter is expressed within the pancreas (due to pancreatic expression of the Cre-recombinase in the P48-Cre animals), two series of 6 hourly intraperitoneal injections of cerulein can induce pancreatitis (75 μg/kg, total 12 injections in 48 h) [30].
7. Perform bioluminescence imaging prior to treatment and 30 h posttreatment using the methodology described as above for imaging the xenografts [30] (see Note 15).

4 Notes

1. Substrate sequence for the construction of the reporter for any protease should be determined considering the specificity of the substrate and based on published literature.

2. The selection of a specific cell line for creating a reporter-expressing stable line should be based on the specific biological question being interrogated and the intrinsic activity and detectability of the proteases and the substrates in the cell line. The cell lines should be selected based on literature search and experimentally.

3. Clonal selection can be done by limited dilution method in 96-well plates. Alternately for picking up the clones, complete growth media with antibiotic is aspirated from the tissue culture dishes containing single-cell clones. Transfer sterilized filter paper discs pre-wetted in trypsin to the clones using sterilized forceps in a laminar flow hood. After 4–5 min, using
sterilized forceps transfer the discs from the tissue culture plate to 24-well plate containing complete growth media with half the concentration of antibiotic used for clonal selection. Generally 12–24 single-cell clones are picked. Forceps can be sterilized by ethanol or by heating. Wait for the ethanol to evaporate before using the forceps to pick up the clones.

4. The G418 concentration for stable clone selection should be empirically decided. For maintaining the stable cell lines, it is preferable to use half the concentration of G418 than that is used for selection.

5. Perform all the bioluminescence acquisitions at 37 °C as the N-Luc and C-Luc fragments used in the construction of the complementation-based reporters have been optimized in mammalian cells at the physiological temperature (37 °C).

6. The reporter is based on the complementation of the light-generating enzyme luciferase, and it works only in live cells under physiological conditions. Methods wherein cell lysates are used to measure the bioluminescence signal may be incompatible with this reporter system and may not be able to yield a desired signal to background (under different treatment conditions).

7. The EnVision system is built with ultrasensitive luminescence detection technology. Even 0.01–0.1 s measurements for each well are usually sufficient. Also, the bioluminescence signal from GloSensor cAMP reagent is stable for a prolonged period of time and facilitates measurement of the protease activity for long-term studies.

8. We have found that 400 μg luciferin/mouse gives the best signal-to-background bioluminescence readings for split firefly luciferase-based reporters. However amount of luciferin can be increased to 150 mg/kg body weight (i.e., 3.5–4 mg/mouse with 20–25 g average mouse weight) in case of smaller tumors or tumors generated from cell lines expressing very low levels of reporter.

9. Generally 4–5 mice can be used in an experimental group for bioluminescence data acquisition on the IVIS imaging system. A stage level, i.e., the distance of the CCD camera from the subject, is selected and kept consistent for the entire experiment. Changing the stage level would lead to variations in photon counts, making it hard to analyze the data.

10. It is important to test 2–3 different concentrations of the drug in the mouse tumor xenograft model to find the optimal concentration which gives highest-fold change over vehicle control without being toxic to animals.

11. For analyzing the bioluminescence data, it is advisable to create similar ROIs for each tumor. The same ROI can be copied
and pasted for each tumor for counting total photon flux for all the time points. However, the positioning of the ROI can be adjusted so that it covers the tumor without overlapping with the ROI of any adjacent tumor.

12. The photon flux should be acquired without any saturated pixels. Data from saturated pixels cannot be accurately analyzed.

13. Since luciferin bioavailability and peak intra-tumoral concentration vary from mouse to mouse, we consider only the maximum photon flux emitted from each tumor for analysis. Since each tumor may show the maximum emittance at different time points, it is helpful to perform sequential reads with a delay between each read.

14. As a limitation of sensitivity of the imaging system, a minimal background signal is always observed in nude and SCID mice. If using a mouse strain with hair, the optical scatter can be minimized by shaving off the hair which will reduce the background.

15. Any data obtained using bioluminescence images acquired in a mouse xenograft model should be independently validated by biochemical methods such as Western blotting or immunohistochemistry (IHC). For validation of the bioluminescence data for Caspase 3/7, tumor tissue should be analyzed using antibodies specific for activated Caspase 3 or PARP antibodies.

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