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Dual Reporter Bioluminescence Imaging with NanoLuc and Firefly Luciferase

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Abstract

Bioluminescence imaging is a powerful, broadly utilized method for non-invasive imaging studies in cell-based assays and small animal models of normal physiology and multiple diseases. In combination with molecular engineering of cells and entire organisms using luciferase enzymes, bioluminescence imaging has enabled novel applications including studies of protein-protein interactions, ligand-receptor interactions, cell trafficking, and drug targeting in mouse models. We describe use of a novel luciferase enzyme derived from *Oplophorus gracilirostris*, NanoLuc, in cell-based assays bioluminescence imaging of tumor-bearing mice. We also combine NanoLuc with another luciferase enzyme, firefly luciferase, to image multiple signal transduction events in one imaging session.

Keywords

NanoLuc; bioluminescence imaging; Firefly luciferase; *Oplophorus gracilirostris*

Introduction

Over the past decade, bioluminescence imaging has emerged as a powerful, widely utilized method for non-invasive imaging studies in cell-based assays and small animal models of normal physiology and multiple diseases. As compared with other whole-animal imaging modalities, provides unparalleled sensitivity for detecting molecular and cellular events in vivo [1]. Relatively low cost and straightforward operation of commercial bioluminescence imaging instruments make this technology readily accessible to investigators without specific expertise in imaging. In combination with molecular engineering of cells and entire organisms, bioluminescence imaging has enabled novel applications including studies of

protein-protein interactions, ligand-receptor interactions, cell trafficking, and drug targeting in mouse models [2–6].

Firefly luciferase (FL) remains the most commonly used reporter enzyme for bioluminescence imaging. Bioluminescence from FL remains relatively constant over an approximately 10–20 minute time period, enhancing reproducibility of data from independent cohorts of mice imaged at different times [7]. Luciferin, the substrate for FL, can be delivered readily into mice via intraperitoneal injection, a simple method that facilitates imaging multiple animals at one time. Despite the strengths and widespread use of FL, relying solely on this enzyme for bioluminescence imaging poses a few important limitations. FL provides a single imaging signal, restricting imaging studies to a single molecular event or population of cells. This luciferase requires ATP as a co-factor, making the imaging signal susceptible to changes in intracellular metabolism. Due to reduced concentrations of ATP in the extracellular space, FL functions most efficiently and produces the highest signal when expressed within intact cells. Finally, the relatively large size (about 60 kDa) of FL imposes steric constraints that frequently limit use of this enzyme as a fusion tag for other proteins.

As an alternative or complement to FL and other beetle luciferases, we and other investigators have used different luciferases, such as *Renilla* or *Gaussia* (GL) [6, 8, 9]. In particular, GL has advantages of ATP-independence and smaller size (about 20 kDa), making this luciferase a better choice for imaging events in the extracellular space or fusing to another protein as an imaging reporter [6, 10]. Since the substrate for GL (coelenterazine) is distinct from luciferin and FL, GL provides a second, independent imaging signal for molecular and cellular imaging. However, imaging with GL is limited by substantial oxidation of coelenterazine in serum, decreasing overall sensitivity relative to FL and preventing imaging in some organs, such as the liver. In addition, the combination of GL and coelenterazine exhibits flash kinetics, requiring animals to be imaged immediately after injecting substrate. GL also emits blue light with peak admission at 480 nm, which restricts penetration through animal tissues for *in vivo* imaging.

To provide another bioluminescence imaging signal to complement FL in cell-based assays and living animals, we investigated a recently reported luciferase enzyme, referred to as NanoLuc, developed from the deep sea shrimp *Oplophorus gracilirostris* [11, 12]. NL shares some limitations of GL, including emission of blue light with peak emission of 478 nm and shorter period of bioluminescence emission in animals. NL also is a small (< 20 kDa), ATP-independent enzyme with advantages for generating bioluminescent fusion proteins and imaging extracellular events. A key benefit of NL over GL is that the former enzyme utilizes a novel substrate, furimazine, which has markedly lower background signal than coelenterazine. Here we report our method for expressing NanoLuc in mammalian breast cancer cells for cell-based assays in addition to bioluminescence imaging studies in living mice. We also show our technique for imaging NL and FL sequentially in animal models, focusing on our method for imaging TGF- β signaling through its receptor TGF- β R1 and activation of the TGF- β promoter as an example. More broadly, these same imaging principles can be applied to a wide range of signaling events or cell tracking studies,

expanding capabilities for molecular and cellular bioluminescence imaging studies in cell-based assays and mouse models of disease pathogenesis and therapy.

Materials

Molecular Biology

1. Secreted NanoLuc plasmid pNL.1.3 (Promega) (see Note 1)
2. Firefly luciferase pGL4 plasmid (see Note 2)
3. Expression vectors with constitutive promoters for expression in mammalian cells
4. Expression vector and packaging constructs for producing lentiviral vectors
5. Enzymes, buffers, and equipment for PCR
6. Restriction digests for DNA and ligations
7. TGF- β promoter-NL reporter plasmid (Promega) (see Note 3)
8. FL complementation reporter for kinase activity of TGF- β R1 [13]

Cell Culture

1. HEK 293 T cells or other cells that can be transfected easily for preparation of lentiviruses
2. Desired cell lines for experiments in cell-based assays and mouse models (see Note 4)
3. Cell culture supplies: media appropriate for selected cell types, plasticware, and incubators

Cell-based Imaging

1. 96 –well plates with black sides, clear bottom, and lid (Corning, Product #3904 or similar)
2. Multichannel pipettes for volumes from 1 to 200 μ l.
3. Sterile pipette tips with low adherence coating adherence.
4. Sterile 1x phosphate-buffered saline (PBS) solution

¹.NL vectors are available with either secreted or intracellular NL. We use secreted NL because we determined this version of the enzyme produced higher bioluminescence than the intracellular enzyme. Using secreted NL also gives the option to measure levels of the enzyme in blood. NL can be obtained in vectors without or with constitutive mammalian promoters and a variety of different drug selection markers.

².Vectors for codon optimized firefly luciferase are available in pGL4 vectors with or without different mammalian promoters or drug selection markers.

³.We describe reagents used for imaging TGF- β signaling at levels of receptor activation and transcription with FL and NL, respectively. FL and NL reporters can be used to image defined components of other signaling pathways of interest to other investigators.

⁴.We use human MDA-MB-231 breast cancer cells (ATCC) because they form primary and metastatic tumors efficiently in mice, but any desired mammalian cell line will work for cell based assays. Using a cancer cell line that forms tumor xenografts in mice is necessary for imaging studies in mice.

5. Stock solution furimazine substrate for NL (currently sold as Nano-Glo® Luciferase Assay System, Promega)
6. D-luciferin powder (Promega or other vendor) (see Note 5)
7. Recombinant TGF- β
8. TGF- β inhibitors SB461542 or SD208 (Tocris or other vendor)
9. Bioluminescence imaging system with high sensitivity and software for data quantification and analysis (IVIS, Perkin-Elmer, or similar system)

Animal Imaging

1. Appropriate mouse strain for experimental system (see Note 6)
2. Small animal shaver (i.e. Wahl compact cordless trimmer or similar instrument)
3. Depilatory solution (i.e. Nair)
4. 28–30 gauge insulin syringe for intravenous tail vein injections and intraperitoneal injections in mice
5. Restraint device for tail vein injection (optional) (Braintree Scientific or other vendor) (see Note 7)
6. Bioluminescence imaging instrument with isoflurane anesthesia (IVIS or similar instrument)

Methods

Construct cells stably expressing NL and FL imaging reporters (see Note 8).

1. For constitutively expressed or inducible NL and FL reporters, stable cells can be generated through methods including transfection or lentiviral transduction with selection for cells stably expressing reporters (see Note 7). We refer readers to standard texts of cell and molecular biology procedures for techniques used to transfer reporters to lentiviral vectors if desired and isolate stable cell lines.
2. Verify expression of reporter construct(s) in stable cell lines. This can be accomplished by qRT-PCR, Western blotting, and/or bioluminescence imaging assays. We refer readers to standard texts for methods used in qRT-PCR and Western blotting. For functional assays using bioluminescence imaging, see description below for cell-based assays.

⁵For cell-based assays, readers may opt to use the Nano-Glo® Dual-Luciferase® Reporter Assay System (Promega), which includes substrates for both NL and FL and a reagent to quench luminescence from FL. Separate stock solutions of each substrate (furimazine and luciferin) are needed for animal imaging studies

⁶Immunocompromised mice are needed for human tumor xenografts. We use NSG mice (The Jackson Laboratory) since these highly immunocompromised mice facilitate growth of human cancer cells.

⁷While optional, we find that a restraint device improves success of tail vein injections in mice.

⁸We use lentiviral transduction to generate populations of cancer cells expressing imaging reporter genes of interest for cell-based assays and mouse studies. Cell-based assays can be performed by transient transfection using transfection protocols optimized for specific reagents and cell types. Cells stably expressing imaging reporters are needed for animal studies.

Cell-based Assays

1. If using separate stock solutions of furimazine and luciferin for cell imaging studies, prepare a stock solution of 15 mg/ml luciferin in PBS and sterile filter. Stocks of luciferin should be stored at -20°C .
2. Plate cells at 1.5×10^4 cells per well in 96 well, black wall plates one day before experiments (see Note 9).
3. For assays with secreted NL, collect 5 μl of culture medium from stably expressing cells and transfer to a round-bottom, black wall 96 well plate to measure NL bioluminescence. Collect the same volume of culture medium from cells not expressing NL to use as a control (see Note 10).
4. To measure bioluminescence from secreted NL, add 45 μl PBS with a 1:1000 dilution of furimazine and image plate on an IVIS with open filter, smallest field-of-view (FOV) to capture the entire plate, large binning, and acquisition time of 30 seconds (see Note 11).
5. Measure NL by adding furimazine at a 1:1000 dilution from the stock solution and mix gently by tapping the side of the well or briefly placing the plate in a shaker (see Notes 12 and 13).
6. After acquiring NL images, add 10 μl PBS with 150 $\mu\text{g}/\text{ml}$ final concentration of D-luciferin (Promega) to each well.
7. Incubate cells for five minutes at 37°C and then measure bioluminescence from FL with 595 nm long pass filter to exclude light from NL.
8. Measure photon flux values from NL in either culture medium or cells using region-of-interest tools on the IVIS and by subtracting bioluminescence from cells not transfected with the reporter (see Note 14).

⁹Black wall plates prevent light emitted by cells in one well from shining into adjacent wells. We typically use cells at 70–90% confluence on the day of imaging, so cell density may need to be optimized for different types of cells. We generally do not starve cells prior to imaging assays because growth factor deprivation can reduce bioluminescence from FL due to depletion of ATP. For experiments regulating the TGF- β signaling pathway, we added varying concentrations of recombinant TGF- β or small molecule inhibitors overnight. If starvation is necessary to activate a signaling pathway with a defined stimulus, we plate cells in normal growth medium and allow cells to adhere to the plate for at least two hours before changing to starvation medium.

¹⁰Medium from control wells is needed to quantify background luminescence produced by oxidation of furimazine in medium with serum. Background can be reduced by decreasing amounts of serum in medium and/or performing assays with PBS.

¹¹Acquisition parameters likely will need to be adjusted based on amount of bioluminescence in different samples to avoid saturating the detection camera since saturated pixels cannot be quantified accurately. If an exposure gives saturated pixels, then shorten the acquisition time and/or use smaller binning. For an IVIS, do not use acquisition times less than one second since smaller exposure times are not accurate. Use longer acquisitions and/or larger binning to improve detection of a weak signal.

¹²These steps are written for sequential imaging with separate NL and FL substrates added sequentially. Investigators may opt to perform cell-based imaging with the Nano-Glo® Dual-Luciferase® Reporter Assay System, which measures FL signal first and then quenches this signal to allow measurement of NL. The Nano-Glo® Dual-Luciferase® Reporter Assay System should be used according to the manufacturer's specifications.

¹³Serum containing medium modestly increases background bioluminescence from the NL substrate. If the imaging signal is dim, NL signal can be measured by removing culture medium and assaying with furimazine diluted in PBS.

¹⁴Investigators optionally may subtract background bioluminescence from control cells not expressing FL. We typically find the background signal for this luciferase to be negligible.

Animal studies

1. All animal studies should be approved by the institutional committee for care and use of animals prior to performing experiments. For orthotopic breast tumor xenografts, implant 5×10^5 MDA-MB-231 cells co-expressing reporters for NL and FL into 4th inguinal mammary fat pads of NSG mice (see Note 15) [REF].
2. Begin imaging studies when tumors reach 8–10 mm diameter. Since the signal duration is shorter for NL, we image this reporter first.
 1. To image NL activity, inject 5 µg (0.25 mg/kg) furimazine (about 40X dilution of Nano-Glo substrate) in 100 µl sterile PBS via tail vein and image mice on an IVIS Spectrum as quickly as possible after injecting the substrate, preferably within 30 seconds (see Note 16).
 2. Image NL with an open filter, large binning, and acquisition times from 1–60 seconds depending on size of tumor and relative intensity of bioluminescence.
 3. FL imaging can be performed 30 minutes or more after NL. Inject D-luciferin at 150 mg/kg intraperitoneally and wait 10 minutes before imaging.
 4. Image FL signal with open filter, large binning, and acquisition times from 1–120 seconds as determined by tumor size and anticipated strength of signal [REF].
 5. To measure serum levels of secreted NL, collect 20 µl blood samples by retro-orbital puncture or other appropriate location for collection of venous blood. Transfer blood to tubes with 2 µl of 20 mM EDTA to prevent blood clotting (see Note 17).
 6. Centrifuge blood samples at top speed in a microfuge for 10 minutes and collect 10 µl of plasma for analysis. Analyze amounts of secreted NL as described for measuring this enzyme in culture medium except for adding 40 µl of PBS with NL substrate diluted 1:1000 (see Note 18). Quantify imaging data using ROI tools on the IVIS software.

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¹⁵Investigators may use separate populations of cells expressing NL or FL reporters, respectively, rather than cells co-expressing both reporters. NL and/or FL reporters may be expressed constitutively for purposes such as tracking cell numbers and localization or regulated reporters to analyze specific signaling pathways. We typically use 6–10 week-old female NSG mice, although other ages, sexes, or mouse strains may be used as appropriate. Other sites for implantation of cancer cells may be selected as appropriate for the experimental question and model system.

¹⁶To reduce time between injection of furimazine and imaging, we anesthetize each mouse prior to intravenous injection and immediately transfer the animal to the stage of the IVIS. The requirement for tail vein injection and immediate imaging means that only one animal is imaged at a time. Maintaining a consistent, short time interval between injection and image acquisition is essential to generate reproducible data since the NL signal decreases over the course of minutes in animals.

¹⁷NL is stable in serum, so samples do not need to be processed immediately after collection but preferably should be analyzed the same day.

¹⁸To control for background signal, it is essential to have plasma from the same strain of mouse without implanted NL cells.

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Figure 1. Dual luciferase imaging of TGF- β signaling in cell-based assays.

A) Schematic of NL and FL reporter constructs. Expression of NL is regulated by a promoter with a SMAD binding element (SBE), so typical TGF- β signaling increases bioluminescence. The BTR FL reporter utilizes luciferase complementation to detect phosphorylation of target substrates by the activated receptor, TGF- β R1. TGF- β signaling decreases bioluminescence from this reporter. B) A549 cells expressing both reporters were treated with either 10 ng/ml TGF- β (panel B) or increasing concentrations of the TGF- β receptor inhibitor SD208 (panel C). B) Graph shows mean values + SEM for fold induction of the NL reporter. Images show bioluminescence imaging and a Western blot of cells treated with vehicle or TGF- β , respectively. C) Graph shows mean values + SEM for activation of the firefly luciferase reporter by inhibition of TGF- β R1 kinase activity.

Figure 2. Imaging TGF- β signaling with NL and FL in living mice.

Mice were implanted with A549 lung cancer cells expressing NL and FL reporters for monitoring TGF- β signaling. We treated mice with either 100 ng TGF- β or 50 mg/kg SD208 by intraperitoneal injection ($n = 5$ per group). A) Bioluminescence images of SBE-NL activity show representative mice from each group prior to treatment and at times 6 and 24 hours after treatment. Pseudocolor scale shows range of depicted photon flux values. B) Firefly luciferase imaging performed immediately after NL imaging shows induction of signal in mice treated with an inhibitor of TGF- β signaling. C) Graph shows mean values + SEM for NL signal. Data were normalized to values at time 0 prior to treatment. D) Graph shows mean values + SEM for firefly luciferase activity in mice treated with either TGF- β or SD208 normalized to values from the same mice prior to treatment. Note that activation of TGF- β signaling increases the NL reporter, while inhibition of the pathway decreases the NL reporter and increases the FL signal. This strategy allows quantification of two different aspects of the same signaling pathway *in vivo*. NanoLuc data for 6 and 24 hour time points were normalized to corresponding photon flux values prior to treatment. Graph shows mean values + SEM for percent change relative to untreated animals in each group at 6 and 24 hour time points. *, $p < 0.05$; **, $p < 0.01$ relative to pre-treatment values.