Chapter 5

Measuring IL-1β Processing by Bioluminescence Sensors I: Using a Bioluminescence Resonance Energy Transfer Biosensor

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Abstract

IL-1β processing is one of the hallmarks of inflammasome activation and drives the initiation of the inflammatory response. For decades, Western blot or ELISA have been extensively used to study this inflammatory event. Here, we describe the use of a bioluminescence resonance energy transfer (BRET) biosensor to monitor IL-1β processing in real time and in living macrophages either using a plate reader or a microscope.

Key words BRET, IL-1β, Sensor, Bioluminescence, Macrophage

1 Introduction

Inflammation is a physiological process in response to tissue injury or pathogen infection [1]. IL-1β, a proinflammatory cytokine, constitutes one of the key components involved in the biological cascade leading to inflammation. Different cell types, such as macrophages and monocytes, synthesize IL-1β as an inactive precursor [2]. Pro-IL-1β is processed to its active form by the protease caspase-1 and then is secreted to the extracellular space where it initiates the inflammatory response after binding to the IL-1 receptor located on neighboring cells [2, 3]. Caspase-1 activation is controlled by the assembly of Nod-like receptors (NLR) into multiprotein complexes termed inflammasomes [3]. A role for IL-1β has been reported in different pathologies including cancer, type 2 diabetes, gout, or Alzheimer's disease. Due to its predominant role in various pathophysiological processes, IL-1β processing has been studied employing antibodies against IL-1β in techniques such as Western blot or ELISA. These approaches have been extensively used for decades to detect IL-1β processing, and they are commonly used as an indirect method to detect NLR assembly into

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active inflammasomes in response to stimulation. However, these tools are limited in their temporal resolution and are not compatible for in situ IL-1β detection. We recently engineered a biosensor based on bioluminescence resonance energy transfer (BRET) that allows monitoring IL-1β processing in real time and in living cells, including macrophages. The precursor pro-IL-1β has been fused at its extremes to the donor (RLuc8) and the acceptor (Venus) of BRET, leading to an energy transfer between the two BRET partners (Fig. 1). We found that this biosensor has similar properties than its endogenous homolog pro-IL-1β and that it is cleaved by caspase-1 which leads to a decrease in BRET signal [4]. Using this biosensor, we were able to analyze IL-1β processing in real time by monitoring BRET variation in different macrophage cell lines. This tool is also compatible with microscopy to visualize such process on single cell as primary macrophage. This makes this biosensor an interesting tool for simple detection of IL-1β processing in situ.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials. We do not add sodium azide to the reagents.

2.1 Expression of the Bioluminescence Sensor in Macrophage Cell Lines

1. Transfection reagent to transfect macrophage cell lines as J774A.1 or immortalized bone marrow derived macrophages. We use TransIT-Jurkat (Mirus) since it gives us good transfection on macrophages, but an equivalent reagent can be also used.
2. Complete cell media appropriate to the cell line in culture. For example, Dulbecco's Modified Eagle's Medium (DMEM) with 10 % of heat-inactivated fetal calf serum (FCS) for J774A.1.
3. Opti-MEM® culture media (Life Technologies) or an equivalent media without serum.
4. Plasmids coding for the IL-1β bioluminescence sensor and for RLuc8 (*see* **Note 1**).

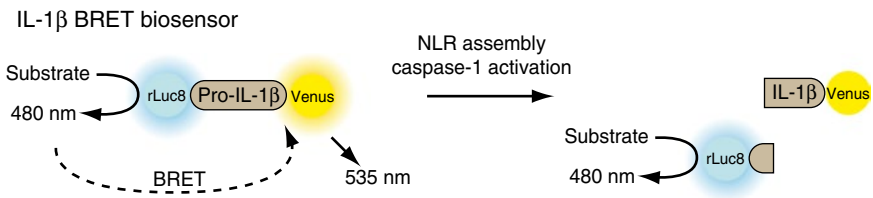

Fig. 1 Schematic representation of the IL-1β BRET biosensor. Upon caspase-1 activation, the BRET donor (rLuc8) and acceptor (Venus) molecules get apart, leading to a decrease of the net BRET signal

2.2 BRET Recording Using a Plate Reader

1. 96-well plate, white, flat and micro-clear bottom, cell culture treated, sterile with lids (*see* **Note 2**).
2. White backing tape to be stuck on the bottom of the 96-well plate the day of recording. It increases the luminescence signal by reflection.
3. A plate reader for luminescence recording equipped with two emission filters close to 480 nm and 535 nm.
4. HBS solution: 147 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 13 mM d-glucose, pH 7.4.
5. Multichannel pipettes.
6. Coelenterazine-h (*see* **Note 3**).

2.3 BRET Recording by Microscopy and Data Analysis

1. 35 mm dishes adapted for luminescence recording and treated for cell culture (e.g., we use μ-Dish from ibidi).
2. Coelenterazine-h (*see* **Note 3**).
3. Microscope and camera adapted for luminescence recording (e.g., our setup was the Olympus LV200 bioluminescence imaging system) and equipped with two emission filters close to 480 nm and 535 nm (*see* **Note 4**). To identify cells expressing the biosensor, filters for excitation and emission of Venus are also required (excitation close to 515 nm and emission close to 528 nm).
4. Software for image acquisition.
5. Software for data analysis, we use ImageJ (NIH), but equivalent software is also suitable.

3 Methods

Carry out all procedures with sterile and pyrogen-free material in biological safety cabinets Class II at room temperature unless otherwise specified.

3.1 Macrophage Transfection and Priming

1. Cells are plated 24 h before transfection. To use TransIT-Jurkat reagent, cells are plated to get 60–70 % confluence on day of transfection (*see* **Note 5**).
2. Transfection of macrophages can be performed following manufacturer instructions. Briefly, for one well of a 96-well plate, use 0.1 μg of DNA, 9 μl Opti-MEM, and 0.3 μl TransIT-Jurkat. Add DNA-transfection reagent complexes on well containing 50 μl of complete cell culture media.
3. Incubate cells with transfection mixture for 4–5 h at 37 °C and 5 % CO_2, then remove transfection reagent containing media, and replace it with fresh cell media.

4. BRET experiments can be performed the following day. If required, prime macrophages by adding 1 μg/ml of lipopolysaccharide to the culture media and incubated the cells for 4 h at 37 °C and 5 % CO_2.

3.2 Measuring IL-1β Processing in Real Time Using a Plate Reader

1. Pre-warm the plate reader at 37 °C.
2. Set up the plate reader controlling software according to your assay (*see* **Note 6**).
3. Predilute coelenterazine-h at 25 μM in HBS. Prepare 10 μl of solution per well to read (*see* **Note 7**).
4. Wash cells two times with 100 μl HBS per well and take care to not detach cells.
5. Keep cells in 40 μl of HBS per well.
6. Cell detachment can be checked using a transmitted light microscope.
7. Stick the white backing tape at the bottom of the plate.
8. Add 10 μl of prediluted coelenterazine-h per well to get a 5 μM final concentration.
9. Insert the plate in the plate reader.
10. Incubate for 6 min to allow the luminescence signal to reach a steady state (*see* **Note 8**).
11. Read the luminescence signal at 480 nm and 535 nm, and determine the gain according to the sensitivity of the plate reader (*see* **Note 9**). As a basal control, use nontransfected cells.
12. Initially use cells transfected with RLuc8 alone to record the luminescence signal at 480 nm and 535 nm in the same experimental conditions set above (*see* **Note 10**).
13. Then record luminescence signal at 480 nm and 535 nm from macrophages before any stimulation to determine the basal BRET signal.
14. BRET value, expressed in milliBRET units (or mBU), can be determined with the following equation:

$$\text{BRET (mBU)} = \left(\left(\frac{\text{Lum(535 nm)}}{\text{Lum(480 nm)}} \right)^{\text{IL 1}\beta\text{ sensor}} - \left(\frac{\text{Lum(535 nm)}}{\text{Lum(480nm)}} \right)^{\text{RLuc 8 only}} \right) \times 1000$$

3.3 Monitoring Real-Time IL-1β Processing in Individual Macrophages

1. Pre-warm the microscope incubator at 37 °C.
2. Predilute coelenterazine-h at 200 μM in HBS. Prepare 100 μl of solution per well to read (*see* **Note 7**).
3. Wash the cells two times with 1 ml HBS and take care to not detach cells.

4. Keep cells in 0.9 ml of HBS per dish.
5. Place the dish in the microscope incubator and use appropriate objective to focus the cells.
6. Define the field and cells that you want to monitor.
7. Take a picture in bright field of your cells and after direct excitation of Venus (excitation close to 515 nm and emission close to 528 nm) to visualize cells that are transfected (*see* **Note 11**).
8. Gently add 100 μl of prediluted coelenterazine-h.
9. Incubate for 6 min to allow the luminescence signal to reach a steady state (*see* **Note 9**).
10. Sequentially record pictures at 480 nm and 535 nm, and stimulate the cells when required (*see* **Note 12**).
11. Export your pictures in tiff format for ImageJ analysis.
12. Using ImageJ software, define that division by 0 = 0. Go to Edit menu > options > misc, and in the "Divide by zero" window introduce the value "0.0" or "NaN".
13. Open the tiff pictures acquired at 480 nm and 535 nm.
14. Apply a median filter of 1 pixel for both pictures.
15. Remove background signal of the same area for both images. Define a field where no cells are present in the 480 nm picture and measure the maximum signal intensity (go to Analyze menu > Measure). Using the ROI manager tools (go to Analyze menu > Tools), repeat a similar measurement on the same field of the 535 nm pictures. Then subtract the correspondent value to the entire picture (go to Process menu > Math > Subtract).
16. Save the two pictures in tiff format with a different file name.
17. Divide the 535 nm pictures by the 480 nm pictures. Go to Process menu > Image calculator. Select the right picture for picture 1 and 2 and select "divide" from the drop-down menu.
18. BRET signals can be represented using a continuous 256-pseudocolor lookup table. Go to Image menu > Lookup tables and select the appropriate LUT.

4 Notes

1. EF-1a promoter is more appropriate than CMV promoter for protein expression in macrophages. Plasmid backbone size affects transfection efficiency (the smaller, the better).
2. 96-well plate with white bottom could be used but will not allow to visualize cells during the different steps of the experiment. BRET recording using a plate reader can be performed on other format plate (i.e., 384-well plate) depending on the assay and/or luminescence signal strength.

3. Coelenterazines are poorly soluble in water and must be resuspended in ethanol or methanol preparing a stock solution at 1 mM. Keep stock solution at −20 °C and protect it from light. For prolonged stability, resuspend coelenterazine in acidified ethanol (10 ml of ethanol and 200 μl of 3 N HCl) and store at −80 °C.
4. Any microscope can be used for BRET recording but will require an appropriate camera for bioluminescence detection and a light-tight enclosure.
5. If cells are less confluent or more confluent than 60–70 % on the day of transfection, it will increase cell death or reduce transfection efficiency, respectively. A cell line stably expressing the bioluminescence sensor can be also used to get more reproducible luminescence signal. Lentivirus can be used to produce such cell lines.
6. The detection of the donor and acceptor luminescence signal of a given well have to be recorded successively before switching to another well recording.
7. Prediluted solution of coelenterazine is susceptible to oxidation by air and thus should not be prepared in advance.
8. During the first 6 min following coelenterazine-h addition, luminescence signal will increase exponentially and might cause inappropriate BRET value (especially if the exposure time for each filter is ≥ 1 s). Thus, it is recommended to wait for the luminescence signal to reach a plateau.
9. Gain must be adjusted to get the widest dynamic range and the best sensitivity for a given plate reader. Alternatively, if possible, perform an automatic gain adjustment. These values of gain will stay approximately the same for each experiment and will just have to be adjusted depending on the transfection efficiency.
10. For a given set of filters, plate reader, and experimental conditions, the ratio $\left(\frac{\text{Lum}(535\text{nm})}{\text{Lum}(480\text{nm})}\right)^{\text{RLuc 8 only}}$ for the donor only willstay approximatively the same during each experiment and could be determined once.
11. Direct excitation of Venus using a laser after addition of coelenterazine-h results in a high background fluorescence. Always excite Venus before addition of the substrate.
12. As mentioned for the BRET recording using a plate reader, exposure time and gain amplification of signal have to be determined to get the best signal/background ratio.

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