Direct Quantification of PTD Transduction Using Real-Time Monitoring

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Chapter 28

Direct Quantification of PTD Transduction Using Real-Time Monitoring

Mi-Sook Lee and Song Her

Abstract

Protein transduction domains (PTD or cell-permeable proteins) have attracted much attention as drug carriers because of their ability to penetrate cellular membranes. Although numerous PTD have been identified and their properties elucidated, their mechanism of action has not been fully understood due to the absence of a reliable quantification method. This chapter provides a direct method for quantifying cellular transduction of PTD in vitro and in vivo using bioluminescence imaging (BLI). This methodology exploits noninvasive techniques to create an environment suitable for the real-time imaging of PTD transduction and is therefore a promising tool for studying the mechanism of PTD transduction and the in vivo application of new therapeutic candidates.

Key words Drug carrier, Protein transduction domains, Cell-permeable proteins, Cellular transduction, Bioluminescence imaging, Real-time imaging

1 Introduction

Protein transduction domains (PTD) have attracted increasing attention in intracellular therapeutic protein delivery (1), and quantifying cellular transduction of PTD is important for comprehending both their mode of transduction and the efficacy of new drugs (2). Current approaches for quantifying PTD transduction, including flow cytometry and confocal fluorescence microscopy, are based on the fluorescent labeling of peptides, but flow cytometry can lead to false-positive results originating from cell surface-bound peptides (3). Also, fluorescence imaging by confocal fluorescence microscopy, which can monitor the subcellular localization of peptides and discriminate between internalized and extracellular fluorescent peptides, is limited by statistical problems (4). Alternatively, direct peptide detection by MALDI-TOF using isotope-labeled peptide was recently reported (5, 6). Although this method allows for the direct quantification of cell-permeable

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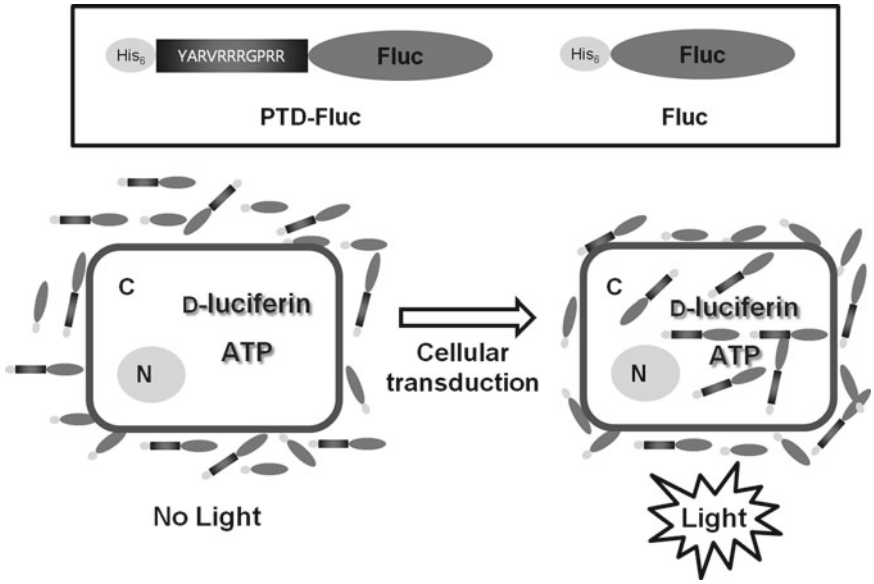

Fig. 1 Schematic illustration of bioluminescence in living cells following PTD-Fluc transduction. *Inset*: Structures of PTD-Fluc containing 11 amino acids (YARVRRRGPRR) and Fluc as a control. After PTD-Fluc transduction, luminescence is emitted by an ATP-dependent luciferin–luciferase reaction (*C* cytosol, *N* nucleus)

proteins and the discrimination of extracellular membrane-bound and intracellular peptides, it can only be used with cell lysates and is not suitable for in vivo measurements.

Bioluminescence imaging (BLI) based on luciferase activity is a rapid and sensitive method for in vitro and in vivo studies of ongoing biological phenomena. The most commonly used luciferase for BLI is firefly luciferase (Fluc), which requires ATP-Mg^{2+} and oxygen in the presence of the substrate, D-luciferin, to produce bioluminescence (7). Using firefly luciferase-tagged PTD (PTD-Fluc), we quantified internalized PTD by the real-time monitoring of ATP-dependent luciferase activity in vitro and in vivo (Fig. 1). This chapter provides a basic protocol for the real-time quantification of PTD transduction using BLI.

2 Materials

2.1 Luciferase Assay of Purified Protein

1. Purified PTD-Fluc (8) (see Note 1).
2. Purified Fluc (8) (see Note 1).
3. Luciferin substrate solution: Combine 1 mM D-luciferin (Xenogen, Alameda, CA, USA) (see Subheading 2.2), 3 mM ATP (ATP disodium salt), and 15 mM $MgSO_4$ in 30 mM HEPES (pH 7.8) using fresh, deionized ATP-free water. Store the solution at −20°C in polypropylene or glass tubes.
4. 96-well black microplate with a clear bottom.

2.2 PTD-Fluc Transduction In Vitro

1. HeLa cells or other biologically relevant cell lines of interest.
2. Dulbecco's modified Eagle's medium (DMEM) complete media: DMEM supplemented with 10% (v/v) fetal bovine serum and 1× (v/v) pen/strep (100× antibiotic solution: 10,000 U of penicillin and 10,000 U of streptomycin).
3. 1× Phosphate-buffered saline without Mg^{2+} and Ca^{2+}.
4. D-Luciferin firefly (potassium salt, MW = 318.42, #XR-1001; Xenogen) stock solution: Reconstitute a 1.0 g of D-luciferin in 33.3 ml of sterile 1× PBS without Mg^{2+} and Ca^{2+} to make a 30 mg/ml (0.1 M) stock solution. Filter sterilize through a 0.2-μm syringe filter. Store in aliquots at −20°C and protect from light (see Note 2).
5. Syringe filter, 0.2 μm.

2.3 PTD-Fluc Transduction In Vivo

1. Male 4-week-old ICR mice weighing approximately 30 g each (SPF grade).
2. 0.5-cc, 27-gauge insulin syringes.

2.4 BLI Equipment (Xenogen)

1. Isoflurane anesthesia chamber.
2. IVIS-200.
3. Caliper/Xenogen IVIS® Living Image software, version 3.0.

3 Methods

3.1 Cell-Free Luciferase Assay

Prior to the in vitro or in vivo analysis of PTD transduction, the following protocol using PTD-Fluc and Fluc should be carried out to accurately determine the activity and concentration of each protein.

1. Bring the luciferin substrate solution to room temperature before starting.
2. Transfer 10 μl each of PTD-Fluc and Fluc in Ni-NTA elute buffer to a 96-well black microplate. Commercially available purified Fluc can be used as a standard for calibration (see Note 3).
3. Add 90 μl of D-luciferin substrate solution and use the substrate solution without purified protein as a blank.
4. Immediately acquire an image using the IVIS-200 imaging system (*see* Subheading 3.2, step 7).
5. Generate a luciferase standard curve for light emission (photons/second, p/s) vs. concentration (ng/ml).
6. Based on the standard curve, determine the concentration of each purified protein (see Note 4).

3.2 Transduction of PTD-Fluc In Vitro

The following quantification protocol based on the ATP-dependent luciferase reaction allows the direct and real-time measurement of transduction in live cells without interfering with surface-bound PTDs (Fig. 1).

1. Seed HeLa cells in a 96-well black microplate in 100 μl of DMEM complete media at 10,000 cells per well.
2. Culture the cells in a humidified atmosphere at 37°C under 5% CO_2 for 20–24 h.
3. Prepare 100 μl of fresh PTD-Fluc dissolved in DMEM complete media to a final concentration of 1–500 nM. Also, prepare fresh Fluc solution in DMEM complete media (see Note 5).
4. Remove the media from the cells and wash them once with pre-warmed DMEM complete media, taking care not to detach the cells.
5. Add 100 μl of PTD-Fluc or control Fluc simultaneously using a multichannel pipette to the appropriate wells (see Notes 6 and 7).
6. At suitable time points, wash the cells twice with 1× PBS without Mg^{2+} and Ca^{2+} to remove surface-bound proteins (see Note 8).
7. To image Fluc, add 100 μl of D-luciferin stock solution to each well at a final concentration of 150 μg/ml using a multichannel pipette.
8. Image initially at 1–5 s, 10 bin, f/4 using Living Image software, version 3.0. The imaging times and binning can then be adjusted accordingly (see Note 9).

3.3 Transduction of PTD-Fluc In Vivo

The results of real-time in vivo monitoring of PTD transduction are illustrated in Fig. 3.

1. Prepare 100 μl of fresh PTD-Fluc dissolved in PBS (50 μM stock solution) to final dose range of 0.1–10 μM/kg body weight. Also prepare fresh Fluc in PBS at the same concentration as PTD-Fluc (see Note 5).
2. Inject 100 μl of PTD-Fluc or Fluc intraperitoneally into unanesthetized male ICR mice using a 27-gauge needle (see Notes 10 and 11).
3. Immediately after injection of each protein, administer 30 mg of D-luciferin/kg body weight intraperitoneally using a 27-gauge needle (see Note 12).
4. Ten minutes after the injection of D-luciferin, anesthetize the mice in an isoflurane induction chamber for 1–2 min (mean time until loss of the righting reflex) and then transfer to an IVIS-200 imaging chamber (see Note 13).
5. Take a brief test image at 1 min, 10 bin, f/8. Use this image to estimate the exposure time and binning needed for subsequent

images (usually a 1–5-min exposure). Quantify the bioluminescence (p/s) within a region of interest encompassing the specific area of bioluminescence.

6. Repeat steps 3–5 every 2 h (see Note 14).

4 Notes

1. Hexahistidine (His_6)-tagged PTD-Fluc and Fluc expressed in *Escherichia coli* BL21 (DE3) cells harboring pRSET-PTD-Fluc or pRSET-Fluc should be purified using an Ni-NTA Fast Start Kit (#30600, Qiagen) (8, 9). Although affinity tags using His_6 are powerful and convenient tools for the purification of recombinant proteins, you can also use genetically engineered fusion partners such as glutathione *S*-transferase (GST), FLAG, or maltose-binding protein (MBP) (10); however, you should check whether the tag has a deleterious effect on the biological properties of the recombinant protein (e.g., effects on solubility and biological activity).
2. D-Luciferin (potassium salt) can be dissolved up to a concentration of 50 mg/ml (62.8 mM); it precipitates out at 60 mg/ml.
3. To establish a standard luciferase calibration curve, make a series of luciferase solutions (10 μl each) ranging in concentration from 1 pg/ml to 1 ng/ml in elution buffer. Alternatively, each sample can be serially diluted by a factor of 10.
4. Keep the purified proteins (if possible, maintain at >10 μM since a high concentration will help prevent a loss of protein stability) in an Ni-NTA elute buffer supplemented with 10% glycerol (#30600, Qiagen) at −80°C in small aliquots for up to a few days. Do not thaw and refreeze; whenever possible, use either fresh or one-time-thawed proteins.
5. Make sure that the activity and concentration of the PTD-Fluc protein are accurate and essentially no different from those of the control (Fluc). You should always perform a cell-free luciferase assay (see Subheading 3.1) and check the activity and concentration of each sample prior to running PTD transduction experiments.
6. Treating the PTD proteins at the same time is important. PTD-Fluc may appear to be internalized very rapidly (within minutes), although the response to treatment is dependent on cell type. Figure 2 illustrates the real-time monitoring of PTD transduction in living cells.
7. Check the viability of the cells under a microscope before proceeding to the next step. The criteria for determining the maximal PTD-Fluc concentration depend on cellular viability.
8. If you wish to test for PTD transduction in a matter of minutes, this step can be skipped (i.e., go on to BLI) because

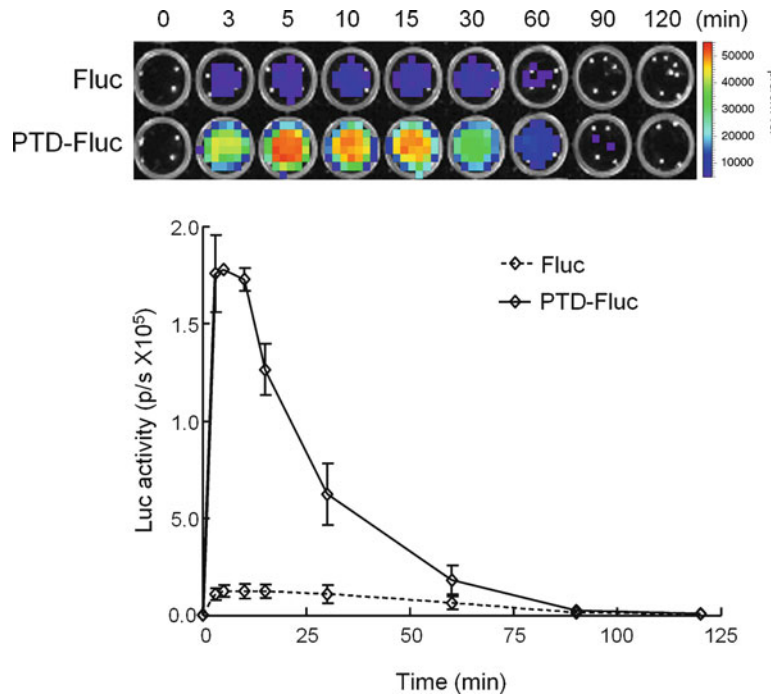

Fig. 2 Real-time imaging and quantification of PTD-Fluc transduction. Time-course representative image (*upper panel*) and quantification of luciferase activity in the presence of 40 nM Fluc or PTD-Fluc (*lower panel*). The data represent the mean ± SEM for each assay, performed in triplicate with three independent experiments. *Colored bars* indicate the bioluminescence signal intensity (p/s/cm²/sr) (Reproduced with permission from ref. 8)

ATP-free extracellular or surface-bound PTDs may not affect the luciferase reaction. This may be confirmed by comparing the results of PTD-Fluc and control (Fluc) transduction (Fig. 2).

9. The level of bioluminescence is directly proportional to the exposure time, depending on the level of PTD-Fluc transduction. If you are unsure of what exposure time to use, start with low-sensitivity settings and increase as necessary (typical range, 0.5–1 min).
10. In general, the maximum intraperitoneal injection volume should not exceed 10 ml/kg body weight for adult mice.
11. Intraperitoneal administration is the injection of a substance into the peritoneal cavity. Note that intraperitoneal delivery is difficult to perform correctly, as you can easily misplace the dose into the intestine, gut, urinary bladder, muscle, or other organs. To avoid puncturing the abdominal viscera, hold the animal with its head tilted downward and insert the needle quickly into the lower left of the midline umbilicus.

12. The first image capture after PTD-Fluc injection should be delayed by approximately 15 min because it takes time to reach peak luminescence output from intact cells after D-luciferin injection (Fig. 3).

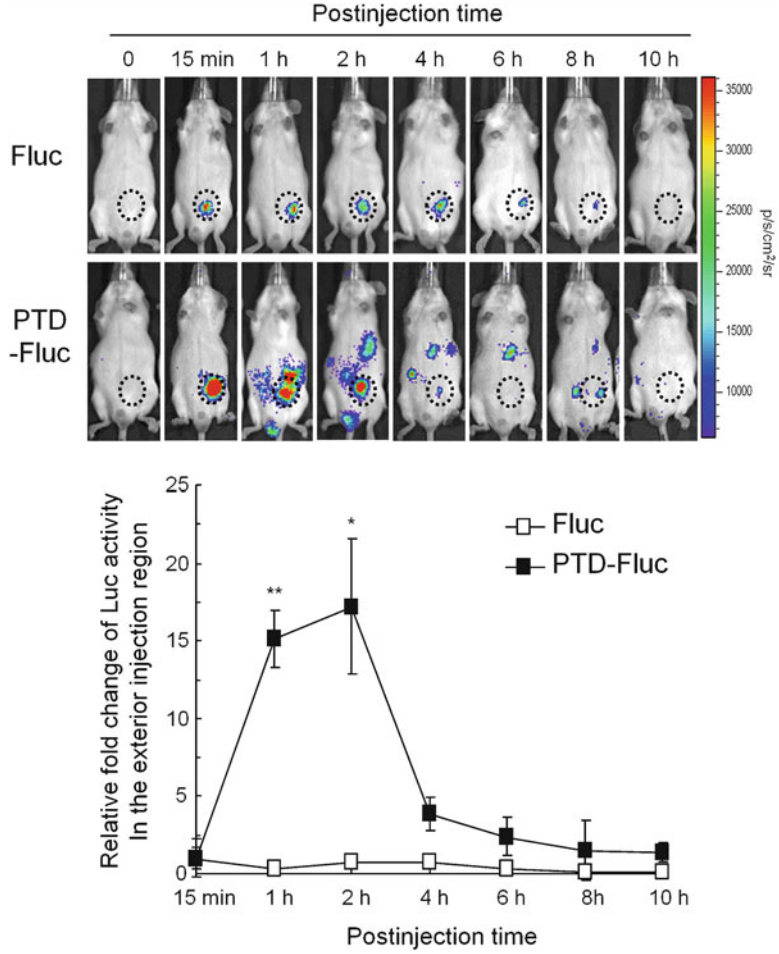

Fig. 3 In vivo real-time imaging and quantification of PTD-Fluc transduction. ICR mice were injected intraperitoneally once with 10 μM/kg PTD-Fluc or Fluc. Next, the mice were given an injection of D-luciferin at 30 mg/kg after 15 min, 1, 2, 4, 6, 8, and 10 h. Anesthesia and BLI were performed as in Subheading 3.3, steps 4 and 5. Representative images from the same mouse in the Fluc-treated group ($n=4$) and PTD-Fluc-treated group ($n=6$) are displayed as pseudocolor images of peak bioluminescence, with variations in color representing the light intensity at a given location (*upper panel*). *Red* represents the most intense light emission, while *blue* corresponds to the weakest signal. The *colored bar* indicates the bioluminescence signal intensity (p/s/cm^2/sr). The mice were imaged with an integration time of 3 min at a binning of 10. The *dotted circle* indicates the region of injection. Quantification of in vivo tracking by measuring the exterior luciferase activity (*lower panel*). Exterior activity was calculated by subtracting the activity in the *dotted circle* from the total activity. *Error bars* represent the SEM. *Significantly different from the Fluc-injected control group ($p<0.05$); **$p<0.01$ (Reproduced with permission from ref. 8)

13. The mice should be anesthetized with 3.0% isoflurane delivered in 100% oxygen in a gas anesthesia induction chamber prior to imaging. Once the mice have been anesthetized, they should be moved onto the imaging stage; anesthesia should be maintained with 1.0–3.0% isoflurane in 100% oxygen inside the IVIS-200.
14. One of the advantages of BLI is that a mouse can be imaged repetitively (e.g., before and after drug administration). This strategy allows each mouse to serve as its own control, which reduces experimental variation.

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