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Noninvasive In Vivo Monitoring of Extracellular Vesicles

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Abstract

Extracellular vesicles (EVs) including exosomes and microvesicles are nanometer-sized vesicles released by cells to deliver lipids, cellular proteins, mRNAs, and noncoding RNAs, thereby facilitating intercellular communication without direct cell-to-cell contacts. Due to their nanoscale size, EVs have been visualized under microscopy in vitro. We here describe a strategy to label EVs with *Gaussia* luciferase for noninvasive bioluminescence imaging and monitoring of systemically administered EVs in vivo.

Keywords

Extracellular vesicles; Microvesicles; Exosomes; *Gaussia* luciferase; Bioluminescence; Coelenterazine

1 Introduction

Cells release various types of extracellular vesicles (EVs) ranging from 40 to 4,000 nm in diameter, which are capable of transporting lipids, DNAs, RNAs, and proteins to recipient cells. These vesicles include exosomes (40–100 nm), microvesicles (100–1,000 nm), and apoptotic bodies (50–4,000 nm) [1, 2]. Since EVs are diffuse within the extracellular matrix and taken up by cells, they can mediate intercellular communication between neighboring cells, as well as cells at distal sites [3–9]. Importantly, cancer cells were found to produce an abundant amount of EVs containing a select subset of cellular proteins, mRNAs and noncoding RNAs, thereby modulating normal cells in their microenvironment to promote angiogenesis and tumor progression [10–20]. On the other hand, since EVs are small and poorly immunogenic, several recent studies have engineered EVs to shield therapeutic genes/drugs for targeted delivery to tumors, even overcoming biological barriers such as the blood–brain barrier [21–26]. Until recently, EVs have only been visualized under high-resolution microscopy due to their nanometer size, and localizing EVs in vivo has been largely unachievable [27].

Photinus pyralis luciferase (firefly; Fluc), *Renilla reniformis* luciferase (sea pansy; Rluc), and *Gaussia princeps* luciferase (marine copepod; Gluc) are among the most commonly used luciferases in biomedical research [28]. Unlike reporters conjugated to fluorescent proteins, such as CD63-GFP for EVs [19, 29], luciferase-based reporters do not need an excitation source for their subsequent light emission. Instead, luciferases emit bioluminescence via conversion of their respective substrate with either ATP and Mg^{2+} (Fluc; *D*-luciferin), or oxygen alone (Rluc and Gluc; coelenterazine). This makes luciferase an ideal reporter for in vivo studies where specialized equipment for excitation of reporter proteins is not required.

Gluc is unique from other luciferases such that it is naturally secreted and capable of emitting flash bioluminescence (480 nm peak) that is over 1,000-fold more sensitive than Rluc and Fluc [30]. Combining these features and the fact that Gluc only requires oxygen as its cofactor for bioluminescence reaction, we engineered a membrane-bound (mb) variant of Gluc fused to a biotin acceptor peptide (BAP), termed mbGluc-BAP [31], for EV labeling. By overexpressing mbGluc-BAP in human embryonic kidney (HEK) 293T cells followed by EV isolation, one can successfully track systemically administered EVs in athymic nude mice via in vivo bioluminescence imaging or by ex vivo analysis of tissues.

2 Materials

2.1 EV Reporter Construct and Donor Cells

1. EV reporter construct: pCSCW2-mbGluc-BAP-IRES-GFP, a lentivirus vector encoding Gluc fused to the transmembrane domain of platelet-derived growth factor receptor (PDGFR) and a biotin acceptor protein (mbGluc-BAP) as well as GFP, separated by an internal ribosomal entry site (IRES), all under the control of the constitutively active cytomegalovirus (CMV) promoter [32].
2. EV donor cells: Human embryonic kidney 293T (293T) cells obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). These cells are cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C and 5 % CO₂.
3. Filter: 0.22 µm polyethersulfone filter (EMD Millipore, Billerica, MA, USA).
4. EV-depleted FBS: Using ultracentrifugation equipment described (*see* Subheading 2.2, **items 1–3**), centrifuge FBS at $100,000 \times g$ for at least 16 h at 4 °C. Collect supernatant (EV-depleted FBS) and filter it through a 0.22 µm filter to further minimize EV contamination of the FBS. Aliquot EV-depleted FBS into 50 ml canonical tubes and store at –80 °C.
5. EV-isolation medium: Supplement DMEM with prepared 10 % EV-depleted FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin.
6. Polybrene: 10 mg/ml in tissue culture grade water.
7. Epifluorescence microscope capable of detecting GFP expression.

2.2 EV Isolation Components

1. Ultracentrifuge: Optima L-90K (Beckman-Coulter, Indianapolis, IN, USA).
2. Rotor: Type 70Ti rotor (Beckman-Coulter).
3. Ultracentrifuge tubes, seal former, tube sealer: Quick-Seal® tubes (Cat#: 342413, Beckman-Coulter), seal former and a tube sealer (Beckman-Coulter).
4. Filter: 0.22 µm polyethersulfone filter (EMD Millipore).
5. 60 ml syringes.
6. Double filtered phosphate buffered saline (PBS): 8 g/L NaCl, 0.2 g/L KCl, 1.78 g/L Na₂HPO₄·2 H₂O, 0.27 g/L KH₂PO₄, adjust to pH 7.4 and filter twice through a 0.22 µm filter.

2.3 Gluc Activity Assay

1. A luminometer such as the Dynex MLX Microtiter Plate Luminometer (Vienna, VA, USA).
2. 96-well white or black microtiter plates.
3. Coelenterazine (CTZ), the Gluc substrate: Reconstitute CTZ to a stock concentration of 5 mg/ml with methanol pre-supplemented with a few drops of HCl to minimize CTZ oxidation and precipitation. Aliquot CTZ and store at -20 °C. Pre-warm CTZ to room temperature before use and protect from light.

2.4 In Vivo Bioluminescence Imaging

1. Animals: Immunodeficient athymic nude mice or any other mouse strain of choice.
2. An optical imaging system such as the IVIS® Spectrum connected to XGI-8 Anesthesia System (Perkin-Elmer, Waltham, MA, USA).
3. Isoflurane (Baxter, Deerfield, IL, USA).
4. XGI-8 Anesthesia System (Perkin-Elmer).
5. Sterile PBS: Autoclaved or 0.22 µm filtered PBS.
6. Coelenterazine: prepare as described in **item 3** in Subheading 2.3. Alternatively, for more stable Gluc signal during imaging, prepare fresh CTZ before each injection according to “Inject-A-Lume” kit manual (Nanolight, Pinetop, AZ, USA).
7. 1.5 ml microcentrifuge tubes.
8. Insulin syringe needles: 29½ gauge, 0.3 ml.
9. Heating device: 43 °C water in a 500 ml beaker to induce vasodilation of tail veins.

3 Methods

3.1 Generate EV Donor Cells

1. Seed 0.5×10^6 HEK 293T cells per well in fresh culturing medium in a 6-well tissue culture plate (34.8 mm diameter).
2. When 60–70 % confluence is reached, transduce the cells with lentiviral vectors encoding mbGluc-BAP-IRES-GFP in 3 ml medium supplemented with 1 μ l polybrene (10 mg/ml) for each well (*see Note 1*).
3. 48 h post-transduction, examine GFP reporter expression under an epifluorescence microscope to determine transduction efficiency. At least 80–90 % of cells should be transduced to generate sufficient EVs labeled with mbGluc-BAP (*see Note 2*).

3.2 EV Isolation and Gluc Activity Assay

1. Add 2×10^6 of 293T cells expressing mbGluc-BAP per 150 mm plate in fresh medium.
2. When the cells reach approximately 50 % confluence, gently aspirate the growth medium and replace with 20 ml of fresh EV-isolation medium.
3. Incubate the cells for another 48 h at 37 °C, 5 % CO₂ to generate conditioned medium containing EVs.
4. Collect conditioned medium in 50 ml canonical tubes and centrifuge at $300 \times g$ for 10 min at 4 °C to remove cell debris.
5. Collect supernatant in separate 50 ml canonical tubes and centrifuge at $2,000 \times g$ for 10 min at 4 °C to remove additional cell debris.
6. Prepare a 60 ml syringe attached to a 0.22 μ m filter followed by a 16 gauge needle.
7. Insert the assembled filtering syringe into a Quick-Seal® tube and filter the supernatant through the syringe (*see Note 3*).
8. Weigh all supernatant-containing Quick-Seal® tubes and adjust to equal weight by removing excess supernatant as needed and seal the tubes with a sealer. Mark one side of each tube with a marker pen for later orientation purposes (Fig. 1a).
9. In a Type 70 Ti rotor, place tubes with equal weight diagonally opposite to one another for balance. Note orientation of the marked tubes since EV pellets will

¹Determine the amount of lentivirus required for transduction based on multiplicity of infection (MOI). 293T cells are highly prone to lentiviral infection, and a MOI of 5 transducing units per cell is recommended. Alternatively, cells can be transfected with plasmids encoding mbGluc-BAP-IRES-GFP for transient expression.

²Transduced cells can be further sorted for GFP expression by fluorescence-activated cell sorting (FACS) to ensure a high percentage of mbGluc-BAP positive cell population. The transduced/FACS sorted cells can also be frozen for later experiments. Low percentage of positive cells will yield a low amount of mbGluc-BAP-labeled EVs, making them unsuitable for subsequent imaging experiments.

³Same filtering syringe can be reused between filtrations. Detach syringe from the filter first before removing the plunger between filtrations—this helps in maintaining filter integrity. When the syringe becomes difficult to depress, replace the filter.

be collected from the side away from center of the rotor (Fig. 1b). Apply Quick-Seal[®] spacers and lid for the rotor, centrifuge at $100,000 \times g$ for 80 min at 4 °C.

10. Gently remove sealed tubes from the rotor with minimal agitation (*see* Note 4).
11. Locate EV pellet at lower side of the tube wall—the side away from the center of the rotor (Fig. 1c).
12. While holding the tube firmly to prevent sudden movement of the tube, carefully cut the tube stem with a razor blade to create an opening (Fig. 1c, 1st cut).
13. Using a Pasteur pipette, gently aspirate most of the supernatant without disturbing the pellet. Focus on the pellet during this procedure to ensure that the pellet is not lost.
14. Cut the tube from the midsection with a razor blade or a pair of scissor (Fig. 1c, 2nd cut).
15. Position the tube upright for 2 min to allow remaining supernatant from the wall to settle to the bottom.
16. Aspirate remaining supernatant with a pipette without disturbing the pellet (*see* Note 5). The pellet may or may not be readily visible at this stage (Fig. 1d).
17. Using a 200 µl tip, resuspend EV pellets by pipetting up and down in 100 µl of double filtered PBS per tube.
18. Situate the tubes on ice to keep original pellet spot covered in PBS for 30 min. This step allows better EV recovery from the tubes.
19. Resuspend the pellet by trituration with a pipette and transfer the EV sample into a 1.5 ml microfuge tube.
20. Determine protein concentration of the EV sample using preferred protein quantification methods (*see* Note 6).
21. Store the samples at −80 °C.

3.3 Gluc Assay on Isolated EVs

1. Dilute CTZ stock (5 mg/ml) in PBS at 1:1,000 and protect from light.
2. Dilute isolated EVs in PBS at 1:100. Add 10 µl of diluted EVs per well in triplicates into a 96-well white microtiter plate.
3. Set the luminometer to dispense 50 µl CTZ and acquire the signal for 10 s per well, with 2 s integration time.

⁴EV pellets detach easily from tube wall when the tubes undergo sudden movement. Use extra caution when handling the tubes from this step and on.

⁵Try to reduce remaining supernatant as much as possible by hand pipetting when approximately 500 µl supernatant is left. Residual supernatant will result in a significant increase in protein concentration (i.e., FBS contamination) and affect Gluc signal in subsequent assays.

⁶Dilute the EV samples 1:5, 1:10, and 1:20 in PBS to estimate EV protein concentration. An average EV concentration of 0.5–1 µg/µl is expected.

4. A 100,000–200,000-fold increase in signal should be observed in mbGluc-BAP EVs when compared to negative or blank controls, thereby validating successful EV labeling (*see* Note 7).

3.4 In Vivo EV Bioluminescence Imaging

1. For each animal, prepare 100 µg of EVs in a total volume of 200 µl with PBS in a 1.5 ml microfuge tube.
2. Place an animal in a restrainer. Warm the tail with 43 °C water in a beaker for a few minutes until tail veins are readily visible for injection.
3. Inject the EV mixture into the tail vein by using an insulin syringe needle (*see* Note 8).
4. For each animal to be imaged, prepare Gluc substrate by adding 20 µl CTZ (5 mg/ml) to 130 µl of PBS in a 1.5 ml microfuge tube, and mix well by pipetting up and down (*see* Note 9).
5. 15–30 min following EV injection, anesthetize the animal by isoflurane (1–1.5 % at 2 L/min) using the XGI-8 Anesthesia System.
6. Inject 150 µl CTZ mixture via tail or retro-orbital vein with an insulin syringe needle (*see* Note 10).
7. Immediately place the animal in the IVIS[®] Spectrum connected to the XGI-8 Anesthesia System for continued general anesthesia.
8. Immediately following CTZ administration, image the animal for bioluminescence signal by IVIS[®] Spectrum (*see* Note 11).

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⁷EVs derived from parental 293T cells exhibit no Gluc activity and can therefore be used as a negative control.

⁸EVs have a short half-life of <30 min in mice. Therefore timing following EV administration is critical to minimize a loss of EV signal during imaging.

⁹When “Inject-A-Lume” kit (Nanolight) is used, apply 150 µl of “Fuel-Inject” diluent to NanoFuel[™] vial, and mix well until solution becomes clear.

¹⁰When using the “Inject-A-Lume” kit, use only 70–75 µl of the CTZ mixture per 25 g mouse. Do not use more than 80 µl of the CTZ mixture from “Inject-A-Lume” kit per 25 g mouse since it is lethal. Timing is absolutely critical following CTZ administration to ensure optimal capture of Gluc signal from mbGluc-BAP labeled EVs. Highest signal can be detected at 1 min post-CTZ injection followed by a rapid loss of signal within 5 min thereafter.

¹¹Allow 1 min post-CTZ injection to achieve highest Gluc signal intensity. Due to the short half-life of both EVs and CTZ, it is recommended to prepare one animal each for either dorsal (Fig. 2a) or ventral side (Fig. 2b) imaging for optimal imaging signal.

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Fig. 1.

EV isolation procedure. (a) Following conditioned medium collection into ultracentrifuge tubes, place a seal former onto each tube stem. Seal the tubes with a tube sealer by applying gentle, downward pressure until the seal former rests on top of the tube shoulder. Do not press the seal former beyond the tube shoulder since it will result in leakage during centrifugation. Using a permanent marker, mark one side of the tubes (*both top and bottom*) for orientation purposes. (b) Place sealed tubes with equal weight opposite to one another in the rotor. Make sure all pre-marked tubes have the same orientation for later EV isolation purposes. Secure the tubes with metal spacers, and firmly seal the rotor with the rotor lid. (c) Location of EV pellet and suggested cut sites for staged aspiration to remove supernatant. (d) Intact EV pellet after the supernatant is removed

Fig. 2.

In vivo bioluminescence imaging of EVs. **(a)** Dorsal side of nude mice administered with either PBS (control) [changed order as read from *left to right*] or mbGluc-BAP EVs. The spleen of mbGluc-BAP injected mouse exhibited a prominent bioluminescence signal when compared to the control at 30 min post-EV injection. **(b)** Ventral side of nude mice showing control and mbGluc-BAP EV localizing predominately to the liver and possibly spleen at 30 min post-EV injection. Although not readily visible here, PBS-injected mouse typically exhibited low level bioluminescence from oxidation of CTZ in the liver