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***In vivo* Bioluminescence Imaging of Luciferase-labeled Cancer Cells**

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[Abstract] Over the past decade, *in vivo* bioluminescent imaging has emerged as a non-invasive and sensitive tool for studying ongoing biological processes within living organisms (Contag *et al.*, 1997; Contag *et al.*, 1998). Based on the detection and quantitation of the photons produced by the oxidation of luciferin by luciferase enzymes (Harvey, 1927), this technique has proved to be particularly useful in analyzing cancerous cells and monitoring tumor growth (Edinger *et al.*, 1999; Sweeney *et al.*, 1999; Vidal *et al.*, 2015), providing a cost-effective insight into how the disease progresses *in vivo*, without the need of serial sacrifice of animals. This protocol describes in detail the procedure of obtaining luciferase-tagged tumors in immunocompromised mice that can be studied by bioluminescent imaging through the use of an IVIS Spectrum imager.

Materials and Reagents

1. Falcon™ Standard 10 cm² Tissue Culture Dishes (Corning, catalog number: 353003)
2. Syringe Filter 0.45 µm strainer (Corning, catalog number: CLS431225)
3. 22Rv1 prostate cancer cells (ATCC, catalog number: CRL-2505)
4. NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG™) mice (Jackson Lab, catalog number: 00557)
5. Phoenix-Ampho cells (ATCC, catalog number: CRL-3213)
6. pLenti CMV Puro LUC (w168-1) (Addgene, catalog number: 17477)
7. Helper pCMV-VSV-G (Addgene, catalog number: 8454) and pMD2.G (Addgene, catalog number: 12259)
8. One Shot Stbl3 Competent *E. coli* (Thermo Fisher Scientific, Invitrogen™, catalog number: C7373-03)
9. LB Broth Medium (Thermo Fisher Scientific, BioReagents™, catalog number: BP1426-2)
10. Ampicillin (Sigma-Aldrich, catalog number: A9393)
11. QIAprep Spin Miniprep Kit (QIAGEN, catalog number: 27106)
12. RPMI 1640 Medium (Thermo Fisher Scientific, Gibco™, catalog number: 11875119)
13. Dulbecco's Phosphate-Buffered Saline (DPBS) 1x (Corning, catalog number: 21-031)
14. jetPEI® DNA Transfection Agent (Polyplus-transfection, catalog number: 101-10N)
15. Penicillin-Streptomycin antibiotic (PenStrep) (Thermo Fisher Scientific, Gibco™, catalog number: 15140122)

16. Polybrene® (Santa Cruz Biotechnology, catalog number: 134220)
17. Isoflurane (Baxter, catalog number: 1001936040)
18. Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Corning, catalog number: 354230)
19. D-luciferin (PerkinElmer, catalog number: 770504)

Equipment

1. Thermo-block (Thermo Fisher Scientific, model: Isotemp™ Digital and Analog Dry Bath Incubator)
2. Shaker incubator (Eppendorf AG, New Brunswick Scientific, model: Innova 44R Incubator/Shaker)
3. Table-top microcentrifuge (Eppendorf AG, model: centrifuge 5415R)
4. Spectrophotometer (Thermo Fisher Scientific, model: SPECTRONIC™ 200 Spectrophotometer)
5. Incubator (Thermo Fisher Scientific, model: Heracell 150i)
6. Vortex (Scientific Industries, model: Vortex Genie 2)
7. IVIS Spectrum imager (PerkinElmer, model: IVIS Spectrum Preclinical In Vivo Imaging System)

Software

1. Caliper LifeScience Living Image® *in vivo* imaging software (PerkinElmer, catalog number: 128110) (Lumina/Kinetic/XR/100, Living Image V4.1)

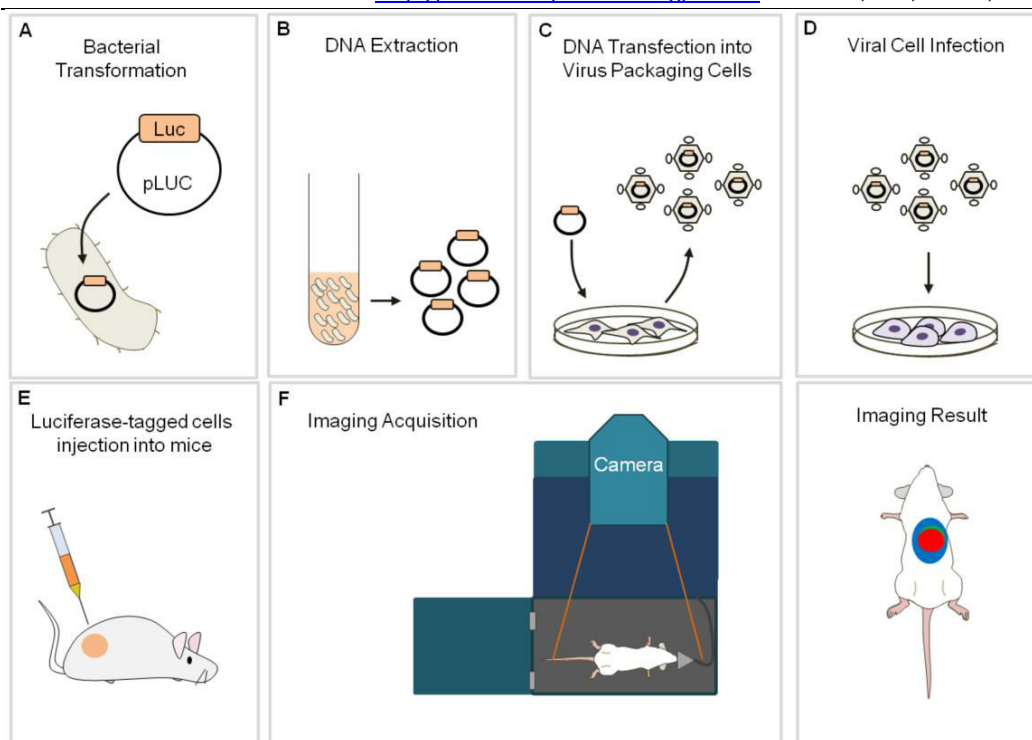


Figure 1. Schematic representation of the steps required to successfully tag cells with luciferase and perform *in vivo* imaging

Procedure

A. Bacterial transformation

Artificially constructed plasmids are the most commonly used vectors for introducing foreign DNA into bacterial cells. In this protocol, we used the commercial plasmid Lenti CMV Puro LUC, which contains the firefly luciferase gene, and was transformed into a culture of Stbl3 competent cells.

1. Transfer 50 μ l of Stbl3 competent cells in a 1.5 ml Eppendorf tube placed on ice .
2. Add between 100 ng and 500 ng of plasmid into tube and resuspend with Stbl3 competent cells (pipet to resuspend, do not use vortex).
3. Incubate tube on ice for 30 min, gently resuspend every 10 min.
4. Place tube in a thermo-block for exactly 45 sec at 42 °C, and immediately put back into ice for 2 min.
5. Add 800 μ l of LB-Medium with no antibiotic into tube, and gently resuspend the sample with a pipet (do not use vortex).
6. Grow up bacteria in a shaker incubator at 37 °C speed 200 rpm for 2-3 h.
7. Plate 100 μ l of transformation mixture on LB-agar plates with 100 μ g/ml of ampicillin.
8. Place plate in incubator at 37 °C overnight.

9. Pick a single colony and inoculate a flask containing 50 ml of LB-Medium + 100 µg/ml of ampicillin.
10. Incubate overnight at 37 °C in shaker incubator speed 200 rpm.

B. DNA extraction

There are several methods that can be used to purify plasmid DNA depending on the size of the bacterial culture and its corresponding plasmid yield, and a handful of kits available from varying manufacturers to do it. Here, we perform a miniprep (miniprep) using the QIAprep Spin Miniprep Kit, which allows the isolation of a small-scaled sample of up to 20 µg plasmid DNA from the transformed bacteria. Briefly, in this protocol the isolation of DNA from bacteria relies upon the use of columns and centrifugation steps in which DNA is sheared, extracted, and precipitated. Follow protocol of manufacturer.

C. Transfection of DNA into viral packaging cells

Depending on the plasmid, several packaging cells can be used to generate the desired virus. In this protocol we used Phoenix-Ampho cells, which are a second-generation retrovirus producer cell line that is also capable of a long-term stable production of lentiviruses when helper DNA (CMV and DMV) is present. Helper DNA encodes viral genes that increase viral replication and packaging.

1. Seed 3×10^6 Phoenix-Ampho cells in 8 ml of RPMI supplemented with 10% FBS and 1% PenStrep in a 10 cm² culture plate 24 h before transfection.
2. In a first 1.5 ml Eppendorf tube, add 6 µg of DNA, 3 µg of each helper (CMV and DMV) and fill up to 250 µl of 150 mM NaCl (per sample to transfect).
3. In a second 1.5 ml tube add 24 µl of jetPEI and 226 µl of 150 mM NaCl (per sample to transfect).
4. Vortex the tubes for 10 sec.
5. Add the Jetpei + NaCl into the Eppendorf tube with the DNA and vortex for 10 sec.
6. Incubate mixture for 15 min at room temperature.
7. Remove media of plate and add 9.5 ml of fresh RPMI supplemented with 10% FBS and 1% PenStrep to Phoenix-Ampho cells (see step C1).
8. Add 500 µl drop by drop, of the mixture (step C5) to the plate and incubate overnight at 37 °C.
9. After 24 h change the medium and add fresh RPMI supplemented with 10% FBS and 1% PenStrep.
10. After 48 h collect and filter the medium with a 0.45 µm strainer into a 50 ml Falcon tube.

Note: Viral concentration can be measured after this step by serial dilution to obtain quantitative information. Various techniques can be used to quantify virus concentration which include traditional methods (e.g. plaque assay) and commercial kits (e.g. ELISA or Q-PCR).

D. Viral particle cell infection

The viral particles produced in the previous step are used to infect cells. Virus will infect cells which hold a receptor that the virus can bind to. Therefore, the efficiency of viral infection will greatly depend on the cell type. In this protocol, we infected 70% confluent 22Rv1 prostate cancer cells growing in 10 cm² culture dishes with 10 ml of media.

1. Add Polybrene to cultured cells to a final concentration of 10 µg/ml.

Note: Polybrene is an efficient infection reagent used to introduce viral vectors into mammalian cells. Polybrene acts by neutralizing the charge repulsion between virions and the cell surface (Davis et al., 2004).

2. Using a 5 ml pipet, add 4 ml of filtered viral particles to cultured cells.

3. Centrifuge 10 cm² plate at 250 x g for 5 min.

Note: This step helps the physical contact of viruses with surface of cells increasing infection efficiency.

4. Place cells in 37 °C, 5% CO₂ incubator for 18-24 h.

Note: Infection efficiency can be affected by incubation time. Troubleshooting using different incubation times can be performed to identify most efficient incubation time for a specific cell type.

5. Aspirate the medium and add 10 ml of fresh RPMI supplemented with 10% FBS and 1% PenStrep. Incubate at 37 °C for 24 h.

6. Select infected 22Rv1 prostate cancer cells by adding Puromycin at a final concentration of 2 µg/ml to culture media.

Note: Puromycin concentration will depend on cell type.

7. Replace medium containing Puromycin every 3 days. Cells that have incorporated the pLenti CMV Puro LUC will grow up under antibiotic selection conditions.

8. Check that the cells have incorporated the luciferase plasmid.

Note: Multiple methods can be used to test successful luciferase. Examples are detection of luciferase by PCR or measuring luciferase activity upon D-Luciferin exposure (see Figure 2).

E. Injection of luciferase-tagged cells into immunocompromised mice

In this protocol we performed subcutaneous injection of luciferase-tagged tumor cells. However, alternative injection sites such as the mouse prostate (orthotopic), which promotes a prostate microenvironment, and subrenal capsule injection, which enhances successful engraftment and preserves tumor heterogeneity, can be performed based on investigator preference (Hidalgo et al., 2014; Siolas et al., 2013).

Note: Conduct all animal procedures in compliance with protocols approved by the institutional animal care committee. This protocol has been conducted at our institution under a specific Animal Care Committee in accordance and compliance with all relevant regulatory and institutional agencies, regulations and guidelines.

1. Harvest attached cells by removing culture media, washing cells with 10 ml 1x DBPS two times and trypsinizing cells using 2 ml 0.05% Trypsin. Recover 2 ml trypsinized cells to 15 ml tube containing 4 ml RPMI supplemented with 10% FBS and 1% PenStep.
2. Count cells using a hemocytometer.
3. Mix 10^6 cells suspended in 100 μ l of RPMI supplemented with 100 μ l of extracellular matrix (1:1 ratio) and place on ice.
4. Anesthetize mice prior to subcutaneous injection in a chamber supplying 5% (v/v) inhaled isoflurane in 1 L/min of oxygen.
5. Using a 25 gauge needle and a 1 ml syringe, inject 250 μ l of cell and extracellular matrix suspension subcutaneously into the flanks of immunodeficient NSG mice.

F. Bioluminescence imaging

The IVIS Spectrum imager expresses the bioluminescent signal in photons per second and displays it as an intensity map. The luminescence, which is the consequence of the photon flux emitted by the luciferase-expressing cells, directly correlates to the size of the tumor and can be measured at the site of injection using a region of interest (ROI) tool. The optical emission image can be adjusted to provide optimal contrast and resolution without affecting quantitation using the Living Image[®] *in vivo* imaging software.

1. Prepare a fresh stock solution of D-Luciferin at 15 mg/ml in 1x DPBS.
2. Anesthetize the mice using isoflurane vaporizer and place them inside the camera box of the IVIS Spectrum imager.
3. Run a background image of the mice.
4. Place the mice in dorsal recumbency (abdomen face up) and inject 150 mg/kg of D-Luciferin (from step F1) intra-peritoneally (ip) 5 min before imaging. The preferred region of injection is the mouse lower right quadrant of the abdominal region.
5. Taking into account the time dependent D-Luciferin uptake by tumor cells, run sequential images of the mice every 2 min until luminescence saturation is reached.

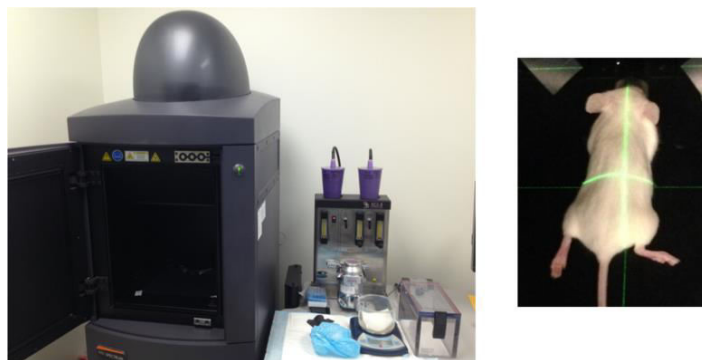


Figure 2. Image illustrates the IVIS spectrum imager and a mouse placed inside the camera box

Representative data

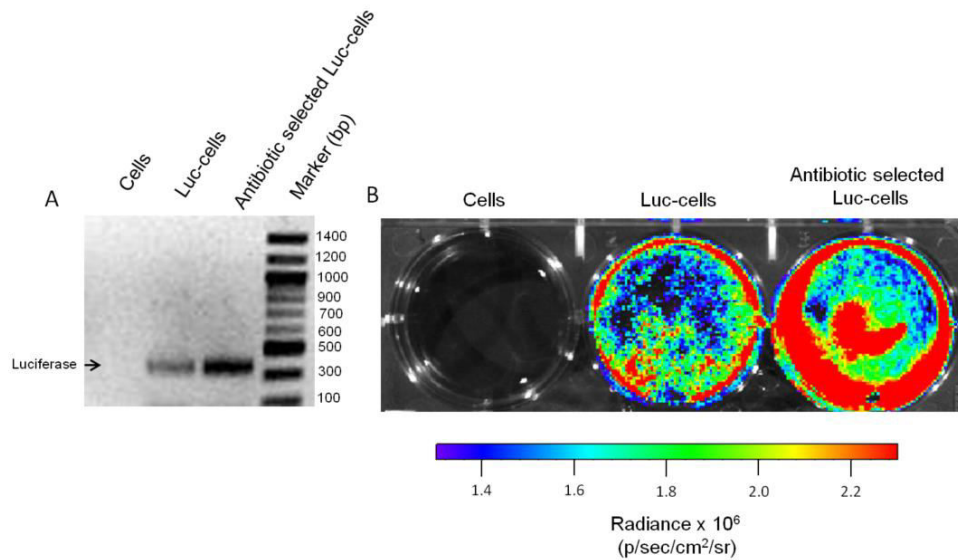


Figure 3. Luciferase detection in cells. Multiple methods can be used for testing the success of cell infection, which include but are not limited to: A. Genetic detection of luciferase gene by PCR and/or B. Testing luciferase activity.



Figure 4. Subcutaneous injection of cells in mice. A. Lift the skin over the back of the neck to make a tent. B. Insert the needle at the tent base, holding it parallel to the animal's body to avoid puncturing underlying structures. C. Aspirate to create a light vacuum and ensure that the needle has not entered a blood vessel. Slowly inject the cell and extracellular matrix suspension.

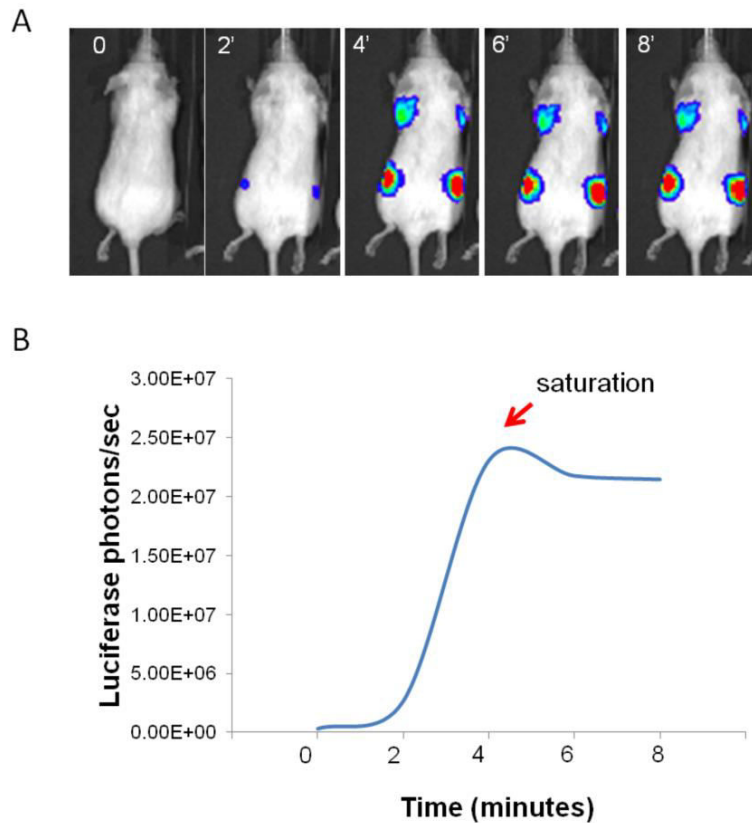


Figure 5. Bioluminescence *in vivo* imaging. A. Representative luciferase time course (every 2 min) imaging of mice bearing subcutaneous tumors injected with luciferin. B. Graph illustrates the bioluminescence saturation of the whole mouse (displayed in A.) obtained until time point 8 min, when the experiment was decided to be concluded.

Acknowledgments

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