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In Vivo Bioluminescence Imaging of Tumor Progression in the Lewis Lung Carcinoma Orthotopic Mouse Model – A Comparison Between the Tail Vein Injection and Intranasal Instillation Methods

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ABSTRACT

Metastasis remains a leading cause of cancer-related mortality, yet its study has been constrained by the lack of reliable animal models that faithfully replicate this complex process. Syngeneic models for studying lung cancer (LC) metastasis are limited, with the Lewis lung carcinoma (LLC) model being the most commonly employed. The conventional LLC orthotopic model involves injecting LLC cells intravenously (i.v.) via the tail vein into syngeneic C57BL/6 mice. However, this model has significant drawbacks, such as tumor development in multiple anatomical sites, incomplete lung tumor penetrance, and challenges in monitoring lung tumor growth. This article highlights the advantages of using luciferase-expressing LLC cells combined with bioluminescence imaging (BLI) to quantify tumor progression in live animals. We demonstrate that both white- and black-furred C57BL/6 mice can be used for BLI. Finally, we propose that intranasal (i.n.) instillation of LLC cells offers a valuable alternative to the traditional i.v. tail vein injection method, particularly for its simplicity and improved reproducibility. Although the LLC i.n. model does not recapitulate the metastasis process via the blood vascular route, it is an effective model for studying tumor seeding within the lungs and is particularly useful for analyzing the impact of the lung microenvironment on tumor initiation and progression.

Basic Protocol 1: LLC intravenous i.v. injection method
Support Protocol 1: *In vivo bioluminescence imaging (BLI)*
Basic Protocol 2: LLC intranasal (i.n.) instillation method

KEYWORDS

In vivo models, Lung cancer, Metastasis, Lewis lung carcinoma, Bioluminescence imaging

INTRODUCTION

Lung cancer (LC) is the leading cause of cancer death worldwide (Bray et al., 2018). Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all the LC cases (Herbst et al., 2018), and therefore represents the LC subtype where reliable animal models are the most needed. Among the different categories of animal models of LC, syngeneic murine models entail the injection of immunologically compatible cancer cells into immunocompetent mice. As a result, tumor growth, tumor microenvironment, and immune responses can be evaluated within the natural organ environment, more closely mimicking human pathophysiological conditions. However, the availability of syngeneic models to study LC metastasis is essentially limited to the Lewis lung carcinoma model. The Lewis lung tumor originated spontaneously as a carcinoma of the lung of a C57BL/6 mouse and was discovered by Dr. Margaret R. Lewis of the Wistar Institute in 1951 (Sugiura & Stock, 1955). The highly tumorigenic but poorly immunogenic LL/2 or LLC1 (hereafter called LLC) cell line was established in 1980 by Bertram et al., (Bertram & Janik, 1980; Mosely et al., 2017; Lechner et al., 2013). By employing whole-exome sequencing, a recent study reveals that LLC cells originated from a male mouse belonging to C57BL/6L (a transitional strain between C57BL/6J and C57BL/6N), which is genotypically very close to the C57BL/6J genome (He et al., 2024). The authors hypothesized that the high mutation burden observed in LLC cells is likely due to the accumulation of mutations during long-term laboratory passaging and the currently available LLC cells are a hypermutated version of the original LLC cells isolated from a C57BL/6L mouse. LLC cells have several of the major mutations found in human NSCLC, such as *Kras*^{G12C}, *Trp53*, and *Cdkn2a* mutations, and can form orthotopic lung tumors in immunocompetent hosts. Therefore, the LLC model represents a valuable mouse model closely resembling human NSCLC (Justilien & Fields, 2013).

LLC cells are typically injected intravenously (i.v.) in the lateral tail vein of C57BL/6 mice, which frequently, but not consistently, generates lung metastases after a few weeks, depending on the number of cells injected (Shrestha et al., 2019; Fidler, 1970). One common problem with this approach is the multiorgan dissemination of the cancer cells (Miki et al., 2000). In addition, the i.v. delivery route of tumor cells can result in sudden death caused by thromboembolism (Schuh, 2004). This has led to criticism of the tail vein injection method to model lung cancer or breast cancer metastasis (Shrestha et al., 2019; Rashid et al., 2013). Due to the high variability in lung tumor incidence and differing disease kinetics between mice, the conventional LLC i.v. model often requires a relatively high number of mice to achieve statistical significance between experimental groups. Therefore, if the lungs are the only targeted organ, more directed modes of cancer cell administration can reduce the growth of unwanted metastases in other tissues, even though they do not employ the vascular route from the primary tumor to the metastatic site.

Recently, alternatives to the i.v. tail vein approach have been developed, utilizing various routes of administration for LLC cells ranging from invasive (e.g., intrapulmonary and intratracheal injections) (Weiss et al., 2015; Tanaka et al., 2023) to non-invasive (e.g., intranasal instillation) (Tanaka et al., 2023) techniques. An advantage of intrapulmonary implantation, by directly puncturing through the intercostal space to the lung parenchyma, is that it avoids thoracotomy. Still, the method can lead to pneumothorax, intrathoracic hemorrhage, and hemoptysis, which increase the risk of death during or after cancer cell inoculation. The intranasal (i.n.) route is an effective, non-invasive, and widely used technique employed for the delivery of various substances to mice including cells, drugs, gene therapy or immunotherapy, allergens, and pathogens to the upper and lower airways (Chau et al., 2018; Southam et al., 2002). In addition to its simplicity, the i.n. route of administration offers the advantage of delivering the cells under mild anesthesia without the need for any surgical operations. LLC cells administered through this route form tumors primarily around bronchioles and alveoli thereby mimicking some of the characteristics of human NSCLC (Tanaka et al., 2023).

In this article, Basic Protocol 1 describes the conventional i.v. injection method using either non-labeled or luciferase-expressing LLC cells. Support Protocol 1 describes how to monitor tumor growth in live animals by bioluminescence imaging (BLI) using an *in vivo* imaging system (IVIS). Finally, Basic Protocol 2 outlines the alternative LLC i.n. instillation method.

CAUTION: All animal studies must be conducted using protocols approved by the Institutional Animal Care and Use Committee (IACUC), adhering to all local, institutional, and governmental regulations concerning the care and use of live laboratory animals.

BASIC PROTOCOL 1: LLC intravenous (i.v.) injection method

We first used the conventional tail vein approach and i.v. delivery of LLC cells in C57BL/6 albino mice. These mice carry a mutation in the tyrosinase gene, and their fur is white rather than black (Townsend et al., 1981; Hughes et al., 2007). The tail vein of albino mice is usually more visible than that of black-furred C57BL/6 mice, facilitating easier injections. However, black-furred C57BL/6 is the most used strain for knockout and transgenic mice, and these mice can also be used. The following protocol details the cell culture and i.v. injection procedure using non-labeled or luciferase-expressing LLC cells and C57BL/6 albino or black recipient mice.

Materials

Male or female (≥ 6-8-week-old) C57BL/6J albino mice (JAX, cat. no. 000058), C57BL/6J mice (JAX, cat. no. 000664), or any knockout or transgenic mouse on the C57BL/6 background can be used.
LLC-GLF (Hosoya et al., 2018) or other commercially available luciferase-expressing LLC cells (e.g., ATCC, cat. no. CRL-1642-LUC2). The original LLC cells (ATCC, cat. no. CRL-1642) can be used if *in vivo* BLI is not desired.
DMEM (Gibco, cat. no. 11965-092)
Fetal bovine serum (Life Technologies, cat. no. 26140079)
Penicillin/streptomycin (P/S) (Gibco, cat. no. 15140-122)
0.25% Trypsin/EDTA (Gibco, cat. no. 25200056)
1X PBS (Gibco, cat. no. 14190-144)
Trypan blue (Gibco, cat. no. 15250-061)
EDTA (Mediatech, cat. no. 46-034-CL)
1X HBSS (Gibco, cat. no. 14065-056)

75 cm^2 (T-75) cell culture flasks (Falcon, cat. no. 353136)
50 mL conical tube (BioPioneer, cat. no. CNT-50R)
70 µm cell strainer (FisherBrand, cat. no. 22-363-548)
Deltaphase Heating pad (Braintree, cat. no. 39DP)
Alcohol pads (Medline, cat. no. MDS090670)
U-100 insulin syringes with 29G needles (Fisher Scientific, cat. no. 14-841-32)
Mouse restrainer (Plas Labs, cat. no. 551-BSRR)

Cell culture and preparation

NOTE: Upon receiving LLC cells, expand them and freeze aliquots to ensure the consistent use of low-passage cells consistently throughout the study. Cells should be thawed approximately one week before the injection day and maintained in culture for at least two passages. Ideally, the last passage should be made 1-2 days prior to the injection day, and the cells should be in the logarithmic growth phase when they are harvested and injected. Maintaining consistency in cell preparation will ensure reproducibility across independent experiments.

1. Culture LLC cells in DMEM medium supplemented with 10% FBS and 1% P/S in the desired cell culture flask (e.g., T-75) according to the ATCC's recommendation.
   The number of flasks required is determined by the desired number of cells injected per mouse. Typically, between 0.25 to 1 x 10^6 cells per mouse are used, depending on the preferred tumor latency (see SUPPORT PROTOCOL 1 and Figure 3 for the kinetic of tumor growth obtained after tail vein injection of 0.25, 0.5, and 1 x 10^6 luciferase-expressing LLC cells in C57BL/6J albino mice).
2. After approximately two passages and at 60-70% confluency, wash cells with 1X PBS and detach cells using 0.25% Trypsin/EDTA (1mL for a T-75, <5 min at 37°C).
3. Wash cells with 1X PBS containing 2 mM EDTA and centrifuge at approximately 125 x g for 5 min at 4°C.
4. Resuspend cells in 1X HBSS without EDTA and pass through a 70 µm cell strainer.
5. Count the cell number using a hemocytometer and check the viability using trypan blue.
6. Ensure that > 90% of the cells are viable.
7. Resuspend cells in ice-cold sterile HBSS at the desired concentration (e.g., 5 x 10^6 cells/mL to inject 1 x 10^6 cells in 200 µL per mouse).
8. Keep the cells on ice before and during the i.v. injection to maintain cell viability and prevent clumping, which could lead to embolism after injection.

i.v. injection

NOTE: It is strongly advised that new users of this technique anesthetize the animals during the procedure until they have gained sufficient proficiency. Always refer to your institution's specific IACUC guidelines as recommendations for i.v. injections may differ depending on institutional protocols and regulations. We recommend using insulin syringes without dead volume (e.g., U-100 insulin syringes with 29G needles, Fisher Scientific, cat. no. 14-841-32) to ensure an accurate measurement of the volume delivered. The graduations on these syringes are typically in insulin units (IU) but can easily be converted to microliters by multiplying the IU by 10 (e.g., 20 IU equals 200 µL). The scale along the syringe is clear, allowing for accurate measurement, with each graduation representing 20 µL.

9. Prior to injection, warm the mice for 5-10 minutes to dilate the veins. Mice may be warmed by placing them in a specific warming box or by using a heating pad placed under the cage.

The Deltaphase Heating Pad (Braintree Scientific, Cat. No. 39DP) is a fixed-temperature heating pad, designed to maintain a steady surface temperature of approximately 37°C (98.6°F) without requiring any additional temperature setting adjustments. For most applications, this heating pad does not require external temperature control; it's preset to provide a stable warming environment suitable for small animals during procedures. If you are using a warming box in addition to the Deltaphase Heating Pad, you will generally only need to set the temperature on the warming box if it has its own adjustable thermostat, as the heating pad itself should maintain its preset temperature. However, always monitor temperature if both devices are used simultaneously to prevent overheating.

10. Place the mouse in a mouse restrainer (e.g., Broome restrainer) of the appropriate size (Fig. 1A).
    Make sure the restrainer is secure but not overly tight to avoid causing stress or injury to the animal. The duration of restraint should be minimized, and the restrainer should be cleaned regularly to prevent stress caused by pheromones or cross-contamination.
    [*Figure 1 near here]
11. Disinfect the tail with an alcohol pad. The tail vein of albino mice is generally easier to see than that of black-furred C57BL/6 mice, which may require enhanced skills or adjustments in lighting to ensure accurate needle placement.
12. Load the syringe without any air bubbles and inject the desired number of cells (e.g., 1 x 10^6 cells suspended in a maximum volume of 200 µL of PBS) into the lateral tail vein by keeping the needle and syringe parallel to the tail (Fig. 1B-D).
    Injecting the cells slowly is crucial to prevent cell lysis when passing through the narrow 29G needle. It is recommended to use one syringe and needle per animal. When positioned correctly, there should be no resistance when pressing down on the plunger. If resistance or swelling at the injection site is encountered, remove the needle and reinsert it higher than the initial site. It is recommended to start at the mid-length of the tail and move upwards.
13. After the injection, remove the needle and apply gentle pressure to the injection site to stop the bleeding.
14. Return the mouse to its cage and observe it to ensure that bleeding stops. If the mouse has been anesthetized, monitor it closely during the recovery process.
15. Monitor the mice over the next few days for morbidity.
16. Monitor the body weight (BW) of the mice regularly, with increased frequency starting 2-3 weeks following cell inoculation, as an indicator of tumor development. Any animals exhibiting signs of significant distress, including a BW loss >20% of baseline BW, hunching posture, inability to eat or drink, difficulty breathing, or moribundity, must be removed from the study and humanely euthanized to minimize suffering.
17. If luciferase-labeled LLC cells have been used, tumor growth can be monitored by *in vivo* bioluminescence imaging as described in Support Protocol 1 below.

SUPPORT PROTOCOL 1: *In vivo* bioluminescence imaging (BLI)

This protocol can be used in continuation with Basic Protocols 1 and 2. Here we demonstrate the advantages of using luciferase-expressing LLC cells and BLI to monitor lung tumor growth non-invasively and longitudinally in live animals. As shown in Figure 2, it is recommended to first test the luciferin substrate and verify the luciferase activity of the luciferase-expressing LLC cells *in vitro*. If the bioluminescent signal is weak, luciferase-expressing cells should be enriched prior to the study. This can be achieved through methods like FACS-sorting for GFP-positive cells or antibiotic selection, depending on the specific cell line used. This will increase the proportion of luciferase-expressing cells in the population, resulting in a stronger signal and more reliable results. In Figure 3, we performed BLI 0, 1, 7, 14, 21, and 28 days after the inoculation of luciferase-expressing LLC cells and show the kinetic of tumor growth obtained after tail vein injection of 0.25, 0.5, and 1 x 10^6 cells in C57BL/6J albino mice. Black-furred C57BL/6 mice can also be used but the fur attenuates the bioluminescent signal. As shown in Figure 4, hair removal by shaving or depilation can overcome this issue and increase the signal at the shaved area.

[*Figures 2, 3 ,4 near here]

Materials

Isoflurane (VetONE, cat. no. 13985-528-60)
Medical oxygen tank (Airgas, cat. no. 0215)
Hair removal cream (optional) (e.g., Veet or Nair)
D-Luciferin, Potassium Salt (GOLDBIO, cat. No. LUCK-100)
1X PBS (Gibco, cat. no. 14190-144)

IVIS® Lumina *In Vivo* Imaging System (Revvity Health Sciences, Inc.)
Isoflurane vaporizer/machine (Veterinary Anesthesia Systems, cat. no. VAS 2001R)
Small animal weighing scale
Alcohol pads (Medline, cat. no. MDS090670)
U-100 insulin syringes with 29G needles (Fisher Scientific, cat. no. 14-841-32)

Image Acquisition

NOTE: Outlined below are the key steps for successful BLI using the IVIS® Lumina II (Fig. 5A) and Living Image® software (version 4.2) both from PerkinElmer, Inc. (now named Revvity Health Sciences, Inc.). The IVIS system can image up to 3 to 5 mice at once depending on the instrument used (Fig. 5B). For more detailed instructions, please refer to the user manuals specific to the versions of the IVIS® Lumina device and Living Image® software that you are using.
[*Figure 5 near hee]

1. Record the body weight of the mice before imaging. If black-furred C57BL/6 mice are used, remove hair in the thoracic region of the mice by shaving (using a razor or an electric shaver) or depilation (using a depilatory cream). Shaving requires ~5-10 minutes per animal.
   Shaving the mice the day before imaging is recommended to prevent false-positive signals caused by skin inflammation immediately following hair removal.
2. Turn on the computer and the IVIS device.
3. Open Living Image® Software. Select the user ID, enter the password if needed, and click on "OK".
4. The IVIS Acquisition Control Panel appears at the bottom right. Click on the box that says "Initialize" (Fig. 5C). When initialization is complete and the CCD camera reaches -90 °C, the temperature box will turn green.
5. While the machine is warming up you can inject the mice with luciferin.
6. Disinfect the injection site with alcohol and inject D-luciferin intraperitoneally (i.p.) at 150 mg/kg (e.g., prepare a fresh stock solution at 15 mg/mL in 1X PBS, sterilize the solution by passing it through a 0.22 μm filter, and inject 10 μL/g of body weight). Three to five mice should be injected with D-luciferin at a time, depending on the capacity of the IVIS machine used.
7. Prepare the anesthesia system (Fig. 5D). Check the isoflurane level in the vaporizer to ensure there is enough for the procedure. Refill the vaporizer with isoflurane if the level is low. Turn the oxygen to the ON position. Turn the evacuation pump button ON. Set isoflurane to 2.5-3.5% in oxygen.
8. Approximately 10 min after the luciferin injection, place the mice into the clear Plexiglas anesthesia chamber and turn the Induction Chamber button ON (Fig. 5D).
9. When the mice are fully anesthetized, turn the IVIS Flow Manifold button to ON and transfer the anesthetized mice to the nose cones inside the imaging chamber by placing them on their backs with ventral side up.
10. Close the door of the imager and maintain anesthesia with a 2% isoflurane during the imaging process.
11. The strength and kinetics of bioluminescence emission can vary between different luciferins. Some luciferins may generate a brighter initial signal with a shorter duration, while others may produce a less intense but more prolonged signal. It is recommended to perform a pilot experiment to assess both the intensity and kinetics of the luciferin used in your specific experimental conditions. Generally, peak emission for D-luciferin occurs ~10-15 minutes after i.p. injection and is cleared in ~2 hours. Acquire image data when maximum luciferin is in circulation (~10-15 minutes after luciferin injection). GoldBio D-luciferin is ultrapure and specifically optimized for use in BLI, ensuring consistent and high signal intensity. While cheaper commercially available D-luciferins may appear cost-effective, their lower purity can lead to variability in experimental results, reduced signal intensity, and less stable signals.
12. On the IVIS Acquisition Control Panel, click "Acquire" (Fig. 5E). Select the sensitivity by choosing an exposure time (e.g., 30-60 sec), Binning (e.g., Medium), and F/stop (e.g., 1). These parameters depend on the strength of the signal. For a strong signal, decrease exposure time first, if necessary, lower Binning, and last, increase the F/stop. Do not lower time below 5 sec. For a weak signal increase exposure time, set Binning to Large, and decrease F/stop if not already on 1. The imaging time should be between one to five min. Make sure "Overlay" is checked (for both luminescent and photographic images).
13. Set the proper field of view and ensure the animals are entirely within the grid.
14. A Box appears asking: "Do you want to enable autosave". Click on "YES" and choose a folder to save your data.
15. The "Edit Image Labels" box will appear. You can give a name to the saved label and include relevant details to the comments box.
16. Collect images at the same time after injection for every mouse to insure consistency and reproducibility.
17. After the image acquisition, the system status will read as "Idle".
18. Turn the IVIS Flow Manifold button to OFF and place mice back into their cage where they should awake quickly.
19. Repeat steps 8-18 as necessary for any additional mice.
20. When finished, set isoflurane to 0.
21. Turn the oxygen to the OFF position.
22. Turn the Evacuation pump button to OFF.
23. Turn off the power supply on the back of the IVIS device.
24. Transfer your files to an USB key, log out of computer, and turn off the monitor.

25. Clean the anesthesia chamber, the inside of the IVIS, and the glass nosecones using a non-abrasive disinfectant or cleaning agent that is safe for the equipment and the animals (e.g., 70% ethyl alcohol/30% deionized water solution). *Attention: Certain cleaning compounds may contain phosphorescent materials, which could interfere with BLI. Contact Revvity (formerly Xenogen) technical support for a list of tested and approved cleaning compounds.*

Image Analysis

26. Open one of the acquired images in the Living Image® software. The image will appear on screen, and a "Tool Palette" will appear in the upper right corner of the screen.
27. To combine all the images into a single composite image, click on "Browse" and select the desired files. Highlight (left-click) files and click "Load as a group".
28. In the Tool Palette, click on Image Adjust and deselect "Individual" in the drop-down menu. This puts all the images on the same scale.
29. In the Tool Palette, select the preferred region of interest (ROI) tool (e.g., oval) from the drop-down menu (Fig. 5F).
30. Place the selected shape over mouse #1 then move it over all the mice to confirm that it fits each mouse.
31. Once you have found the best shape for all the mice, place it over mouse #1 and right-click to duplicate it (Fig. 5G).
32. A second ROI will appear. Move it to mouse #2. Repeat this process for all mice.
33. In the Tool Palette, click on "Measure ROIs".
34. Your data will appear. You can now export, save, and analyze your data in another software (e.g., GraphPad Prism).

BASIC PROTOCOL 2: LLC intranasal (i.n.) instillation method

This protocol can be an alternative to Basic Protocol 1 and can be integrated with Support Protocol 1. Here we evaluate the i.n. instillation method to administrate LLC cells as a potentially superior alternative to the conventional i.v. tail vein approach. In addition to its simplicity, the i.n. route of administration offers the advantage of delivering the cells directly to the lungs, non-invasively, using a micropipette while the animals are under mild anesthesia. The anesthesia prevents the mouse from sneezing or coughing out the administered material while remaining light enough to allow for largely normal breathing, facilitating the delivery of the cells to the lungs. A volume > 35 µL is necessary for optimal delivery to the lower respiratory tract and the most commonly used volume for i.n. instillation is 50 µL (Turner et al., 2011). A delivery volume of 50 µL allows ~55% of the dose to reach the lungs (Southam et al., 2002). As a result, we opted to modify the protocol recently published by Tanaka et al., (Tanaka et al., 2023) and deliver 1 x 10^6 LLC cells to the mice in two separate i.n. treatments, each consisting of 0.5 x 10^6 cells in 50 µL of PBS (one in the morning and one in the afternoon on the same day) to minimize the potential administration errors among mice that may arise from a single dosing.

Materials

Male or female (≥ 6-8-week-old) C57BL/6J albino mice (JAX, cat. no. 000058), C57BL/6J mice (JAX, cat. no. 000664), or any knockout or transgenic mouse on the C57BL/6 background can be used.
LLC-GLF (Hosoya et al., 2018) or other commercially available luciferase-expressing LLC cells (e.g., ATCC, cat. no. CRL-1642-LUC2). The original LLC cells (ATCC, cat. no. CRL-1642) can be used if *in vivo* BLI is not desired.
DMEM (Gibco, cat. no. 11965-092)
Fetal bovine serum (Life Technologies, cat. no. 26140079)
Penicillin/streptomycin (P/S) (Gibco, cat. no. 15140-122)
0.25% Trypsin/EDTA (Gibco, cat. no. 25200056)
1X PBS (Gibco, cat. no. 14190-144)
Trypan blue (Gibco, cat. no. 15250-061)
EDTA (Mediatech, cat. no. 46-034-CL)
1X HBSS (Gibco, cat. no. 14065-056)

75 cm^2 (T-75) cell culture flasks (Falcon, cat. no. 353136)
50 mL conical tube (BioPioneer, cat. no. CNT-50R)
70 µm cell strainer (FisherBrand, cat. no. 22-363-548)
P100 or P200 micropipette with sterile tips
Isoflurane vaporizer/machine (Veterinary Anesthesia Systems, cat. no. VAS 2001R)

Cell culture and preparation

Please refer to the *NOTE* in Basic Protocol 1 for critical information on LLC cell culture and preparation, including guidance for expanding, freezing, and maintaining low-passage cells throughout the study to ensure consistent experimental results. Maintaining consistent culture conditions is essential for ensuring the reproducibility of the data.

1. Culture LLC cells in DMEM medium supplemented with 10% FBS and 1% P/S in the desired cell culture flask (e.g., T-75) according to the ATCC's recommendation. In this protocol, 1 x 10^6 LLC cells are administrated to the mice in two separate i.n. treatments of 0.5 x 10^6 cells each. One treatment is administered in the morning and the other in the afternoon on the same day. Therefore, it is necessary to double the number of cell culture flasks to accommodate the two separate treatments. Calculate the number of cell culture flasks needed to grow enough LLC cells based on the number of mice in your experiment and the desired number of cells per administration.
2. After approximatively two passages and at 60-70% confluency, wash cells with 1X PBS and detach cells using 0.25% Trypsin/EDTA (1mL for a T-75, <5 min at 37°C).
3. Wash cells with 1X PBS containing 2 mM EDTA and centrifuge at approximately 125 x g for 5 min at 4°C.
4. Resuspend cells in 1X PBS without EDTA and pass through a 70 µm cell strainer.
5. Count the cell number using a hemocytometer and check the viability using trypan blue.
6. Ensure that > 90% of the cells are viable.
7. Resuspend cells at the desired concentration (e.g., 10 x 10^6 cells/mL to administrate 0.5 x 10^6 cells in 50 µL per mouse) in ice-cold sterile PBS.
8. Keep cells on ice before and during the i.n. treatment to maintain cell viability and prevent clumping.

i.n. instillation

NOTE: Always refer to your institution's specific IACUC guidelines as recommendations for i.n. administrations may differ depending on institutional protocols and regulations.

9. Place up to five mice into the anesthesia chamber at a time.
10. Set isoflurane to 2.5-3.5% in oxygen and wait ~1-2 minutes until the mouse is unresponsive but maintains normal breathing.
11. Confirm anesthesia by gently pinching the hind paw and observing for no response.
    The duration of anesthesia should be minimized, and the anesthesia chamber should be cleaned regularly to prevent stress caused by pheromones or cross-contamination.
12. Once anesthetized, remove the mouse from the chamber and place it on its back in the palm of your hand (Fig. 6A).

[*Figure 6 near here]

13. Position your thumb under the mouse's jaw and throat, gently tilting its head back (Fig. 6B). This posture will aid in better inhalation and help prevent the mouse from gagging or swallowing during the procedure.
14. Load a P100 or P200 micropipette with 50 µL of LLC cells in PBS and position the pipette tip gently over the mouse's nostrils (Fig. 6C).
15. Administer the LLC cell suspension drop by drop, allowing the mouse to inhale each drop before adding more (Fig. 6D). Alternate nostrils if needed for even distribution. Avoid placing the entire volume in one nostril at once to reduce the risk of the liquid entering the digestive system instead of the respiratory tract.
    If some of the cell suspension were misdirected into the esophagus and entered the digestive tract, the primary consequence would be a reduction in the number of cells reaching the lungs, likely resulting in a decreased tumor burden in the lungs. To mitigate this risk, we recommend administering LLC cells in two separate i.n. treatments, one in the morning and one in the afternoon on the same day. This approach minimizes the likelihood of significant administration errors in individual mice, which could otherwise skew the results.
16. Ensure the mouse fully inhales the cell suspension (the solution should not leak out) (Fig. 6E) If a mouse sneezes back some of the cell suspension, use your micropipette to re-aspirate it and re-administer it to the mouse. If the leaked cell suspension cannot be recovered, estimate the volume lost and re-administer an equivalent amount to ensure the proper dosage. Record any instances of leakage or re-administration in your notes, as these events could influence data interpretation. For additional troubleshooting methods, refer to Table 3: Troubleshooting Guide for i.n. Instillation.
17. Place the mouse on its back in its cage and monitor it to ensure normal recovery and breathing.
18. Repeat the procedure in the afternoon and monitor the mice over the next few days for morbidity.
19. Monitor the mice's BW regularly, with increased frequency starting 2-3 weeks following cell inoculation, as an indicator of tumor development. Any animals exhibiting signs of significant distress, including a BW loss >20% of baseline BW, hunching posture, inability to eat or drink, difficulty breathing, or moribundity, must be removed from the study and humanely euthanized to minimize suffering.
20. If luciferase-labeled LLC cells have been used, tumor growth can be monitored by BLI (see Support Protocol 1).
21. In Figure 7, we show the result of an experiment performed as described in this protocol (Basic Protocol 2) using i.n. instillation of luciferase-labeled LLC cells and BLI.

[*Figure 7 near here]

COMMENTARY

Background Information

The lungs are one of the most common target organs for cancer metastasis (Nguyen et al., 2009), occurring in up to 50% of patients diagnosed with malignant tumors that have metastasized to other sites (Mohammed et al., 2011). Among the various mouse models for studying metastasis, the LLC model has been referenced in over 5,000 publications on PubMed since its establishment in 1956. The tail vein injection technique is commonly used to administrate LLC cells in syngeneic mice even though it does not recapitulate the exact vascular route from the primary tumor to the metastatic site (Khanna & Hunter, 2005). Despite the popularity of the tail vein approach and i.v. delivery of cancer cells, this model is associated with several limitations such as sudden death due to thromboembolism (Shrestha et al., 2019; Rashid et al., 2013), tumor development in multiple anatomical sites (Miki et al., 2000), and challenges in monitoring lung tumor growth without advanced imaging equipment. Therefore, it can be quite expensive, time-consuming, and challenging to reproduce.

More targeted methods of LLC cell administration could minimize the growth of unwanted metastases outside the lungs and may represent valuable alternatives to the conventional i.v. tail vein approach. For instance, Tanaka et al., recently demonstrated that LLC cells administered via i.n. instillation form tumors exclusively within the lungs, predominantly around the bronchioles and alveoli (Tanaka et al., 2023), thereby replicating certain characteristics of NSCLC. In our study, we employed both the conventional LLC i.v. model and the more recently developed LLC i.n. model and provide detailed step-by-step instructions for the successful implementation of both models. We also provide a detailed protocol for using luciferase-expressing LLC cells and BLI to monitor lung tumor growth. This technique is beneficial in orthotopic models, where tumors develop within internal organs and are not as easily measurable as they are in subcutaneous transplantation models. Mice on a white or albino background are preferred for optical imaging because their lack of pigmentation reduces light absorption and scattering, thereby enhancing the clarity and accuracy of the imaging results (Chen & Thorne, 2012). The absence of melanin allows for better light penetration, improving the detection of bioluminescent or fluorescent signals from labeled cells or tissues. This makes albino mice particularly well-suited for *in vivo* imaging applications, such as tracking tumor progression in real time, where high signal sensitivity is crucial. Black-furred C57BL/6 mice can also be used but the fur attenuates the bioluminescent signal (Ji et al., 2020). However, hair removal by shaving or depilation can increase the signal in the shaved area while the fur attenuates the signal elsewhere (Wu et al., 2001).

In this study, we used white- and black-furred C57BL/6 for *in vivo* BLI after either i.v. or i.n. administration of luciferase-expressing LLC (LLC-GLF) cells. These cells were generated using a lentivirus vector to express luciferase and GFP (green fluorescent protein) reporters as previously described (Godebu et al., 2014; Hosoya et al., 2018). Several commercially available luciferase-expressing LLC cell lines (e.g., ATCC, cat. no. CRL-1642-LUC2) are suitable for use with the protocols outlined in this article. The strategy adopted in this study for *in vivo* BLI is similar to the one used in other studies (Zheng et al., 2010). One of the critical advantages of *in vivo* BLI is its ability to monitor tumor growth non-invasively and longitudinally in live animals (Close et al., 2011). This is made possible by the relatively non-toxic nature of D-luciferin, which can be administered repeatedly at different time points without significantly affecting the animals or tumor growth (Tiffen et al., 2010). However, like other imaging techniques, *in vivo* BLI also has its limitations (Close et al., 2011). Some of these limitations together with potential issues encountered with the i.v. and i.n. modes of cell administration are addressed in the Troubleshooting section below.

Critical Parameters

All animal studies must receive approval from the IACUC before being conducted in any laboratory. Proficiency in mouse handling and i.v. or i.n. administration techniques is crucial for the successful implementation of the LLC i.v. or i.n. models, respectively. It is critical to keep the cells on ice before and during the i.v. or i.n. treatments to maintain cell viability and prevent clumping, which could lead to thromboembolism after i.v. injection. It is recommended to establish a growth curve for LLC cells and regularly monitor their *in vitro* growth rate to ensure consistent and reproducible tumor development *in vivo*.

Troubleshooting Tables

Tables 1, 2, and 3 outline some commonly encountered issues during tail vein injection, *in vivo* BLI, and i.n. instillation, respectively, along with their likely causes and suggested solutions for overcoming them.

[*Tables 1, 2, 3 here]

Statistical Analysis

Researchers are advised to conduct pilot experiments using either statistical software (e.g., GraphPad Prism) or consult a biostatistician to accurately determine the sample size and ensure the appropriate number of experimental mice are used for their studies. All animal studies should be adequately powered to achieve statistically significant results with the smallest number of animals. However, as mentioned above, the LLC i.v. model may necessitate a relatively high number of mice to reach statistical significance between treatment groups because of the issues frequently encountered in this model (e.g., sudden death due to thromboembolism and high variability in the incidence of lung tumors). Sample sizes in the LLC i.v. and i.n. models should be determined based on preliminary studies and the effect of the treatment or genetic manipulation under investigation.

Understanding Results

In this study, we compared the i.v. and i.n. modes of LLC cell administration using luciferase-expressing LLC cells and BLI to monitor lung tumor growth in live animals. We found that when 1 x 10^6 cells are administrated, the kinetics of tumor growth is comparable between the two models and mice should be euthanized 3 or 4 weeks after cell inoculation (see Fig. 3 and 7). Similarly to that observed in the i.v. model with 1x10^6 cells (Fig. 3B), 4 out of 6 mice treated i.n. with 1x10^6 cells had a detectable bioluminescent signal in the 3rd week after LLC cell administration (Fig. 7B). To investigate whether the two mice that had no detectable signal developed lung tumors or not, we harvested the lungs and determined the presence of macroscopic tumors at the surface of the 5 lung lobes for each mouse with naked eyes. To our surprise, all the mice (6/6) had visible tumor nodules on 2 or more lung lobes (Fig. 7C). Next, we performed H&E staining on FFPE lung tissue sections following standard protocols as previously described (Wang et al., 2023) and evaluate the presence of microscopic tumors after whole slide scanning using QuPath software (Bankhead et al., 2017). All the mice (6/6) presented microscopic and macroscopic lung tumors on H&E-stained sections (Fig. 7D). Therefore, *in vivo* BLI is extremely useful for monitoring tumor growth in live animal and determining the most suitable endpoint for the experiment in both the LCC i.v. and i.n. models. However, false negatives can occur, and we highly recommend performing a final tumor assessment on H&E-stained sections to accurately quantify the number and size of lung tumors at the end point of an experiment. *In vivo* BLI and tumor quantification on H&E-stained lung sections can be complementary techniques. BLI allows real-time tracking of tumor progression, while H&E staining provides precise quantification of metastatic nodules in the lungs at the experiment's endpoint.

We demonstrated that white- and black-furred C57BL/6 mice can be used for *in vivo* BLI. Although black fur can attenuate the bioluminescent signal and hair removal is typically considered optional for optical imaging (Puaux et al., 2011), we found that hair removal significantly enhances signal detection at the shaved area (Fig. 4). Therefore, nearly any knockout or transgenic mouse that is on the C57BL/6 background can be utilized in these models and for *in vivo* BLI. Although LLC cells are presumed to have originated from a male mouse (He et al., 2024) and may contain Y chromosome antigens, both male and female recipient mice have historically been used in this model. In this study, we utilized male and female mice and found no significant gender bias in our observations.

Based on the data generated in both the LLC i.v. and i.n. models, we propose that the i.n. model represents a valuable alternative to the conventional i.v. model. In the i.v. model, we observed tumor development in multiple anatomical sites including the injection site, the legs, the oral cavity, the liver, and the bladder (Fig. 8), which may negatively impact tumor development in the lungs. Although we never observed any tumor development in the upper airways, including the nose, nasal cavity, pharynx, trachea, or other organs apart from the lungs in approximately 100 mice in the i.n. model, further evaluation of this model and the i.n. administration method for other tumor cell lines is warranted.

[*Figure 8 near here]

Time Considerations

In both the i.v. and the i.n. models, mice should typically be euthanized 3 or 4 weeks after inoculating of 1 x 10^6 LLC cells. As shown in Figure 3, using fewer cells (e.g., 0.5 or 0.25 x 10^6 cells) will significantly delay tumor growth kinetics. *In vivo* BLI typically takes 2-3 hours for two groups of mice (n=6 mice/group) when using an IVIS system equipped with 3 nose cones, allowing for the simultaneous imaging of 3 mice. For optimal efficiency, experimental group sizes should be in multiples of 3 (e.g., n=3, 6, or 9 mice/group). If the system has 5 nose cones, imaging time can be further reduced.

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AUTHOR CONTRIBUTIONS

Miki Yamada-Hara: Investigation (lead), data curation (lead), formal analysis (equal), methodology (equal), visualization (equal), and writing – original draft preparation (equal); **Naoki Takahashi:** Investigation (supporting), formal analysis (supporting), data curation (supporting); **Ji Won Byun:** Investigation (supporting), formal analysis (supporting), data curation (supporting); **Liping Zeng:** Investigation (supporting), formal analysis (supporting), data curation (supporting); **Zhihe Wang:** Investigation (supporting), formal analysis (supporting), data curation (supporting); **Arisachi Tanaka:** Investigation (supporting), formal analysis (supporting), data curation (supporting); **Zahra Malakoutikhah:** Investigation (supporting), formal analysis (supporting), data curation (supporting); **Tomoko Hayashi:** Resources (equal) and supervision (supporting); **Nicholas JG Webster:** Funding acquisition (supporting), resources (equal), and supervision (supporting); **Eyal Raz:** Funding acquisition (equal), conceptualization (equal), project administration (equal), supervision (lead), and writing – review and editing (supporting); and **Samuel Bertin:** Funding acquisition (equal), conceptualization (equal), project administration (equal), data curation (equal), formal analysis (equal), methodology (equal), visualization (equal), writing – original draft preparation (equal), and writing – review and editing (lead).

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data, tools, and material (or their source) that support the protocol are available from the corresponding author upon reasonable request.

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FIGURE LEGENDS

Figure 1. Lateral tail vein injection technique. A) Gently place the mouse in the restrainer and hold the tail at mid-length. Using your non-dominant hand, position your index finger and thumb around the tail just above the injection site to apply gentle pressure. Hold the syringe and needle in your dominant hand and position the needle at a shallow angle (around 10-20 degrees) to the tail. **B)** Insert the needle into the vein, parallel to the tail, moving towards the mouse's body. You should feel little to no resistance when the needle enters the vein. **C, D)** If necessary, tape can be used to secure the restrainer to the bench to prevent movement during the injection procedure. Note: The two red marks on the mouse's tail were made using a Sharpie marker for identification purposes and are not related to the injection procedure.

Figure 2. Validation of the luciferase activity of LLC cells *in vitro*. LLC-GLF cells were plated in triplicate in a 48-well plate and serially diluted as indicated. After adding D-luciferin to the cells at a final concentration of 150 µg/mL, allow the cells to incubate with luciferin for 5-10 minutes at 37°C. Measure luminescence by IVIS imaging using the appropriate exposure time (e.g., 45 seconds).

Figure 3. Assessment of lung tumor growth by BLI in the LLC i.v. model. A) Age- and sex-matched C57BL/6J albino mice were randomly assigned to three groups (n=6 mice/group). The mice were injected i.v. into the lateral tail vein with the indicated number of LLC-GLF cells on day 0, and tumor growth was monitored by BLI, as shown in this schematic overview of the study design. **B)** Representative photos of tumor signals quantified by BLI in the three experimental groups and at the different time points. The luminescence intensity is shown in a blue-to-red spectrum. **C)** IVIS radiance was recorded on day 28 after the LLC cell injection. Data are presented as mean ± SEM. **D)** Survival curves of mice in the three experimental groups. Values are expressed as a percentage of survival.

Figure 4. Comparison of lung tumor growth in albino and wild-type (WT) C57BL/6J mice in the LLC i.v. model. A) C57BL/6J albino and WT mice were injected i.v. into the lateral tail vein with the indicated number of LLC-GLF cells on day 0, and tumor growth was assessed by BLI, as shown in this schematic overview of the study design. **B)** Representative photos of tumor signals quantified by BLI on day 21 after the LLC cell injection in the three experimental groups (n=1 mouse/group is shown).

Figure 5. BLI using the IVIS® Lumina II and Living Image® software. A) Overview of the IVIS® Lumina II Imaging System. **B)** Interior view of the imaging chamber. This system has three nose cones to provide anesthesia, enabling the simultaneous imaging of up to three mice. **C)** Open the Living Image® software, and in the Acquisition Control Panel that appears, (1) click on "Initialize". (2) The temperature box will turn green once the initialization process is complete, indicating that the imaging system is ready to use. **D)** Overview of the anesthesia system. (1) Check the isoflurane level and (2) refill the vaporizer with isoflurane if the level is low. (3) Turn the oxygen to the ON position. (4) Turn the evacuation pump button ON. (5) Set isoflurane to 2.5-3.5%. (6) Place the mice into the anesthesia chamber, and (7) turn the Induction Chamber button ON. (8) When the mice are anesthetized, turn the IVIS Flow Manifold button to ON and transfer the mice to the nose cones inside the imaging chamber. **E)** In the Acquisition Control Panel, (1) click on "Acquire". (2) Select the sensitivity by choosing the appropriate exposure time, Binning, and F/stop. (3) Ensure "Overlay" is checked to acquire both luminescent and photographic images. **F)** In the Tool Palette, select the preferred ROI tool (e.g., oval) from the drop-down menu. **G)** Create an ROI of the desired size and right-click to duplicate it. Transfer the anesthetized mice to the nose cones inside the imaging chamber by placing them on their backs with ventral side up and confirm that the ROIs fit each mouse.

Figure 6. i.n. instillation technique. A) Place the anesthetized mouse in the palm of your hand. **B)** Position your thumb on the mouse's jaw and throat to prevent a gag reflex or swallowing during the procedure. **C)** Use a P100 or P200 micropipette loaded with 50 uL of LLC cells in PBS and position the pipette tip gently over the mouse's nostrils. **D)** Administrate the cells dropwise until **E)** the full dose is inhaled.

Figure 7. Assessment of lung tumor growth by BLI in the LLC i.n. model. A) Age- and sex-matched WT C57BL/6J mice (n=6 mice) were treated i.n. under light anesthesia (isoflurane) with the indicated number of LLC-GLF cells on day 0, and tumor growth was evaluated by BLI, as shown in this schematic overview of the study design. **B)** Representative photos of tumor signals quantified by IVIS at day 26 post LLC cell injection and after shaving. **C)** Representative photos of the 5 lung lobes for each mouse with visible

macroscopic tumors. **D)** Representative photos of H&E-stained lung sections. The areas within the red borders were considered positive for lung tumors after image analysis using QuPath software. Scale bars, 2 mm.

Figure 8. Potential problems encountered with the LLC i.v. model. A) Tumor development in multiple anatomical sites including the injection site, **B)** the legs, **C)** the oral cavity, **D)** the liver, and **E)** the bladder as observed here in C57BL/6J albino mice by the naked eye (A and B) or BLI (C-E).

Table 1. Troubleshooting Guide for Tail Vein Injection

Problem	**Possible Cause**	**Solution**
Difficulty finding the vein	Vasoconstriction, room temperature is too cold	Warm the tail gently with a warm compress or warm water to promote vasodilation. Use albino mice rather than black-furred mice.
Swelling at the injection site	Injection outside the vein due to incorrect needle placement	Ensure proper restraint of the mouse and stabilize the tail to minimize movement during injection.
No tumor development	Poor cell viability or cell lysis during the tail vein injection	Keep the cells on ice before and during the i.v. injection. Inject the cells slowly. Consider using wider (e.g., 27-28G) needles.
Sudden death	Embolism	Keep the cells on ice before and during the i.v. injection to prevent clumping. Filter the solution before injection by passing cells through a cell strainer (e.g., 40 or 70 μm) and ensure cells are resuspended well.

Table 2. Troubleshooting Guide for *In Vivo* BLI

Problem	**Possible Cause**	**Solution**
Weak or no bioluminescent signal	Poor luciferase expression in the cells	Verify luciferase activity *in vitro* before *in vivo* studies. Ensure cells are properly transduced or transfected.
	Delayed imaging after luciferin injection	Perform imaging promptly (typically within 10-15 min post-luciferin injection).
	Inadequate image acquisition settings (e.g., short exposure time or low Binning)	Increase exposure time, set Binning to Large, and/or decrease F/stop.
False-positive signal	Skin inflammation due to shaving or depilation	Perform shaving or depilation the day before imaging to reduce inflammation artifacts.
	Inadequate image acquisition settings (e.g., high exposure time or high Binning)	Decrease exposure time, lower Binning, and/or increase the F/stop.
False-negative signal	Luciferase gene silencing over time	Ensure cells are actively expressing luciferase before injection. Consider fresh transduction/transfection if necessary.

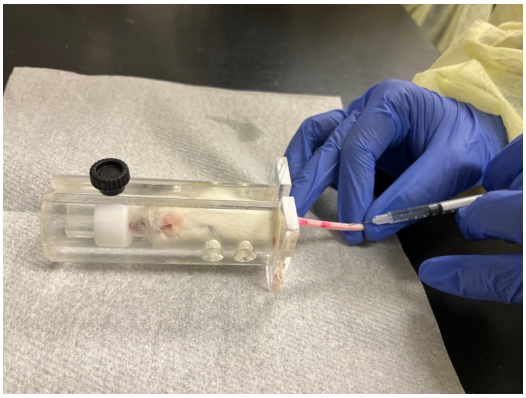
	Inadequate image acquisition settings (e.g., short exposure time or low Binning)	Increase exposure time, set Binning to Large, and/or decrease F/stop.

Table 3. Troubleshooting Guide for i.n. Instillation

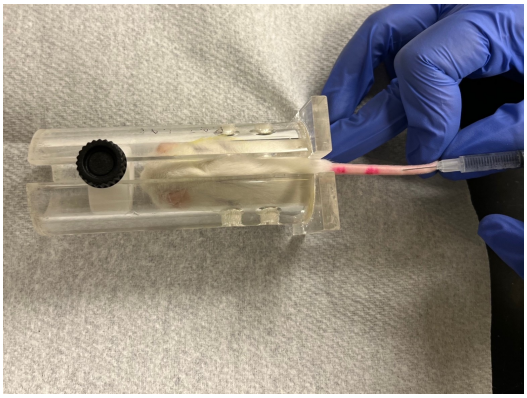
Problem	**Possible Cause**	**Solution**
Mouse sneezing or coughing out the cell suspension	Improper anesthesia depth or too rapid delivery of cells	Ensure the mouse is fully anesthetized. The heart rate should have slowed down but the mouse should be breathing normally, indicating an appropriate level of sedation for the procedure. Administer the solution slowly to minimize discomfort and avoid triggering reflexes.
Mouse swallowed the cell suspension instead of inhaled it	Insufficient anesthesia time or depth. Cells may have entered the esophagus instead of the respiratory tract	Ensure the mouse is fully anesthetized. Position your thumb on the mouse's jaw and throat to prevent a gag reflex or swallowing during the procedure. Deliver cells at a slower pace to allow proper inhalation.
Inconsistent tumor development between mice	Inconsistent dosing or variability in cell viability	Ensure all mice receive the full 50 μL dose each time. Ensure accurate cell counts and viability checks before administration.

Figure 1

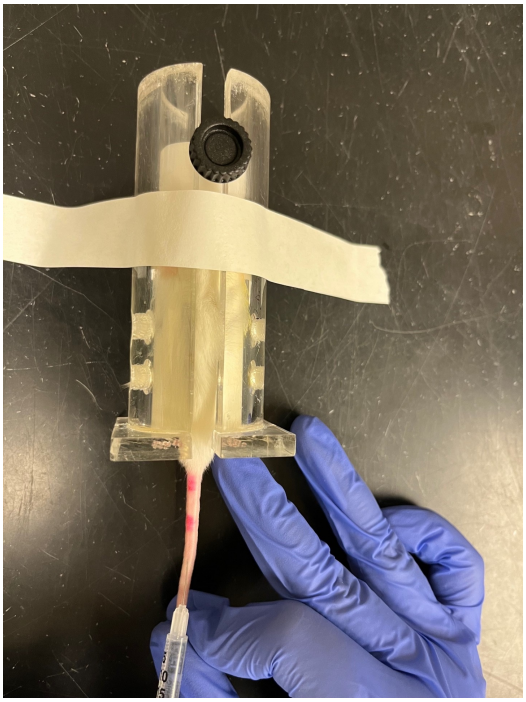
A



B



C



D

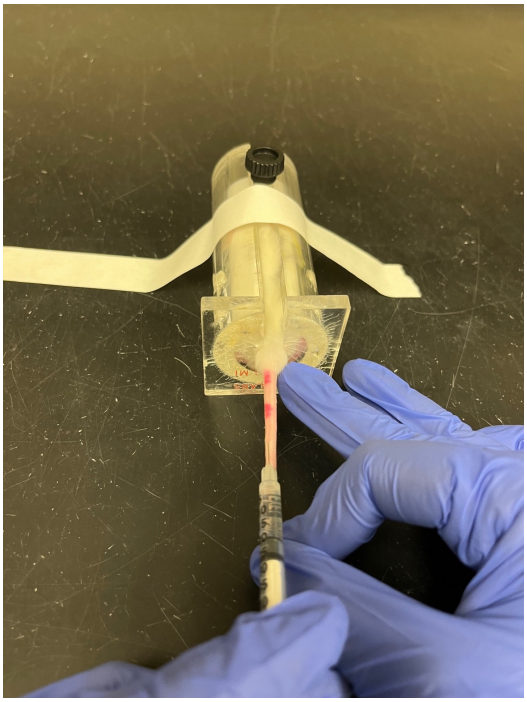


Figure 2

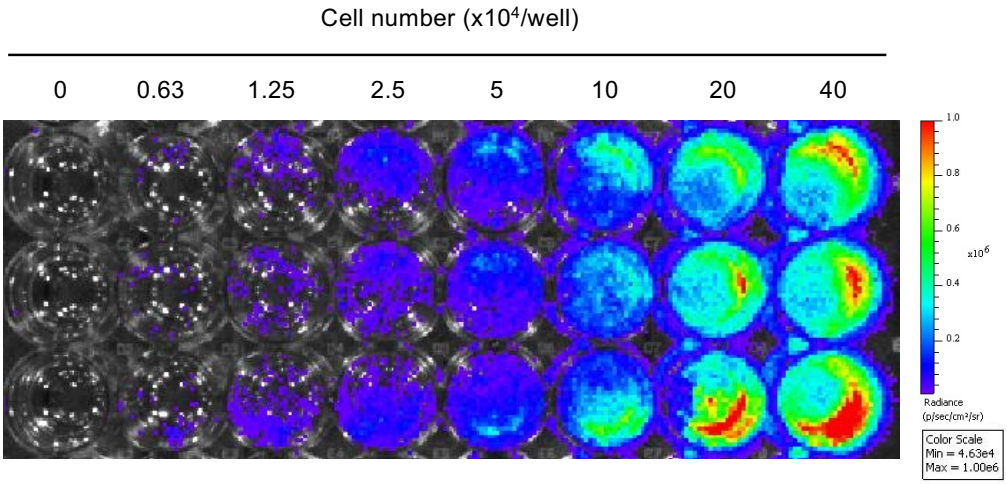

Cell number (x10⁴/well)
0
0.63
1.25
2.5
5
10
20
40
1.0
0.8
0.6
x10⁶
0.4
0.2
Radiance
(p/sec/cm²/sr)
Color Scale
Min = 4.63e4
Max = 1.00e6

Figure 3

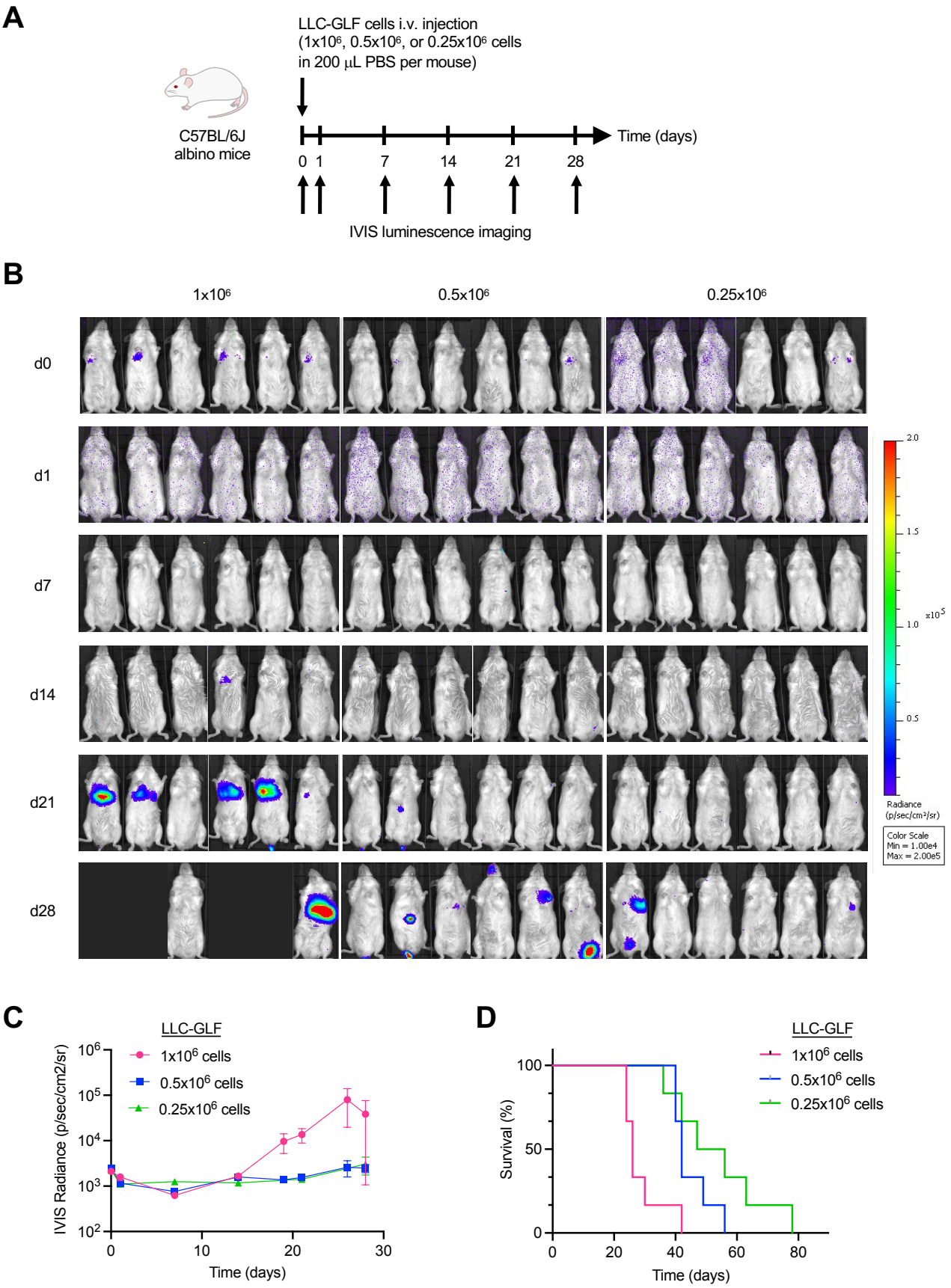
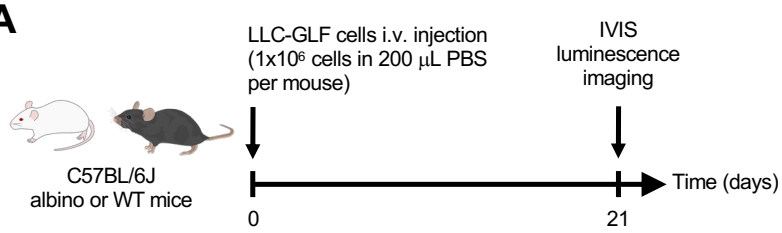

A
LLC-GLF cells i.v. injection
(1x10⁶, 0.5x10⁶, or 0.25x10⁶ cells
in 200 μL PBS per mouse)
C57BL/6J
albino mice
Time (days)
0 1 7 14 21 28
IVIS luminescence imaging
B
1x10⁶
0.5x10⁶
0.25x10⁶
d0
d1
d7
d14
d21
d28
Radiance
(p/sec/cm²/sr)
Color Scale
Min = 1.00e4
Max = 2.00e5
C
LLC-GLF
1x10⁶ cells
0.5x10⁶ cells
0.25x10⁶ cells
IVIS Radiance (p/sec/cm2/sr)
Time (days)
D
LLC-GLF
1x10⁶ cells
0.5x10⁶ cells
0.25x10⁶ cells
Survival (%)
Time (days)

Figure 4

A


LLC-GLF cells i.v. injection
(1x10⁶ cells in 200 μL PBS
per mouse)
IVIS
luminescence
imaging
C57BL/6J
albino or WT mice
Time (days)
0
21

B

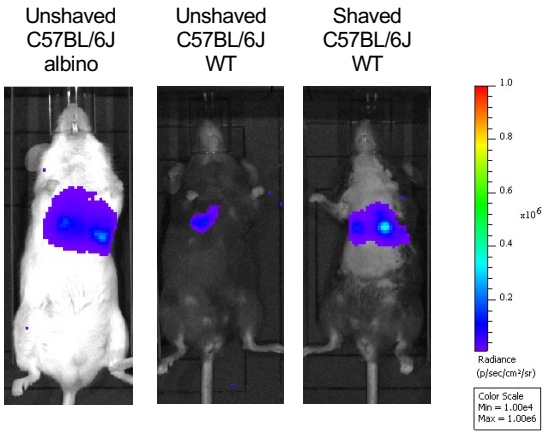
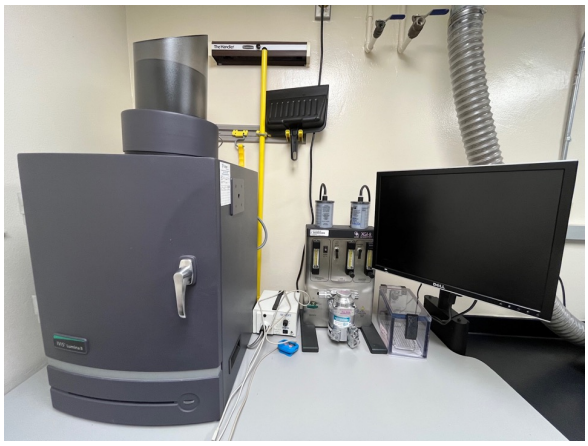

Unshaved
C57BL/6J
albino
Unshaved
C57BL/6J
WT
Shaved
C57BL/6J
WT
1.0
0.8
0.6
x10⁶
0.4
0.2
Radiance
(p/sec/cm²/sr)
Color Scale
Min = 1.00e4
Max = 1.00e6

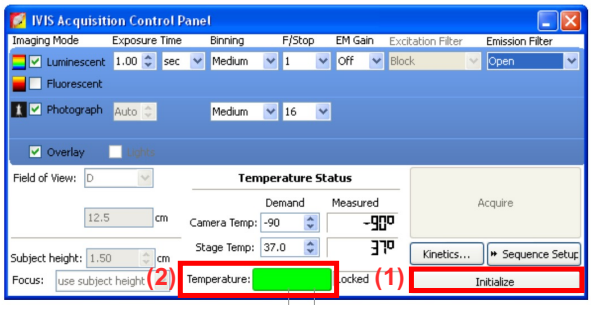
Figure 5

A

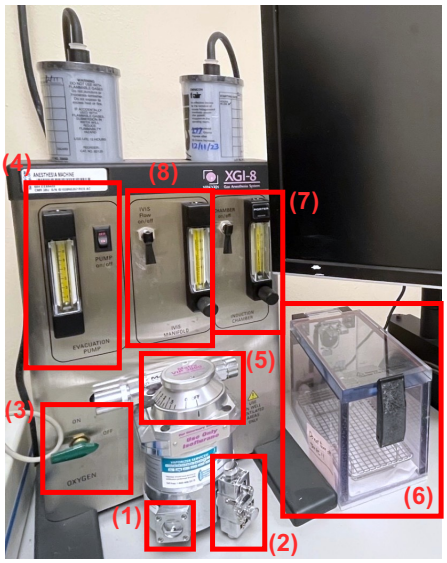


B

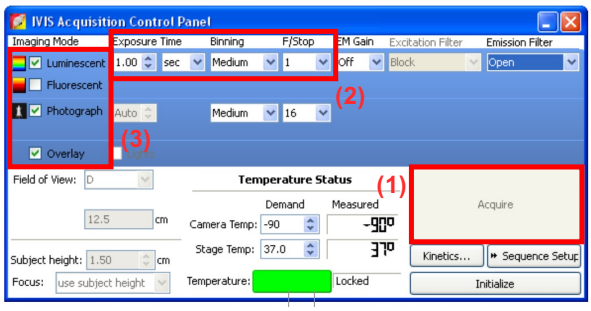
C



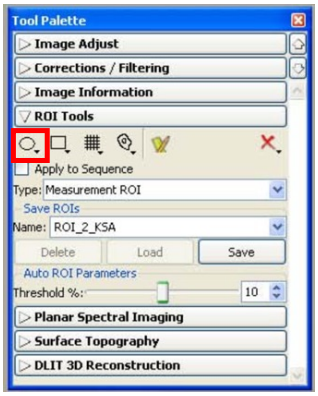
D



E



F



G

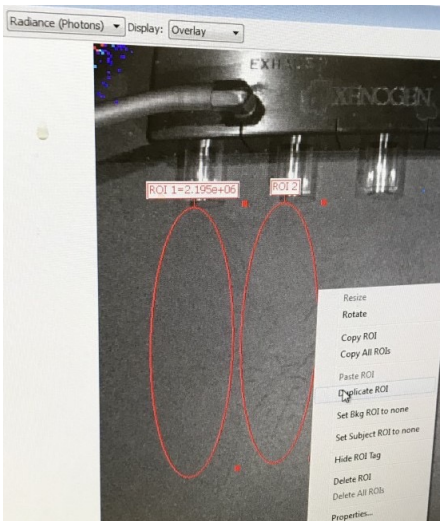


Figure 6

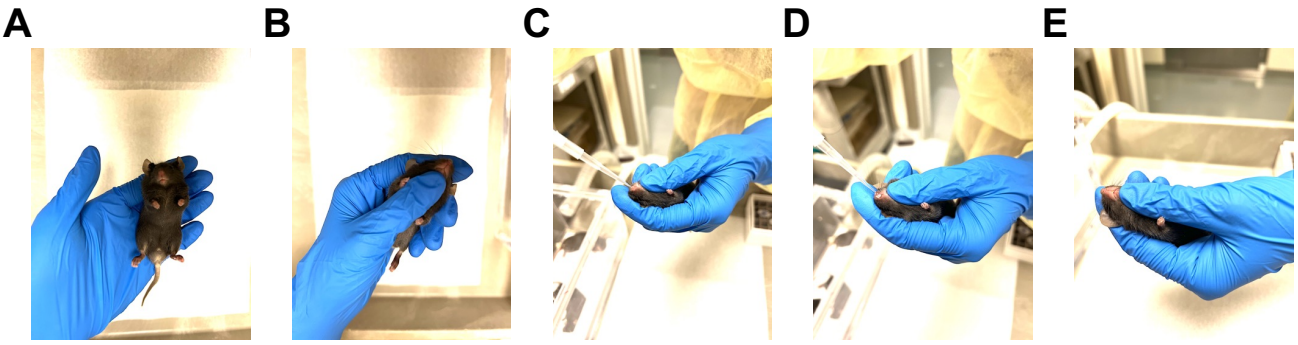
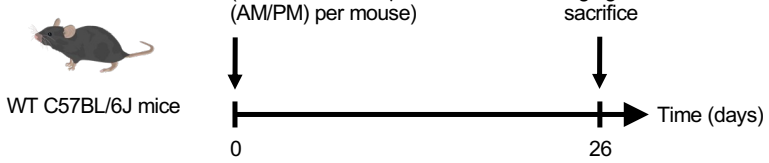
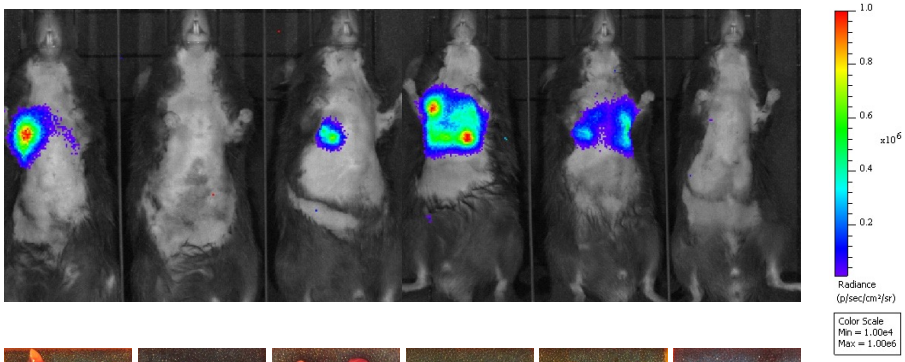


Figure 7

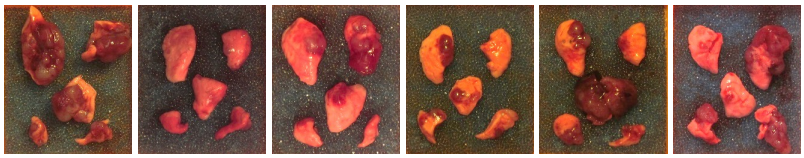
A



B



C



D

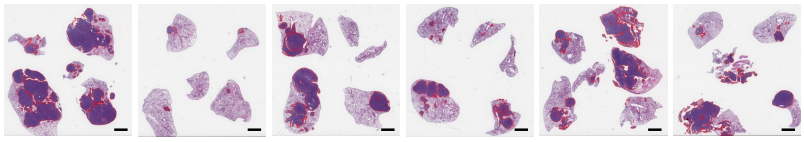


Figure 8