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Monitoring breast cancer growth and metastatic colonies formation in mice using Bioluminescence

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TITLE:

Monitoring Breast Cancer Growth and Metastatic Colony Formation in Mice Using Bioluminescence

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KEYWORDS:

Breast cancer, Animal model, Lung metastasis, Lentivirus, Luciferase, Bioluminescence, Orthotopic, Tumor growth.

SUMMARY:

Here, we describe a noninvasive monitoring method involving luciferase and green fluorescent protein expression in various breast cancer cell lines. This protocol provides a technique to monitor tumor formation and metastatic colonization in real time in mice.

ABSTRACT:

Breast cancer is a frequent heterogeneous malignancy and the second leading cause of mortality in women, mainly due to distant organ metastasis. Several animal models have been generated, including the widely used orthotopic mouse models, where cancer cells are injected into the mammary fat pad. However, these models cannot help monitor tumor growth kinetics and metastatic colonization. Cutting-edge tools to monitor cancer cells in real time in mice will significantly advance the understanding of tumor biology.

Here, breast cancer cell lines stably expressing luciferase and green fluorescent protein (GFP) were established. Specifically, this technique contains two sequential steps initiated by measuring the luciferase activity *in vitro* and followed by the implantation of the cancer cells into mammary fat pads of nonobese diabetic-severe combined immunodeficiency (NOD-SCID) mice. After the injection, both the tumor growth and metastatic colonization are monitored in real time by the noninvasive bioluminescence imaging system. Then, the quantification of GFP-expressing metastases in the lungs will be examined by fluorescence microscopy to validate the observed bioluminescence results. This sophisticated system combining luciferase and fluorescence-based detection tools evaluates cancer metastasis *in vivo,* which has great potential for use in breast

cancer therapeutics and disease management.

INTRODUCTION:

Breast cancers are frequent types of cancer worldwide, with approximately 250,000 new cases diagnosed each year in the United States[1]. Despite its high incidence, a new set of anticancer drugs has significantly improved breast cancer patient outcomes[2]. However, these treatments are still inadequate, as many patients experience disease relapse and metastatic spread to vital organs[2], which is the primary cause of patient morbidity and mortality. Therefore, one of the main challenges in breast cancer research is identifying the molecular mechanisms regulating the formation of distal metastases to develop new means to inhibit their development.

Cancer metastasis is a dynamic process in which cells detach from the primary tumor and invade neighboring tissues through the blood circulation. Thus, animal models in which the cells undergo a similar metastatic cascade can facilitate the identification of the mechanisms that govern this process[3,4]. Additionally, these *in vivo* models are essential for developing breast cancer therapeutic agents[5,6]. However, these orthotopic models cannot indicate the actual tumor growth kinetics as the effect is only determined upon termination. Therefore, we established a luciferase-based tool to detect tumor development and metastatic colonization in real time. Additionally, these cells express GFP to detect the metastatic colonies. This approach is relatively simple and does not involve any invasive procedures[3]. Thus, combining luciferase and fluorescence detection is a helpful strategy to advance the preclinical studies of breast cancer therapeutics and disease management.

PROTOCOL:

All mouse experiments were carried out under the Hebrew University Institutional Animal Care and Use Committee-approved protocol MD-21-16429-5. In addition, the Hebrew University is certified by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

1. Cell line maintenance

NOTE: The human breast cancer cell lines (MCF-7, MDA-MB-468, and MDA-MB-231) were used in this protocol.

1.1. Culture all the breast cancer cell lines in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C in a humidified carbon dioxide (5% CO_2) incubator.

NOTE: Check the cell density regularly; split and expand for future usage when they reach 70% confluency.

2. Virus preparation

2.1. Treat the HEK293T cells with 1 mL of trypsin until they detach.

2.2. Add 10 mL of DMEM (10% FBS) to neutralize the trypsin activity, and transfer the cell suspension to a new 15 mL centrifuge tube.

2.3. Centrifuge at ~150 × *g* for 5 min to sediment the cells. Discard the supernatant after the centrifugation and add 1 mL of fresh DMEM medium.

2.4. Determine the cell concentration and seed 1.2×10^6 cells/well in a six-well plate.

2.5. On the following day, prewarm all the necessary reagents, including transfection reagent, serum-free DMEM, envelope plasmid (VSV-G), lentivirus packaging plasmid (ΔVPR), and pLX304 Luciferase-V5 blast plasmid or FUW GFP plasmid.

2.6. In a 1.5 mL autoclaved centrifuge tube, mix 50 µL of serum-free DMEM with 0.3 µg of VSV-G plasmid, 1 µg of ΔVPR, and 1.2 µg of pLX304 Luciferase-V5 blast plasmid or FUW GFP plasmid.

2.7. After thoroughly mixing these plasmids with the medium, add 5 µL of the transfection reagent, mix gently, and incubate the mixture at room temperature for 15 min.

2.8. Following incubation, add the mixture dropwise to the HEK293T cells.

2.9. After 24 h, replace the growth medium with 2 mL of (30% FBS) DMEM. On the following day (48 h post-transfection), harvest the medium containing the viruses ("pLX304 Luciferase-V5 or the FUW GFP viruses").

NOTE: The use of 30% FBS enhances the virus production efficiency.

2.10. To avoid any HEK293T cell residues, pass the virus-containing medium through a 0.45 µm syringe filter or centrifuge at ~150 × *g* for 5 min, and collect the supernatant.

NOTE: For long-term storage, keep the working aliquots of the virus at -80 °C.

3. Establishing cells stably expressing GFP and luciferase ("GFP^+ Luc^+ cells")

3.1. The day before infecting the cells, seed 8×10^5 cells per well in a six-well plate.

3.2. After overnight incubation, replace the growth medium with fresh medium containing 8 µg/mL of polybrene. Add 200 µL of FUW GFP viruses dropwise to the cells.

3.3. Optional: To enhance the virus efficiency, centrifuge the plate at 560 × *g* for 30 min (37 °C) (spin infection).

3.4. Incubate the cells for 48 h, and verify the GFP expression by fluorescence microscopy.

3.5. Sort the GFP-expressing cells by fluorescence-activated cell sorting (FACS) (GFP^+) (**Figure 1A**).

3.6. Infect the GFP-sorted cells with the pLX304 Luciferase-V5 blast viruses as described in step 3.2.

3.7. Treat the cells with blasticidin (10 µg/mL) 30 h post-infection to enrich for pLX304 Luciferase-V5 blast-expressing cells (GFP^+, Luc^+ cells). Then, every two days, replace with fresh medium containing blasticidin. Additionally, as a control, treat naïve cells with the same blasticidin-containing medium.

NOTE: A clear difference between the infected and control cells should be observed after a few days of blasticidin treatment. This effect is cell line-dependent and usually takes ~8–10 days. A poor survival rate will indicate a low viral production yield. If so, produce a new batch of viruses as low efficiency may affect future experiments.

4. Validating *in vitro* luciferase activity

4.1. Grow the MCF-7, MDA-MB-468, and MDA-MB-231 GFP^+ Luc^+ cells in a 15 cm plate to 80% confluency. Harvest the cells by trypsinization, as described in steps 2.1–2.2.

4.2. Seed an increasing number of cells in each well (0.1, 0.5, 1, 2, 3, 4 × 10^4) into a black 96-well plate.

NOTE: Black 96-well plates are more suited for measuring luciferase levels as white or transparent plates will produce autoluminescence signals. As a control, use phosphate-buffered saline (PBS) alone in one well to ensure there is no autoluminescence from PBS.

4.3. Fill all the wells with 100 µL of DMEM and incubate for 16–24 h.

4.4. Prepare luciferin solution in PBS at a 1.5 mg/mL concentration. Aliquot and store this luciferin solution at -20 °C.

4.5. Wash the cells once with PBS gently, add 100 µL of luciferin solution into each well, and wait for 2 min. Finally, measure the luciferase activity in all the breast cancer cells using bioluminescence.

NOTE: Examining *in vitro* GFP and luciferase expression prior to animal experiments is crucial. Additionally, blank wells are used to subtract the background.

5. Injecting mice with GFP^+ Luc^+ cells

5.1. Transfer 5 × 10^6 (MCF7 and MDA-MB-468) or 2 × 10^6 (MDA-MB-231) GFP^+ Luc^+ cells into 200 µL or 100 µL PBS, respectively.

5.2. Before injection, clean the sterile biological hood with 5% disinfectant solution (see the **Table of Materials**). Then, anesthetize the mice with filtered (0.2 µm) air containing 4% isoflurane at an airflow rate of 1 L/min for 2−3 min.

NOTE: It is essential to confirm proper anesthetization; pinch the mouse's toe and look for any response.

5.3. Place a cone over the anesthetized mouse head in a supine position. Apply vet ointment to its eyes to prevent dryness while under anesthesia.

5.4. Wipe the abdominal area of the mouse, above the mammary gland, with ethanol using a cotton swab and lift the 4^{th} mammary gland with forceps.

5.5. Insert the needle 27 G x 3/4 (0.4 x 19 mm) under the fat pad and slowly inject 100 µL of the cell suspension.

NOTE**:** A round bulge will appear under the skin. In addition, an improper injection may lead to deviation in the tumor growth rate or absence of tumor in the same experimental group.

5.6. After the injection procedure, take the mice out of the hood and transfer them to a new cage. Monitor the mice until they return to consciousness.

NOTE: Ensure that all the used needles and syringes are discarded in the sharps box.

6. Measuring the luciferase levels in GFP^+ Luc^+ mice

6.1. Before the bioluminescence detection, restrain the conscious mouse by holding its neck with the left hand. Then, tilt the hand to the left, resulting in the mouse face upward with the lower body in a supine position.

6.2. Inject 100 µL of luciferin (30 mg/mL) intraperitoneally (i.p.) into the abdominal surface of the mouse in the lower-left abdominal quadrant using a 1.0 mL syringe with needle size 27 G x 3/4 (0.4 x 19 mm).

NOTE: The tip of the needle should not be inserted more than 3–5 mm from the abdominal wall, as it might penetrate visceral organs. Additionally, it is recommended to perform i.p. injection without anesthesia as luciferin distribution in the body of anesthetized mice is slower than in conscious mice. Thus, monitoring luciferase levels in conscious mice is a faster procedure.

6.3. Keep the mouse for 7 min without anesthesia followed by 3 min within the anesthesia chamber before measuring the tumor kinetics.

NOTE: The incubation time varies between the different experiments, cell lines, and species to species. Thus, it is recommended to calibrate the incubation time before starting the experiments.

6.4. Anesthetize the mice as described in steps 5.2–5.3.

6.5. Open the software (**Table of Materials**) during the incubation, initialize the imaging system, and click the **Initialize** button.

NOTE: Be aware that the camera will take ~10 min to cool down and reach -90 °C. Additionally, as background control, measure the luciferase levels in naïve mice (*i.e.,* GFP^+ mice injected with cells that do not express the luciferase gene).

6.6. Keep the setup in auto exposure using the following settings: exposure time auto, 60 s; Binning Medium, F/Stop 1; Excitation filter blocked; Emission filter open. When **Initialization** ends, select **Imaging Wizard** | **Bioluminescence** and then click **Next | Open Filter** | select **Mouse** in the image subject.

6.7. In **Field View**, select stage **C** (10 cm) and **Subject Height**: 1.50 cm.

6.8. Click **Image Setup Stage** to **C**, and before clicking the **Acquire** button, ensure that the mouse is placed on the stage in the proper supine position.

6.9. Close the door, click the **Acquire** button, and wait for an image to appear on the screen.

NOTE: With the **Auto-Exposure** option, a strong signal takes 5–20 s depending on the signal; a weak signal will take around 60 s.

6.10. Repeat this step for the other mice.

6.11. To detect lung metastasis, cover the strong signal of the primary tumor using thick black cardboard paper and expose only the ventral side of the lungs towards the camera. Capture the image using the same parameters described above.

7. Acquiring *ex vivo* image using bioluminescence and fluorescence

7.1. Euthanize the mice using carbon dioxide (CO_2) inhalation in the desiccator and dissect the mice using autoclaved scissors and forceps.

7.2. Perfuse the mice using 0.9% saline, harvest the organs, and rinse with 1x PBS to eliminate bloodstains from the organ.

7.3. Transfer the rinsed organ to a Petri dish and place it into the stage of the bioluminescence machine. Apply the same bioluminescence setting as described in steps 6.2–6.6.

NOTE: Due to the decrease in luciferase signal, this step is time-limiting. Thus, after euthanizing mice, immediately visualize the organ by bioluminescence.

7.4. For GFP images, apply the same setting as described in step 6.3, using **Fluorescence GFP filter** instead of **Bioluminescence**.

7.5. Take lung images using a stereo microscope to examine the presence of GFP^+ colonies.

8. Bioluminescence data analysis

8.1. Double-click the software and select **Open** from the **File** menu.

8.2. Open all the files and minimize them. Ensure all the units are **Radiance (Photons)**. Additionally, click **Apply to All** to keep the same parameters for all the images.

8.3. From the **View** menu, select the **Tool Palette**, which will open a new window.

8.4. From the **Tool Palette** window, select the **ROI Tools** tab and in **Type**, choose the **Average Bkg ROI**.

8.5. From the **ROI tools**, select **Circle**, and draw a small circle on the thoracic area of the mouse.

8.6. Double-click on the circle, select the **Use as BKG for future ROIs** option from the **Background ROI** tab, and click **Done**.

9. Measuring the total flux

9.1. From the **Tool Palette** window, select the **ROI Tools** tab and in **Type**, choose the **Measurement of ROI**.

9.2. From the **ROI tools**, select **Circle**, and draw a big circle on the primary tumor of the mouse.

9.3. Right-click **Copy ROI** and right-click **Paste ROI** into every individual file of each mouse.

9.4. Click **Measure ROIs** from the **ROI Tools** tab, which will open a new window named **ROI Measurements**. From this tab, keep the **Measurements Types** as **Radiance (Photons)** and **Image Attributes** as **All Populated Values**.

9.5. Export the file as **Measurements File (*.txt)** or **Csv (*.csv) format**.

9.6. Open the exported data in a spreadsheet and take the **Total Flux (p/s)** and the values for the weeks.

NOTE: This step can be used to measure different groups. For example, mice treated with a vehicle *vs.* those treated with a drug.

9.7. Generate an XY plot, where the **Time** is presented along the X-axis and **Total Flux (p/s)** along the Y-axis. For each week, take the **Total Flux (p/s)** parameter for each sample group and determine any significant differences using the non-parametric Student's *t*-test.

REPRESENTATIVE RESULTS:

We generated breast cancer cell lines (MDA-MB-231, MCF-7, and MDA-MB-468) expressing GFP and luciferase vectors. Specifically, this was achieved by a sequential infection. First, the breast cancer cell lines were infected with a lentivirus vector expressing fluorescent GFP. The GFP-positive cells (GFP^+) were sorted 2 days post-infection (**Figure 1A,B**) and infected with the pLX304 Luciferase-V5 vector. Then, blasticidin was used to select for luciferase to generate the indicated (GFP^+, Luc^+) cells. To validate the *in vitro* luciferase activity, we demonstrated a cell number-dependent increase in the luciferase activity levels (**Figure 1C**). In addition, a linear correlation was found between the luciferase activity and the cell number (**Figure 1D**).

To confirm luciferase detection in the mice, all three GFP^+, Luc^+ breast cancer cell lines were injected into the mammary fat pad of female NOD/SCID mice. Then, the mice were subjected to bioluminescence reading every two weeks to determine the tumor growth kinetics. We found that tumor growth kinetics varies between the cell lines; it is faster in the more aggressive MDA-MB-231 and slower in the less aggressive cell lines MCF-7 and MDA-MB-468 (**Figure 2**).

Next, the fluorescence readings of the isolated tumors generated by the MDA-MB-231 cell line were obtained. Specifically, 6 weeks post injection, the tumors were harvested from the mice to confirm the GFP fluorescence; the tumors were found to maintain their GFP expression (**Figure 3A**). The next goal was to determine whether metastatic colony formation could be assessed in real time in the lung of a living mouse using the bioluminescence machine; positive bioluminescence readings were obtained from the lung of the whole mouse (**Figure 3B**). To verify that these were positive metastatic colonies, the lung was harvested, and the metastatic colonies were observed for GFP and bioluminescence (**Figure 3C**).

FIGURE AND TABLE LEGENDS:

Figure 1: Validation of GFP expression and luciferase activity in cells. (**A**) The GFP cells were sorted by FACS. Representative images of MDA-MB-231 non-GFP and GFP^+ cells. (**B**) MDA-MB-231, MCF-7, and MDA-MB-468 cells were infected with GFP-expressing virus, followed by FACS sorting. An image of each cell line is represented in brightfield (left) and GFP(right). The cells were captured under a Nikon Eclipse 80i microscope at 10x magnification. Scale bars = 100 µm. (**C**) The bioluminescence due to luciferase activity in each cell was determined by a luminometer. An increasing number of cells (as in **A**) were seeded in a black 96-well plate. The color bar represents the intensity of luminescence. (**D**) An XY plot demonstrating the luciferase activity of MDA-MB-

231 cells (as measured in **C**). Abbreviations: GFP = green fluorescent protein; SSC-A = area of side-scattered peak; GFP^+ = GFP-positive; FACS = fluorescence-activated cell sorting.

Figure 2: Kinetics of tumor growth was determined by the bioluminescence machine. *In vivo* tumor growth kinetics were determined weekly in NOD-SCID mice, and the representative images were captured using bioluminescence for (**A**) MDA-MB-231, (**B**) MCF-7, (**C**) MDA-MB-468. The color bar represents the intensity of luminescence. (**D**) Quantification of the luminescence activity is presented as total flux. (**E**) MDA-MB-231 mice; individual reading represented as a plot.

Figure 3: Different approaches to validate tumor formation and lung metastasis. (**A**) The mice were perfused, and the tumors generated from MDA-MB-231 cells were harvested. The GFP levels in the tumors were measured by the bioluminescence machine. (**B**) Lung metastasis in the whole mice, as shown by the bioluminescence. (**C**) To confirm the presence of metastases, the lungs were harvested and observed under SMZ18 Nikon Stereomicroscope (brightfield and GFP). Bioluminescent-Luc-samples were taken immediately after euthanizing the mice. The color bar represents the intensity of luminescence. Abbreviations: GFP = green fluorescent protein; Luc = luciferase; BLI = bioluminescence imaging.

DISCUSSION:

Animal-based experiments are obligatory for cancer research[7–9], and indeed many protocols have been developed[3, 6,10–14]. However, most of these studies determined the biological effect only at the end of the experiments, and thus the impact on tumor growth kinetics or metastasis colonization remains undetermined. Here, we provide a noninvasive dual bioluminescence approach by inoculating cells expressing GFP and luciferase into the mammary fat pad. Using this powerful tool, tumor development and metastasis can be monitored in mice in real time[14]. However, this technique contains a few critical steps, which demand extra caution. For example, one of the critical steps for the success of this experiment is to verify the efficiency of infection by monitoring the luciferase and GFP expression levels in the cells before mouse injection. Thus, the blasticidin dosages [15] and the lentiviral production[16] protocol should be optimized for each cell line to increase the experimental efficiency.

A few technical issues may affect the bioluminescence signal in the *in vivo* experiment. These issues include the mouse's movement during the bioluminescence reading, which may interfere with the image quality and thus affect the tumor kinetic curves. Thus, the animals must be fully anesthetized after the substrate injection and during the entire procedure. Additionally, placing multiple animals in the machine simultaneously may lead to inconsistency in luminescence reading as mice with a high signal can mask those of less intensity. Therefore, the luminescence readings must be taken individually for each mouse.

When conducting the *in vitro* bioluminescence reading, it is vital to replace the culture medium with PBS, as the medium contains serum and other supplements that may interfere with the signal. Additionally, it is necessary to eliminate the background reading by measuring the luminescence signal of a sample that only contains PBS (no cells).

This protocol describes a noninvasive technique to measure breast cancer cell growth and metastases. Specifically, this paper describes the injection of breast cancer cell lines, expressing both GFP and luciferase into the mouse mammary fat pad. This combination provides a quick and reliable method to measure metastatic colonization *in vivo* and *ex vivo*.

Despite the clear advantages of this method, it has some limitations. The primary constraint is the need for a bioluminescence machine, as this is a relatively expensive machine and therefore not always available. In addition, each read is time-consuming, and thus the machine can be overbooked and unavailable. Another limitation refers to the protocol itself. To detect the bioluminescence signal in the *ex vivo* samples, it is recommended to euthanize the mice and examine the sample immediately. This step is a time-limiting stage and is not feasible for a large set of experiments.

In conclusion, this noninvasive bioluminescence tool is highly sensitive to detecting tumor development and metastasis in mice. This protocol is not restricted to breast cancer and could be applied to other carcinomas such as lung and pancreatic cancer. Furthermore, because it is noninvasive, it can be applied to measure the efficacy of anticancer drugs[12] and their effects on tumor growth kinetics in real time.

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DISCLOSURES:

All authors have disclosed that they do not have any conflicts of interest.

REFERENCES:

1. Waks, A. G., Winer, E. P. Breast cancer treatment: A review. *JAMA*. **321** (3), 288–300 (2019).
2. Jin, X., Mu, P. Targeting breast cancer metastasis: *Breast Cancer: Basic and Clinical Research*. **9** (Suppl 1), 23–34 (2015).
3. Saha, D. *et al.* In vivo bioluminescence imaging of tumor hypoxia dynamics of breast cancer brain metastasis in a mouse model. *Journal of Visualized Experiments: JoVE*. (56) 3175 (2011).
4. Rashid, O. M. *et al.* Is tail vein injection a relevant breast cancer lung metastasis model? *Journal of Thoracic Disease*. **5** (4), 385–392 (2013).
5. Fantozzi, A., Christofori, G. Mouse models of breast cancer metastasis. *Breast Cancer Research*. **8** (4), 212 (2006).
6. Kocatürk, B., Versteeg, H. H. Orthotopic injection of breast cancer cells into the mammary fat pad of mice to study tumor growth. *Journal of Visualized Experiments: JoVE*. (96) 51967 (2015).
7. Baker, M. The whole picture. *Nature*. **463** (7283), 977–979 (2010).

8. Wang, Y., Tseng, J.-C., Sun, Y., Beck, A. H., Kung, A. L. Noninvasive imaging of tumor burden and molecular pathways in mouse models of cancer. *Cold Spring Harbor Protocols*. **2015** (2), 135–144 (2015).
9. Kim, J. E., Kalimuthu, S., Ahn, B.-C. In vivo cell tracking with bioluminescence imaging. *Nuclear Medicine and Molecular Imaging*. **49** (1), 3–10 (2015).
10. Paschall, A. V., Liu, K. An orthotopic mouse model of spontaneous breast cancer metastasis. *Journal of Visualized Experiments: JoVE*. (114) (2016).
11. Morten, B.C., Scott, R.J., Avery-Kiejda, K.A. Comparison of Three Different Methods for Determining Cell Proliferation in Breast Cancer Cell Lines. *Journal of Visualized Experiments*. (**115**) 54040 (2016).
12. Zimmerman, M., Hu, X., Liu, K. Experimental metastasis and CTL adoptive transfer immunotherapy mouse model. *Journal of Visualized Experiments: JoVE*. (**45**) 2077 (2010).
13. Lv, X. *et al.* Orthotopic transplantation of breast tumors as preclinical models for breast cancer. *Journal of Visualized Experiments: JoVE*. (**159**) doi: 10.3791/61173 (2020).
14. Cheng, R. Y. S. *et al.* Studying triple negative breast cancer using orthotopic breast cancer model. *Journal of Visualized Experiments: JoVE*. (**157**), doi: 10.3791/60316 (2020).
15. Bajikar, S. S. *et al.* Tumor-suppressor inactivation of GDF11 occurs by precursor sequestration in triple-negative breast cancer. *Developmental Cell*. **43** (4), 418–435.e13 (2017).
16. Khatib, A. *et al.* The glutathione peroxidase 8 (GPX8)/IL-6/STAT3 axis is essential in maintaining an aggressive breast cancer phenotype. *Proceedings of the National Academy of Sciences of the United States of America*. **117** (35), 21420–21431 (2020).

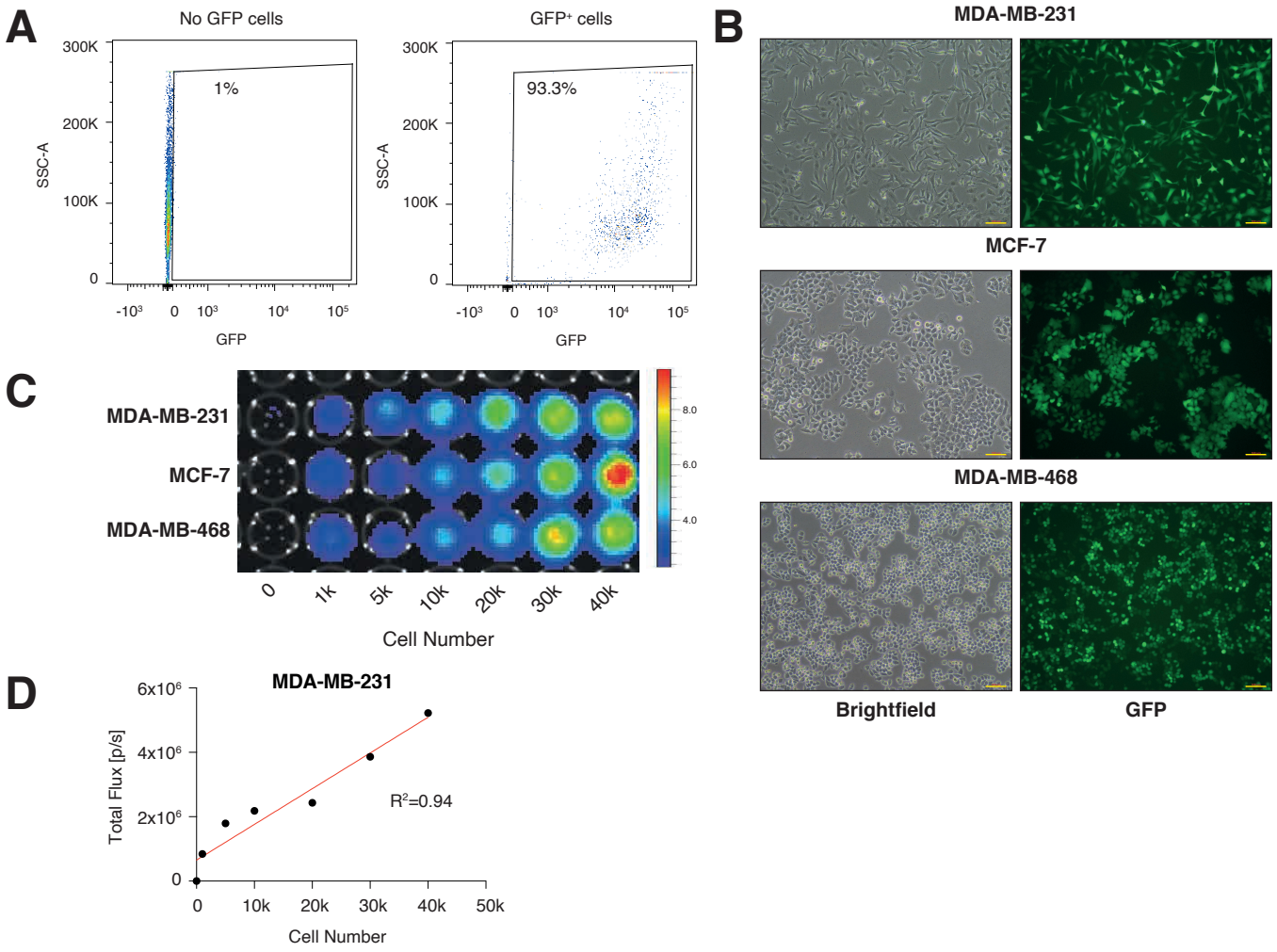

Figure 1

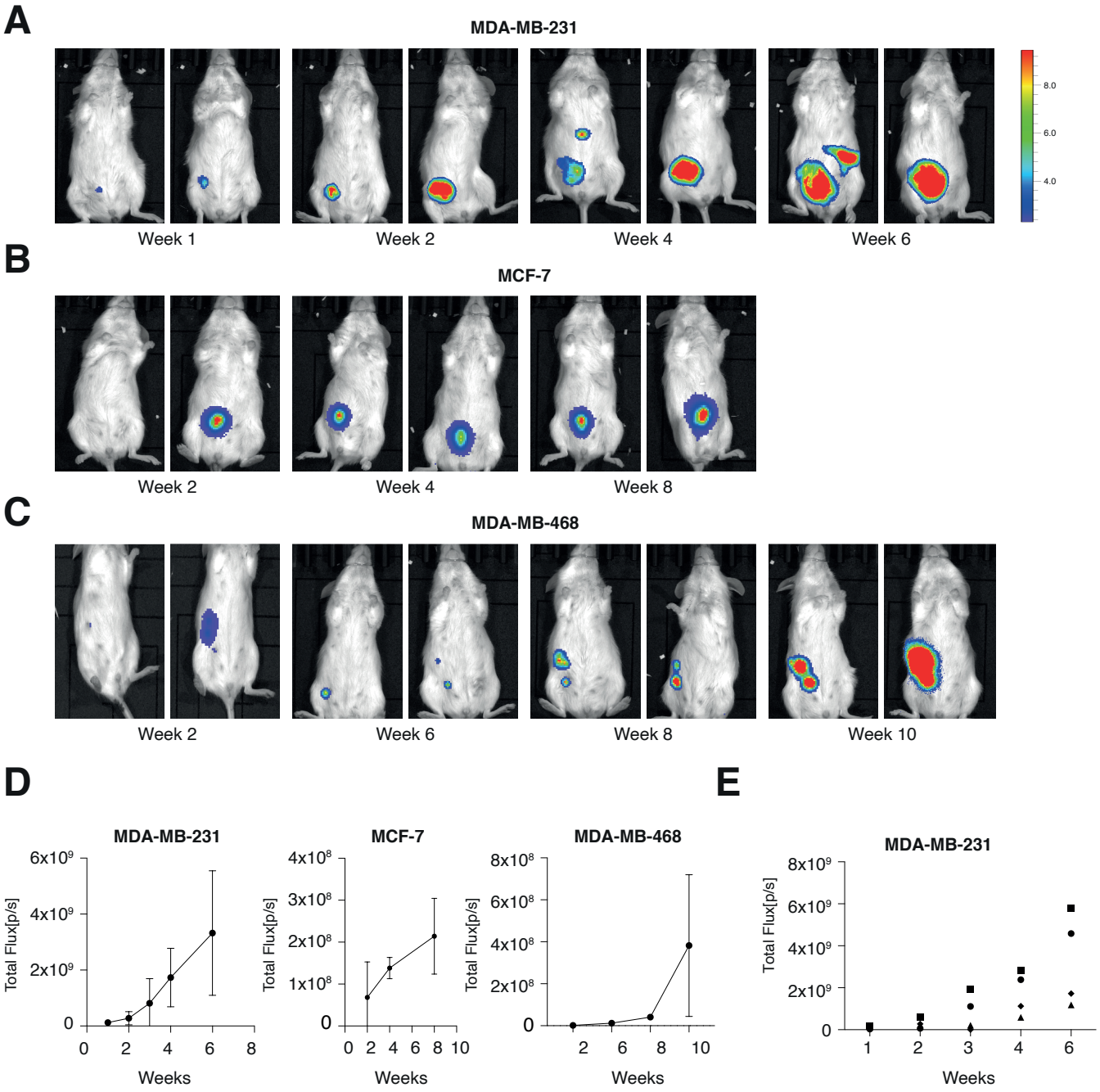

Figure 2

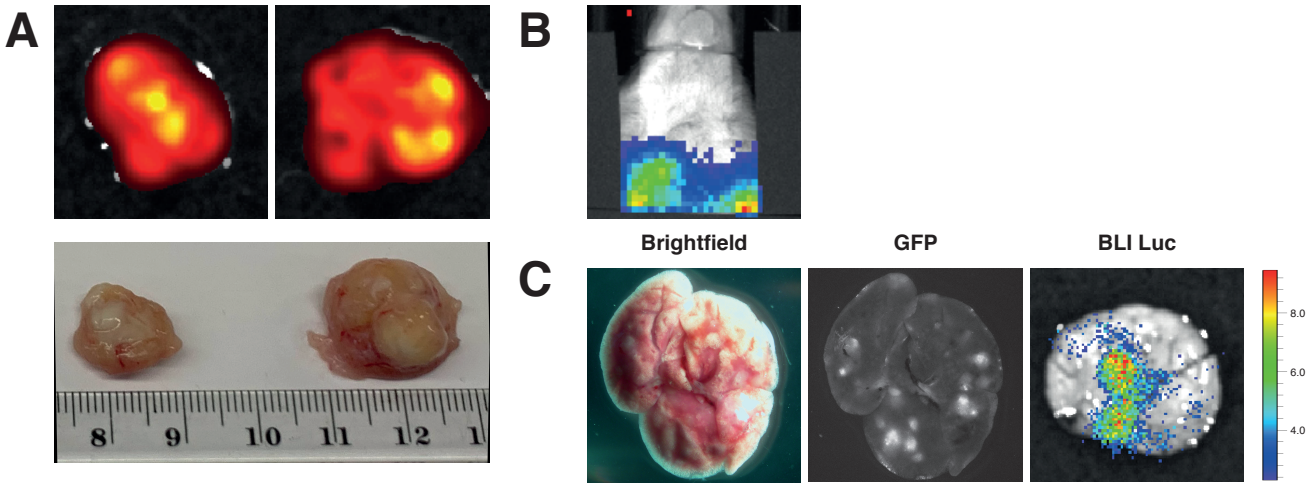

Figure 3

RESPONSE TO REVIEWERS

We have addressed all the reviewers' comments one by one, and responses are given below. The comments were extremely constructive and helpful, and by addressing them, we now have a much clearer to understand our protocol in the revised manuscript. In addition, we have added few figures to enlighten our protocol more precise way. We thank all the reviewers and editor for their valuable comments.

To help the reviewers, the changes are done by authors are marked in red in the revised manuscript file.

Editorial comments: Changes to be made by the Author(s): *1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.*

We thoroughly checked the spelling and the grammar issues throughout the manuscript. Then we subject the revised manuscript to further editing processes.

2. Please revise the following lines to avoid overlap with previously published work: 78-80.

We incorporated the changes in the revised manuscript.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript (this includes the figures and the legends) and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents, e.g., IVIS Spectrum In Vivo Imaging System, Perkin Elmer, USA etc

We checked the manuscript and removed all commercial language.

4. Being a video-based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:
a) What happened to the mice after the study? Please specify the euthanasia method without highlighting it.

We added the euthanasia method as step 7.1 in the revised manuscript.

b) Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

Thank you for the suggestion we have incorporated in the manuscript in step 5.3.

c) For survival strategies, discuss post-surgical treatment of animals, including recovery conditions and treatment for post-surgical pain.

We added a few more steps (step 5.6-7) where we discuss the post-injection procedure (step 5.6).

d) Discuss maintenance of sterile conditions during survival surgery.

We have mentioned this point in our manuscript in the step 5.2

e) Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.

We added a step where we discuss the post-injection procedure (step 5.6).

f) Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.

We added a step where we discuss the transfer of the mice to a new cage. We emphasis that the transfer will only take after the return of consciousness (step 5.7).

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We have changed the protocol section accordingly to the editor suggestion.

6. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

As per the suggestion from the editor, we modified the protocol section in our manuscript.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should

be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

In our manuscript we elaborated the protocol section with more specific details.

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

As suggested, we simplified the protocol portion in the throughout protocol section.

9. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.

The paper format is completely modified throughout the manuscript according to the JOVE instruction to authors.

10. Please discuss all figures in the Representative Results. However, for figures showing the experimental setup, please reference them in the Protocol.

We discussed all the figures in the representative results in our revised manuscript.

11. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

We have included and described the representative results of our revised manuscript.

12. Are there any limitations of this technique? Please add to the discussion.

We have incorporated limitations of the technique in the discussion section of our revised manuscript.

13. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal

names. Make sure all references have page numbers or if early online publication, include doi.

As per the jove model, the reference section modified accordingly in our manuscript.

14. Please add all items (plasticware, glassware, buffers, solvents, equipment, software etc) in the Table of Materials so that it serves as a handy reference for users to get everything ready for the protocol. Please sort the Materials Table alphabetically by the name of the material.

We added all the items in the revised manuscript.

Reviewers' comments:

Reviewer #1:

Manuscript Summary: This manuscript describes a useful protocol for expressing luciferase and GFP in cancer cells as a means for monitoring tumor growth and metastasis in vivo/ex vivo in real time.

Major Concerns: None.

Minor Concerns:

Minor suggestions are as follows: - The authors describe in the Abstract the idea that luciferase expression can be used to facilitate non-invasive imaging of tumor growth in living mice, while GFP expression can be used to facilitate the microscopic ex vivo analysis of disseminated tumor cells in animal tissues. This dual use aspect of the system is unique and has advantages over more commonly used, single mode systems involving only luciferase expression, which works well for imaging tumor growth in living mice but is not as useful for microscopic ex vivo analyses that require spatial resolution. I suggest that the authors spend some effort in the Discussion to discuss this aspect of their system in more detail.

The authors often use the word "transfection" or its derivatives when describing lentiviral infections. In some circles, the word "transfect" is reserved for non-viral introduction of DNA or related species into cells, while viral delivery is termed "transduction." I would consider using "transduction" in the current manuscript for this reason.

We would like to thank the reviewer for his comment, we changed “transfection” into “infection” throughout the manuscript.

In the caption for Figure 3A, the authors state that GFP expression is being monitored by IVIS imaging. This image appears to instead reflect the measurement of luminescence by luciferase. Please clarify.

In the “representative results” chapter we elaborate on the methods and emphasis that this is fluorescence reading.

There are some minor typographical errors in the manuscript that should be corrected during copy-editing.

As we wrote to the editor, we thoroughly checked the spelling throughout the manuscript. Then we subject the revised manuscript to further editing processes.

Reviewer #2:
Manuscript Summary:
The authors are proposing to create a JOVE video and protocol showing how to use human breast cancer cells expressing both a fluorescent protein (GFP) and the firefly luciferase gene in combination with IVIS imaging to monitor primary mammary tumor formation and spontaneous metastasis. Though JOVE publications exist that combine a fluorescent protein (GFP) and luciferase-mediated real-time detection of tumors in mice, this protocol would add to this existing literature in a meaningful way. Use of IVIS imaging to monitor primary breast cancer growth has not been demonstrated in a JOVE video, despite the clear power of this approach.

Major Concerns:
I support this article being published, but feel the authors need significantly increase the detail in their protocol before the article is accepted. This will help ensure others can use this approach. I detail my suggestions below:

We would like to thank the reviewer for his valuable comments, we significantly changed the protocol section and added additional details.

Step 2.2 needs more detail:
Which luminometer was used and what were the settings?

We have included in the protocol section of revised manuscript with more details and setting (steps 6).

They should explain why they used a black-well dish (it is an important detail so the reader should know why).

In step 4.3 we elaborate on the black well plate usage in the protocol section.

There is no description of control cells or wells in this step. Do they subtract background signals?

Yes, we subtract the background see step 4.5.

There is no indication of what they consider to be adequate luciferase activity? It is important to indicate how they ensure an adequate percentage of cells is expressing luciferase and also what level of expression they consider adequate for in vivo experiments. Weak expression makes metastases hard to detect.

In step 6.2 we added a note that indicates that a control mouse (injected with cells not expressing the luciferase genes), are needed to be examined to eliminate any background readings.

Is it important to use a dilution series here and if so why? Did they check for linear increases in luciferase activity or are they just making sure the cells are positive?

In the “Representative Results” chapter we elaborated on the importance of the series dilution. In addition, in figure 1D we added a graph representing that the readings vs. cell number gave a linear correlation.

Step 2.3 does not contain adequate details:
Am I correct that this is an injection through the skin into the mammary gland using anatomical landmarks to locate the mammary gland? Or is this a surgical procedure in which the mammary gland is exposed for the injection. Both of these protocols are common and it is not clear from the text as written. I am assuming it is just an injection, but the authors write "the mammary gland is wiped with ethanol prior to injection" which may confuse a reader into thinking the gland is exposed. I assume they meant to say the skin above the gland is wiped with ethanol? If this is just an injection, more details are still necessary to help the reader.

We modified the protocol accordingly to make this point clearer, as we did not apply any surgical procedure. The cells were injected at the 4th of the Abdominal mammary gland (Step 5.4).

Is hair removed from injection site?

No, we inject the cells directly without removing the hair.

Which fatpad is injected?

We have incorporated the protocol portion of the manuscript in step 5.4.

There is no mention of restraint (physical or chemical). Are the mice anaesthetized?

We have included a complete anaesthetize procedure in the protocol portion of the manuscript in step 5.2.

What landmarks are used to ensure the injection is in the fatpad. How is the mouse positioned during the injection?

To visualize the correct mammary fatpad the skin above the gland is wiped with ethanol (step 5.4). This step is important as to verify the correct injection region. After the injection there will be a round bulge appearing under the skin. The mice were kept in supine position during the injection as described in step 5.3.

While some of this will be visualized in the video, it is critical to describe it well to ensure others can do this technique properly.

We have described all the protocol in a more elaborate way in our manuscript.

If this is a surgical procedure extensive details on the surgical procedure are necessary.

In this manuscript we only describe the injection procedure and not surgical.

Section 2.4 does not contain adequate details:

We have included all the details in our revised manuscript see step 6.

Lines 121-123 belong in this section not section 2.3.

We moved these specific lines to the respective paragraph in the revised manuscript (step 6).

The incubation time following luciferin injection is critical and should be kept consistent. Perkin Elmer recommends imaging no sooner than 10 minutes post injection, but they also suggest an optimization be done to determine the appropriate window of time post luciferin that the mice should be imaged. This can change depending on the mouse strain, cell lines used, the expression level of luciferase and the location of the tumors. Authors should note that this optimization should be done.

First, we thank the reviewer for the valuable comment. Indeed, the incubation time differs from cell line to cell and varies from species to species and most importantly the expression level will determine the kinetics of the signal therefore, we recommend calibration by their own before starting experiments for respective cell lines (step 6.3).

Details on how the lucifierin is injected should be more extensive including how mouse is re-strained, where the injection is made, and needle size. Are the mice anesthetized then injected with luciferin. These details are critical when trying to do a procedure for the first time, and not all steps can be included in the video.

In the revised manuscript we elaborate on the luciferin injection procedure in step 6.2 and 6.3.

How the mice are positioned in the IVIS machine is also critical and should be explained. Positioning can greatly influence the detection of smaller tumors (especially metastases)

After the IP procedure the mice are anesthetized. During the bioluminescent detection the mice are still placed with a cone over their head in a supine position (steps 6.5 and 5.3).

What settings are used during for image acquisition? Stage height and exposure time are not described. Is this an auto exposure? What are the other settings set to?

We have included all the details in the updated protocol section of our revised manuscript (steps 6.6- 6.13).

They should indicate the importance of using a control mouse that was not injected with luciferase positive cells to help distinguish background signal from real signal

As the reviewer suggested, we indicate the importance of control mice in the revised manuscript (Step 6.6).

There is no description of how the data is processed or analyzed. Do they use ROIs or are they measuring total body flux? The processing of the IVIS images is a critical step to ensure the data is accurate and differences can be detected. Though the authors are not showing the use of this to compare tumor growth between 2 experimental samples, this is presumably the use of this procedure. So it is critical to describe how to analyze and compare images from different mice and different experimental groups. Is there any normalization here? If two or more groups were compared how would statistics be done.

We elaborated in more detail the data processed (Step 9). Specifically, in step 9.6 we describe the usage of different experimental samples.

Minor Concerns:

- Lines 71 and 72 the authors write the cells were stably transfected, yet they appear to be using lentivirus to stably transduce the cells- If so, it is important they use the proper terminology and make it clear that the cells were stably transduced with lentivirus that delivered GFP or luciferase.

We thank the reviewer for the valuable comment. We changed the word from “transfection'' to “infection” throughout the protocol.

- Line 81- should probably say "determine cell number"? Not cell density

We changed the word from “cell density” to “cell number” in our revised manuscript.

- Line 92- Did the authors really use a .22uM syringe filter to remove cell debris? Most protocols use 0.44 as 0.22 can reduce viral titres. If 0.22 is accurate no change is necessary.

The reviewer is correct. We changed the manuscript to 0.44µm (step 2.11).

- How was the data is Figure 2D generated? Is this an average of raw flux for each mouse at each timepoint? A dot for each mouse at each timepoint mice should be shown to demonstrate the variability that is expected in this experimental model. They should consider individual traces and then a best-fit line. Alternatively, they can plot each mouse's signal as dot plots. As indicate above, most often this approach would be used to compare experimental groups, so its important they discuss how they would process data and what statistical tests would be used to compare groups.

As the reviewer suggestion we add a figure representing each mice signal from the mice in our revised manuscript (Figure 2E).

- In 3A they say mice were perfused. With what?

At the end of the experiment day all the mice were perfused using 0.9% saline and then selected organs were harvested for further analysis (step 7.2).

- They mention a few critical steps in the discussion that should be included in the protocol itself:
They mention that ensuring high infection efficiency is a critical step, but they do not describe how they do this in their procedure. This point was raised above. In my comments on Section 2.2.

We have included it in the revised protocol section of our manuscript (Step 3.7).

They mention use of a blank in the in vitro luciferase step but this is not in protocol.

We incorporated the use of blank in the in vitro luciferase activity in our revised manuscript (Step 4.5).

- They do not describe uses for this procedure very well. What actual experiments would be done using this approach? Most researchers would not just measure tumor growth of a single cell line.

We described each and every step in the revised manuscript.

- There are existing JOVE papers that describe similar approaches to this one, including a few that use breast cancer cells and some that couple fluorescence with bioluminescence. This manuscript and procedure is still worthy of publication in my opinion, but the authors should acknowledge the other JOVE publications and explain why their procedure is different or has slightly different uses. Please note I am not suggesting you need to explain why your approach is superior (or the others inferior), more that you explain how yours differs.

We add a few references describing other protocols