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