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Multimodal Bioluminescent and PET/CT Imaging of Multiple Myeloma Bone Marrow Xenografts in NOG Mice

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TITLE:

Multimodal Bioluminescent and Positronic-emission Tomography/Computational Tomography Imaging of Multiple Myeloma Bone Marrow Xenografts in NOG Mice

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KEYWORDS:

multiple myeloma, xenografts, bioluminescence, PET/CT, tumor microenvironment, bone marrow

SUMMARY:

Here we use bioluminescent, X-ray, and positron-emission tomography/computed tomography imaging to study how inhibiting mTOR activity impacts bone marrow-engrafted myeloma tumors in a xenograft model. This allows for physiologically relevant, non-invasive, and multimodal analyses of the anti-myeloma effect of therapies targeting bone marrow-engrafted myeloma tumors *in vivo*.

ABSTRACT:

Multiple myeloma (MM) tumors engraft in the bone marrow (BM) and their survival and progression are dependent upon complex molecular and cellular interactions that exist within this microenvironment. Yet the BM microenvironment cannot be easily replicated *in vitro*, which

potentially limits the physiologic relevance of many *in vitro* and *ex vivo* experimental models. These issues can be overcome by utilizing a xenograft model in which luciferase (LUC)-transfected 8226 MM cells will specifically engraft in the mouse skeleton. When these mice are given the appropriate substrate, D-luciferin, the effects of therapy on tumor growth and survival can be analyzed by measuring changes in the bioluminescent images (BLI) produced by the tumors *in vivo*. This BLI data combined with positronic-emission tomography/computational tomography (PET/CT) analysis using the metabolic marker 2-deoxy-2-(^{18}F)fluoro-D-glucose (^{18}F-FDG) is used to monitor changes in tumor metabolism over time. These imaging platforms allow for multiple noninvasive measurements within the tumor/BM microenvironment.

INTRODUCTION:

MM is an incurable disease made up of malignant plasma B-cells that infiltrate the BM and cause bone destruction, anemia, renal impairment, and infection. MM makes up 10% - 15% of all hematological malignancies[1] and is the most frequent cancer to involve the skeleton[2]. The development of MM stems from the oncogenic transformation of long-lived plasma cells that are established in the germinal centers of lymphoid tissues before eventually homing to the BM[3]. The BM is characterized by highly heterogeneous niches; including diverse and critical cellular components, regions of low pO_2 (hypoxia), extensive vascularization, complex extracellular matrices, and cytokine and growth factor networks, all of which contribute to MM tumorgenesis[4]. Thus, the development of a disseminated MM xenograft model characterized by tumors that are strictly engrafted in the BM would be a very powerful and clinically relevant tool to study MM pathology *in vivo*[5,6]. However, numerous technical hurdles can limit the effectiveness of most xenograft models, making them costly and difficult to apply. This includes problems associated with consistent and reproducible tumor engraftment within the BM niche, a prolonged time to tumor development, and limitations in the ability to directly observe and measure changes of tumor growth/survival without having to sacrifice mice during the course of the experiment[7,8].

This protocol uses a modified xenograft model that was initially developed by Miyakawa *et al.*[9], in which an intravenous (IV) challenge with myeloma cells results in “disseminated” tumors that consistently and reproducibly engraft in the BM of NOD/SCID/IL-2γ(null) (NOG) mice[10]. The *in situ* visualization of these tumors is achieved by the stable transfection of the 8226 human MM cell line with a LUC allele and serially measuring the changes in the BLI produced by these engrafted tumor cells[6]. Importantly, this model can be expanded to utilize various other LUC-expressing human MM cell lines (*e.g.*, U266 and OPM2) with a similar propensity to specifically engraft in the skeleton of NOG mice. The identification of the tumors by bioluminescent imaging of the mice is followed by measuring the uptake of radiopharmaceutical probes (such as ^{18}F-FDG) by PET/CT. Together, this allows for additional characterization of critical biochemical pathways (*i.e.*, alterations in metabolism, changes in hypoxia, and the induction of apoptosis) within the tumor/BM microenvironment. The major strengths of this model can be highlighted by the availability of a wide range of radiolabeled, bioluminescent and fluorescent probes and markers that can be used to study MM progression and pathology *in vivo*.

PROTOCOL:

All animal procedures described below were approved by the Institutional Animal Care and Use Committee (IACUC) of the Greater Los Angeles VA Healthcare system and were performed under sterile and pathogen-free conditions.

1) Preparation of Luciferase-expressing 8226 Cells (8226-LUC)

1.1) Maintain the human MM cell line, 8226, in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere containing 95% air and 5% carbon dioxide (CO_2).

1.2) Generate stable LUC-expressing reporter 8226 cells by transfecting 1 x 10^6 8226 cells with a luciferase reporter vector followed by a selection with hygromycin (100 - 350 mg/mL) as previously described[6].

1.3) Maintain the cells under standard sterile culturing conditions for 2 - 3 weeks, changing the medium every other day, until the establishment of a stably transfected 8226 cell line has been generated.

1.4) Confirm the *in vitro* luciferase activity in 1 x 10^6 transfected cells/100 µL of phosphate buffered saline (PBS) by adding the luciferase substrate, D-luciferin (150 µg/mL working solution in prewarmed culture medium), and measuring the bioluminescence of the cells in a test tube or 96-well plate using a luminometer[6].

2) Orthotopic Xenograft Model

2.1) Maintain NOG mice (4 - 6 weeks old, male or female) under sterile/pathogen-free conditions in an appropriate animal care facility.

2.2) Prepare the 8226 LUC-transfected cells for IV injection into the NOG mice (~5 x 10^6 cells/mouse) on the day of the experiment. Wash the cells 3x in ice-cold PBS, count them, and resuspend them in ice-cold PBS (minus antibiotics and FBS) at a final concentration of 5 x 10^6 cells/200 µL of PBS) in a sterile test tube. Keep the cells on ice and challenge the mice as soon as possible (within 30 - 120 min).

2.3) Anesthetize the mouse (with isoflurane, 1% - 3% in air at 0.5 - 1 L/min) and place the animal in a supine position on a heating pad with the head facing away. Check the level of anesthetization by pinching the toe. Apply ophthalmic ointment to the eyes.

2.4) Inject the 8226-LUC cells into the mice through the tail vein (200 µL/injection) using a 1 mL insulin syringe and a 26-G needle.

Note: A heat lamp can be used to warm the tail, thereby dilating the vein and increasing the efficacy of injections.

2.5) Return the animal to their home cage and monitor the animals until they have fully recovered.

2.6) Confirm the engraftment of 8226-LUC cells in the mouse skeleton by measuring the BLI in anesthetized mice (described below and shown in **Figure 1A**).

Note: BM exudates can also be stained for human CD45 expression using flow cytometry (**Figure 1B**) or by the immunohistochemistry of harvested bone (**Figure 1C**).

3) Measurement of BLI *In Vivo*

Note: Typically, measurable BLI from engrafted tumors in mice can be observed first between 10 - 20 days postchallenge, but this may need to be experimentally determined.

3.1) Anesthetize the mouse (with isoflurane, 1% - 3% in air at 0.5 - 1 L/min) and check the level of anesthetization by pinching the toe. Apply ophthalmic ointment to its eyes.

3.2) Give the animal an IP injection (200 µL/injection) of "*in vivo*-grade" D-luciferin substrate (30 mg/mL diluted in sterile saline).

3.3) Measure the BLI within 5 - 10 min, following the injection of the luciferin substrate (although the BLI activity can be observable in the mice for up to 45 - 60 min), by placing the anesthetized animal in a supine position in a small-animal imaging system (**Figure 2**). Select luminescent and X-ray analysis and acquire images (**Figure 3**).

3.3.1) Measure the average radiance (photons/s/cm^2/steradian) in selected regions of interest (ROIs) using imaging software (**Figure 4).**

3.3.2) Return the animal to its home cage and monitor it until it has fully recovered (approximately 15 - 20 min).

4) Treatment of the Mice with Targeted Therapy

4.1) Measure the baseline BLI and randomize the animals into treatment groups (4 - 8 mice/group).

4.2) Treat the mice with an IP injection (200 µL/injection) of temsirolimus (0.2 - 40 mg/kg mouse) using a regimen of five daily IP injections followed by 2 days of rest and an additional five daily injections[11].

4.3) Measure the luciferase activity (photons/s/cm^2/steradian) 2x per week as described in section 3 and plot the change of the BLI over time. Changes in tumor BLI can be presented as serial images of the mice (**Figure 5B**).

Note: It is recommended that daily monitoring of the animals be performed until they present the following symptoms (typically within 45 - 60 days of the initial challenge): weight loss (loss of more than 10% relative to the weight prior to implantation) and/or hind limb paralysis, at which time they should be euthanized using a CO_2 chamber followed by cervical dislocation.

5) PET/CT Analysis Using ^{18}F-FDG to Measure Changes in Tumor Metabolism

5.1) Fast the animals in their home cage by removing their food for 24 h prior to the experiment to avoid excess non-specific uptake of ^{18}F-FDG.

5.2) Prepare ^{18}F-radiolabeled FDG PET probes (50 - 100 µCi/injection in sterile saline) on the day of the experiment. Record the time and activity of the ^{18}F-FDG probe with a dose calibrator.

Note: Because ^{18}F has a relatively short half-life (~2 h), a careful preparation and planning for the use of these probes must be established.

5.3) Anesthetize the animal (with isoflurane, 1% - 3% in air at 0.5 - 1 L/min) and place it in a supine position on a heating pad with the head facing away. Check the level of anesthetization by pinching the toe. Apply ophthalmic ointment to its eyes.

5.4) Inject the probe *via* the tail vein (100 µL/injection) using a shielded 1 mL insulin syringe and a 26-G needle.

Note: A heat lamp can be used to warm the tail, thereby dilating the vein and increasing the efficacy of injections.

5.5) Measure the residual radioactivity in the needle/syringe in a dose calibrator and note the activity and time.

Note: The standardization of mouse incubation, dosage, and timing is essential to ensure data reproducibility and comparability.

5.6) Maintain the mice under anesthesia and at 37 °C using a heating pad during the whole period of probe uptake (~45 - 90 min).

Note: It is important that the mice remain quiescent and at 37 °C to avoid non-specific uptake of the probe.

5.7) Stagger the injections of the ^{18}F-FDG probe to occur at approximately 20- to 30-min intervals to ensure similar labeling times in the mice.

Note: Proper workflow and timing of the probe injections will result in optimal and reproducible imaging conditions.

5.8) Measure the ^{18}F-FDG activity in a PET/CT small-animal imaging system in selected regions of interest that correspond to the engrafted tumors (**Figure 6**).

Note: Initially, a 10-min static PET exposure and a 2-min CT exposure are recommended, but the exact exposure times may need to be experimentally determined.

5.9) Return the animals to their home cages and monitor them until fully recovered. House the mice in a dedicated return room for 24 h while the probe decays to background levels.

Note: Because of the short half-life of ^{18}F, multiple measurements using a fresh probe can be made as frequently as 2x per week.

REPRESENTATIVE RESULTS:

In initial pilot studies, IV injections of 8226-LUC cells into NOD/SCID mice did not develop BM-engrafted MM tumors, although squamous MM tumors were easily formed (100% success rate). In contrast, IV challenges with 8226 cells into NOG mice generated (within 15 - 25 days) tumors in the skeleton (and only rarely formed tumors in non-skeletal tissue, such as the liver or spleen). Since tumors in the skeleton could not be visually confirmed by physically examining the animals, other methods had to be considered to confirm successful tumor engraftment and the location of the tumors within the BM (**Figure 1**). Similar to the reports by Miyakawa *et al.*[9], human IgG, produced by 8226 cells, could be detected in the serum of challenged NOG mice using ELISA (data not shown), although these data only confirmed engraftment and not the location or extent of the tumors. Serial imaging of multiple animals shows the distribution of BM-engrafted MM tumors (**Figure 4**). The BLI produced by these engrafted tumor cells can then be serially and non-invasively measured to assess changes in tumor growth and survival in mice treated with the mTOR inhibitor temsirolimus (**Figure 5**). Finally, PET/CT analysis for the uptake of ^{18}F-FDG in the tumors was used to demonstrate temsirolimus-mediated changes in glucose metabolism and that this correlated with changes in tumor growth (**Figure 6**). However, by stably transfecting 8226 cells with a luciferase allele, in conjunction with an optical imaging/X-ray analysis, the exact location and distribution of MM tumors in the mouse skeleton could be quickly and non-invasively determined.

FIGURE LEGENDS:

Figure 1: Confirmation of the engraftment of 8226-LUC cells in the mouse skeleton. (**A**) NOG mice were challenged with 5 x 10^6 8226-LUC2 cells IV (mouse on the left) or with saline (mouse on the right). After 15 days, the mice were given an IP injection of D-luciferin substrate and imaged using a small-animal imaging system. (**B**) The mice were sacrificed, the femurs were collected, and the BM cellular exudate was harvested. The expression of human CD45 was determined by flow cytometry using a PE-conjugated anti-human CD45 antibody. (**C**) This panel shows an immunohistochemistry of serial sections of mouse femurs stained for hypoxia using pimonidazole (top panels) or human CD45 (bottom panels) in mice challenged with 8226 (right panels) or saline (control; left panels). The asterisk indicates the location of a large blood vessel in the serial sections. The scale bar = 100 µm.

Figure 2: Anesthetized NOG mice showing positioning in the imaging system prior to measuring the BLI signal from bone marrow-engrafted MM tumors.

Figure 3: Set-up of the optical imaging and X-ray analysis of the bioluminescent signal collected from NOG mice. Once the animals have been successfully challenged and the engraftment of tumors in the BM has been confirmed, the mice can be randomized into treatment groups. At various times during the course of the experiment, the animals can be monitored for changes in the BLI. Because the procedure is noninvasive, the frequency of monitoring could be altered to reflect the expected growth of the tumors, as well as how quickly the cancer cells will respond to therapy.

Figure 4: Serial imaging of multiple animals showing the distribution of bone marrow-engrafted MM tumors. The regions of interest (ROI) for each mouse are identified.

Figure 5: Changes in the BLI of engrafted tumors in NOG mice during an anti-MM therapeutic regimen. (**A**) This panel shows a change in the average radiance (p/s/cm^2/ser) in temsirolimus (20 mg/kg mouse)-treated NOG mice challenged IV with 5 x 10^6 cells. Once the mice were observed to be positive for engrafted tumors, they were randomized into various treatment groups. The mice were given five IP injections daily, followed by 2 days of rest and, then, an additional five IP injections of temsirolimus (the bars on the X-axis indicate the days of treatment) or vehicle control. On various days, the mice were given IP injections of "*in vivo*-grade" D-luciferin, and the BLI was measured using an optical imaging platform. The values represent the average radiance (p/s/cm^2/ser) ± a 95% confidence interval. (**B**) This Kaplan–Meier plot shows the change in percentage of the surviving mice over time. Mice that had reached the endpoint criteria (*i.e.*, a loss of > 10% body weight or hind-limb paralysis) were humanely euthanized. (**C**) This panel shows representative images of mice taken on days 28, 35, and 40, showing changes in the LUC activity.

Figure 6: Serial imaging and relative changes in the ^{18}F-FDG uptake by PET/CT. (**A**) This panel shows serial imaging of the same mouse for bioluminescence (optical imaging/X-ray) imaging and ^{18}F-FDG uptake by PET/CT. BLI was measured in anesthetized mice, and the PET/CT imaging of the same mouse was performed 24 h later. The mouse fasted overnight and, on the day of the study, received an IV injection of 50 µCi ^{18}F-FDG. The mice were maintained under anesthesia during the probe uptake incubation (60 min) and, then, were analyzed for ^{18}F-FDG uptake by PET/CT analysis. Tumors (T1 and T2) are indicated. H = heart, K = kidney, and B = bladder. (**B**) This panel shows relative changes (in a percentage of untreated control tumors) in the ^{18}F-FDG uptake in control mice (no treatment) or temsirolimus (20 mg/kg mouse)-treated mice (measured at days 22, 35, and 40). The data is presented as the mean (*n* = 5 mice) ± 1 S.D.

DISCUSSION:

Despite a variety of preclinical xenograft models of MM[6,9,11-13], the ability to study the tumor/BM interactions within the BM microenvironment remains difficult[14]. The techniques described here

allow for the rapid and reproducible engraftment of 8226-LUC tumors cells in the skeleton of NOG mice.

The critical steps in this protocol involved the establishment of luciferase-expressing MM cell lines and the verification of a stable expression of luciferase *in vitro*[15]. Once the cell lines have been established, NOG mice are challenged by IV injection, and the engraftment of tumors to the skeleton is confirmed by measuring the BLI activity *in vivo* (typically within 10 - 20 days postchallenge) (**Figure 1A**). The use of NOG mice is important because other immunodeficient strains (such as NOD/SCID) do not typically form BM-engrafted tumors. The relatively high success rate of implantation (> 90%) and the short interval to observe a positive BLI signal makes this model ideal for a high-throughput and longitudinal analysis of anti-MM therapeutic modalities (**Figure 4**). Another very important aspect of this model is that the BM-engrafted MM cell lines maintain their morphological and immunologic features of the parent cell lines (*e.g.*, 8226, U266); they have consistent and reproducible growth patterns and exhibit characteristics of patient diseases, such as increasing serum human IgG paraprotein levels and the formation of bone lesions.

The advantage of this strategy is the utilization of these powerful imaging techniques to repeatedly study biochemical and molecular components of the tumor/BM microenvironment over the course of the disease progression and in response to anti-tumor therapies. In this experiment, we found that targeting mTOR activity in mice with BM-engrafted myeloma tumors resulted in a decrease in bioluminescent measurements that could be observed over time. Furthermore, we found that changes in the uptake of the ^{18}F-FDG probe (**Figure 6B**) in these same tumors were correlated to changes in the bioluminescent signal (**Figure 5B**). Another critical component of this procedure is that multiple biochemical variables can be measured within the tumor over time. This is especially important because tumor progression is a dynamic process characterized by changes in tumor physiology and microenvironment characteristics. Thus, this procedure, because of its non-invasive nature and the relatively short half-life of the probes, allows for multiple longitudinal analyses of changes in the tumor response to therapy.

After mastering the technique, the future applications of the technique present a great deal of flexibility. For example, the use of other bioluminescent or fluorescent tags (*e.g.*, fluorescently tagged antibodies) could also be utilized, as could various other ^{18}F-labeled probes and radiopharmaceutical compounds to study specific biochemical pathways or processes in the tumor/BM microenvironment. Because of the short half-life of many of these radionucleotides, multiple serial measurements could be made during the course of a specific experiment to study drug uptake, distribution, kinetics, and decay. Using this xenograft model, the ability of MM to utilize anaerobic glycolysis and other metabolic pathways (*i.e.*, fatty acid synthesis) by using other probes (*e.g.*, ^{18}F-fluoro-2deoxyglucose [^{18}F-FDG] and ^{18}F-fluoro-4-thia-palmitate [FTP])[16,17], as well as the ability to measure the anti-proliferative effects of treatment using another widely used probe (*i.e.*, 3'-deoxy-3'-[^{18}F]fluorothymidine [^{18}F-FLT]) can be included in the experimental designs. In addition, it may be possible to directly measure cell death by using ^{18}F-Annexin B1 to measure apoptosis *in vivo*[18]. Many of these compounds are commercially available.

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DISCLOSURES:
The author Kevin Francis is an employee of Perkin-Elmer.

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Figure 1

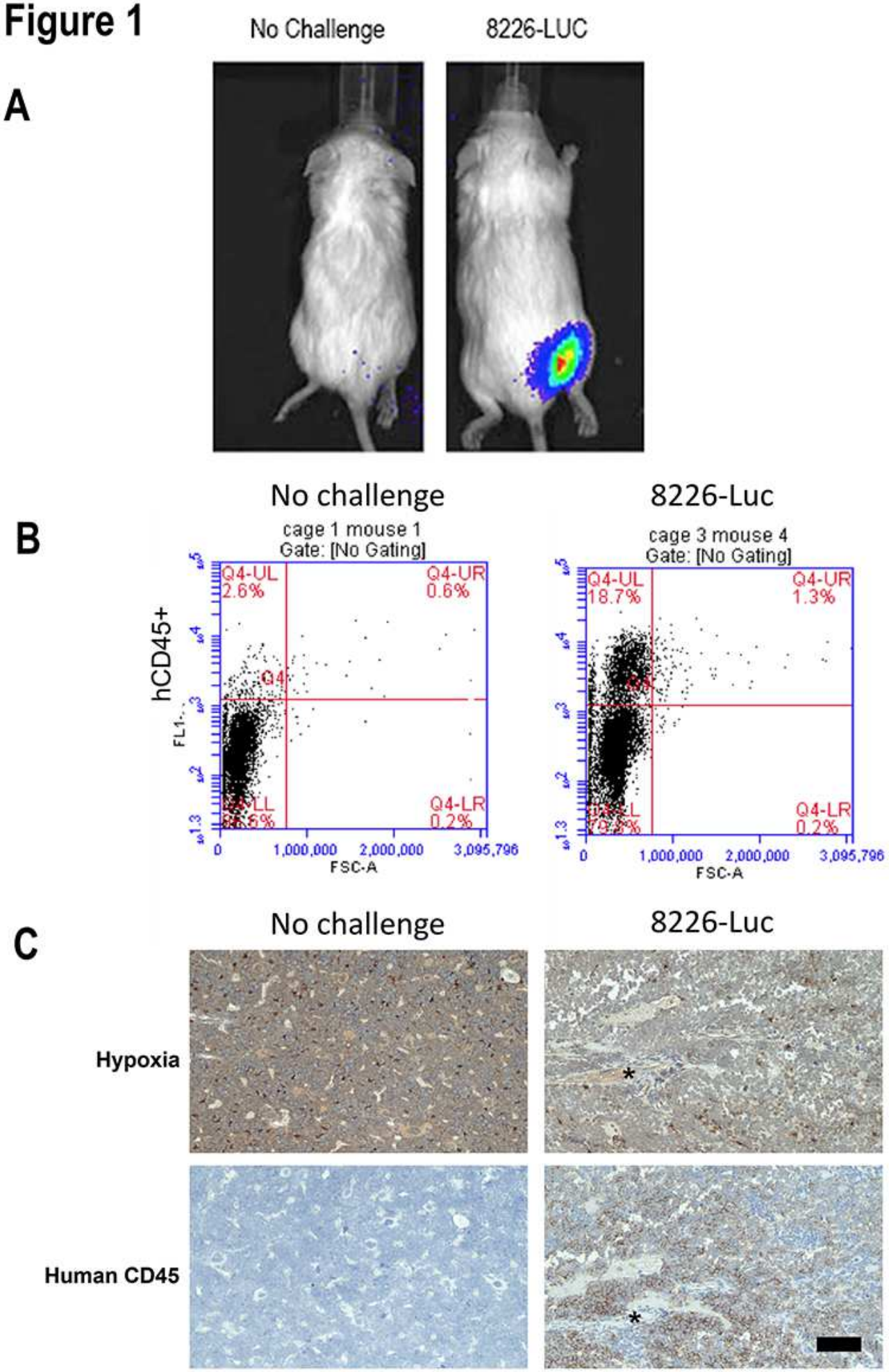

Figure 2

Figure 3

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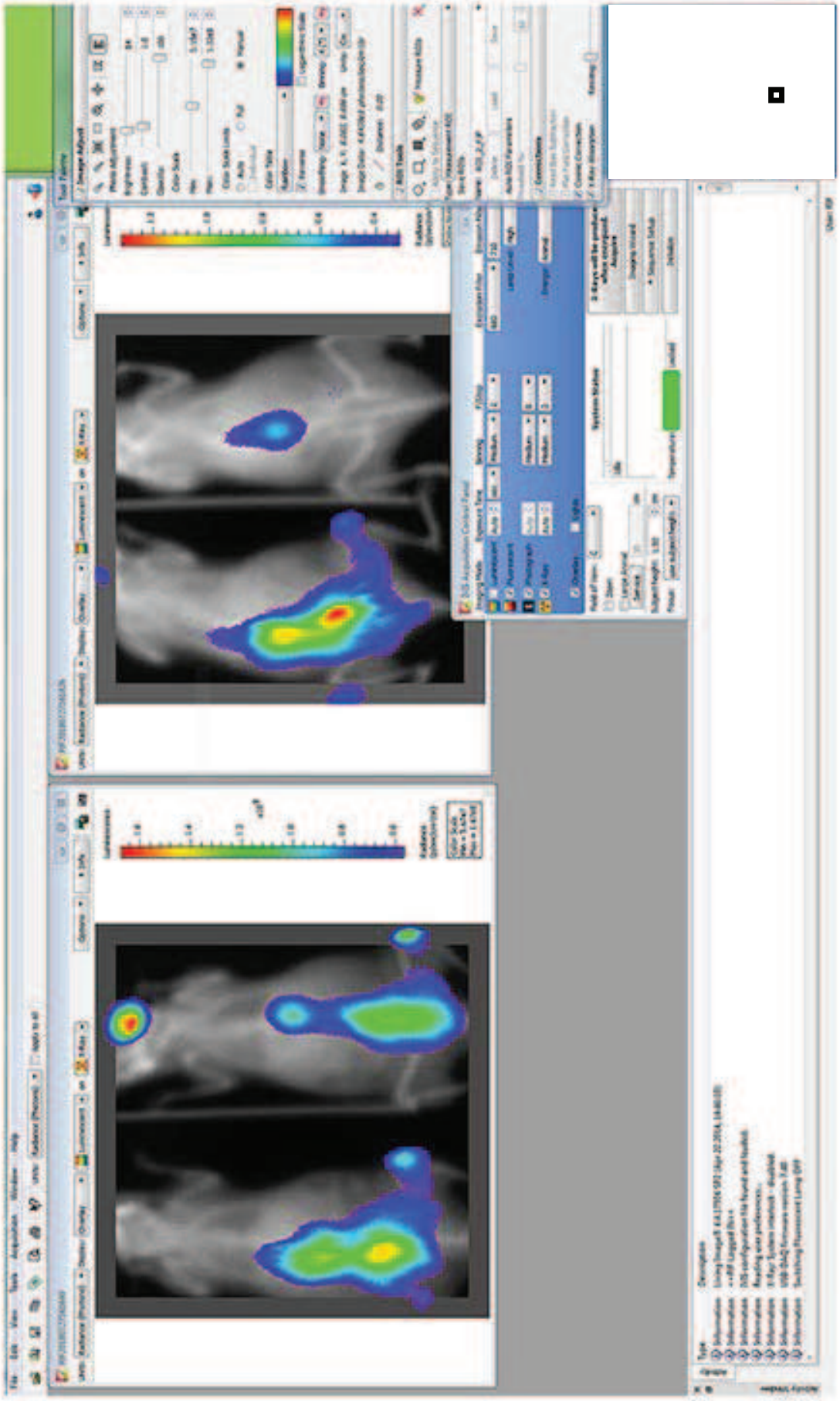

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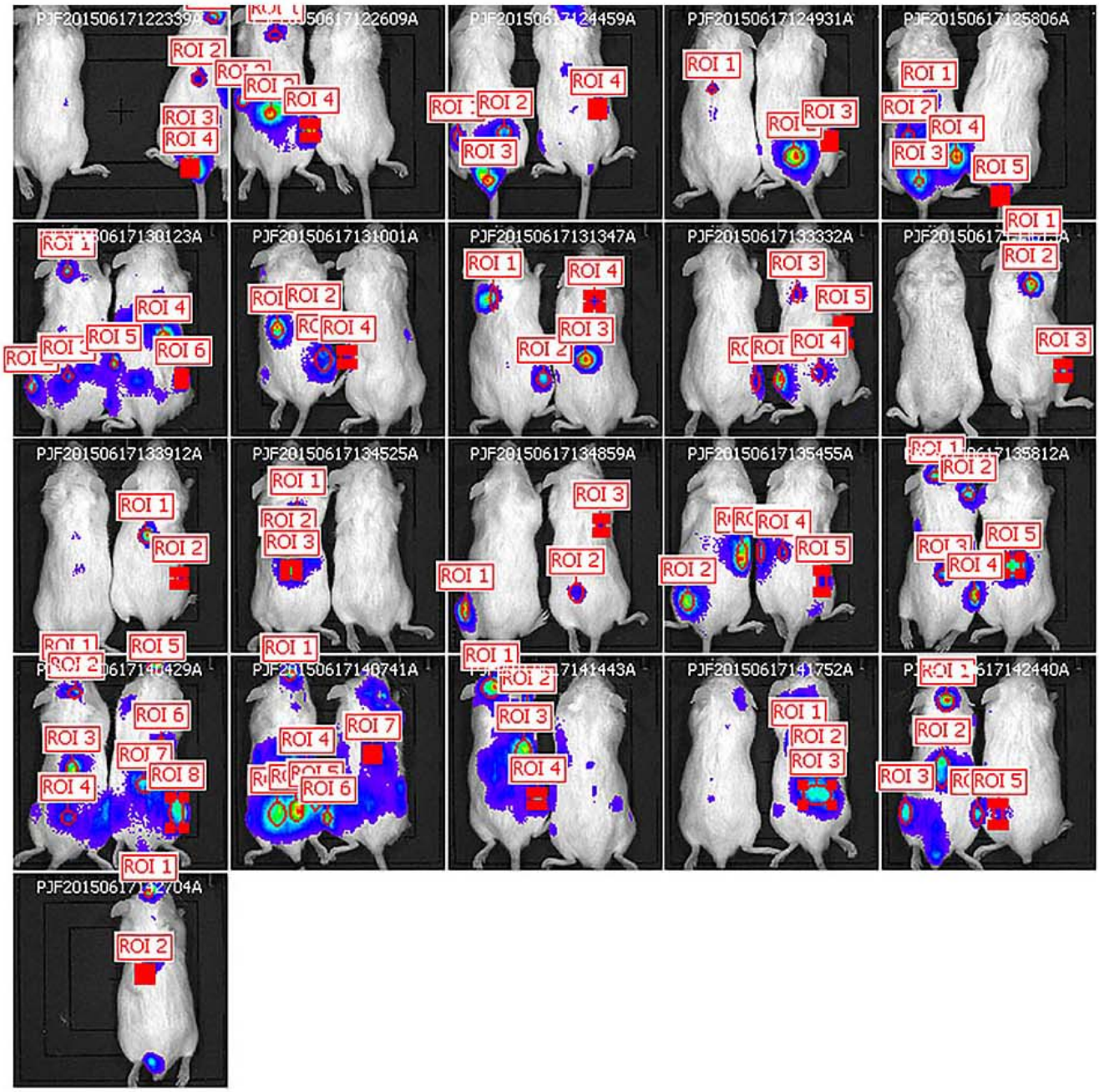

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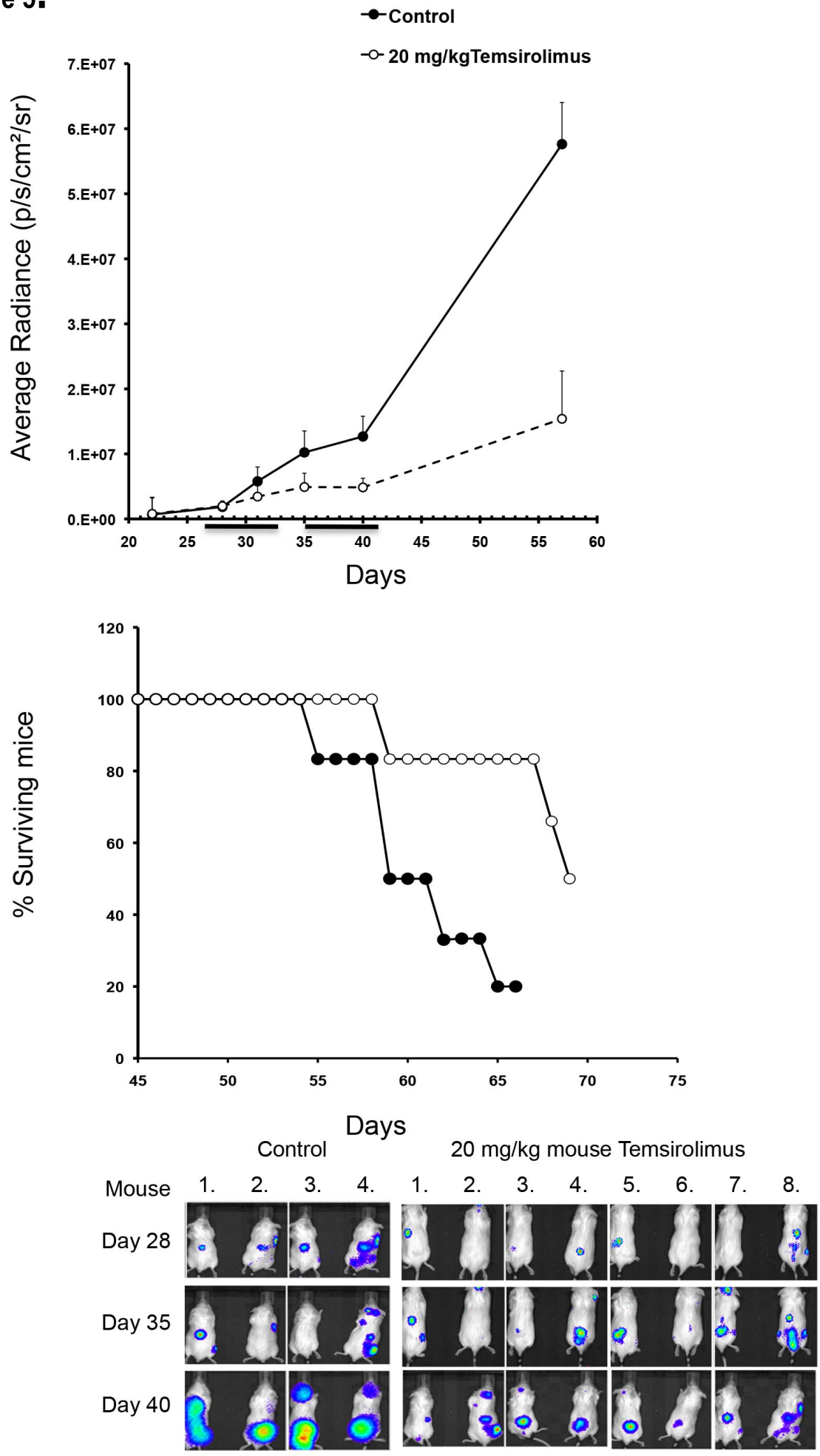
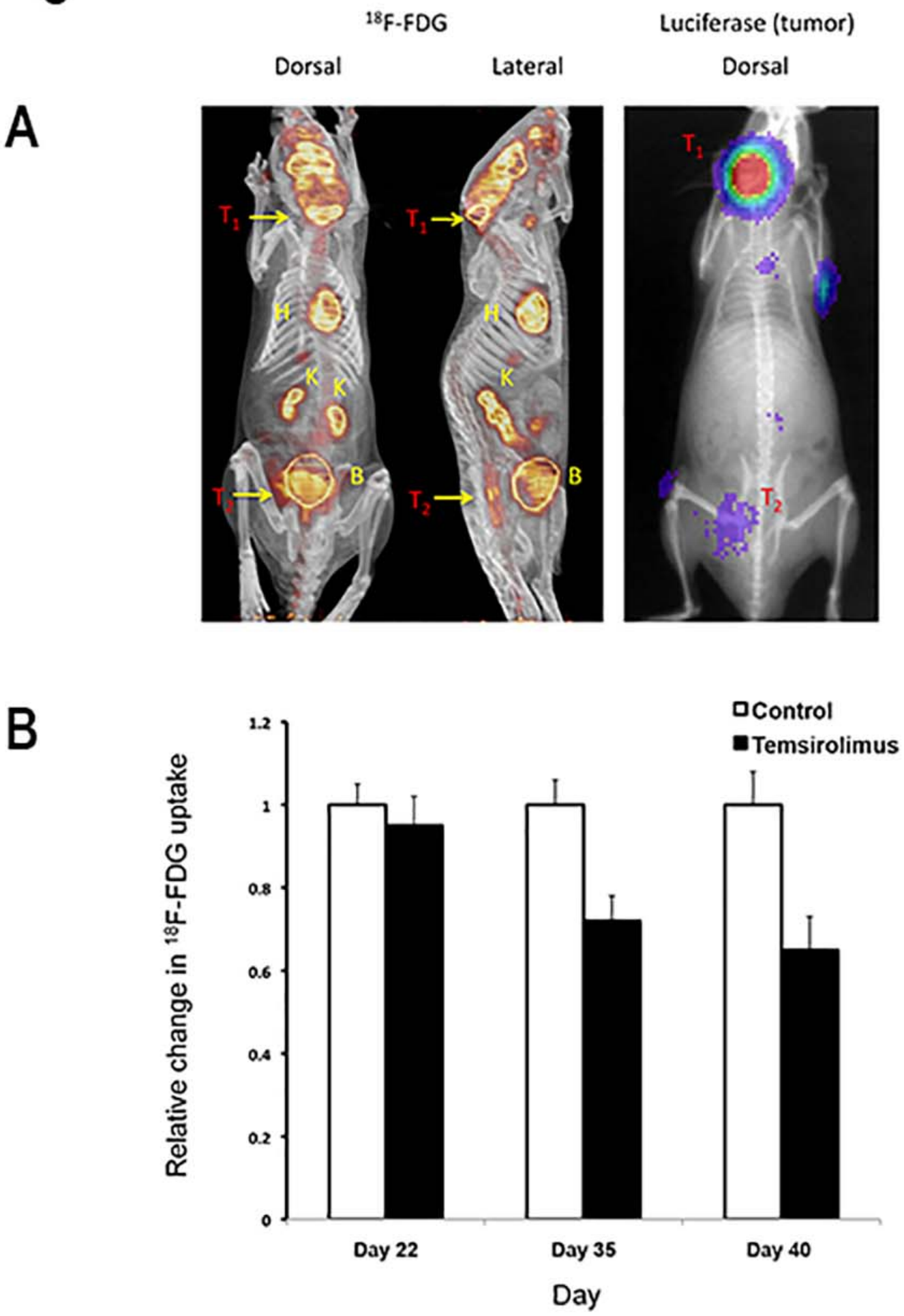


Figure 6



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
8226 human myeloma cell line	ATCC	CCL-155	
NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ Mice (NOG)	Jackson Labs	5557	
VivoGlo Luciferin substrate	Promega	P1041	
Hypoxyprobe-1Kit	HPL	HP1-100	
PE-CD45 (clone H130)	BD Biosciences	555483	Used for flow cytometry to identify human CD45+ tumor cells in BM exudate
rabbit anti-human CD45 (clone D3F8Q)	Cell Signaling Technology	70527	Primary antibody used for Immunohistochemistry of excised bone
Goat Anti-rabbit IgG (HRP conjugated)	ABCAM	ab205718	Seconday antibody used for Immunohistochemistry of excised bone
Dual-Luciferase Reporter Assay System	Promega	E1910	
pGL4.5 Luciferase Reporter Vector	Promega	E1310	
IVIS Lumina XRMS In Vivo Imaging System	Perkin Elmer		
Sofie G8 PET/CT Imaging System	Perkin Elmer		

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Signature:	Patrick Frost
Date:	2/25/2018

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Dear Editors,

Please find our item-by-item response to each of the concerns raised by the reviewers.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: We have proofread the manuscript to ensure that spelling/grammar issues have been resolved.

2. Please revise lines 202-206 to avoid previously published text.

Response: We have carefully reviewed the current manuscript and revised the text as appropriate.

3. Please submit the figures as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif, .pgn, or .psd, please ensure that the image is 1920 pixels x 1080 pixels or 300 dpi.

Response: The figures are now saved as .eps files. We hope that this is correct.

4. Figure 1: Please line up the panels (panel labels) better.

Response: Figure 1 has been modified as requested.

5. Figures 4/6: Please provide a high-resolution figure showing only the images of multiple animals. Please do not use the screen shot.

Response: Figure 4 has been modified as requested. We now believe that Figure 6 and 7 essentially replicate the same information, so we have removed the original Fig 6 from this manuscript and have renumbered the “previous” Fig 7 to the “new” Fig 6.

6. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

Response: The table of the essential supplies, reagents and equipment has been changed as requested.

7. Please provide an email address for each author.

Response: We have provided the email address for each author.

8. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

Response: We have rephrased the Short Abstract as requested.

9. Please revise the Introduction to include a clear statement of the overall goal of this method.

Response: We added a clear statement as to the overall goal of our protocol.

10. Please define all abbreviations before use.

Response: We have defined all abbreviations before use throughout.

11. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

Response: We have used SI abbreviations for all units.

12. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Response: This has been corrected.

13. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: This has been corrected.

14. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: GIBCO, Lonza, Nucleofector Kit, Promega, Jackson Laboratories, VivoGlo, Perkin-Elmer IVIS, etc.

Response: This has been corrected.

15. Please revise the protocol to contain only action items that direct the reader to do something. 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.”

Response: This has been corrected.

16. 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.

Response: This has been corrected by adding references to our previously published manuscript that details how to perform these procedures.

17. 1.2/1.4: Please add more details to this step. This step does not have enough detail to replicate as currently written. Alternatively, add references to published material specifying how to perform the protocol action.

Response: This has been corrected by adding references to our previously published manuscript that details how to perform these procedures.

18. 3.3: The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

Response: We have removed the discussion from this section and moved it the DISCUSSION section as requested.

19. Line 133: Should 90 be 90%?

Response: This has been corrected. It now reads “90%”.

20. 4.2: Please specify the drugs/therapy given to the mice.

Response: This information has been provided.

21. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response: We have highlighted the area that we believe are the essential steps of the protocol in yellow.

22. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Response: We believe we have ensured that this highlighted section forms a cohesive narrative.

23. 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.

Response: We have added the requested paragraph.

24. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

Response: This has been corrected.

25. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

Response: This has been corrected.

26. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

Response: This has been corrected.

We thank the reviewer for their careful review of this manuscript and have made every attempt to address each of the issues and concerns raised below.

Specific responses to Reviewer #1:

Minor Concerns:
1) The discussion should be expanded somewhat. What is the advantage of performing both BLI and PET scan? What is the draw back of these assays? BLI has for example a lower resolution than PET scan and is only semi-quantative.

Response: We have expanded on the discussion and apologize for the lack of clarity in our description of the advantages of performing bout BLI and PET scan. First, we would point out that directly measuring changes or the tumors engrafted in the skeleton of mice is difficult to assess in vivo. However, by transfecting MM cells with a luciferase allele, the location and progression of MM tumors can be easily, non-invasively and longitudinally monitored over the course of the experiment.

2) The 5TMM series, referred to in ref 11 are not xenograft models but syngenic models.

Response: The reviewer is correct that the 5TMM system is not a xenograft model. We only wished to highlight the fact that clinically relevant xenograft models of MM are difficult to develop and so we have clarified this point.

3) This also leads to another drawback of the proposed model which is not discussed: the fact that immunotherapy cannot be assessed in an immune deficient model.

Response: We agree that immunotherapy cannot be assessed immune deficient models. We would argue that our focus is more directed towards studying “drug-based” anti-MM therapies and how that impacts on marrow engrafted tumor cells, rather than a focus on development of specific immunotherapeutic strategies. For this reason, we felt that a discussion of using this model for immunotherapy was outside of the scope of the current study.

Specific responses to Reviewer #2:

1) The manuscript is littered with typing errors and grammar mistakes, making it very difficult to read and demonstrating poor proofreading.

Response: We apologize for the errors and have made every effort to correct these problems.

2) Line 182: Is it really 10 x 10^6 cells? This would equal 10^7 cells, which seems quite a lot for a volume of 200 µl. If this number however holds true, why do the authors not write 10^7 cells then?

Response: We agree with the reviewer that this range is very high. Because this is a “procedure” manuscript, we initially wanted to make it clear that some of the initial procedures (such as challenging mice with 8226 tumors) evolved over time. In our first experiments, we found that using less than about 4X10^6 cells resulted in a much lower rate of engraftment in the bone marrow. The percentage of successful engraftment (approaching 100%) increased with

increasing number of cells (up to about 10^7 cells). We also found that there was some “operator” variation in the ability to successfully make IV injections into the mice, so we initially tried to present the manuscript with a range of cells to challenge mice. However, as the reviewer makes clear, we agree that we probably made our description of the procedure less clear. As we have refined our techniques, we think that 5X10^6 cells/200 ul injection is suitable and so we have rewritten the manuscript to clarify this important point.

3) Line 100-101: Here the authors state that they used 5-10 x 10^6 cells. Not only is this contradictory to line 182, but also VERY critical. The authors have to ensure using equal cell numbers for every animal to make their results interindividually comparable. While it for sure is not possible to inject the very same amount of cells in each experiment, this range is way too huge (10 x 10^6 cells is a 100% increase compared to 5 x 10^6 cells! Please take a stand to this fact.

Response: The reviewer makes a very important point that is well taken. As stated above, we have revised our manuscript to indicate that we believe that challenging mice with 5X10^6 cells should result in >90% tumor engraftment.

4) Line 187: Staining for hypoxia and CD45. Please state which primary and secondary antibodies were used.

Response: This information has been added to the manuscript and materials file.

5) Figures:

Fig. 1B+C) The column "No cells" should be labeled "Control" or "RPMI" or "PBS" or "saline", whatever the control solution consisted of - according to line 103 I guess it was PBS, according to line 183 one could guess it was "saline" (sodium chloride or PBS?).

Fig 1B presents Luc in the first column and control in the second column, for fig 1C it is vice versa. Please match.

Response: Figure 1B and C have been revised. To clarify, our “control” images are from mice that were not challenged with 8226LUC cells. This is demonstrated in Fig 1A with a mouse lacking BLI signals (as would be expected). The mice were then sacrificed and the long bones were harvested and the bone marrow exudate was stained for huCD45 by flow cytometry (shown in Fig 1B). Fig 1 C shows the results of IHC of serial sections from harvested long bones that were stained for hypoxia and CD45. These experiments were designed as “proof of principle” for BM engrafted tumors.

Fig. 3, 4 and 6: It does not become clear to me why the authors decided to take photographs of the monitor. It looks to me as if these images were taken with a smartphone standing in front of a computer, which does not support the scientific context of the manuscript. Please explain the specific advantage of presenting the images in that particular manner (e.g. it is important to see the computer mouse on the right or chamber on the left on Fig. 3), or otherwise submit regular screenshots if the aim of these images is to present the software interface.

Response: This is an oversight on our sight. We had thought to indicate the steps of the imaging steps as would be observed by someone setting up the procedure. However, the reviewer is correct and we should have done a better job so we regret the confusion. Figures 3, 4, and 6

have been revised. Figure 3 and 4 show screen captures for software interfaces. The previous version figure 6 was removed altogether and our previous figure 7 is now figure 6.

Fig. 5:
This figure has such a poor resolution that it is difficult to decipher. Consider submitting a high-resolution TIF file.

Please expand the poor labeling. 100 nmol/mouse of what? What is HIF-PA? This abbreviation is neither introduced, nor is the rationale of applying HIF-PA explained. Please state clearer that HIF-PA was the treatment in your representative results.

Response: Figure 5 has been completely redone. First, we made an error and had presented previously published data for a hypoxia inducible factor (HIF) polyamide. In this experiment, we had used a variety of drug treatments and inadvertently included the wrong data. Figure 5A now presents the change in average radiance over time for animals treated with 20 mg/kg mouse of Temsirolimus (5 daily IP injections, followed by 2 days rest and an additional 5 IP injections). In our previously published work, we have found this concentration and treatment regimen with temsirolimus to be effective. In Figure 5B, we now present a Kaplan–Meier plot showing the % surviving animals, which supports the hypothesis that temsirolimus treatment kills tumor cells and prolongs survival. Finally we present representative images of mice treated with temsirolumus or vehicle control.

Line 144: Fig. 5B is not a plot. The plot is Fig. 5A. Rather use an expression such as "BLI visualization" for fig. 5B.

Response: This has been corrected.

Why do the authors show BLI images for day 22, 35 and 40 in Fig. 5B, but Fig. 5A presents an additional data point for day 28? Is there a reason why day 28 is missing in Fig. 5B?

Response: This was a typo and day 22 data should have been written as day 28 as now if presented in our new Fig 5C.

Fig. 7:
Delete the legend "Co-registration of tumors…" from within the image. This is already conveyed in the legend (line 212-214).

Response: This has been corrected.

Materials table:

Response: The materials table has been filled in with the appropriate information.

Minor Concerns:
==========
1) Line 125: replace "mouse" with "body weight"

Response: This has been revised as suggested.

2) Line 75-76: "in a way that is scientifically significant". Significances have to be calculated, in this context I suggest to rather write "scientifically sound".

Response: This has been revised as suggested.

3) Line 109: "Inject the mice". You do not inject mice, but rather cells into mice.

Response: This was an unclear statement on our part and has been corrected to state: Inject the cells into the mice through the tail vein.

4) Line 247: "the future applications of the technique are almost limitless in their scope." Please find a less striking phrase.

Response: The line has been revised to now state: After mastering the technique, the future applications of the technique present a great deal of flexibility. For example,

Specific responses to Reviewer #3:

1) Major concerns: Immune system plays an important role in multiple myeloma pathogenesis. The NOG mouse strain is immunocompromised. Therefore, is limited in providing comprehensive information regarding the interaction of myeloma cells with its microenvironment. It is essential to draw a comparison between intravenous disseminated mouse model versus "orthotopic" mouse model. Orthotopic mouse model would be described as tumor cells directly ingrafted in the long tumor bone such as the tibia.

Response: The reviewer raises a point that is very well taken. However, we would argue that basically all xenograft-type models of MM are performed in immunocompromised mice and each of the various strategies (be they SQ, IV disseminated, or physically injected into the tibia) will have their own set of pros and cons that must be weighed individually as to how well each meets the scientific and experimental relevance of the hypotheses being tested. Our model is in no way perfect, but we do believe that it provides a significant level of clinical relevance, reproducibility and scientific flexibility for examining the *in vivo* anti-tumor responses of various anti-MM therapies. More importantly, we believe that using non-invasive BLI and PET/CT strategies to study the bone marrow/MM microenvironment is very powerful and exciting tool.

2) Another major concern is that the study is using FDG to demonstrate tumor engraftment in the brain. The challenge with this design is that brain has a high level of physiological uptake of FDG. This would make it challenging to discern the tumor uptake from the background tissue uptake.

Response: We believe that there was some confusion. We have used optical imaging of the BLI produced by the luciferase-expressing tumor cells, in conjunction with X-ray analysis to demonstrate and identify where the tumors cells have engrafted in the mouse skeleton. We don't recommend that ^{18}F-FDG should be used to demonstrate tumor engraftment, as there seems to be uptake in other tissues (specifically the brain, heart, kidney and bladder). In our hands, we have found that the MM tumors tend to develop in the skull, spine, pelvis, and long bones of the mice. We agree that 18F-FDG is taken up in the brain, and would also agree that it will be very difficult to study FDG uptake in MM tumors that reside in the skull. However as shown in our figure, uptake in other, non-skull localized tumors can be differentiated from background tissue

uptake.

Minor Concerns:
The figures are low resolution. The axes are insufficiently labeled.

Response: The figures have been remade with greater resolution and the axes labels have been corrected.

Further Editorial Comments (from 8/13/2018):

1. The editor has formatted the manuscript as per the journal's style. Please retain the same.
   Response: The formatting has been maintained as requested.

2. Please address all specific comments marked in the manuscript.

   Response: This has been completed (please see above).

3. 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.

   Response: The requested paragraph has been included at the end of the Discussion Section.

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   Response: We do not believe that any figures have been previously published. We have regenerated Figure 5 to include non-published data.

5. Figure 1: Please include a scale bar for the panel C.

   Response: The requested scale bar has been added.

6. Please upload better quality figures especially 5 and 6 as these appear blurry.

   Response: We have uploaded better quality figures of Figure 5 and 6.

Sincerely Yours

Patrick Frost, PhD.