Johns Hopkins Department of Dermatology
Cancer Research Building II, Suites 209 & 210
1550 Orleans Street
Baltimore, MD 21231
410-955-8662 T
410-955-8645 F

JOHNS HOPKINS
MEDICINE

December 29, 2013

Dear Editors,

Thank you for the review of our manuscript for JoVE. Below is a list of the revisions that were made in response to the comments by the Editors and Reviewer 1 and 2, point-by-point:

EDITORIAL COMMENTS:

1) All of your previous revisions have been incorporated into the most recent version of the manuscript. Please download this version of the Microsoft word document from the "file inventory" to use for any subsequent changes.

RESPONSE: This was done as suggested.

2) Please disregard the comment below if all of your figures are original.
If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

RESPONSE: All figures will be original figures.

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

RESPONSE: This was done as suggested.

4)"2.7) Inoculate 2 µL of $1x10^3$ CFU of bioluminescent *S. aureus* Xen36 into the knee joint space." This process is not described in enough detail. How are the bacteria injected into the joint space?

RESPONSE: The new step 2.9 (Page 5, Line 213) was changed to:
"Using a micropipette, pipette 2 µL of $1x10^3$ CFU of bioluminescent *S. aureus* Xen36 onto the tip of the implant within the knee joint space."

5) Should the mouse in 4.1 be anesthetized?

RESPONSE: Step 4.1 (Page 5, line 282) was changed to "Place anesthetized LysEGFP mice into an imaging chamber."

5) Step 2.9 refers to something that should be done prior to surgery, but is presented after the surgery is complete. Please present action in chronological order

RESPONSE: This step was moved to step 2.2 (Page 5, lines 187-189) and the steps were reordered so that they are in chronological order.

6) Some issues regarding Notes:

-The Note before 2.1 contains protocol steps that use the wrong verb tense.

RESPONSE: The Note before 2.1 (Page 14, lines 173-182) was changed to the present verb tense.

-"2.5) ...into the intramedullary canal using". This sentence fragment should be finished with "Titanium K-wires." and not left with that information only in the "note".

RESPONSE: The word "using" was a mistake and was deleted. This sentence (Page 5, Lines 205-207) was revised to end with "…into the intramedullary canal."

-Note in step 2.9 contains information which should be included as a step at the appropriate time. "At the end of the experiments, all animals were euthanized ..."

RESPONSE: This information is now included in step 2.11 (page 5, Lines 225-228).

-Note in 5.2.2 does not appear to be optional or additional information and should be included as part of the protocol in the appropriate location.

RESPONSE: Notes have been removed and worked into the steps 5.2/5.2.1/5.2.2 in the appropriate locations. The steps have been reworded slightly to better reflect software selections and accommodate the smooth inclusion of these notes into the text.

REVIEWER #1 COMMENTS:

Minor Concerns:
Four other references should be included referring to probe development:
Ning, X. et al. Maltodextrin-based imaging probes detect bacteria in vivo with high sensitivity and specificity. Nat. Mater. 10, 602-607 (2011).

Panizzi, P. et al. In vivo detection of Staphylococcus aureus endocarditis by targeting pathogen-specific prothrombin activation. Nat. Med. 17, 1142-1146 (2011).

Kong, Y. et al. Imaging tuberculosis with endogenous beta-lactamase reporter enzyme fluorescence in live mice. Proc. Natl Acad. Sci. USA 107, 12239-12244 (2010).

van Oosten, M. et al. Real-time in vivo imaging of invasive- and biomaterial-associated bacterial infections using fluorescently labelled vancomycin. Nat. Commun. 4:2584 doi: 10.1038/ncomms3584 (2013)

RESPONSE: As suggested, these 4 references have been added to the statement referring to probe development (Page 12, Lines 533-536).

REVIEWER #2 COMMENTS:

Major Concerns:

1) One specific conclusion that is not supported by the data is correlation between bioluminescence signal and the bioburden (CFU). This information is in particular important, as authors indicate the strain of bacteria (Xen29) used to generate current data will be replaced with a different *S. aureus* strain (Xen36). Authors need to provide the survival and growth rate of Xen36 *in vivo* and the correlation of bioluminescence signal to CFU.

RESPONSE: In 3 of our prior published manuscripts, we have provided information about the survival and growth rate of Xen36 *in vivo* (Pribaz, J et al. 2012 *J. Orthop. Res* 30:335–340[19]) and we enumerated CFU from the joint/bone tissue and implants and the numbers of CFU closely approximate the bioluminescent signals taken at the same time points (Niska, JA, et al. 2012 *Antimicrob Agents Chemother* 56: 2590-2597 [17] and Niska, JA, et al. 2013. *Antimicrob Agents Chemother* 57: 5080-5086[18]). To include this information in the current manuscript, the following sentence was added to the REPRESENTATIVE RESULTS section "*In vivo* bioluminescent and fluorescent imaging" in the second to last sentence (Page 10, Lines 435-437):

"Our previous work demonstrated that the *in vivo* bioluminescent signals closely approximated the numbers of *ex vivo* CFU isolated from the joint/bone tissue and adherent to the implants [17, 18]."

2) Important control group appears to be missing. Please show the survival / persistence of test organism with no foreign body implant in the knee.

RESPONSE: Although we appreciate this suggestion to add this control group, a control group without an implant would model *S. aureus* septic arthritis rather than an orthopaedic implant *S. aureus* infection. Since the purpose of this manuscript is to provide the methods to model an orthopaedic implant infection, this experimental group is not indicated for this study.

3.1) Fig . 1. Bioluminescence signal appears to increase over time, reaching a peak around day 19. However, the neutrophil signal seems to disappear rapidly. Do the authors have any explanation as to why there is no recruitment of neutrophils to the site of infection with the progression of infection?
3.2) What is the average life span of activated and non-activated neutrophils in this mouse strain LysEGFP?
3.3) What is the repeated exposure X-ray irradiation on neutrophil production?
PLEASE SEE THE SPECIFIC RESPONSES TO EACH OF THESE QUESTIONS, FOLLOWED BY REVISED TEXT IN THE MANUSCRIPT, BELOW:

3.1) RESPONSE: In our previous work, we compared 4 different bioluminescent *S. aureus* strains (ALC2906, Xen29, Xen36 and Xen40) in LysEGFP mice using this same orthopaedic implant infection model without any X-ray irradiation (Pribaz, J et al. 2012 J. Orthop. Res 30:335–340[19]). We observed some differences in EGFP-neutrophil signals among the different *S. aureus* strains, suggesting different strains induce varying degrees neutrophilic inflammation. However, all of the neutrophil EGFP signals decreased to background levels by 14-21 days after infection and remained at background levels through day 42 when the experiment was arbitrarily terminated. Although we do not know exactly why the neutrophil signals decreased, it is likely that the infection over time becomes more chronic, and as this occurs, perhaps the immune response is no longer dominated by neutrophil infiltration as with other chronic infections. This subject is area of ongoing investigation in our lab and is beyond the purpose of this manuscript, which is focused on presenting the methods of using noninvasive *in vivo* imaging to monitor the infection and neutrophil infiltration in a mouse model of orthopaedic implant infection.

3.2) RESPONSE: During a *S. aureus* skin wound infection in LysEGFP mice, we found that neutrophils survived 3-fold longer (4.96 ± 0.38 days) in *S. aureus*-infected wounds compared with uninfected wounds (1.58 ± 0.31 days) (Kim, M-H, et al. 2011. *Blood* 117: 3343-3352[23]). The increased life-span is likely due to survival signals such as TLR2 and PGE2 as we previously described (Granick, JL, et al. 2013. *Blood* 122:1770-1778). In the *S. aureus* wound infection model, we identified that neutrophil infiltration involved 3 main mechanisms: (1) robust neutrophil recruitment from the circulation, (2) prolonged neutrophil survival at the site of infection and (3) the homing of KIT+ progenitor cells to the abscess, where they locally give rise to mature neutrophils (Kim, M-H, et al. 2011. *Blood* 117: 3343-3352[23]). From these prior studies, we expect that similar mechanisms will contribute to neutrophil infiltration to the site of the orthopaedic implant *S. aureus* infection and the neutrophils will survive longer in infected mice compared with uninfected mice.

3.3) RESPONSE: We did not measure the effect of X-ray irradiation on neutrophil production but the X-ray irradiation likely had minimal impact on neutrophil production or the kinetics of the neutrophil recruitment to the site of infection because neutrophil fluorescence in this model had the same kinetics in non-irradiated mice (Pribaz, J et al. 2012 J. Orthop. Res 30:335–340[19]) as irradiated mice (Niska, JA, et al. 2012. *PLOS ONE* 7(10): e47397[16]).

To include the responses to major concern 3 (3.1, 3.2, 3.3) in the manuscript, the following text has been added to the Discussion page 11, lines 504-516:

"One interesting finding that should be pointed out is that we observed that the EGFP-neutrophil fluorescent signals decreased to background levels by 14-21 days and remained at background levels for the duration of the experiment despite the presence of bioluminescent bacteria. It is unlikely that the X-ray irradiation impacted neutrophil survival as we observed similar kinetics of the neutrophil signals in non-irradiated mice[19]. In our previous work involving a model of S. aureus infected wounds, neutrophil infiltration involved a combination of robust neutrophil recruitment from the circulation, prolonged neutrophil survival at the site of infection and the homing of KIT+ progenitor cells to the abscess, where they locally give rise to mature neutrophils[23]. It is likely that similar processes contributed to neutrophil infiltration in the orthopaedic implant S. aureus infection model. Although it is unknown why the neutrophil signals decreased in the orthopaedic infection model, it could be that the immune response changed over time as this infection progressed from an acute to chronic infection and this is a subject of future investigation."

4) Page 11 line 485. Although stated to be the case by the authors, there is no monitoring of bacterial burden in this study. In order to demonstrate this, it is essential to establish in vivo correlation between bioluminescence signal and CFU for Xen29 and Xen36.

RESPONSE: Please see the above response to major concern #1.

Minor Concerns:
1) Page 4, line145. Please confirm if lux operon is integrated into bacterial chromosome or plasmid.

RESPONSE: This sentence was changed to read: "Note: *S. aureus* Xen36[21] is a genetically engineered *S. aureus* strain that contains a modified *lux* operon derived from *Photorhabdus luminescens*, which is integrated into a stable native plasmid found in this bacterial strain."

2) Page 4. No method given for cultivation and preparation of bacterial inocula.

RESPONSE: We have provided the method for cultivation and preparation of the bacterial inocula in the Protocol steps 1.1 to 1.7 (page 4, lines 141-163).

3) Page 5. Please indicate whether the animals in control group (uninfected) were subjected to the same surgical procedure as test group.

RESPONSE: We thank Reviewer 2 for pointing this out. The following Note was added to step 2.7:
"Note: In control uninfected mice, 2 µL of sterile saline is added without any bacteria."

4) What is the limit of detection of test organism and neutrophils in this model?
RESPONSE: Based on our previous work we can accurately detect between $1x10^2$ and $1x10^3$ bacteria using *in vivo* bioluminescent imaging. We have not measured the limit of detection of neutrophils. To include this in the manuscript, the following sentence as added to the Figure 1 Legend, page 11, lines 470-471:
"The limit of detection of the bacterial burden using *in vivo* bioluminescent imaging is between $1x10^2$ and $1x10^3$ CFU."

5) Page 5, line 225 (3.1). Were the images acquired from dorsal or ventral side of animal?

RESPONSE: Images were acquired from the ventral side. The following was added to step 3.1 (Page 6, lines 233-234):
"3.1) Anesthetize LysEGFP mice (e.g., 2% inhalation isoflurane) and place them with ventral side up into an imaging chamber."

6) Page 5, line 228. (3.2). Which series of IVIS imaging system is used in the study?

RESPONSE: Page 6, line 237, in step 3.2, the following IVIS system was used as is mentioned: "3.2) Perform *in vivo* bioluminescent imaging using the IVIS Spectrum optical whole animal *in vivo* imaging system (PerkinElmer, Inc.)."

7) Page 6, line 233. (3.2). How long were the animals imaged per session?

RESPONSE: The following Note has been added to steps 3.2 and 3.3 (Page 6, lines 242 and 247, respectively):
3.2) "Note: For *in vivo* bioluminescent imaging, mice are typically imaged between 1 to 5 minutes."
3.3) "Note: For *in vivo* fluorescent imaging, mice are typically imaged between 0.5 seconds."

Thank you for the review of our manuscript. We hope that we adequately addressed all of the concerns of the reviewers.

Sincerely,

Lloyd S. Miller, M.D., Ph.D.
Associate Professor
Department of Dermatology
Department of Medicine, Division of Infectious Diseases
Department of Orthopaedic Surgery