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In Vivo Mouse Model of Spinal Implant Infection

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Corresponding Author:	Benjamin Kelley, MD UCLA Los Angeles, CA UNITED STATES
Corresponding Author's Institution:	UCLA
Corresponding Author E-Mail:	bkelley@mednet.ucla.edu
Order of Authors:	Benjamin V. Kelley
	Stephen D. Zoller
	Danielle Greig
	Kellyn Hori
	Nicolas Cevallos
	Chad Ishmael
	Peter Hsiue
	Rishi Trikha
	Troy Sekimura
	Thomas Olson
	Ameen Chaudry
	Michael M. Le
	Anthony A. Scaduto
	Kevin P. Francis
	Nicholas M. Bernthal
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TITLE:

In vivo Mouse Model of Spinal Implant Infection

AUTHORS:

Benjamin V. Kelley[1], Stephen D. Zoller[1], Danielle Greig[1], Kellyn Hori[1], Nicolas Cevallos[1], Chad Ishmael[1], Peter Hsiue[1], Rishi Trikha[1], Troy Sekimura[2], Thomas Olson[2], Ameen Chaudry[2], Michael Le[2], Anthony A. Scaduto[1], Kevin P. Francis[1], Nicholas M. Bernthal[1]

[1]Department of Orthopaedic Surgery, University of California Los Angeles, Los Angeles, CA, USA
[2]David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA, USA
Corresponding Author: Nicholas M. Bernthal

Email Address:
bkelley@mednet.ucla.edu
SZoller@mednet.ucla.edu
DGreig@mednet.ucla.edu
KHori@mednet.ucla.edu
NCevallos@g.ucla.edu
CIshmael@mednet.ucla.edu
PHsiue@mednet.ucla.edu
RishiTrikha6@gmail.com
TSekimura@mednet.ucla.edu
TEOlson@mednet.ucla.edu
AChaudry@mednet.ucla.edu
MMLe@mednet.ucla.edu
TScaduto@mednet.ucla.edu
NBernthal@mednet.ucla.edu

KEYWORDS:

Spine, implant infection, *Staphylococcus aureus*, bioluminescent bacteria, orthopaedic surgery, bacterial infection, osteomyelitis

SUMMARY:

The protocol describes a novel in vivo mouse model of spinal implant infection where a stainless-steel k-wire implant is infected with bioluminescent *Staphylococcus aureus* Xen36. Bacterial burden is monitored longitudinally with bioluminescent imaging and confirmed with colony forming unit counts after euthanasia.

ABSTRACT:

Spine implant infections portend poor outcomes as diagnosis is challenging and surgical eradication is at odds with mechanical spinal stability. The purpose of this method is to describe a novel mouse model of spinal implant infection (SII) that was created to provide an inexpensive, rapid, and accurate in vivo tool to test potential therapeutics and treatment strategies for spinal implant infections.

In this method, we present a model of posterior-approach spinal surgery in which a stainless-steel k-wire is transfixed into the L4 spinous process of 12 week old C57BL/6J wild-type mice and inoculated with 1 x 10^3 CFU of a bioluminescent strain of *Staphylococcus aureus* Xen36 bacteria. Mice are then longitudinally imaged for bioluminescence in vivo on post-operative days 0, 1, 3, 5, 7, 10, 14, 18, 21, 25, and 35. Bioluminescence imaging (BLI) signals from a standardized field of view are quantified to measure in vivo bacterial burden.

To quantify bacteria adhering to implants and peri-implant tissue, mice are euthanized and the implant and surrounding soft tissue are harvested. Bacteria are detached from the implant by sonication, cultured overnight and then colony forming units (CFUs) are counted. The results acquired from this method include longitudinal bacterial counts as measured by in vivo *S. aureus* bioluminescence (mean maximum flux) and CFU counts following euthanasia.

While prior animal models of instrumented spine infection have involved invasive, ex vivo tissue analysis, the mouse model of SII presented in this paper leverages noninvasive, real time in vivo optical imaging of bioluminescent bacteria to replace static tissue study. Applications of the model are broad and may include utilizing alternative bioluminescent bacterial strains, incorporating other types of genetically engineered mice to contemporaneously study host immune response, and evaluating current or investigating new diagnostic and therapeutic modalities such as antibiotics or implant coatings.

INTRODUCTION:

The purpose of this method is to describe a novel mouse model of spinal implant infection (SII). This model was designed to provide an inexpensive and accurate tool to flexibly assess the effect of host, pathogen, and/or implant variables in vivo. Testing potential therapeutics and treatment strategies for spinal implant infections in this model is aimed at guiding research development prior to application in larger animal models and clinical trials.

Implant related infection after spine surgery is a devastating complication and unfortunately occurs in approximately 3–8% of patients undergoing elective spine surgery[1-5] and up to 65% of patients undergoing multilevel or revision surgery[6]. Treatment of spinal implant infections often requires multiple hospitalizations, multiple surgeries, and prolonged antibiotic therapy. SIIs portend poor patient outcomes including neurological compromise, disability, and an increased risk of mortality. Management of SII is extremely expensive, costing upwards of $900,000 per patient[7].

Staphylococcus aureus is the most common virulent pathogen of SII[8-11]. Bacteria can seed the hardware directly during surgery, through the wound during the postoperative period, or later via hematogenous spread. In the presence of metal implants, *S. aureus* form biofilm that protects the bacteria from antibiotic therapy and immune cells. While removal of infected hardware may help effectively eradicate an infection, this is frequently not feasible in the spine without causing destabilization and risking neurologic compromise[12].

In the absence of explanting infected hardware, novel approaches are needed to prevent, detect, and treat SII. Historically, there have been limited animal models of SII to efficiently assess the safety and efficacy of novel therapies. Previous animal models of SII require large numbers of animals and collection of data points requiring euthanasia including colony counting, histology, and culture[13-15]. Lacking longitudinal in vivo monitoring, these models only provide one data point per animal and are therefore expensive and inefficient.

Previous work studying a mouse model of knee arthroplasty infection established the value and accuracy of noninvasive in vivo optical imaging to longitudinally monitor infection burden[16]. The detection of bioluminescence allows bacterial burden to be quantified over a longitudinal time course in a single animal humanely, accurately, and efficiently. Moreover, prior studies have demonstrated a high correlation between in vivo bioluminescence and CFUs adherent to implants[17]. The capacity to track infection over time, has led to a more nuanced understanding of implant related infection. In addition, monitoring longitudinal infection in this way, has allowed the effectiveness of antibiotic therapy and novel antimicrobials to be accurately assessed[16-18].

Leveraging these tools, we developed and validated a model of postoperative spinal implant infection. In the method presented, we utilize an inoculum of bioluminescent *S. aureus* Xen36 to establish an in vivo mouse model of SII to longitudinally monitor bacterial burden[16-18]. This novel model provides a valuable tool to efficiently test potential detection, prevention, and treatment strategies for SII prior to their application in larger animal models and clinical trials.

PROTOCOL:

All animals were handled in strict accordance with good animal practice as defined in the federal regulations as set forth in the Animal Welfare Act (AWA), the 1996 Guide for the Care and Use of Laboratory Animals, PHS Policy for the Humane Care and Use of Laboratory Animals, as well as the institution's policies and procedures as set forth in the Animal Care and Use Training Manual, and all animal work was approved by the University of California Los Angeles Chancellor's Animal Research Committee (ARC).

1. *S. aureus* bioluminescent strain choice

1.1 Use the bioluminescent *S. aureus* strain Xen36 as the inoculum of interest.

NOTE: This strain was derived from the parental strain *S. aureus* ATCC-49525, which is a clinical isolate from a septic patient. *S. aureus* Xen36 uniquely utilizes a *lux*ABCDE operon, which is optimized and integrated into the host's native plasmid.[19] As a result, the Xen36 strain is capable of producing a blue-green bioluminescent light with a peak wavelength emission of 490 nm. This emission signal is only produced by living metabolically active bacterial organisms.

2. Preparation of *S. aureus* for inoculation

2.1 Add 200 µg/mL kanamycin to Luria Broth plus 1.5% agar to isolate *S. aureus* Xen36 from potential contaminants, utilizing the kanamycin resistance gene linked to the *lux* operon[19].

2.2 Streak *S. aureus* Xen36 bacteria onto tryptic soy agar plates (tryptic soy broth [TSB] plus 1.5% agar) and incubate at 37 °C for 12-16 h.

2.3 Isolate single colonies of *S. aureus* Xen36 and individually culture in TSB for 12-16 h at 37 °C in a shaking incubator (200 rpm).

2.4 Dilute resultant culture at a 1:50 ratio.

2.5 Culture for additional 2 h at 37 °C to isolate midlogarithmic phase bacteria.

2.6 Pellet, resuspend, and wash bacteria in phosphate buffered saline (PBS) three times.

2.7 Estimate a single bacterial inoculum (1 x 10^3 CFU/2 µL) by measuring the absorbance at 600 nm. The ideal OD600 is between 0.650 and 0.750.

NOTE: The optimal concentration of Xen36 for the establishment of a chronic infection was found to be 1 x 10^3 CFU. Lower dosing of bacteria was cleared by the host immune system and higher dosing caused wound breakdown. Wound breakdown does not differentiate between a deep implant infection and a superficial wound infection and is therefore avoided in this model (**Figure 1**)[20].

3. Mice

3.1 Use 12-week-old male C57BL/6J wild-type mice.

3.2 House mice in cages with a maximum of 4 at a time.

3.3 Keep water available at all times. Maintain a 12-hour light/dark cycle and do not perform experimentation during the dark phase of the cycle.

3.4 Use alfalfa-free chow for feeding due to potential interference with fluorescent signaling.

3.5 Have research or veterinary staff assess mice daily to ensure the well-being of the animals throughout the entirety of the experiment.

4. Mouse surgical procedures

4.1 Induce anesthesia by placing mice in an isoflurane (2%) chamber for approximately 5 minutes. Confirm appropriate depth of anesthesia by monitoring respirations to remain rhythmic and slower than when awake and not changing in response to noxious stimuli (e.g., surgical manipulation, toe pinch).

4.2 Transfer anesthetized mice to a preparation station and remove hair from the sacrum to the upper thoracic spine with rodent clippers.

4.3 Clean and sterilize the skin with triple washes of alternating betadine solution and isopropyl alcohol.

4.4 Transfer anesthetized and sterilized mice in the prone position to a sterile surgical bed maintaining anesthesia with administration of inhaled isoflurane (2%) via nose cone.

4.5 Maximally flex the hips and identify the position of the knee at the level of the spine to approximate the lumbar 4 vertebral body.

4.6 Make a longitudinal 2 cm incision through skin with a 15-blade surgical scalpel.

4.7 Palpate the spinous processes to confirm midline and continue the incision down to bone.

4.8 Dissect subperiosteally on the right side of the L4 spinous process, extending laterally to the transverse process.

4.9 Pass an absorbable braided suture size 5-0 cephalad and caudad to the L4 body through the fascia and leave open, in preparation for future closure.

4.10 Using a 25 G spinal needle, ream the spinous process of L4 using a 25 G spinal needle and insert a 0.1 mm diameter, 1 cm long "L-shaped" surgical grade stainless steel implant along the lamina with the long arm laying cephalad.

4.11 Inoculate the implant with 1 x 10^3 CFUs/2 µL bioluminescent *S. aureus* Xen36, taking care to ensure all solution contacts the implant.

4.12 Tie the previously passed absorbable suture immediately following inoculation to ensure containment of inoculum on the implant.

4.13 Close skin in running fashion with absorbable suture.

4.14 Administer pain medicine via subcutaneous injection of buprenorphine (0.1 mg/kg) immediately postop and then every 12 hours for 3 days thereafter.

4.15 Recover mice on a heating pad and monitor for return to normal activity.

4.16 Obtain postoperative radiographs to confirm appropriate placement of implant.

5. Longitudinal In vivo bioluminescence imaging to measure bacterial burden

5.1 Anesthetize mice with inhaled isoflurane (2%). Confirm appropriate depth of anesthesia by monitoring respirations to remain rhythmic and slower than when awake and not changing in response to noxious stimuli (e.g., surgical manipulation, toe pinch).

5.2 Remove hair from the sacrum to the upper thoracic spine with rodent clippers.

5.3 Load mice onto field of view of bioluminescent imaging platform to perform in vivo bioluminescence imaging (BLI)[19].

5.4 Capture bioluminescent signal over a 5 min acquisition time. Utilize medium binning settings with a 13 cm field of view.

5.5 Repeat steps 5.1-5.4 on postoperative days 0, 1, 3, 5, 7, 10, 14, 18, 21, 25, and 35 (or other days based on specific experimental design) to monitor bacterial burden.

5.6 Present BLI data via color scale and overlay on a grayscale photograph. Isolate a standard ovoid region of interest (ROI) using BLI software to quantify BLI in total flux (photons per second) or mean maximum flux (photons/second/centimeter^2/steradian).

6. Quantify bacteria adherent to implants and surrounding tissue

6.1 Euthanize mice on POD 35 or alternative post-operative date of choice with exposure to carbon dioxide. Confirm euthanasia with secondary cervical dislocation.

6.2 Sterilize the dorsal skin according to Step 4.3 and position the mouse prone on a sterile surgical field.

6.3 Sharply incise the previous incision using a 15-blade surgical scalpel.

6.4 Use sterile scissors to bluntly dissect to the L4 spinous process and identify the surgical implant.

6.5 Use a needle driver to gently twist and remove the implant from its position in the L4 spinous process.

6.6 Using sterile forceps and scissors, harvest approximately 1 g of spinous process bone and soft tissue immediately surrounding the surgical implant and place in 1 mL of TSB in small conical rhino tube with 4 sharp homogenizing beads.

6.7 Record weight of soft tissue by weighing conical tube before and after harvest.

6.8 Place the implant in 0.5 mL of 0.3% Tween-80 in TSB and sonicate for 10 min.

6.9 Vortex the resulting implant suspension for 5 min and culture overnight for 12-16 h.

6.10 Homogenize the soft tissue and spinous processes previously placed in 1 ml TSB surrounding the implant using homogenizer.

6.11 Vortex the resulting soft tissue suspension for 5 min and culture overnight for 12-16 h.

6.12 After overnight culture, count CFU from the implant and surrounding tissues, respectively. Express value as total CFU/g harvested for soft tissue and CFU/mL for sonicated implant.

REPRESENTATIVE RESULTS:

The procedure presented here was used to assess the efficacy of antibiotic regimens in an in vivo mouse model of SII. Specifically, the efficacy of combination vancomycin and rifampin antibiotic therapy was compared to vancomycin monotherapy and untreated infected controls.

Prior to surgery, mice were randomized to either combination therapy, monotherapy, or infected control. A statistical power analysis was performed to calculate sample size. Anticipated means of mean maximum flux $1 \times 10^5 \pm 3.2 \times 10^4$ and 1.4×10^5 were used to determine sample size, which were calculated as N=10 in each group. Mice underwent surgical implantation, inoculation with *S. aureus* Xen36, and were measured for in vivo S. aureus bioluminescence on POD 0, 1, 3, 5, 7, 10, 14, 18, 21, 25, and 35. On POD 35, mice were sacrificed and CFUs for implant-adherent and surrounding tissue bacteria were quantified.

Mice in the monotherapy group received a therapeutic dose of vancomycin (110 mg/kg twice daily) delivered subcutaneously. This dose was selected to approximate the area under the curve for typical human exposure for vancomycin[21-23]. Mice in the combination therapy group received a therapeutic subcutaneous dose of vancomycin (110 mg/kg twice daily) and rifampin (25 mg/kg daily)[24]. Mice in the infected control group received sham injections of sterile saline. Treatment for all groups was performed from postoperative days 7 to 14.

Effect of antibiotic therapy on BLI

Infected control mice had BLI signals peaking on POD 10 that remained above 1.0×10^5 photons/s/cm^2/sr until sacrifice, successfully modeling a chronic SII (**Figure 2**). Mice treated with vancomycin monotherapy had significantly lower BLI signal compared to infected control, with a 2-fold reduction from POD 10–21 ($p<0.03$). After POD 21, there was no significant difference in BLI between monotherapy and infected control groups. Mice treated with vancomycin-rifampin combination therapy had an even lower BLI signal, which was 20-fold lower than infected control on POD 10. A significant reduction persisted until POD 28 ($p<0.01$). After POD 28, there was no significant difference in BLI between groups. There was no significant difference in BLI between any of the three groups at final imaging on POD35.

CFUs from implants and surrounding tissue

Mice were sacrificed on POD 35. Implants and surrounding tissue were harvested and processed for CFU counting (**Figure 3**). No significant difference was observed in CFUs between infected control, monotherapy, or combination therapy groups.

FIGURE AND TABLE LEGENDS:

Figure 1. Wound breakdown in high dose *S. aureus* Xen36 inoculum. Images of the dorsal skin of mice inoculated with *S. aureus* Xen36 during a spine implant infection. (**A**) Mouse inoculated with $1x10^3$ CFUs, and intact dorsal skin. (**B**) Mouse inoculated with 1×10^4 CFUs, and evidence of considerable wound breakdown. Figure adapted and reprinted with permission from Dworsky et al.[25]

Figure 2. Measurement of bacterial burden using in vivo bioluminescence. 1×10^3 CFU of S. aureus possessing the bioluminescent construct in a stable plasmid (Xen36) were inoculated into the L4 spinous process of mice (n = 10 mice per group) in the presence of a stainless steel implant. (**A**) Bacterial counts as measured by in vivo S. aureus bioluminescence (mean maximum flux [photons/s/cm2/sr] ± sem [logarithmic scale]), with a flow diagram of the experimental protocol below. On POD 7, antibiotic administration began with vancomycin, a combination of vancomycin and rifampin or a sterile saline control. Antibiotic administration was stopped on POD 14. On POD 35, mice were sacrificed and CFUs from the implant and surrounding tissue were measured. (**B**) Representative in vivo S. aureus bioluminescence on a color scale overlaid on top of a grayscale image of mice. Figure adapted and reprinted with permission from Hu et al.[25]

Figure 3. Confirmation of bacterial burden using CFU counts. At POD 35, mice were sacrificed, pins were sonicated, tissue was homogenized, and bacteria were cultured and counted. Figure adapted and reprinted with permission from Hu et al.[25].

DISCUSSION:

Implant related infections in the spine portend poor outcomes for patients[1-5]. Unlike many other areas in the body, infected hardware in the spine frequently cannot be removed due to the risk of instability and neurologic compromise. This unique challenge in the setting of biofilm bacteria resistant to systemic antibiotic therapy necessitate novel approaches to treatment[12]. Previous research in novel treatments for SII has been limited by expensive, inefficient animal models. To better study these infections and efficiently assess the efficacy of potential treatments, we developed a noninvasive longitudinal mouse model of SII using in vivo bioluminescence imaging.

Prior animal models of instrumented spine infection required ex vivo tissue analysis to evaluate results, requiring large cohorts and were unable to monitor infection over time[13,14,26]. In contrast, the mouse model of SII presented in this paper leverages BLI to reliably monitor bacterial burden over time[16-18]. This novel approach enables investigators to assess the response of bacteria and the host to antibiotics, coatings, or immune modulation.

Critical steps in the protocol include: appropriate preparation of Xen36 *S. aureus* and estimation of single inoculum 1×10^3 CFUs/2 µL; precise surgical implantation, inoculation, and closure; in vivo bioluminescent imaging; and confirmation of bacterial burden using CFU counts.

Modifications to the model may include alternative bioluminescent bacterial strains, longer term time points, or the use of integration of other types of genetically engineered mice, such as those expressing green fluorescent protein in myeloid cells (Lys-EGFP) to contemporaneously measure neutrophil infiltration with in vivo fluorescence imaging[20]. Additional methodologies may be utilized to complement those described in the protocol to address processes such as vertebral osteolysis, disc degeneration, soft tissue infection, and implant biofilm infection. Techniques that provide quantitative outcomes in these processes may include but are not limited to: micro-computed tomography, magnetic resonance imaging, real time quantitative PCR, serology, histology, immunohistochemistry, and/or variable pressure scanning electron microscopy.

This model has several limitations. First, the model of spine surgery is a gross simplification compared to clinical practice. In contrast to extensive decompression and multilevel fusion surgeries typical of high-risk spine surgery patients, the model surgery involves minimal bone resection with a single stainless-steel implant. As clinical spine implants have multiple different materials, these may have different susceptibilities to bacterial infection and biofilm formation. In addition, as with all animal models, the host response to infection of mice is different than that of humans.

In the future, this model could be used to assess treatment of SII with other antibiotic delivery modalities including vancomycin powder, antibiotic loaded beads, or coated implants. In addition, this model may be used to study the mechanistic basis of the host response to SII.

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DISCLOSURES:
The authors have no conflicts of interest to disclose.

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Figure 1

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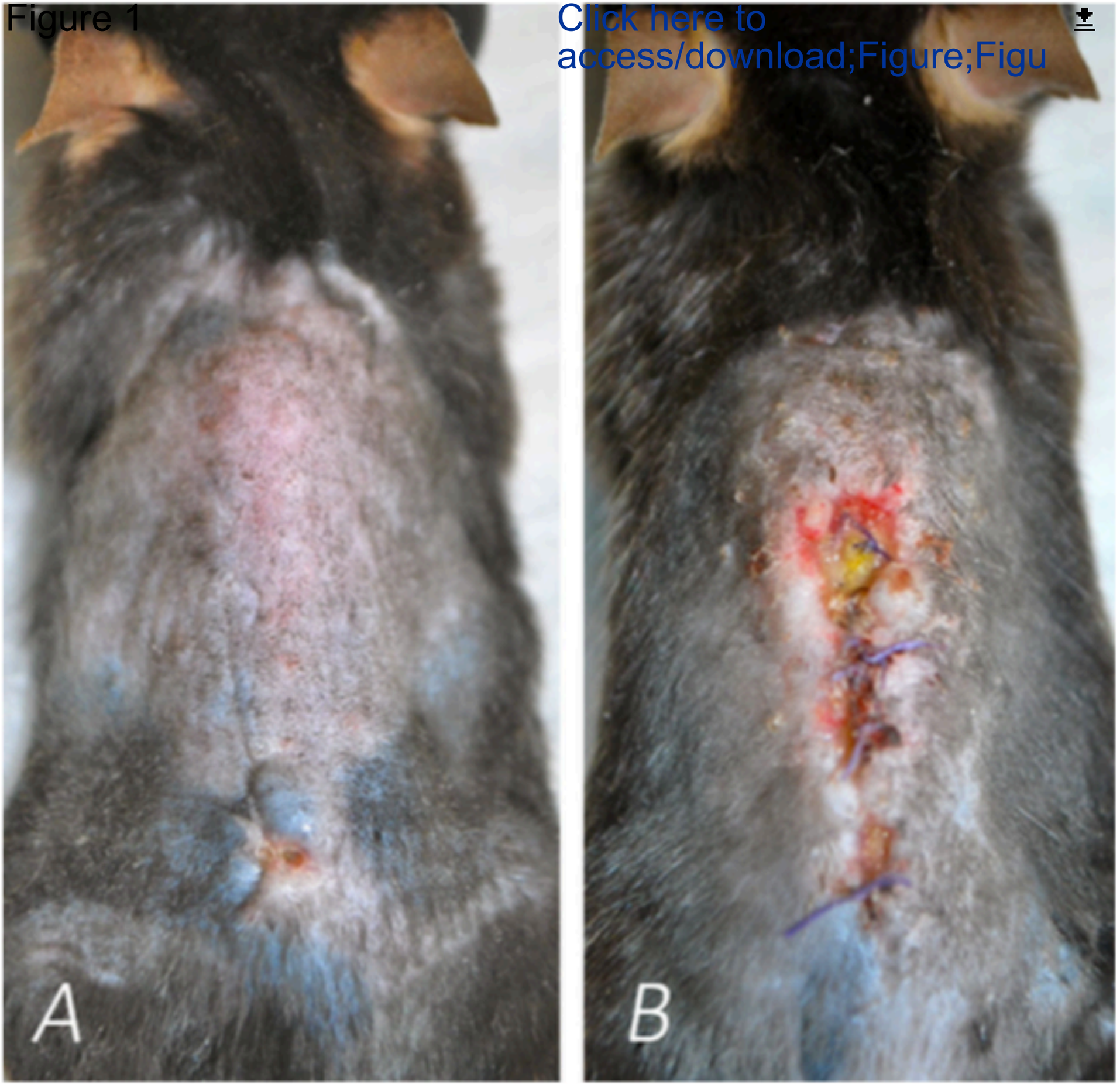

Figure 2

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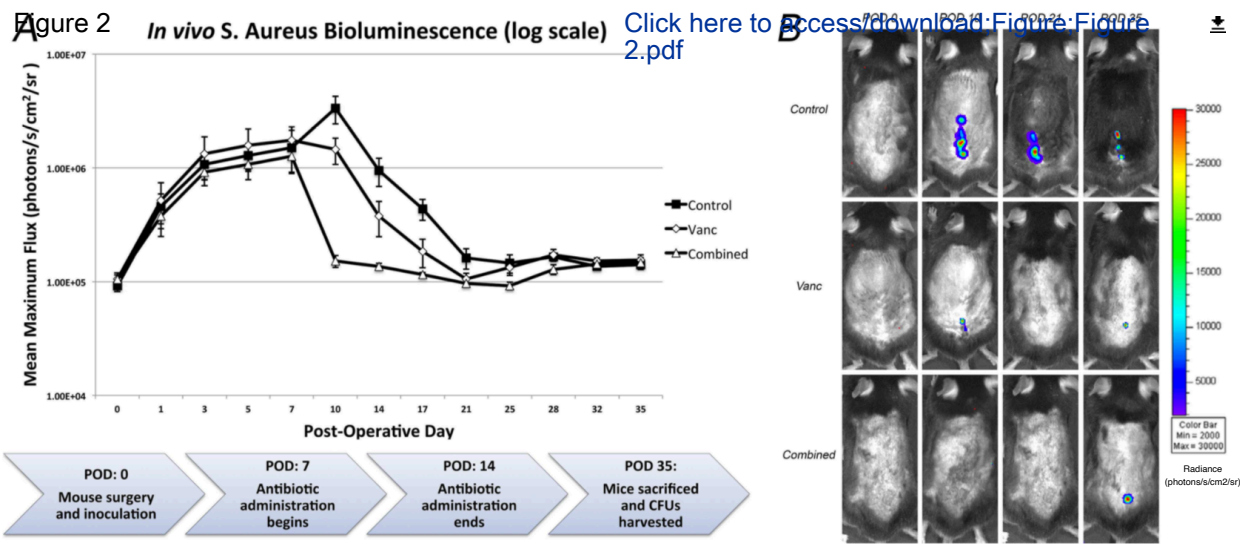
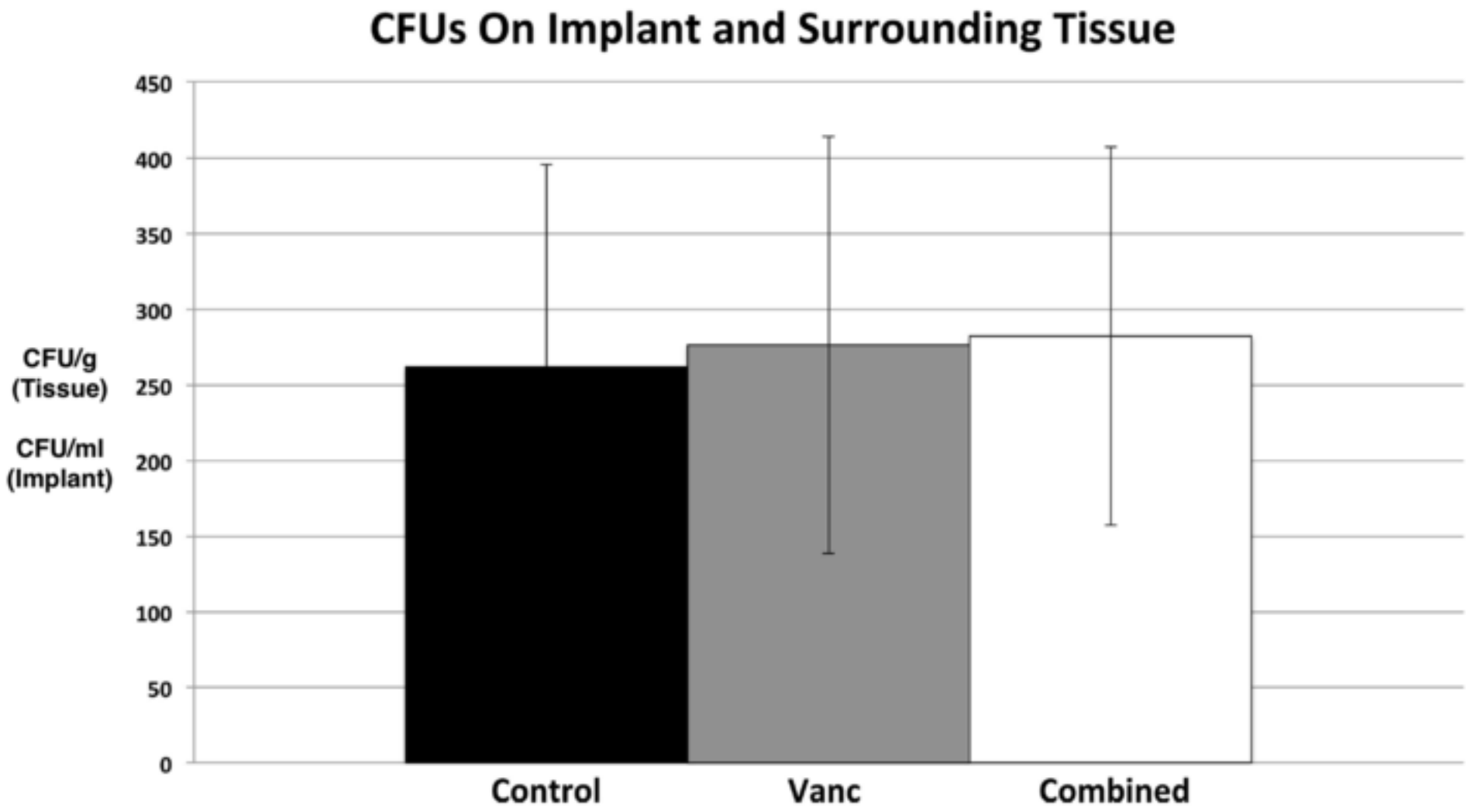


Figure 3



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Biomate 3S UV-VIS Spectrophotometer	Thermo Scientific
Bioshield 720+ swinging bucket rotor	Thermo Scientific
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Staphylococcus aureus - Xen36	Perkin Elmer
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Vortex mixer VX-200	Labnet Internation
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Comments/Description

120 g capacity, 0.1 mg readability, backlit LCD, internal adjustment, metal base

BD Bacto Tryptic Soy Broth (Soybean-Casein Digest Medium)
Spectrophotometer; Thermo Scientific; BioMate 3S; Six-position cell holder; Spectral bandwidth: 1.8nm; Long-life xenon lamp; Store up to 40 test methods; 16L x 13W x 9 in. H; Rotor, Swinging bucket; Thermo Scientific; BIOShield 720 high speed; Capacity: 4 x 180mL (0.72L); Angle: 90 deg. ; Max. speed/RCF: 6300rpm/7188 x g; Max. radius: 16.2cm
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Single 150L
The IVIS Lumina X5 high-throughput 2D optical imaging system combines high-sensitivity bioluminescence and fluorescence with high-resolution x-ray into a compact system that
Shaker, Incubated; Thermo Scientific; Digital; MaxQ 4450; Speed 15 to 500rpm +/-1rpm; 5 deg. C above ambient to 80 deg. C; 120V 50/60Hz

PBS, Phosphate Buffered Saline, 1X Solution, pH 7.4

24 x 1.5/2.0mL rotor with ClickSeal biocontainment lid
Centrifuge, Benchtop; Thermo Scientific; Sorvall Legend X1R (Refrigerated), 1L capacity; Max. Speed/RCF 15,200rpm/25,830 x g; CFC-free cooling -10C to +40C; 120V 60Hz
Staphylococcus aureus - Xen36 bioluminescent pathogenic bacteria for in vivo and in vitro drug discovery. This product was derived from a parental strain from the American Type
Sterilizer, Benchtop; Heidolph; Tuttnauer; Model 2540E; Self-contained design with refillable reservoir controls water purity for sterilization; 120V 50/60Hz; 1400w. With

Tween 80, Fisher BioReagents, Non-ionic detergent for selective protein extraction

120V touch or continuous mixer, 230V: 0 - 2,850 rpm,120V: 0 - 3,400 rpm

0.9% Sodium Chloride Injection, USP

JoVE60560R1 Rebuttal Document

"In Vivo Mouse Model of Spinal Implant Infection"

Dear Editorial Committee,

Thank you very much for your thoughtful comments. The following changes described in **bold** have been made to the revised manuscript according to your comments enumerated here:

Editorial comments:

1. With the new revisions (and formatted per JoVE guidelines, see attached), the protocol is over 2.75 pages, our limit for filming. Please highlight 2.75 pages or less, including headers and spacing, for filming.

Protocol highlighted in yellow with less than 2.75 pages included for filming

2. It may be best to address reviewer 1's comments about tracking other outcomes. The protocol does not need to be altered, but a few sentences in the Discussion could be added.

Reviewer #1:

The manuscript by Kelley et al entitled "In Vivo Mouse Model of Spinal Implant Infection" is a preliminary study of potential interest. The strength of the study is its focus on the spine, which is underrepresented in the literature of musculoskeletal infections due to technical challenges in generating reproducible quantitative outcomes and the health and wellness of experimental animals during the infection study period. Thus, a rigorous JoVE article with surgical details with radiology on how to reproducibly generate SII of a particular segment (e.g. L4-L5) with quantitative outcomes on vertebral osteolysis, disc degeneration, soft tissue infection and implant biofilm could be a high-impact article. Unfortunately, this study does not go beyond longitudinal BLI and CFU outcomes, which are now routine in this field.

The following was added to the manuscript:

Line 334: Additional methodologies may be utilized to complement those described in the protocol to address processes such as vertebral osteolysis, disc degeneration, soft tissue infection, and implant biofilm infection. Techniques that provide quantitative outcomes in these processes may include but are not limited to: micro-computed tomography, magnetic resonance imaging, real time quantitative PCR, serology, histology, immunohistochemistry, and variable pressure scanning electron microscopy.

Sincerely,

Ben Kelley and co-authors

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Publisher of new article	My JOVE Corp.
Author of new article	Benjamin Kelley, Stephen Zoller, Nicholas Bernthal
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