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TITLE:
A Mouse Model to Assess Innate Immune Response to *Staphylococcus aureus* Infection

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KEYWORDS:
Staphylococcus aureus; innate immunity; neutrophils; wound healing; inflammation; whole-animal imaging

SUMMARY:
An approach is described for real-time detection of the innate immune response to cutaneous wounding and *Staphylococcus aureus* infection of mice. By comparing LysM-EGFP mice (which possess fluorescent neutrophils) with a LysM-EGFP crossbred immunodeficient mouse strain, we advance our understanding of infection and the development of approaches to combat infection.

ABSTRACT:
Staphylococcus aureus (*S. aureus*) infections, including methicillin resistant stains, are an enormous burden on the healthcare system. With incidence rates of *S. aureus* infection climbing annually, there is a demand for additional research in its pathogenicity. Animal models of infectious disease advance our understanding of the host-pathogen response and lead to the development of effective therapeutics. Neutrophils play a primary role in the innate immune response that controls *S. aureus* infections by forming an abscess to wall of the infection and

facilitate bacterial clearance; the number of neutrophils that infiltrate an *S. aureus* skin infection often correlates with disease outcome. LysM-EGFP mice, which possess the enhanced green fluorescent protein (EGFP) inserted in the Lysozyme M (LysM) promoter region (expressed primarily by neutrophils), when used in conjunction with *in vivo* whole animal fluorescence imaging (FLI) provides a means of quantifying neutrophil emigration noninvasively and longitudinally into wounded skin. When combined with a bioluminescent *S. aureus* strain and sequential *in vivo* whole animal bioluminescent imaging (BLI), it is possible to longitudinally monitor both the neutrophil recruitment dynamics and *in vivo* bacterial burden at the site of infection in anesthetized mice from onset of infection to resolution or death. Mice are more resistant to a number of virulence factors produced by *S. aureus* that facilitate effective colonization and infection in humans. Immunodeficient mice provide a more sensitive animal model to examine persistent *S. aureus* infections and the ability of therapeutics to boost innate immune responses. Herein, we characterize responses in LysM-EGFP mice that have been bred to MyD88-deficient mice (LysM-EGFP×MyD88$^{-/-}$ mice) along with wild-type LysM-EGFP mice to investigate *S. aureus* skin wound infection. Multispectral simultaneous detection enabled study of neutrophil recruitment dynamics by using *in vivo* FLI, bacterial burden by using *in vivo* BLI, and wound healing longitudinally and noninvasively over time.

INTRODUCTION:

Staphylococcus aureus (*S.* aureus) accounts for the majority of skin and soft tissue infections (SSTIs) in the United States[1]. The incidence of methicillin-resistant *S. aureus* (MRSA) infections has increased steadily over the past two decades[2], motivating the study of the mechanisms of persistence and the discovery of new treatment strategies. The standard of care for MRSA infections is systemic antibiotic therapy, but MRSA has become increasingly resistant to antibiotics over time[3] and these drugs can diminish the host's beneficial microbiome, causing negative health effects, especially in children[4]. Preclinical studies have examined alternative strategies to treat MRSA infections[5], but translating these approaches to the clinic has proved challenging due to emergence of virulence factors that thwart host immune responses[6]. To dissect the host-pathogen dynamics that drive *S. aureus* SSTIs, we combine noninvasive and longitudinal readouts of the number of neutrophils recruited to the wound bed with kinetic measures of bacterial abundance and wound area.

Neutrophils are the most abundant circulating leukocyte in humans and the first responders to a bacterial infection[7]. Neutrophils are a necessary component for an effective host response against *S. aureus* infections due to their bactericidal mechanisms, including production of reactive oxygen species, proteases, antimicrobial peptides and functional responses including phagocytosis and neutrophil extracellular trap production[8,9]. Human patients with genetic defects in neutrophil function, such as chronic granulomatous disease and Chediak-Higashi syndrome, show an increased susceptibility to *S. aureus* infection. In addition, patients with genetic (such as congenital neutropenia) and acquired (such as neutropenia seen in chemotherapy patients) defects in neutrophil numbers are also highly susceptible to *S. aureus* infection[10]. Given the importance of neutrophils in clearing *S. aureus* infections, enhancing their immune capacity or tuning their numbers within a *S. aureus* lesion may prove an effective strategy in resolving infection.

Over the past decade, transgenic mice with fluorescence neutrophil reporters have been developed to study their trafficking[11,12]. Combining neutrophil reporter mice with whole animal imaging techniques permits spatiotemporal analysis of neutrophils in tissues and organs. When combined with bioluminescent strains of *S. aureus*, it is possible to track the accumulation of neutrophils in response to *S. aureus* abundance and persistence in the context of bacterial virulence factors that directly and indirectly perturb neutrophil numbers in affected tissue[13-16].

Mice are less susceptible to *S. aureus* virulence and immune evasion mechanisms than humans. As such, wild-type mice may not be an ideal animal model to investigate the efficacy of a given therapeutic to treat chronic *S. aureus* infection. MyD88-deficient mice (*i.e.,*, MyD88$^{-/-}$ mice), an immunocompromised mouse strain that lacks functional interleukin-1 receptor (IL-1R) and Toll-like receptor (TLR) signaling, show greater susceptibility to *S. aureus* infection compared to wild-type mice[17] and an impairment in neutrophil trafficking to a site of *S. aureus* infection in the skin[18]. Development of a mouse strain that possesses a fluorescent neutrophil reporter in MyD88$^{-/-}$ mice has provided an alternative model for investigating the efficacy of therapies to treat *S. aureus* infection compared to current neutrophil reporter mice.

In this protocol, we characterize *S. aureus* infection in the immunocompromised LysM-EGFP×MyD88$^{-/-}$ mice, and compare the time course and resolution of infection with the LysM-EGFP mice. LysM-EGFP×MyD88$^{-/-}$ mice develop a chronic infection that does not resolve, and 75% succumb to infection after 8 days. A significant defect in initial neutrophil recruitment occurs over 72 hours of the inflammatory phase of infection, and 50% fewer neutrophils recruit during the latter stage of infection. The increased susceptibility of the LysM-EGFP×MyD88$^{-/-}$ mice makes this particular strain a rigorous preclinical model to evaluate the efficacy of new therapeutic techniques targeting *S. aureus* infection compared to current models that utilize wild-type mice, especially techniques aiming to boost the innate immune response against infection.

PROTOCOL:

All mouse studies were reviewed and approved by the Institutional Animal Care and Use Committee at UC Davis and were performed according to the guidelines of the Animal Welfare Act and the Health Research Extension Act.

1. Mouse Source and Housing

1.1. Derive LysM-eGFP mice on a C57BL/6J genetic background as described previously[12]. Derive LysM-EGFP×MyD88$^{-/-}$ mice by crossing LysM-EGFP mice with MyD88$^{-/-}$ mice on a C57BL/6J background.

1.2. House mice in a vivarium. For these studies, animals were housed at the University of California, Davis in groups prior to surgery and single housed following surgery. Use mice between the ages of 10-16 weeks for this model.

2. Bacterial Preparation and Quantification

2.1. Remove the bioluminescent *S. aureus* strain of interest from -80 °C storage to thaw on ice. Streak on a 5% bovine blood agar plate. Incubate the streaked plate in a humidified incubator at 37 °C overnight (16 h).

Note: In this protocol, the ALC2906 SH1000 strain was used. This strain contains the shuttle plasmid pSK236 with the penicillin-binding protein 2 promoter fused to the *luxABCDE* reporter cassette from *Photohabdus luminescens*[18].

2.2. Prepare tryptic soy broth (TSB) by mixing 0.03 g of TSB powder per mL of pure water, and autoclave TSB to sterilize. When cooled, add any necessary antibiotics using sterile technique. In this protocol, add 10 µg/mL of chloramphenicol[18] to the TSB to select for expression of the pSK236 shuttle plasmid, which contains the bioluminescence luxABCDE cassette.

2.3. Pick 3-4 separate colonies from the *S. aureus* plate into TSB with 10 µg/mL chloramphenicol to start an overnight culture. Incubate bacteria on an incubating shaker at 37 °C overnight (16 h).

2.4. Start a new bacterial culture from the overnight culture by diluting a sample 1:50 into TSB with 10 µg/mL chloramphenicol. Culture in an incubating shaker at 200 rpm and 37 °C.

2.5. Two hours after splitting the *S. aureus*, monitor the optical density at 600 nm (OD_{600}) on a spectrophotometer. Observe the OD_{600} *vs.* time to find mid-logarithmic phase growth. For the ALC2906 SH1000 strain, an OD_{600} of 0.5 is mid-logarithmic and corresponds to a concentration of $1x10^8$ CFU/mL (**Figure 1**).

2.6. When OD_{600} is 0.5, wash bacteria 1:1 with ice-cold DPBS. Centrifuge the bacteria for 10 min at 3000 x g and 4 °C. Carefully decant the supernatant and add additional chilled DPBS and vortex thoroughly. Centrifuge once more for 10 min at 3000 x g and 4 °C.

2.7. Carefully decant the supernatant. Resuspend the bacterial pellet at a desired concentration. For these studies, collect 3 mL of ALC2906 SH1000 and resuspend in 1.5 mL of PBS, correlating to a bacteria concentration of about $2×10^8$ CFU/mL. Keep bacteria on ice until use.

2.8. To verify bacteria concentration, dilute 100 µL of the bacterial sample 1:10,000 and 1:100,000 in PBS. Plate 20 µL aliquots on an agar plate. Incubate at 37 °C in a humidified incubator for 16 h. Count CFUs by gross examination and calculate a bacterial concentration the following day.

3. Excisional Skin Wounding and Inoculation with *S. aureus*

3.1. Administer 100 µL of 0.03 mg/mL buprenorphine hydrochloride (~0.2 mg/kg) to each mouse *via* intraperitoneal injection.

3.2. Twenty minutes post-injection, place 2-4 mice in a chamber with 2-3 LPM oxygen with 2-4% isoflurane. Once mice are fully anesthetized, transfer the mice one at a time to a nose cone connected to 2-3 LPM oxygen with 2-4% isoflurane. Verify mice are fully anesthetized by firmly pinching each rear paw between a thumb and forefinger. Proceed to the next step if the animal does not respond to the pinch.

3.3. Shave a 1-inch by 2-inch section on the back of the mouse with electric clippers and clear the area of fur clippings using a clean wipe or gauze. Avoid using depilatory lotion because it may cause excess inflammation.

3.4. Clean the back of the mouse first with 10% povidone-iodine soaked gauze and then with a 70% ethanol soaked gauze. Clean the area in a spiral pattern, moving outward from the center of the surgical area. Wait approximately 1 min for the surgical area to dry prior to surgery.

3.5. Hold the shaved back of the mouse loosely between two fingers and firmly press a sterile 6 mm punch biopsy at the center of the prepared surgical area. Do not pull the skin taut.

3.5.1. Twist the punch biopsy to create a circular outline on the skin that fully cuts through the skin in at least one section of the outline. Be careful not to cut into the underlying fascia or tissue.

3.5.2. Use sterile scissors and forceps to cut through the epidermis and dermis following the circular pattern imprinted by the punch biopsy.

4. *S.* aureus Inoculation

4.1. Fill a 28 G insulin syringe with the desired bioluminescent bacterial inoculant. In this study, administer a concentration of 1×10^8 CFU/mL (50 µL). Do not administer more than 100 µL of volume.

4.2. Inject 50 µL of inoculant between the fascia and tissue in the center of the wound on the back of the mouse. Ensure that the inoculant forms a bubble at the center of the wound with minimal leakage or dispersion.

4.2.1. Pull the dermis to the side, hold the syringe nearly parallel to the tissue, and slowly push the syringe into the tissue until a sudden decrease in resistance is felt, which indicates piercing of the fascia. Carefully lead the syringe into the center of the wound and dispense the inoculant slowly. Remove the syringe slowly from the animal.

4.3. Inject the same volume of sterile PBS into the wounds of uninfected animals as described above.

4.4. Return the animal to its cage. Place the cage under a heat lamp or on top of a heating pad, and monitor the animal until recovery from anesthesia.

5. *In vivo* BLI and FLI

5.1. Initialize the whole animal imager through the instrument software. Anesthetize mice in a chamber receiving 2-3 LPM oxygen with 2-4% isoflurane. Deliver anesthesia to the nosecones inside the imager.

5.2. Place the wounded and infected mouse into the imager. Position the mouse such that the wound is as flat as possible. Use the following sequence set-up to image the mice.

5.2.1. Select **Luminescence** and **Photograph** as the imaging mode. The exposure time is 1 min at small binning and F/stop 1 (luminescence) and F/stop 8 (photograph). The emission filter is **Open**. Click the **Acquire** button to record the image.

5.2.2. Select **Fluorescence** and **Photograph** as the imaging mode. The exposure time is 1 s at small binning and F/stop 1 (Fluorescence) and F/stop 8 (photograph) with an excitation wavelength of 465/30 nm and an emission wavelength 520/20 nm with a high lamp intensity. Click the **Acquire** button to record the image.

5.3. Return the animal to its cage and monitor until recovery from anesthesia.

5.4. Image mice daily as described above.

6. Image Analysis

6.1. Open images to be quantified.

6.2. Place a large circular region of interest (ROI) over the entire wound area including surrounding skin for each mouse in the image. The neutrophil *in vivo* FLI EGFP signals and *in vivo* BLI *S. aureus* signals extend beyond the wound edge after several days and was included in these studies (**Figure 2A** and **2B**). Click **Measure ROI** and record values for mean flux for each mouse. Plot the mean flux of each signal (p/s) *versus* time.

6.3. If absolute numbers of neutrophils or *S. aureus* in the wound are desired, perform the following experiments.

6.3.1. To correlate neutrophil numbers to the *in vivo* FLI EGFP signals, wound C57BL/6J mice as described above, and transfer a range of bone marrow-derived neutrophils ($5x10^5$ to $1x10^7$)

from LysM-EGFP or LysM-EGFPxMyD88$^{-/-}$ donors directly on top of different wounds. Image as described above and correlate the *in vivo* FLI EGFP signals to the known quantity of neutrophils.

6.3.2. To correlate *S. aureus* CFU to the *in vivo* BLI signals, wound and infect mice as described above. On day 1 post-infection record *in vivo* BLI signals from the mice and immediately euthanize and chill carcasses. Excise the wound, homogenize the tissue, and plate bacterial dilutions on agar for overnight incubation. The next day, count colonies to determine CFU per wound.

6.4. To measure wound healing fit a circular ROI over the wound edge and measure the area of the wound (cm^2) and plot the fractional change from baseline *versus* time (**Figure 2C**).

7. Statistics

NOTE: All data are presented as mean ±SEM. p <0.05 were considered statistically significant

7.1. Determine differences between two groups on a single day using the Holm-Sidak method, with alpha = 0.05, and analyze each time point individually, without assuming a consistent SD.

7.2. Compare differences between multiple groups on the same day by one-way ANOVA with the Tukey multiple-comparisons posttest. Survival between experimental groups was analyzed by the Mantel-Cox method.

REPRESENTATIVE RESULTS:

LysM-EGFP×MyD88$^{-/-}$ mice are more susceptible to *S. aureus* infection than LysM-EGFP mice

The strain of *S. aureus* used in this study, ALC2906[18], was constructed with a plasmid that contains the *lux* construct that produces bioluminescent signals from live and actively metabolizing bacteria. When inoculated into mice, *in vivo* bioluminescence imaging (BLI) techniques can be used to permit the longitudinal measurement of bacteria burden within an infected wound. Both LysM-EGFP×MyD88$^{-/-}$ mice and LysM-EGFP mice were wounded and infected with 1 x 10^7 CFU of *S. aureus* in the wound to compare their susceptibility to *S. aureus* infection. LysM-EGFP×MyD88$^{-/-}$ mice showed greater susceptibility to infection than LysM-EGFP mice demonstrated by 80% lethality of LysM-EGFP×MyD88$^{-/-}$ mice during the first 8 days of infection compared to 0% lethality of LysM-EGFP mice during the entire 15-day duration of the experiment (**Figure 3A**). Both strains of mice lost ~5% body weight following infection, but LysM-EGFP mice recovered original weight by day 2, while LysM-EGFP×MyD88$^{-/-}$ mice did not recover lost weight (**Figure 3B**). Wound closure in the presence of *S. aureus* infection was not different between the two strains; however, sterilely wounded LysM-EGFP×MyD88$^{-/-}$ had a significant defect in wound healing compared to LysM-EGFP mice (**Figure 3C**), as previously reported[19]. Whole animal imaging was used to measure bacterial burden daily, and as early as day 1, LysM-EGFP×MyD88$^{-/-}$ wounds had a higher bacterial burden than LysM-EGFP wounds. LysM-EGFP mice controlled infection and decreased *in vivo* BLI signals in the wound, while LysM-EGFP×MyD88$^{-/-}$ mice exhibited an increase in bacterial burden that plateaued on day 4

until animal death (**Figure 3D**,**E**). Together, these data demonstrate the increased susceptibility of LysM-EGFP×MyD88$^{-/-}$ mice to *S. aureus* infection compared to LysM-EGFP mice.

Neutrophil trafficking is impaired in *S. aureus* infected LysM-EGFP×MyD88$^{-/-}$ mice compared to LysM-EGFP mice

The LysM-EGFP mouse produces green fluorescent neutrophils due to a EGFP protein inserted downstream of the Lysozyme M promoter[12]. This mouse has been utilized to study longitudinal *in vivo* neutrophil trafficking in response to dermal *S. aureus* infection[13,14,20-22]. To compare neutrophil kinetics to wounds produced on LysM-EGFP and LysM-EGFP×MyD88$^{-/-}$ mice, a 6 mm full thickness skin wound was administered on the dorsum, and mice were imaged daily. A 40% decrease in neutrophil trafficking to wounds was observed in LysM-EGFP×MyD88$^{-/-}$ compared to LysM-EGFP mice (**Figure 4A,C**). When 1 x 10^7 CFU of *S. aureus* was introduced immediately after wounding, neutrophil trafficking was attenuated in both strains of mice, but LysM-EGFP×MyD88$^{-/-}$ mice were more sensitive to *S. aureus* infection with respect to neutrophil recruitment. Infection caused a 50% decrease in initial neutrophil trafficking to LysM-EGFP×MyD88$^{-/-}$ wounds compared to a 15% decrease observed in LysM-EGFP wounds (**Figure 4B**). LysM-EGFP mice also recruited significantly more neutrophils to the wound during the later stages of infection (**Figure 4A,C**) compared to LysM-EGFP×MyD88$^{-/-}$ mice, which were unable to increase neutrophil numbers even in the presence of sustained bacterial burden. Together, these data demonstrate that LysM-EGFP×MyD88$^{-/-}$ have a defect in neutrophil recruitment, which is consistent with their increased susceptibility to *S. aureus* infection.

Figure 1: Correlation between OD_{600} and CFU counts for ALC2906.

3-4 ALC2906 SH1000 colonies were picked from an agar plate and transferred into TSB with 10 µg/mL chloramphenicol for overnight culture. The next day, the suspension was split 1:50 into TSB with 10 µg/mL chloramphenicol and cultured. Optical density at 600 nm (OD_{600}) was measured in regular intervals after 2 hours using a spectrophotometer. At each measurement the bacteria was diluted 1:100,000 in ice cold PBS and aliquoted onto an agar plate for overnight incubation. CFUs were counted the following day to calculate the initial concentration and correlated to OD_{600}. N=3 with 4 different OD_{600} measurements per experiment.

Figure 2: Representative region of interests (ROIs) for data analysis.

To analyze *in vivo* BLI and *in vivo* FLI signals, large, equivalent sized ROIs were centered over the wound and total flux (photons per second) was measured. To measure wound closure, an ROI was fit to the wound edge and area (cm^2) was measured.

Figure 3: LysM-EGFP×MyD88$^{-/-}$ mice are more susceptible to *S. aureus* infection compared to LysM-EGFP mice.

LysM-EGFP and LysM-EGFP×MyD88$^{-/-}$ mice were administered a 6mm wound on the dorsum and infected with 1×10^7 CFU of bioluminescent *S. aureus* or sterile saline. Animals were monitored daily and (**A**) survival and (**B**) weight were recorded. Animals were imaged daily to measure (**C**) wound size and (**D**) bacterial luminescence. (**E**) Representative bioluminescent images are depicted from infected LysM-EGFP and LysM-EGFP×MyD88$^{-/-}$ mice. Scale bar = 3

mm. Data represent 7-16 mice per group for A, C, & D and 3 mice per group for B and are expressed as mean ±SEM. * p =0.05, ** p =0.01, ***p =0.001, $^{****}p$ <0.0001 between infected (**A, B, & D**) or uninfected (**C**) groups. Statistical significance was determined using Mantel-Cox test (**A**) and the Holm-Sidak method (**B-D**), with alpha = 0.05, and each time point was analyzed individually, without assuming a consistent SD.

Figure 4: LysM-EGFP×MyD88$^{-/-}$ mice have defective neutrophil recruitment to infected wounds.

LysM-EGFP and LysM-EGFP×MyD88$^{-/-}$ mice were administered a 6mm wound on the dorsum and infected with 1 x 10^7 CFU of bioluminescent *S. aureus* or sterile saline. Animals were imaged daily to measure (**A,B**) neutrophil content. (**C**) Representative fluorescent images are depicted from infected and non-infected LysM-EGFP and LysM-EGFP×MyD88$^{-/-}$ mice. Scale bar = 5 mm. Data represent 8–16 mice per group and are expressed as mean ±SEM. *p<0.05, ** p <0.01, and *** p <0.001 between infected groups and $^{\#\#}$ p <0.01 between uninfected groups (**A**) or as depicted on the graph (**B**). Statistical significance was determined using the Holm-Sidak method (**A**), with alpha = 0.05, and each time point was analyzed individually, without assuming a consistent SD, and one-way ANOVA (**B**), with the Tukey multiple-comparisons post-test.

DISCUSSION:

S. aureus infection models that utilize bioluminescent *S. aureus* infection in a fluorescent neutrophil reporter mouse in conjunction with advanced techniques of whole animal *in vivo* optical imaging have advanced our knowledge of the innate immune response to infection. Previous studies using the LysM-EGFP mouse have shown that up to 1 x 10^7 neutrophils recruit to an *S. aureus* infected wound over the first 24 hours of infection[14], and wound-recruited neutrophils extend their half-life from 1.5 days to 5 days in response to a *S. aureus*-infected wound[22]. A survival tactic adapted by mice to combat infection is trafficking of hematopoietic stem and progenitor cells (HSPC) to an infected wound from the bone marrow where they expand into bactericidal EGFP$^+$ neutrophils in a TLR2/MyD88/PGE_2 dependent manner[21]. In addition, extramedullary granulopoiesis provides an essential source of neutrophils to rescue *S. aureus* infected MyD88$^{-/-}$ mice from lethal sepsis[13]. Adoptive transfer of HSPC from LysM-EGFP mice into wild type mice makes it possible to examine the process of local neutrophil production and its importance in combatting *S. aureus* within a wound[13,21]. It is also possible to calibrate precisely the number of neutrophils in the wound; neutrophil number in wounds correlates linearly with EGFP signal from 1 x 10^6 to greater than 1 x 10^7 neutrophils, and the limit of detection is about 1 x 10^6 neutrophils in a 6 mm wound[14].

In our representative results we compare longitudinal neutrophil and *S. aureus* kinetics in the wounds of LysM-EGFP and LysM-EGFP×MyD88$^{-/-}$. To our knowledge, this is this first study that compares the bacterial burden and neutrophil trafficking between wild type and immunocompromised mice longitudinally through resolution of infection. Of particular interest is the degree of neutrophil recruitment attenuation caused by *S. aureus* infection in the MyD88$^{-/-}$ strain, which elicited a 15% decrease in neutrophil trafficking in wild type mice compared to a 50% decrease in MyD88$^{-/-}$. *S. aureus* is able to evade the host's immune response by producing a number of virulence factors known to indirectly inhibit neutrophil trafficking (*i.e.,* α-toxin,

Panton-Valentine leukocidin, and Chemotaxis inhibitory protein[23,24]), and our results indicate that MyD88 signaling, likely through TLR2, TLR4, and IL-1R, is critical to combat the immune defects caused by *S. aureus* virulence factors. Virulence factor-knockout *S. aureus* strains can be used in this model to characterize their effect on myeloid cell trafficking[13]. Further, when studied in conjunction with the LysM-EGFP×MyD88$^{-/-}$ mouse, this model highlights the role of MyD88 signaling in combatting the pathogenic effects of virulence factors.

There are several critical steps in this protocol that if performed improperly can introduce variability into studies. The wounding and infection procedures are straightforward compared to other mouse disease models, but it is not without its intricacies. Skin punch biopsies are very sharp and can easily cut into the spinotrapezius muscle below the skin. Damaging this muscle and its fascia alters neutrophil recruitment and increases the likelihood of an improper and more invasive *S. aureus* infection that is not centered within the wound. Mice with significant damage to the spinotrapezius muscle should be excluded from studies.

Another source of error in this model comes from changes in the bioluminescent *S. aureus* strain over time, which can alter *in vivo* BLI signals and growth kinetics. The ALC2906 strain contains a shuttle plasmid that contains the modified *lux* operon from *Photorhabdus luminescens* construct that is required to produce the bioluminescent signals, which can be discarded by the *S. aureus* bacteria over time[25]. In a broth culture without selection, 97% of SH1000 colonies produced bioluminescent signals on day 3. This frequency dropped to 53% on day 5 and 21% on day 10[26]. Thus, at later time points during the infectious course, the *in vivo* BLI signals will likely begin to slightly (<1 log difference) underestimate the actual *in vivo* bacterial burden. It is important to freeze down fresh glycerol stocks of *S. aureus* with antibiotic selection to maintain the plasmid upon arrival and replacing working stocks of bacteria frequently (*i.e.,* every three months) can help prevent loss of the bioluminescent construct during culture maintenance. Newer strains of bioluminescent *S. aureus* contain a stably integrated bioluminescent construct[27,28] and are likely an improvement over the ALC2906 strain used in this model.

While this model is useful to study localized dermal *S. aureus* infections, it has limitations. The *in vivo* BLI signals of bacterial burden is limited to the wound and adjacent skin. The model does not provide a reliable readout for deep invasive infections such as sepsis, and animals must be euthanized to measure dissemination of bacteria in to the bloodstream or kidneys[13]. Newer and brighter engineered bioluminescent strains have been generated that can permit the detection of *in vivo* BLI signals from internal organs[27,28]. In addition, longitudinal detection and monitoring of neutrophil trafficking cannot be measured in other commonly acquired *S. aureus* infections, including respiratory and blood infections. Reduced sensitivity due to tissue autofluorescence is also a limitation of this model. The recently developed Catchup mouse utilizes a TdTomato RFP under the Ly6G promoter and may have a higher signal to noise ratio than the LysM-EGFP mouse due to reduced tissue auto fluorescence in the RFP channel[11].

The potential crosstalk between *in vivo* BLI and FLI emission signals in this model is worthy of discussion but is negligible. If we perform experiments without light excitation and only collect

in vivo FLI EGFP signals using the 520/20 filter, we do not observe any appreciable signals from infected mice, demonstrating that collected signals are from *in vivo* FLI EGFP signals and not from overlap of *in vivo* BLI signals of the bacteria (data not shown). This is primarily due to the signal collection time, which is only 1 second for *in vivo* FLI and 1 minute for *in vivo* BLI, and a specific excitation wavelength filter of 465/30 nm and emission filter of 520/20 nm. These settings allow for optimal detection of *in vivo* FLI EGFP signals without contribution from *in vivo* BLI signals. This is best demonstrated when comparing the order of magnitude of the y-axis between **Figure 3D** and **Figure 4A**. The signals from the *in vivo* BLI is about 100-fold less than the *in vivo* FLI of EGFP signals, indicating that the *in vivo* BLI signals are negligible and contribute to less than 1% of the signals observed from *in vivo* FLI.

We expect future applications of this model will help researchers discover and characterize *S. aureus* virulence factors, and serve as a pre-clinical animal model to test novel therapeutics that aim to clear *S. aureus* infection by boosting the innate immune response.

DISCLOSURES:

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Figure 1

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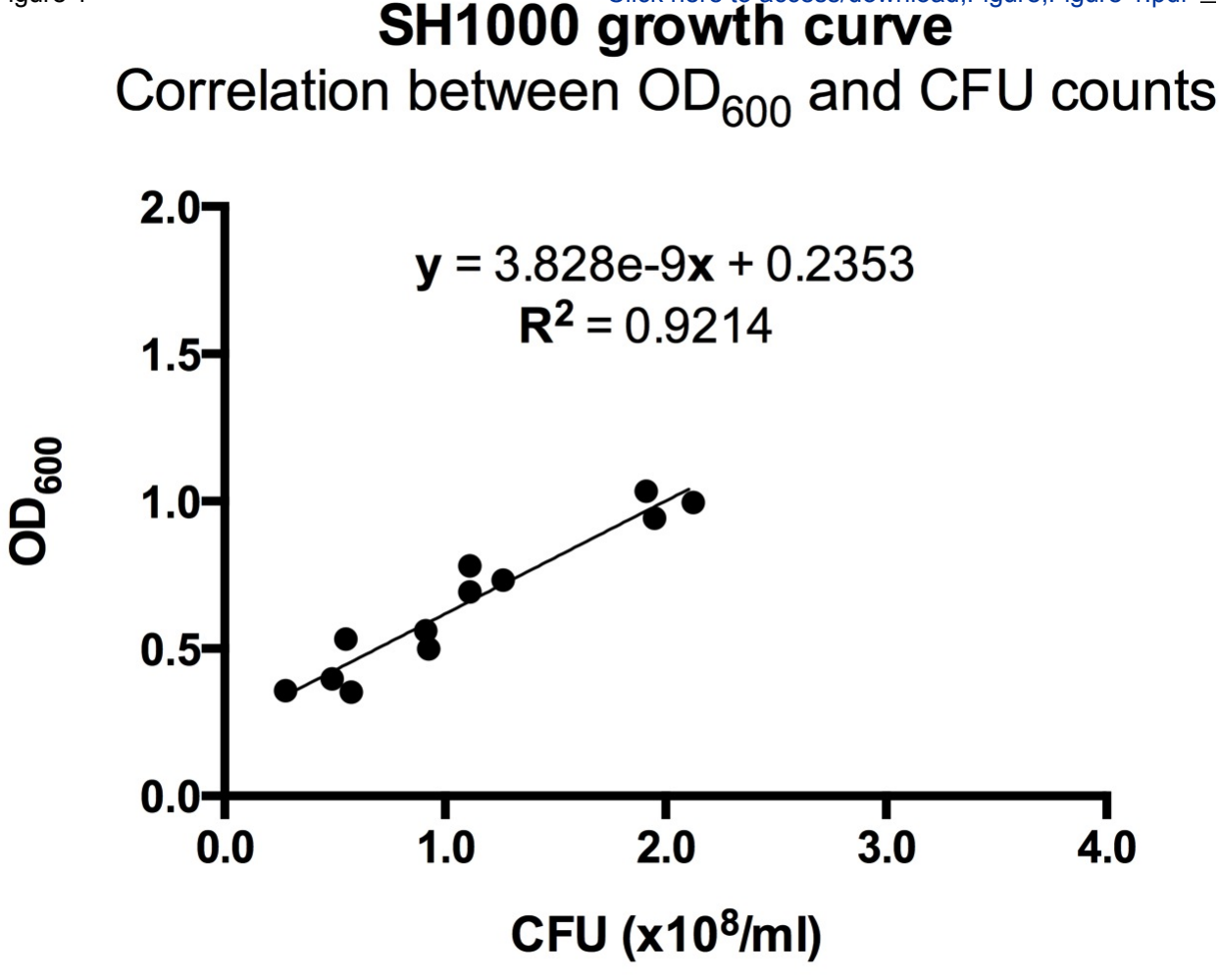
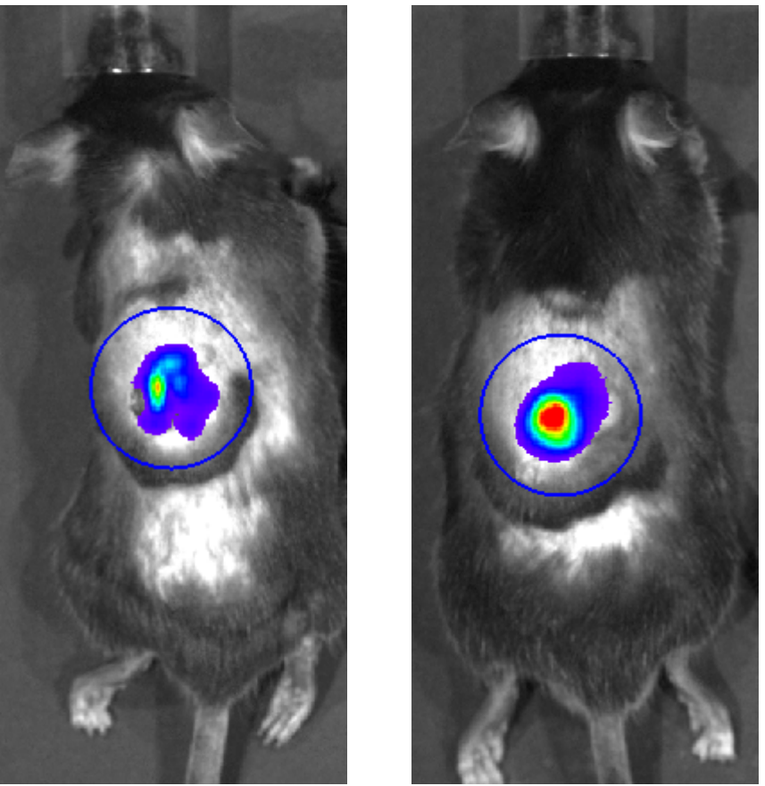


Figure 2

A

Bioluminescence
Bacteria Burden

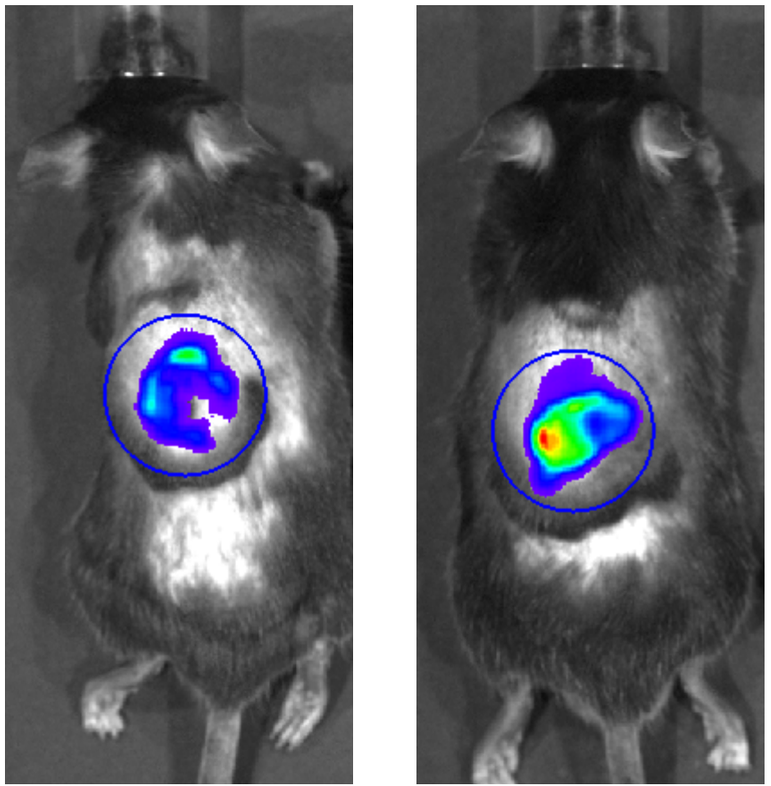
Day 1 Day 5



B

EGFP Fluorescence
Neutrophil Content

Day 1 Day 5



C

Photograph
Wound Healing

Day 1 Day 5

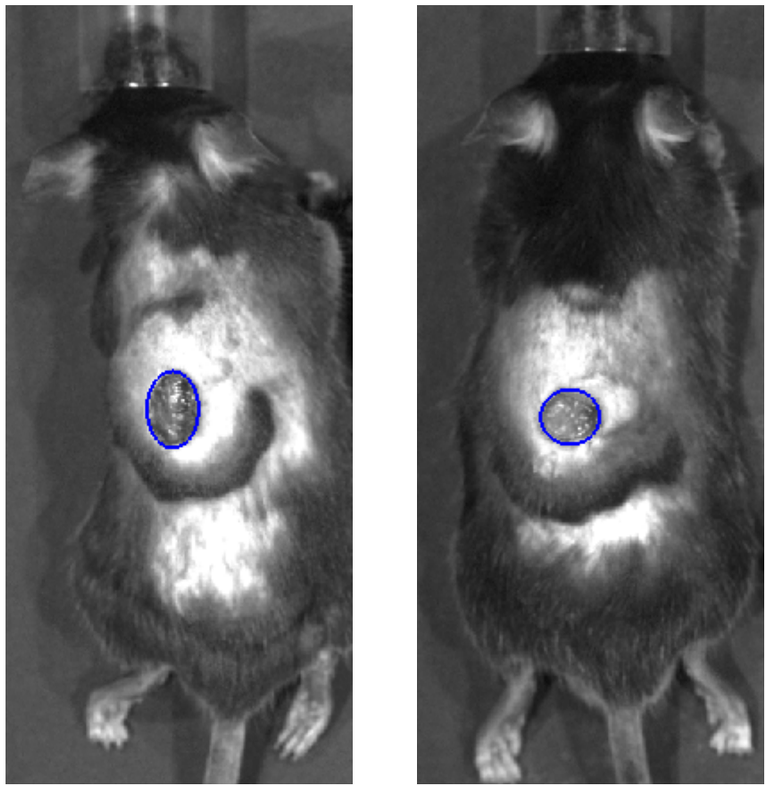


Figure 3

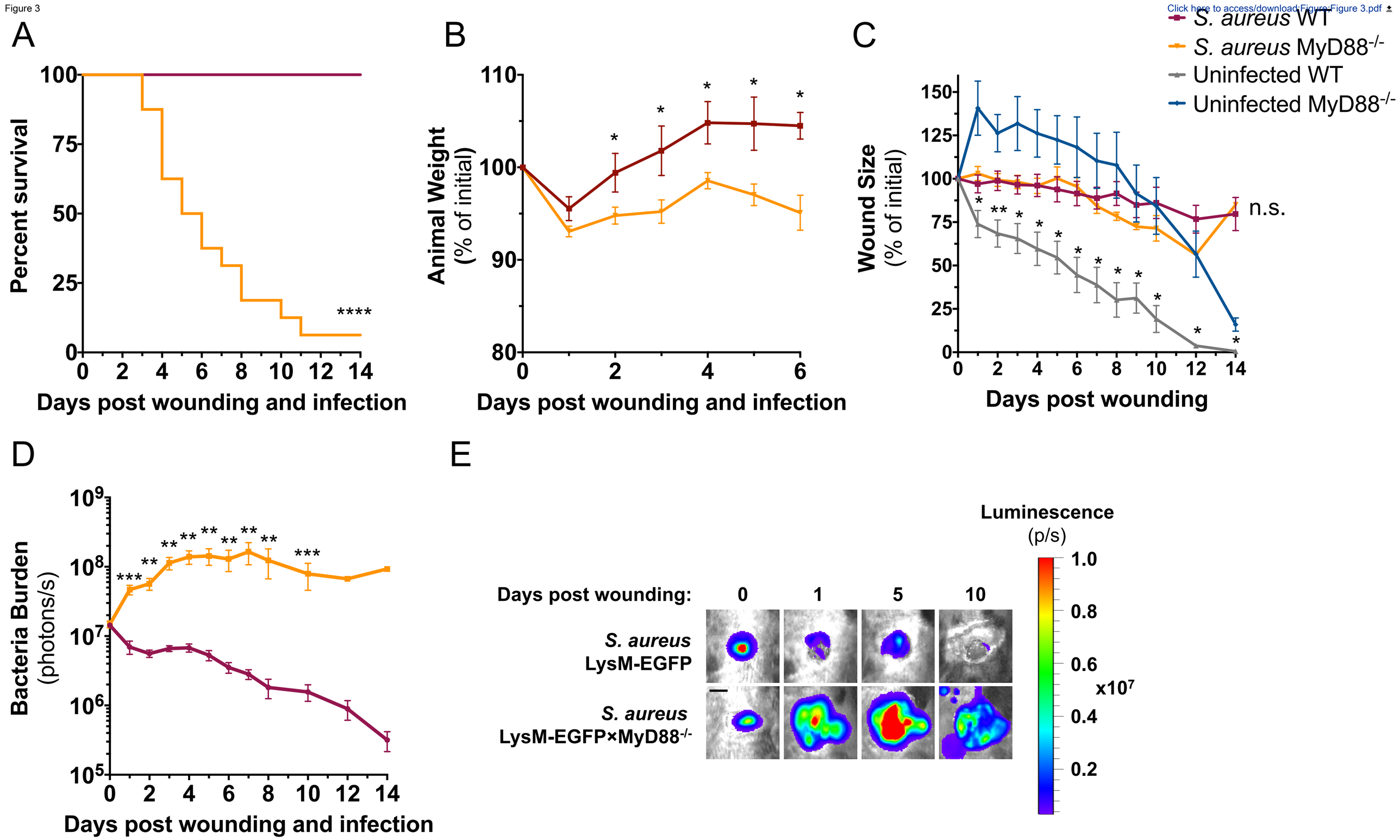
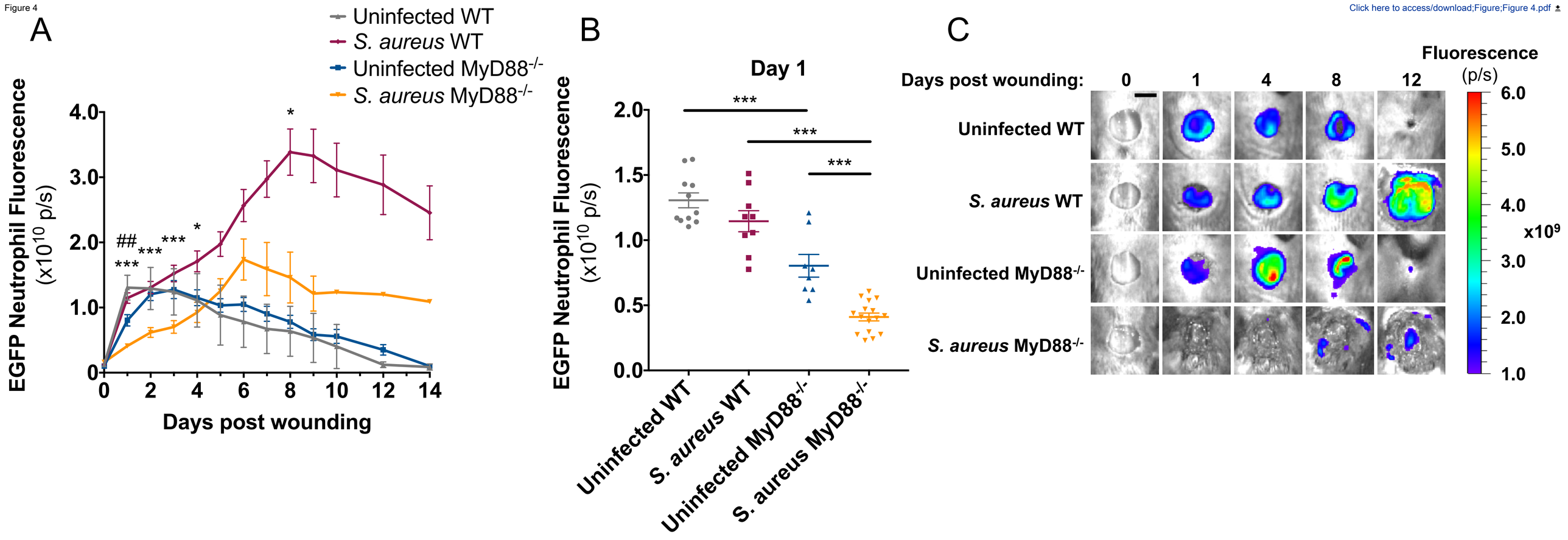


Figure 4



Name of Material/ Equipment	Company
14 mL Polypropylene Round-Bottom Tube	Falcon
6mm Disposable Biopsy Punch	Integra Miltex
Bioluminescent *S. aureus*	Lloyd Miller, Johns Hopkins
Bovine Blood Agar, 5%, Hardy Diagnostics	VWR
Buprenoprhine hydrochloride injectable	Western Medical Supply
C57BL/6J Mice	Jackson Labratory
Chloramphenicol (crystalline powder)	Fisher BioReagents
DPBS (1X)	Gibco
Insulin Syringes	Becton, Dickson and Company
IVIS Spectrum In Vivo Imaging System	Perkin Elmer
Living Image Software – IVIS Spectrum Series	Perkin Elmer
LysM-eGFP Mice	Thomas Graff Albert Einstein College of New York
Microvolume Spectrophotometer	ThermoFisher Scientific
MyD88 KO Mice	Jackson Labratory
Non-woven sponges	AMD- Ritmed Inc
Povidone Iodine 10% Solution	Aplicare
Prism 7.0	GraphPad Software
Tryptic Soy Broth	Becton, Dickson and Company

Catalog Number	Comments/Description
352059	
33-36	
ALC 2906 SH1000	
10118-938	
7292	0.3 mg/mL
000664	
BP904-100	
14190-144	
329461	.35 mm (28 G) x 12.7 mm (1/2'')
124262	
128113	
NA	
ND-2000	
009088	
A2101-CH	5 cm x 5 cm
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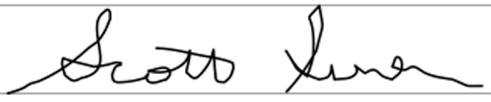
Signature:	 Date: 8/28/18

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Response to reviewers' comments for JoVE manuscript 59015:

We wish to thank the reviewers for their detailed comments, which we have used to improve the manuscript. We have carefully considered each comment and provide our point-by-point responses below indicated in red. We have added 2 figures to the manuscript. Figure 1 shows the correlation between *Staphylococcus aureus* optical density and concentration, and Figure 2 provides examples of ROIs drawn for image analysis.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.
Addressed

• **Protocol Detail:** 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 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.
Addressed
1) 1.3: 10-16 weeks?
Addressed
2) 2.3: Which antibiotic at what concentration?
Addressed (10 µg/ml chloramphenicol)
3) 2.4: What is the shaker speed?
Addressed (.134xG or 200RPM)
4) 2.7: Do you have a reference for the relation between OD and CFU?
Yes, this is added to new figure 1.
5) 2.8: Dilute in TSB? How are the CFUs counted? Under a microscope? Mention magnification if so.
Addressed (colonies counted by gross examination)
6) 3.2: What is the oxygen flow rate?
Addressed (2-3 Liters per minute)
7) 3.3: Do you mean "depilatory" instead of dilapidation? Please revise this step for grammar.
Addressed
8) 3.4: % povidone-iodine?
Addressed (10%)
9) 4.2: how do you relate the photon counts to bacterial concentration? Was this calibrated?
These instructions were removed from the manuscript. Bacteria concentration is determined through other methods (OD600 and serial dilutions and plating, which are described in the manuscript), and this process is used by us as a sanity check to verify bioluminescent signal, and isn't required in the manuscript.
10) 4.4: Mention needle gauge, inoculant max volume, needle angle and depth to ensure that a bubble is formed.

Addressed
11) 4.5: Are there are any post-procedure steps? Please add a step to mention that the animal is monitored until recovery from anesthesia.
Addressed
12) 5.1: Mention oxygen flow rate.
Addressed
13) 5.2: Are there are any post-procedure steps? Please add a step to mention that the animal is monitored until recovery from anesthesia.
Addressed

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Mention of commercial sounding language was removed from the manuscript.
• **Table of Materials:** 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/software in separate columns in an xls/xlsx file. Please include items such as bioluminescent S. aureus strain, imaging instrument, animal strain, software etc.
Table of materials was updated.
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This manuscript is composed entirely of original data.

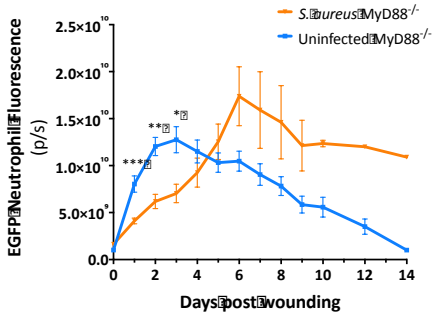
Comments from Peer-Reviewers:

Reviewer #1:

Manuscript Summary:
This manuscript describes the development of a bioluminescent model to track neutrophils

migration to a wound infected with staphylococcus aureus.

Major Concerns:
Can the authors better explain the results in Figure 2A and B? There does not seem to be much difference in neutrophils flow between uninfected and infected MyD88-/- mice in panel A. What are the repercussion of these data on the described model?
There is a significant difference in trafficking to the wound between the two strains on days 1, 2, & 3 (see below). We point to this defect in neutrophil recruitment as a reason for the increased susceptibility of MyD88$^{-/-}$ mice to succumb to *S. aureus* infection.



Minor Concerns:
1) Some sentences are broken and will need editing (see end of first paragraph in Introduction)
Addressed
2) section 1.3 "ages 10-16": weeks?
Addressed
3) 4.1 and 4.2: more details should be given about the protocol for bioluminescence. Also would be helpful to quote some ROI values for different bacterial concentrations. as examples
These instructions were removed from the manuscript. Bacteria concentration is determined through other methods (OD600 and serial dilutions and plating, which are described in the manuscript), and measuring the *in vivo* bioluminescence imaging (BLI) signals provides a real time estimate of the bacterial abundance and spatial density.
4) I think the discussion of the critical steps and pitfalls provided in the Discussion section should be extended, and some of this information should also be included in the Methods.
Content was added to the discussion. Specifically, the pitfalls of using the ALC2906 SH1000 strain were added.

Reviewer #2:

Manuscript Summary:
This is a nice method paper on the use of bioluminescent S. aureus and LysM-EGFP mice to track bacteria and neutrophils during subcutaneous infection. Adequate details are provided to allow the reader to use the method to study host immune responses to staph in vivo.

Minor concerns:
1) Page 3 line 143 - Did the authors use antibiotic selection to maintain optimal bioluminescence after thawing from -80? If not, how quickly is plasmid lost in vitro?
In these studies, thawed bacteria were streaked onto blood agar without antibiotics. Bioluminescence imaging has confirmed that >90% of the colonies produce bioluminescent

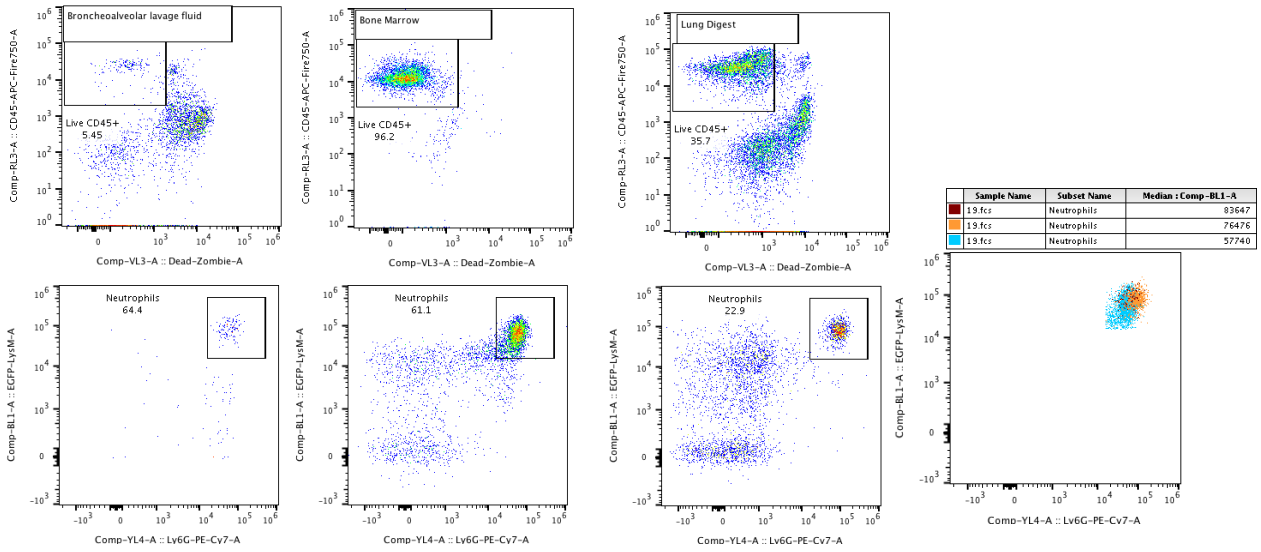
signals. Since 3-4 colonies are chosen for overnight culture in antibiotics, at least one bioluminescent positive colony is nearly guaranteed to be expanded in overnight culture.
A previous publication from our laboratory (Bernthal et al, *PLoS ONE (*2010)) using the same strain of SH1000 grown in broth without selection, showed that 97% of SH1000 colonies produced bioluminescent signals on day 3. This frequency dropped to 53% on day 5, and 21% on day 10. Thus, at later time points during the infectious course, the *in vivo* BLI signals will likely begin to slightly (<1 log difference) underestimate the actual *in vivo* bacterial burden. This information has been added to the manuscript.

2) Page 4 line 207 - Please define ROI

Region of interest, now written out and abbreviation defined.

3) Page 5 line 255 - How stable is LysM expression level? Does it change with inflammation and recruitment to tissues? This is important because neutrophil number is standardized based on imaging of neutrophils harvested from blood or bone marrow of naïve mice?

Expression of LysM-EGFP increases in the cytosol of neutrophils as they as mature in the bone marrow, and thus is expressed at slightly higher levels in distal tissues where mature cells traffic. In the plots below, the expression of LysM-EGFP is compared between neutrophils in the bone marrow, neutrophils migrating through the lung parenchyma, and neutrophils that trafficked to



the alveolar space. Bone Marrow neutrophils expressed 25% less EGFP than lung neutrophils and 30% less EGFP than BALf neutrophils. When correlating EGFP to neutrophil number, bone marrow neutrophils are used because of the higher quantity that can be isolated. This information has been added the to the manuscript. Because of this difference in EGFP expression between bone marrow and blood neutrophils, it is possible that the number of neutrophils in the wound is overestimated by up to 20%.

4) Page 9 line 397- How rapidly are plasmids lost in vivo and what is a reasonable max length for an experiment given the plasmid is not stably maintained?

This is an important point and additional commentary has been added to the discussion.

Reviewer #3:

Manuscript Summary:
They present standardized approach to monitor bacterial infection of the skin and its progression in a specialized immunocompromised animal model that has GFP neutrophils. This would allow the investigators to correlate PMN with bacteria burden.

Major Concerns:
1) Some explanation of potential crosstalk between the light produced by the bacteria and any impact on GFP signal. Since bioluminescence is generally emitting around 560 nm it is unlikely to be excited by the GFP but since both are colocalized it should be at least addressed. How does this impact cell numbers or is it controlled for. I am worried that the light being produced by the bioluminescence microbes is also able to exciting GFP and thus cause overlap.
The potential crosstalk between *in vivo* BLI and FLI emission signals in this model is worthy of discussion but is negligible. If we perform experiments without light excitation and only collect *in vivo* FLI EGFP signals using the 520/20 filter, we do not observe any appreciable signals from infected mice, demonstrating that collected signals are from *in vivo* FLI EGFP signals and not from overlap of *in vivo* BLI signals of the bacteria (data not shown). This is primarily due to the signal collection time, which is only 1 second for *in vivo* FLI and 1 minute for *in vivo* BLI, and a specific excitation wavelength filter of 465/30 nm and emission filter of 520/20 nm. These settings allow for optimal detection of *in vivo* FLI EGFP signals without contribution from *in vivo* BLI signals. This is best demonstrated when comparing the order of magnitude of the y-axis between Figure 3D and Figure 4A. The signals from the *in vivo* BLI is about 100-fold less than the *in vivo* FLI of EGFP signals, indicating that the *in vivo* BLI signals are negligible and contribute to less than 1% of the signals observed from *in vivo* FLI.
This is an important point and additional commentary has been added to the discussion.
2) Step one on the mouse housing states that your have to derive LysM-eGFP mice and then cross with MyD88-/- from Jax. Seems that for this to be broadly used that the resulting strain should be deposited at Jax or another resource to have more investigators use their model in this protocol.
Upon publication of this manuscript, we will offer to donate this strain to The Jackson Laboratory. In addition, we have made our LysM-eGFP strain available to all investigators who request them and would be willing to provide the LysM-EGFPxMyD88$^{-/-}$ strain as well.

Line 202 - Is it accurate that bacteria concentration is determined prior to injection. It maybe interesting to stress as typically we estimate inoculum by absorbance measures and confirm by overnight incubation of serial dilutions. Here serial dilutions are not being done either.
We use the same technique. Absorbance is used to estimate bacteria concentration. After bacteria is washed and resuspended in PBS, we incubate serial dilutions to confirm concentrations. We updated sections 2.5 and 2.8 to include this information in the manuscript.

Line 129-131 - may need actual protocol number of animal protocols approved depending on the journal requirements.
We do not believe this is required by JoVE, but will be added per request by the editor.

Minor Concerns:
Line 53 - "knocked in" should be replaced, as it is more slang than clear "inserted in" instead.
“knocked in” replaced with “inserted in”

Line 72. There is punctuation missing
punctuation added
Line 87, NET abbreviation is not needed as it is only used once
“NET” removed
Line 182 - may need to add some text on how fully anesthetized is determined since this is a methods paper.
Additional information added in section 3.2
Line 187 - "by be inflammatory" maybe should be "may cause inflammation".
This section was rewritten and should be clearer.
Line 204 - should read more correctly "… image the total bioluminescence signal from the plate for 1 minute." Removed from the submission
Line 207 - pretty sure it is more accurately the LivingImage software not the Spectrum Software.
Use of commercial language is not permitted by JoVE, so this was removed from the submission, but is accurately described in the list of materials.
Line 247 - probably should be PerkinElmer now a days.
Use of commercial language is not permitted by JoVE, so this was removed from the submission, but is accurately described in the list of materials.

Line 366 - not sure what is meant by "dark wild type mice"
The word dark is removed for clarity
Line 403 - needs a comma between citations
Comma added

Reviewer #4:

Manuscript Summary:
Anderson et al convincingly presents a robust method to simultaneously monitor the kinetics of a bacterial infection and the immune response in a longitudinal fashion. They convincingly present the method that includes a breeding strategy to create a knockout reporter strain (LysM-EGFPxMyD88-/-) followed by a wound infection using a bioluminescent bacteria strain they generate (ALC2906 SH1000-Lux). This method presented, albeit not novel, is an important example for the scientific community to replicate in other studies to simultaneously monitoring wound injury with bioluminescent and fluorescent reporters.

Major Concerns:
1. It is not clear why the authors chose to use such a wide range of subjects in Figure 1 (N=3-16).
Longitudinal animal weights were only recorded for a subset of animals (n=3). The other graphs all correspond to 7-16 mice per group. I added this information into the figure legend.
2. The image resolution in both figures 1e and 2c are unacceptable for an imaging paper.
This is the best image resolution possible from the IVIS device and have been widely published by us and others (Cho et al. 2010 & Kim et al. 2011) These images can be removed from the figure set if desired by the editor.
3. Is the measurement of Flux (P/s) a max, median or mean? More in-depth description of the analysis techniques is needed in this manuscript. A potential addition to increase the utility of this manuscript would include a figure to outline how the ROI was drawn and the data

interpreted. This is essential since it is well known that optical imaging analysis is highly variable.
The measurement is a mean flux of photon detection. This was added to section 6.2. Figure 2 was added to show methods used to draw ROIs.
4. BLi and FLi is not quantitative, it is semi-quantitative at best. I would recommend that use of the word quantitatively (line 118) be modified accordingly.
Addressed by removal
5. More thorough description of the bacterial strain used.
More detail is provided in section 2.1 regarding the bacteria strain.
Minor Concerns:
None

References

Bernthal, N. M., Stavrakis, A. I., Billi, F., Cho, J. S., Kremen, T. J., Simon, S. I., et al. (2010). A mouse model of post-arthroplasty Staphylococcus aureus joint infection to evaluate in vivo the efficacy of antimicrobial implant coatings. PLoS ONE, 5(9), e12580. http://doi.org/10.1371/journal.pone.0012580

Cho, J. S., Pietras, E. M., Garcia, N. C., Ramos, R. I., Farzam, D. M., Monroe, H. R., et al. (2010). IL-17 is essential for host defense against cutaneous Staphylococcus aureus infection in mice. The Journal of Clinical Investigation, 120(5), 1762–1773. http://doi.org/10.1172/JCI40891

Kim, M. H., Granick, J. L., Kwok, C., Walker, N. J., Borjesson, D. L., Curry, F. R. E., et al. (2011). Neutrophil survival and c-kit+-progenitor proliferation in Staphylococcus aureus-infected skin wounds promote resolution. Blood, 117(12), 3343–3352. http://doi.org/10.1182/blood-2010-07-296970