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TITLE:

Assessing Biofilm Dispersal in Murine Wounds

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SUMMARY:
Here, we describe *ex vivo* and *in vivo* methods for assessing bacterial dispersal from a wound infection in mice. This protocol can be utilized to test the efficacy of topical antimicrobial and anti-biofilm therapies, or to assess the dispersal capacity of different bacterial strains or species.

ABSTRACT:
Biofilm-related infections are implicated in a wide array of chronic conditions such as non-healing diabetic foot ulcers, chronic sinusitis, reoccurring otitis media, and many more. Microbial cells within these infections are protected by an extracellular polymeric substance (EPS), which can prevent antibiotics and host immune cells from clearing the infection. To overcome this obstacle, investigators have begun developing dispersal agents as potential therapeutics. These agents target various components within the biofilm EPS, weakening the structure, and initiating dispersal of the bacteria, which can theoretically improve antibiotic potency and immune clearance. To determine the efficacy of dispersal agents for wound infections, we have developed protocols that measure biofilm dispersal both *ex vivo* and *in vivo*. We use a mouse surgical excision model that has been well-described to create biofilm-associated chronic wound infections. To monitor dispersal *in vivo*, we infect the wounds with bacterial strains that express luciferase. Once mature infections have established, we irrigate the wounds with a solution

containing enzymes that degrade components of the biofilm EPS. We then monitor the location and intensity of the luminescent signal in the wound and filtering organs to provide information about the level of dispersal achieved. For *ex vivo* analysis of biofilm dispersal, infected wound tissue is submerged in biofilm-degrading enzyme solution, after which the bacterial load remaining in the tissue, versus the bacterial load in solution, is assessed. Both protocols have strengths and weaknesses and can be optimized to help accurately determine the efficacy of dispersal treatments.

INTRODUCTION:

The rise of antibiotic resistance worldwide is leading to a lack of antibiotic options to treat a variety of bacterial infections[1]. In addition to antibiotic resistance, bacteria can gain antibiotic tolerance by adopting a biofilm-associated lifestyle[2]. A biofilm is a community of microorganisms that are protected by a matrix of polysaccharides, extracellular DNA, lipids, and proteins[3], collectively called the extracellular polymeric substance (EPS). As the antibiotic resistance crisis continues, new strategies that prolong the use of, or potentiate the efficacy of, antibiotics are sorely needed. Anti-biofilm agents are one promising solution[4].

Amongst the different anti-biofilm strategies that have been proposed, the utilization of dispersal agents, which target different components of the biofilm EPS, are at the forefront of therapeutic development[5]. Glycoside hydrolases (GH) are one such class of dispersal agent. GH are a large class of enzymes that catalyze the cleavage of different bonds within the polysaccharides that provide structural integrity to the EPS. Our group, as well as others, have shown that GH can effectively degrade biofilms, induce dispersal and improve antibiotic efficacy for a number of different bacterial species, both *in vitro* and *in vivo*[6-11].

With a growing interest in biofilm dispersal, it is important to develop effective methods that assess dispersal efficacy. Here, we present a detailed protocol for the treatment of biofilm-associated wound infections with a dispersal agent in mice, and the assessment of dispersal efficacy, *in vivo* and *ex vivo*. The overall goal is to provide effective methods that can be used with preclinical models to measure biofilm dispersal effectively and efficiently.

A murine surgical excision infection model was used in these studies to establish a biofilm-associated infection. We have used this model for over 15 years and published our observations extensively[7,9,12-21]. In general, this is a non-lethal infection model where bacteria remain localized to the wound bed and are biofilm-associated (bacteria seen in aggregates surrounded by EPS), setting up a chronic infection that lasts up to 3 weeks. However, if mice are immunocompromised (with Type 1 diabetes for example), they can become more susceptible to developing a fatal systemic infection in this model.

In this report, we provide protocols for assessing the dispersal of bacteria from a wound, both *in vivo* and *ex vivo*. Both protocols can be used to examine the efficacy of a dispersal agent and have their own strengths and weaknesses. For example, assessing dispersal *in vivo* can provide important, real-time information about the spread of bacteria to other parts of the body after dispersal, and how the host responds. On the other hand, assessing dispersal *ex vivo* may be

more desirable for screening multiple agents, doses, or formulations, as the tissue can be divided into multiple sections that can be tested separately, thus reducing the number of mice required. When assessing multiple agents, we typically measure dispersal first *in vitro* as previously described [6,9,22]. We then test the most effective *ex vivo* and reserve *in vivo* testing for a limited number of very promising agents.

PROTOCOL:

This animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Texas Tech University Health Sciences Center (protocol number 07044). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

1. Preparing bacteria for mouse infections

NOTE: Here we describe infecting mice only with *Pseudomonas aeruginosa*. However, other bacterial species may be used to cause infection. Bacterial strains and materials are detailed in the **Table of Materials**.

1.1. Use *Pseudomonas aeruginosa* wild-type strain PAO1 carrying the luminescence plasmid pQF50-lux[23] for these experiments as previously described[7].

1.2. Grow *P. aeruginosa* strains in baffled Erlenmeyer flasks, with shaking at 200 rpm, in Luria-Bertani (LB) broth at 37 ˚C for 16 to 20 h. Add a final concentration of 100 µg/mL of ampicillin to the overnight culture of PAO1(pQF50-lux) for maintenance of the plasmid.

1.3. Subculture *P.* aeruginosa by adding 100 µL of the overnight culture to an Erlenmeyer flask containing 10 mL of LB broth with a final concentration of 100 µg/mL ampicillin. Then grow for 2-2.5 h at 37 °C with shaking, to an $OD_{600\ nm}$ of 0.4, and then serially dilute (1:10) three times for an infecting dose of approximately 10^4 cells.

2. Preparation of biofilm dispersal enzyme

NOTE: For this study we use a 10% solution of equal parts amylase and cellulase (5% of each), which will be referred to as “GH”, for the dispersal treatment.

2.1. Use a 10% GH solution (w/v) of amylase (from *Bacillus subtilis*) and fungal cellulase (from *Aspergillus niger*) diluted in 1x phosphate buffer saline (PBS). Use PBS as the vehicle control treatment.

2.2. Warm all treatments to 37 °C for 30 min for enzyme activation.

3. Experimental animals and preoperative setup

3.1. House mice, 5 litter mates per cage, in environment-controlled conditions, with 12 h light/dark cycles and proper access to food and water.

3.2. Preferably, use female Swiss Webster mice, between 8 and 10 weeks old.

3.3. Weigh mice to determine the proper amount of anesthesia to administer.

3.4. Administer anesthesia by the intraperitoneal injection of 100 mg/kg ketamine 10 mg/kg xylazine.

3.5. Apply eye cream (e.g., Refresh P.M.) carefully onto the eye using a cotton swab to reduce dryness.

3.6. Monitor physiologic parameters including respiratory rate, skin coloration, and body temperature throughout the surgery.

4. Dorsal full thickness excision surgery

4.1. Assess surgical plane of anesthesia by toe pinch and monitoring of respiratory rate and effort. Shave the dorsal surface and apply a depilatory cream for 10 min (or as per product's instructions), after which it was gently removed, ensuring the skin was dry.

4.2. For pain management, administer 0.05 mL of lidocaine subcutaneously into the area to be excised, and inject 0.02 mL of buprenorphine subcutaneously into the scruff of the neck. Wait 10 min to ensure pain management was reached.

4.3. Before excision, disinfect the dorsal surface using an alcohol swab and draw a 1.5-cm diameter circle towards the posterior portion of the back. Maintain a sterile surgical field throughout the procedure and used autoclaved instruments and sterile gloves. To limit contamination, conduct the surgery by a lit Bunsen burner and use clean tools for each animal.

4.4. Administer a full-thickness, excisional skin wound to the level of panniculus muscle with surgical scissors.

4.5. After excision, immediately cover wounds with a semipermeable polyurethane dressing.

4.6. Inject approximately 10^4 CFU of bacteria in 100 µL PBS under the dressing, onto the wound bed, to establish an infection.

NOTE: Wounds can also be infected with multiple species of bacteria and/or fungi simultaneously, or by placing pre-formed biofilms[12], or even debridement tissue extracted from patients[19], onto the wound bed.

4.7. After infection, place mice into cage with heating pads and monitor until they regained

their righting reflex.

NOTE: Ensure that mice do not get cold as this can lead to death.

4.8. Establish wound infections for 48 h.

4.9. Inspect adhesive dressings daily for tears and areas of non-adherence and replaced if needed.

5. *In vivo* dispersal treatment

NOTE: Here we describe administration of the dispersal agents by applying a series of 3 topical wound irrigation solutions (**Figure 1**). However, the protocol can be adapted for other types of delivery such as the application of gels, creams or dressings.

5.1. Place mice under isoflurane anesthesia (at 3% and 1 L per minute of oxygen) while administering the treatments.

5.2. Prepare three 1 mL syringes, with 25 G needles, containing 200 µL of treatment for each mouse.

5.3. Inject 200 µL of enzyme solution or PBS control onto the wound by carefully lifting and pinching the skin with forceps, slightly above the wound toward the head of the animal. Carefully pierce the syringe through the lifted skin and slowly inject the solution between the dressing and the wound bed. The dressing utilized in this study often adheres to both the wound bed and the surrounding tissue.

5.3.1. To ensure that the entire wound bed is covered by solution, gently raise the dressing with forceps, pulling it away from the wound bed, while slowly injecting the solution.

5.4. After treatment, place mice back in their cages for 30 min.

5.5. After 30 min of exposure to the treatment, re-anesthetize the mice with isoflurane and aspirate the solution with a syringe, making sure to puncture in the same area as before.

NOTE: This aspirated solution contains dispersed bacterial cells, which may be saved for various downstream analyses.

5.6. Administer the second and third irrigation treatments as the first, using a new syringe each time.

6. *In vivo* dispersal imaging and analysis

NOTE: If a luminescent strain of bacteria is utilized to initiate infection, an *In Vivo Imaging System*

(IVIS) can be used to visualize dispersal from the wound bed.

6.1. Image mice immediately before and after treatment and at 10 and 20 h post-treatment (**Figure 2 and Supplemental Figure 1**).

6.2. Perform imaging while mice were under anesthesia by placing them individually in the IVIS and imaging each one for 5 s at a wavelength of 560 nm. Save images for further analysis.

NOTE: Optimal wavelength and exposure time will depend on the reporter used and the IVIS instrument and imaging software.

6.3. For analysis, open images in the IVIS software and select the area to be analyzed in the Tool Palette.

6.4. To account for background, place a circle on the background of a photo and then place the same sized circle on top of the wound bed for each mouse.

6.5. To upload measurement values, select **View -> Region of Interest (ROI) measurements** and upload the table of measurements to a spreadsheet. Subtract the background circle reading from each of the wound bed measurements to calculate luminescence for each sample. Calculate percent luminescence changed by:
ROI pre-treatment-ROI post-treatment/ ROI pre-treatment) x 100.

NOTE: Control and treated mice should be analyzed simultaneously to normalize for variables that may affect image acquisition or radiance.

7. Assessing dispersal by determining CFU

7.1. Humanely euthanize mice by the intraperitoneal injection of 0.2 mL of pentobarbital sodium (e.g., Fatal Plus) under anesthesia with 100 mg/kg ketamine 10 mg/kg xylazine.

7.2. Harvest wound bed tissue by first gently removing the dressing with sterile forceps, and then cutting around the perimeter of the wound bed with sterile surgical scissors. Gently lift one end of the wound bed with forceps while using scissors to cut/tease the sample away from the muscle layer.

7.3. Place tissue from the wound bed in pre-labeled and pre-weighed 2 mL homogenization tube containing 1 mL of PBS. Weigh the tubes again after inserting the samples, and then gently invert 3-5 times to rinse off any dispersed or loosely adhered bacteria, and remove the PBS. Add 1 mL of fresh PBS.

7.4. In order to harvest the spleens, place mice in a supine anatomical position and secure by pinning their extremities to a dissection surface. Soak the area to be dissected with 70% ethanol in order to disinfect the area and keep the fur from contaminating the sample.

7.5. Carefully make a small incision perpendicular to the midline with surgical scissors in the dermis of the lower abdomen, making sure to pull the skin up and away from the internal organs. Continue the incision interiorly, along the mid-line, until the sternum was reached. Once the peritoneal cavity was exposed and the internal organs were visible, gently separate the spleen from the connective tissue and place in a separate homogenization tube.

7.6. Weigh spleen and rinse with PBS, as described for the wound bed tissue.

NOTE: Other organs of interest can be similarly harvested.

7.6.1. When making the initial incision, take care to avoid puncturing the intestines, or other organs.

7.7. Homogenize samples in 2 mL pre-filled bead mill tubes using a benchtop homogenizer at 5 m/s for 60 s, twice.

7.8. To enumerate CFU, serially dilute samples and spot-plate on selective agar. For serial dilution, pipette 100 µL of the sample into 900 µL of PBS in a 1.5 mL tube, vortex, and repeat 5 times. Pipette 10 µL of each dilution onto an agar plate as shown in **Figure 3**.

NOTE: If more precise CFU quantitation is required, spread 100 µL of each dilution of the sample across separate agar plates.

8. *Ex vivo* assessment of dispersal (Figure 4)

8.1. Wound mice and infect with approximately 10^4 PAO1 as described above, and then euthanize 48 h after infection.

8.2. Carefully remove the dressing with forceps, using sterile technique and without disturbing the wound bed.

8.3. Use sterile surgical scissors to cut the perimeter of the wound bed away from the uninfected skin by using forceps to lift one end of the wound bed and scissors to excise infected wound tissue from the muscle layer underneath and surrounding uninfected tissue. Divide wound bed samples into equal parts or use whole.

8.4. Place wound tissue into empty, pre-weighed 2 mL bead mill homogenizing tubes. Then weigh the tubes again after adding the sample. Calculate the weight of the sample by:
weight after adding sample – weight before adding sample.

8.5. Add 1 mL of PBS, and gently invert tube 3-5 times to rinse the sample and remove any planktonic cells. Remove and discard the PBS.

8.6. Add 1 mL of GH treatment (or PBS as a negative control) to the sample and incubate for 2 h at 37 °C with shaking at 80 rpm.

8.7. Remove the biofilm dispersal solution and place into a separate 1.5 mL tube. This contains the dispersed cells.

8.8. Add 1 mL of PBS to the remaining tissue sample and gently invert 3-5 times to rinse off any remaining dispersed cells. Remove and discard the PBS.

8.9. Add 1 mL of PBS to the remaining tissue and homogenize at 5 m/s for 60 s using a benchtop homogenizer.

8.10. Serially dilute the solution of dispersed cells and the homogenized tissue sample by placing 100 µL of sample into 900 µL of PBS in a 1.5 mL tube, and repeating five times for a total of 6 dilutions.

8.11. Spot-plate 10 µL of each dilution onto media selective agar (e.g., Pseudomonas Isolation Agar for *P. aeruginosa*) and incubate the plates at 37 °C overnight.

8.12. Count the CFU and calculate the 'percent dispersed' as (CFU in supernatant/ (CFU in supernatant+ CFU in biofilm tissue)) x 100.

REPRESENTATIVE RESULTS:

In this experiment, 8–10 week old female Swiss Webster mice were infected with 10^4 CFU of PAO1 carrying the luminescence plasmid pQF50-lux. As described above, an infection was allowed to establish for 48 h prior to administering 3 x 30 min treatments of either PBS (vehicle control) or 10% GH (treatment) to digest the biofilm EPS. Mice were imaged pre-treatment, directly after treatment (0 h) and at 10 h and 20 h post-treatment. **Figure 2A** and **Supplemental Figure 1** show an established infection within the wound bed generating a bright bioluminescent signal. The dispersal of bacteria out of the wound bed can be visualized immediately after the GH treatment (0 h), but not after PBS treatment. It should be noted that in previous studies, we detected bacteria in the blood and spleen as early as 5 h post GH treatment[7]. Bacterial dissemination into the organs can also be seen by placing the mouse on its side for imaging, as shown in the lower panel of **Figure 2A**.

Images from both the PBS and GH-treated mice demonstrated an increase in luminescent signal at 20 h post-treatment compared to pre-treatment (**Figure 2B**). We hypothesize this is due to the mechanical disruption of the biofilm caused by the irrigation of solution. At 20 h post-treatment, the mice were euthanized, and the wound beds and spleens were collected to enumerate CFU/gram of tissue. While the bacterial loads in the wound beds were similar (**Figure 2C**), bacteria were only detected in the spleens of GH-treated mice (**Figure 2D**), suggesting disseminated spread of the dispersed bacteria.

Previously, we have shown that GH treatment can break-up aggregates of bacteria, improving

CFU counts[21]. This may explain the slight increase of bacterial load in the wound beds of the GH-treated mice. Lastly, although the GH-treated wound beds had slightly higher CFU/gram, there was a lower percent bioluminescent change. These results suggest the importance of confirming luminescence with CFU counts. These representative results give an example of data that can be collected by utilizing the protocols described above.

FIGURE AND TABLE LEGENDS:

Figure 1. Establishing biofilm-associated wound infections and assessing dispersal agent efficacy *in vivo*. Dressings were checked for tears or loss of adhesion to the skin of the mouse prior to treating. Compromised dressings were replaced. Mice were placed under anesthesia immediately prior to treatment administration. 200 µL of enzyme solution, or a vehicle control, was injected into the space between the wound bed and dressing. The dressing was gently raised using forceps to ensure the entire wound was saturated in solution. The treatment was left on the wound bed for 30 min. After 30 min, the treatment was aspirated with a syringe, and another 200 µL of treatment was added. This was repeated for a total of 3 treatments. To measure dispersal in real time, mice were imaged using IVIS prior to treatment, immediately post-treatment (0 h), 10 h, and 20 h post-treatment. At 20 h post-treatment, the mice were euthanized, and the wound beds and spleens were collected. The samples were rinsed in PBS and then homogenized in fresh PBS two times at 5 m/s for 60 s. The samples were then serially diluted and spot plated. CFU were enumerated and CFU/gram were calculated. The level of dispersal of bacteria from the wound can either by assessed by IVIS or by determining the number of CFU that spread systemically to the spleen.

Figure 2. Representative data demonstrate how to assess dispersal *in vivo.* Wounds infected with 10^4 PAO1(pQF50-lux) for 48 h were treated with either PBS, or 10% GH (biofilm dispersal enzyme solution). PBS treatment was used as a vehicle control. The wound beds were treated with 3 x 30 min treatments. Mice were imaged using IVIS prior to treatment, immediately post-treatment, 10 h post-treatment, and 20 h post-treatment. It should be noted that the side-view images are from different PBS and GH-treated mice than those shown in the overhead-view images **(A)**. The ROIs for each pre-treatment wound bed and each 20 h post-treatment wound bed were recorded. Percent change from pre-treatment was calculated as (ROI pre-treatment-ROI post-treatment/ ROI pre-treatment) x 100 **(B)**. At 20 h post-treatment, mice were euthanized. The wound beds and spleens were collected and weighed to determine CFU/gram **(C and D, respectively)**. N=3.

Figure 3. Serial dilution and spot plating. Homogenized sample was vortexed and 100 µL was transferred to a 1.5 mL Eppendorf tube containing 900 µL of PBS. The sample was vortexed and 100 µL was transferred to another 1.5 mL Eppendorf tube with 900 µL of PBS. This process was repeated 5 times. Starting with the last dilution tube, 10 µL of sample was spot plated onto the agar plate in spot 10^{-8}. This was continued up to dilution 10^{-3}. Typically, the 10^{-3} dilution results in a lawn of bacteria within the spot. Some organs, which have low bacterial loads, may require spot plating up to the 10^{-1} dilution. Repeat for each tissue sample. Place agar plate in an incubator at 37 ˚C for 24-48 h, or grow under conditions that are optimal for the bacterial species of interest. To enumerate CFU, count individual colonies at the lowest dilution possible, and

multiply by the appropriate dilution factor to determine CFU/mL or use tissue weight to calculate CFU/g of tissue.

Figure 4. Measuring dispersal from infected tissue *ex vivo*. Mice were euthanized and the infected wound tissue was aseptically removed. The infected tissue was placed into a 2 mL bead mill homogenizing tube and rinsed briefly with PBS. 1 mL of dispersal agent was added and the sample was incubated for 2 h at 37 ˚C with shaking at 80 rpm. After incubation, the solution containing the dispersed cells was removed and placed into a separate 1.5 mL tube. The remaining tissue was rinsed with 1 mL of PBS. 1 mL of fresh PBS was added to the tissue and homogenized at 5 m/s for 60 s. The enzyme solution and homogenized tissue were then serially diluted and spot plated onto selective agar. The CFU were enumerated and dispersal was calculated as: CFU from enzyme solution/ (CFU from enzyme solution + CFU from tissue) x 100.

Supplemental Figure 1. Measuring *in vivo* dispersal. Wounds were infected with 10^4 PAO1(pQF50-lux) for 48 h and then treated with either PBS, or 10% GH (biofilm dispersal enzyme solution). PBS treatment was used as a vehicle control. The wound beds were treated with 3 x 30 min irrigation treatments. Mice were imaged using IVIS prior to treatment, immediately post-treatment, 10 h post-treatment, and 20 h post-treatment **(A)**. At 20 h post-treatment, mice were euthanized. The wound beds and spleens were collected and weighed to determine CFU/gram **(B and C, respectively)**. N=3.

DISCUSSION:

Here we describe protocols that can be utilized to study the efficacy of biofilm dispersal agents. These protocols can be easily adapted to use with different types of dispersal agents, bacterial species or *ex vivo* samples, including clinical debridement samples. This protocol also provides a clinically relevant model to collect and study dispersed bacterial cells. The phenotypes of dispersed bacterial cells have been shown to be distinct from those of either planktonic or biofilm cells [5,24-26]; however, the phenotypes of bacteria dispersed *in vivo* have yet to be described. If dispersal agents are to be used therapeutically, determining their efficacy, as well as understanding the phenotype that these cells adopt, is important. Additionally, this protocol could be used to study how variations in EPS structure or alteration of signaling pathways affect dispersal. For example, dispersal of *P. aeruginosa* strains with mutations in different exopolysaccharide components (e.g., Pel, Psl, Alginate) could be compared to that of wild-type strains.

Overall, this protocol can be broken into four main sections: (1) establishing an infection (2) administering treatment or (3) collecting infected tissue for *ex vivo* treatment and (4) determining dispersal efficacy. The critical part of section 1 is the successful establishment of the infection in the wound bed. If luminescent strains, which require antibiotics to maintain plasmid stability, are being utilized it is imperative to add the appropriate antibiotics when culturing the strains in preparation for infection. It should also be considered that the plasmids may not be maintained during infection in the animal (without antibiotic pressure), thus assessment of bacterial load should not completely rely on IVIS imaging, but be confirmed by CFU. The inoculating dose is also a critical step to initiate infection. For *P. aeruginosa* and *S. aureus,* an

infecting dose of 10^{4-5} CFU is typically used, but the optimal dose may differ depending upon the bacterial and mouse species used.

The critical part of section 2 is ensuring the wound bed is covered by the dressing throughout the development of infection and treatment. If the dressing is not adhered properly, the treatment can leak from the wound bed and may be ineffective. The dressing is also important for keeping the wound bed protected from potential contaminants, and impairing contractile healing by the mouse, which is essential for mimicking human wound healing. Lastly, the critical part of section 3 is to properly handle the collected samples. To enumerate CFU, the infected tissue should be rinsed with 1 mL of PBS prior to the addition of another 1 mL of PBS for homogenization. More fibrous organs, such as the lungs, may require more thorough homogenization.

The primary limitations of these protocols include the required close monitoring of the dressing and the utilization of IVIS to image dispersal and make assumptions about dispersal efficacy. For long-term studies, the re-growth of fur can be problematic because it can impede dressing adherence to the wound site. Dressings often require replacement, and their removal can lead to accidental debridement of the wound and opportunity for contamination. To visualize dispersal from the wound bed, a bioluminescent bacterial strain must be used. If the bacterial species of interest lacks a bioluminescent reporter, this option is not feasible. Another limitation is the potential of the dispersal treatment to induce bacteremia, which can lead to death of the mouse. However, we have seen that dispersed cells can be effectively killed by antibiotics *in vivo*[7]. The representative results depicted in **Figure 2** illustrate a limitation of IVIS for measuring dispersal efficacy. First, a luminescent strain of the bacterial species of interest must be utilized. The strain must produce a luminescent signal that is bright enough to detect through multiple layers of tissue in order to visualize dispersal from the wound bed to other parts of the body. It has been suggested that a minimum of roughly 10^6 bacteria are required to produce a luminescent signal strong enough to detect by IVIS[27]. A decrease in luminescent signal has also been described when bacteria enter stationary phase[28]. This can cause a disconnect between ROIs measured and CFU enumerated[29]. This limitation can lead to false assumptions when there is not a signal strong enough for detection, but bacteria are still present. As shown in **Figure 2B**, the PBS and 10% GH treatments resulted in increased luminescence post-treatment. This is a curious and consistent observation, which has also been seen after dispersing cells *in vitro* (Cláudia Marques**,** Binghamton University, personal communication). It is possible that the physical disruption of the biofilm from administering the treatment simply spreads out cells that were tightly packed, resulting in increased light signal. Alternatively, dispersal treatment could 'awaken' biofilm cells which were previously metabolically inactive, resulting in increased bioluminescence. Either way, it is crucial to confirm the results of IVIS imaging with CFU data. Lastly, there is a heterogeneous distribution of bacteria within infected wound tissue[30,31]. Therefore, if the tissue is divided *ex vivo* to provide more samples, it should not be assumed that the bacterial load in each wound section is identical.

There are modifications and troubleshooting that can be conducted to overcome the limitations of these models. First, the amount of time allotted for exposure of the depilatory cream may need to be adjusted depending upon the mouse species used. For example, 7 min is typically

used for Swiss Webster mice, while C57BL/6 mice may need up to 10 min of exposure to successfully remove the fur. This of course also depends on the product used, and exposure times should never exceed the manufacturer's instructions. If a long study (4+ days) is being conducted, depilatory cream may need to be reapplied to remove re-grown fur and ensure a proper seal of the dressing. Next, if a luminescent signal is difficult to detect, the exposure time can be increased during IVIS imaging. The inoculating dose and infection time can also be adjusted. It is important to use an infecting dose that is not too high that it overwhelms the mouse, but is also not too low that the bacteria are immediately cleared by the immune response.

In this protocol, we describe treating 48 h-old wound infections with dispersal agents, as this is a time point we have shown infections caused by *P. aeruginosa* and/or *S. aureus* are established and biofilm-associated [12,15,16]. However, depending upon the bacterial species, other time points may be more optimal. Ideally, the bacterial load in the wound should plateau once an infection is established. For example, we have seen that with both *P. aeruginosa* and *S. aureus,* the bacterial load in the wound reaches approximately 10^8 CFU/g of tissue by 48 h post-infection and remains at that level until wound closure. **Figure 1** illustrates treatment with a solution requiring 3 x 30 min washes. However, if the dispersal agent was in another form, the washes would not be necessary. For example, a cream, gel or engineered dressing could simply be placed on top of the wound bed. Lastly, the wound can be infected in a variety of ways including the inoculation of planktonic bacterial cells, preformed biofilms[12], or even infected debridement tissue from another animal or human[19,32]. We have seen that transplanting a preformed biofilm or debridement sample on to the wound bed results in more successful poly-microbial infections than when multiple species are inoculated in their planktonic form[12].

Overall, these protocols describe methods to study biofilm dispersal and the evaluation of therapeutic biofilm dispersal agents. These models allow for the development of complex poly-microbial biofilm-associated infections that mimic clinically relevant human infections. Our hope is that these protocols will be utilized and refined to further the development of anti-biofilm dispersal agents for therapeutic use.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1-revised

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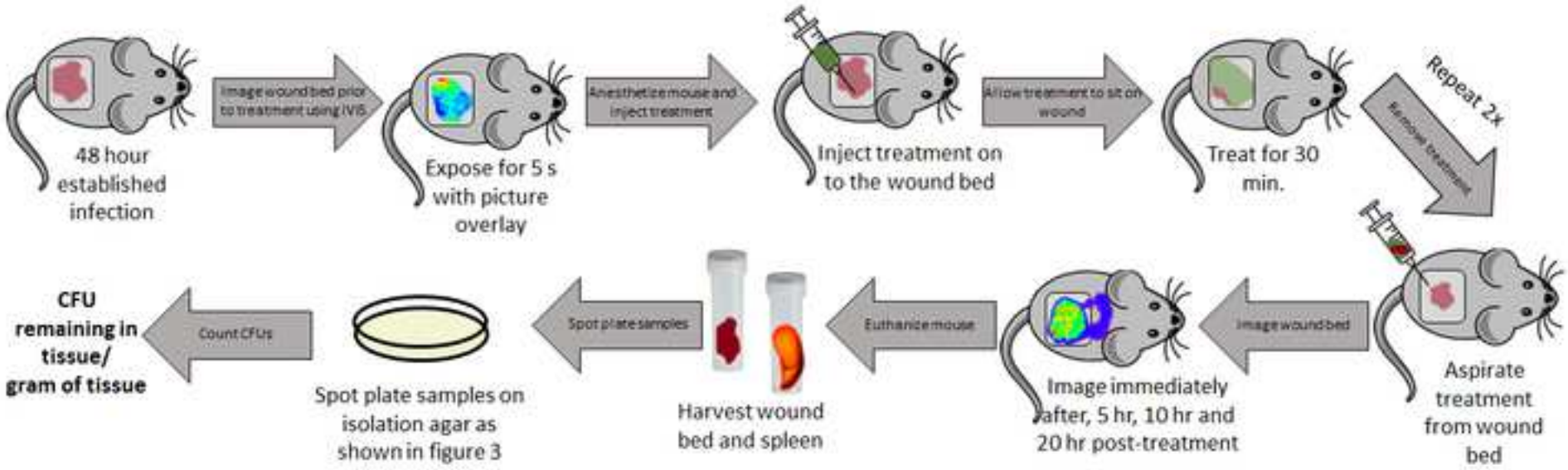

Figure 2-revised

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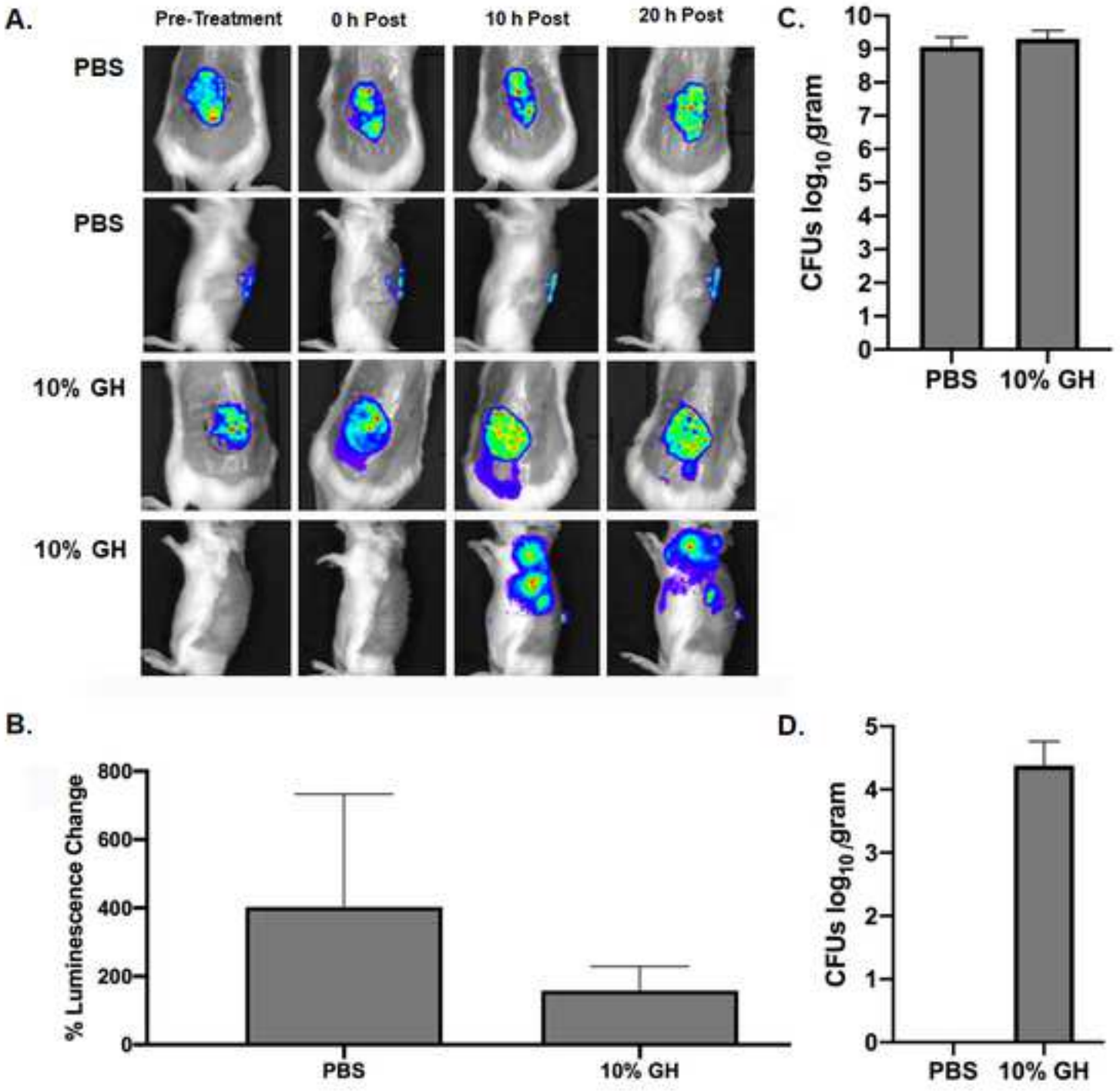


Figure 3

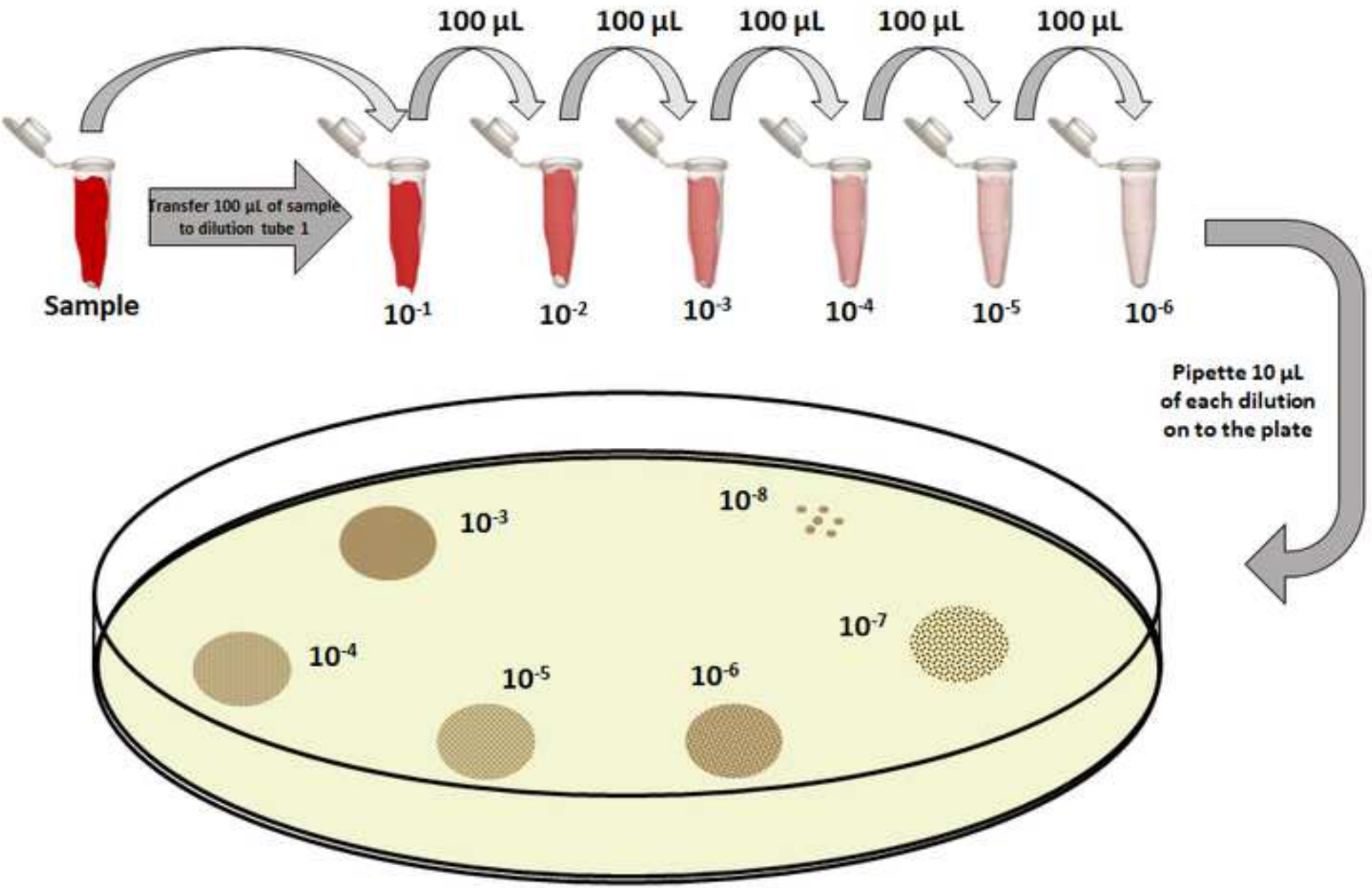
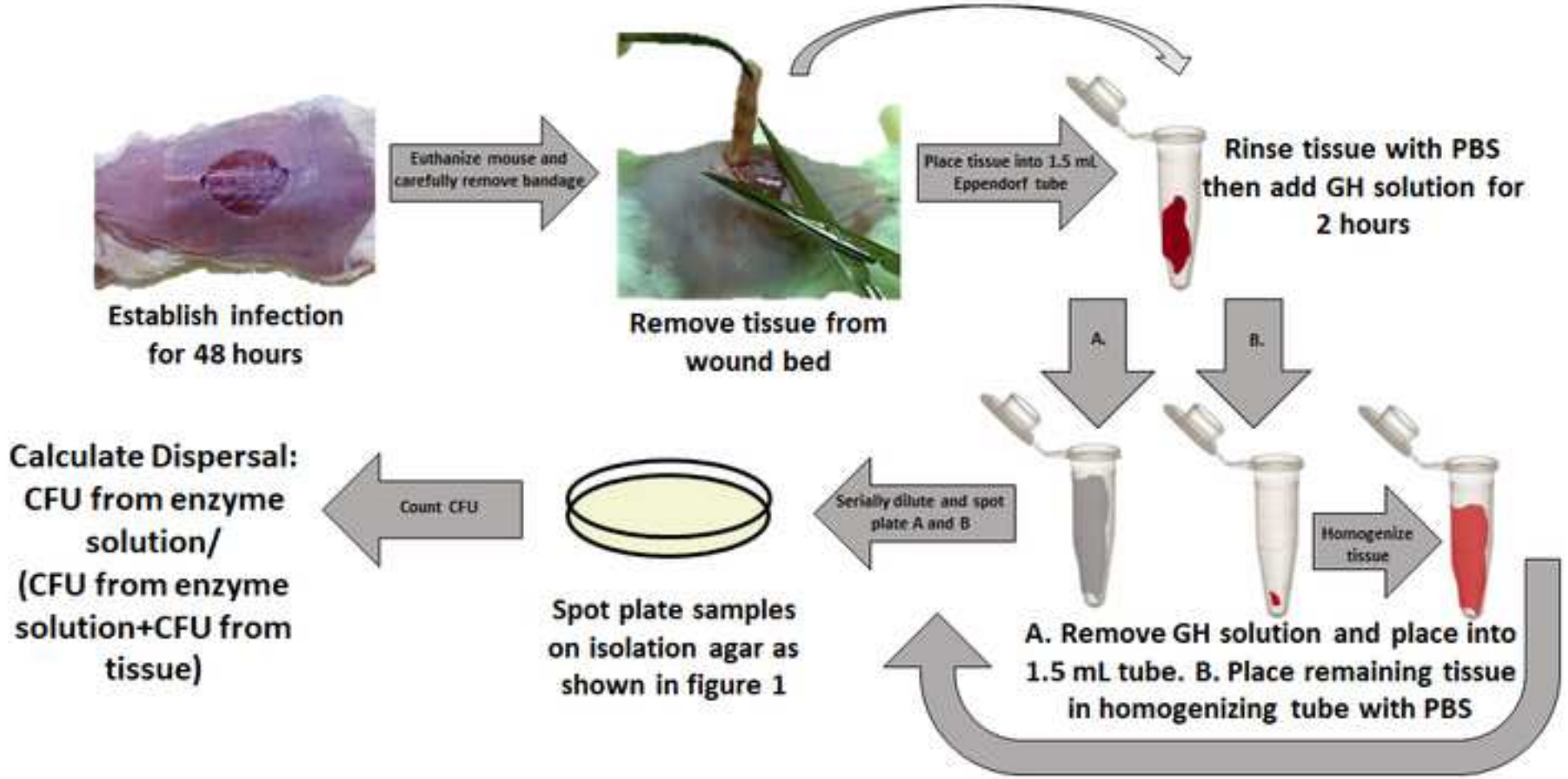


Figure 4-revised



Supplemental Figure

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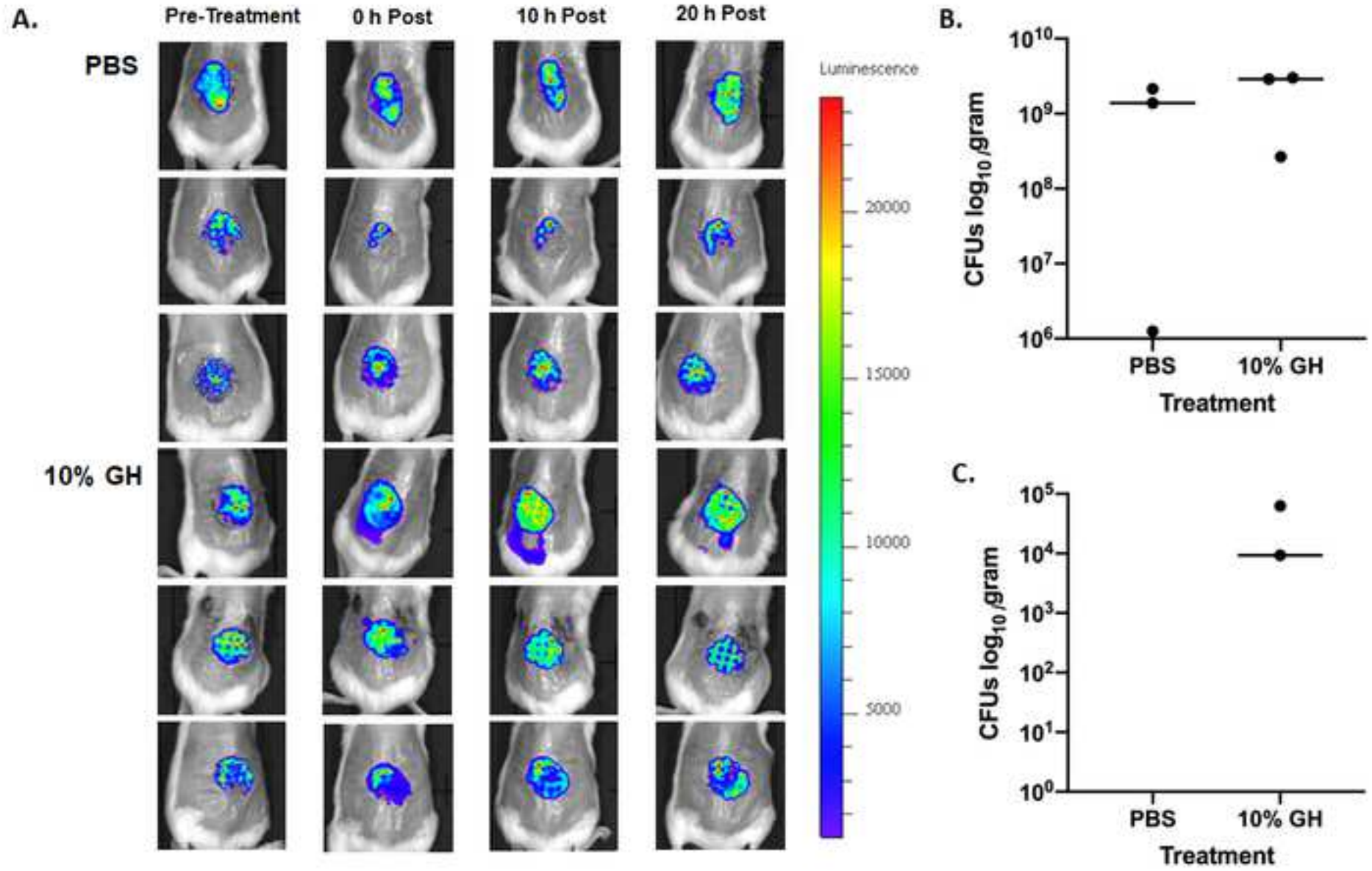

1.5 mL microcentrifuge tube	Fisher	14823434
25G 58 in needle	Fisher	14823434
Ampicillin Sodium Salt	Fisher	BP1760-5
Amylase	MP Biomedicals	2100447
Buprenorphine SR-LAB 5 mL (1 mg/mL)	ZooPharm	RX216118
Cellulase	MP Biomedicals	2150583
Depilatory cream	Walmart	287746
Dressing Forceps, Serrated Tips	Fisher	12-460-536
Erlenmeyer flasks baffled 125 mL	Fisher	101406
FastPrep-24 Benchtop Homogenizer	MP Biomedicals	6VFV9
Fatal Plus	Vortech Pharmaceuticals	0298-9373-68
Homogenizing tubes (Bead Tube 2 mL 2.4 mm Metal)	Fisher	15340151
Isoflurane	Diamond Back Drugs	
Ketamine hydrochloride/xylazine hydrochloride solution C-IIIN	Sigma Aldrich	K4138

LB broth, Miller	Fisher	BP1426-2
Lidocaine 2% Injectable	Diamond Back Drugs	2468
Meropenem	Sigma Aldrich	PHR1772-500MG

Name	**Company**	**Catalog Number**
Non-sterile cotton gauze sponges	Fisher	13-761-52
PAO1 pQF50-lux bacterial strain	Ref [13]	N/A
Petri dishes	Fisher	PHR1772-500MG
Phosphate Buffer Saline 10x	Fisher	BP3991
Polyurethane dressing	Mckesson	66024007
Pseudomonas isolation agar	VWR	90004-394
Refresh P.M.	Walmart	
Sterile Alcohol Prep Pads	Fisher	22-363-750
Straight Delicate Scissors	Fisher	89515
Swiss Webster mice	Charles River	551NCISWWEB
Syring Slip Tip 1 mL	Fisher	14823434

Use to complete serial dilutions of samples
Attaches to 1 mL syringe
Make a 50 mg/ mL stock solution and add 100 µL to 10 mL of LB broth for both overnight and subculture
Make a 5% w/v solution, vortex- other dispersal agents can be used
Use as pain mainagement- may use other options
Add 5% w/v to the 5% w/v amylase solution, vortex, activate at 37 °C for 30 min- other dispersal agents can be used
Use a small amount to massage into the hair follicles on the back of the animal and allot 10 min to remove hair
Can use other forms of forceps
Use to grow overnights and sub-cultures of bacteria
Use 5 m/s for 60 s two times to homogenize tissue
Inject 0.2 mL intraperitaneal for each mouse
Used to homogenize samples for plating
Use as anasethia- other options can also be utilized to gain a surgical field of anasethia

Add 25 g/L and autoclave
Inject 0.05 mL through the side of the marked wound bed area so it is deposited in the center of the mark.
Make 5 mg/mL to add to the GH solution to apply topically and a 15 mg/mL solution to inject

Comments

Use to remove the depilatory cream
PAO1 pgF50-lux was used as the *P. aeruginosa* strain of interest in this paper's representative results
Dilute 10x to 1x prior to use
Cut the rounded edge off and cut the remaining square into 4 equal sections
Add 20 mL/L of glycerol and 45 g/mL to water, autoclave, and pour 20 mL into petri dishes
Use on eyes to reduce dryness during procedure.
Use to clean the skin immediately prior to wounding to disinfect the area
Can also use curved scissors
Other mice strains can be used
Used to administer drugs and enzyme treatment

March 4, 2021

Dear Editor

We are submitting a second revised version of the manuscript "**Assessing Biofilm Dispersal in Murine Wounds**." Two very minor revisions were requested, which included adding a reference (line 109) and deleting unnecessary title segments (lines 186, 218 and 289). We have included marked and unmarked versions of the revised manuscript. The requested changes to the video will be submitted separately.

Thank you very much for the effort and consideration you have given this work.

Regards,

Kendra Rumbaugh