Journal of Visualized Experiments

In vivo investigation of antimicrobial blue light therapy for multidrug-resistant Acinetobacter baumannii burn infection using bioluminescence imaging

--Manuscript Draft--

Manuscript Number:	JoVE54997R2
Full Title:	In vivo investigation of antimicrobial blue light therapy for multidrug-resistant Acinetobacter baumannii burn infection using bioluminescence imaging
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	antimicrobial blue light; multidrug-resistance; Acinetobacter baumannii; burn; mouse model; Infection; bioluminescence imaging
Manuscript Classifications:	2.3.440: Gram-Negative Bacteria; 3.1: Bacterial Infections and Mycoses; 3.1.539: Infection; 3.26.200: Burns; 3.26.808: Soft Tissue Injuries; 3.26.951: Wound Infection
Corresponding Author:	Tianhong, Dai, PhD Massachusetts General Hospital Boston, Massachusetts UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	TDAI@mgh.harvard.edu
Corresponding Author's Institution:	Massachusetts General Hospital
Corresponding Author's Secondary Institution:	
First Author:	Yucheng Wang, MD, PhD
First Author Secondary Information:	
Other Authors:	Yucheng Wang, MD, PhD
	Olivia Harrington
	Clinton K Murray
	Michael R Hamblin, PhD
Order of Authors Secondary Information:	
Abstract:	Burn infections continue to be an important cause of morbidity and mortality. The increasing emergence of multidrug-resistant (MDR) bacteria has led to frequent failure of traditional antibiotic treatment. Alternative therapeutics are urgently needed to tackle MDR. An innovative non-antibiotic approach, antimicrobial blue light (aBL), has shown promising effectiveness against MDR infections. The mechanism of action of aBL is not well understood yet. It is commonly hypothesized that naturally occurring endogenous photosensitizing chromophores in bacteria, e.g., iron-free porphyrins and flavins, etc., are excited by aBL, which in turn produces cytotoxic reactive oxygen species (ROS) through a photochemical process. Unlike another light-based antimicrobial approach, antimicrobial photodynamic therapy (aPDT), the involvement of exogenous photosensitizer for aBL therapy is not required. All it needs to take effect is the irradiation of blue light, therefore, it is simple and inexpensive. The receptors of aBL are the endogenous cellular photosensitizers in bacteria instead of the DNA, thus aBL is believed to be much less detrimental to host cells than ultraviolet-C (UVC) irradiation, which directly causes DNA damage in host cells. In this paper, we have described a protocol to assess the effectiveness of aBL therapy for MDR Acinetobacter baumannii infection in a mouse model of burn injury. By using a

	bioluminescent bacterial strain, we are able to noninvasively monitor the extent of infection in real time in living animals. This technique is also an effective tool for observing the spatial distribution of infections in animals.
Author Comments:	
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	

TITLE:
In vivo investigation of antimicrobial blue light therapy for multidrug-resistant *Acinetobacter baumannii* burn infections using bioluminescence imaging

AUTHORS & AFFILIATIONS:
Yucheng Wang[1,2,3], Olivia Harrington[1], Clinton K Murray[4], Michael R. Hamblin[1], Tianhong Dai[1]

[1]Wellman Center for Photomedicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA
[2]Department of Laser Medicine, Chinese PLA General Hospital, Beijing, China
[3]College of Medicine, Nankai University, Tianjin, China
[4]Infectious Disease Service, Brooke Army Medical Center, Fort Sam Houston, TX, USA

EMAIL ADDRESSES:
Yucheng Wang (wangyucheng87@126.com)
Olivia Harriton (OHARRINGTON@mgh.harvard.edu)
Clinton K. Murray (clinton.k.murray.mil@mail.mil)
Michael R. Hamblin (hamblin@helix.mgh.harvard.edu)
Tianhong Dai (tdai@mgh.harvard.edu)

CORRESPONDING AUTHOR:
Tianhong Dai
tdai@mgh.harvard.edu

KEYWORDS:
Antimicrobial blue light; multidrug resistance; *Acinetobacter baumannii*; burn; mouse model; infection; bioluminescence imaging

SHORT ABSTRACT:
Infections caused by multidrug-resistant (MDR) bacterial strains have emerged as a serious threat to public health, necessitating the development of alternative therapeutics. We present a protocol to evaluate the effectiveness of antimicrobial blue light (aBL) therapy for MDR *Acinetobacter baumannii* infections in mouse burns by using bioluminescence imaging.

LONG ABSTRACT:
Burn infections continue to be an important cause of morbidity and mortality. The increasing emergence of multidrug-resistant (MDR) bacteria has led to the frequent failure of traditional antibiotic treatments. Alternative therapeutics are urgently needed to tackle MDR bacteria.

An innovative non-antibiotic approach, antimicrobial blue light (aBL), has shown promising effectiveness against MDR infections. The mechanism of action of aBL is not yet well understood. It is commonly hypothesized that naturally occurring endogenous photosensitizing chromophores in bacteria (*e.g.*, iron-free porphyrins, flavins, etc.) are excited by aBL, which in turn produces cytotoxic reactive oxygen species (ROS) through a photochemical process.

Unlike another light-based antimicrobial approach, antimicrobial photodynamic therapy (aPDT),

aBL therapy does not require the involvement of an exogenous photosensitizer. All it needs to take effect is the irradiation of blue light; therefore, it is simple and inexpensive. The aBL receptors are the endogenous cellular photosensitizers in bacteria, rather than the DNA. Thus, aBL is believed to be much less genotoxic to host cells than ultraviolet-C (UVC) irradiation, which directly causes DNA damage in host cells.

In this paper, we present a protocol to assess the effectiveness of aBL therapy for MDR *Acinetobacter baumannii* infections in a mouse model of burn injury. By using an engineered bioluminescent strain, we were able to noninvasively monitor the extent of infection in real time in living animals. This technique is also an effective tool for monitoring the spatial distribution of infections in animals.

INTRODUCTION:

Burn infections, which are frequently reported because of cutaneous thermal injuries, continue to be an important cause of morbidity and mortality[1]. The management of burn infections has been further compromised by the increasing emergence of multidrug-resistant (MDR) bacterial strains[2] due to the massive use of antibiotics. One important MDR Gram-negative bacteria is *Acinetobacter baumannii*, which is known to be associated with recent battle wounds and is resistant to almost all available antibiotics[3]. The presence of biofilms at the injured foci has been reported[4,5] and is believed to exacerbate the tolerance to antibiotics and host defense[6,7], causing persistent infections[8,9]. Therefore, there is a pressing need for the development of alternative treatments. In the recently announced *National Strategy for Combating Antibiotic-Resistant Bacteria*, the development of alternative therapeutics to antibiotics has been noted as an action by the government of the United States[10].

Light-based antimicrobial approaches, as indicated by the name, require light irradiation with or without other agents. These approaches include antimicrobial photodynamic therapy (aPDT), ultraviolet-C (UVC) irradiation, and antimicrobial blue light (aBL). In previous studies, they have shown promising effectiveness in killing MDR bacterial strains[11-13]. Among the three light-based approaches, aBL has attracted increasing attention in recent years due to its intrinsic antibacterial properties without the use of photosensitizers[14]. In comparison to aPDT, aBL only involves the use of light, while aPDT requires a combination of light and a photosensitizer. Therefore, aBL is simple and inexpensive[14]. In comparison to UVC, aBL is believed to be much less cytotoxic and genotoxic to host cells[15].

The goal of this protocol is to investigate the effectiveness of aBL for the treatment of burn infections caused by MDR *A. baumannii* in a mouse model. We use bioluminescent pathogenic bacteria to develop new mouse models of burn infections that allow the non-invasive monitoring of the bacterial burden in real time. Compared to the traditional method of body fluid/tissue sampling and subsequent plating and colony counting[16], this technique provides accurate results. The process of tissue sampling could introduce another source of experimental error. Since the bacterial luminescence intensity is linearly proportional to the corresponding bacterial CFU[17], we can directly measure the survival of bacteria after a certain dose of light irradiation. By monitoring the bacterial burden in living animals receiving the light treatment in real time, the kinetics of bacterial killing can be characterized using a significantly reduced number of mice.

PROTOCOL:

All animal procedures are approved by the Institutional Animal Care and Use Committees (IACUC) of Massachusetts General Hospital (Protocol #2014N000009) and are in accordance with the guidelines of the National Institutes of Health.

1. Preparation of bacterial culture

1.1) Add 7.5 mL of Brain Heart Infusion (BHI) medium to a 50-mL centrifuge tube. Seed *A. baumannii* cells in the BHI medium and then incubate the *A. baumannii* culture in an orbital incubator (37 °C) for 18 h.

1.2) Centrifuge the culture of cells at 3,500×g for 5 min, remove the supernatant, and wash the pellets in phosphate-buffered saline (PBS).

1.3) Re-suspend the bacteria pellets in fresh PBS and thoroughly pipette the suspension.

1.4) Collect 100 μL of the bacterial suspension and make a 1:10 dilution using fresh PBS.

1.5) Transfer the dilution to a 1.5-mL semi-micro cuvette and measure the optical density (OD) at a wavelength of 600 nm ($OD_{600\text{-}nm}$).

1.6) Calculate the $OD_{600\text{-}nm}$ of the original (undiluted) suspension in PBS according to the measured $OD_{600\text{-}nm}$ value of the dilution and the dilution factor (10).

1.7) Adjust the original suspension in PBS to $OD_{600\text{-}nm} = 0.6$ (corresponding to a cell density of 10^8 CFU/mL).

2. Mouse model of burn infection caused by bioluminescent *A. baumannii*

2.1） Use adult female BALB/c mice aged 7-8 weeks and weighing 17-19 g. Allow the mice to acclimatize to laboratory conditions for at least 3 days before the start of experiment. Maintain the mice in a 12-h light/dark cycle under a room temperature of 21 ℃and give them food and water *ad libitum*.

2.2 ） Anesthetize the mice with an intraperitoneal injection of a ketamine-xylazine cocktail (100 mg/kg-20 mg/kg). Lightly touch the palpebra of each mouse with a cotton swab; an absence of the palpebral reflex suggests an appropriate anesthetic depth.

2.3 ） Shave the mice on the back to expose as much skin as possible by using a 50-blade hair clipper.

2.4 ） Place the lid of a 35-mm petri dish underneath the abdomens of the mice to keep their backs in a relatively horizontal position.

2.5 ） Boil water in a 250-mL beaker (80% full) using a 10" x 10", 220 VAC hotplate. Immerse a brass block (1 cm × 1 cm cross section) into the beaker until thermal equilibration with the water

is reached. Thermal equilibration usually takes <5 min and is indicated by the re-boiling of the water in the beaker.

2.6) Prior to creating the burn injury, administer pre-emptive analgesics (a subcutaneous injection of 0.1mg/kg buprenorphine) for pain relief.

2.7) Ten minutes after the pre-emptive analgesics, gently press the heated brass block to the shaved area on the back of the mice for 3 s to induce burn wounds.

Note: To avoid thermal injury to the working personnel, wear thermal-resistant gloves when performing the burning procedure.

2.8) Administer 0.5 mL of sterile saline through subcutaneous injection to prevent dehydration.

2.9) Five minutes following the induction of the thermal injury, inoculate 50 μL of bacterial suspension containing 5×10^6 CFU in PBS onto the mouse burns using a pipette. By moving the pipette tip in a zigzag motion on the skin, smear the aliquots on the burns to distribute the bacterial cells in the burned area as evenly as possible.

2.10) Immediately after bacterial inoculation, perform bioluminescence imaging for the infected burns, as described in Section 4.

2.11) Place the mice on a water-heated surgical bed (37 °C, recovery area) until the mice have completely recovered from anesthesia. House the mice in separate cages in a Biosafety Level-2 animal room.

2.12) Administer analgesics (subcutaneous injection of 0.1 mg/kg buprenorphine) twice daily for the first three days after the burn injury.

3. Antimicrobial blue light therapy for *A. baumannii* infection in mice

3.1) Start aBL therapy at 24 h after bacterial inoculation.

3.2) Use a light-emitting diode (LED) with a peak emission at 415 nm for aBL irradiation. Mount the LED on a heat sink to prevent thermal effects on the irradiated area in mice[18]. Fix the LED to an optical support rod with clip connectors to allow the LED to move up and down.

3.3) Turn on the power/energy meter and press the wavelength button to select 415 nm. Reset the power/energy meter to subtract the background (ambient light).

3.4) Place the power/energy meter right under the LED. Wear blue-light-protective goggles. Turn on the LED light and adjust the distance between the LED aperture (a lens that converges the light from the LED) and the light sensor (2 cm in diameter) of the power/energy meter so that the light spot covers the whole area of the light sensor.

3.5) Carefully adjust the position (level) of the LED and record the reading of the power/energy meter. Calculate the irradiance according to the reading: Irradiance = Reading (W)/Area (cm^2). Move the LED to a position where the irradiance is 100 mW/cm^2 and then fix the clip connectors.

3.6) Turn off the LED. Measure the distance between the LED aperture and the light sensor of the power/energy meter.

3.7) Anesthetize the mice with an intraperitoneal injection of a ketamine-xylazine cocktail (100 mg/kg-20 mg/kg). An absence of the palpebral reflex suggests an appropriate anesthetic depth.

3.8) Randomly divide the mice into an aBL-treated group (n = 10) and an untreated control group (n = 10).

3.9) For the aBL-treated group, cover the eyes of mice with aluminum foil to avoid overexposure to light. Place the mouse burns directly under the LED, with the lid of a 35-mm petri dish underneath the abdomens of the mice to keep their backs in a horizontal position.

3.10) Replace the power/energy meter mentioned in step 3.5 with a mouse on a square petri dish. Adjust the height of the mouse back to a position where the distance between the LED aperture and the surface of the mouse burn is equal to that between the LED aperture and the level of the light sensor of the power/energy meter (as discussed in step 3.5).

3.11) Irradiate the infected burns at an irradiance of 100 mW/cm^2. Deliver aBL in doses of 36 J/cm^2 until a total dose of 120 J/cm^2 is reached (*e.g.*, 0, 36, 72, 96, and 120 J/cm^2). After each light dose, perform bioluminescence imaging for the mice, as discussed in Section 4.

3.12) For the untreated control group, perform bioluminescence imaging of the mouse burns, as discussed in Section 4, using the same time intervals as used for the aBL-treated group.

3.13) After aBL therapy, perform bioluminescence imaging, as discussed in Section 4, daily for the first 3 days and then on alternate days to monitor the temporal bio-burden of infections in mice.

4. Bioluminescence imaging of infections in mice

4.1) Image the mice using a low-light imaging system that includes an intensified charge-coupled device camera, a camera controller, a specimen chamber, and an image processor[19].

4.2) Anesthetize the mice with an intraperitoneal injection of a ketamine-xylazine cocktail (100 mg/kg-20 mg/kg). Lightly touch the palpebral of the mice with a cotton swab; an absence of the palpebral reflex suggests an appropriate anesthetic depth.

4.3) Start the live imaging software. In the control panel that appears, click ″Initialize.″ Wait until the color of the ″Temperature″ box turns green, indicating that the temperature of the stage in the specimen chamber has reached 37 °C.

4.4) Place the mice on the stage (37 °C) in the specimen chamber of the imaging system, with the infected burns directly under the camera.

Note: The bioluminescence of the bacteria could decrease when the burns become dry. Therefore, it is recommended to moisturize the mouse burns with PBS before imaging.

4.5) In the control panel, put a check mark next to "Luminescence." Select "Auto exposure" so that the exposure time for imaging will be optimized by the live imaging software based on the bioluminescence intensity.

4.6) Select "C" from the "Field of View" drop-down list. Select the "Scan mid range" option to let the software determine the focal distance. Put a check mark next to ″Overlay.″

4.7) Click "Acquire" to capture the image. In the "Edit Image Label" box, click "OK;" an "Image Window" and "Toll Palette" will appear.

4.8) Set Auto ROI parameters for auto-selection.

4.9) Quantify the bioluminescence intensity as relative luminescence units (RLUs) and display the bioluminescence in a false-color scale ranging from pink (most intense) to blue (least intense)[19,20].

4.10) Calculate the survival fraction of the bacteria in mouse burns at varying time points based upon bioluminescence intensity analysis. The survival fraction of bacteria at a given time point = the bioluminescence intensity measured at that time point / the bioluminescence intensity measured right before aBL exposure[17].

5. Euthanasia of the mice

5.1） In case of systematic infections, as indicated by the spread of bioluminescence outside of the burned area, euthanize the mice in both the aBL-treated and the control groups by delivering carbon dioxide (CO_2) compressed gas into a closed cage.

5.1.1) Open the CO_2 tank or valve regulator to initiate the flow of gas. Verify that the regulator reads the correct psi (pounds per square inch) based on instructions posted by the unit, and adjust the regulator to the correct psi as needed, typically no higher than 5 psi.

5.1.2) Fill slowly. The flow rate should displace no more than 30% of the chamber/cage volume per min (for a typical mouse cage, ~2 L/min; for a rat cage, ~7.5 L/min).

5.1.3) Wait approximately 3-5 min for the animal to stop moving or breathing; the eyes should be fixed and dilated. Turn off CO_2 tank or regulator valve to stop the flow of CO_2.

5.1.4) Ensure that the heart is not beating by feeling the chest between the thumb and forefinger. Ensure that there is no blink reflex by touching the eyeball.

5.1.4.1) If there is a heartbeat or blink reflex, repeat the euthanasia process or use scissors to open the chest cavity to create a pneumothorax (the animal must be non-responsive to a toe pinch prior to performing this procedure).

REPRESENTATIVE RESULTS:

The *A. baumannii* strain that we used is an MDR clinical isolate, as reported previously[12,17]. The bacterial strain was made bioluminescent by the transfection of *luxCDABE* opera[11]. Figure 1A shows the successive bacterial luminescence images from a representative mouse burn infected with 5×10^6 *A. baumannii* and exposed to a single aBL exposure at 24 h after bacterial inoculation. A Gram-stain of the histological section of a representative mouse skin burn specimen (harvested at 24 h post-inoculation) demonstrated the presence of *A. baumannii* biofilms on the surface of the infected burn (Figure 1B). As shown in Figure 1A, the bacterial luminescence was almost eradicated after an exposure of 360 J/cm^2 aBL was delivered (60 min of irradiation at an irradiance of 100 mW/cm^2). Figure 1C is the dose-response curve of the mean bacterial luminescence from mouse burns infected with 5×10^6 *A. baumannii* and treated with aBL at 24 h after bacterial inoculation (n = 10). To achieve a 3-log$_{10}$ inactivation of *A. baumannii* in mouse burns, approximately 360 J/cm^2 aBL was required. The bacterial luminescence of the mouse burns unexposed to aBL remained almost unchanged during an equivalent period of time (data not shown; $P < 0.001$).

FIGURE LEGEND:

Figure 1. aBL inactivation of bacteria in infected mouse burns. (A) Successive bacterial luminescence images from a representative mouse burn infected with 5×10^6 CFU of *A. baumannii* and exposed to 360 J/cm^2 aBL at 24 h after bacterial inoculation. (B) Gram-stained histological section of a representative mouse skin burn showing the presence of *A. baumannii* biofilms (arrows) in the mouse burn. The skin sample was harvested at 24 h after bacterial inoculation. (C) Dose-response curve of mean bacterial luminescence of mouse burns infected with 5×10^6 *A. baumannii* and treated with an exposure of 360 J/cm^2 aBL at 24 h (n = 10) after bacterial inoculation. Bars: standard deviation.

DISCUSSION:

aBL is a novel method for treating infections. Since its mechanism of action is completely different from that of chemotherapy, it is more of a physiotherapy. The agent that mediates the antimicrobial effect is blue light irradiation (400-470 nm). With the development of blue LEDs, we gained access to an effective and simple light-based antimicrobial approach for MDR infections.

In this protocol, we have described the development of a mouse model of burn infections caused by a bioluminescent strain of MDR, *A. baumannii*. With the use of bioluminescent bacteria, the extent of infection can be non-invasively monitored in real time in living animals via bioluminescent imaging. The use of engineered bioluminescent strains of bacteria and the low-light imaging technique creates an efficient technique for monitoring infections in real-time during antimicrobial therapy. This method can also be used in the investigations of infections caused by other microbial species and located at other sites. Besides the efficacy assessment of antimicrobial approaches, this method can also be used to track the progress of infection.

By using this mouse model, we demonstrated that aBL (415 nm) successfully inactivated bacteria in established infections (Figure 1A and C). Prior to aBL therapy, clusters of bacteria were observed in the established infections (Figure 1B), which is a feature of biofilms. Biofilms are more tolerant of traditional antibiotics and host defense compared to their planktonic counterparts[6,7] and are frequently associated with persistent infections[8,9]. The representative results are promising in that 415-nm aBL is biofilm-penetrating. In addition, together with previous

reports[29-32], our results demonstrate that the effectiveness of aBL persists regardless of the drug-resistance profile of bacteria.

The protocol described here involves three main procedures: (1) the development of a mouse model of burn infections, (2) aBL therapy, and (3) bioluminescence imaging. While developing a mouse model of burn infections, we noted that there were several factors that affect the extent of infection and the subsequent effectiveness of aBL: (1) The burning time affects the wound depth and the proliferation of bacteria. When the burning time was increased from 3 to 7 s, the bacterial luminescence was much stronger (indicating a higher extent of infection) at 24 h post-inoculation, and the eradication of infection required much higher aBL exposures (> 360 J/cm^2). (2) The inoculum of the bacteria is a key parameter for the development of infections. A higher bacterial inoculum usually results in a higher extent of infection, while a sufficiently low inoculum frequently fails to develop stable infections in mice. In the latter condition, bacterial luminescence usually becomes undetectable soon after bacterial inoculation. (3) The interaction between bacteria and hosts is dependent upon the bacterial species. We also used *P. aeruginosa* to develop an infection model. We found that, under the same conditions (*i.e.*, burning time and bacterial inoculum), the infections caused by *P. aeruginosa* progressed much more rapidly than *A. baumannii* infections, and sepsis was always observed in mice within 48 h post-inoculation[25].

For the execution of aBL therapy, there are several important points that need to be addressed: (1) Proper light irradiance is required for the maximized efficacy of aBL therapy. (2) The surface of the burn in the mice should be placed as horizontally as possible. A failure to appropriately position the burn surface can compromise the efficacy of aBL therapy. (3) During light exposure, it is suggested that the eyes of mice be protected with aluminum foil, especially when a laser is used as the light source. (4) During light exposure, care should be taken to monitor the mice in case they awaken from anesthesia. In this case, a small additional dose of anesthetics should be administrated to keep the animals anesthetized. (5) Both aBL-treated mice and untreated mice should be placed on a heating bed to maintain the body temperature when under anesthesia. During the process of bioluminescence imaging, the bioluminescence of bacteria could decrease when the burns become dry. Therefore, it is recommended to moisturize the mouse burns with PBS before imaging.

There are also some limitations of the techniques discussed in this protocol: (1) For the purpose of monitoring of the extent of infection in real time, bioluminescent bacterial strains must be used. Therefore, before a clinical strain can be tested in the animal model, it must be genetically modified by the transfection of the *lux CDABE* operon[11]. (2) The effectiveness of aBL is related to the wavelengths[33] and bacterial species/strains[34] used. The blue wavelengths, together with other parameters, should be further optimized for inactivating different bacterial species/strains. (3) We only investigated superficial infections in mice. For deep-seated infections, the topical delivery of aBL may not be able to reach the infections, so interstitial light delivery may be needed[35]. (4) There is a sensitivity limitation of the imaging system, especially when imaging deep infections[19]. As a result, even when the pixels of bioluminescence are completely eliminated, there might still be viable bacterial cells remaining, allowing bacterial regrowth to occur. An extended exposure to aBL is recommended after the elimination of bacterial luminescence in order to prevent bacterial regrowth.

ACKNOWLEDGEMENTS:
This work was supported in part by the Center for Integration of Medicine and Innovative Technology (CIMIT) under the U.S. Army Medical Research Acquisition Activity Cooperative Agreement (CIMIT No. 14-1894 to TD) and the National Institutes of Health (1R21AI109172 to TD). YW was supported by an ASLMS Student Research Grant (BS.S02.15). We are grateful to Tayyaba Hasan, PhD at the Wellman Center for her co-mentorship for YW.

DISCLOSURES:
The authors declare that they have no competing financial interests.

REFERENCES:
1 Gibran, N. S. *et al.* Summary of the 2012 ABA Burn Quality Consensus conference. *J Burn Care Res.* **34** (4), 361-385, doi:10.1097/BCR.0b013e31828cb249 (2013).
2 Sommer, R., Joachim, I., Wagner, S. & Titz, A. New approaches to control infections: anti-biofilm strategies against gram-negative bacteria. *Chimia (Aarau).* **67** (4), 286-290 (2013).
3 Peleg, A. Y., Seifert, H. & Paterson, D. L. Acinetobacter baumannii: emergence of a successful pathogen. *Clin Microbiol Rev.* **21** (3), 538-582, doi:10.1128/cmr.00058-07 (2008).
4 Uppu, D. S. *et al.* Amide side chain amphiphilic polymers disrupt surface established bacterial bio-films and protect mice from chronic Acinetobacter baumannii infection. *Biomaterials.* **74** 131-143, doi:10.1016/j.biomaterials.2015.09.042 (2016).
5 Schaber, J. A. *et al.* Pseudomonas aeruginosa forms biofilms in acute infection independent of cell-to-cell signaling. *Infect Immun.* **75** (8), 3715-3721, doi:10.1128/iai.00586-07 (2007).
6 Hoiby, N., Bjarnsholt, T., Givskov, M., Molin, S. & Ciofu, O. Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents.* **35** (4), 322-332, doi:10.1016/j.ijantimicag.2009.12.011S0924-8579(10)00009-9 [pii] (2010).
7 Lebeaux, D., Ghigo, J. M. & Beloin, C. Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. *Microbiol Mol Biol Rev.* **78** (3), 510-543, doi:10.1128/MMBR.00013-1478/3/510 [pii] (2014).
8 Akers, K. S. *et al.* Biofilms and persistent wound infections in United States military trauma patients: a case-control analysis. *BMC Infect Dis.* **14** 190, doi:10.1186/1471-2334-14-190 1471-2334-14-190 [pii] (2014).
9 Burmolle, M. *et al.* Biofilms in chronic infections - a matter of opportunity - monospecies biofilms in multispecies infections. *FEMS Immunol Med Microbiol.* **59** (3), 324-336, doi:10.1111/j.1574-695X.2010.00714.xFIM714 [pii] (2010).
10 *National strategy on combating antibiotic-resistant bacteria*, <https://www.whitehouse.gov/sites/default/files/docs/carb_national_strategy.pdf> (2014).
11 Dai, T. *et al.* Photodynamic therapy for Acinetobacter baumannii burn infections in mice. *Antimicrob Agents Chemother.* **53** (9), 3929-3934, doi:AAC.00027-09 [pii]10.1128/AAC.00027-09 (2009).
12 Zhang, Y. *et al.* Antimicrobial blue light therapy for multidrug-resistant Acinetobacter baumannii infection in a mouse burn model: implications for prophylaxis and treatment of combat-related wound infections. *J Infect Dis.* **209** (12), 1963-1971, doi:10.1093/infdis/jit842jit842 [pii] (2014).
13 Dai, T. *et al.* Ultraviolet C light for Acinetobacter baumannii wound infections in mice: potential use for battlefield wound decontamination? *J Trauma Acute Care Surg.* **73** (3), 661-667, doi:10.1097/TA.0b013e31825c149c (2012).

14 Dai, T. *et al.* Blue light for infectious diseases: Propionibacterium acnes, Helicobacter pylori, and beyond? *Drug Resist Updat.* **15** (4), 223-236, doi:10.1016/j.drup.2012.07.001 S1368-7646(12)00046-5 [pii] (2012).
15 Yin, R. *et al.* Light based anti-infectives: ultraviolet C irradiation, photodynamic therapy, blue light, and beyond. *Curr Opin Pharmacol.* **13** (5), 731-762, doi:10.1016/j.coph.2013.08.009 S1471-4892(13)00155-0 [pii] (2013).
16 Haisma, E. M. *et al.* Inflammatory and antimicrobial responses to methicillin-resistant Staphylococcus aureus in an in vitro wound infection model. *PLoS One.* **8** (12), e82800, doi:10.1371/journal.pone.0082800 (2013).
17 Wang, Y. *et al.* Antimicrobial Blue Light Inactivation of Gram-Negative Pathogens in Biofilms: In Vitro and In Vivo Studies. *J Infect Dis.* **213** (9), 1380-1387, doi:10.1093/infdis/jiw070, (2016).
18 Chen, D., Shen, Y., Huang, Z., Li, B. & Xie, S. Light-Emitting Diode-Based Illumination System for In Vitro Photodynamic Therapy. *Int J Photoenergy.* **2012** (2), doi:10.1155/2012/920671,(2012).
19 Demidova, T. N., Gad, F., Zahra, T., Francis, K. P. & Hamblin, M. R. Monitoring photodynamic therapy of localized infections by bioluminescence imaging of genetically engineered bacteria. *J Photochem Photobiol B.* **81** (1), 15-25, doi:10.1016/j.jphotobiol.2005.05.007 (2005).
20 Hamblin, M. R., Zahra, T., Contag, C. H., McManus, A. T. & Hasan, T. Optical monitoring and treatment of potentially lethal wound infections in vivo. *J Infect Dis.* **187** (11), 1717-1725, doi:JID30226 [pii]10.1086/375244 (2003).
21 Rowan, M. P. *et al.* Burn wound healing and treatment: review and advancements. *Critical Care.* **19** 243, doi:10.1186/s13054-015-0961-2 (2015).
22 Marx, D. E. & Barillo, D. J. Silver in medicine: The basic science. *Burns.* **40** (Supplement 1), S9-S18, doi:10.1016/j.burns.2014.09.010 (2014).
23 Heyneman, A., Hoeksema, H., Vandekerckhove, D., Pirayesh, A. & Monstrey, S. The role of silver sulphadiazine in the conservative treatment of partial thickness burn wounds: A systematic review. *Burns.* **42** (7), 1377-1386, doi:10.1016/j.burns.2016.03.029 (2016).
24 Roberts, J. A. *et al.* Individualised antibiotic dosing for patients who are critically ill: challenges and potential solutions. *Lancet Infect Dis.* **14** (6), 498-509, doi:10.1016/s1473-3099(14)70036-2 (2014).
25 Dai, T. *et al.* Blue light eliminates community-acquired methicillin-resistant Staphylococcus aureus in infected mouse skin abrasions. *Photomed Laser Surg.* **31** (11), 531-538, doi:10.1089/pho.2012.3360 (2013).
26 Uppu, D. S. *et al.* - Amide side chain amphiphilic polymers disrupt surface established bacterial bio-films and protect mice from chronic Acinetobacter baumannii infection. *Biomaterials.* **74** 131-143, doi:10.1016/j.biomaterials.2015.09.042 (2016).
27 Donlan, R. M. & Costerton, J. W. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev.* **15** (2), 167-193 (2002).
28 Olsen, I. Biofilm-specific antibiotic tolerance and resistance. *Eur J Clin Microbiol Infect Dis.* doi:10.1007/s10096-015-2323-z (2015).
29 Song, H. H. *et al.* Phototoxic effect of blue light on the planktonic and biofilm state of anaerobic periodontal pathogens. *J Periodontal Implant Sci.* **43** (2), 72-78 (2013).

30 Rosa, L. P., da Silva, F. C., Viana, M. S. & Meira, G. A. In vitro effectiveness of 455-nm blue LED to reduce the load of Staphylococcus aureus and Candida albicans biofilms in compact bone tissue. *Lasers Med Sci.* **31** (1), 27-32, doi:10.1007/s10103-015-1826-2 (2015).
31 Guffey, J. S. & Wilborn, J. In vitro bactericidal effects of 405-nm and 470-nm blue light. *Photomed Laser Surg.* **24** (6), 684-688, doi:10.1089/pho.2006.24.684 (2006).
32 Enwemeka, C. S., Williams, D., Enwemeka, S. K., Hollosi, S. & Yens, D. Blue 470-nm light kills methicillin-resistant Staphylococcus aureus (MRSA) in vitro. *Photomed Laser Surg.* **27** (2), 221-226, doi:10.1089/pho.2008.241310.1089/pho.2008.2413 [pii] (2009).
33 Bumah, V. V., Masson-Meyers, D. S., Cashin, S. E. & Enwemeka, C. S. Wavelength and bacterial density influence the bactericidal effect of blue light on methicillin-resistant Staphylococcus aureus (MRSA). *Photomed Laser Surg.* **31** (11), 547-553, doi:10.1089/pho.2012.3461 (2013).
34 Maclean, M., MacGregor, S. J., Anderson, J. G. & Woolsey, G. Inactivation of bacterial pathogens following exposure to light from a 405-nanometer light-emitting diode array. *Appl Environ Microbiol.* **75** (7), 1932-1937, doi:10.1128/AEM.01892-08AEM.01892-08 [pii] (2009).
35 Kim, M. *et al.* Optical lens-microneedle array for percutaneous light delivery. *Biomedical Optics Express.* **7** (10), 4220-4227, doi:10.1364/boe.7.004220 (2016).

Figure 1A

Click here to download Figure Fig. 1A.tif

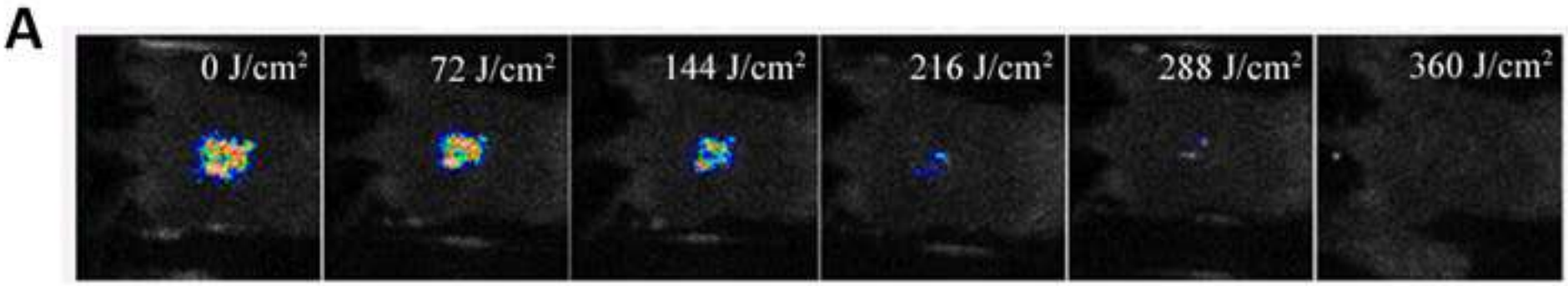
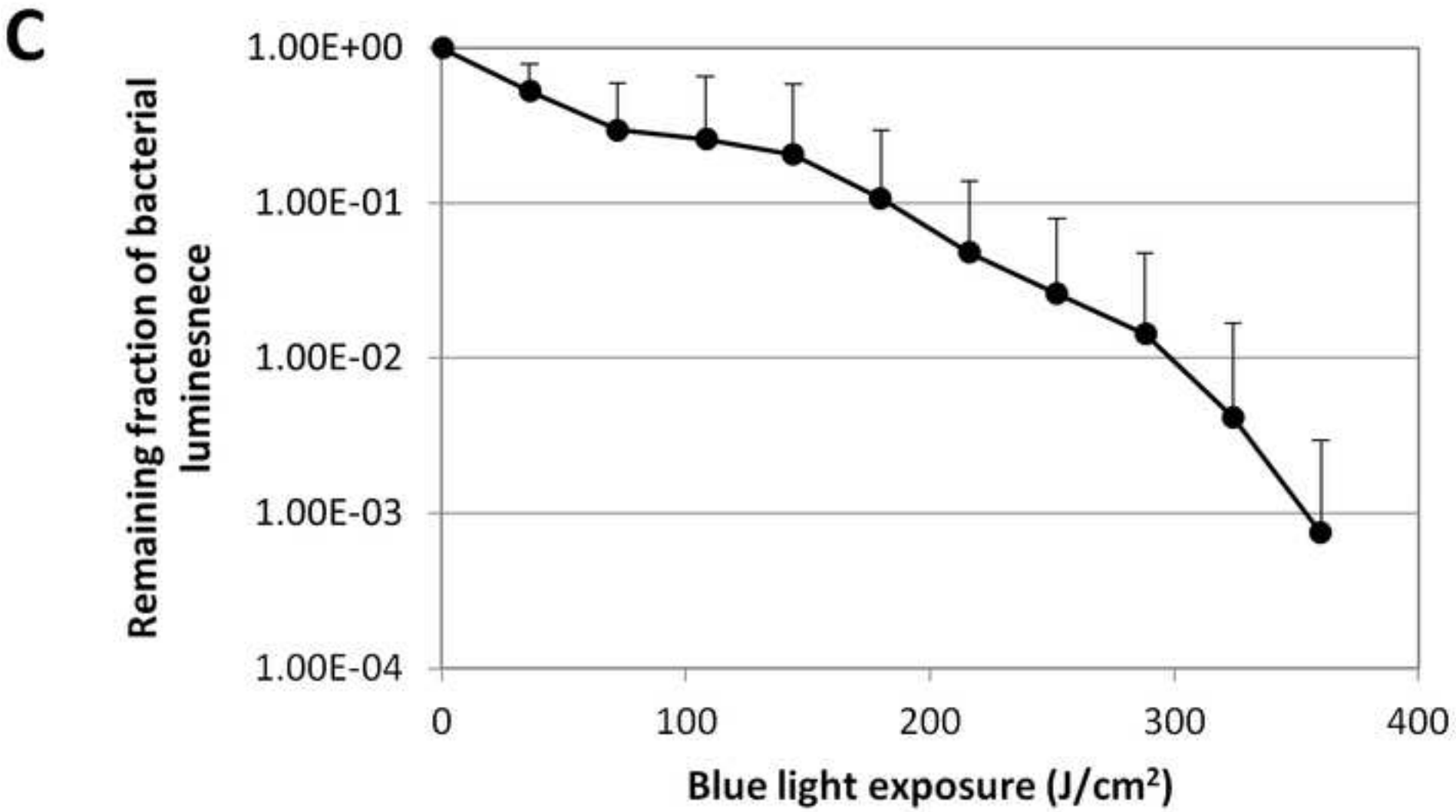


Figure 1B

Click here to download Figure Figure 1B.tif

Figure 1C

Click here to download Figure Fig. 1C.tif



Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment
JoVE_Materials TD_rev.xls

Name of Material/ Equipment	Company	Catalog Number
IVIS	PerkinElmer Inc, Waltham, MA	IVIS Lumina Series III
Light-emitting diode LED	VieLight Inc, Toronto, Canada	415 nm
Power/energy meter	Thorlabs, Inc., Newton, NJ	PM100D
Mouse	Charles River Laboratories, Wilmington, MA	BALB/c
Acinetobacter baumannii	Brooke Army Medical Center, Fort Sam Houston, TX	Clinical isolate
Insulin Syringes	Fisher Scientific	14-826-79
Sodium Chloride	Fisher Scientific	721016
Phosphate Buffered Saline, 1X Solution	Fisher Scientific	BP24384
Brain Heart Infusion	Fisher Scientific	B11059
Falcon 15mL Conical Centrifuge Tubes	Fisher Scientific	14-959-70C
Benchtop Incubated Orbital Shakers	Laboratory Supply Network, Inc, Atkinson, NH	Incu-Shaker Mini
Inoculating Loops	Fisher Scientific	22-363-605
Fisher Scientific Redi-Tip Pipet Tips, 1-200µL	Fisher Scientific	02-707-502
Thermo Scientific Sorvall Legend X1 Centrifuge	Fisher Scientific	75-004-220
Brass Block	Small Parts, Inc., Miami, FL	10 mm by 10 mm
Extreme Dragon PBI/Kevlar High-Heat Gloves	Superior Glove Works Ltd, Cheektowaga, NY	PBI83514
Greiner dishes	Sigma-Aldrich Co. LLC	P5112-740EA
Corning Digital Hot Plate	Cole-Parmer Instrument Company, LLC	UX-84301-65
Mouse/Rat Thin Line Water Heated Surgical Bed	E-Z Systems	EZ-211

Comments/Description
Pre-clinical in vivo imaging
Light source for illumination
Light irradiance detector
7-8 weeks age, 17-19 g weight
Engineered luminescent strain
BD Lo-Dose U-100 Insulin Syringes for injection
0.9% Sodium Chloride
A standard phosphate buffer used in many biomolecular procedures
Bacterial culture medium
For bacterial suspension centrifuge
For culturing of bacteria
For smearing bacterial inoclum on burn surface of mice
Pipet Tips
For bacterial suspension seperation
For creation of burns in mice
Heat Resistant Gloves
35 mm ×10 mm
10" x 10", 220 VAC, for boiling water
Prevents heat loss and hypothermia during surgery

JOVE JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT - UK

Title of Article: *In vivo* investigation of antimicrobial blue light therapy for multidrug-resistant *Acinetobacter baumannii* burn infections using bioluminescence imaging

Author(s): Yucheng Wang, Olivia Harrington, Clinton K Murray, Michael R Hamblin, Tianhong Dai

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: [X] Standard Access [ ] Open Access

Item 2 (check one box):

[X] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution 3.0 Agreement (also known as CC-BY), the terms and conditions of which can be found at: http://creativecommons.org/licenses/by/3.0/us/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and

(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the

Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including,

without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

AUTHOR:

Name:	Tianhong Dai
Department:	Wellman Center for Photomedicine
Institution:	Massachusetts General Hospital
Article Title:	Antimicrobial blue light therapy for multidrug-resistant bacterial infections in mouse burns
Signature:	Tianhong Dai
Date:	05/12/2016

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy as a PDF to the *JoVE* submission site upon manuscript submission (preferred);
2) Fax the document to +1.866.381.2236; or
3) Mail the document to *JoVE* / Atn: *JoVE* Editorial / 1 Alewife Center Suite 200 / Cambridge, MA 02140

For questions, please email editorial@jove.com or call +1.617.945.9051.

MS # (internal use):

RESPONSES TO THE EDITOR AND REVIEWERS' COMMENTS:

- ***JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.***

This comment has been implemented.

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

All figures used in this manuscript are new ones that have not been published.

- ***Formatting: Introduction, 3rd paragraph – Please include citations for these claims.***

Done.

- ***Please copyedit the manuscript for numerous grammatical errors and awkward phrasing, some of which are described below. Such editing is required prior to acceptance and should be performed by a native English speaker.***

-***Line 59 – "sequel of"***
The ″sequel of″ has been replaced with ″consequence of″.

-***Line 69 – United government?***
The "United government" has been replaced with ″Government of the United States″.

-***Line 76 – "relatively burgeoning weapon" – please clarify***
The sentence has been rewritten.

-***Line 83 – "We have been able to gain access to the power given by bioluminescent pathogenic bacteria" – please rephrase; it is unclear how these bacteria have a power to which one would gain access***
This sentence has been rephrased.

-***Line 90 – "In virtue"***
The "In virtue of" has been replaced with ″Through″.

-***Line 92 – "using minimized number of mice"***
The phrase has been changed to ″significantly reduced number of mice″.

-3.2 – "Optical Support Rod" should be lower case; otherwise this gives the impression of a brand name; "the LED move up"
The comment has been implemented and the typo has been corrected. Thanks.

-2.2, 3.7, 4.2 – "a fair anesthetic depth" – use "appropriate" instead.
Done.

-3.10 – "altitude" – use "height" instead; "positon" typo
Thanks. The comment has been implemented and the typo has been corrected.

-3.11 – use "dose" instead of "aliquots"
We still think "aliquots" is a more appropriate word here.

-Line 253 – "Silver ions…is"
This sentence has been deleted with the whole paragraph.

-Line 264 – "we can access to"
Corrected as *″we can have the access to″*. Thanks for catching this typo.

-Line 294 – "inoculum frequently fail"
The typo has been corrected. Thanks for catching it.

-Line 298 – "found that under the same conditions" – thought is incomplete
Corrected.

-Line 306 – "are suggested being protected"
Corrected as *″it is suggested the eyes of mice be protected ″*.

-Line 312 – "the following issue is worth attention: (1) the" – no need to number a single point, so delete the 1.
Done.

-Line 318 – "expended" – wrong word
The sentence has been rephrased.

-The discussion section must be extensively edited for clarity and proper construction of lists.

The section has been significantly revised in accordance with the editor's comment.

• Additional detail is required:

-2.5 – How long does thermal equilibration take?
The thermal equilibration takes several minutes and could be determined by the re-boiling of the water. This statement has been added to the manuscript.

-3.11 – Are doses given on the same day or different days? How long does each treatment last?
Doses are given on the same day. Light is continuously delivered only with intervals for bioluminescence imaging. The treatment duration depends on the total light exposure required to eradicate infection and light irradiance. For *eg.*, in our study, a maximum of 360 J/cm^2 was delivered, which was a total 60-min irradiation at a fluence rate of 100 mW/cm^2.

-From the discussion, suggestions like protecting the eyes of mice with foil or moistening the skin with PBS prior to imaging should appear in the protocol section.
We thank the editor for the constructive suggestion, which has been implemented.

Reviewer #2:

Major Concerns:

- ***Experimental procedure and methods are logical and correct sufficiently. However, this paper have little novelty in idea, method and conclusion because similar studies have been published already especially in burn wound model and pathogen (Acinetobacter baumannii). There are many original and review papers about bioluminescence of bacterial infection. There is no evidence or***

experiment to biofilm in Fig B although it is fully supposed.

We thank the reviewer's comments and totally understand the reviewer's concern. We have published a paper regarding the use of the antimicrobial blue light against acute *Acinetobacter baumannii* infections using burn mouse model. Meanwhile, we are aware that there have been many published papers on the bioluminescence of bacterial infection. However, what we are discussing in this paper are **the details of an experimental protocol** to assess the effectiveness of antimicrobial blue light therapy for infection in a mouse model of burn injury. The use of bioluminescent bacterial strain herein works as a convenient and suitable tool for the assessment, however it is not the main goal of our work.

Fig 1B representatively shows the presence of biofilms in the mouse burns 24 h post-inoculation, while Fig 1A demonstrates that 24-h infection in mouse burns was eradicated after an exposure of 360 J/cm^2 aBL had been delivered. As a result, Fig 1A and Fig 1B together indirectly indicate that aBL therapy was effective against biofilms. Since aBL is not only used to treat biofilm-associated infections, the experiment of biofilm is not fully discussed.

- ***After aPDT, figure of burn wound is necessary to compare with the first infected wound (Fig B).***

Thanks for the constructive suggestion. After blue light treatment, the bioluminescence of bacteria was eradicated, indicating an inactivation of the bacteria. However, gram staining only demonstrates the presence of bacteria but not the viability of bacteria, thus we would not expect much difference between the gram-stained samples before and after blue light treatment. However, we would consider to obtain the gram-stained samples several days after the blue light treatment, which would show the re-distribution of the bacteria and compare them with the first infected wound.

Minor Concerns:

- ***Reference should be corrected according to submission guideline***

Done.

Reviewer #4:

Minor Concerns*:*

Two minor points:

- ***Page 4, line 115: Authors write "Use adult female BALB/c mice..." Is it not possible to use male mice? And about other mouse strains? Please discuss.***

Yes, it is possible to use male mice. The reason why we chose female mice is that they are less aggressive, and, therefore, are less prone to scratch and thus disruption of wounds.

Since the pigments of skin would absorb the photons emitted from the bioluminescent bacteria, it is better to use albinic mice for the burn infection model to minimize the error of bioluminescence monitoring. As a result, BALB/c strain was chosen for our study.

- ***Page 9, line 297 - 299: Authors report "We have also studied other bacterial species...bacterial inoculum). The infections ..." Please check this phrase. I think that the dot should be removed.***

Thanks for catching this typo. The dot has been removed.