INTRODUCTION TO THE REVISED SUBMISSION

This application is the resubmission of the 2010 OTA Basic Research Grant #12, which was reviewed by the OTA Research Committee in November, 2010. The resubmission has been substantially revised in response to the reviewers' comments, which are reasonable and helpful.

We are pleased that both the reviewers noted that the application was *"very innovative"* and addressed *"a real problem"*; the proposed technology *"could potentially be a very valuable approach to this very serious problem"*.

One critique from the reviewers was that they thought *"this project is already funded seems"*. As a matter of fact, the proposal is a completely new one that has not received funding. The PI initiated the idea of the proposal just recently and has just published a letter to editor in the journal of Photodiagnosis and Photodynamic Therapy discussing the potential of photodynamic therapy (PDT) for surgical site infections (Hamblin MR, **Dai T**. Can surgical site infections be treated by photodynamic therapy? Photodiagnosis Photodyn Ther. 2010; 7:134-136.). The PI has a finished fellowship entitled ″Photodynamic therapy for multi-drug resistant wound infections *in vivo* (01/01/2009-12/31/2009) ″. The objective of the fellowship project was to investigate the use of PDT for *A. baumannii* **burn infection**, so there is no overlap between the two projects.

In addition to the revisions made in accordance with the reviewers' comments, we have further improved the research proposal by making the following two major changes:

(1) We will use a more clinically relevant rat model to study deep incisional infections. In brief, standard incision measuring 4 cm in length and 5 mm lateral and parallel to the vertebral column will be carried through the skin. The incision will be then continued to a depth of approximately **1 cm** into the underlying paraspinous muscles. Sterile sand (≈100 mg) will be introduced into each wound as an infection-potentiating foreign body and the wounds will be inoculated with 50 μL of suspension containing a defined number of bacteria cells.

(2) When the light penetration of topical delivery may be insufficient to reach the infected wound sites of deep incisional infections, we will use a diode laser coupled with an optical fiber and controlled by an optical fiber movement device to deliver the light interstitially into the wound sites.

Response to reviewer 1 (*the original critiques were cited using Italic typography*)**:**

- *Does the genetic modification make them more susceptible to PDT?*

We have carried out tests with the bioluminescent strains of *Pseudomonas aeruginosa*, *Acinetobacter baumannii* as well as their parent strains, and no noticeable difference was found in terms of the bacterial susceptibility to PDT and the bacterial virulence. We have addressed this point in the current re-submission.

- *Budget: Unrealistic- unless they have some other source of money for the project? We do see this in the PI's CV: Wellman Center for Photomedicine, Bullock-Wellman Fellowship Award (Dai, Tianhong--PI). Photodynamic Therapy for Multi-Drug Resistant Wound Infections In Vivo. ($60,000, 1/1/09-12/31/09)*

We have adjusted the budget by removing the budget for hiring a part-time technician and replacing the money on the purchase of supplies. The Bullock-Wellman Fellowship project mentioned by the reviewer has already been finished. The objective of this fellowship project was to evaluate the efficacy of PDT for *A. baumannii*

burn infections, so there is no overlap between the two projects.

- *Does the project have Institutional Review Board approval? I imagine it does- it already seems to be funded.*

Yes, we have Institutional Animal Care and Use Committee (IACUC) approvals ready for the studies of phototherapy of localized wound infections. However, the proposal of PDT for surgical site infections is a new one and has not been funded. There is no overlap between the current application and the previous fellowship project of the PI.

Response to reviewer 2:

- *... the title speaks about "prophylaxis" but no mention of this is made in the experiment ...*

In the study design, PDT will be initiated at varying time points from 30 min to 48 h after the inoculation of bacteria. To apply PDT shortly after the inoculation of bacteria (i.e., 30 min after bacterial inoculation) is applicable as prophylaxis or the interruption of very early stages of infection. The longer periods, on the other hand, will be especially important for established chronic infections. We have emphasized this point in the current resubmission.

ABSTRACT OF RESEARCH PLAN

INVESTIGATOR NAME/INSTITUTION	PROJECT TITLE
INSTITUTION: Massachusetts General Hospital Harvard Medical School	**Antimicrobial Photodynamic Therapy for Prevention and Treatment of Surgical Site Infections**

Abstract of research plan: Please provide a 250 word abstract with 5 underlined phrases for project summary, to fit in the box below.
Avoid summaries of past accomplishments and the use of the first person. The abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application.

The hypothesis of this proposal is: **Antimicrobial photodynamic therapy (PDT) can be used as an alternative approach for prevention and treatment of multi-drug resistant surgical site infections (SSIs)**. To test this hypothesis, we propose the following two specific aims:

Aim 1: Determine the prophylactic and therapeutic efficacies of PDT for surgical wounds in rodents infected with *Staphylococcus aureus*, *Pseudomonas aeruginosa*, or *Acinetobacter baumannii*, which are the pathogens frequently encountered in SSIs. We have developed a protease-stable polycationic photosensitizer that can efficiently kill a panel of pathogens. In addition, we have unique access to an ***in-vivo* bioluminescence imaging** technique and use it to monitor in real time the extent of infection in living animals. PDT will be initiated at varying time points post-infection to investigate its efficacies for both prophylaxis and treatment of SSIs. The efficacies found with PDT will be compared with those of gentamicin, a commonly used antimicrobial agent for SSIs.

Aim 2: Investigate the **effects of PDT on leukocytes**, which are important in the local defense mechanism against dissemination of infections. The effects of PDT on the viability, phagocytosis, and reactive oxygen species (ROS) production of leukocytes will be investigated.

Successful completion of the proposed work will provide the foundation required to assess the efficacies (Aim 1) and the possible side effects (Aim 2) of PDT for SSIs. If PDT can be shown to be both effective and safe, this research will address the serious problem of emerging drug-resistant bacteria by providing a much-needed alternative treatment for SSIs.

A) SCIENTIFIC AIMS. Provide a concise statement of the aims of the proposed research. (Not to exceed 400 words) This section should include the research question, the hypothesis and scientific aims.

Surgical site infections (SSIs) caused by bacteria that are resistant to multiple classes of antibiotics are an important and increasing problem in modern orthopaedic surgery [1-3]. The hypothesis of this proposal is: **Antimicrobial photodynamic therapy (PDT) can be used as an alternative approach for prevention and treatment of multi-drug resistant SSIs.** To test this hypothesis, we propose the following two specific aims:

Aim 1: Determine the prophylactic and therapeutic efficacies of PDT for surgical wounds in rodents infected with *Staphylococcus aureus*, *Pseudomonas aeruginosa,* or *Acinetobacter baumannii,* which are the pathogens frequently encountered in SSIs. In this section, we will investigate both superficial incisional SSIs and deep incisional SSIs by using mouse and rat models, respectively. We have developed a protease-stable polycationic photosensitizer (conjugates between polyethyleneimine (PEI) and chlorine-e6) that can efficiently kill a panel of pathogenic bacteria when activated by red light [4]. In addition, **we have unique access to an *in vivo* bioluminescence imaging technique and use it to continuously monitor in real time the extent of infection in living animals** [5]. PDT will be initiated at varying time points after bacterial inoculation to investigate its efficacies for both prophylaxis and treatment of SSIs. Red light at the wavelength of 660-665 nm will be delivered either topically using a non-coherent light lamp (for superficial incisional SSIs) or interstitially using a diode laser coupled with an optical fiber (for deep incisional SSIs). The efficacies found with PDT will be compared with those of gentamicin, a commonly used antimicrobial agent for SSIs [6].

Aim 2: Evaluate the effects of PDT on leukocytes, which are important in the local defense mechanism against dissemination of infections. The experiments will be performed *in vitro* using rodent leukocyte cultures and *in vivo* using rodent wounds, respectively. The effects of PDT on the viability, phagocytosis, and reactive oxygen species (ROS) production of leukocytes will be evaluated.

Successful completion of the work proposed in this application will provide the foundation required to assess the efficacies (Aim 1) as well as the potential side effects (Aim 2) of PDT for SSIs, and will help establish protocols for the use of this treatment option. If PDT can be shown to be both safe and effective, this research will address the serious problem of emerging antibiotic resistant bacteria by providing a much-needed alternative treatment for SSIs.

B) BACKGROUND & SIGNIFICANCE. Discuss pertinent previous publications on research topic. Explain what is known and unknown and how proposed work will make an impact. Also, explain why the results of the proposed work may be important and how it may lead to future investigation. Provide a statement as to the relevance of the project to OTA's Mission Statement. (Not to exceed 400 words)

B.1. SSIs caused by drug resistant bacteria pose health threat.

SSIs account for 22% of healthcare acquired infections (HAIs) and **33% of SSIs are orthopaedic infections** [1]. Despite advances in surgical wound care and management, infections remain a leading cause of mortality and morbidity in surgical patients [1-3]. Many SSIs are treated with topical or systemic antibiotics. Unfortunately, due to the widespread use of such antibiotics, a growing number of bacterial strains have developed resistance to all clinically significant antibiotics, leading to what has been called the "end of the antibiotic era" [7]. **Recently, a dangerous new mutation (named NDM-1) that makes some bacteria resistant to carbapenems, which are the antibiotics used as a last resort when common antibiotics have failed, is being found in patients in the United States** [8-9]. As a result, there is a pressing need for the development of alternative treatment regimens.

B.2. Antimicrobial PDT may offer a promising treatment option for multi-drug resistant SSIs.

One promising and innovative approach to kill and eliminate antibiotic resistant bacteria that are infecting tissue is antimicrobial PDT. PDT is based on the concept that a non-toxic photosensitizer (PS) can be localized preferentially in certain tissues or cells, and subsequently activated by visible light to produce reactive oxygen species (ROS) that can kill target cells (Fig. 1) [11,12]. All studies that have examined the killing of antibiotic resistant bacteria by PDT have found them to be equally as susceptible as their naïve counterparts [13,14]. Moreover, it has not as yet been possible to artificially induce resistance to PDT in any bacterium where it has been tested [15]. Because the delivery of visible light to living tissue is almost by definition a localized process, PDT for infections is likely to be applied exclusively to localized infections. SSIs are essentially localized infections. Therefore, antimicrobial PDT is an ideal method of treating such infections.

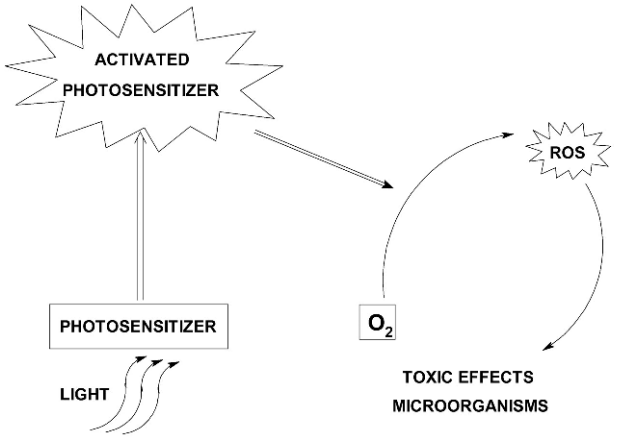


Fig. 1. Schematic depiction of photodynamic inactivation of microorganisms.

The proposed study serves as the initial efforts in the pursuit of PDT for SSIs. Thus, the first and most important impact is through opening a branch of study on a new treatment regimen. Given the significant drawbacks in current treatments, this new technology has great potential. If PDT can be shown to be both safe and effective, public health would benefit tremendously including possible life saving intervention for those unfortunate people who die of multi-drug resistant SSIs.

C) PREVIOUS WORK DONE ON THE PROJECT. Describe thoroughly preliminary studies performed by members of the research team relative to the topic of this proposal. These preliminary studies should be provided chronologically. Pertinent publications should have a short summary of the publication to distill the project for the reviewer. (Not to exceed 800 words and/or two pages)

C.1. Bioluminescent strains of bacteria.

We have unique access to the power given by bioluminescent pathogenic bacteria to develop new rodent models of wound infections. These bacterial strains were rendered bioluminescent by stably transforming the entire *Photorhabdus luminescens* lux operon into the bacterial cells [22]. By using a luminescence plate reader in combination with a colony formation assay, a good linear correlation between the bacterial luminescence and the numbers of colony forming units (CFU) of the bioluminescent bacterial strains was found (Fig. 2, *A. baumannii* as the sample strain).

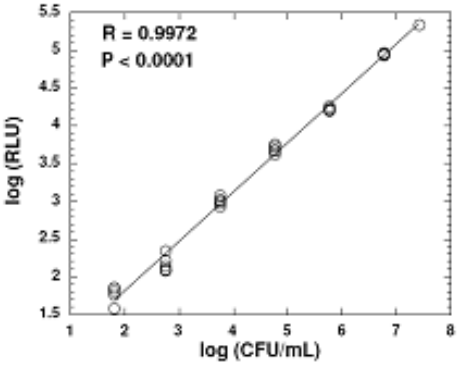


Fig. 2. Linear correlation between bacterial luminescence (RLU) and colony forming units (CFU/mL) of *A. baumannii.*

As the bacterial luminescence intensity is linearly proportional to the bacterial CFU, the extent of infection can be monitored in real time by using a photon counting ICCD camera. This method is a significant improvement on the traditional use of survival or body fluid sampling and subsequent plating and colony counting. The first method suffers from the disadvantage of being wasteful of animals, and does not really address the question of where the bacteria are in the animal, while the second method suffers from the disadvantage that tissue sampling introduces another source of experimental error, is laborious and does not give real-time results.

We have also carried out tests with the bioluminescent strains of bacteria as well as their parent strains, and no noticeable difference was found between them in the bacterial susceptibility to PDT and the bacterial virulence.

C.2. Mouse models of soft-tissue infections and abscesses using bioluminescent bacteria.

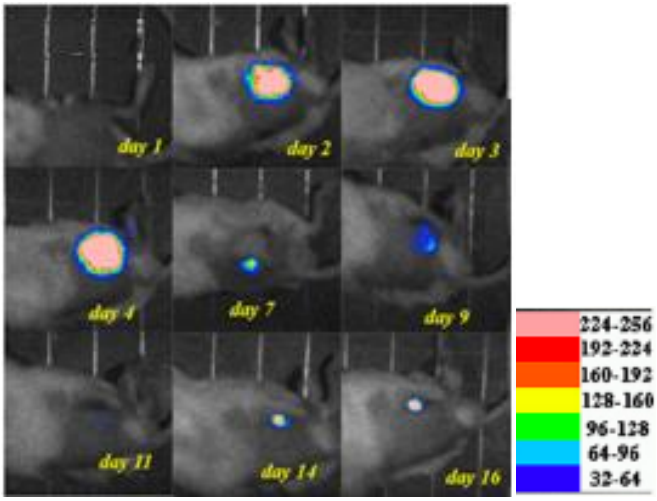


Fig. 3. Series of bioluminescence images resulting from the injection of 10^6 bioluminescent *S. aureus* into the thigh muscle of a neutropenic mouse.

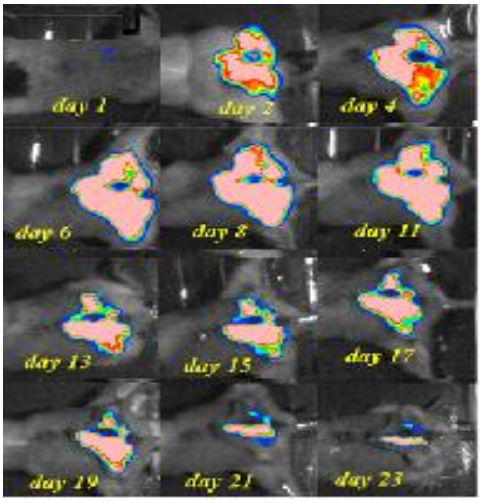


Fig. 4. An abscess generated in a normal mouse by injecting 10^6 bioluminescent *S. aureus* into a pocket on the back containing a 3-cm coiled monofilament nylon suture.

Fig. 3 shows a series of bioluminescence images resulting from the injection of 10^6 CFU of bio-luminescent *S. aureus* 8352-4 into the thigh muscle of a neutropenic mouse. By the second day the bacteria has multiplied > 1000-fold and remains at similar levels for 3 days before declining between days 7-11. Interestingly, by day 14 the infection has returned in a lesser but more tightly localized form, consistent with

abscess formation. This *S. aureus* abscess has been observed to last up to 29 days after infection.

Fig. 4 shows an abscess generated in a normal (non-neutropenic) mouse by injecting 10^6 bioluminescent *S. aureus* CFU into a pocket on the back containing a 3-cm coiled monofilament nylon suture in a model first described by Edlich *et al* [24]. The growth of the bacteria is both dramatic and long lasting, with massive infection still present at 19 days after the initial injection.

C.3. Polycationic conjugate PS.

We have discovered a method of targeting polycationic PS conjugates to microorganisms and subsequent illumination with red light produces up to 6-log$_{10}$ (99.9999%) of inactivation of micro-organisms *in vitro* [4]. The method is based on the covalent attachment of PS to polycationic peptides, such as polyethyleneimine (PEI), that can bind to and penetrate microorganisms (Fig. 5). Cross-linked PEI (high molecular weight 10,000–25,000) is reacted with chlorine-e6 (c_{e6}) (Frontier Scientific, Logan, UT) and 1-ethyl-3-(3-dimethylaminopropyl) -carbodiimide hydrochloride (Sigma-Aldrich Co., St. Louis, MO) in borate buffer (0.1 M, pH = 8.5), for 24 h in the dark. The crude conjugate is purified by precipitation from acetone at −20°C followed by exhaustive (2 days, three changes) dialysis (MW cutoff 2 kDa) against distilled water containing 0.1% Triton X-100. The substitution ratio is calculated, from the absorption spectrum of the conjugate (0.1 M NaOH, 1% SDS), to be an average of 1 c_{e6}/PEI-chain, assuming that the absorption coefficient of conjugated c_{e6} is the same as that of free c_{e6} (ε_{400nm} =150,000 $M^{-1}cm^{-1}$) and the trinitrobenzene sulfonic acid assay for free amino groups.

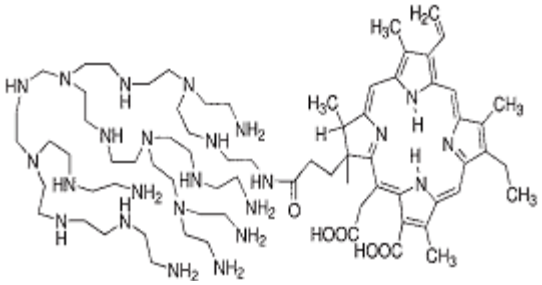
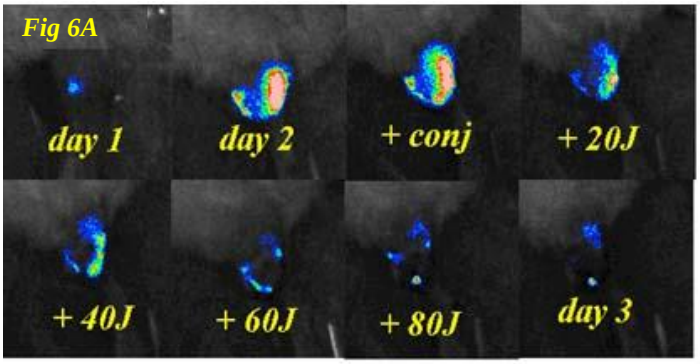


Fig. 5. Chemical structure of polyethyleneimine chlorine-e6 (PEI-c_{e6}) conjugate.

C.4. PDT for soft-tissue infections in mice.

Our initial experiments on PDT of soft-tissue infections used an injection of 10^6 bioluminescent *S. aureus* into the thigh muscle of a neutropenic mouse. The next day 100 µL of a 200 µM solution of PS was injected into the infected area as 5 aliquots of 20µL, distributed around the center of the infection as determined from the luminescence image. Thirty (30) minutes later the mouse was illuminated with a surface spot from the 665-nm diode laser at a fluence rate of 100 mW/cm^2. After each aliquot of 20 J/cm^2 had been delivered the mouse was imaged to construct the series of images shown in Fig. 6A, and the derived pixel counts shown in Fig. 6B. There is a small but significant drop in luminescence on adding the PS into the muscle but a pronounced light-dose dependent drop in luminescence after illumination until 95% is gone after 80 J/cm^2 of light. Gratifyingly, by the next day the infection had not returned, nor indeed in succeeding days. The mouse thigh healed normally and it regained full use of the affected leg.



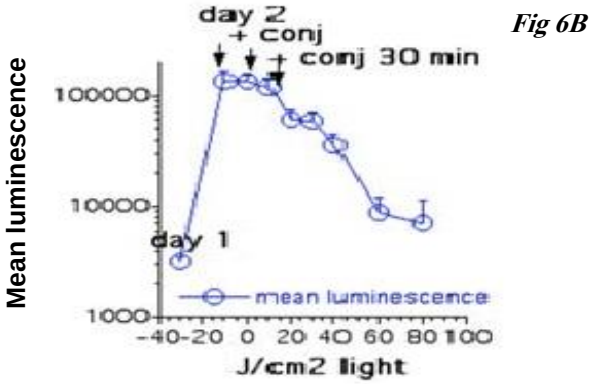


Fig. 6. (A) Series of bioluminescence images resulting from a representative mouse infected with 10^6 bioluminescent *S. aureus* on day 1 and treated with PDT on day 2. (B) Mean bioluminescence values from 5 infected mice treated with PDT as described in panel (A). Bars: Standard deviation.

D) METHOD. Give details of your research plan, including how the results will be analyzed. For each specific aim mentioned in "A" show how your plan is expected to fulfill the aim. Please include a timeline for completion of this study as well as a justification for number of specimens proposed. (Not to exceed 1500 words and/or 4 pages**)**

D.1. Determine the prophylactic and therapeutic efficacies of PDT for SSIs in rodents.

We will investigate both superficial incisional SSIs and deep incisional SSIs by using mouse and rat models, respectively. The efficacies found with PDT will be compared with those of gentamicin, a commonly used antimicrobial for SSIs [6].

D.1.1. Light delivery devices. For superficial incisional SSIs, we will use a non-coherent light source (LumaCare LC-122, Newport Beach, CA) to deliver light topically to the infected wound sites (Fig. 6A). The LumaCare light source can produce the entire spectrum of visible light, and the probes filter and focus the light to specific wavelengths. We will use a 660-nm wavelength (red light) probe in the study.

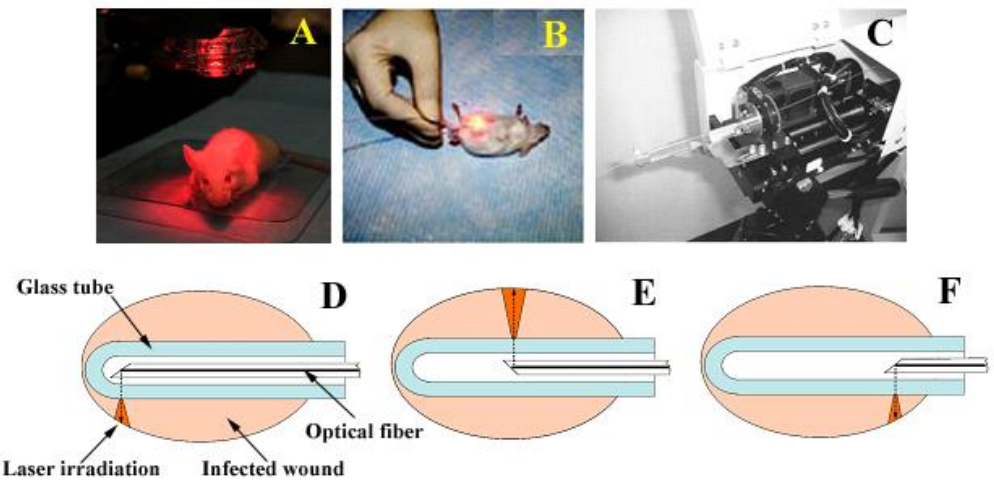


Fig. 7. A) Topical delivery of light to superficial incisional SSIs in rodents. B) Interstitial delivery of light into deep incisional SSIs in rodents. C) Optical fiber movement device for interstitial light delivery. D-F) Schematic depiction of interstitial light delivery during PDT. A laser-proof glass tube is implanted in an infected wound. An optical fiber, whose tip is cut and polished at an angle of about 45°, is inserted into it. Laser beam is delivered in a direction nearly perpendicular to the axis of the optical fiber. The fiber tip rotates at 12 rpm and performs a reciprocal motion at 18 mm/min. Laser beam rotation is observed in the infected wound when the fiber moves from (D)→(E)→(F).

For deep incisional SSIs, we will use a 665-nm wavelength (red light) diode laser (BWF-665-1; B&W Tek, Inc., Newark, DE) to interstitially deliver light into the infected wound sites (Fig. 7B). The laser is coupled into a 200-μm optical fiber. In order to expose light from the inside of the infected wound, a laser-proof glass tube (external and internal diameters, 0.762 mm and 0.254 mm, respectively; Accu-Glass LLC, St. Louis, MO) will be inserted into the wound site, and then the optical fiber will be guided into it (Figs. 7D, 7E, 7F). The distal tip of the fiber is cut and polished at an angle of about 45°, so that the laser beam will be delivered approximately perpendicular to the optical axis of the fiber. By using a fiber movement device (Fig. 7C), the fiber tip will be rotated clockwise and counter-clockwise alternately at a speed of 12 rpm (rotations per min) and perform a reciprocal linear motion from one end to the other of the infected wound at a speed of 18 mm/min. The light dose will be expressed as the integrated energy per unit cm along the moving trajectory during reciprocal motions of the fiber tip. The fiber movement device is shown in Fig. 7C. It has two motors for the independent fiber movement, one is for the rotation and the other is for the reciprocal linear motion of the fiber. The range of the reciprocal linear motion could be set in the range of 0-100 mm according to the size of the infected wound.

D.1.2. Choice of bacteria. We will study three bacterial species: *S. aureus*, *P. aeruginosa*, and *A. baumannii,*

which are the pathogens frequently encountered in SSIs. These bacterial strains are rendered bioluminescent by stably transforming the entire Photorhabdus luminescens lux operon into the bacterial cells. The *S. aureus* strain we will use is 8325-4 (ATCC). The bioluminescent strain of *P. aeruginosa* is derived from ATCC 19660 (strain 180). The *A. baumannii* strain was isolated from an infected US service member deployed overseas. Studies in our lab confirmed that bioluminescence gene transformation neither changes the susceptibility of bacteria to PDT nor the bacterial virulence.

D.1.3. Animal models of SSIs. We will investigate both superficial incisional SSIs and deep incisional SSIs by using mouse and rat models, respectively. The **superficial incisional SSIs** in mice will be produced as previously described [23]. In brief, a 1-cm length of bacterial-contaminated silk suture will be inserted under the skin of the mid-back of each shaved mouse. An incision will be made along the length of the suture down to, but not into, the panniculus carnosus. The **deep incisional SSIs** in rats will take place also using a protocol of a previous study [27]. A standard incision measuring 4 cm in length and 5 mm lateral and parallel to the vertebral column was carried through the skin. The incision will be then continued to a depth of approximately 1 cm into the underlying paraspinous muscles. Sterile sand (100 mg) will be introduced into each wound as an infection-potentiating foreign body. Both of the two wound types will be inoculated with 50 μL of suspension containing a defined number of bacterial cells.

We will first study how the luminescent organisms behave in the rodent models of SSIs. Specifically what is the effect of bacterial inoculum on development of local infection, sepsis, and wound healing? We will test inocula of from 10^4 to 10^8 CFU and mice will be followed daily for luminescence signal, body weight, appearance (ruffled fur is a good indicator of systemic infection), survival and wound healing. For each CFU inoculum, a group of 7 rodents will be tested. When the behaviors of the untreated SSIs have been defined we will progress to testing of PDT in these models.

D.1.4. Bioluminescence imaging set-up. The set-up (Hamamatsu Photonics, Bridgewater, NJ) consists of an intensified CCD camera mounted in a light-tight specimen chamber, fitted with a light-emitting diode, a set-up that allows for a background gray-scale image of the entire rodent to be captured (Fig. 8). In the photon-counting mode, an image of the emitted light from the microorganisms is captured using an integration time of 2 min. By use of ARGUS software (Hamamatsu), the luminescence image is presented as a false-color image superimposed on top of the grayscale reference image. The ARGUS software also calculates the total pixel values from the luminescence images of the infected area.

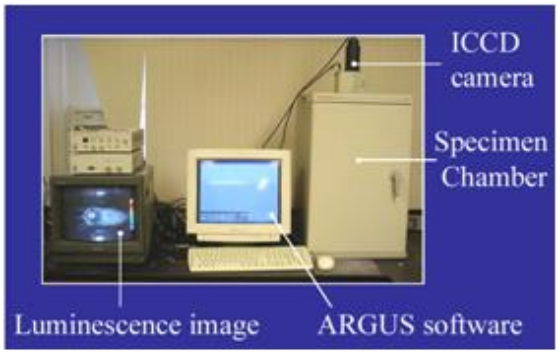


Fig. 8. Bioluminescence imaging set-up

D.1.5. PDT for SSIs. PS (PEI-c_{e6}) will be added at varying time points of 30 min, 24 h, or 48 h after bacterial inoculation. To apply PDT shortly after bacterial inoculation (i.e., 30 min post-inoculation) is applicable as prophylaxis or the interruption of very early stages of infection, while the longer periods will be especially important for established infections. PS will be injected percutaneously (for superficial incisional SSIs) or subcutaneously (for deep incisional SSIs) as 50-100 μL of a solution in phosphate-buffered saline (PBS) (200-400 μ*M* PS equivalent). After a further 30 min, the infected wound sites will be illuminated either topically (for superficial incisional SSIs) using a 660-nm non-coherent lamp (as shown in Fig. 5A) or interstitially (for deep incisional SSIs) using a 660-nm diode laser coupled with an optical fiber (as shown in Figs. 5B-5F). Animals will be given total light doses of up to 240 J/cm^2. For each combination of bacteria species and time point of PS addition, 7 rodents will be used. In addition, for each bacteria species, a group of 7 rodents without PDT will serve as the non-treated controls, respectively.

D.1.6. Gentamicin prophylaxis and treatment of SSIs (positive control). We will investigate an alternative topical antimicrobial treatment (gentamicin) to compare its effects on bacterial infection with those of PDT. About 0.16 mL (for superficial incisional SSIs) or 1.6 mL (for deep incisional SSIs) of 50-mg/ml aqueous gentamicin solutions (a dose equal to 20 mg/kg-rodent-body-weight [28]) will be injected into the depth of infected surgical wounds at varying time points of 30 min, 24 h, or 48 h after bacterial inoculation. For each

condition, 7 rodents will be used.

D.1.7. Follow-up and statistical analysis. Rodents (including untreated controls) will undergo luminescence imaging at 24 hourly intervals after PDT. Blood samples will be withdrawn from the orbital plexus and cultured on Brain-Heart Infusion (BHI) plates for determining the presence of bacteria in blood stream. Rodents will also be followed for survival, body weight, and wound healing. When rodents die they will be dissected and organ samples (i.e., liver) taken for dissociation and determination of bacterial numbers and sectioned for hematoxylin-eosin (H&E) staining for tissue damage.

Values will be presented as means and standard deviations. Areas under the curves representing the time courses of bioluminescence intensity, wound area, and body weight will be calculated using numerical integration. Differences in the areas under the curves between different rodent groups will be compared for statistical significance using a Student's *t*-test. Survival analysis on different rodent groups will be performed using the Kaplan-Meier method. Survival curves were compared, and the differences in the survival rates will be tested for significance by the use of a log-rank test. *P*-values of < 0.05 are considered significant.

D.2. Aim 2. Evaluate the effects of PDT on leukocytes.

It is suggested that polycationic PS can be selectively bound to bacteria, therefore, the side effects of PDT on the surrounding tissues and cells would be minimal. However, to the best of our knowledge, this has not been rigorously tested. In this section, we will investigate the effects of PDT on leukocytes, which are important in the local defense mechanism against dissemination of infections.

D.2.1. Effects of PDT on the viability of leukocytes *in vitro*. Leucocytes will be isolated from rat venous blood as previously described [29]. One hundred (100) µL mixed solutions of leucocytes ($\approx 10^5$ cells) and PS (10 µM PS equivalent) in PBS will be incubated for 30 min and then irradiated in a 24-well plate with the pre-determined effective *in vitro* antimicrobial light dose. Sixteen (16) µL of the irradiated solution will be mixed with 4 µL of 0.4% trypan blue immediately after the irradiation, and then dropped onto a hemacytometer. Total number of leucocytes and the number of trypan blue-stained leucocytes (dead leucocytes) will be counted and the percentage of surviving leucocytes after PDT will then be calculated. The experiments will be performed in triplicate.

D.2.2. Effects of PDT on the phagocytosis and ROS production of leucocytes *in vitro*. Two hundred (200) µL rat venous blood will be mixed with 10 µL PS-PBS solutions (10 µM PS equivalent). After 30 min incubation, the mixed solution will be irradiated in a 24-well plate with the pre-determined effective *in vitro* antimicrobial light dose. In addition, 200 µL rat venous blood without PDT will serve as the control. The experiments will be performed in triplicate.

Phagocytosis will be evaluated using heat-killed *S. aureus* labeled with propidium iodide (PI) at 5%, 2.4×10^9 cells/mL. To do this, a mixture of 100 µL of PDT-treated or non-treated rat venous blood, 100 µL of PI-labeled *S. aureus*, and 800 µL of PBS will be prepared in a plastic tube. The tube will be incubated in a shaking water bath at 37 °C for 30 min, and then 2 mL of ethylene diamine tetraacetic acid (EDTA) will be added to terminate phagocytosis. Leukocytes will be isolated and resuspended in 1.0 mL of 3 mM EDTA in PBS. PI-labeled *S. aureus* fluorescence of leucocytes will be studied by flow cytometry [30]. Phagocytosis will be determined by assessing the percentage of cells staining positive for PI.

To assess the **leucocytes ROS production**, 100 µL PDT-treated or non-treated rat venous blood will be mixed with 100 µL phorbol-myristate acetate (PMA, 2 µg/mL) in PBS to activate leucocytes or with 100 µL PBS for measurement of the basal production of ROS by resting cells. The tubes will then be incubated for 30 min at 37 °C. Afterwards, 2 µg of dihydrohodamine 123 (DHR) will be added and the incubation will be continued for additional 30 min. The cells will be chilled in ice to stop reaction. Leukocytes will be isolated and prepared for flow cytometry to measure intracellular DHR fluorescence of leucocytes [30]. Histograms of the fluorescence intensity will be constructed for each tube and the geometric mean of the fluorescence intensity of DHR in that population of cells will be determined.

D.2.3. Histological evaluation of leukocytes in the infected wounds before and after PDT *in vivo*. We will use superficial incisional SSIs in mice and deep incisional SSIs in rats infected with *S. aureus* as the representative infection types in this section. For each infection type, rodents will be sacrificed immediately after PDT (n=7) or at 24 h after PDT (n=7). In addition, another group of 7 rodents without PDT will serve as

non-treated control. Tissue of infected lesion will be extracted and processed for H&E staining. Accumulation of leukocytes in the infected sites and morphological changes of leukocytes will be evaluated microscopically.

D.2.4. Statistical analysis. All data will be taken with three replicates for *in vitro* studies and 7 replicates for *in vivo* animal studies, respectively, for each independent experiment. The results will be presented as mean values and standard deviation. Significant differences in experiments will be calculated using a Student's *t*-test. *P*-values of < 0.05 are considered significant.

D.3. Timeline for completion of the proposed study (Table 1)

Table 1. Timeline for completion of the proposed study

Aims/Tasks	Month											
	1	2	3	4	5	6	7	8	9	10	11	12
Aim 1. Prophylactic and therapeutic efficacies of PDT for SSIs in rodents	×	×	×	×	×	×	×	×	×	×	×	×
1) PDT for superficial incisional SSIs in mice	×	×	×	×	×							
2) PDT for deep incisional SSIs in rats					×	×	×	×	×			
3) Gentamicin treatment of SSIs									×	×	×	×
Aim 2. Effects of PDT on leukocytes				×	×	×	×	×	×	×	×	×
1) Effect of PDT on the viability of leukocytes *in vitro*				×	×							
2) Effect of PDT on the phagocytosis and ROS production of leukocytes *in vitro*						×	×	×				
3) Histological evaluation of leukocytes before and after PDT *in vivo*									×	×	×	×

D.4. Vertebrate animals

Based on our prior work involving rodent infection models, a power analysis is used to compute the number of rodents to be used for analysis of variance. We have calculated that 7 rodents per group will provide a power of at least 0.8 with α=0.05 to correctly determine a 10% difference between different groups.

Male BALB/C mice and Sprague-Dawley rats will be used for the studies of superficial incisional SSIs and deep incisional SSIs, respectively. Totally, 273 mice and 273 rats will be used over the whole period of study:

- Section D.1.3: 105 mice and 105 rats (15 groups of 7 mice/rats involving 3 bacterial species and 5 inocula);
- Section D.1.5: 84 mice and 84 rats (9 groups of 7 mice/rats involving 3 bacterial species and 3 different PS addition time, and 3 groups 7 mice/rats involving 3 bacterial species without treatment);
- Section D.1.6: 63 mice and 63 rats (9 groups of 7 mice/rats involving 3 bacterial species and 3 different application time points of gentamicin);
- Section D.2.3: 21 mice and 21 rats (2 groups of 7 mice/rats involving two animal scarification time points, and one group of 7 mice/rats as non-treated controls).

The animal care facilities and programs of the Massachusetts General Hospital (MGH) meet the requirements of the law and NIH regulations. Animal Care Protocols have been approved for these studies: Protocol # 2006N000096 MGH (for studies using mice) and Protocol # 2006N000220 MGH (for studies using rats).

E) REFERENCES. (Not to exceed two pages)

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SALARIES AND WAGES (List all personnel for whom money is requested)	% Of Time on this project	Requested from OTA Funds (Omit Cents)
	%	$
	%	
	%	
	%	
Fringe Benefits _____% of Salaries and Wages Salaries and Wages plus Fringe Benefits	TOTAL	

PERMANENT EQUIPMENT (Justification to be appended)	
Subtotal	

CONSUMABLE SUPPLIES (Exclude animals and animal care)	
Chemical and reagents	$6,008
Disposable and plastic ware	$3,834
Subtotal	$9,842

ANIMALS AND ANIMAL CARE	
273 Female BALB/C mice @$18.15 each; 273 female Sprague-Dawley rat @$20.40 each.	$10,620
Animal housing for 14 days (273 mice at 5 mice/cage=55 cages @$1.13/cage/day, 273 rats at 2 rats/cages=137 cages@1.13/cage/day)	$3,038
Subtotal	$13,658

ALL OTHER EXPENSES	
Domestic travel for the PI to attend a scientific meeting to present results	$1,500
Subtotal	$1,500

TOTAL DIRECT COSTS $25,000