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bio-protocol www.bio-protocol.org/e4302 Bio-protocol 12(02): e4302. DOI:10.21769/BioProtoc.4302

Rapid *in vitro* and *in vivo* Evaluation of Antimicrobial Formulations Using Bioluminescent Pathogenic Bacteria

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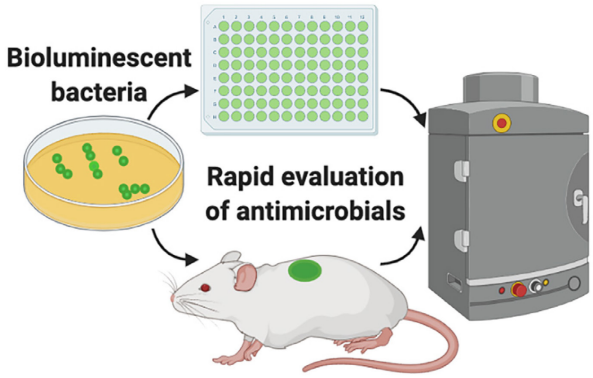
[Abstract] Basic and translational research needs rapid methods to test antimicrobial formulations. Bioluminescent bacteria and advanced imaging systems capable of acquiring bioluminescence enable us to quickly and longitudinally evaluate the efficacy of antimicrobials. Conventional approaches, such as radial diffusion and viable count assays, are time-consuming and do not allow for longitudinal analysis. Bioluminescence imaging is sensitive and gives vital spatial and temporal information on the infection status in the body. Here, using bioluminescent *Pseudomonas aeruginosa*, we describe an *in vitro* and an *in vivo* approach to rapidly evaluate the antimicrobial efficacy of the host-defense peptide TCP-25.

Graphic abstract:



Evaluation of antimicrobials using bioluminescent bacteria.

Keywords: *In vivo* imaging, *In vivo* models, Antimicrobials, Bioluminescent, Infection

[Background] Both in basic research and in the pharmaceutical industry, there is a high demand for *in vitro* and *in vivo* models that can provide quick and reproducible results when testing antimicrobial formulations. Availability of bioluminescent bacteria and advanced imaging systems capable of acquiring bioluminescence originating from *in vitro* experiments or experimental animal models enable us to

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quickly and longitudinally evaluate the efficacy of antimicrobials. Conventional *in vitro* approaches, among many others, include radial diffusion and viable count assays (Balouiri *et al*., 2016). For *in vivo* evaluation, CFU (colony-forming unit) enumeration of the animal tissue is the standard practice. Although all these approaches have their own advantages, they are time-consuming as microbiological culturing protocols require 24 to 72 h before results are known. In particular, *in vivo* evaluation requires that animals be sacrificed for each experimental time point, which renders this process laborious and longitudinal analysis unattainable.

Bioluminescence imaging does not require an external excitation light source. Therefore, for *in vivo* applications, it is preferred over fluorescence imaging, which often generates a high background, as tissues in the body produce autofluorescence upon excitation with an external source (Choy *et al.*, 2003).

The additional significant advantage is that *in vivo* bioluminescence imaging gives vital information on spatial distribution of the infection throughout the body (Karimi *et al*., 2016). Infection development from the original inoculation site can be tracked and its spread can be visualized and quantified. If required, this method can also be combined with fluorescently labeled drugs, so that signals from bioluminescent bacteria and fluorescent drugs can be acquired simultaneously and co-registered, to study their interactions (van Oosten *et al*., 2013; Puthia *et al*., 2020). This method can easily be adapted for different antimicrobial products such as gels, aqueous solutions, dressings, and implants. We have successfully used bioluminescent versions of the clinically important pathogenic bacteria *Staphylococcus aureus* and *Pseudomonas aeruginosa* for the initial *in vitro* screening of antimicrobial formulations (Puthia *et al*., 2020). Employing IVIS imaging, these bioluminescent bacteria were further used by us to evaluate the *in vivo* efficacy of an antimicrobial formulation in a mouse model of infection using a subcutaneous implant (Strömdahl *et al*., 2021).

Here, using bioluminescent *P. aeruginosa*, we describe an *in vitro* and an *in vivo* approach to rapidly evaluate the antimicrobial efficacy of the host-defense peptide TCP-25 (thrombin-derived C-terminal peptide). TCP-25 is a thrombin-derived C-terminal peptide (TCP-25, GKYGFYTHVFRLKKWIQKVIDQFGE) that shows strong antimicrobial (Puthia *et al*., 2020) and antiendotoxic properties (Saravanan *et al*., 2018). *In vivo*, TCP-25 protects against *P. aeruginosa*-induced sepsis and LPS-mediated shock (Papareddy *et al*., 2010; Kalle *et al*., 2012). Bioluminescent *P. aeruginosa (P. aeruginosa* Xen41) is derived from the isolate PAO1 and contains the *luxCDABE* operon of *Photorhabdus luminescens,* which is constitutively expressed (Winson *et al*., 1998; Dusane *et al*., 2017). Using bioluminescence imaging, we performed longitudinal analysis in both *in vitro* and *in vivo* assays, to obtain rapid and valuable results.

Materials and Reagents

1. Bacterial inoculation loop (Copan, catalog number: 8177CS20H)
2. Bacterial culture tube, Screw cap, 12 mL (Sarstedt, catalog number: 60.9922.937)
3. Clear flat bottom 96 well microplate (Greiner Bio-One, catalog number: 655101)
4. Mepilex® Transfer (Mölnlycke Heath Care, catalog number: 294800)

5. Biopsy punch, 6 mm (Kai, catalog number: BP-60F)
6. Petri dish (Sigma-Aldrich, catalog number: P5981)
7. Alcohol wipes (Cutisoft wipes, BSN Medical, catalog number: 204364)
8. Gauze swab (SELEFA, ONRMED, Finlan, catalog number: 222002)
9. BALB/c mice, 8-12 weeks old, male or female (JANVIER LABS, France, BALB/cJRj)
10. *P. aeruginosa* Xen41 (PerkinElmer, catalog number: 119229)
11. BD Bacto Todd Hewitt Broth (Fisher Scientific, catalog number: 249210)
12. TCP-25 (GKYGFYTHVFRLKKWIQKVIDQFGE)

    As an antimicrobial drug, we used the thrombin-derived peptide TCP-25 (synthesized by Biopeptide, San Diego, CA, USA).
13. VICRYL suture (Johnson & Johnson, Belgium, catalog number: V452H)
14. Endotoxin free water (Sigma, catalog number: TMS-011-A)
15. Isoflurane gas (Forane, Baxter, catalog number: CA2L9100)
16. Tris buffer (10 mM, pH 7.4)
17. Ketamine (Ketaminol, Intervet, catalog number: 511519)
18. Xylazine (Rompun vet, BAYER, catalog number: 023572)
19. Depilatory cream (Veet Hair removal Cream, Reckitt Benckiser, catalog number: 5701092103888)
20. TCP-25 solution (see Recipes)
21. TH broth (see Recipes)

Equipment

1. Surgical scissors (Aesculap, catalog number: BC162R)
2. Tweezers (Aesculap, catalog number: BD217R)
3. Shaking incubator (Innova 42, Fisher Scientific, catalog number: 11330025)
4. In vivo imaging system (IVIS spectrum, PerkinElmer, catalog number: 124262)
5. Cordless hair clipper (Aesculap, catalog number: GT416)
6. Spectrophotometer (Genesys 20, Thermo Scientific)
7. Centrifuge (Sigma 1-6 compact centrifuge, Sigma)

Software

1. Living Image 4.5.5 Software (PerkinElmer)
2. Prism, version 8.3.0 (GraphPad Software, LLC.)

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Procedure

A. Preparation of bacteria for *in vitro* and *in vivo* experiments

1. Using a bacterial inoculation loop, pick a colony of *P. aeruginosa* Xen41 from a stock culture plate and inoculate it in 5 mL of TH broth held in a bacterial culture tube. Keep the cap of the tube loose and incubate in a shaking incubator at 37°C overnight.
2. The next morning, to refresh the culture, take 100 µL from the overnight culture and add to a new bacterial culture tube with 5 mL of TH broth. Keep the cap of the tube loose and incubate in a shaking incubator at 37°C.
3. After 1 h of incubation, take the tube and measure OD at 620 nm in a spectrophotometer. The aim is to achieve an OD of 0.4 so, if its less, place the tube back in the incubator and measure the OD after every 15 min, until it reaches 0.4. Then, take the tube and prepare for washing.
   Note: The use of a spectrophotometer that accepts 10-15 mL culture tubes helps, as OD can be measured without taking a separate sample.
4. Centrifuge for 10 min (3,000 × *g*, room temperature) and remove the supernatant. Add 500 µL of Tris buffer (10 mM Tris, pH 7.4) to the pellet and dissolve by mixing with a pipette. Centrifuge again for 10 min (3,000 × *g*, room temperature). Remove the supernatant, add 300 µL of Tris buffer to the pellet, and mix with a pipette to obtain a ~10^9 CFU/mL bacterial suspension.
   Note: For each bacterial strain, the OD vs. CFU relationship may be different. Thus, growth curves should be prepared for each bacterial strain.

B. *In vitro* assay to determine antimicrobial efficacy

Figure 1 illustrates the experimental set-up and workflow of the *in vitro* assay.

Note: We use the IVIS imaging system for data acquisition of the in vitro assay with bioluminescent bacteria in a 96-well plate format. Any other luminometer can be used for the plate format assay. The added advantage of the IVIS imaging is that it can also produce a heat map image of each well representing the total flux emitted by the bacteria.

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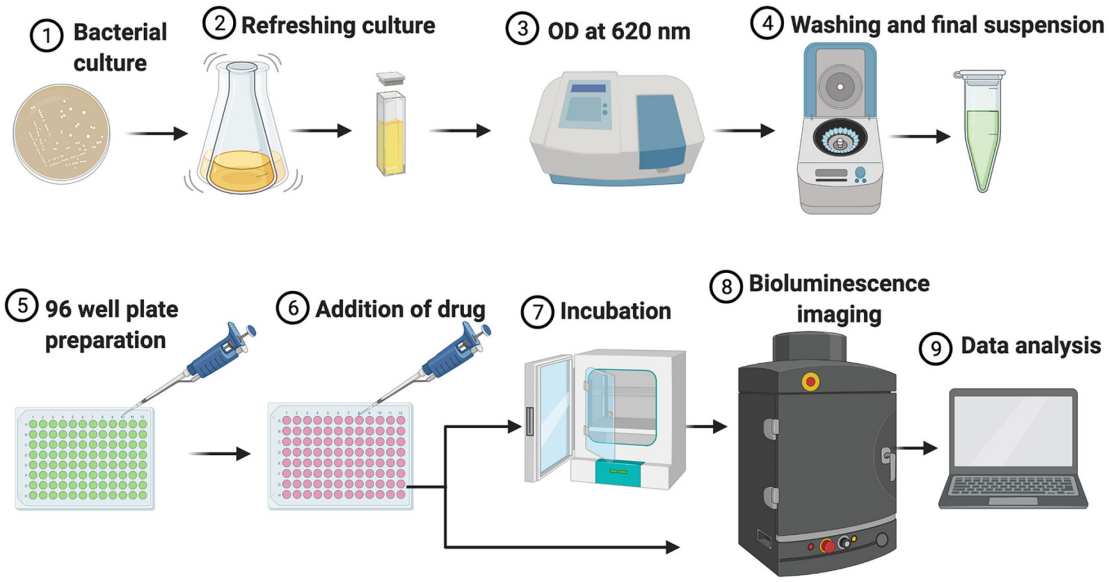

Figure 1. Illustration of the experimental set-up and workflow for the *in vitro* assay.

1. From the bacterial suspension obtained in Step A4, prepare a suspension of 2 × 10^8 cfu/mL in Tris buffer.
2. Add 50 µL (*i.e.*, 10^7 CFU/mL) of the suspension to each well of a clear 96-well plate.
   Note: Use duplicates or triplicates for each condition according to the experimental plan.
3. Add 250 µL of TCP-25 solution prepared in Tris buffer. Here, to study dose response, we use 5 µM, 10 µM, 20 µM, 50 µM, and 100 µM final concentrations of TCP-25. For controls, add 250 µL of Tris buffer. Mix with a pipette gently to avoid bubble formation.
   Note: At this step, in place of the TCP-25 solution, 100 to 250 µL of any other test formulation can be added. If a drug is solubilized in a different buffer than Tris, include the relevant buffer control group in the experimental plan. Use a multichannel pipettor to add the formulation, as this will allow for quick data acquisition to study rapid antimicrobial activity of the drug.
4. Transfer the plate to the IVIS imaging system. Keep plate aligned with the horizontal 'field of view' lines on the stage.
   Note: Read the IVIS spectrum user manual before using the IVIS. Start the Living Image software at least 15-20 min prior to imaging and initialize it. The initialization process brings the camera temperature down to -90°C. Stage heating should be set to 37°C.
5. Set parameters in the IVIS acquisition control panel. Select luminescent (imaging mode), auto (exposure), and C (field of view). Click 'Acquire' to start imaging. Immediately after imaging, keep the plate in the incubator (37°C). For kinetic analysis, the plate can be left on the stage too.
   Note: We do imaging for 2, 5, 15, 30, 60, and 120 min. As most buffers do not support bacterial growth, additional supplements or growth mediums should be tested and used for experiments requiring longer incubation periods.
6. Move to data analysis.

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C. Preparation of PU discs for subcutaneous implantation

Figure 2 illustrates the experimental set-up and workflow of the *in vivo* assay.

Note: Perform this step in a class II biosafety cabinet or use sterile conditions. Discs can be coated with other peptides or drugs as well. Optimize and test the coating method for each compound.

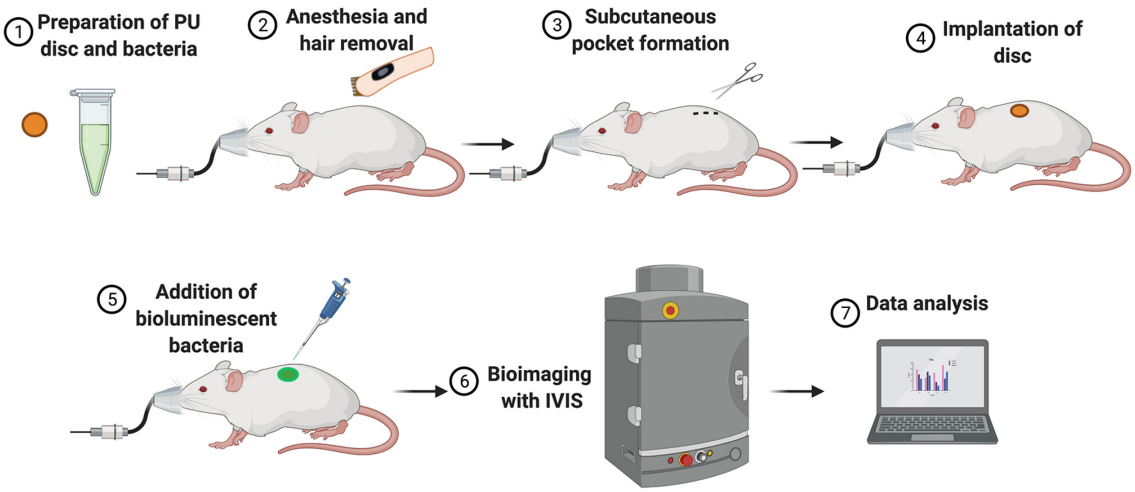

Figure 2. Illustration of the experimental set-up and workflow for the *in vivo* assay.

1. Remove the protective plastic coverings from the polyurethane (PU) dressing (Mepilex Transfer). Cut PU discs of 6 mm in diameter with a biopsy punch.
2. Solubilize the TCP-25 in sterile water to prepare stock solutions (5.0 and 0.5 mg/mL). Use 20 µL of each stock solution to prepare discs with 100 µg and 10 µg TCP-25, respectively.
3. Keep PU discs in a petri dish and add 20 µL of the stock solution to each PU disc. Add only 20 µL of sterile water to the control discs. Leave the petri dish in a biosafety cabinet and air dry them at room temperature (20°C ± 1°C) for 12 h.
4. After coating, store discs immediately at -80°C in a moisture free box.

D. Subcutaneous implantation of PU disc in mice

Note: Disc implantation can be performed under general anesthesia either via parenteral (e.g., ketamine and xylazine cocktail) or inhalation routes (e.g., isoflurane gas). Since this procedure requires a short time (3-5 min), anesthesia with isoflurane is preferable and safer for the animal. Anesthesia with isoflurane requires an anesthesia system capable of delivering isoflurane to the mouse through a mouse nose cone.

1. In an induction chamber, anesthetize BALB/c mice (8-10 weeks old) with a mixture of 4% isoflurane and oxygen (flow rate: 1.5 L/min). Once anesthetized, remove the mice from the induction chamber and place on the table. Immediately adjust the nose cone to the mouse and maintain anesthesia with 2% isoflurane and oxygen mixture.

Note: Induction of anesthesia can be achieved in the induction chamber of the IVIS or in any other isoflurane-oxygen delivery system. BALB/c mice are preferred for bioluminescence imaging as dark pigments interfere with emitted signals. If unavoidable, C57BL/6 or mice with darker coat color can be used with optimized protocols. Isoflurane is toxic and its use requires following standard safety protocols for your facility.

2. Using hair clippers, shave hair from the back of the mouse, in an area slightly below the interscapular region. Clean the skin with an alcohol wipe and dry it with sterile gauze.
   Note: Fur removal is necessary, as mouse hair can interfere with luminescence signals during imaging. Hair removal can also be achieved with depilatory creams. Depilatory creams should be used at least 2-3 days prior to the disc implantation. If using mice with darker coat color, hair removal with depilatory creams gives better results.
3. Using scissors, under aseptic conditions, make a 5-10 mm long cut on the skin of the mouse's back. Use the tip of the scissors to create a small subcutaneous pocket under the skin. With the help of a tweezer, insert a PU disc in the subcutaneous pocket.
4. Grasp the wound edge with a tweezer and, with the help of a pipettor, add 50 µL of *P. aeruginosa* Xen41 bacterial suspension (1×10^7 CFU) to the PU disc. Close the skin wound with two simple interrupted sutures using Vicryl.
   Note: In our experience, an inoculum of 1×10^6 to 1×10^7 CFU of P. aeruginosa Xen41 gives a good detectable signal during imaging. Factors such as luminescent bacterial strain, mice strain, and mice coat color may affect the signal intensity and, therefore, inoculum size should be optimized in pilot experiments. Skin wounds can also be closed with mouse skin staples or with tissue glue. Use a fresh bacterial suspension. After preparation of the bacterial suspension, keep it on ice for a maximum of 1-2 h.
5. Return the mice to the cages.
   Note: If using general anesthesia or if the procedure takes longer, a heating pad underneath the mice should be used to maintain their body temperature.

E. *In vivo* imaging with IVIS

Note: Read the IVIS spectrum user manual before using the IVIS. Start the Living Image software at least 15-20 min prior to imaging and initialize it. The initialization process brings the camera temperature down to -90°C. Stage heating should be set to 37°C.

1. In the induction chamber, anesthetize mice using 4% isoflurane-mixed oxygen.
   Note: Read the IVIS spectrum user manual before using the IVIS.
2. Transfer the mice to the IVIS imaging chamber. Position them in a prone position and ensure that their noses are inside the nose cones. Immediately start 2% isoflurane supply to the chamber to maintain anesthesia.
   Note: A maximum of five mice can be imaged at once. Unless imaging one mouse at a time, including mice from different experimental groups in a single imaging snapshot is appropriate.

3. Select appropriate parameters for imaging. On the IVIS acquisition control panel, set luminescent (imaging mode), auto (exposure), and D (field of view for 5 mice), or C (field of view for 3 mice).
   Note: The 'imaging wizard' option can be used to change imaging mode or other parameters.
4. Click 'acquire' in the IVIS acquisition control panel to start imaging.
   Note: Increase exposure time to acquire weak bioluminescent signals. In vivo imaging time points should be determined according to the need of the project. In this model, we usually image 15 min, 3, 6, 24, and 48 h after disc implantation. Frequency of imaging should be kept to the minimum, as anesthesia has adverse effect on the health of mice.

Data analysis

Note: For both in vitro and in vivo experiments, data acquired by IVIS is analyzed and quantified using the Living Image Software. Data acquired from IVIS should be saved for subsequent analysis. Depending on the Living Image Software availability, data can be analyzed later in another computer.

1. Start the Living Image program and open the saved file. Each image, along with its metadata is saved in an individual folder. Open the raw (.txt file) to see an overlay image which is generated from a photographic image and a bioluminescence image. From the tool palette, select a region of interest (ROI) on each well of the plate, for the *in vitro* experiment, and on each mouse, for the *in vivo* experiment. The ROI is in an area where luminescent signals are observed or expected. Pick a circle ROI for each well of the 96 well plate. For mice, we chose a circle ROI covering the implanted disc area, but larger than the implanted disc diameter. More than one ROI can be selected in one image. The shape and size of the ROI from one area can be copied and applied to other areas or images.
2. To obtain the bioluminescence data, select radiance (photons) as units on the main image view and in the ROI measurements window. Click measure ROIs and the measurement data will appear in a new window.
3. Copy raw data to GraphPad Prism or Excel for statistical analysis. Figure 3 shows results from bioluminescence imaging of a microplate. Figure 4 shows results from *in vivo* bioluminescence imaging.
4. Data are presented as means ± SEM or SD. Compare the means between two groups with a Mann-Whitney test. Compare means between more than two groups with a Kruskal-Wallis test. Depending on the experimental plan, other appropriate statistical methods can also be used.
   Note: Read the Living Image Software User's Manual for details on ROI, data measurement, and data display.

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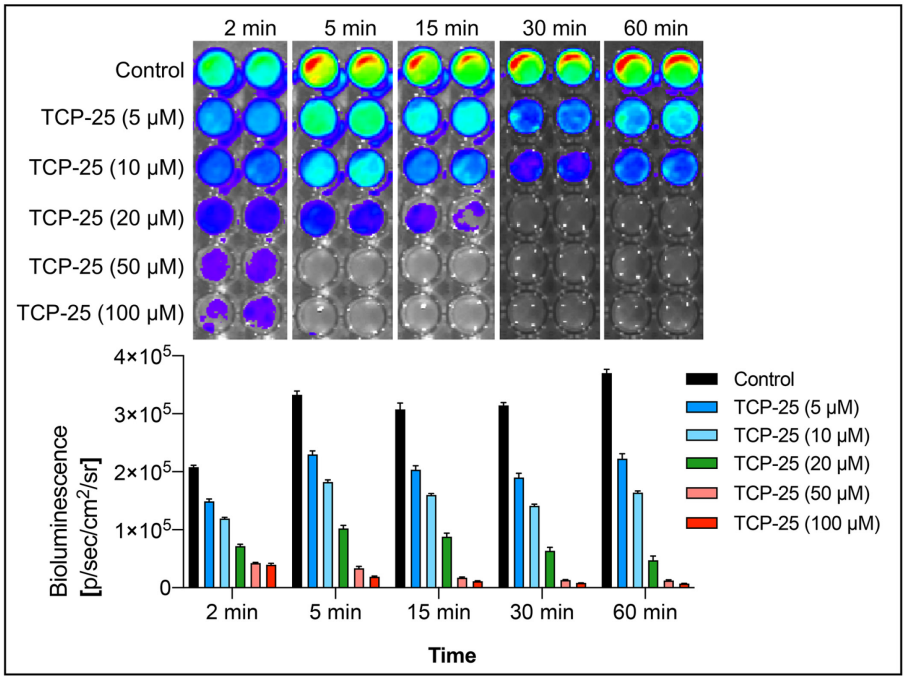

Figure 3. *In vitro* evaluation of antimicrobial efficacy of TCP-25.

In a microplate, bioluminescent *P. aeruginosa* Xen41 was incubated with increasing concentrations of TCP-25 for various time points and the bioluminescence emission imaged using IVIS. Representative light emission intensity heat-map shows the total flux emitted by the bacteria in each well. The bar chart shows quantification of the bioluminescence intensity emitted by the bacteria.

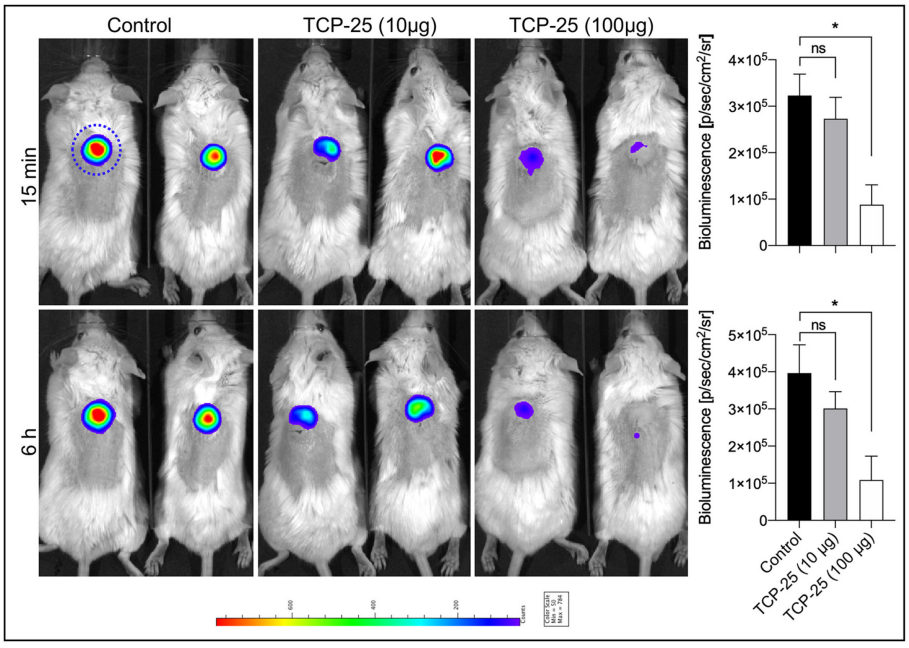

Figure 4. *In vivo* evaluation of TCP-25 coated PU disc in a mouse model of subcutaneous implant infection.

TCP-25 coated PU discs were subcutaneously implanted and infected with bioluminescent *P. aeruginosa* Xen41. Intensity of the emitted bioluminescence was visualized using IVIS. Representative light emission intensity heat-map overlays are shown for time points 15 min and 6 h. The blue dotted line denotes the region of interest for the quantification. The bar chart shows quantification of the bioluminescence intensity emitted by bacteria. Data are presented as the mean ± SEM (n = 4). P values were determined using a Kruskal-Wallis test followed by Dunn's post test. *$P \leq 0.05$; ns, not-significant.

Notes

Experiments described here using bioluminescent bacteria are reproducible. Sometimes, there can be issues with low luminescent emission from the bacteria. The experimenter should check if the bacterial culture is maintained in a selective media. In addition, results might vary with different strains of mice, thu the *in vivo* assay should be optimized in pilot experiments with new mice strains.

Recipes

1. TH broth
   a. Dissolve 30 g of BD Bacto Todd Hewitt Broth powder in 1 L of purified water.
   b. Autoclave at 121°C for 15 min.
   c. Cool to room temperature before use.
2. TCP-25 solution
   a. Add required amount of TCP-25 in an Eppendorf tube.
      Note: We usually make 1 mg/mL or 5 mg/mL fresh stock solutions.
   b. Add Tris buffer (10 mM, pH 7.4).
   c. Vortex the solution briefly.
   d. Centrifuge for 1 min (2,575 × *g*, room temperature) to remove air bubbles.

Acknowledgments

We thank Drs. Kristina Hamberg and Sofia Almqvist, Mölnlycke Health Care AB (Göteborg, Sweden) for providing the Mepilex PU material. The authors thank Ann-Charlotte Strömdahl for excellent technical assistance We acknowledge BioRender's assistance in creating cartoons. We have received grants from the Swedish Research Council (project 2017-02341, 2020-02016), Edvard Welanders Stiftelse and Finsenstiftelsen (Hudfonden), the Torsten Söderberg, Crafoord, and Österlund Foundations, Stiftelsen Lars Hiertas Minne, the Royal Physiographic Society of Lund, and the Swedish Government Funds for Clinical Research (ALF). This protocol is derived from the original research paper (Strömdahl *et al.*, 2021).

This protocol is adapted from our previous work (Puthia *et al.*, 2020; doi:

10.1126/scitranslmed.aax6601).

Competing interests

Dr. Schmidtchen is a founder of in2cure AB, a company developing anti-inflammatory peptides for therapeutic applications.

Ethics

Animal experiments were performed according to Swedish Animal Welfare Act SFS 1988:534 and were approved by the Animal Ethics Committee of Malmö/Lund, Sweden.

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