Study of *in vivo* catheter biofilm infections using pediatric central venous catheter implanted in rat

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Venous access catheters used in clinics are prone to biofilm contamination, contributing to chronic and nosocomial infections. Although several animal models for studying device-associated biofilms were previously described, only a few detailed protocols are currently available. Here we provide a protocol using totally implantable venous access ports (TIVAPs) implanted in rats. This model recapitulates all phenomena observed in the clinic, and it allows bacterial biofilm development and physiology to be studied. After TIVAP implantation and inoculation with luminescent pathogens, *in vivo* biofilm formation can be monitored *in situ*, and biofilm biomass can be recovered from contaminated TIVAP and organs. We used this protocol to study host responses to biofilm infection, to evaluate preventive and curative antibiofilm strategies and to study fundamental biofilm properties. For this procedure, one should expect ~3 h of hands-on time, including the implantation in one rat followed by *in situ* luminescence monitoring and bacterial load estimation.

INTRODUCTION

Medical devices such as peripheral or central venous catheters (CVCs), urinary catheters, or prostheses are nowadays essential to modern medicine, and they greatly improve patients' healthcare. However, these devices are prone to contamination by microbial pathogens, leading to biofilm formation and biofilm-related infections that are extremely difficult to eradicate owing to the high tolerance of biofilms to antibiotics and host immune defenses[1]. Currently, there is no efficient method for early biofilm detection, prevention or eradication besides traumatic and costly removal or replacement of contaminated devices[2–5]. Therefore, there is a dire need for specific and timely detection of biofilm formation on medical devices. However, the development of new strategies aiming at the prevention or eradication of chronic and nosocomial biofilm infections requires relevant biofilm models and approaches to provide a better understanding of the dynamics of biofilm formation and physiology.

Device-associated biofilm animal models

A large number of *in vitro* models have been used to study biofilm formation and physiology or for large-scale anti-biofilm drug screening[6–11]. Although they are useful, these *in vitro* models do not reproduce the complex interactions occurring in the device-associated infection milieu comprising microorganisms, the host and the contaminated abiotic surface. Alternatively, animal models of medical device–associated infections provide better access to important parameters when characterizing pathogenic biofilms, host factors and other biotic signals. Early foreign body infection models used diverse animals that were subcutaneously implanted with different devices such as tissue cages, beads, pacing devices, cement or polyethylene disks, fabric to mimic cardiac valves, surgical mesh, vascular grafts and vascular catheters (see, e.g., Van Wijngaerden *et al.*[12] and Lebeaux *et al.*[10] for reviews). Although animal models with subcutaneously implanted foreign bodies provided insights into host- and microorganism-associated parameters, they often required precolonization of the devices. In clinical settings, the contaminations associated with the use of implants often originate from an external source, thus limiting the clinical relevance of these models. Several subcutaneously implanted mouse CVC biofilm models were described using luminescent variants of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Candida albicans* for real-time monitoring of biofilm formation[13–16] and dissemination[17]. While providing useful information, these catheter models are not connected to blood circulation, and therefore they lack exposure to key host factors, such as venous blood circulation or humoral factors.

In the late 1990s, a few CVC models designed to study bacterial biofilm formation *in vivo* were developed. One of the first of these used a vascular catheter inserted into the jugular vein of a rat and was developed to study *S. aureus* and *S. epidermidis* biofilm-related infections and to evaluate the efficacy of several antimicrobial agents, including antibiotics and antimicrobial peptides[7,10,11,18–21]. Other CVC models using different hosts include a mouse model catheterized in the jugular vein used to study the role of alternative SigB in *S. aureus* virulence[22], or a precatheterized (jugular vein) mouse model to study the eradication efficacy of lysostaphin against *S. aureus* biofilm[23]. In rabbits, port access venous catheters inserted in the jugular vein were used to test several antibiotics against *S. aureus*[24]. Finally, several animal models were also developed to study *C. albicans* biofilm-related infections, which is an important causative agent of CVC-related infections[25–28]. All these studies technically required restraint jackets for animals and required animal killing in order to assess and to quantify colonization and infection. Moreover, many of these animal models were developed to study short-term biofilm infections.

Protocol development and optimization

To better understand device-associated biofilm infections, our laboratory has optimized a long-term CVC model using commercial pediatric TIVAPs inserted in rat jugular veins[29–31]. Unlike other venous catheters used in other *in vivo* studies, TIVAPs are closed devices that are accessible through a port implanted subcutaneously and connected to the central venous system via a

catheter usually inserted into the jugular or subclavian vein. In our model, the TIVAP is subcutaneously implanted with the port secured in a pocket, at the dorsal midline toward the end of thoracic vertebrae. To reduce host sacrifice and to allow real-time monitoring of biofilm formation on the TIVAP and associated pathologies in the living host, we use bioluminescent variants of clinically relevant bacteria forming biofilms; these biofilms are formed on biomaterials that are introduced into the port by puncturing the silicone septum using Huber needles. Next, bacterial colonization and biofilm formation are monitored, without further invasive intervention, as a function of luminescence using a charge-coupled device (CCD) camera. We optimized the inoculum size to be 1×10^4 colony-forming units (c.f.u.) per 50 µl per port for *Escherichia coli* and 1×10^6 c.f.u. per 50 µl for *S. aureus* and *P. aeruginosa*. Increased bioluminescence signals were correlated to higher bacterial titers within TIVAPs. At these dosages, we were able to measure the signals associated with chronic biofilms up to 120 d. This animal model was successfully used to study the colonization of venous catheters by clinically relevant pathogens (*S. aureus*, *S. epidermidis*, *P. aeruginosa* and *E. coli*)[29–33].

Biofilm biomass formed in the TIVAP can be determined quantitatively by collecting the bacteria, as discussed in detail below and plating on suitable agar plates. We obtained counts of viable bacteria ranging from 7.6 to 8.3 log c.f.u./ml (port) and 7.5–9.2 log c.f.u./ml (catheter) in the TIVAP. The bacterial load in the organs (lungs, heart, spleen, kidneys, liver or blood) can also be determined by plating tissue homogenates. To complement microbiological methods assessing biofilm pathogenesis in the rat TIVAP model, we used electron microscopy to visualize biofilm structures growing on the lumen of the catheter and on the silicone septum of the TIVAP. Furthermore, we also optimized cyclophosphamide-based immunosuppression to study the consequences of TIVAP-associated biofilm infection in immunocompromised hosts (**Supplementary Method 1** and **Supplementary Fig. 1**).

Advantages and limitations of the model

This model presents several advantages over other foreign body and CVC *in vivo* models of infection. Total implantation enables the inoculation of the device with clinically relevant bacteria (and potentially fungi) into the lumen through an external route, which is clinically more relevant compared with precolonized, subcutaneously implanted device models or models that use a hematogenous route of infection. In addition, uncontrolled external contaminations are reduced compared with other CVC animal models in which the catheter hub is external. This is of utmost importance when monitoring biofilms for long-term (chronic) infection, in which the probability of uncontrolled contamination from external sources increases. Furthermore, central venous access catheter animal models allow systemic and antibiotic lock curative therapies (described in **Supplementary Method 2**, **Supplementary Table 1** and **Supplementary Fig. 2**), as well as prophylactic strategies, to be assessed. Being totally implanted under the skin, the TIVAP animal model avoids the use of restraint jackets, which are usually used in other CVC animal models. Moreover, the TIVAP animal model allows clinically important phenomena such as localized port pocket infection and hematogenous colonization to be studied (see **Supplementary Methods 3** and **4**). Although bioluminescence was used in several *in vivo* models of foreign body–related infection[34–36], this TIVAP animal model was the first to describe biofilm-associated infections as a function of bioluminescence in a CVC connected to blood circulation[30]. As opposed to a recent short-term CVC-associated biofilm mouse model for evaluating the efficacy of lysostaphin alone or in combination with oxacillin against bioluminescent *S. aureus* biofilm[37], our model allows the dynamics of *in vivo* biofilm progression to be monitored over a long period (model of chronic infection). The use of bioluminescence reduces the number of used animals. We showed that our TIVAP rat model recapitulates all phenomena observed in clinical TIVAP-associated biofilm infections, and that it can also be used to study the fundamental mechanisms of *in vivo* biofilm formation.

This model also presents some limitations. Besides being an expensive technique, it requires extensive training to acquire optimal technical expertise. It is also labor-intensive, and it requires everyday monitoring and manipulations to notably avoid thrombosis and clogging of the TIVAP. In addition, the technique is so far restricted to rats or rabbits because of the difficulty of adapting it to mice because of the large size and weight of clinically used TIVAPs.

Relevance and applications of the *in vivo* TIVAP biofilm model from a clinical point of view. Clinical applications of the TIVAP model include the following:

(i) Evaluation of current procedures used to handle TIVAP in clinical settings, including skin disinfection procedures, TIVAP patency maintenance[29] and so on.

(ii) Study of the different routes of TIVAP colonization in addition to classical endo-luminal colonization, including the following: extraluminal colonization of the catheter; extraluminal colonization of the port leading to subcutaneous port pocket infection (which can be assessed as described in **Supplementary Method 3**), a clinical situation in which repeated intradermal needle punctures during access to TIVAP may lead to subcutaneous bacterial port pocket infection[38] (20% of TIVAP-implanted rats inoculated with *P. aeruginosa* or *S. aureus* developed subcutaneous infection around port[30]); catheter tip colonization via a hematogenous route of infection (which can be assessed as described in **Supplementary Method 4**), a situation observed when TIVAP-implanted patients suffer from bloodstream infection: TIVAP-implanted rats injected with *S. aureus* in the tail vein showed colonization of TIVAP catheter tip[30]. Furthermore, we observed, in our TIVAP rat model, chronic colonization of implanted devices with occasional catheter-related bloodstream infections as detected in CVC-bearing patients[30]. Thus, our rat TIVAP model successfully reproduced clinical situations such as biofilm-related bloodstream infections, organ colonization and port-pocket infections.

(iii) Investigation of controlled chronic infection and recurrence of infection after conservative treatment.

(iv) Development of early biofilm detection methods ('biofilm biomarkers'). Such biomarkers are currently lacking, and their identification could strongly facilitate medical decisions.

(v) Evaluation of prophylactic strategies including novel catheter lock solutions; the catheter lock therapy corresponds to the use of highly concentrated antimicrobial (often antibiotic)

solutions that dwell inside the catheter for at least 12–24 h (ref. 39). Another strategy is catheter treatment with an anti-adhesive procedure[29]; we modified the surface of commercial TIVAPs composed of silicone and titanium, using methyl cellulose (MeCe) and polyethylene glycol (PEG), two macromolecules with described anti-adhesive activities[40,41]. Using our rat model of biofilm infections inoculated with bioluminescent bacteria, we showed that an anti-adhesive approach could constitute an efficient prophylactic strategy to control infections in medical devices. In addition, anti-biofilm strategies against biofilm-related infection can be used, such as vaccination.

(vi) Evaluation of curative strategies against difficult-to-treat pathogens (*P. aeruginosa*, methicillin-resistant *S. aureus*, possibly *C. albicans*), for which the current clinical recommendation in cases of device-related infection is costly and traumatic removal of the device. These curative strategies include curative catheter lock solutions that are active on already developed infectious biofilms[30,31,33] (**Supplementary Method 2**, **Supplementary Table 1** and **Supplementary Fig. 2**). We used EDTA as adjuvant in combination with gentamicin as a lock solution to eradicate all the bacterial biofilms tested using our rat TIVAP model[31], and L-arginine, a basic amino acid, combined with gentamicin to eradicate *S. aureus* and *E. coli in vivo* biofilms[33]. Additional strategies include novel anti-biofilm molecules and other alternative strategies (ultrasonic waves, or photodynamic or phage therapies).

(vii) Evaluation of anti-thrombotic treatments, as thrombosis is observed using this model[29]. Previous studies showed that biofilm-colonized implanted catheters are at higher risk of causing thrombosis[42]. We used our model to demonstrate the better patency in the TIVAP coated with antiadhesive coatings compared with control untreated TIVAPs[29].

Relevance and applications of the *in vivo* TIVAP biofilm model from a fundamental point of view. Fundamental applications of the TIVAP model include the following:

(i) The study of biofilm physiology *in vivo* and in a clinically relevant model amenable to transcriptomic, proteomic or metabolomic analyses. Biofilm-specific properties such as biofilm-associated production of molecules[32] or biofilm-specific increased recalcitrance to antimicrobial agents and to the immune system can be studied using this model.

(ii) Evaluation of the *in vivo* role of biofilm-promoting factors identified *in vitro*.

(iii) *In vivo* study of clinically relevant biofilm-forming microorganisms other than bacteria, in particular *C. albicans*, which is one of the main causes of catheter-related infection.

(iv) Investigation of the impact of the implanted host immune system on the development of *in vivo* biofilms[30] (**Supplementary Method 1** and **Supplementary Fig. 1**) and, reciprocally, study of the impact of biofilm development on the immune system.

Overview of the procedure stages

The procedure described is designed to study bacterial biofilms using a CVC called TIVAP inserted in rat jugular vein. One animal study takes between 2 and 3 weeks, but it may be longer depending on the question to be addressed; for example, immunosuppression of animals will add another 4 d to a study. A trained engineer, PhD student, post-doctoral fellow or researcher can perform all stages of the procedure. Reporting of our animal experiments follows the ARRIVE guidelines[43].

The study can be broadly divided into the following six stages (see flowchart of the different steps in **Fig. 1**).

- *Presurgical procedures (Steps 1–3)*: after reception of the rats, they are transferred two per cage and housed in the animal facility. They are given painkillers in specialized gels before surgery.
- *Surgical procedure (TIVAP implantation; Steps 4–29)*: the TIVAP is implanted subcutaneously with the catheter inserted into the jugular vein.
- *Postsurgical care (Steps 30–39)*: animals are allowed to recover from surgery before injecting bacteria in the port. During this time of recovery, the patency of TIVAP is maintained by the 'flush and draw' technique to mimic clinical TIVAP use, and the temperature and weight of the animals are regularly monitored.
- *Bacterial challenge (Steps 40–49)*: 4 d post surgery, TIVAP is contaminated via controlled bacterial inoculation through the port by bacteria that are allowed to adhere to the device surface (internal) for a certain duration of time before the inoculum is removed.
- *Postchallenge care and monitoring (Steps 50–52)*: the biofilm is allowed to form on the device for 1, 3–4 or 10 d, or even 120 d, depending on the question to be addressed. Blood sampling is done at day +4 (before inoculation to ensure TIVAP sterility); day +7/+8; and day +14 to analyze the TIVAP-mediated infection. Animals are monitored for the clinical symptoms.
- *Euthanasia and sampling (Steps 53–75)*: on the day of experiment termination, animals are euthanized and the TIVAP and organs are aseptically removed for analysis.

Experimental design

Laboratory facilities. All the described animal work was done in the Institut Pasteur animal facilities, which are accredited by the French Ministry of Agriculture to perform experiments on live rodents (accreditations A75-15 27, issued on 12 November 2004, and A75-15 04, issued on 22 May 2008). Work on animals was performed in compliance with French and European regulations on care and protection of laboratory animals (European Commission directive 2010/63; French law 2013-118, 06 February 2013). The protocols used in this study were approved by the ethics committee of 'Paris Centre et Sud N°59' (reference 2012-0045). All methods are performed in a Biosafety Level 2 facility, containing a class II biosafety cabinet. Aseptically collected samples (TIVAPs, organs or blood) from animals can be processed in the main laboratory inside a class II biosafety cabinet to maintain sterility. The animal room is cleaned with disinfectant every day until the end of a study.

Controls. Proper controls must be used in the study. For each study, three uninfected animals with implanted TIVAPs should be used as biofilm-negative controls. For testing antimicrobials, 1× PBS or another appropriate reagent control must be used to compare the efficacy of the tested compounds.

Anesthesia optimization. Anesthetics are important to sedate rats and to reduce discomfort during surgery, as well as during

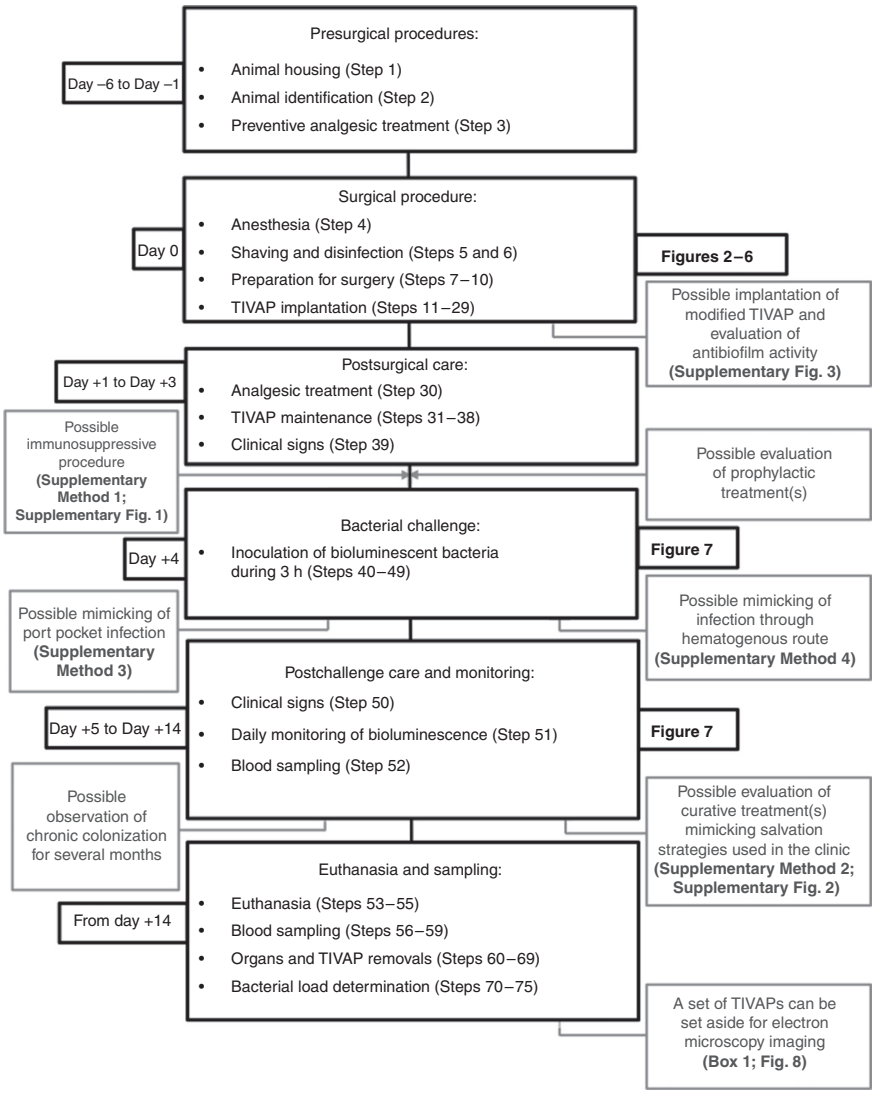

Figure 1 | Schematic representation of the six different major stages of the *in vivo* model of TIVAP-related infection. Steps in black correspond to regular procedures of implantation, contamination and monitoring of colonization (**Figs. 2–7**). Steps in gray correspond to potential supplementary or different procedures that can be applied to the regular model (**Fig. 8**; **Box 1**; **Supplementary Methods 1–4** and **Supplementary Figs. 1–3**).

follow-up procedures. For example, rats treated with cyclophosphamide have different susceptibilities to anesthesia (isoflurane)-induced hypoxia; therefore, empirical determination of chemical anesthesia and/or isoflurane dosages is required. We have optimized a ketamine-xylazine-acepromazine mix (7:2:1 (vol/vol/vol)) that is injected intraperitoneally (500 µl of cocktail per 300 g of rat weight). Acepromazine keeps the rats calm and allows additional injections during the surgery, if needed. Isoflurane concentration for healthy rats was optimized to 3% for the induction of anesthesia and for the maintenance of the anesthetic state at an oxygen flow rate of 0.8 liters/min and air flow rate of 0.4 liters/min to get a total debit of 1.2. Isoflurane concentration for immunosuppressed rats was optimized to 1.5–2.0% for the induction of anesthesia and 1.5% for the maintenance of the anesthetic state at an oxygen flow rate of 0.6 liters/min and air flow rate of 0.4 liters/min to get a total debit of 1.0 liters/min.

Isoflurane is an inhalation anesthetic found under several trade names, including Aerrane, Floran, Florane and Isothane. It provides a quick, easy and long-lasting effect when provided continuously to the research animals. Prolonged exposures in animals need to be regulated and monitored closely to prevent death. Injectable anesthetics, such as ketamine-xylazine-acepromazine mixtures, can be used for long-term (up to 45 min) sedation of rats. Ketamine, xylazine and acepromazine are controlled substances, which require secured storage and proper records of use.

Note that isoflurane is a halogenated ether. It is a colorless liquid anesthetic with a pungent odor. High doses of isoflurane exposure in research animals have been demonstrated to have fetal toxic effects. Pregnant women are recommended not to use it unless other means of anesthesia are not available and, in such a case, special masks need to be worn. Only an approved anesthetic respirator system approved by the ethical committee should be used. Also make sure to work in a well-ventilated area when using isoflurane or any other inhalation anesthetics.

Choice of animals. We chose the Crl:IGS (CD/SD) variant of Wistar rats for our study, as they are recommended by Charles River Laboratories for studying surgical models and infectious diseases. The rats are 250–275 g at reception day and gain ~50 g by the day of surgery. Note: Wistar rats may also be used for the study.

Choice of microbes. Luminescent variants of four clinically relevant pathogens, *E. coli*, *P. aeruginosa*, *S. aureus* and *S. epidermidis*, were used (*S. aureus* Xen36, Xen30, Xen31 and *S. epidermidis* Xen43 from Caliper; *P. aeruginosa* Lm1, a bioluminescent derivative of the PAK clinical strain[44]; and *E. coli* EAEC 55989 (ref. 45), transformed with stable plasmid pAT881)[46]. *E. coli* and *P. aeruginosa* strains were grown in LB medium; *S. aureus* Xen36 and *S. epidermidis* Xen43 were cultured in tryptic soy broth supplemented with 0.2% (wt/vol) glucose at 37 °C. Other relevant bacteria may also be studied within this model. Although bioluminescence is an efficient tool for following *in situ* colonization of the device and potential dissemination, it is also possible to estimate end point bacterial load using nonbioluminescent microbial variants.

Optimization of inoculum size and volume. The inoculum dose was optimized to 10^4 cells for *E. coli*, and 10^6 cells for *P. aeruginosa* and *S. aureus*. For *S. epidermidis*, the maximum dose that could be used was 10^8 cells, but it did not lead to bioluminescent-detectable

colonization. Currently, no other bioluminescent strain of *S. epidermidis* is available[16]. The volume of the inoculum was optimized to 50 µl per TIVAP. The dead volume of the port is 250 µl. This ensures that no bacteria are flushed into the bloodstream at the time of inoculation.

Antibacterial agents. The described methods were successfully used in our model to test the antibiofilm activity of cefazolin, gentamicin, EDTA, ethanol (70% vol/vol) and L-arginine, which were injected alone or in certain combinations in catheter lock solutions[30,31,33]. Moreover, our model was successfully used to evaluate the antiadhesion characteristics of biomimetic glycocalyx-like polymers such as methylcellulose[29]. Empirically, the effect of any relevant antimicrobial agents or antiadhesive molecules can be evaluated using our model. *In vitro* concentration of the antimicrobial agents and the procedures for grafting antiadhesive molecules on silicone and/or titanium need to be verified before *in vivo* evaluation in the rat TIVAP model.

MATERIALS

REAGENTS

- Rats. We have used CD/SD (IGS:Crl) male rats (Charles River) weighing 275–300 g **! CAUTION** Any experiments involving live rats must conform to relevant institutional and governmental regulations. This protocol, as described, is in compliance with French and European regulations on care and protection of laboratory animals (European Commission directive 2010/63; French law 2013-118, 6 February 2013). The protocols used in this study were approved by the ethics committee of 'Paris Centre et Sud N°59' (reference 2012-0045).
- Sterile 1× PBS (cat. no. 10010-023, Gibco)
- Sterile water (cat. no. 15230-07, Gibco)
- Kanamycin (cat. no. B5264-1G, Sigma-Aldrich)
- Cefazolin (Sigma-Aldrich, cat. no. C5020-500MG)
- Vancomycin (Sigma-Aldrich, cat. no. 94747-5G)
- Gentamicin (Sigma-Aldrich, cat. no. G1914-5G)
- Lysogeny broth (LB medium (Invitrogen, cat. no. 12795-027)
- L-Arginine (Sigma-Aldrich, cat. no. A5006-100G)
- Agar degranulated (Difco, cat. no. 214530)
- Tryptic soy broth (TSB) (Difco, cat. no. 211825)
- Tryptic soy agar (TSA) (Difco, cat. no. 236950)
- Ethanol, 70%, vol/vol (made from absolute alcohol; Sigma-Aldrich, cat. no. 34935-1L) **! CAUTION** Ethanol is a flammable substance; follow institutional instructions for storage and handling.
- Sterile heparin, 500 IU/ml, 5-ml bottle (Sanofi Aventis, cat. no. 512507)
- Ketamine, Imalgen1000 (Merial SAS, cat. no. IMA004) **! CAUTION** Ketamine is a controlled substance, which requires secured storage and proper records of use. Avoid accidental administration to humans. Should such an exposure occur, notify a physician immediately.
- Xylazine (Rompun, 2% (wt/vol); Bayer Healthcare, cat. no. ROM001) **! CAUTION** Xylazine is a controlled substance, which requires secured storage and proper records of use. Avoid accidental administration to humans. Should such an exposure occur, notify a physician immediately.
- Acepromazine (injectable), Calmivet solution (Sanofi Aventis, cat. no. CAL226) **! CAUTION** Acepromazine is a controlled substance, which requires secured storage and proper records of use. Avoid accidental administration to humans. Should such an exposure occur, notify a physician immediately.
- Betadine gel, 10% (wt/wt), tube of 100 g (Meda Pharmaceuticals)
- Anesderm 5% (wt/wt), tube of 5 g (Pierre Fabre)
- Betadine solution, bottle of 120 ml (Vetoquinol)
- Eye drops, Ocrygel (cat. no. 843TVN)
- Gel, MediGel sucralose, 56 g (Fisher Scientific, cat. no. TPP2270)
- Virkon, 1% (vol/vol) (Reltyon)
- Sodium pentobarbital, Dolethal (Alcyon, cat. no. 6847542)
- Isoflurane, IsoVet 1,000 mg/g, inhalation vapor (Schering-Plough; Imaxio, cat. no. IsoVet 1,000 mg/g) **! CAUTION** Pregnant women are recommended not to use it unless other means of anesthesia are not available and, in such cases, special masks need to be worn. Only an approved anesthetic respirator system approved by the ethical committee should be used. Also make sure to work in a well-ventilated area when using isoflurane or any other inhalation anesthetics.
- Buprecare (ibuprofen) (Axience)
- Cyclophosphamide monohydrate (Sigma-Aldrich, cat. no. C0768-5G) **! CAUTION** Cyclophosphamide is toxic and immunosuppressant. Cyclophosphamide is an antineoplastic (anti-cancer) drug categorized as an alkylating agent. Its side effects depend on dosage and include lowered blood counts, sterility in males and females, pregnancy defects and or discoloration of skin and nails. Use of a chemical hood and gloves is highly recommended when using this compound.
- Sodium cacodylate trihydrate (Sigma-Aldrich, cat. no. C0250-100G) **! CAUTION** Na-cacodylate is a derivative of arsenic. It is highly toxic when inhaled, ingested or on contact with skin or eyes. It is described as a possible carcinogen and teratogen. Using the chemical hood and gloves is highly recommended when using this compound.
- Ruthenium red (Sigma-Aldrich, cat. no. R2751-1G)
- Glutaraldehyde, 25% (wt/vol) (Sigma-Aldrich, cat. no. G5882)

EQUIPMENT

- CCD camera (IVIS 100; Xenogen)
- Vet abc (SCIL, cat. no. ABC vet 2.0)
- Surgical hood (Faster-air, cat. no. FASTER FLOWFAST H18)
- Laminar air flow (Faster-air, cat. no. Faster BH2006)
- Hot bead sterilizer (Fine Science Tools (FST), cat. no. 18000-50)
- Beads (FST, cat. no. 18000-51)
- Micro-spring scissors (1; FST, cat. no. 15007-08)
- Fine scissors (1; FST, cat. no. 14502-14)
- Fine forceps (2; FST, cat. no. 11245-30)
- Blunt forceps (1; FST, cat. no. 11000-14)
- Hemostats (2; FST, cat. no. 130008-12)
- Silk thread, 22.5 m (FST, cat. no. 18020-30)
- Curved forceps (FST, cat. no. 91197-00)
- Suture Vicryl 3-0 polyglactin absorbable, box of 36 (Ethicon, cat. no. V497H)
- Suture Dafilon 3/0 polyamide non-absorbable, box of 36 (B. Braun, cat. no. C0935239)
- Scalpel (Swann-Morton, cat. no. 0510)
- TIVAP, POLYSITE micro 2000, 5F (Perouse Medical, cat. no. 2105 ISP)
- Sterile champ (LCH, cat. no. CSO-02NT)
- Sterile gloves (size 7–8, medium; LCH, cat. no. STP641)
- Sterile gauze pad (LCH, cat. no. CNST-470)
- Huber needle, straight 7/10, 22 gauge, 30 mm, box of 50 (Perouse Medical, cat. no. 512507)
- Respiratory mask, FFP2 (Fisher Scientific, cat. no. 19-130-4825)
- Sterile gown (BARRIER, cat. no. 98000622)
- Temperature controller, with rectal probe (Physitemp Instruments, cat. no. TCAT-2LV)
- Thermocage, animal warming system (Datestand, cat. no. MK3)
- IPTT-300, electronic chip introducer and chip (Plexx, cat. no. BV 11059)
- Wireless reader with round Implantable Micro Identification (IMI) probe (Plexx, cat. no. DAS-7006R)
- Mozer Max45 clipper (Moser-AnimalLine, cat. no. 1245-0066)
- Isoflurane vaporizer, TAG (TEM, cat. no. TAG1100)
- Conical tubes, 50 ml (Fisher Scientific, cat. no. 14-432-22)
- Conical tubes, 15 ml (Fisher Scientific, cat. no. 14-959-70C)
- Microtubes (Fisher Scientific, cat. no. 05-402-24B)
- gentleMACS Octo dissociator (Miltenyi Biotec, cat. no. 130-095-937)
- gentleMACS M tubes (Miltenyi Biotec, cat. no. 130-096-335)
- Cell strainer, 70 µm Nylon (BD Falcon, cat. no. 3523350)
- ULTRA-TURRAX T25 digital homogenizer (IKA, cat. no. 0010001502)
- NeyTech Ultrasonik, 44–48 kHz (NeyTech)

REAGENT SETUP

Cacodylate solution, 0.07 M Cacodylate solution is prepared by mixing 10.5 ml of 0.2 M Na-cacodylate (pH 7.4), with 19.5 ml of sterile distilled water; prepare it while wearing gloves and working under a chemical hood. Prepare this fresh every time from stock solution (0.2 M) store at 4 °C. Stock solution can be stored at 4 °C for up to 1 year.

Electron microscopy (EM) fixative solution EM fixative solution is a mixture (vol/vol/vol) of 6% (vol/vol) glutaraldehyde, 0.15% (wt/vol) ruthenium red and 0.2 M Na-cacodylate, pH 7.4. EM fixative solution should be stored for <1 week at 4 °C.

PROCEDURE

! CAUTION All methods are performed in a Biosafety Level 2 facility containing a class II biosafety cabinet. Aseptically collected samples (TIVAPs, organs or blood) from animals can be processed in the main laboratory inside a class II biosafety cabinet to maintain sterility.

Presurgical procedures ● TIMING 30 min per rat

1| *Day –6, reception of animals*. Acclimatize rats to a 12-h day/night cycle for 1 week before use with commercial feed/tap water *ad libitum*.

! CAUTION The animal room should be cleaned with disinfectant every day until the end of a study.

2| *Day –5* (10 min per rat). Insert an electronic chip in the flank region of rats to monitor the temperature during the course of the study. Monitor the weight of rats on a regular basis.

3| *Day –2* (10 min per rat). Feed all the rats with MediGel sucralose (one cup (56 g) per cage per day) without ibuprofen for rats to habituate to the mixture, followed by MediGel+ibuprofen gel (0.05 mg/kg rat) on day –1.

▲ CRITICAL STEP If ibuprofen is included in the first dose of MediGel sucralose, most of the rats do not eat the painkiller and it can lead to health issues post surgery. MediGel with ibuprofen can be prepared on day –2 and stored at 4 °C, but it should be brought to room temperature (~22 °C) before feeding the rats.

Surgical procedure (TIVAP implantation, day 0) ● TIMING ~55 min

4| *Anesthesia* (2 min per rat, 5–6 min for a rat to sleep). First sedate the rats using an isoflurane box (3% isoflurane at an oxygen flow rate of 0.8 l/min and air flow rate of 0.4 l/min; **Fig. 2**, panel **a**). Next, anesthetize the rats using a 500-µl cocktail (per 300 g of rat weight) of ketamine-xylazine-acepromazine mix (7:2:1 (vol/vol/vol)) injected intraperitoneally.

5| *Shaving* (3–4 min per rat). Once the rat is fully unconscious, closely shave the dorsal side of the rat (**Fig. 3a**) with an electric clipper to remove hair from the neck up to the lower end of the thoracic vertebrae and from the left to the right end of the body. Next, turn the rat on its back and shave the neckline carefully without cutting the skin (**Fig. 3b**). Remove any excess hair with the help of a clean paper towel.

▲ CRITICAL STEP If the rat is not completely asleep, any procedure including shaving can be stressful, leading to death of the rat during the procedure.

6| *Disinfection* (5 min per rat). Transfer the rat onto a sterile sheet. Gently clean the shaved area (both dorsal and ventral sides), as well as the surrounding area in two steps: first, wash with Betadine soap using a sterile gauze pad or any other sterile pad of absorbent material; and second, disinfect with Betadine solution. Repeat the cycle three times. Allow the Betadine to dry for 5 min (**Fig. 3c**).

▲ CRITICAL STEP Betadine has been previously shown to kill bacteria in seconds to minutes (as per US Centers for Disease Control (CDC) guidelines[47]).

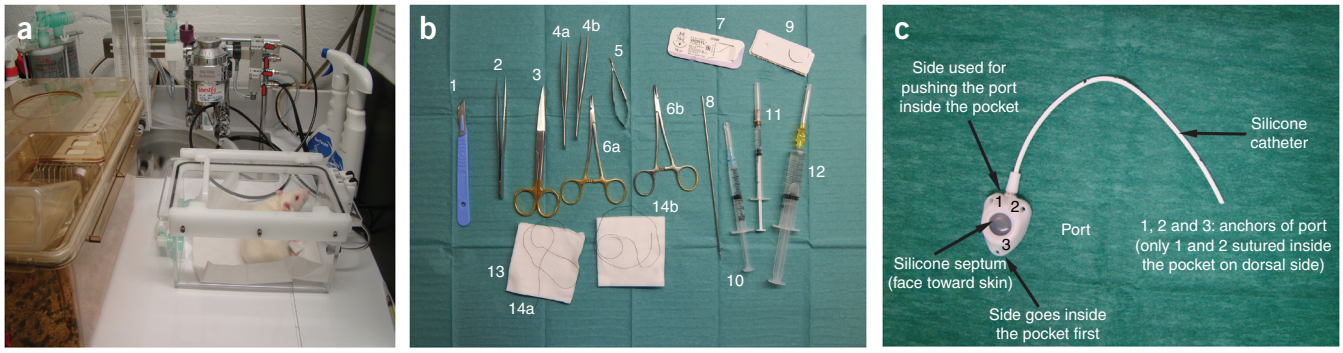

Figure 2 | Preparation for surgery. (**a**) A CD/SD (IGS:Crl) rat was briefly kept in an isoflurane box to calm down, and it was injected intraperitoneally with a chemical anesthesia mixture to complete sedation and analgesia before starting the procedure. (**b**) Surgery instruments were prepared and kept on a sterile sheet in the surgical hood. (1) Sterile scalpel, (2) forceps, (3) scissors, (4a and 4b) forceps for vein clearing and holding the vein for catheter insertion, (5) spring scissors for vein incision, (6a and b) hemostat forceps, (7) polyglactin absorbable suture for securing the port inside the pocket, (8) tunneling rod for passing the catheter under the skin from the dorsal incision to the ventral incision, (9) polyamide nonabsorbable suture, (10) syringe containing 1× PBS and attached Huber needle, (11) syringe with heparin (500 IU/ml), (12) syringe with 1× PBS for cleaning and moistening the incisions, (13) sterile gauge pads and (14a and 14b) silk thread to clamp the catheter on the vein post insertion. (**c**) Labeled TIVAP showing port with anchors (two of which are used to suture), silicone septum and silicone catheter, which is inserted in the vein. Work on animals was performed in compliance with French and European regulations on the care and protection of laboratory animals (European Commission directive 2010/63; French law 2013-118, 6 February 2013). The protocols used in this study were approved by the ethics committee of 'Paris Centre et Sud N°59' (reference 2012-0045).

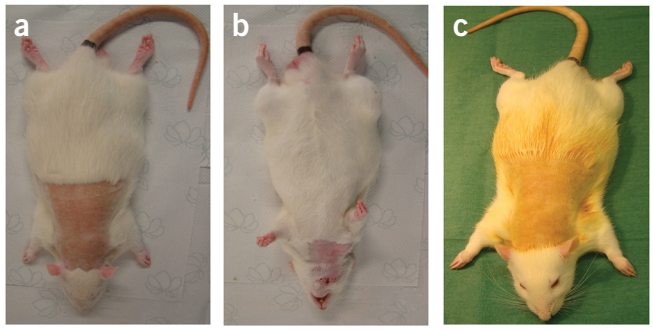

Figure 3 | Shaving and disinfection procedures. Rat preparation for surgery was performed under laminar air flow using a surgical hood, and aseptic conditions were maintained throughout the surgical procedure. (**a**,**b**) Rat was shaved on the dorsal side from the neck to the lower end of the thoracic vertebrae and along the neckline of the ventral side. (**c**) Disinfection of skin using Betadine soap and solution. Work on animals was performed in compliance with French and European regulations on care and protection of laboratory animals (European Commission directive 2010/63; French law 2013-118, 6 February 2013). The protocols used in this study were approved by the ethics committee of 'Paris Centre et Sud N°59' (reference 2012-0045).

7| *Preparation for surgery* (~15 min; **Fig. 2**). It takes ~15 min for a rat to be ready for surgery (**Fig. 2a**); meanwhile, arrange and prepare the surgery area (**Fig. 2b**). For this, first clean the surgical hood with 70% (vol/vol) ethanol and spread out a sterile champ/sheet.

8| On the sheet, place on the left corner a pair of scissors, hemostats (two), spring scissors, fine forceps (two), two pieces of silk thread ~20 cm long, curved forceps, sterile gloves, the TIVAP (with its kit open) and a scalpel. On the right hand side, keep sterile gauze pads, a syringe with 1× PBS (50 ml, from the TIVAP kit), Vicryl suture, monofilament suture, a 1-ml syringe with a Huber needle filled with 1× PBS (from the TIVAP kit) and a 1-ml syringe filled with sterile 300 µl of heparin (**Fig. 2b**).

9| Turn on the 37 °C thermocage system for storing the rat after surgery.

10| Turn on the instrument sterilizer.
▲ **CRITICAL STEP** Keep everything you need for the surgery handy, as the rats may start to wake up if there are any delays in the process of surgery, and they may be stressed.

11| *Surgery* (~30–45 min per rat; **Figs. 4–6**). Once the rat is disinfected in Step 6, place the rat on the surgical sheet prepared in Step 7 with the dorsal side up and tail away from you. Take some 70% (vol/vol) ethanol and wipe the body of the rat once more. Wear sterile gloves and avoid touching any surrounding areas.

12| Make an incision (~1.5 cm) on the dorsal line at the upper end of the thoracic vertebrae with a scalpel (**Fig. 4a**).
▲ **CRITICAL STEP** Incise the skin (both epidermis and dermis) just enough to expose the subcutaneous tissues. A pocket is made in the soft subcutaneous tissues. Avoid making a very deep incision, as it results in local inflammation. Pulling the skin outward while making the incision prevents deep incisions.
? TROUBLESHOOTING

13| Use scissors to make a subcutaneous pocket inside the incision by opening and closing the scissors slowly. Make the pocket big enough to easily insert the port of TIVAP (**Fig. 4b**,**c**).

14| Take the port and insert the pointed end first while pushing the wider part with your thumbs. Take care that the septum side of the port is facing the skin to allow access for injections (**Fig. 4d–f**). Once it is inserted, secure the TIVAP by suturing the anchors on the TIVAP's rear portion (using a hemostat to hold the suture and blunt forceps to hold inside of the skin) with Vicryl 3.0 nonabsorbable suture (**Fig. 4g–l**).

15| Insert the Huber needle into the port by puncturing the septum (**Fig. 5a**), and gently turn the rat on its back (**Fig. 5b**). Place a wet gauze pad (in 1× PBS) on the incision.
▲ **CRITICAL STEP** It is important to keep a wet pad of gauze on the incision throughout the process to prevent it from drying out.

16| Make an incision ~1–1.5 cm in the neck on the right side with a scalpel through the skin (**Fig. 5c**). Gently try to tear the peritoneum beneath the skin incision, and also tear the surrounding fat and connective tissues underneath with a fine forceps to expose the vein (**Fig. 5d**,**e**). Keep the area wet and clean with 1× PBS.

17| *Locate the vein* (**Fig. 5f**,**g**). The left external jugular vein is situated superficially under the mandibular salivary glands, and it forms an 'inverted Y' junction with the right jugular vein; the tail of the 'inverted Y' joins the subclavian vein. Keep the area and veins wet with 1× PBS at all times.
▲ **CRITICAL STEP** It is important to keep the vein wet. If the vein dries out, it will lose its flexibility and it will be difficult to make a cut in the vein and to insert the catheter.

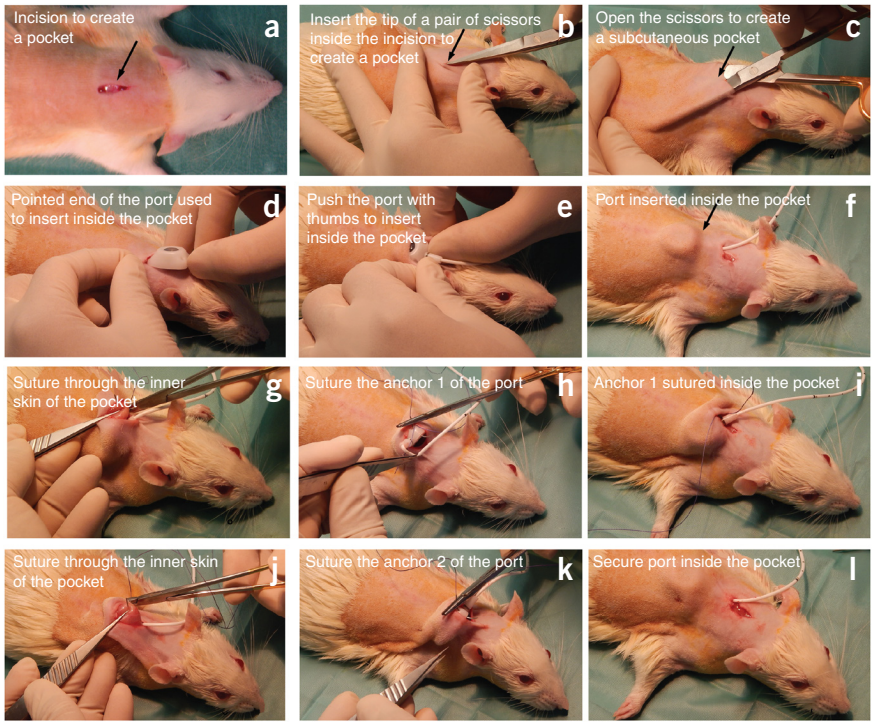

Figure 4 | TIVAP surgical implantation (Steps 12–14). (**a**–**l**) An incision was made along the dorsal line at the upper thoracic vertebrae (**a**), after which a port pocket is created (**b**,**c**), the TIVAP is inserted in the subcutaneous pocket (**d**–**f**), and it is secured by sutures (**g**–**l**). See **Figure 2c** regarding the anchors 1, 2 and 3. Work on animals was performed in compliance with French and European regulations on care and protection of laboratory animals (European Commission directive 2010/63; French law 2013-118, 06 February 2013). The protocols used in this study were approved by the ethics committee of 'Paris Centre et Sud N°59' (reference 2012-0045).

18| Use curved forceps to pass two black silk threads under the external jugular vein (**Fig. 6a**). Use these silk threads in Step 26 (after inserting the catheter in the external jugular vein) to clamp one on the proximal end (to tie the incised vein) and another on the distal end (to secure the catheter inside the external jugular vein) of the jugular vein with hemostats.

19| Next, try to clean the area surrounding the vein, and remove any extra tissues that might be attached to the jugular vein. Keep the vein and surrounding area wet with 1× PBS.

20| After exposing the vein, slightly and gently tilt the rat sideways on the left, and insert the blunt end of the tunneling rod (provided in the TIVAP kit) through the incision made on the dorsal side (used for securing port) and push out from the ventral incision in the neck region. On the pointed end (on dorsal side), connect the tip of the catheter and pull it along the tunneling rod on the ventral side so as to have catheter tunneled under the skin ready to be inserted in the jugular vein (**Fig. 6b**).

21| Cut the catheter to a length of 4.5 cm (the catheter has markings 1 cm apart) at an angle not more than 30° (**Fig. 6c**). This is the length of the catheter to be inserted in the external jugular vein to reach the top of the right atrium.
▲ **CRITICAL STEP** Cutting the catheter at a higher angle will allow easy insertion of the catheter in the jugular vein, but it will lose patency quickly because of suction of the vein against the bigger tilted cut while pulling the blood with syringe.

22| Make sure that the port and catheter are filled with 1× PBS. For this, fill the TIVAP by slowly pushing the piston of the syringe with the Huber needle inserted into the port.
▲ **CRITICAL STEP** Before inserting the catheter in the jugular vein, it is important to make sure that there is no air in the TIVAP, as it can kill the rat within a few minutes.

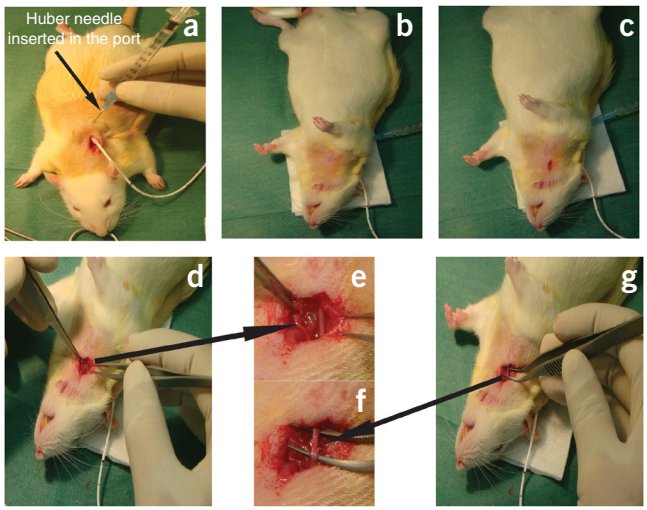

Figure 5 | TIVAP surgical implantation (Steps 15–17). (**a**) Huber needle inserted in the port. (**b**) Rat flipped on its back. (**c**) 1- to 1.5-cm incision in the neck region. (**d**,**e**) Jugular vein exposed after removal of surrounding tissues without curved forceps underneath; **e** shows an enlarged view. (**f**,**g**) Exposed jugular vein after removal of surrounding tissues with curved forceps underneath; **f** shows an enlarged view. Work on animals was performed in compliance with French and European regulations on care and protection of laboratory animals (European Commission directive 2010/63; French law 2013-118, 6 February 2013). The protocols used in this study were approved by the ethics committee of 'Paris Centre et Sud N°59' (reference 2012-0045).

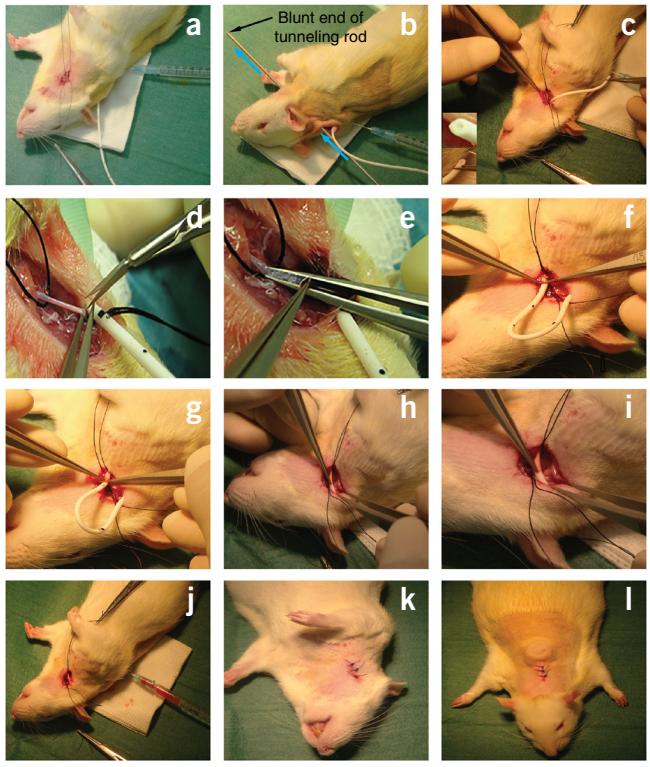

Figure 6 | TIVAP surgical implantation (Steps 18–27). (**a**) Cotton threads inserted under the jugular vein to secure the catheter. (**b**) Tunneling rod inserted for passing the catheter under the skin from the dorsal to the ventral side. Blue arrows indicated the direction of insertion of the rod from the ventral incision coming out of the dorsal (neck) incision to pull the catheter. (**c**) Catheter cut at a slant (inset showing the correct cut). (**d**) Jugular vein incised using micro-spring scissors. (**e**) Dilation of the jugular vein using forceps. (**f–i**) Catheter inserted in the jugular vein. The catheter is inserted into the vein (**f**) and pushed until reaching the right atrium (**g**,**h**). (**i**) enlarged view of **g**. (**j**) Flush and draw blood to check the patency of TIVAP. (**k**,**l**) Surgical wounds closed by suturing ventral (**k**) and dorsal (**l**) incisions. Images in **h–j** are adapted from Chauhan *et al.*[31] with permission from the American Society for Microbiology. Work on animals was performed in compliance with French and European regulations on care and protection of laboratory animals (European Commission directive 2010/63; French law 2013-118, 06 February 2013). The protocols used in this study were approved by the ethics committee of 'Paris Centre et Sud N°59' (reference 2012-0045).

23| While gently holding the vein with a fine forceps, carefully make a partial cut (longitudinal or horizontal) in the external jugular using micro-spring scissors (**Fig. 6d**).
▲ **CRITICAL STEP** There may be lots of blood flow from the vein. In this case, take a wet gauze pad and press it against the vein for 2–3 min or until the bleeding has stopped. Keep the vein wet using 1× PBS.

24| Dilate the vein carefully by inserting closed forceps a little bit. Once the forceps are inside, slowly release to open it (**Fig. 6e**), and at the same time hold the upper part of the incision with one forceps and insert the catheter using another forceps (**Fig. 6f**). Slightly push the catheter inside the vein (~4.5 cm; **Fig. 6g–i**), followed by flushing 100 µl of 1× PBS in the vein.
▲ **CRITICAL STEP** This step should be done as early as possible, as the vein may be dehydrated and become inflexible. This step is very tricky and requires lots of practice in order to correctly insert the catheter. Furthermore, there can be blood loss leading to the death of the rat; hence, the insertion must be quick.
? TROUBLESHOOTING

25| Try to gently draw the blood (**Fig. 6j**) to ensure the reflux and then flush ~500–600 µl 1× PBS and lock with 250 µl heparin (500 IU/ml). Remove the Huber needle from the port.
? TROUBLESHOOTING

26| Tie the black thread on the distal end of the vein to secure the catheter, and tie the proximal thread to block the incised end of the vein.
▲ **CRITICAL STEP** The thread should not be too tight, as it can create pressure and prevent the drawing of blood. In addition, it should not be loose, as the catheter may slip out of the vein.

27| Clean the surgery area and close the ventral (**Fig. 6k**; three or four stitches) and dorsal (**Fig. 6l**; four or five stitches) incisions by making simple interrupted sutures. Apply antiseptic cream (such as Betadine) and local anesthetic lidocaine cream (such as Anesderm).

28| Transfer the rat to a 39 °C chamber until it wakes up.

29| Finally, transfer the rat into a new cage with a new water bottle. Place a cup of MediGel+ibuprofen (0.05 mg/kg rat) in the cage.

Postsurgical care: day +1 to day +3 ● TIMING ~10 min per rat per day
30| *Feeding analgesic*. Place a new cup of MediGel+ibuprofen (0.05 mg/kg rat) in the rat cage on day +1 after surgery.

31| *TIVAP maintenance—'flush and draw'* (4 min anesthesia, 5 min flush and draw per rat). To maintain the patency of the catheters, the TIVAP is flushed every day until the day of the bacterial challenge. To do this, first transfer the rat to the isoflurane box until the induction of anesthesia.

32| Meanwhile, place a sterile sheet in the laminar air flow, and place the nosepiece connected to the isoflurane system on it.

33| Place the nose of the anesthetized rat in the nosepiece such that the tail is away from you.

34| Clean the skin on and surrounding the port with Betadine solution and 70% (vol/vol) ethanol, and allow it to air-dry for 4–5 min.

35| Gently insert the Huber needle connected to a 2-ml syringe filled with 1× PBS into the port by puncturing the silicone septum of the port.

36| Slowly turn the rat on its back, keeping the needle inside the port.

37| Carefully inject ~100 µl of 1× PBS, and then check blood reflux by slowly pulling the piston of the syringe. Flush TIVAP with 1.0–1.5 ml of 1× PBS, followed by locking the TIVAP with 250 µl of heparin (250 IU/ml).

38| Clean the skin with Betadine solution, and then put the rat back in the cage.
▲ **CRITICAL STEP** If the rat is not anesthetized completely, the movement of neck may block the blood reflux. If isoflurane system is not available, chemical anesthesia can be given to rats. In this case, 250 µl of anesthesia per 500 g of rat is sufficient.

39| Monitor the clinical signs such as local inflammation around the port, weight loss and fever. If clinical signs are not normal—i.e., weight loss, fever or torpidity are observed—then euthanize the rat.
▲ **CRITICAL STEP** Local inflammation can be due to several reasons, including forced injection through the port, infection or too much local injury from surgery.
? TROUBLESHOOTING

Bacterial challenge: day +4 ● TIMING 30 min per rat
40| *Bacteria preparation*. One day before bacterial challenge (**Fig. 7**), start overnight culture of bioluminescent *E. coli* 55989 or *P. aeruginosa* in LB medium and *S. aureus* (Xen36, Xen30 or Xen31) or *S. epidermidis* Xen43 in tryptic soy agar/broth at 37 °C with shaking at 140 r.p.m.

41| Spin 5 ml of culture at 6,000*g* and 22 °C for 10 min to pellet the bacteria, and resuspend in 5 ml of 1× PBS. Wash the culture twice in 5 ml of 1× PBS. Finally, resuspend the bacteria in 5 ml of 1× PBS, and measure the optical density at 600 nm (OD_{600}).

42| Dilute the bacterial inoculum in 1× PBS so as to inject 10^4 c.f.u. per 50 µl for *E. coli*, 10^6 c.f.u. per 50 µl for *P. aeruginosa* and *S. aureus* Xen36, and 10^8 c.f.u. per 50 µl for *S. epidermidis* Xen43.

43| Record the weight and temperature of the rat before anesthesia. Clinical symptoms such as local inflammation at the site of port or in the neck region, and any other abnormality such as breathing trouble, weight loss and fever, must be recorded. Any sick rat must not be included in the study, and it should be euthanized.

44| Anesthetize the rat as described in Step 4. Meanwhile, spread the sterile sheet in the laminar air flow. Keep Betadine solution, 70% (vol/vol) ethanol, sterile gauze pads, 50 µl of bacterial inoculum in a 1-ml tuberculin graduated syringe, a 1-ml syringe attached with a Huber needle and filled with 1× PBS, and a 1-ml syringe filled with 300 µl of heparin.

45| Transfer the anesthetized rat to the dark box of an IVIS imaging system with a CCD camera for monitoring bioluminescence before bacterial challenge. Acquire the image using Living Image software (provided with the imaging system). Select the acquisition wavelength for bacterial luminescence, set binning to medium: M (8), open the emission filter and block the excitation. Image the rats for 1 min on both dorsal and ventral sides. At this stage, the absence of luminescence is expected in all the rats.

46| After imaging, transfer the rat to the hood for infection. Follow Steps 33–37, except do not fill the TIVAP with a heparin lock. Remove the syringe by keeping the Huber needle inside the port, and replace it with a syringe to withdraw 150 µl of blood for analysis. Replace this syringe with another one containing bacterial inoculum. Slowly push the inoculum into the port. Normally, this should take 2 min. Uninfected control rats with TIVAPs filled with 1× PBS are used as controls.
▲ **CRITICAL STEP** If the TIVAP is not patent, do not use it for the study. The blood sample is important to check contamination of the TIVAP during the postsurgical maintenance. Care should be taken while taking the needle out of the port to avoid injecting yourself with bacteria. This procedure is always done under the hood if using class II pathogens. Injection of inoculum must be very slow to avoid flushing bacteria into the stream.
? TROUBLESHOOTING

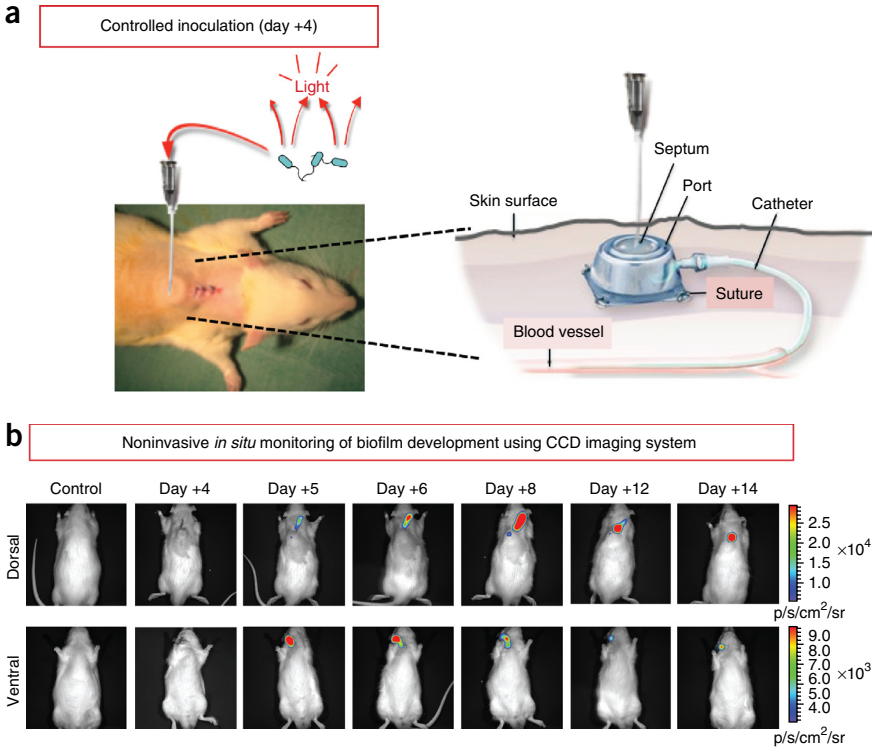

Figure 7 | Bacterial inoculation and monitoring of biofilm formation using bioluminescence. (**a**) The bacterial inoculum is directly injected through the septum of the TIVAP using a Huber needle, and biofilm development is monitored using a CCD camera imaging system allowing the measurement of bioluminescence. (**b**) An example of the images obtained for the colonization of the TIVAP by a bioluminescent clinically relevant strain of *P. aeruginosa* and showing the progressive biofilm development within the TIVAP. The picture of the rat with implanted TIVAP is reproduced with permission from Chauhan *et al.*[30] (copyright 2012) under a Creative Commons Attribution License. Work on animals was performed in compliance with French and European regulations on care and protection of laboratory animals (European Commission directive 2010/63; French law 2013-118, 06 February 2013). The protocols used in this study were approved by the ethics committee of 'Paris Centre et Sud N°59' (reference 2012-0045).

47| After bacterial challenge, again transfer the rat to the dark box of the IVIS imaging system with a CCD camera for monitoring bioluminescence. Keep the imaging settings described in Step 45 and acquire the bioluminescent images to check the initial base signal because of the inoculation in the ports. Usually, no signal is seen at this stage. Use the rats with TIVAPs filled with 1× PBS as uninfected controls.

48| Carefully place the rat back into the cage. Label the cage as class II.

49| *Removal of planktonic bacteria*. Three hours after bacterial inoculation, the planktonic bacteria are removed leaving only the adhered bacteria to form biofilm. For this, repeat the anesthesia procedure as in Step 4. Insert a Huber needle attached to a 1-ml syringe containing 500 µl of 1× PBS, and flush 50–100 µl in the TIVAP very slowly. Replace the syringe with a new 1-ml syringe to withdraw ~300 µl of blood. Remove this syringe with another syringe containing ~250 µl of heparin (500 IU/ml) and lock the TIVAP.

Postchallenge care and monitoring ● TIMING 30 min per rat

50| *Monitoring clinical symptoms*. Carefully monitor and record weight loss, fever, diet loss or any other abnormal behavior. Any rat with a temperature >39 °C ± 0.5 or <34 °C ± 0.5 for continuous 3 d must be euthanized. Any sick rats must be euthanized.

51| *Bioluminescence for biofilm development and pathogenesis* (**Fig. 7**). Biofilm formation inside the lumen of the TIVAP and associated infection is measured as a function of bioluminescence. From day +5 to day +14, transfer the anesthetized rat (either isoflurane or chemical) to the dark box of the IVIS imaging system with a CCD camera for monitoring bioluminescence. Length of exposure can vary depending on the bacteria and the expected infection. Normally, 1-min exposure is sufficient for *E. coli* and *P. aeruginosa*, but for *S. aureus* Xen36 exposure can be about 2–4 min. Control rats are exposed for the same or more time compared with test rats. Maximum biofilm formation should be obtained at day +7/+8. In case of chronic infection experiments, biofilms can be maintained and observed for several months[30].

▲ CRITICAL STEP Exposing control rats is important to evaluate and to measure background coming from different materials such as animal feed or reagents used. It is important to use at least four control rats per study for statistics.

? TROUBLESHOOTING

52| *Blood sampling*. Blood sampling can be done on day +4, day +7/+8 and day +14 to monitor the blood stream infection and associated changes in host factors such as cytokine analysis. Blood (150 µl) can be drawn either from the caudal (tail) vein (option A) or by retro-orbital plexus puncture (option B).

(A) Tail vein ● TIMING 10 min per rat

(i) Anesthetize the rat as described in Step 4. If you are using chemical anesthesia, inject only 200 µl intraperitoneally, as the procedure takes only 10 min for injection.

(ii) Clean the tail with 70% (vol/vol) ethanol, and then rub the tail with a sterile gauze pad to remove the dead scales.
(iii) Approximately 5 cm above the tail end, insert a tuberculin needle and slowly pull out the blood. Collect the appropriate volume of blood and transfer it to a collection tube containing anticoagulant (for viable bacteria count, estimation of immune cell population) or tube without anticoagulant (for cytokine analysis).

(B) Retro-orbital plexus ● TIMING 10 min per rat

(i) Anesthetize the rat as described in Step 4.
(ii) Perform blood sampling, as described earlier[48]. Put the rat on a comfortable table. The retro-orbital plexus is present behind the eye at the lateral canthus. Use a sterile Pasteur pipette to gently and slowly puncture the orbital plexus. While firmly holding the Pasteur pipette near the medial canthus, with gentle rotating movements, insert the tube through the conjunctival membrane. Keep rotating the tube through the orbit until the blood starts to flow. Collect the appropriate volume of blood and transfer it to a collection tube containing anticoagulant (for viable bacteria count, estimation of immune cell population) or tube without anticoagulant (for cytokine analysis).
! CAUTION Technical expertise is required before performing blood sampling. With regard to the presence of the retro-orbital plexus instead of sinus in rats, it is not a preferred method and a minimum of 10 d must be allowed for tissue repair before repeat sampling from the same orbit[49].
▲ CRITICAL STEP Of the circulating blood volume, ~10% of the total volume can be safely removed every 2–4 weeks, 7.5% every 7 d and 1% every 24 h (refs. 50,51).

Euthanasia and sampling ● TIMING ~45 min per rat

53| *Euthanasia* (~5 min per rat). After the last day of observation by bioluminescence, euthanize the animals using a procedure validated by the ethics committee of your institution (CO_2 or chemical euthanasia using intraperitoneal injection of Dolethal (2 ml per rat, 200 mg/ml)).

54| Put the unconscious rat on a sterile sheet in the laminar air-flow system.

55| Clean the body of the rat with 70% (vol/vol) ethanol.

56| *Blood sampling from animals* (~5 min per rat). Use the procedure validated by the ethics committee of your institution (retro-orbital plexus/heart puncture/tail vein). To withdraw blood (volume depends on the experiment planned; 100 µl for c.f.u./ml or 200 µl for cytokine analysis) by heart puncture, keep the anesthetized rat on its back on a sterile clamp.
▲ CRITICAL STEP When blood sampling is desired, take the blood sample from the rat while it is still alive and unconscious, as withdrawing blood from the heart of dead rat is difficult owing to a lack of pumping. Euthanasia by Dolethal takes ~10 min, providing the experimenter with an ample amount of time to sample blood. For euthanasia by CO_2, first anesthetize the rat using 400 µl of a ketamine-xylazine-acepromazine mixture in order to sample blood.

57| Feel the heartbeat with your finger for the place with the fastest beat.

58| Insert a 22-G needle connected to a 1-ml syringe at this point.

59| Withdraw the blood slowly and transfer it to a collection tube containing anticoagulant (for viable bacteria count, estimation of immune cell population) or tube without anticoagulant (for cytokine analysis).

60| *Organ and TIVAP sampling* (~10 min per rat). To aseptically remove the TIVAP and to place it in a sterile Petri plate for imaging with CCD camera imaging system, first clean the body of the rat with 70% (vol/vol) ethanol and cut open the incision site on the dorsal side (made at the time of surgery to create a pocket for the port).
▲ CRITICAL STEP A set of TIVAPs can also be set aside for electron microscopy imaging, as described in **Box 1** (and also see **Fig. 8**).

61| Next, cut the sutures made to secure the TIVAP at the time of surgery. Remove any tissue attached to the TIVAP.

62| After this, gently pull the catheter out of the vein while it is attached to the port, and transfer the whole TIVAP (port plus catheter) to a sterile Petri plate for imaging.

63| To aseptically remove spleen, kidneys, lungs, liver and heart and transfer them to Petri plates, first remove the whole spleen followed by kidneys (both), lungs (whole), liver (a part; the liver is a large organ and one lobe is sufficient for bacterial load enumeration) and heart (whole). As the viable count for organs is done as c.f.u./ml/g of organ, each organ must be weighed before homogenization (see Step 66).

Box 1 | Electron microscopy ● TIMING ~30 min per rat

1. After aseptic removal of colonized TIVAP from rats, cut 1 cm of the catheter tip and dissect the septum from the port using a scalpel.
2. Wash the septum and catheter pieces twice in cacodylate solution and then fix them in EM fixative solution for a minimum of 1 h.
! CAUTION Na-cacodylate is a derivative of arsenic. It is highly toxic when inhaled, ingested or when put in contact with skin or eyes. It is described as a possible carcinogen and teratogen. The use of a chemical hood and gloves is highly recommended when using this compound.
3. Store the samples at 4 °C until they are sent for microscopy to the core facility or experts (not more than 7 d). The imaging should be done on the inner lumen of the catheter and face of the septum inside the port to visualize the biofilm formation.

64| Image all organs and TIVAPs using a CCD camera for bioluminescence signals. Acquire the images of the collected organs and TIVAPs using settings described in Step 45. Acquisition time for the bioluminescence in the organs may vary from 30 s to 1–2 min depending upon the signals. In case of saturated signals, set the acquisition time to automode. Quantify the bioluminescence intensity using the ROI (region of interest) tool in the Living Image software as photons per second per centimeter squared per steradian (p/s/cm^2/sr).

65| *Organ treatment and enumeration of bacterial load* (~10–15 min per rat). After bioluminescence imaging, wash the organs in 4 ml of 1× PBS buffer (in a 50-ml conical tube) before transferring to gentleMACS M tubes containing 5 ml of 1× PBS.

66| Weigh all the organs for analyzing parameters per gram of organ.
▲ CRITICAL STEP Steps 66–69 should be carried out under the laminar air flow (P2 lab).

67| Homogenize the organs using gentleMACS M tubes with the gentleMACS Octo dissociator (Miltenyi Biotec). Alternatively, homogenization of each organ can be done using a T25 digital ULTRA-TURRAX homogenizer (IKA).

68| Pass the homogenized organs through a cell strainer (70 µm nylon, BD Falcon).

69| Serially dilute the homogenates from Step 68 in 1× PBS, and plate 40 µl of each dilution on agar plates and incubate them at 37 °C for colony counts overnight. Plate each dilution in triplicate.

70| *Extraction and quantification of biofilm bacteria from the TIVAP* (~10–15 min per rat). Carefully wipe the TIVAP with 70% (vol/vol) ethanol before extracting intraluminal biofilm bacteria to avoid contamination.

71| Cut the catheter into small pieces and make a slit horizontally to expose the lumen, and transfer to a microtube containing 1 ml of 1× PBS.
▲ CRITICAL STEP Steps 71–75 should be carried out under the laminar air flow (P2 lab).

72| Remove the septum from the port using a sterile scalpel and forceps, cut it into small pieces and transfer it to a separate tube containing 1 ml of 1× PBS.

73| Scratch the cells attached to the titanium body of the port with a pipette tip in 100 µl of 1× PBS and transfer to the same tube as the septum.

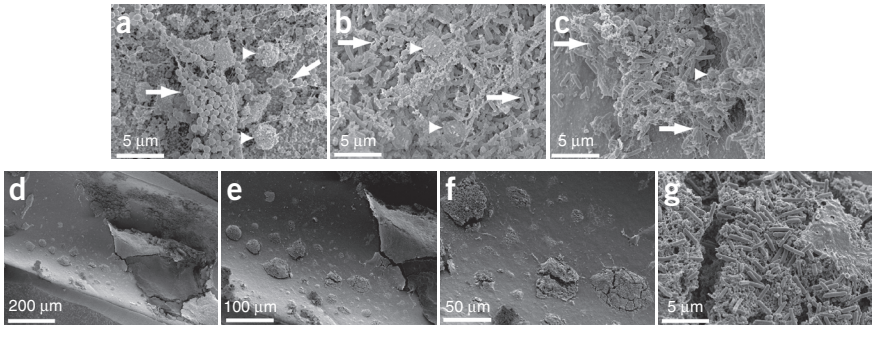

Figure 8 | Biofilm formation in TIVAPs was confirmed by scanning electron microscopy (SEM). TIVAPs implanted in rats and inoculated with *S. aureus*, *P. aeruginosa* or *E. coli* were collected on day 4 after infection (day +8) and analyzed with SEM. Arrows represent bacteria in the biofilm; arrowheads, blood cells. (**a**) TIVAP inoculated with *S. aureus*. (**b**) TIVAP inoculated with *P. aeruginosa*. (**c**) TIVAP inoculated with *E. coli*. (**d**–**g**) SEM imaging to show *P. aeruginosa* biofilm growing inside the lumen of the implanted catheter with typical biofilm microcolonies. The images presented in **d**–**g** represent progressive magnified view of the same catheter. Work on animals was performed in compliance with French and European regulations on care and protection of laboratory animals (European Commission directive 2010/63; French law 2013-118, 6 February 2013). The protocols used in this study were approved by the ethics committee of 'Paris Centre et Sud N°59' (reference 2012-0045).

74| Extract the biofilm formed on the septum and in the lumen of the catheter by vigorously vortexing the tubes for 1 min, followed by transferring them to an ultrasonic water bath (NeyTech Ultrasonik, 44–48 kHz) for 5 min and a second vortexing for 1 min.

75| Then, serially dilute the bacterial suspension from Step 74 in 1× PBS, and plate 40 µl of each dilution on agar plates and incubate at 37 °C for colony counts overnight. Plate each dilution in triplicate. Calculate c.f.u./ml.
? TROUBLESHOOTING

76| Use an Excel sheet to plot c.f.u./ml (as estimated in Step 75) and bioluminescent signals (measured in Step 64) together for correlation.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
24	Catheter is blocked while inserting inside the vein	Catheter is pushed into the wrong direction, and it is entering the right axillary vein	Pull the catheter out slightly and gently rotate it inside the jugular vein. Push the catheter gently in the right direction
25	No blood reflux during surgery	Catheter is too long or too short. Catheter length is optimized to be ~4.5 cm to reach exactly the tip of the right atrium. Longer catheters will reach the inferior vena cava or enter the rat's heart	Pull the catheter out of the vein slowly. Press the vein with a wet gauze pad to prevent blood loss and to prevent the hydration of the vein. Check the length of the catheter and cut it to the right length If the catheter is shorter, then try to change the position of the port so that some length can be gained by reducing the distance between the port and the vein (in this case one may have to optimize the dead volume to avoid flushing of bacteria into the blood stream at the time of challenge)
		The slanting cut made for inserting the catheter has a bigger angle. Too slanted a cut will result in pulling the vein against it and thus blocking the blood draw	Pull out the catheter as above, make the right cut, and then reinsert it
12,39	Local inflammation at the site of port implantation after surgery	Incision was too deep	Only a 1–2-cm incision should be made. Pulling the skin outward while making the incision prevents deep incisions
		Too much injury to the port area	Surgical training is required to keep the injuries to a minimum
46	TIVAP is not patent during postsurgery maintenance	The cut made on the catheter to insert was too slanted	Normally, it is not advisable to make a bigger slant for the ease of insertion, as such cuts result in the vein sticking to the catheter tip while withdrawing blood, but one can try to lift the rats from the back while slightly pushing the rat's chest up to prevent the vein from sticking to the catheter tip, and then opening the lumen or trying to gently and slightly move the catheter without pulling it out of the vein
		The catheter slipped out of the vein because the thread tied to secure it in the vein was too loose	If the catheter has slipped out of the vein, the rat must be excluded from the study
		Flushing was not done properly, resulting in blood clotting inside the TIVAP	These catheters (rats) are unusable for the study unless comparing coating materials for patency

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
51	No bioluminescence signals after infection	Acquisition time was too short	Increase the time of acquisition up to 4 min. In this case, also acquire the bioluminescence for control rats for a longer time to avoid background
		Biofilm formed is not enough to capture the signals	Biofilm capacities of different species and strains can vary. Detailed characterization of *in vitro* biofilm capacities can be performed. Time for bioluminescence acquisition can be adapted to specific *in vitro* biofilm capacities
		Bioluminescence produced by the strain used is too low	The bioluminescence signal produced by bacteria must be high in order to be visible through the skin of the rat and through the TIVAP. Measurement of bioluminescence during *in vitro* biofilm formation within the TIVAP can be performed to ensure that it is strong enough to be detected
75	No biofilm formation at the end of the study	Bacterial inoculum may not be sufficient to form a biofilm	Bacterial inoculum optimization may be needed for your bacterial strains
		Time required for bacteria to adhere may need to be longer	Increase the time to remove planktonic bacteria after injecting inoculum
		Different catheters may behave differently	TIVAP properties may change from one lot to another. Try changing the lot used
		Contamination of the catheters can prevent biofilm formation	Contamination from the rat flora can prevent biofilm formation of your strain. Disinfect the skin of the rat properly to avoid any external contamination

● TIMING

Steps 1–3, presurgical procedures, day –6 to day –1 (6 d): 30 min per rat
Steps 4–29, surgical procedure (TIVAP implantation, day 0): 1–2 d in total; ~55 min per rat per day (Step 7 could be done in parallel with Step 4)
Steps 30–39, postsurgical care: day +1 to day +3: 4 d in total; ~10 min per rat per day
Steps 40–49, bacterial challenge: day +4: 2 d in total; ~30 min per rat (inoculum is started 1 d before the challenge)
Steps 50–52, postchallenge care and monitoring: ~30 min per rat (for Step 52A or 52B, hands-on time is ~10 min per rat)
Steps 53–76, euthanasia and sampling: ~45 min per rat
Box 1, electron microscopy: ~30 min per rat

ANTICIPATED RESULTS

Luminescent signals corresponding to bacterial colonization can be measured within 3 h after the injection of inoculum for *E. coli* and *P. aeruginosa*. For *S. aureus*, the luminescent signal can be measured after 24 h. Exposure time to measure the luminescence can vary between bacterial strains. Normally, 1 min of exposure is sufficient to detect measurable signals for bioluminescent variant strains (used in the study) of *E. coli* 55989 and *P. aeruginosa* PAK, but one can expect an exposure time of 2–4 min for *S. aureus* Xen36 (ref. 30). For S. *epidermidis* Xen43, bioluminescence signals were not obtained for any concentration of inoculum. For *E. coli*, *P. aeruginosa* and *S. aureus* maximum biofilm formation is visualized using bioluminescence or electron microscopy 3–4 d after bacterial challenge (**Figs. 7** and **8**). One can expect to collect ~7.6–8.3 log c.f.u./ml (port) and ~7.5–9.2 log c.f.u./ml (catheter) of biofilm biomass from TIVAP 10 d after bacterial challenge (day +14). An increase in biofilm-associated bioluminescence could be measured up to day 120 post infection, with signals reaching a maximum on day +8. Reduced signals were observed from day +12 onward, indicating the restriction of biofilms to TIVAP colonization (**Fig. 7**)[30]. These results can be correlated to the bloodstream infection, as indicated by the presence of bacteria 4 d after bacterial challenge, whereas clearance of bacteria from the peripheral blood occurs 8 d after bacterial challenge[30]. Thus, this model allows the study of chronic biofilm infection.

In immunosuppressed rats, severe pathology is indicated by weight loss, fever and high bioluminescence as a function of systemic biofilm infection, and it leads to the death of the animals 3 d after bacterial challenge (**Supplementary Fig. 1**)[30]. The blood and organ samples show high bacterial load compared with immune-competent rats.

In curative lock therapy experiments, the bioluminescent biofilms observed 3–4 d after bacterial challenge might disappear just after one instillation of lock solution, such as in the case of gentamicin+EDTA lock. On the contrary, bioluminescence signals indicating the presence of persistent bacteria in the catheter may be seen, as in the case of gentamicin, EDTA and L-arginine alone (**Supplementary Fig. 2**)[31]. Lock therapy may lead to bloodstream and systemic infection in some rats. Thus, the use of systemic treatment in conjunction with lock therapy is required.

In addition to biofilm pathogenesis, molecular mechanisms associated with biofilm can be studied[32]. It was shown using mutants of a bioluminescent variant of *E. coli* 55989 that lipid A palmitoylation did not affect the bacterial adherence to catheters, but rather it enhanced the *in vivo* biofilm survival. Moreover, the palmitoylation led to reduced cytokine triggering *in vivo* in a rat model, indicating the role of palmitoylation in evading host defenses[32].

A modified TIVAP coated with methylcellulose and PEG was used to evaluate the prevention of adhesion of *P. aeruginosa* and *S. aureus* using the rat model, thus demonstrating biofilm inhibition (**Supplementary Fig. 3**)[29].

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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