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Institut Pasteur, Unité de Génétique des Biofilms, Département de Microbiologie

F-75015 Paris, France.

* christophe.beloin@pasteur.fr

ABSTRACT:

Venous access catheters used in clinics are prone to biofilm contamination, contributing to chronic and nosocomial infections. So far, biofilm physiology was mostly studied *in vitro,* due to a relative lack of clinically relevant *in vivo* models. Here, we provide a relevant protocol of totally implantable venous access port (TIVAP) implanted in rats. This model recapitulates all phenomena observed in clinic and allows studying bacterial biofilm development and physiology. 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 anti-biofilm strategies, and to study fundamental biofilm properties. For this procedure, one should expect ~3h00 of hands-on time including the implantation in one rat followed by *in situ* luminescence monitoring and bacterial load estimation.

Keywords: biofilm, catheter-related infection, rat model, bioluminescence

INTRODUCTION:

Medical devices such as peripheral or central venous catheters, urinary catheters or prostheses are nowadays essential to modern medicine and greatly improve patients' healthcare. These devices are however prone to contamination by microbial pathogens leading to biofilm formation and biofilm-related infections that are extremely difficult to eradicate due to the high tolerance of biofilms towards 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 prevention or eradication of chronic and nosocomial biofilm infections require 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 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. These therefore do not provide access to important parameters when characterizing pathogenic biofilms, host factors and other biotic signals. By contrast, only few *in vivo* models have been developed to study bacterial biofilm formation associated with central venous catheter (CVC). One of the first corresponding models used vascular catheter inserted in the jugular vein of rat and was developed to study *Staphyloccus aureus* and *Staphyloccus epidermidis* biofilm-related infections and to evaluate the efficacy of several antimicrobial agents, including antibiotics and anti-microbial peptides [7,10-15]. Other CVC models using different hosts include a mice model catheterized in the jugular vein used to study the role of alternative SigB in *S. aureus* virulence [16], or a pre-catheterized (jugular vein) mice model to study the eradication efficacy of lysostaphin against *S. aureus* biofilm [17]. Using rabbit, port access venous catheters inserted in the jugular vein were used to test several antibiotic against *S. aureus* [18]. Finally, several animal models were also developed to study *Candida albicans* biofilm-related infections, an important causative agent of CVC-related infections [19-22]. All these studies were invasive in nature and required animal killing in order to assess and quantify colonization and infection. As an alternative to these CVC models where the catheter is inserted in

the vascular circulation, subcutaneously implanted mice CVC biofilm models were described using luminescent variants of *S. aureus*, *S. epidermidis* and *Pseudomonas aeruginosa* for real-time monitoring of biofilm formation [23,24] and dissemination [25]. However, these models do not adequately reflect real clinical situation and do not take into account key host factors such as venous blood circulation or humoral factors.

Overview of the proposed *in vivo* catheter biofilm model protocol

To better understand device associated biofilm infections, our laboratory has optimized a long-term central venous catheter model using commercial pediatric Totally Implantable Venous Access Ports (TIVAP) inserted in rat jugular veins [26-28]. Unlike other venous catheters used in other *in vivo* studies, TIVAP are closed devices 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 towards the end of thoracic vertebrae. Luminescent variants of clinically relevant bacteria forming biofilm on biomaterial are introduced into the port by puncturing the silicone septum using Huber needles. Then, bacterial colonization and biofilm formation is monitored, without further invasive intervention, as a function of luminescence using charge-coupled device (CCD) camera. We optimized the inoculum size to be 1 x 10^4 CFU/50 µL per port for *E. coli* and 1 x 10^6 CFU/50 µL *S. aureus* and *P. aeruginosa*. Increased bioluminescence signals were correlated to higher bacterial titers within TIVAP. At these dosages we were able to measure the signals associated with chronic biofilms up to 120 days. 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*) [26-30].

Biofilm biomass formed in the TIVAP can be determined quantitatively by harvesting the bacteria as discussed in details below and plating on suitable agar plates. Counts of viable bacteria ranging from 7.6 to 8.3-log CFU/mL (port) and 7.5 to 9.2- logs CFU/mL (catheter) were obtained 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, electron microscopy was used in order 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.

Relevance and applications of the *in vivo* TIVAP biofilm model

We showed that our TIVAP rat model recapitulates all phenomena observed in clinical TIVAP-associated biofilm infections and can also be used to study the fundamental mechanisms of *in vivo* biofilm formation.

TIVAP applications from a clinical point of view:

1. Evaluation of current procedures used to handle TIVAP in clinical settings, including skin disinfection procedures, TIVAP patency maintenance [26], etc.
2. Study of the different routes of TIVAP colonization in addition to classical endo-luminal colonization including:
   i/ extra-luminal colonization of the catheter;
   ii/ extra-luminal colonization of the port leading to subcutaneous port pocket infection, a clinical situation where repeated intradermal needle punctures during access to TIVAP may lead to subcutaneous bacterial port pocket infection [31] (20% of TIVAP-implanted rats inoculated with *P. aeruginosa* or *S. aureus* developed subcutaneous infection around port [27]);
   iii/ catheter tip colonization via hematogenous route of infection, 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 [27]. 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 [27]. Thus, our rat TIVAP model successfully reproduced clinical situations such as biofilm-related bloodstream infections, organs colonization and port-pocket infections.
3. Investigation of controlled chronic infection and recurrence of infection after conservative treatment.
4. Development of early biofilm detection methods ("biofilm biomarkers"). Such biomarkers are currently lacking and their identification could strongly facilitate medical decisions.
5. Evaluation of prophylactic strategies including:
   i/ 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 to 24 hours [32];
   ii/ catheter treatment with anti-adhesive procedure [26]. We modified the surface of commercial TIVAPs composed of silicone and titanium, using methyl cellulose (MeCe) as well as polyethylene glycol (PEG), two macromolecules with

described anti-adhesive activities [33,34]. 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;

iii/ anti-biofilm strategies against biofilm-related infection such as vaccination.

6. 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 case of device-related infection is costly and traumatic removal of the device. These curative strategies include:

   i/ curative catheter lock solutions active on already developed infectious biofilms [27,28,30]. 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 [28] and L-arginine, a basic amino-acid, combined with gentamicin to eradicate *S. aureus* and *E. coli in vivo* biofilms [30];

   ii/ novel anti-biofilm molecules;

   iii/ alternative strategies (ultrasonic waves, photodynamic or phage therapies).

7. Evaluation of anti-thrombotic treatments since thrombosis is observed using this model [26]. Previous studies showed that biofilm colonized implanted catheters are at higher risk of causing thrombosis [35]. We used our model to demonstrate the better patency in the TIVAP coated with anti-adhesive coatings compared to control untreated TIVAPs [26].

TIVAP applications from a fundamental point of view:

1. 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 [29] or biofilm-specific increased recalcitrance to antimicrobial agents and to the immune system can be studied using this model.
2. Evaluation of the *in vivo* role of biofilm-promoting factors identified *in vitro*.
3. *In vivo* study of clinically relevant biofilm-forming microorganisms other than bacteria, in particular *C. albicans*, one of the main causes of catheter-related infection.
4. Investigation of the impact of the implanted host immune system on the development of *in vivo* biofilms [27] and, reciprocally, study of the impact of biofilm development on the immune system.

Experimental Design

Overview of the procedure stages

The procedure described is designed to study bacterial biofilms using a central venous catheter called TIVAP inserted in rat jugular vein. The study can be broadly divided into 6 stages (see Flow chart of the different steps is presented in **Figure 1)**: One animal study takes between 2-3 weeks but may be longer depending on the question to be addressed, for example immunosuppression of animals will add another 4 days to a study. A trained engineer, PhD student, post-doctoral fellow or researcher can perform all stages of the procedure.

1) *Pre-surgical procedures*: After reception of the rats, they are transferred 2 per cage and housed in animal facility. They are given pain-killers in specialized gels prior to surgery.

2) *Surgery (TIVAP implantation)*: TIVAP is implanted subcutaneously with catheter inserted into the jugular vein.

3) *Postsurgical care*: 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 "flush and draw" technique to mimic clinical TIVAP use, and the temperature and weight of the animals are regularly monitored.

4) *Bacterial challenge*: Four days 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 certain duration of time before the inoculum is removed.

5) *Post challenge care and monitoring*: The biofilm is allowed to form on the device up to day1, day4, day10 or even up to 120 days, depending on the question addressed. Blood sampling is done at day 0 (before inoculation to ensure TIVAP sterility); day4 and day8 to analyze the TIVAP mediated infection. Animals are monitored for the clinical symptoms.

6) *Euthanasia and sampling*: On the day of experiment termination, animals are euthanized and the TIVAP and organs are aseptically removed for analysis.

Laboratory facilities

All the described animal work was done in the Institute Pasteur animal facilities, which are accredited by the French Ministry of Agriculture to perform experiments on live rodents (accreditations A75-15 27, issued 12 November 2004, and A75-15 04, issued 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, 06th 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 (TIVAP, 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, uninfected animal control with implanted TIVAP should be used as biofilm negative controls. For testing antimicrobials, 1X PBS or other appropriate reagent control must be used to compare the efficacy of the tested compounds.

Anesthesia optimization

Anesthetics are critical to sedate rat and reduce discomfort during surgery as well as during 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 ketamine, xylazine and acepromazine mix (7:2:1, v/v) injected intraperitoneally to 500 µL cocktail/300grams 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 maintenance of the anesthetic state at an oxygen flow-rate of 0.8 L/min and air flow-rate of 0.4 L/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 maintenance of the anesthetic state at an oxygen flow-rate of 0.6 L/min and air flow-rate of 0.4 L/min to get a total debit of 1.0 L/min.

Critical step: Isoflurane is an inhalation anesthetics found under several trade names such as aerrane, floran, florane, isothane etc. 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 mixture can be used for long-

term (up to 45 min) sedation of rats. Ketamine and acepromazine are controlled substances, which require secured storage and proper records of usage.

CAUTION: 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 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 well-ventilated area when using isoflurane or any other inhalation anesthetics.

Choice of Animals

We chose Crl:CD/SD variant of wistar rats for our study as they are recommended for studying infectious diseases. The rats are 250-275 grams at reception day and gain ~50 grams 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, i.e. *E. coli, P. aeruginosa, S. aureus* and *S. epidermidis* were purchased (*S. aureus* Xen36, Xen30, Xen31 and *S. epidermidis* Xen43 from Caliper) or gifted (*P. aeruginosa* Lm1, a bioluminescent derivative of the PAK clinical strain [36] and *E. coli* EAEC 55989 [37] transformed with stable plasmid pAT881) [38]. *E. coli* and *P. aeruginosa* strains were grown in Lysogeny broth (LB); *S. aureus* Xen36 and *S. epidermidis* Xen43 were cultured in Tryptic Soy broth (TSB) supplemented with 0.2% glucose at 37°C. Other relevant bacteria may also be studied within this model. While bioluminescence is an efficient tool to follow *in situ* colonization of the device and potential dissemination, it is also possible to estimate end-point bacterial load using non-bioluminescent microbial variant.

Optimization of inoculum size and volume

The inoculum dose was optimized to 10^4 cells for *E. coli*, 10^6 cells for *P. aeruginosa* and *S. aureus.* For *S. epidermidis* the maximum dose that could be used was 10^8 cells but did not lead to bioluminescent-detectable colonization. Currently no other bioluminescent strain of *S. epidermidis* is available [24]. The volume of the inoculum was optimized to 50µL per TIVAP. The dead volume of the port is 250µL. This insures that no bacteria are flushed into the blood stream 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%) and L-arginine, injected alone or in certain combination in catheter lock solutions [27,28,30]. Moreover, our model was successfully used to evaluate the anti-adhesion characteristics of biomimetic glycocalyx-like polymer such as methylcellulose [26]. Empirically, the effect of any relevant antimicrobial agents or anti-adhesive molecules can be evaluated using our model. *In vitro* concentration of the antimicrobial agents and procedures to graft anti-adhesive molecules on silicone and/or titanium need to be verified before *in vivo* evaluation in rat TIVAP model.

MATERIALS

Reagents:

Product	Reference	Manufacturer
Sterile 1X Phosphate buffered Saline (PBS)	10010-023	GIBCO
Sterile water	15230-071	GIBCO
Kanamycin	B5264-1G	Sigma Aldrich
Cefazolin	C5020-500MG	Sigma Aldrich
Vancomycin	94747-5G	Sigma Aldrich
Gentamicin	G1914-5G	Sigma Aldrich
Lysogeny Broth (LB)	12795-027	Invitrogen
L-arginine	A5006-100G	Sigma Aldrich
Agar degranulated	214530	DIFCO
Tryptic Soy broth (TSB)	211825	DIFCO
Tryptic soy agar (TSA)	236950	DIFCO
Ethanol, 70% (made from absolute alcohol)	34935-1L	Sigma Aldrich
Sterile Heparin, 500IU/mL, 5mL bottle,	512507	Sanofi Aventis
Ketamine, Imalgen1000	IMA004	Merial SAS Lyon, Fr
Xylazine, (Rompun™2%)	ROM001	Bayer Healthcare, Germany
Acepromazine (Injectable), Calmivet Solution	CAL226	Sanofi Aventis, Lure, FR
Betadine gel 10%, tube of 100g		Meda Pharma
Anesderm 5%, tube of 5g	Pharmacy	Pierre Fabre, France
Betadine solution, bottle of 120mL		Vetoquinol
Eye drops, Ocrygel®	843TVN	
Gel, medigelsucralose 56gm	TPP2270	Fisher Scientific
Vikron® 1%		Reltyon
Sodium pentobarbital, Dolethal	6847542	Alcyon France
Isoflurane, IsoVet 1000mg/g inhalation vapour (Schering-Plough)	IsoVet 1000mg/g	Imaxio, France

Buprecare® (Ibuprofen)		Axience, France
Cyclophosphamide monohydrate	C0768-5G	Sigma Aldrich
Sodium Cacodylate Trihydrate	C0250-100G	Sigma Aldrich
Ruthenium red	R2751-1G	Sigma Aldrich
Glutaraldehyde 25%	G5882	Sigma Aldrich

Equipments:

Equipments (number needed for the study)	**Catalogue number**	**Manufacturer**
Charged-coupled device CCD Camera (IVIS 100)		Xenogen Corporation, Alameda, CA, USA
Vet abc	ABC vet 2.0	SCIL, Germany
Surgical hood	FASTER FLOWFAST H18	Faster-air, France
Laminar air flow	Faster BH2006	Faster-air, France
Hot bead sterilizer	18000-50	Fine Science Tools (FST)
Beads	18000-51	FST
Micro spring scissors (1)	15007-08	FST
Fine scissors (1)	14502-14	FST
Fine forceps (2)	11245-30	FST
Blunt forceps (1)	11000-14	FST
Hemostats (2)	130008-12	FST
Silk Thread, 22.5 meters	18020-30	FST
Curved forceps	91197-00	FST
Suture Vicryl 3-0 Polyglactin absorbable, box of 36	V497H	Ethicon
Suture Dafilon 3/0 Polyamide non-absorbable, box of 36	C0935239	B Braun
Scalpel	0510	Swann-Morton

TIVAP, Polysite 2000 micro, 5F	2105 ISP	Perouse medical
Sterile champ	CSO-02NT	LCH
Sterile gloves (size 7-8, medium)	STP641	LCH
Sterile gauze pad	CNST-470	LCH
Huber needle, straight 7/10, 22G, 30mm, box of 50	512507	Perouse Medical
Respiratory Mask, FFP2	19-130-4825	Fisher Scientific
Sterile gown	98000622	Barrier, France
Temperature Controller, with rectal probe	TCAT-2LV	Physitemp Instruments Inc, USA
Thermocage, animal warming system	MK3	Datestand Ltd., Manchester, UK
IPTT-300, Electronic chip introducer and chip	BV 11059	PLEXX, Europe
Wireless Reader with round IMI probe	DAS-7006R	PLEXX, Europe
Mozer Max45 clipper	1245-0066	Moser animalline
Isoflurane vaporizer, TAG	TAG1100	TEM (Lormont, France)
Conical tubes , 50mL	14-432-22	Fisher scientific
Conical tubes , 15mL	14-959-70C	Fisher scientific
Microtubes	05-402-24B	Fisher scientific
GentleMACS™ Octo Dissociator	130-095-937	Miltenyi Biotec
GentleMACS™ M tubes	130-096-335	Miltenyi

		Biotec
Cell strainer 70µm Nylon	3523350	BD Falcon
T25 digital ULTRA-TURRAX® homogenizer	0010001502	Ika, France
NEYtech Ultrasonik, 44–48 Khz		NEYtech

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PROCEDURE

Step 1: Pre-surgical Procedures

1A) Day -6, reception of animals: The CD/SD (IGS:Crl) male rats (CharlesRiver) weigh 275–300grams and are acclimatized to a 12 h day/night cycle for one week prior to use with commercial feed / tap water ad libitum. (TIMING: Responsibility of Animal Facility)

1B) Day -5, an electronic chip is inserted in the flank region of rats to monitor the temperature during course of the study. The weight of rats is also monitored on regular basis. (TIMING: 10 min per rat)

1C) Day -2, all the rats are fed with Medigel-sucralose (1cup (56gm) per cage per day) without ibuprofen* for rats to habituate to mixture followed by Medigel+ibuprofen gel on day -1. (TIMING: 10 min per rat)

Critical step: if given directly with the drug, most of the rats do not eat the pain-killer and can lead to health issues post-surgery. Medigel with ibuprofen can be prepared on day -2 and stored at 4°C but brought to room temperature before feeding the rats.

Step 2: Surgical Procedure (TIVAP implantation, day 0) (TIMING: ~ 55min)

2A) *Anesthesia* (TIMING: 2 minutes per rat, 5-6 minutes for a rat to sleep)

Rats are anesthetized using a 500µL cocktail (per 300grams of rat weight) of ketamine, xylazine and acepromazine mix (7:2:1, v/v) injected intraperitoneally.

2B) *Shaving* (TIMING: 3-4 minutes per rat)

Once the rat is fully unconscious, closely shave dorsal side of the rat with an electric clipper to remove hair from the neck up to lower end of the thoracic vertebrae and from left to right end of the body (**Figure 2**). Then, turn the rat on its back and shave the neckline carefully without cutting the skin. 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 rat during the procedure.

2C) *Disinfection*: (TIMING 5 minutes per rat)

Transfer the rat on a sterile sheet. The shaved area (both dorsal and ventral sides) as well as surrounding area is gently cleaned in 2 steps: i) wash with betadine soap using a

sterile gauze pad or any other sterile pad of absorbent material; ii) disinfect with betadine solution. Repeat the cycle 3 times.

2D) *Preparation for surgery*: (TIMING ~15 minutes)

Since it takes ~15 minutes for a rat to be ready for surgery, meanwhile, arrange and prepare the surgery area. For this,

2D.1) Clean the surgical hood with 70% ethanol and spread a sterile champ/sheet.

2D.2) On the sheet, place on left corner a pair of Scissors, hemostats (2), spring scissors, fine forceps (2), 2 pieces of silk thread around 20 cm long, curved forceps, sterile gloves, TIVAP (with its kit open), scalpel and on the right hand side keep sterile gauze pad, a syringe with 1X PBS (50mL, from the TIVAP kit), vicryl suture, monofilament suture, 1 mL syringe filled with 1X PBS with Huber needle (from the TIVAP kit) and 1mL syringe filled with sterile 300µL heparin.

2D.3) Turn on 37°C thermo-cage system for storing the rat after surgery.

2D.4) Turn on instrument sterilizer.

Critical step: keep everything you need for the surgery handy as the rats may start to wake up in case of delay in the process of surgery and may be stressed.

2E) *Surgery*: (TIMING ~30-45 minutes per rat, **Figure 2** and **Figure 3**)

2E.1: Once the rat is disinfected, place the rat on the surgical sheet with the dorsal side up and tail away from you. Take some 70% ethanol and wipe the body of the rat once more. Wear the sterile gloves and avoid touching any surroundings.

2E.2: Make an incision (~1.5cm) on dorsal line at the upper end of thoracic vertebrae with a scalpel.

2E.3: Using a scissor, make a subcutaneous pocket inside the incision by opening & closing the scissor slowly. Make the pocket big enough to easily insert the port of TIVAP.

2E.4: Take the port and insert the pointed end first while pushing the wider part with your thumbs. Once inserted, secure the TIVAP by suturing (using a hemostat to hold the suture and a blunt forceps) inside of the skin with vicryl, 3.0, non-absorbable suture.

2E.5: Insert Huber needle into the port by puncturing the septum and gently turn the rat on its back. Place a pad of gauze pad wet in 1X PBS.

Critical step: It is important to keep a wet pad of gauze pad on the incision throughout the process to prevent its dehydration.

2E.6: Make an incision about 1-1.5cm in the neck on right hand side with scalpel. Gently try to remove the muscles with a fine forceps to expose the vein. Keep the area wet and clean with 1X PBS.

2E.7: Locating the vein: Left Jugular vein is situated superficially under the salivary glands and it forms “inverted Y” junction with the right jugular vein, tail of “inverted Y” joining the subclavian vein. Keep the area and veins wet with 1X PBS, all the time.

Critical step: It is important to keep the vein wet because if dried it will lose its flexibility and it will be difficult to make a cut in the vein as well as tough to insert the catheter in vein.

2E.8: Using a curved forceps pass two black silk threads under the external jugular vein. 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.

2E.9: Then, try to clean the area surrounding the vein and remove any extra tissues that might be attached to jugular vein. Keep the vein and surrounding area wet with 1X PBS.

2E.10: After exposing the vein, slightly tilt the rat (gently) and insert the blunt end of tunneling rod (provided in the TIVAP kit) from the dorsal side and push out from the ventral incision in the neck region. On the pointed end put on the catheter and pull it along the tunneling rod on ventral side so as to have catheter tunneled under the skin ready to be inserted in the jugular vein.

2E.11: Cut the catheter to a length of 4.5 cm (catheter has markings 1cm apart) at an angle not more than 30°. This is the length of catheter to be inserted in the external jugular vein to reach the top of right atrium.

Critical step: Cutting the catheter at a higher angle will allow easy insertion of catheter in the jugular vein but it will lose patency quickly due to suction of vein against the bigger tilted cut while pulling the blood with syringe.

2E.12: Make sure that port and catheter are filled with 1X PBS. For this, fill the TIVAP by slowly pushing the piston of the syringe with Huber needle inserted in to port.

Critical step: It is important to make sure before inserting the catheter in jugular vein that there is no air in the TIVAP as it can kill the rat within few minutes.

2E.13: While gently holding the vein with a fine forceps make a partial cut in the external jugular carefully using a micro spring scissors.

Critical step: There may be lots of blood flow from the vein. In this case take wet gauze pad and press it against the vein for 2-3 minutes or until the bleeding has stopped. Keep the vein wet using 1X PBS.

2E.14: Dilate the vein carefully by inserting closed forceps little bit. Once the forceps is inside slowly release to open it, at the same time hold the upper part of the incision with one forceps and insert the catheter using another forceps. Slightly push the catheter inside the vein (~4.5cm) followed by flushing 100µL 1X 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 most tricky and need lots of practice to insert the catheter. Furthermore there can be blood loss leading to death of rat; hence the insertion must be quick. **TROUBLESHOOT**

2E.15: Try to gently draw the blood to make sure the reflux and then flush about 500-600µL 1X PBS and lock with 250µL heparin (500 IU/mL). Remove the Huber needle form the port. **TROUBLESHOOT**

2E.16: Tie the black thread on the distal end on the vein to secure the catheter and 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. Also, it should not be lose as the catheter may slip out of the vein.

2E.17: Clean the surgery area and suture dorsal (4-5 stitches) and ventral (3-4 stitches) incisions. Put antiseptic cream such as betadine and local anesthetic lidocaine cream such as Anesderm.

2E.18: Transfer the rat to 39°C chamber until wakes up.

2E.19: Finally, transfer the rat into a new cage with new water bottle. Place a cup of Medigel+ibuprofen per cage.

Step 3: Postsurgical care: Day +1 to Day +4

3A) *Feeding Analgesic*: New cup of Medigel + ibuprofen is kept in the cage of the rats on day +1 after surgery.

3B) *"Flush and draw":* (TIMING 4 minutes anesthesia, 5 minutes flush and draw per rat).

3B.1) To maintain the patency of the catheters, the TIVAP is flushed every day until the day of the bacterial challenge. The rat is transferred to the isoflurane box until the induction of anesthesia.

3B.2) Meanwhile, place a sterile sheet in the laminar air flow and place the nose piece connected to the isoflurane system on it.

3B.3) Place the nose of the anesthetized rat in the nose piece such that the tail is away from you.

3B.4) Clean the skin on and surrounding the port with betadine solution and 70% ethanol and allow to air dry for 4-5 minutes.

3B.5) Gently insert the Huber needle connected to a 2mL syringe filled with 1X PBS into the port by puncturing silicon septum of the port.

3B.6) Slowly turn the rat on its back keeping the needle inside the port.

3B.7) Carefully inject about 100µL 1X PBS and then check blood reflux by slowly pulling the piston of the syringe. Flush TIVAP with 1.0 to 1.5mL 1X PBS, followed by locking the TIVAP with 250µL heparin (250IU/mL).

3B.8) Clean the skin with betadine solution and 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 250µL anesthesia per 500 grams rat is sufficient. **TROUBLESHOOT**

3C) *Clinical signs*: Monitor the clinical signs such as local inflammation around the port, weight loss and fever. If clinical signs are not normal then euthanize the rat.

Critical step: Local inflammation can be due to several reasons such as forced injection through the port, infection or too much local injury due to surgery. **TROUBLESHOOT**

Step 4: Bacterial inoculation and monitoring of biofilm formation using bioluminescence: Day +4 (TIMING 30 minutes per rat) (see **Figure 4)**

4A) *Bacteria preparation*: One day before bacterial challenge, start overnight culture of bioluminescent *E. coli* 55989 or *P. aeruginosa* in Lysogeny broth and *S. aureus* (Xen36, Xen30 or Xen31) or *S. epidermidis* Xen43 in Tryptic soy agar broth at 37°C shaking at 140rpm.

4B) Spin 5mL culture at 7500rpm for 10 minutes to pellet bacteria and re-suspend in 5mL 1X PBS. Wash the culture twice in 5mL 1X PBS. Finally, resuspend bacteria in 5mL 1X PBS and measure optical density at 600nm (OD600nm).

4C) Dilute the bacterial inoculum so as to inject 10^4 CFU/50µL for *E. coli*, 10^6 CFU/50µL for *P. aeruginosa* & *S. aureus* Xen36 and 10^8 CFU/50µL for *S. epidermidis* Xen43.

4D) Record the weight and temperature of rat before anesthesia. Clinical symptoms such as local inflammation at the site of port or in the neck region, 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 should be euthanized.

4E) Anesthetize the rat as described under “flush and draw” method (see step 3B). Meanwhile, spread the sterile sheet in the laminar air flow. Keep betadine solution, 70% ethanol, sterile gauze pads, 50µL bacterial inoculum in 1mL tuberculin graduated syringe, 1mL syringe attached with Huber needle and filled with 1X PBS, 1mL syringe filled with 300µL heparin.

4F) Anesthetized rat is transferred to the dark box of IVIS imaging system with a charged coupled device (CCD) camera (Xenogen Corporation, Alameda, CA, USA) for monitoring bioluminescence before bacterial challenge.

4G) After imaging, transfer rat to the hood for infection. Place rat with its nose inside the nose piece and tail away from you. Clean the skin on and surrounding the port with betadine solution and 70% ethanol and allow to air dry for 4-5 minutes. Gently insert the Huber needle connected to a 2mL syringe filled with 1X PBS into the port by puncturing silicon septum of the port. Slowly turn rat on its back keeping the needle inside the port. Slightly inject about 50µL-100µL 1X PBS and then check blood reflux by slowly pulling the piston of the syringe. Remove the syringe keeping the Huber needle inside the port and replace with a syringe to withdraw 150µL blood for analysis. Replace this syringe also with another one containing bacterial inoculum. Slowly push inoculum into the port. Normally this should take 2 minutes.
Critical step: If the TIVAP is not patent, do not use it for the study. The blood sample is important to check contamination of TIVAP during the post-surgical maintenance. Care should be taken while taking out the needle out of the port to prevent 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.

4H) After bacterial challenge, rat is again transferred to the dark box of IVIS imaging system with a charged-coupled device (CCD) camera (Xenogen Corporation, Alameda, CA, USA) for monitoring bioluminescence. The rats (with 1X PBS) are used as uninfected controls.

4I) Rat is then carefully placed back into the cage. Label the cage as Class II.

4J) *Removal of planktonic bacteria*: 3 h after bacterial inoculation, the planktonic bacteria are removed leaving only the adhered bacteria to form biofilm. For this, repeat the anesthesia procedure as above. Insert a Huber needle attached to a 1mL syringe containing 500µL 1X PBS and flush 50-100µL in the TIVAP very slowly. Replace the syringe with new 1mL syringe to withdraw about 300uL blood. Remove this syringe with another syringe containing ~250µL heparin (500IU/ml) and lock the TIVAP.

Step 5: Post challenge care and monitoring: (TIMING: 30 minutes per rat)

5A) *Monitoring Clinical symptoms*: Carefully monitor/record weight loss, fever, diet loss or any other abnormal behavior. Any rat with temperature above 39°C +/- 0.5 or below 34°C +/- 0.5 for continuous 3 days must be euthanized. The sick rats must be euthanized.

5B) *Bioluminescence for biofilm development and pathogenesis* (see **Figure 4)**: Biofilm formation inside the lumen of the TIVAP and associated infection is measured as a function of bioluminescence. Anesthetized rat (either isoflurane or chemical) is transferred to the dark box of IVIS imaging system with a charged coupled device (CCD) camera (Xenogen Corporation, Alameda, CA, USA) for monitoring bioluminescence. Length of exposure can vary depending on the bacteria and the expected infection. The exposure time can vary depending upon bacteria and infection. Normally, 1 minute exposure is sufficient for *E. coli* and *P. aeruginosa* but for *S. aureus* Xen36 exposure can be about 2-4 minutes. Control rats are exposed for the same or more time compared to test rats.

Critical step: Exposing control rat is important to evaluate and measure background coming from different material such as animal feed or reagents used. It is important to use at least 4 control rats per study for statistics.

5C) *Blood sampling*: Blood sampling can be done on day 4 and day 8 post-infection to monitor the blood stream infection and associated changes in host factors such as cytokine analysis. Blood (150µL) can be drawn either from the caudal (tail) vein or by retro orbital plexus puncture.

5C.1) *Tail vein*: (TIMING: 10 minutes per rat).

Anesthetize the rat as described above. In case using chemical anesthesia, inject only 200µL intraperitoneally as the procedure takes only 10 minutes for injection. Clean the tail with 70% ethanol and rub the tail with a sterile gauze pad to remove the dead scales. Approximately 5cm above the tail end insert a tuberculin needle and

slowly pull the blood. Collect the appropriate volume of blood and transfer to a collection tube containing anti-coagulant (for viable bacteria count, estimation of immune cell population) or tube without anti-coagulant (for cytokine analysis).

5C.2) *Retro orbital plexus*: (TIMING: 10 minutes per rat)

Anesthetize the rat as described above. Put the rat on comfortable table. Use a Pasteur sterile pipette to gently and slowly puncturing the orbital plexus. While firmly holding the Pasteur pipette near the sinus, with gentle rotating movements insert the tube through the membrane. Keep rotating the tube through the orbit until the blood starts to flow. Collect the appropriate volume of blood and transfer to a collection tube containing anti-coagulant (for viable bacteria count, estimation of immune cell population) or tube without anti-coagulant (for cytokine analysis).

Critical Step: Of the circulating blood volume, approximately 10% of the total volume can be safely removed every 2 to 4 weeks, 7.5% every 7 days, and 1% every 24 hours [39,40].

CAUTION: Technical expertise is required before performing blood sampling. Regarding the presence of retro orbital plexus instead of sinus in rats, it is not a preferred method and a minimum of 10 days must be allowed for tissue repair before repeat sampling from same orbit [41].

Step 6: Euthanasia and sampling: (TIMING ~45 minutes per rat)

After the last day of observation by bioluminescence:

6A) *Euthanasia:* (TIMING ~5 minutes per rat)

Euthanize the animals using a procedure validated by the ethics committee of your institution (CO_2 or chemical euthanasia using IP injection of Dolethal (2mL/rat, 200mg/mL)). Put the unconscious rat on a sterile sheet in the laminar air flow system. Clean the body of the rat with 70% ethanol.

6B) *Blood sampling on animals*: (TIMING: ~5 min per rat)

Using a procedure validated by the ethics committee of your institution (retro-orbital plexus/heart puncture/tail vein). The blood (volume depends on experiment planned; 100µL for CFU/mL or 200µL for cytokine analysis) is withdrawn by heart puncture. Keep the anesthetized rat on its back on a sterile champ. Feel the heartbeat with your figure for place with fastest beat. Insert at this point a 22G needle connected to a 1mL syringe. Withdraw the blood slowly and transfer to a collection tube containing anti-coagulant (for viable bacteria count, estimation of immune cell population) or tube without anti-coagulant (for cytokine analysis).

Critical Step: When blood sampling is desired, take the blood sample from rat while it is still alive and unconscious, as withdrawing blood from the heart of dead rat is difficult

due to lack of pumping. Euthanasia by Dolethal takes ~10 minutes providing experimenter ample of time to sample blood. In case of euthanasia by CO_2, first anesthetize rat using 400µL ketamine-xylazine-acepromazine mixture to sample blood.

6C) *Organ and TIVAP sampling:* (TIMING: ~10 min per rat)

Aseptical removal of each TIVAP and placement in a sterile Petri plate for imaging with CCD camera imaging system. A set of TIVAPs can be set aside for electron microscopy imaging (see **Figure 5**).

6C.1) Remove the TIVAP aseptically and put it in a labeled sterile Petri plate.

6C.2) Aseptically remove spleen, kidneys, lungs, liver and heart and transfer to Petri plates.

6C.3) All organs and TIVAP are imaged using CCD camera for bioluminescence signals.

6D) *Organ treatment and enumeration of bacterial load*: (TIMING: ~10-15 min per rat)

All the procedure is done under the laminar air flow (P2 lab).

6D.1) After bioluminescence imaging, wash organs in 4mL 1X PBS buffer (in a 50mL conical tube) before transferring to gentleMACS™ M tubes containing 5mL 1X PBS.

6D.2) Weigh all the organs for analyzing parameters per gram of organ.

6D.3) 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).

6D.4) Homogenized organs are passed through cell strainer (70µm nylon, BD Falcon).

6D.5) The bacterial suspension is then diluted serially, plated on agar plates and incubated at 37°C for colony counts.

6E) *Extraction and quantification of biofilm bacteria from TIVAP*: (TIMING: ~10-15 min per rat) All the procedure is done under the laminar air flow (P2 lab).

6E.1) TIVAP are carefully wiped with 70% EtOH before extracting intraluminal biofilm bacteria to avoid contaminant.

6E.2) The catheter is cut into small pieces and a slit is made horizontally to expose the lumen and transferred to a microtube containing 1 mL 1X PBS.

6E.3) The septum is removed from the port using a sterile scalpel and forceps, cut into small pieces and transferred to a separate tube containing 1 mL 1X PBS.

6E.4) Cells attached to the titanium body of the port are scratched in 100 mL 1X PBS and transferred to the same tube as the septum.

6E.5) Biofilm that formed on the septum and in the lumen of catheter is extracted 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 minute.

6E.6) The bacterial suspension is then diluted serially, plated on agar plates and incubated at 37°C for colony counts. CFU/mL and bioluminescent signals (ROI, p/S/cm^2/sr) are plotted together for correlation.

Other related procedures

supp1) Port pocket infection: (TIMING ~20 min per rat)

supp1A) Any inflammation around the port after injecting the bacterial inoculum in the port indicates a pocket infection.

supp1B) From euthanized rat, carefully remove the port from the pocket by opening the incision made at the time of surgery. Cut the sutures used for securing the port in the pocket.

supp1C) Image the rat in the dark box of IVIS imaging system with a charged coupled device (CCD) camera (Xenogen Corporation, Alameda, CA, USA) for monitoring bioluminescence and evaluate the bacterial load in the pocket.

supp1D) Remove all the pus formed in the pocket for CFU estimation.

supp1E) Plate the serial dilutions for *E. coli* and *P. aeruginosa* on LB and for *S. aureus* on TSB.

***supp2) Hematogenous infection*:** (TIMING ~10 min per rat)

supp2A) *S. aureus* Xen36 is used to check the possibility of TIVAP colonization through the venous system.

supp2B) Overnight grown *S. aureus* Xen36 culture is used as inoculum. An inoculum of 5 X 10^8 cells of *S. aureus* Xen36 is centrifuged, washed and resuspended in 500µL 1X PBS.

supp2C) Anesthetize the rat as described above. In case using chemical anesthesia, inject only 200µL intraperitoneally as the procedure takes only 10 minutes for injection.

supp2D) Clean the tail with 70% ethanol and rub the tail with a sterile cotton swab to remove the dead scales. Approximately 5cm above the tail end, inject bacteria into the bloodstream of rat (already containing implanted TIVAP) through the lateral tail vein.

supp2E) Monitor rat before and after injecting bacteria for bioluminescence using IVIS-100 imaging system to check infection and colonization.

supp2F) 3-days after the injection euthanize rats as described earlier in the protocol.

supp2G) TIVAP is removed aseptically to confirm the colonization by bioluminescence and plating CFU/mL.

supp2H) 1-cm tip of the catheter is plated for CFU enumeration as the bacteria colonized only the tip of catheter.

***supp3) Immunosuppression of rats*:** (TIMING ~15-20 min per rat)

supp3A) Rats are injected intraperitoneally with cyclophosphamide.

CAUTION: cyclophosphamide is toxic and immunosuppressant. Cyclophosphamide is an antineoplastic (anti-cancer) drug categorized as an alkylating agent. Its side effects depend upon dosage such as lowered blood counts, sterility in males and females, pregnancy defects and or discoloration of skin and nails. Using the chemical hood and gloves is highly recommended when using this compound. For detailed literature on side effects, consult: *http://www.ncbi.nlm.nih.gov/pubmed/?term=Cyclophosphamide+side+effects*

supp3B) Dose and regimen of cyclophosphamide delivery are optimized by estimating blood total leukocyte count as determined using an animal blood cell counter (Vet abc, SCIL, Germany ABC vet 2.0, Germany). Furthermore, immunosuppressed status of rats is indicated by the physical state of rats such as lack of appetite, reduced weight and loss of hair. One hundred mg/kg body weight of cyclophosphamide was finally selected for giving intraperitoneal injections to rats (n= 4 for each bacterial strain) on day -4 and 50 mg/kg on day -1 of inoculation.

Critical step: An optimized inoculum dose of 10^2 CFU/ 50µL 1X PBS for all bacterial strains (higher inoculum doses lead to the death of animals overnight) is used for TIVAP contamination and is confirmed by plating for CFU/mL. Control catheterized and immunosuppressed rats receive 50µL 1X PBS only.

supp3C) Prior to inoculation of clinical strains, all rats are checked for the absence of infections as for immune-competent rats.

4) Electron microscopy: (TIMING ~30 min per rat)

4A) After aseptic removal of colonized TIVAP from rats, 1 cm catheter of the catheter tip is cut and the septum is dissected from the port using a scalpel.

4B) Septum and catheter pieces are washed twice in cacodylate solution prepared with gloves and under a chemical hood (0.07 M: 10.5mL 0.2M Na-cacodylate pH 7.4 + 19.5 mL sterile D/W) and then fixed in EM fixative solution (for example, a mixture v/v/v of 6% glutaraldehyde, 0.15% Ruthenium red, 0.2M Na-cacodylate pH 7.4).

CAUTION: Na-Cacodylate is a derivative of arsenic. It is highly toxic when inhaled, ingested, or put in 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.

4C) Inner lumen of the catheter and face of the septum inside the port are used to visualize biofilm formation.

4D) Samples are stored at 4°C until sent for microscopy to the core facility / experts (not more than 10 days).

5) In vivo lock therapy :

Antibiotics and their concentration for lock therapy studies were chosen based on recommendations made by IDSA guidelines [4] (Table 1). Efficacy of cefazolin (5,000mg/mL in 5000IU/mL heparin) and gentamicin lock therapies (1.0mg/mL, or 5mg/mL), EDTA (30mg/mL), ethanol (70%), L-arginine (0.4%) alone or in combination is evaluated against bioluminescent *E. coli* 55989, *P. aeruginosa* PAK, MSSA *S. aureus* Xen36, MRSA *S. aureus* Xen30 and *S. epidermidis* Xen43 [27,28,30]. Since 30% mortality was observed associated with antibiotic lock therapy [27], this therapy is always used in conjunction with systemic vancomycin hydrochloride (50mg/kg, for MSSA, MRSA, and *S. epidermidis*) or gentamicin (30mg/kg, for Gram-negative bacteria) subcutaneous injections [42,43]. The 4-day-old biofilm formed inside the implanted TIVAP is locked (200µL) with the above-discussed antibiotics and monitored for biofilm clearance by measuring bioluminescence. Two types of regimen are followed for lock therapy: first is a 5-day lock regimen during which the old lock is replaced by a new one every 24 h for 5 days in conjunction with systemic treatment for 5 days. We also assess 1 day lock regimen with a single instillation dwelling for 7 days in conjunction with 1 day of systemic treatment. Rats with a colonized TIVAP but receiving PBS lock are used as controls.

5A) Anesthetize infected rat and measure the bioluminescence as described above after 4 days of infection in TIVAP.

5B) Rat is transferred in the hood for instilling antibiotic solution in the TIVAP. Instill 200 µL antibiotic solutions in the port slowly. Vancomycin or gentamicin for Gram positive and Gram negative bacteria respectively is injected intraperitoneally to prevent systemic infection due to flushing of the lock into the blood circulation.

5C) Replace the lock every 24 h.

Critical step: It is possible that TIVAP may be blocked because of biofilm formation and old lock may not be retrievable. In this case flush the old lock with PBS and then instill the fresh lock in the port.

5D) Monitor biofilm clearance by bioluminescence imaging every day before replacing the lock.

5E) Euthanize rats after day 7 of the last lock instillation for estimating viable cell counts and electron microscopy analyses as described above.

5F) In case of immunosuppressed rats, they are sacrificed on day 3 post-lock instillation.

TIMING

Step1: day -6 to day-1 (6 days)

Step2: 1-2 days (step 2A to 2E.19: ~55 min per rat per day and step2D: could be done in parallel with step 2A)

Step3: 4 days; 3A to 3C: ~10 min per rat per day

Step 4A to 4J: 2 days; ~30 min per rat (Inoculum is started a day before the challenge)

Step A to 5C ~30 min per rat.

Step 5C.1 /5C.2: ~10 min per rat

Step 6A to 6E ~45 min per rat

1 **TROUBLESHOOTING**

2

Step	Trouble/problem	Possible reason(s)	Solution
2E.14	Catheter blocked while inserting inside the vein.	Catheter pushed into the wrong direction and entering the right axillary vein.	Pull the catheter out slightly and gently rotate it while inside the jugular vein. Push the catheter gently in the right direction.
2E.15	No Blood reflux during surgery.	a) Catheter too long or too short. Catheter length is optimized to be ~4.5cm to reach exactly the tip of the right atrium. Longer catheter will reach inferior vena cava or enter the rat's heart. b) The slanting cut made for inserting the catheter has bigger angle. Too slant cut will result in pulling the vein against it due to pressure and thus blocking the blood draw.	a) Pull the catheter out of the vein slowly. Press the vein with a wet gauze pad to prevent blood loss and prevent the hydration of vein. Check the length of the catheter and cut it to the right length. If the catheter is shorter, than 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). b) Pull out the catheter as above and make the right cut and reinsert it.
3B.8	TIVAP not patent during post-surgery maintenance.	a) The cut made on the catheter to insert was too slanting. b) The catheter slipped out of the vein due to loosely tied thread to secure it in the vein. c) Flushing was not done properly resulting in blood	a) Normally, it is not advisable to make a bigger slant for the ease of insertion but one can try to lift the rats from the back slight to open the channel or try to gently and slightly move the catheter without pulling it out of the vein. b) If the catheter has slipped out of the vein, rat must be excluded from the study.

		clotting inside the TIVAP.	c) These catheters (rats) are unusable for the study unless until comparing coating materials for patency.
3C	Local inflammation at the site of port implantation after surgery.	a) Incision too deep b) Too much injury to the port area	a) Only 1-2cm incision should be made. Pulling the skin outwards while making the incision prevents deep incisions. b) Training is required to keep the injuries to minimum.
5B	No signals after infection.	a) Acquisition time was short b) Biofilm formed is not enough to capture the signals. c) Bioluminescence produced by the strain used is too low.	a) Increase the time of acquisition up to 4 minutes. In this case acquire the bioluminescence for control rats also for longer time to avoid background. b) 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 *in vitro* biofilm capacities. c) 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 TIVAP can be performed to ensure that it is strong enough to be detected.
6E.6	No biofilm formation at the	a) Bacterial inoculum may not be enough	a) Bacterial inoculum optimization maybe needed for

	end of the study.	to form biofilm b) Time required for bacteria to adhere maybe longer. c) Different catheters may behave differently. d) Contamination on the catheters can prevent biofilm formation.	your bacterial strains. b) Increase the time to remove planktonic bacteria after injecting inoculum. c) TIVAP properties may change from lot to another. So, try changing the lot used. d) Contamination from the rat flora can prevent biofilm formation of your strain. Disinfect the skin of the rat properly to avoid any external contamination.

Table 2. TROUBLE SHOOTING

ANTICIPATED RESULTS

1. 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 minutes for *S. aureus* Xen36 [27]. For S*. epidermidis* Xen43 bioluminescence signals were not obtained for any concentration of inoculum. One can expect to harvest ~7.6 to 8.3-log CFU/mL (port) and ~7.5 to 9.2-log CFU/mL (catheter) of biofilm biomass from TIVAP after 10d of infection. Increase in biofilm-associated bioluminescence could be measured up to day 120 post-infection with signals reaching maximum on day 4. Reduced signals were observed from day 8 onwards, indicating restriction of biofilms to TIVAP colonization (**Figure 5**) [27]. These results can be correlated to the bloodstream infection as indicated by presence of bacteria on day 4 whereas clearance of bacteria from the peripheral blood by day 8. Thus, this model allows non-invasive study of chronic biofilm infection.

2. In case of immunosuppressed rats, severe pathology can be seen as indicated by weight loss, fever and high bioluminescence as function of systemic biofilm infection leading to death of animals by day 3 (**Figure 6**) [27]. The blood and organ samples show high bacterial load compared to immune-competent rats.

3. In case of lock therapy experiments, the absence of bioluminescence may be observed just after 1 instillation of antibiotic solution, such as in case of gentamicin+EDTA lock. On contrary, this bioluminescence signals indicating presence of persistent bacteria in the catheter may be seen as in case of gentamicin, EDTA, L-arginine alone (**Figure 7**) [28]. Lock therapy may lead to bloodstream and systemic infection in some rats. Thus, use of systemic treatment in conjunction with lock therapy is required.

4. In addition to biofilm pathogenesis, molecular mechanisms associated with biofilm can be studied [29]. 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 enhanced the *in vivo* biofilm survival. Moreover, the palmitoylation lead to reduced

cytokine trigger *in vivo* in rat model indicating role of palmitoylation in evading host defenses [29].

5. Modified TIVAP coated with methylcellulose and PEG was used to evaluate prevention of adhesion of *P. aeruginosa* and *S. aureus* using the rat model and thus, biofilm inhibition (**Figure 8**) [26].

1

Microorganism	Gentamicin (30mg/mL)	Cefazolin (5mg/mL)	EDTA (30mg/mL)	Ethanol (70%)	Gentamicine+EDTA (5mg/mL+30mg/mL)	Gentamicine +Cefazolin (1mg/mL+ 5mg/mL)
E. coli	Y	N.D.	Y	N.D.	Y	N.D.
P. aeruginosa	Y	N.D.	Y	N.D.	Y	N.D.
MSSA	N.D.	Y	Y	Y	Y	Y
MRSA	N.D.	N.D.	Y	N.D.	Y	N.D.
S. *epidermidis*	N.D.	N.D.	Y	N.D.	Y	N.D.

2 Y: Efficacy tested, N.D.: efficacy not tested

3 **Table 1: Lock therapy concentration (mg/mL)**

LEGENDS TO FIGURES:

Figure 1. Schematic representation of the different steps of the *in vivo* model of totally implantable venous access port related infection. Steps in black boxes correspond to regular procedures of implantation, contamination and monitoring of colonization. Steps in yellow boxes correspond to potential procedures that can be applied to the regular model.

Figure 2. TIVAP surgical implantation. TIVAP were surgically implanted in CD/SD (IGS: Crl) rats. Surgery was performed under laminar air flow using a surgical hood and aseptic conditions were maintained throughout the surgical procedure. For ease, labels in figure correspond to steps described in the procedure under surgery section. (2A) Rat was briefly kept in an isoflurane box to calm down and injected intraperitoneally with a chemical anesthesia mixture to complete sedation and analgesia before starting the procedure. (2B.1 – 2B.2) Rat was shaved on dorsal side from the neck to the lower end of thoracic vertebrae and along the neck line of ventral side. (2C) Disinfection of skin using betadine soap and solution. (2D) Pre-surgical preparations. Instruments and objects required during the surgery kept on a sterile sheet in the laminar air flow. (2E.2) Incision was made along the dorsal line at the upper thoracic vertebrae. (2E.4.1 – 2E.4.2 1 – 2E.4.3) TIVAP inserted in the subcutaneous pocket and secured by sutures. (2E.5.1) Huber needle inserted in the port. (2E.5.2) Rat flipped on its back. (2E.6) 1-1.5cm Incision in neck region (2E.7.1 – 2E.7.2) Jugular vein exposed. 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, 06th 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. TIVAP surgical implantation (contd.). (2E.8) Cotton threads inserted under the jugular vein to secure catheter. (2E.10) Tunneling rod inserted for passing the catheter under the skin from dorsal to ventral side. (2E.11) Catheter cut at a slant (inside caption showing the correct cut). (2E.13) Jugular vein incised using micro spring scissors. (2E.14) Dilation of jugular vein using forceps. (2E.14.1 to 2E.14. 5) Catheter inserted in the jugular vein. (2E.15) Flush and draw blood to check patency of TIVAP. (2E.17.1 – 2E.17.2) Surgical wounds closed by suturing ventral and dorsal incisions. 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, 06th 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 4: Bacterial inoculation and monitoring of biofilm formation using bioluminescence. 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 to measure bioluminescence. Below is shown 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. 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, 06th 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 5. Biofilm formation in TIVAP was confirmed by scanning electron microscopy (SEM). TIVAPs implanted in rats and inoculated with *S. aureus*, *P. aeruginosa* or *E. coli* were harvest on day 5 post-infection and analyzed with scanning electron microscopy. Arrows represent bacteria in biofilm; arrowheads, blood cells. (A) TIVAP inoculated with *S. aureus*, (B) TIVAP inoculated with *P. aeruginosa*, (C) TIVAP inoculated with *E. coli*. (E) Scanning electron microscopy to show *P. aeruginosa* biofilm growing inside the lumen of implanted catheter with typical biofilm micro colonies. (F-G) Magnified view: a *P. aeruginosa* micro colony showing typical biofilm structures. 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, 06th 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. Biofilm led to lethal infection in immunosuppressed rats. (A) TIVAP implanted and cyclophosphamide-treated rats were injected with 10^2 CFU in 100mL of *P. aeruginosa* into the port of TIVAP and photon emission was monitored up to day 3 to evaluate biofilm formation and associated infection. (B) Bacterial load from different organs aseptically removed from dead animals was analyzed, organs were homogenized and were plated on LB agar for viable counts per mL. CFU results are means +/- standard deviations. Number of rats (n) used in the experiment, n = 4. 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, 06th 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 7. *In vivo* lock therapy against *Staphylococcus aureus* biofilm in the implanted TIVAP: 5-day regimen presented. 200µL high dose antibiotics solution was instilled in TIVAP of rats (day 0) to treat methicillin sensitive *S. aureus* biofilm colonization (n = 5). Lock therapy was associated with systemic vancomycin for *S. aureus*. The lock was renewed every 24 h for 5 days, and its efficacy was monitored as photon emissions. (A) Control rats with PBS lock. (B) 5 mg/mL gentamicin lock. (C) 30 mg/mL EDTA alone. (D) Combined gentamicin (5 mg/mL) and EDTA (30 mg/mL) lock. In panels A to D, representative experiments are shown. (E-H) Rats were euthanized after 7 days of treatment, and TIVAP were harvested and monitored for photon emissions. (I) Bacterial cells from TIVAP were harvested and plated on TSB agar for counts of CFU/mL. CFU results are means +/- standard deviations. One-way analysis of variance (ANOVA) with Graphpad Prism version 5.0c was used for statistical analysis. A *P* value of <0.05 was considered significant, **** *P* <0.0001. 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, 06th 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 8. Modified anti-adhesive totally implantable venous access port (TIVAP). (A) Commercially available pediatric TIVAP used in the study, and dismantled TIVAP (a, catheter; b and c, envelope of port; d, sealing ring of port; e, septum; f, titanium port) and anti-adhesive molecules used to modify TIVAP parts. The catheter and septum were modified using methylcellulose (MeCe) derivative and the titanium port was modified using polyethylene glycol (PEG) derivative. (B) Rats with modified or unmodified implanted TIVAPs were inoculated with 10^6 colony-forming units (CFUs) of *Staphylococcus aureus* or 10^3 CFUs of *Pseudomonas aeruginosa* per 50µL of 1X phosphate-buffered saline. Bacteria were allowed to adhere to the TIVAP endoluminal surface for 3 hours (*S. aureus*) or 1.5 hours (*P. aeruginosa*) and biofilms were left to form for 5 days, and TIVAP was extracted to measure bacterial biofilm colonization. Viable bacteria were counted by plating on tryptic soy agar for *S. aur*eus or lysogeny broth agar for *P. aeruginosa*. (Control: unmodified TIVAP and Si-Ti: both silicone and titanium modified). Statistical analysis was performed using 1-way analysis of variance

(ANOVA) with GraphPad Prism software (version 5.0c). Differences were considered significant at $P < 0.05$. *$P \leq 0.01$; **$P \leq 0.001$. 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, 06th February, 2013). The protocols used in this study were approved by the ethics committee of “Paris Centre et Sud N°59” (reference 2012-0045).

1

	Pros of the model			Cons of the model
1	Clinically relevant		1	Labor intensive
2	Only requires basic small animal surgical training		2	Requires a 1-3 month training to acquire optimal technical expertise
3	Totally implanted and no requirement for external containments on the animal		3	Expensive
4	Closed by a septum enabling controlled infections and reduced external uncontrolled contaminations		4	Require daily manipulation (flush/draw) to avoid thrombosis and clogging
5	Allows the *in vivo* study of all clinically relevant catheter pathogens		5	Restricted use in rat due to difficulty to adapt for small-sized mice
6	Allows *in vivo* biofilm monitoring over very long period (model of chronic infection) thus reducing the number of used animals			
7	Biofilm progression can be studied using luminescent bacteria or fungi (*Candida* sp.)			
8	Allows the evaluation of prophylactic and curative anti-biofilm approaches			
9	Allows the evaluation of the contribution of the host immune system in biofilm development			

2

3 **Table 3. Advantages, limitations and adaptations:**

AUTHOR CONTRIBUTIONS STATEMENTS

A.C. and C.B. initiated the development of the model. A.C., JM.G. and C.B. designed the experiments. A.C. performed the experiments. A.C., JM.G. and C.B. wrote the manuscript.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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Protocol overview

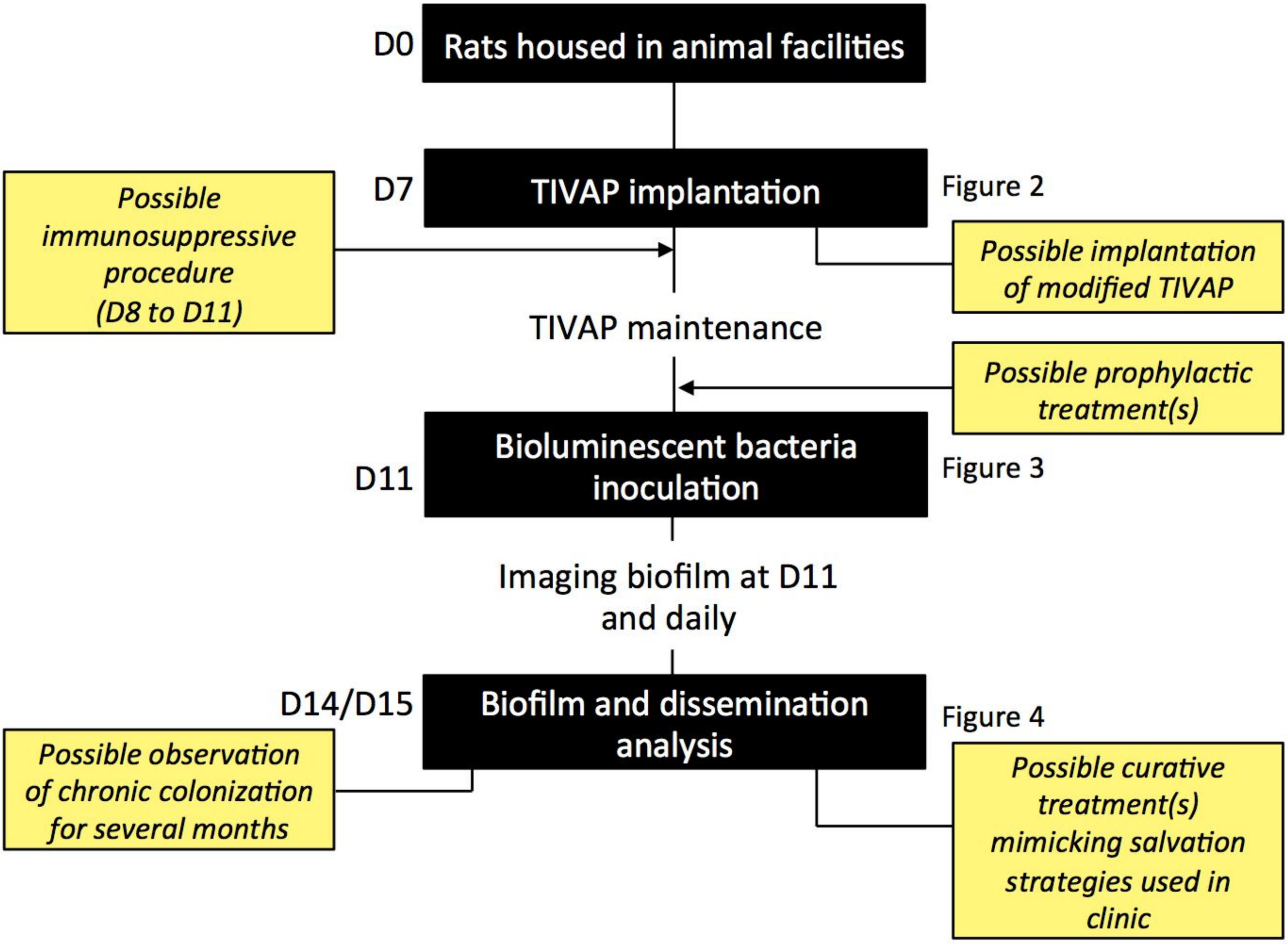
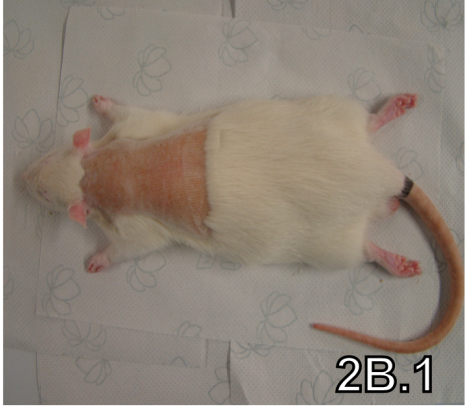


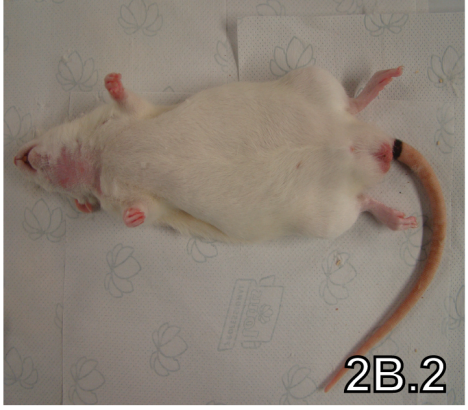
Figure 2

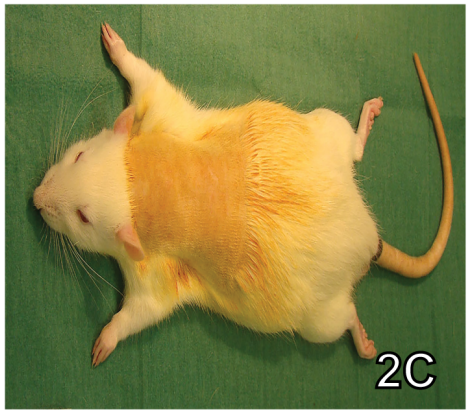
Figure 3

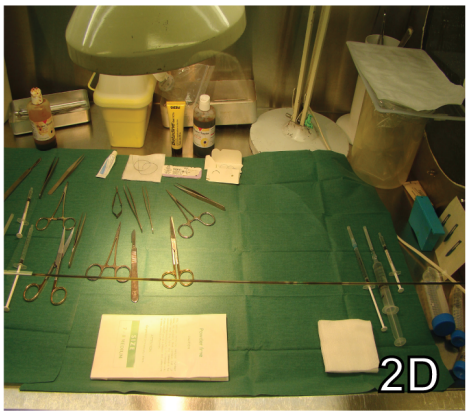
Figure 4

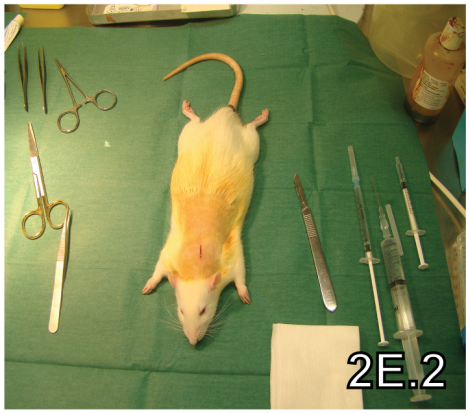
Figure 1

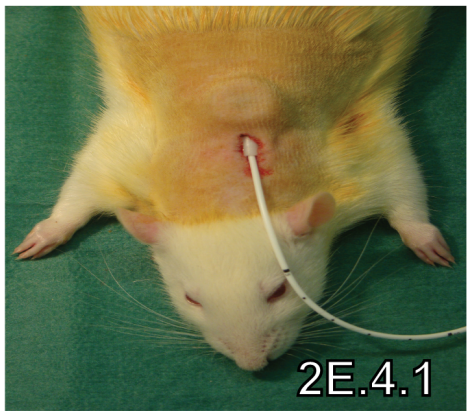


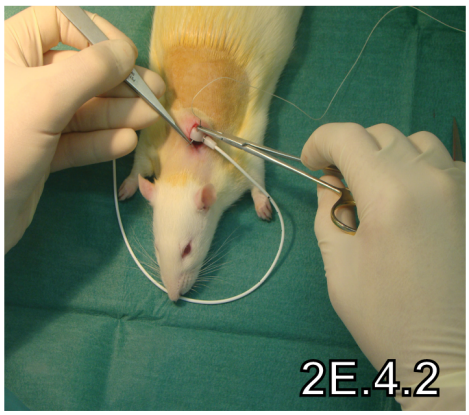


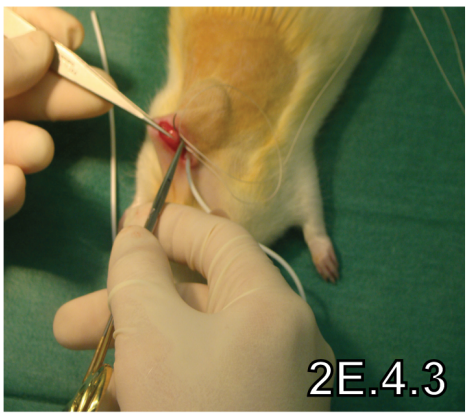


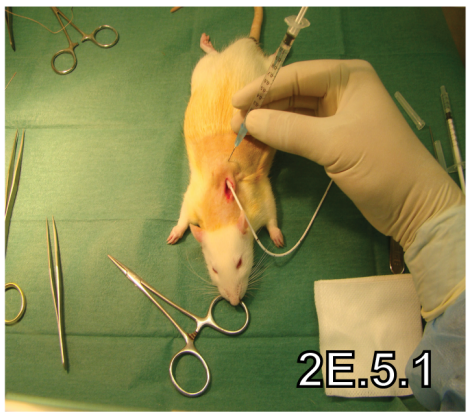


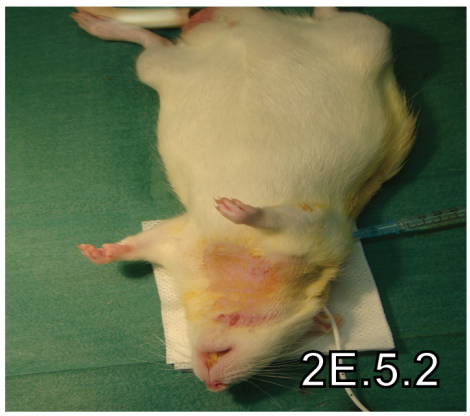


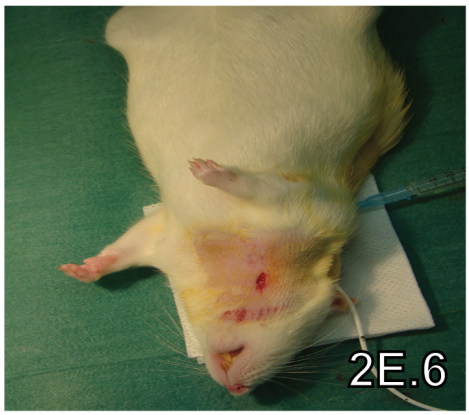


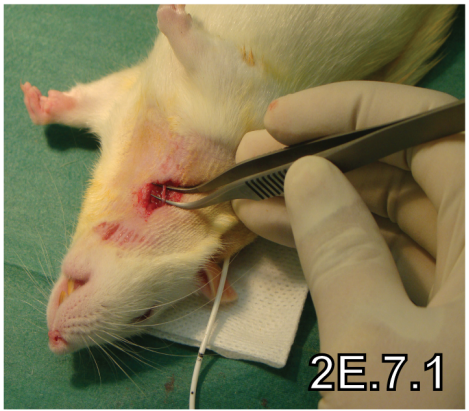












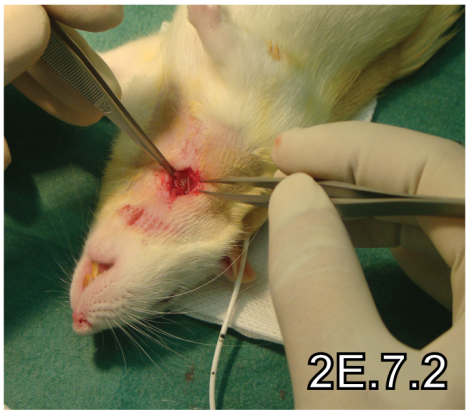
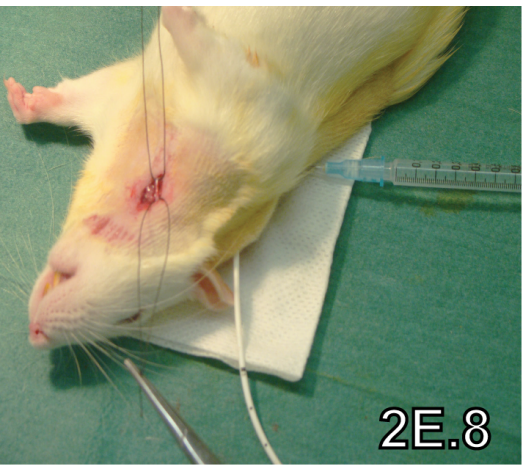


Figure 2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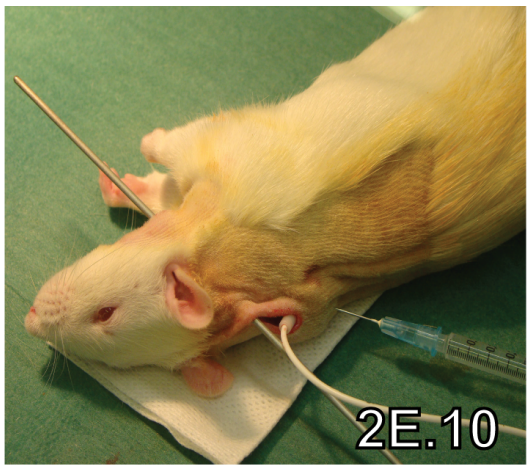
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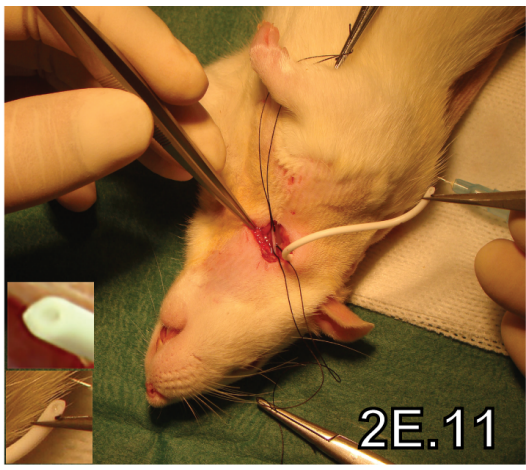
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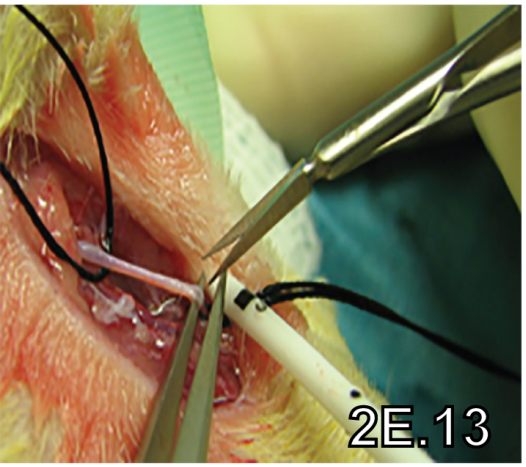
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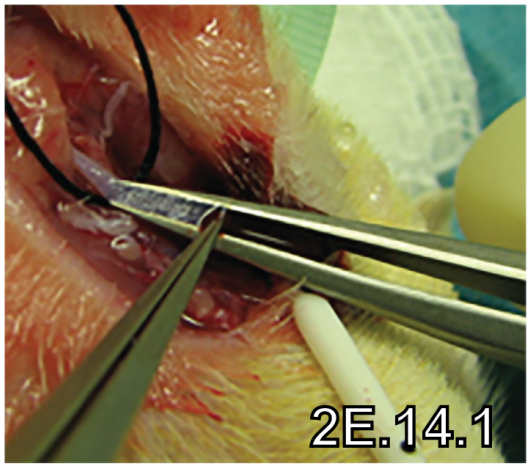
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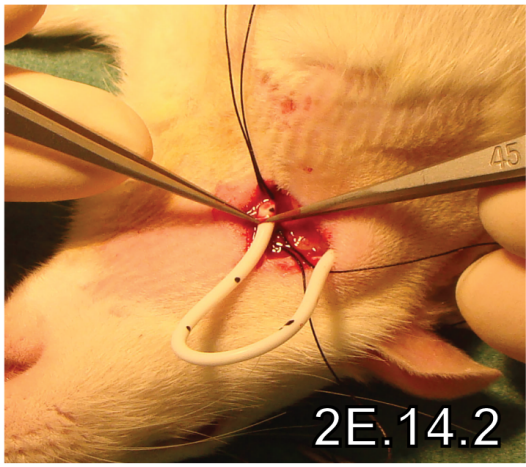
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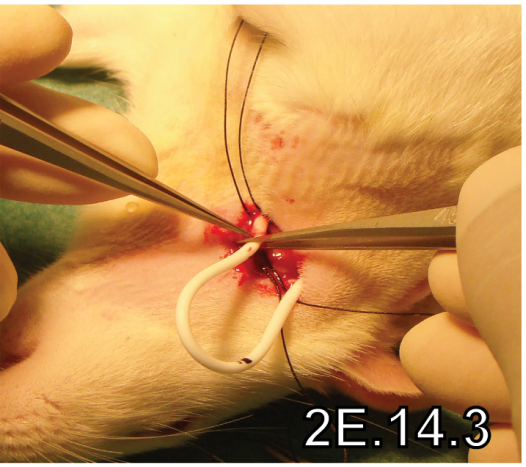
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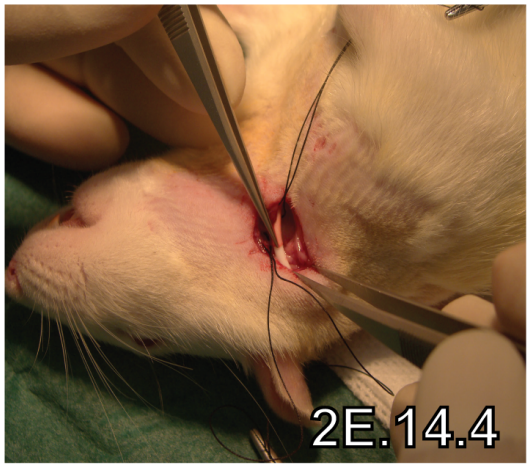
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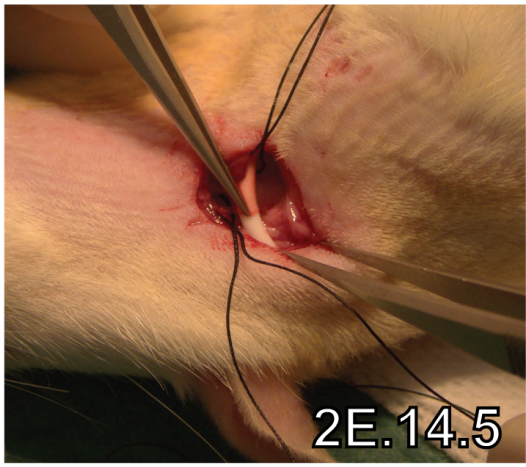
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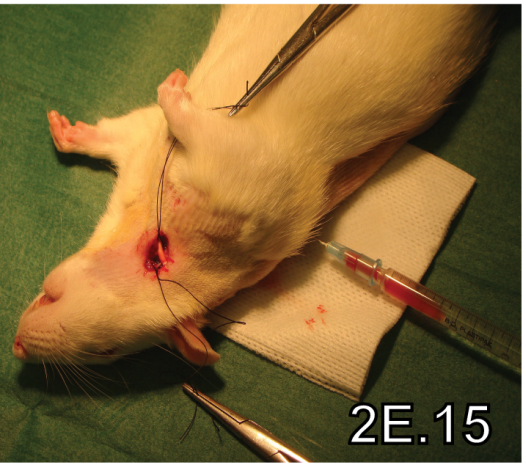
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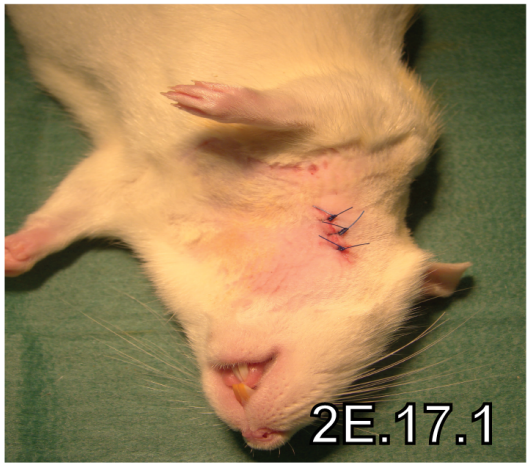

2E.17.1

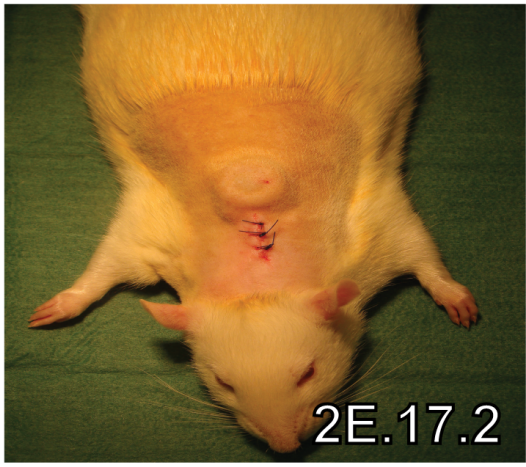
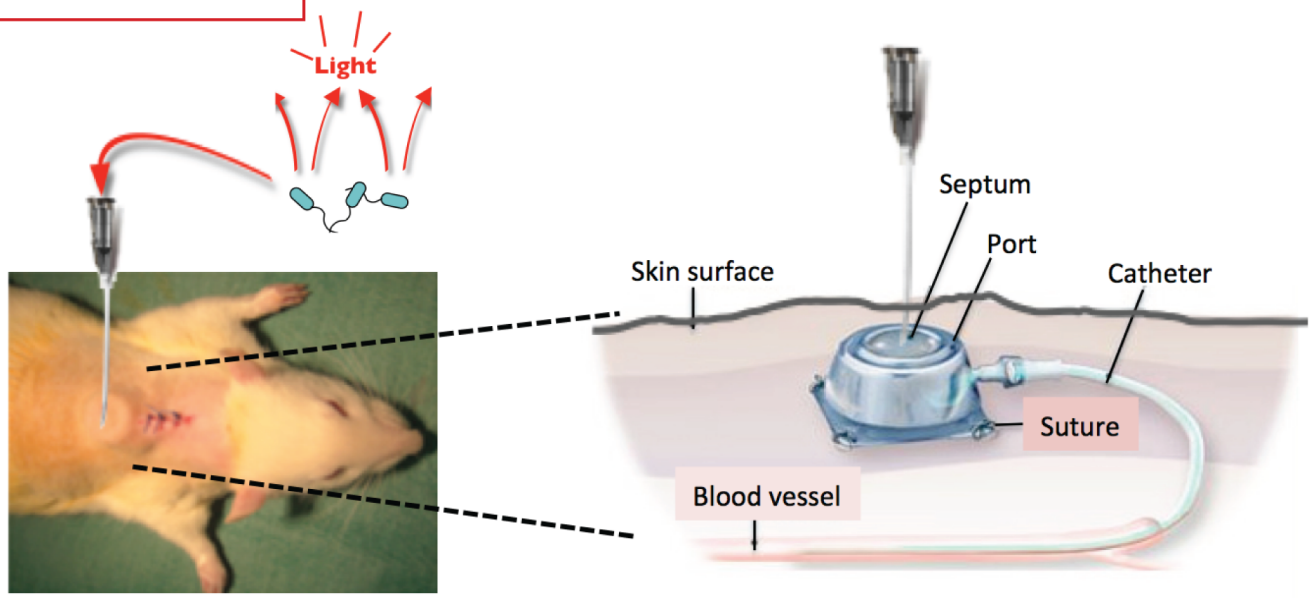

2E.17.2

Figure 3

Controlled Inoculation



Non invasive *in situ* monitoring of biofilm development using CCD imaging system

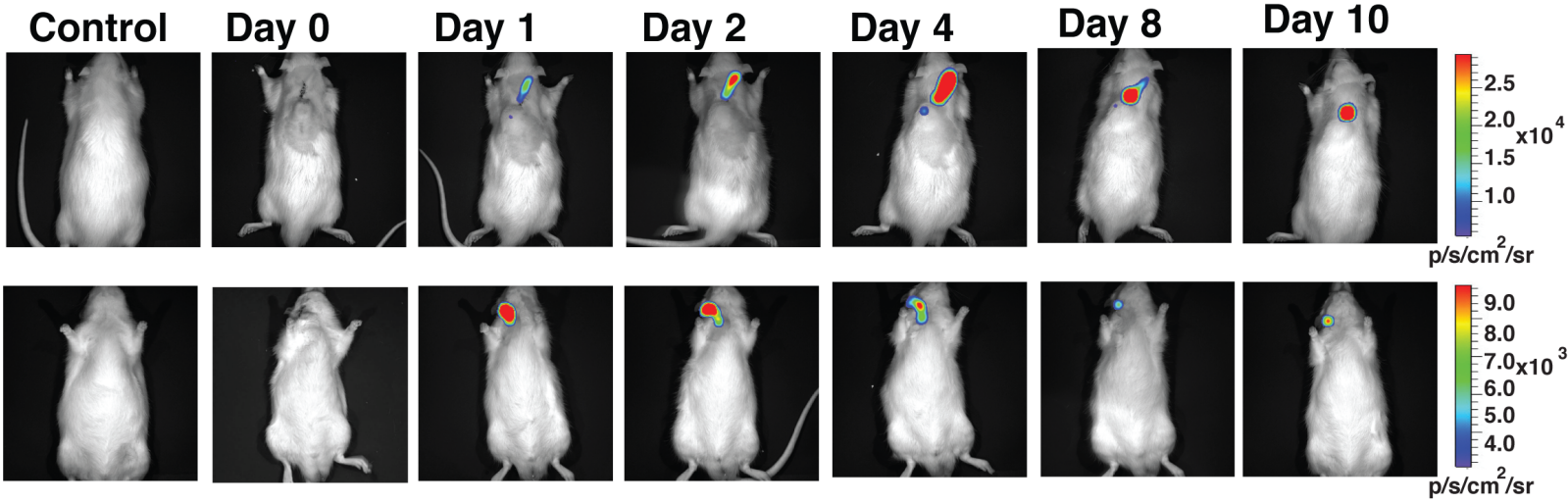


Figure 4

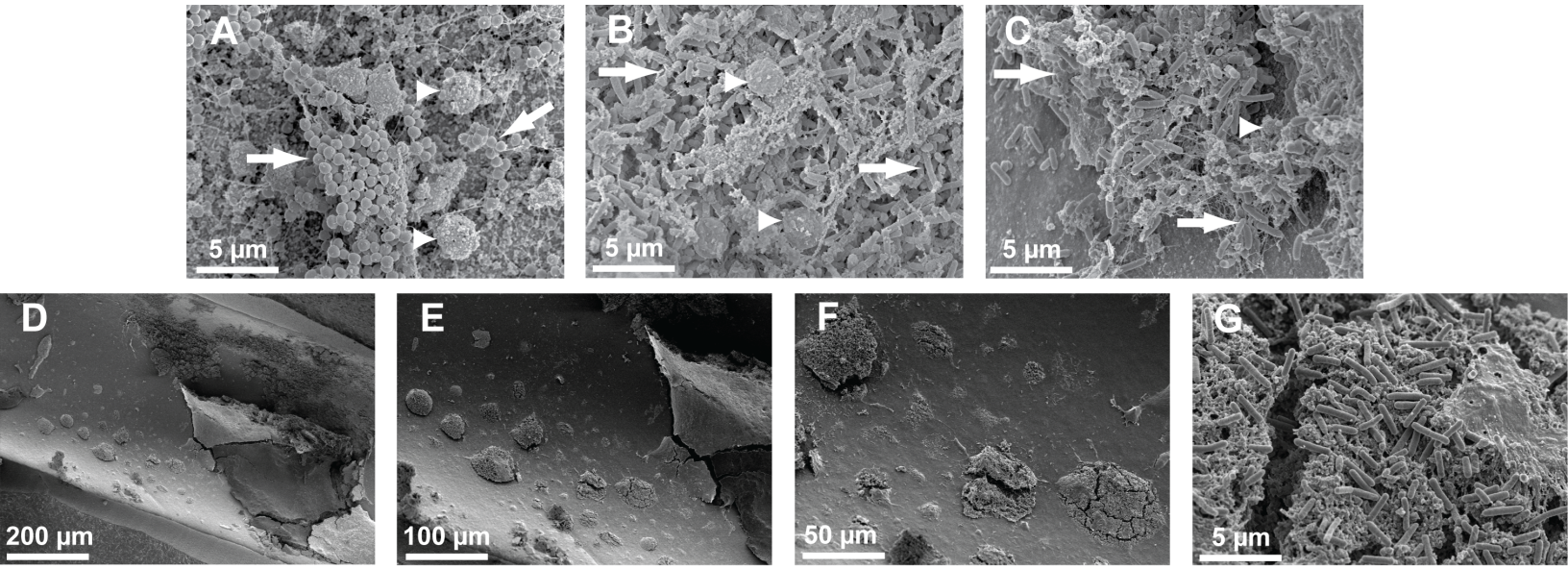
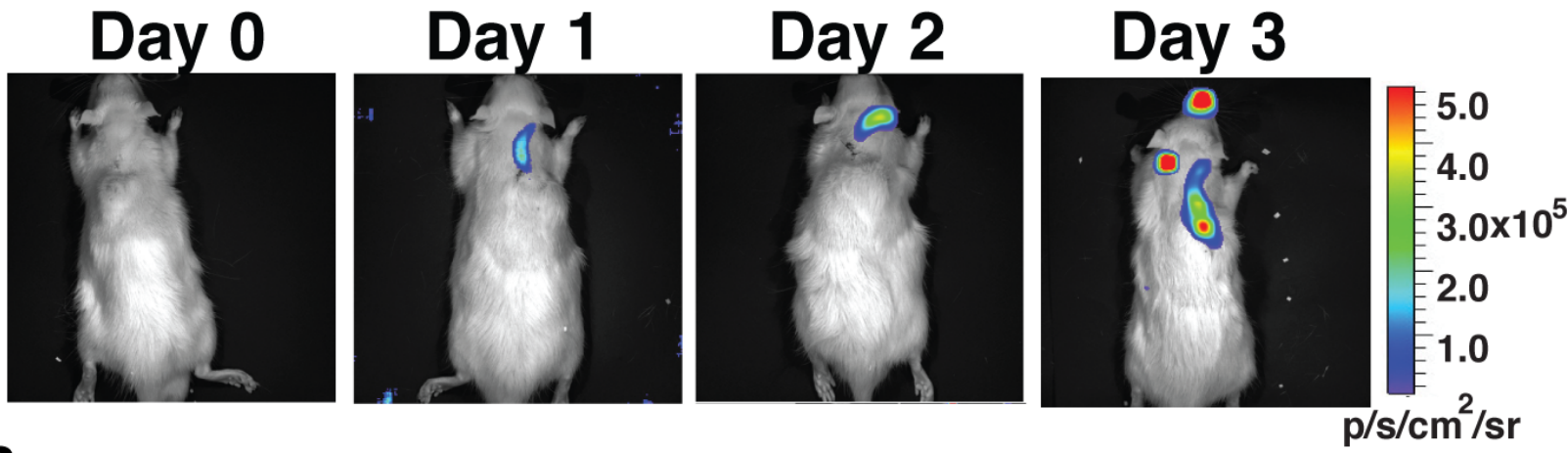


Figure 5

A


Day 0
Day 1
Day 2
Day 3
5.0
4.0
3.0x10^5
2.0
1.0
p/s/cm^2/sr

B

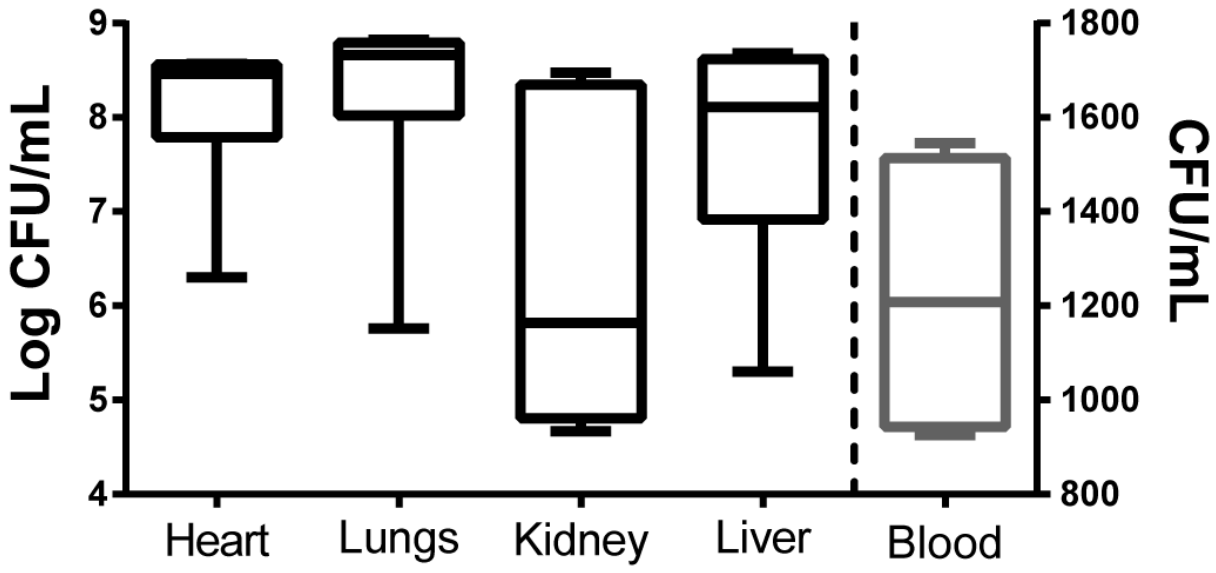
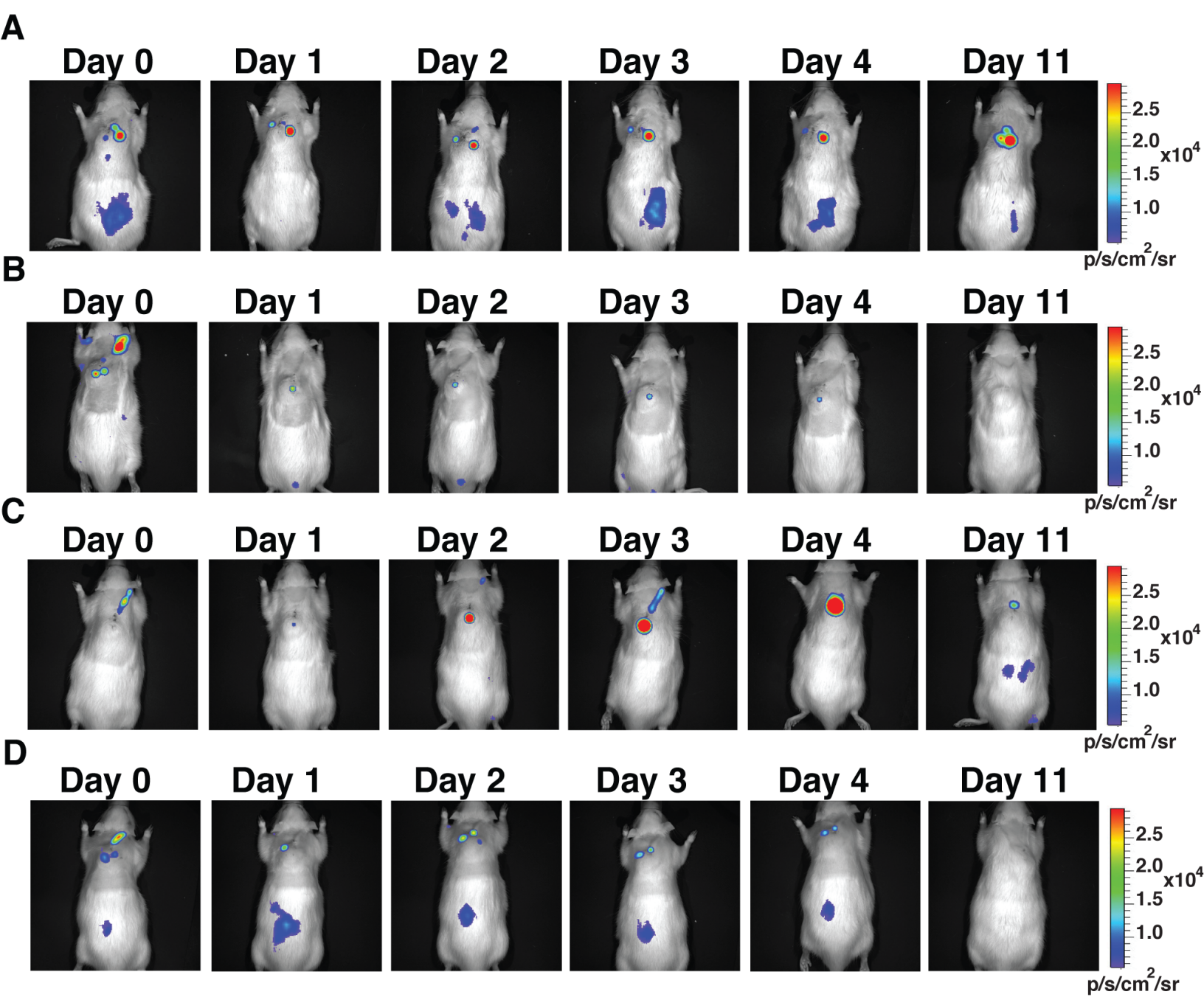
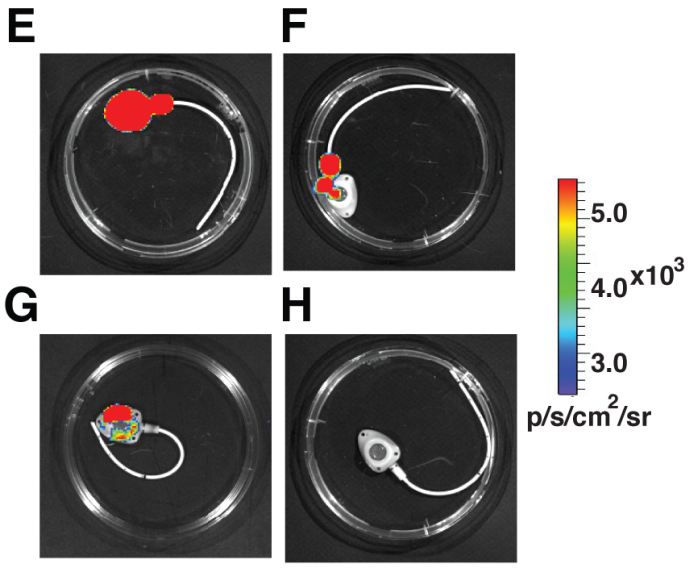

Log CFU/mL
9
8
7
6
5
4
1800
1600
1400
1200
1000
800
CFU/mL
Heart
Lungs
Kidney
Liver
Blood

Figure 6


A
Day 0
Day 1
Day 2
Day 3
Day 4
Day 11
2.5
2.0
x10^4
1.5
1.0
p/s/cm^2/sr
B
Day 0
Day 1
Day 2
Day 3
Day 4
Day 11
2.5
2.0
x10^4
1.5
1.0
p/s/cm^2/sr
C
Day 0
Day 1
Day 2
Day 3
Day 4
Day 11
2.5
2.0
x10^4
1.5
1.0
p/s/cm^2/sr
D
Day 0
Day 1
Day 2
Day 3
Day 4
Day 11
2.5
2.0
x10^4
1.5
1.0
p/s/cm^2/sr


E
F
G
H
5.0
4.0
x10^3
3.0
p/s/cm^2/sr

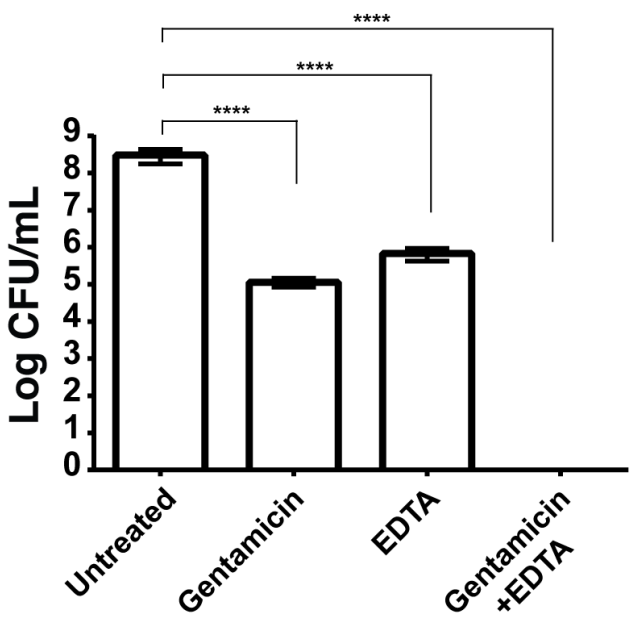
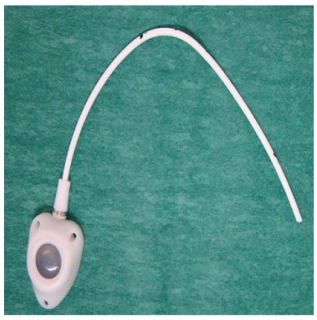
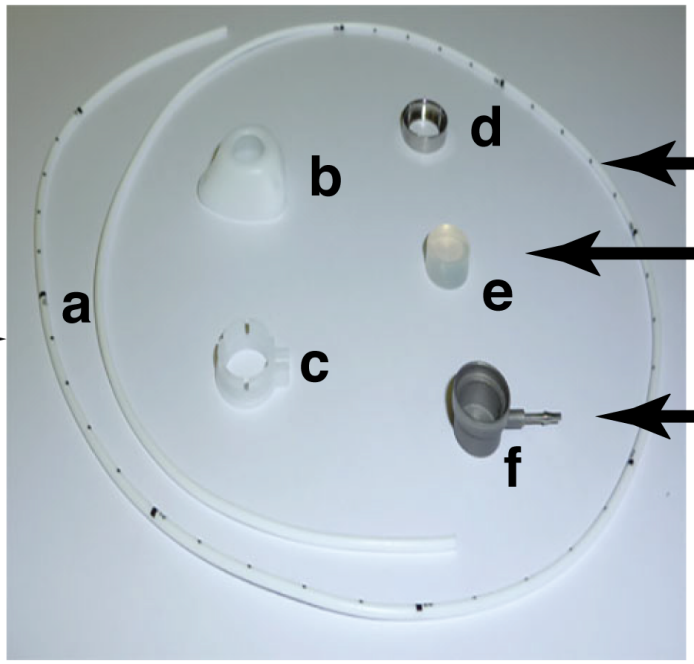

I
****
****
****
Log CFU/mL
9
8
7
6
5
4
3
2
1
0
Untreated
Gentamicin
EDTA
Gentamicin +EDTA

Figure 7

A




a
b
c
d
e
f
Methyl Cellulose
(MeCe)
Poly Ethylene Glycol
(PEG)

B

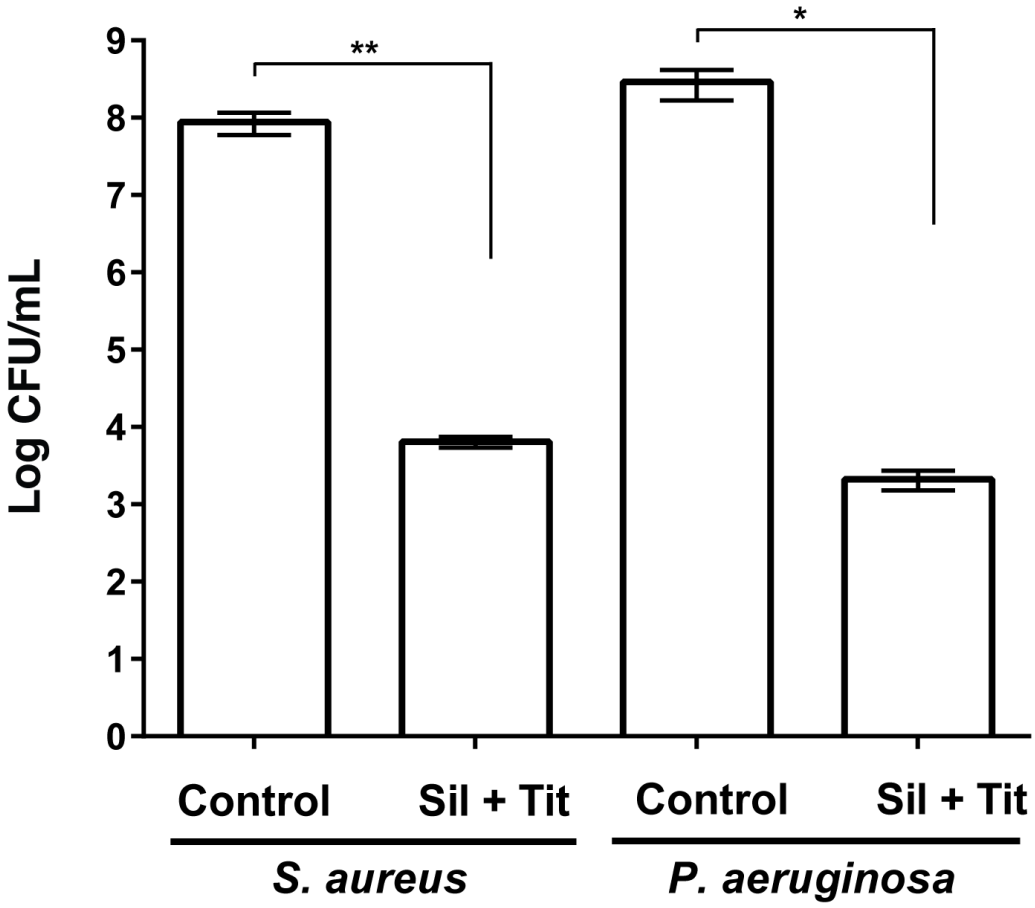

Log CFU/mL
0
1
2
3
4
5
6
7
8
9
**
*
Control
Sil + Tit
Control
Sil + Tit
S. aureus
P. aeruginosa

Figure 8