Video Article

Candida albicans Biofilm Development on Medically-relevant Foreign Bodies in a Mouse Subcutaneous Model Followed by Bioluminescence Imaging

Soňa Kucharíková[1], Greetje Vande Velde[2], Uwe Himmelreich[2], Patrick Van Dijck[1]

[1]Department of Molecular Microbiology, Laboratory of Molecular Cell Biology, Institute of Botany and Microbiology, VIB, KU Leuven

[2]Biomedical MRI Unit/ MoSAIC, Department of Imaging & Pathology, KU Leuven

Correspondence to: Patrick Van Dijck at Patrick.VanDijck@mmbio.vib-kuleuven.be

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Abstract

Candida albicans biofilm development on biotic and/or abiotic surfaces represents a specific threat for hospitalized patients. So far, *C. albicans* biofilms have been studied predominantly *in vitro* but there is a crucial need for better understanding of this dynamic process under *in vivo* conditions. We developed an *in vivo* subcutaneous rat model to study *C. albicans* biofilm formation. In our model, multiple (up to 9) *Candida*-infected devices are implanted to the back part of the animal. This gives us a major advantage over the central venous catheter model system as it allows us to study several independent biofilms in one animal. Recently, we adapted this model to study *C. albicans* biofilm development in BALB/c mice. In this model, mature *C. albicans* biofilms develop within 48 hr and demonstrate the typical three-dimensional biofilm architecture. The quantification of fungal biofilm is traditionally analyzed *post mortem* and requires host sacrifice. Because this requires the use of many animals to perform kinetic studies, we applied non-invasive bioluminescence imaging (BLI) to longitudinally follow up *in vivo* mature *C. albicans* biofilms developing in our subcutaneous model. *C. albicans* cells were engineered to express the *Gaussia princeps luciferase* gene (*gLuc*) attached to the cell wall. The bioluminescence signal is produced by the luciferase that converts the added substrate coelenterazine into light that can be measured. The BLI signal resembled cell counts obtained from explanted catheters. Non-invasive imaging for quantifying *in vivo* biofilm formation provides immediate applications for the screening and validation of antifungal drugs under *in vivo* conditions, as well as for studies based on host-pathogen interactions, hereby contributing to a better understanding of the pathogenesis of catheter-associated infections.

Video Link

The video component of this article can be found at http://www.jove.com/video/52239/

Introduction

Candida albicans is a commensal organism, which can be found at different sites of healthy individuals, for example on the skin or as a part of the gastrointestinal and vaginal flora. However, in hospitalized, and especially immunocompromised patients, it may cause a wide range of infections [1]. In such individuals, the weakened immune system allows *Candida* cells to disseminate into the bloodstream and to invade deeper tissues causing life-threatening infections. In addition, the presence of abiotic substrates such as central venous and urinary catheters, artificial heart valves and joints may provide a niche for *Candida* attachment [2]. Adhesion to such substrates is a prerequisite for further biofilm development, which represents a layer of yeast and hyphal cells embedded in extracellular polymeric material, mainly consisting of polysaccharides [2]. *C. albicans* catheter –associated infections are associated with high mortality rate. A general characteristic of biofilms is their decreased susceptibility to known antifungals, such as azoles [3,4]. Only newer classes of antifungal drugs, such as echinocandins and liposomal formulation of amphotericin B proved to be active against catheter-associated infections [5-7]. Because of biofilm resilience to antifungals, therapeutic approaches are very limited, often leading to catheter removal and its subsequent replacement as a sole solution.

Most of our current understanding of *C. albicans* biofilm development originates from *in vitro* studies on abiotic substrates such as polystyrene, or plastics used for the manufacture of above-mentioned devices, *i.e.,* silicone, polyurethane [2]. These models are quite advanced and try to mimic the situation *in vivo* as closely as possible. However, these systems do not involve the continuous blood flow and the immune system of the host. This resulted in the development of *in vivo* model systems, such as the central venous catheter (CVC) model [8-10], the denture stomatitis model of oral candidiasis [11] and a murine model for catheter-associated candiduria [12]. Additionally, *C. albicans* biofilm development was studied *in vivo* on the mucosal surfaces, such as those from the vagina [13] and oral cavity [14]. Our laboratory contributed with the establishment of a subcutaneous *C. albicans* biofilm model, which is based on the implant of infected catheter pieces on the back of Sprague Dawley rats [15]. This model was successfully used in our laboratory to test biofilm susceptibility to fluconazole and echinocandin drugs [5,16], to study the effect of combinatorial therapy of diclofenac and caspofungin [17]. More recently, we adapted this system for use in BALB/c mice [18,19]. In comparison with other *in vivo* models, the main advantage of this subcutaneous model is the possibility to study multiple biofilms per animal developed inside the lumen of implanted catheter pieces.

To reduce the number of laboratory animals, we have adapted this model to study the development of *C. albicans* biofilms non-invasively by using bioluminescence imaging (BLI) [18,19]. This method proved to be a powerful technique, which can be used to quantify biofilms by measuring the specific BLI signal at the region of interest (in our case the area of implanted catheters), avoiding animal sacrifice. In comparison to bacteria, which can express both the gene and the substrate required for the bioluminescence reaction due to the introduction of a specific *lux* operon [20], most of the eukaryotic organisms, including *C. albicans,* are dependent on the heterologous expression of a luciferase gene coupled with the external administration of a specific substrate, such as D-luciferin or coelenterazine [21]. Probably due to the presence of the fungal cell wall and *C. albicans* morphogenesis, the intracellular delivery of the substrate for the luciferase enzyme was a main challenge [21]. In order to solve this problem, Enjalbert *et al.* [22] engineered a strain where a synthetic *C. albicans* codon-optimized version of the gene for the naturally secreted *Gaussia princeps* luciferase (*gLuc*) was fused to to the *C. albicans PGA59* gene, a GPI- anchored cell wall protein. Because of the presence of luciferase at the cell wall, problems concerning the intracellular availability of the substrate could be avoided. This particular system was used to study superficial infections caused by *C. albicans* [22]. Very recently, BLI was also used to follow the progression of oropharyngeal candidiasis and its possible treatment [23]. Such findings support the use of BLI as a promising technique to study infections caused by free-living cells but also device-associated infections.

In this study, we describe the *C. albicans* biofilm development on polyurethane catheter pieces in BALB/c mice and its quantification using BLI. We provide a detailed protocol of *in vitro* colonization of polyurethane catheters during the period of adhesion followed by implantation in mice and subsequent biofilm development in live animals. Apart from measuring the BLI signal emitted by the *C. albicans* cells, we also determine the colony forming units for comparison with the standard technique for biofilm fungal load quantification.

Protocol

NOTE: All animal experiments were approved by the ethical committee of KU Leuven (project number 090/2013). Maintain animals in accordance with the KU Leuven animal care guidelines.

1. *C. albicans* Growth

1. Twenty-four hours before the initiation of the animal experiment, prepare YPD plates by adding 10 g of yeast extract granulated, 20 g of bacteriological peptone, and 15 g of granulated agar. Make up volume to 900 ml with Milli-Q water and autoclave.
2. Add 50 ml of sterile 40% glucose. Mix thoroughly and pour into Petri dishes. Leave agar plates to cool and solidify.
   NOTE: In this study, use two strains, namely wild type *C. albicans* SC5314 *–gLuc* negative strain *(referred to as WT)and C. albicans* SKCA23 strain, which is a wild type *C. albicans* SC5314 transformed with Clp10::Act1p-gLUC59 plasmid [22].In this strain (named SKCA23-*ACTgLuc*) *gLuc* was fused to the endogenous *PGA59* gene under the control of *ACT1* (actin) promoter (this promoter is active in the yeast, as well as hyphal stage of fungal growth). This strain can be requested from the laboratory of Prof. Patrick Van Dijck, KU Leuven, Leuven, Belgium. Plasmid Clp10::Act1p-gLUC59 plasmid [22] was kindly donated by Prof. C. d'Enfert, Institute Pasteur, Paris, France.
3. Maintain both strains in glycerol stock and store at -80 °C.
4. Prior to any experiment streak strains onto an YPD plate. Incubate plate at 37 °C overnight.

2. Catheter Pieces Preparation

1. Before the experiment, determine how many catheters are needed. It is possible to implant up to 6 catheter pieces per mouse (3 catheters on the left and 3 catheter on the right side of the animal (**Figure 2A**).
2. Twenty-four hr prior to the animal surgery, open the package containing triple-lumen catheter under the biological safety cabinet. Remove all unnecessary parts with sterile tweezers and cut the part attached to the catheter with a sterile scalpel. Place ruler under the plastic package and cut polyurethane catheter pieces of exactly 1 cm according to the scale on the ruler **(Figure 1**).
   NOTE: It is important to mention that this type of catheter piece is not phosphorescent and therefore suitable for BLI [18,19].
3. Place a maximum of 15 cut catheter pieces (step. 2.2) into 2 ml microcentrifuge tubes. Always prepare 3 pieces extra, which are used to enumerate the amount of attached *Candida* cells on the device after the period of adhesion.
4. Supplement catheters with approximately 1.8 ml of 100% fetal bovine serum (FBS).
5. Vigorously vortex and add additional 100-200 µl of 100% FBS. Completely cover all catheter pieces with serum.
6. Incubate at 37 °C overnight.

3. Animals and Suppression of the Immune System

1. Keep female BALB/c mice (approximately 8 weeks of age) in individually ventilated filter top cages with free access to standard food and water *ad libitum*.
2. Initiate suppression of the immune system 24 hr before animal surgery by adding dexamethasone (0.4 mg/L) to the drinking water of animals. In order to avoid any bacterial contamination of the host, supplement the drinking water with antibiotic, *e.g.*, ampicillin sodium powder (0.5 g/L).
3. Keep immunosuppression of animals during the entire experiment (up to 6 days).

4. *Ex vivo C. albicans* Adhesion on FBS-coated Polyurethane Substrates

1. Transfer each serum-coated catheter piece into a fresh 1.5 ml microcentrifuge tube.
2. Scrape off some *C. albicans* cells grown on YPD plates (Step 1) and suspend them in 1 ml of PBS.
3. Dilute *Candida* cells (1:100) in a separate microcentrifuge tube. Take 10 µl of the diluted sample and apply it on a cell counting chamber. Count at least 16 small squares.
4. Prepare *Candida* cells (each strain separately) in RPMI 1640 medium to a final concentration of 5 x 10^4 cells/ml.

5. Add 1 ml of cell suspension to each serum-coated catheter piece.
6. Vigorously vortex and ensure that the catheters are submerged in the medium and not floating on top.
7. Incubate catheters at 37 °C for 90 min (period of adhesion).
8. Remove catheters with sterile tweezers and wash them twice with 1 ml of PBS. During this step make sure that the washing fluid goes through the lumen by keeping the catheter in vertical position while very gently flushing the catheters. Importantly, do not use a strong flow which may lead to the removal of attached cells.
9. Transfer each washed catheter to a clean microcentrifuge tube (one piece per tube).
10. Place on ice and keep there until surgery.

5. Anesthesia

1. Prepare anesthesia by mixing 75 µl of ketamine (100 mg/ml) with 100 µl of medetomidine (1 mg/ml) and 825 µl of sterile saline. Administer intraperitoneally (i.p.) 60-80 µl of anesthetic cocktail per 10 g body weight, resulting in a dose of 45-60 mg/kg ketamine and 0.6-0.8 mg/kg medetomidine.
2. For reversal of anesthesia, dilute 50 µl atipamezole (5 mg/ml) in 4.95 ml saline, administer i.p. 100 µl per 10 g body weight as antidote, resulting in a dose of 0.5 mg/kg.
3. After injection of anesthesia, place the animal into a separate cage and wait until it is fully asleep.
4. Confirm proper anesthetization of the animal by light skin pinch and toe pinch, which do not cause any damage to the skin. Any observed movement indicates that the animal is not sufficiently anesthetized to perform surgery. If this happens, wait a couple of minutes longer until the animal does not show any signs of movement upon skin or toe pinch.

6. Animal Surgery

1. Transfer anesthetized animal from the cage on a clean tissue placed on the heating pad, pre-warmed to 37 °C (**Figure 2A**, (1)).
   NOTE: A cheaper alternative is to use electrically heated blankets. It is also possible to use isothermic pads, which must be warmed up in the microwave prior to the animal surgery.
2. Apply ophthalmic ointment on the eyes.
3. Shave the lower back of the animal with an electric razor. Remove all animal hairs and transfer animal on a clean tissue. Disinfect the skin (*e.g.*, with 1% iodine isopropanol or 0.5% chlorhexidine in 70% alcohol) and leave the disinfected area to dry for approximately 1 min (**Figure 2A**, (2)).
4. Make a small incision in the skin (one on the left and one on the right side of the animal) (approximately 0.5–1 cm) (**Figure 2A**, (3)).
5. Dissect the subcutis with a scissor to create two subcutaneous tunnels. Each tunnel should be approximately 1.5 cm long and 1 cm wide.
6. Insert three catheter pieces, previously infected with *Candida*, in each tunnel. Ensure that the catheters lie next to each other in a horizontal arrangement and that they do not cover each other to enable the implantation of six catheter fragments in total (**Figure 2A**, (4)).
7. Close the incisions with sutures. Alternatively, use Dermabond to close the wound.
8. Disinfect the wound very gently with 0.5% chlorhexidine in 70% alcohol or with iodine isopropanol (1%).
9. Apply local anesthetic (xylocaine gel, 2%) directly on the wound.
10. Administer reversal of anesthesia (protocol 5, step 2): intraperitoneally 100 µl per 10 g body weight.
11. Transfer animal to a clean cage previously placed on a heating plate. Keep the animal separate and warm until the animal is completely awake. Meanwhile, continue with the operation and implant of the next animal. Once all operated animals are fully awake. Transfer to one cage. Monitor animals regularly.

7. Bioluminescence Imaging: Preparation of Coelenterazine (CTZ), the Substrate for *G. princeps luciferase*

1. Prepare fresh coelenterazine (CTZ) stock solution by dissolving 5 mg/ml CTZ in acidified ethanol or according to manufacturer's instructions.
2. Prepare 1.2 mM working solution by diluting the stock solution 1:10 in sterile PBS.
   NOTE: Inject 100 µl CTZ working solutions subcutaneously in the area surrounding the catheters. Use insulin syringes for subcutaneous injection of CTZ.
3. Always keep CTZ in the dark (*e.g.*, cover microcentrifuge tubes containing CTZ with aluminum foil). Store the stock solution at -80 °C for the duration of the experiments.

8. Bioluminescence Imaging

1. Initialize the BLI camera.
2. Anesthetize the animals using an induction box. Use a gas mixture of isoflurane in oxygen, N_2O/ O_2, or air at 2-3%.
3. After induction, maintain anesthesia in the induction box and in the imaging chamber at 1.5-2%.
4. Before starting the imaging session, place the imaging plate in position A, which corresponds to a FOV of 10 cm. Ensure that the right position of the anesthesia outlets and animal by placing a sleeping animal in the box.
5. Take few photographs until animal is in the desired imaging position, in the FOV right under the camera.
6. Prepare two insulin syringes each containing 100 µl of the CTZ working solution.
7. Place one animal on the bench and keep it asleep by means of a nose cone providing gas anesthesia.
8. Bring the needles of the syringes in a place surrounding catheters subcutaneously and inject the CTZ simultaneously on top of the catheters.
9. Immediately after injection, place the animal on the warm plate in the camera box and start the bioluminescence image acquisition.
10. Acquire consecutive scans with acquisition times ranging from 20 to 60 sec (depending on the signal intensity) until the maximum signal intensity is reached. During the acquisition of the next frame, measure the BLI signal intensity of the previously acquired frames by placing a ROI over each catheters trio and measuring the photon flux through this ROI.

11. Repeat from step 7 for the next animal(s).
12. After imaging, return animals to their cage. Repeat BLI during the course of an experiment for longitudinal, non-invasive follow-up of biofilm formation.
13. Analyze the BLI data using Living Image software. Place a rectangular ROI of fixed size over each catheter trio and measure the photon flux (radiance) through each ROI. Repeat this for every animal.
14. Report the BLI signal intensity of each catheter trio as photon flux per second. Represent the BLI data on a logarithmic scale by plotting the mean and SD of the photon flux per second for each group. Perform statistical analysis on the $\log_{10}$ transformed data.

9. Catheter Explant

1. Prepare microcentrifuge tubes containing 1 ml of PBS, one for each catheter device and keep them on ice.
2. Euthanize the animals by cervical dislocation (**Figure 2B**, (1)).
3. Disinfect the skin of the back with 0.5% chlorhexidine in 70% alcohol or with iodine isopropanol (1%).
4. Make an incision (approximately 3 cm) above the catheters.
5. Cut subcutaneous tissue and remove the catheter fragments one by one from under the subcutaneous tissue using sterile tweezers (**Figure 2B**, (2)).
6. Handle catheter gently in vertical position and wash it twice with 1 ml of sterile PBS. Place each catheter piece into a separate microcentrifuge tube (prepared in Step 1).

10. Quantification of Biofilm-associated Cells by Colony Forming Units Count (CFUs) and Statistical Analyses of Results

1. Sonicate catheters previously placed into 1 ml of PBS for 10 min at 40,000 Hz in a water bath sonicator and place them on ice.
2. After sonication, vigorously vortex for 30 sec and place again on ice.
3. Prepare two additional microcentrifuge tubes containing 900 µl of PBS.
4. Make 1:10 and 1:100 dilutions from your original sample (the one which contains the catheter) and keep all microcentrifuge tubes on ice.
5. Plate 100 µl of the original samples, 1:10 and 1:100 dilutions on YPD agar plates in duplicate.
6. Incubate plates for 2 days at 37 °C and count CFUs.
7. Count the colonies grown on YPD agar plates (countable amount of colonies, maximum 300 colonies/plate) and multiply them by the proper dilution factor (1x-original, 10x or 100x dilution). Further multiply each catheter piece by 10x, which corresponds to the final amount of colonies in 1 ml tube containing the catheter. Bring the final amount of colonies to $\log_{10}$ cells/catheter piece with standard deviation (SD). Express data as a mean ± SD.
8. Place all values for each catheter and specific group into a spreadsheet and/or statistical program, to perform the above-mentioned calculations and statistical analyses. Indicate specific groups to compare, *i.e.* WT *vs.* mutant or 2 days *vs*. 6 days biofilm formation and perform unpaired t-test and ANOVA with Tukey post-test on the log10-transformed data.
    1. Express the level of significance by a *p* value. In this study indicate significance if *p* value <0.05, represented as follows: * *p* <0.05, ** *p* <0.005, *** *p* <0.0005.

Representative Results

In this study, we show the surgical procedure of catheter implant and explant during *in vivo C. albicans* biofilm development in a mouse. Moreover, we display the quantification of mature biofilms not only by classical CFUs enumeration but also by BLI.

As shown in **Figure 1A**, non-phosphorescent polyurethane catheter pieces were cut into 1 cm devices and subsequently coated with serum. This step is very important because it allows *Candida* cells to attach to the substrate more rapidly in comparison with non-serum coated implants [15]. It is important to mention that prior to any *C. albicans* experiment we first documented the background luminescence of our devices [18]. This step is crucial before performing any BLI because high phosphorescence of the device will interfere with the evaluation of the specific bioluminescent signal intensity and signal kinetics from *gLuc*-expressing cells. Next, *C. albicans* cells are incubated with the serum-coated catheter pieces. Catheter fragments are then implanted on the back part of the animal after the subcutaneous incision (**Figure 2A**). In this *in vivo* set up, two incisions are performed (one on the right and another one on the left side of the back of a mouse) followed by formation of subcutaneous tunnels inside each incision (**Figure 2A** (3)). Subsequently, three devices, previously infected with *Candida* cells, are implanted inside each operation site (**Figure 2A** (3 and 4)). This set up allows us to study up to six biofilms per animal. It is noteworthy that two operation sites provide a possibility to study biofilm formation by two different strains of interest, for example wild type and mutant in the same animal. **Figure 2B** displays the wound containing catheters prior to the device explant and subsequent washing steps. Biofilms developed inside the back part of the animal were assessed for the bioluminescence signal measurements. One of the representative animals displaying the bioluminescence signal together with rectangles characterizing the regions of interest (ROIs) is shown in **Figure 3**. After the last BLI time point, catheters are explanted, sonicated and subsequently vortexed and further assessed for the biofilm-forming cells quantification by CFUs. Data analyses demonstrating the Log_{10} CFUs obtained from each catheter piece after biofilm development and explantation are shown in **Figure 4A**. Next to that, CFUs were compared with the BLI signal intensity and these data are shown in **Figure 4B**. Ninety min after implantation a clear BLI signal is produced by the *ACTgLuc*-expressing biofilms whereas there in the wild type strain, hardly any light is produced; The intensity of the light detected from the *ACTgLuc*-expressing biofilms is significantly increasing as the biofilm is formed, here following the same mouse (**Figure 4C**). This increase in light follows the same trend as the increase in CFUs per biofilm (**Figure 4A**). In our experiments we also observed that a normal wild type strain (not engineered to express luciferase) also results in the production of light upon addition of the substrate. However, the photon flux was significantly lower than that obtained for the engineered strain.

Taken together, our data illustrate that BLI is a powerful technique to monitor and quantify *in vivo* mature *C. albicans* biofilm formation in a subcutaneous mouse model.

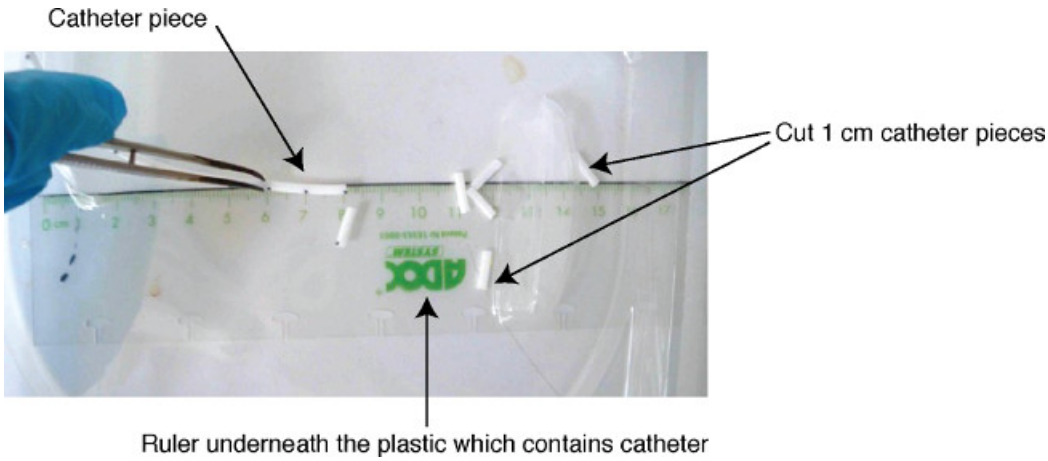

Figure 1: Preparation of polyurethane devices. Polyurethane part of the catheter cut into 1 cm pieces. Plastic pocket containing catheter is open under the sterile conditions and all parts, except catheter are removed from the package. Secondly, place ruler under the plastic pocket and cut exactly 1 cm polyurethane pieces. Such devices are subsequently distributed to microcentrifuge tubes (max 15 pieces/tube) and submerged in 100% fetal bovine serum followed by overnight incubation at 37 °C.

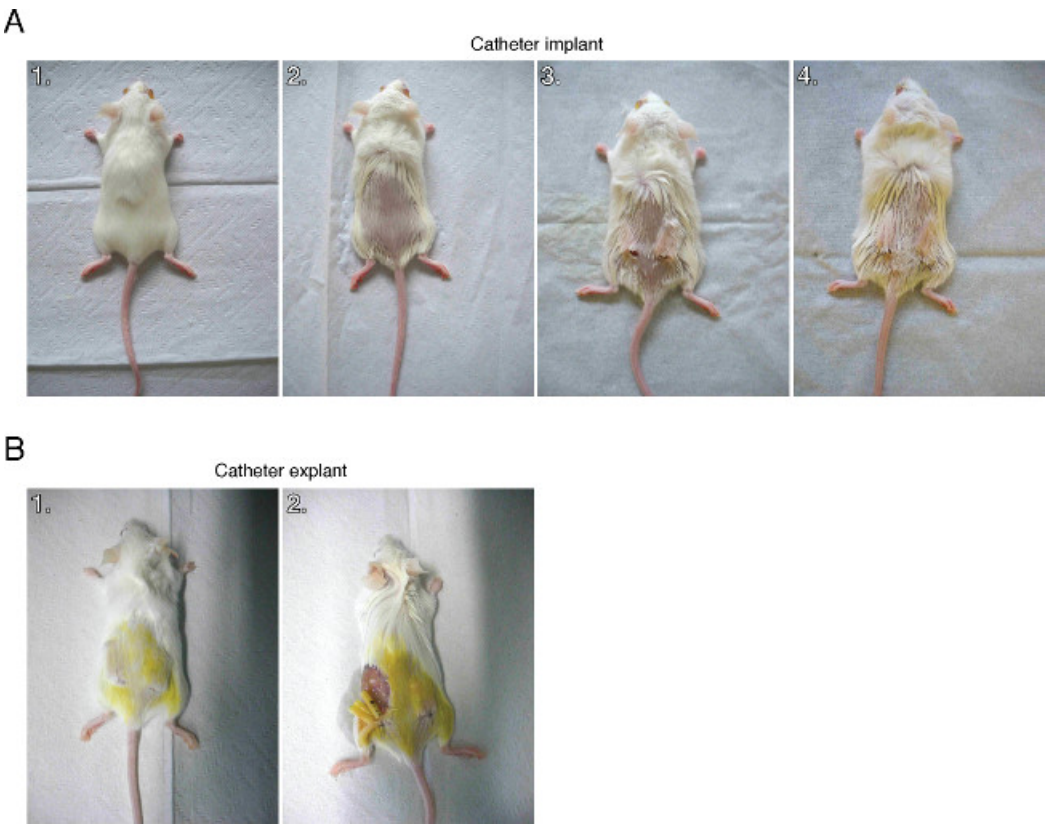

Figure 2: Major steps during the animal surgery. (A) Procedure of catheter implant. (1) Place anesthetized animal on a warm pad containing paper tissue and apply the ophthalmic ointment on eyes. (2) Shave lower part of the back and disinfect. (3) Create two small (approx. 0.5 cm) incisions through the skin on the left and on the right side of the back. Create subcutaneous tunnel inside each incision and place 3 catheters inside. (4) Close the wound with sutures and place animal on a warm pad to recover. **(B)** Procedure of catheter removal. (1) Place sacrificed animal on a pad and disinfect the operated side containing catheters. Cut the wound right above the catheters. (2) Take out each catheter gently and wash twice with 1 ml of PBS. Place to the separate microcentrifuge tube.

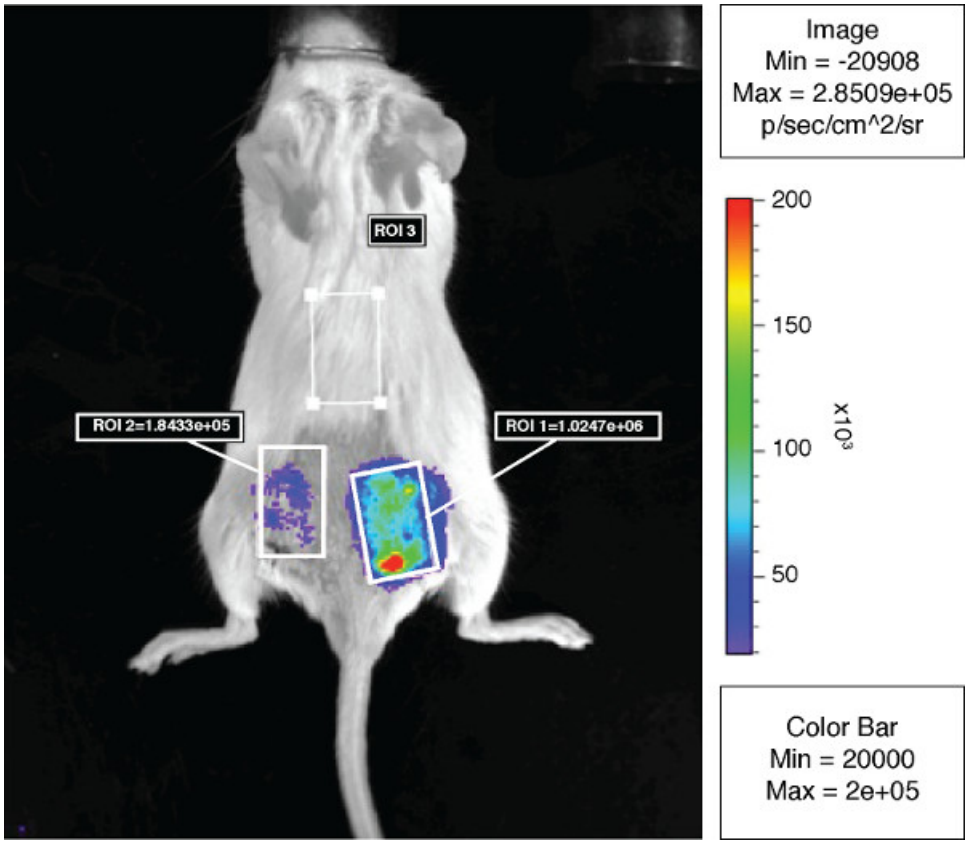

Figure 3: Bioluminescence signal measurement. *In vivo* BLI image from one representative mouse imaged after 6 days of biofilm development. The quantification of signal intensity is performed by placing a region of interest (ROI) (rectangular) around the catheters followed by measurement of the photon flux per second through every ROI.

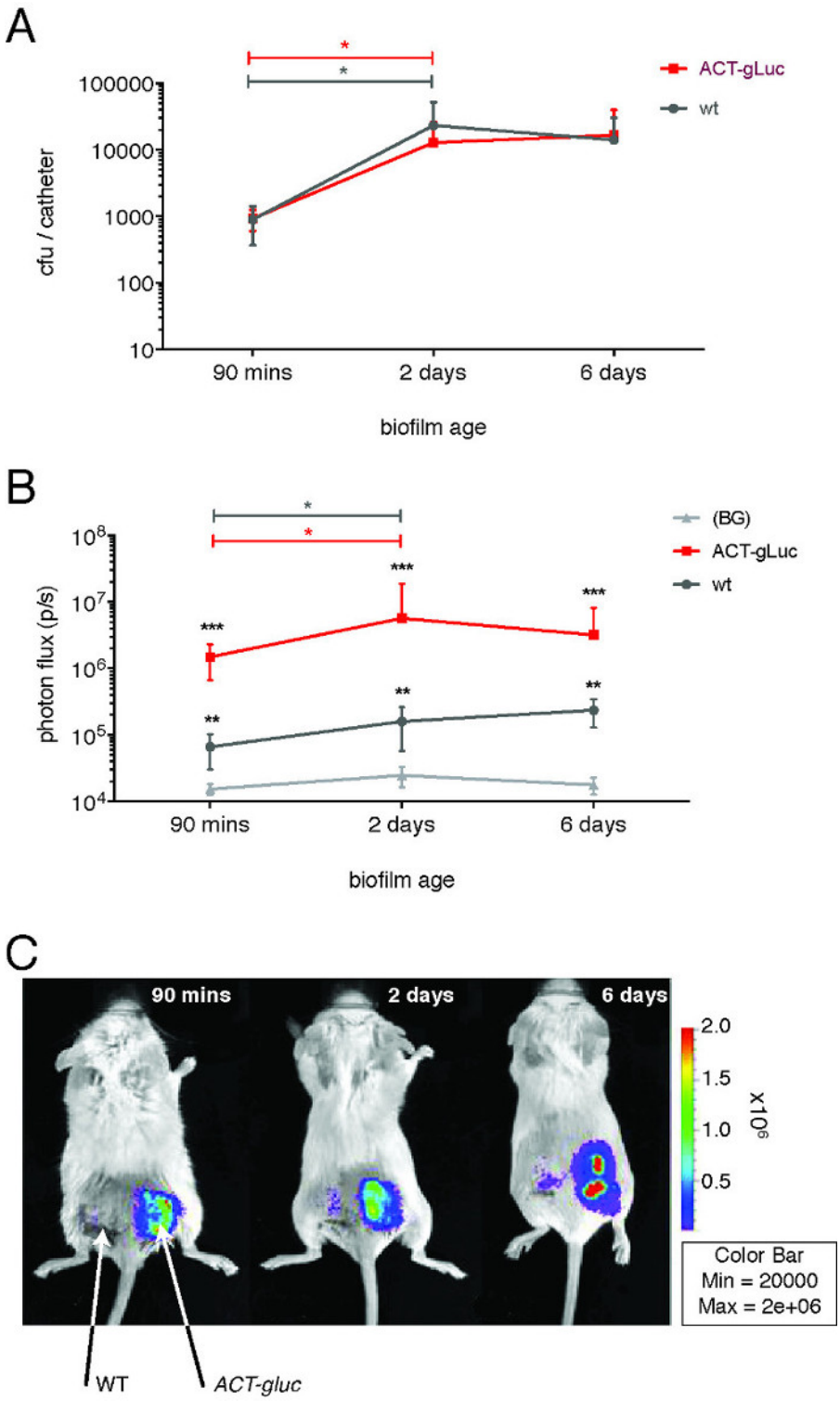

Figure 4: Quantification of mature *Candida albicans* biofilms by colony forming units (CFUs) and by BLI. (A) *In vivo* mature *C. albicans* SKCA23-*ACTgLuc* and SC5314 (WT) biofilm quantification by Log10 CFUs after 90 min, 2 days and 6 days of biofilm development. **(B)** Bioluminescence signal intensity quantification from *in vivo* biofilms formed by SKCA23-*ACTgLuc* and by *C. albicans* WT. Signal was determined after 90 min (period of adhesion), 2 days and 6 days of biofilm development. As a control, we used mice that were implanted with non-colonized catheters and that were subcutaneously injected with CTZ. The photon flux obtained in these mice results in our background signal (BG). Data were expressed as mean ± standard deviation (SD). Statistical significance is indicated as follows: * p <0.05, ** p <0.005, *** p <0.0005. **(C)** *In vivo* bioluminescence images from one representative mouse that was implanted with catheter fragments containing wild type *C. albicans* cells on the left side and *ACTgLuc*-expressing cells on the right side. The mouse was imaged at three different time periods, *i.e.* 90 min, 2 days and 6 days after implantation of the catheter fragments.

Discussion

The use of animal models, and especially rodent models, for studies dedicated to microbial biofilms is very important as the host immune system is an essential factor in biofilm formation that *in vitro* models cannot account for. In this study, we describe a relatively straightforward subcutaneous *C. albicans* biofilm mouse model, which can be easily adopted in a research laboratory and does not require strong technical skills. This model was originally developed to study *Staphylococcus epidermidis* biofilm formation in a rat [24].

In the presented model, serum-coated polyurethane catheters were challenged with *Candida* cells *ex vivo* during the period of adhesion (90 min, 37 °C). This first *in vitro* step might be considered as a limiting factor because of the lack of the immune system at the very first stages of the biofilm infection process. Following adhesion and before catheter implant, non-device associated cells are removed by washing. Avoiding the washing step after the period of adhesion may create uncontrollable amount of cells associated with the device. Because of this reason, we suggest to wash catheters prior to the implant and therefore, to initiate its development from adhered cells. After this initial adhesion period, catheters contain approximately 2.0-2.5 Log_{10} CFUs/device spread alongside the catheter lumen [15]. During this stage *Candida* forms germ tubes, which are attached onto the substrate.

In order to resemble the situation in hospitalized patients of whom the immune system is often compromised, we immunosuppressed the rats in our subcutaneous model system [15]. Partial impairment of the immune system of a rat, prior to the device implant and throughout the period of biofilm development, resulted in increased reproducibility of biofilm-forming cells retrieved from catheters implanted in the same host and also from devices obtained from additional animals. Because of these findings we suggest to immunosuppress the mice before catheter implant and also during the period of biofilm formation. However, our results show that the variability in immunocompetent mice is much less compared to that observed in rats, which makes that mice model system also suitable for studying the role of the host immune system on biofilms. In our original rat model and also in the same model translated to mice, mature *C. albicans* biofilms develop within 48 hr demonstrated by the similar amount of CFUs and biofilm architecture between 2 and 6 days [15,18,19].

The subcutaneous biofilm model was successfully used to follow biofilm development by several mutants [15]. It has been shown previously by Nobile *et al.* [25] that *C. albicans bcr1Δ/bcr1Δ* failed to form biofilms in a central venous catheter (CVC) model. This strain displayed rudimentary biofilm features in our subcutaneous model pointing to the fact that the phenotypic changes found in the CVC model could be reproduced in the subcutaneous model [15].

In comparison with other existing models, *i.e.*, CVC model or denture stomatitis model [8,9,11], the subcutaneous model allows to follow biofilm development in multiple catheter pieces obtained from one animal, thereby reducing the number of animals needed for biofilm studies. Despite these advantages, a shortcoming may be the lack of blood flow that may lead to certain deprivation of nutrients in the biofilm. Therefore, in term of nutrient supplies and environmental conditions, the subcutaneous model is more related to device-associated infections developed on joint prostheses, voice prostheses and pacemakers than the ones formed on intravenous catheters. Even more importantly, translating the rat subcutaneous biofilm system to mice made this animal model compatible with BLI, which further reduces costs and most importantly, the number of necessary animals. Furthermore, translating the rat model to mice enables the use of transgenic mouse models to study host factors relevant to catheter-associated infection studies.

The major advantage of using BLI to quantify biofilms in live animals lies not only in the non-invasive character of this method, but also in its ability to provide dynamic information on the development of an infection. In this study, *gLuc* expressed at the cell wall of *C. albicans* allowed better accessibility and direct interaction with the substrate coelenterazine, which are crucial for a detection of bioluminescent signal *in vivo.* In the study of Vande Velde *et al.* [18], we were able to follow the bioluminescent signal from *C. albicans* biofilms developed on foreign bodies *in vitro*. The BLI signal intensity strongly corresponded with the data obtained from additional biofilm quantification techniques, *i.e.,* CFUs determination and XTT reduction assay. Next to these results, we showed that the bioluminescent signal from *in vivo* biofilms was in agreement with the amount of biofilm-associated cells recovered from explanted biofilms. Additionally, Vande Velde *et al.* [18] demonstrated that BLI can be used to follow biofilm development by a wild type and also by *C. albicans* bcr1Δ/bcr1Δ(biofilm-deficient strain) in the same animal at the same time. Such findings strongly support the use of BLI during the studies dedicated to assessing the time course of biofilm development and infection in the same animal.

Herewith, we described an experimental procedure for *in vivo* subcutaneous implant of *Candida*-infected devices with subsequent monitoring of *in vivo* biofilm development by BLI. Taken together, BLI showed to be a reliable technique, which allowed us to follow an infection over time avoiding animal sacrifices at each time point of data analyses.

Disclosures

The authors declare that they have no competing financial interests.

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