Towards non-invasive monitoring of pathogen–host interactions during Candida albicans biofilm formation using in vivo bioluminescence

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Summary

Candida albicans is a major human fungal pathogen causing mucosal and deep tissue infections of which the majority is associated with biofilm formation on medical implants. Biofilms have a huge impact on public health, as fungal biofilms are highly resistant against most antifungals. Animal models of biofilm formation are indispensable for improving our understanding of biofilm development inside the host, their antifungal resistance and their interaction with the host immune defence system. In currently used models, evaluation of biofilm development or the efficacy of antifungal treatment is limited to ex vivo analyses, requiring host sacrifice, which excludes longitudinal monitoring of dynamic processes during biofilm formation in the live host. In this study, we have demonstrated for the first time that non-invasive, dynamic imaging and quantification of in vitro and in vivo C. albicans biofilm formation including morphogenesis from the yeast to hyphae state is feasible by using growth-phase dependent bioluminescent C. albicans strains in a subcutaneous catheter model in rodents. We have shown the defect in biofilm formation of a bioluminescent bcr1 mutant strain. This approach has immediate applications for the screening and validation of antifungals under in vivo conditions, for studying host–biofilm interactions in different transgenic mouse models and for testing the virulence of luminescent C. albicans mutants, hereby contributing to a better understanding of the pathogenesis of biofilm-associated yeast infections.

Introduction

Fungal infections have become increasingly important for environmental, animal and human health (Fisher et al., 2012). In humans, fungal infections caused by yeasts (e.g. Candida albicans, Cryptococcus neoformans) and molds (e.g. Aspergillus fumigatus) have emerged over the past two decades and become more and more significant in clinical practice (Nucci and Marr, 2005; Warnock, 2006; Chen et al., 2010). C. albicans is part of the commensal flora of the digestive system and vaginal tract of healthy individuals, but it is also an opportunistic pathogen under certain conditions. C. albicans is the most often isolated species in fungal infections (Wisplinghoff et al., 2004; Enoch et al., 2006; Kim and Sudbery, 2011; Pemán and Salavert, 2012). When the host defences are weakened, infections can occur that range from superficial to systemic and even life threatening candidiasis (Wisplinghoff et al., 2004; Kim and Sudbery, 2011). The emergence of fungal diseases is related to an increase in severely ill or immune compromised patients over the past three decades (Eggimann et al., 2003a,b; Nucci and Marr, 2005; Giri and Kindo, 2012). The growing use of implanted medical devices is another reason why the incidence of fungal infections has steadily increased, as many of these infections are emerging from biofilms formed on medical implants (Nucci and Marr, 2005; Pfaffer and Diekema, 2010; Giri and Kindo, 2012). They are a major problem in hospitals (Douglas, 2003) as cells in such biofilms are difficult to treat since they are often tolerant to the classical antifungal drugs (Hawser and Douglas, 1995; Lewis et al., 2002). A major challenge in Candida biofilm research is the development of suitable models that account for host factors at different infection sites and that allow to evaluate the potential and efficacy of novel antifungal treatment strategies under in vivo conditions (Nett and Andes, 2006).
Among others, an intravenous catheter model (Andes et al., 2004) and a subcutaneous catheter model (Řičicová et al., 2010) allow in vivo Candida biofilm research and have greatly facilitated antifungal testing in small animals (Schinabeck et al., 2004; Kuchariková et al., 2010; 2013). These models have in common that fungal load in biofilms is analysed post mortem, requiring host sacrifice and enumeration of microorganisms from individual biofilms to evaluate the efficacy of antifungal treatment. Also for studying biofilms formed under in vivo conditions, evaluation of biofilm properties (architecture, morphology) are limited to ex vivo techniques such as microscopic analysis. This approach requires the use of many animals, is labour intensive and only provides one point per animal and therefore does not enable monitoring of the true time-course of biofilm formation and pathogenesis in vivo.

Different imaging modalities are available that enable non-invasive and repeated imaging of targeted cells in living organisms, but especially bioluminescence imaging (BLI) has emerged as a powerful new method to analyse infectious diseases in animal models and microbial viability in particular (Hutchens and Luker, 2007). BLI is based on the detection of visible light (photons) that is produced by an enzymatic oxidation of a substrate, catalysed by luciferase enzymes. It is one of the few imaging methods that can non-invasively quantify cell viability. BLI provides the opportunity to serially and quantitatively monitor infection and microbial load in a single host over time (Sjölander and Jonsson, 2007). Although BLI is well established for imaging different (microbial) cell populations (Contag et al., 1995; Hutchens and Luker, 2007), it is particularly challenging for fungal pathogens (d’Enfert et al., 2010; Brock, 2012). Unlike bacteria that can be genetically engineered to express all necessary components for the bioluminescence reaction from the lux operon, fungal and other eukaryotic cells depend on external substrate administration for the bioluminescence reaction. Because of their cell wall, the diffusion of the necessary substrate for the light producing reaction is limited, hampering detection of the resulting weak BLI signal in vivo, especially from deeper infection sites (Doyle et al., 2006). The permeability of hyphal cells to the firefly luciferase substrate luciferin is even more limited resulting in the inability to quantifiable image them during infection (Doyle et al., 2006). This was an important obstacle given the importance of the yeast-to-hyphae transition in C. albicans virulence (Biswa et al., 2007). A Gaussia princeps luciferase (gLuc) enzyme that is located extracellularly has made BLI of hyphal cells possible, thereby showing substantial improvement in the use of BLI as a tool to monitor superficial C. albicans infections (Enjalbert et al., 2009). Additionally, yeast and hyphal cell growth could each be visualized in vitro by tagging luciferase to cell wall proteins that are specifically expressed in either of these morphological forms (Enjalbert et al., 2009). However, the possibility to image the time-course of biofilm formation and morphology transitions during biofilm growth in vitro and in vivo has not been investigated before. As imaging biofilms in catheters inside the animal poses extra challenges due to light scattering, we explored the feasibility to non-invasively image C. albicans biofilm development in vivo by using BLI in a subcutaneous catheter model. Furthermore, as the yeast-to-hyphae transition is a key factor involved in C. albicans biofilm formation and pathogenicity, we investigated if the time-course of C. albicans biofilm morphogenesis under in vitro and in vivo conditions can be monitored with BLI. We also used BLI to monitor the effect on biofilm formation of the C. albicans Bcr1 gene which plays a central role in in vitro and in vivo substrate adhesion and biofilm formation, by using a bioluminescent BCR1 deletion strain.

Results

Minimizing background signal for BLI

Catheters are often made radio-opaque to enable the assessment of the catheter position inside the body. For example, barium sulfate filler is added to make the catheter visible under fluoroscopy and on X-ray images. However, barium sulfate phosphoresces. Therefore, we tested different intravenous catheters for background luminescence when imaged with BLI and found two out of three to be highly phosphorescent in vitro (Fig. 1 panel A and Fig. S1), which is a confounding factor for evaluating the specific bioluminescent signal and signal kinetics from gluc-expressing C. albicans biofilms in vitro.

To assess the relevance of this phosphorescence background signal from the catheters for BLI in vivo, catheters containing biofilms of ACTgLuc-expressing C. albicans and empty catheter pieces were implanted subcutaneously. After catheter implantation and after 2 and 6 days of biofilm maturation, the mice were imaged by BLI. Images acquired sequentially before and after opening the scanner door and with or without addition of coenzyme terazine (CTZ) – the substrate used for the bioluminescence reaction – were compared. At all time points, we detected a clearly visible signal from empty implanted Arrow catheters without addition of CTZ (Fig. 1 panels B and C). This signal increased significantly every time the animals were exposed to light (i.e. when opening the scanner door), indicating that the detected signal originated from phosphorescence of the catheter pieces. Next, we opened the door and added CTZ to the catheters and compared the specific BLI signal from biofilms of ACTgLuc-expressing cells with light emanating from empty catheters. Unlike for the empty catheters, the BLI

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signal intensity from catheters containing ACTgLuc-expressing cells increased significantly above the phosphorescence signal upon CTZ addition (Fig. 1 panels B and C). However, the additional increase in luminescence upon CTZ addition was relatively small compared with the increase in catheter phosphorescence upon opening of the scanner door. Performing the same in vivo experiment using non-phosphorescent catheters (Certofix),
no phosphorescent background signal could be detected after exposing the animals to light (Fig. 1 panel E). A significant BLI signal above background could clearly be visualized and quantified only upon addition of CTZ to catheters containing biofilms formed by *ACTgLuc*-expressing *Candida* cells (Fig. 1 panels D and E). Therefore, and to avoid complications in signal analysis due to different kinetics of the phosphorescence and *ACTgLuc*-induced BLI signals, we have used non-phosphorescent catheters (Certofix) for all further *in vitro* and *in vivo* BLI experiments. Biofilms formed on the non-phosphorescent catheters showed the same properties regarding biofilm formation (data not shown) and were therefore comparable to biofilms formed on the catheters used in previous studies (Kuchariková et al., 2010; Říčicová et al., 2010).

### BLI of *in vitro* biofilm formation

CTZ is known to undergo some auto-oxidation in the presence of oxygen, contributing to a background BLI signal (Shimomura et al., 1993; Tannous et al., 2005). Therefore, we determined the minimum number of *ACTgLuc*-expressing *C. albicans* cells in suspension that resulted in a detectable BLI signal, significantly above the background signal originating from auto-oxidation of CTZ alone or incubated with non-luminescent wt cells. The specific BLI signal strongly...
correlated with the amount of ACTgLuc-expressing C. albicans cells (R² = 0.998).

To investigate the feasibility of using BLI for imaging biofilm development, we first evaluated the BLI signal intensity from C. albicans biofilms formed on the bottom of polystyrene cell culture plates in vitro. ACTgLuc-expressing and wt C. albicans cells were allowed to adhere to the bottom of 96- and 24-well cell culture plates and incubated for mature biofilms to be formed. BLI was performed after 90 min (period of adhesion) and after 2 and 6 days of mature biofilm formation. At every imaging time point, the metabolic activity of the biofilm forming cells (in 96-well plates) and colony-forming units (cfu) (in 24-well plates) were quantified. At all imaging time points, the BLI signal from ACTgLuc-expressing biofilms formed on 96-well plates was significantly higher than from wt biofilms (Fig. 2, panel B). Two days after adhesion, the BLI signal was significantly increased for ACTgLuc-expressing biofilms, which is consistent with mature biofilm development at this stage (Kucharíková et al., 2011). The noticeable finding that the BLI signal from wt biofilms was also increased can probably be attributed to the increase of organic matter in the extracellular matrix, which might stimulate CTZ auto-oxidation. The BLI signal of 6-day-old biofilms was decreased, which can be explained by a limitation in nutrients, which corresponds to the metabolic activity of the cells in the biofilm measured by the XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) assay. Overall, the BLI data was in agreement with the metabolic activity of the biofilm forming cells as measured by the XTT reduction assay (Fig. 2, panel B).

For biofilms formed in 24-well plates, nutrients are less likely to be a limiting factor for biofilm development compared with biofilms formed in 96-well plates. Indeed, the BLI signal intensity from biofilms formed by ACTgLuc-expressing C. albicans as well as from wt cells increased significantly after 2 and 6 days of mature biofilm formation, which was corroborated by a significant increase of cfu over time (Fig. 2, panel C). Importantly, the BLI signal from ACTgLuc-expressing C. albicans biofilms was at every imaging time point significantly higher than the background BLI signal measured from wt C. albicans biofilms, for the same amount of cfu per biofilm (Fig. 2, panel C).

We next evaluated the BLI signal intensity from biofilms formed inside polyurethane catheter pieces in vitro. Similar to the results for biofilms formed on cell culture plates, the specific BLI signal from ACTgLuc-expressing biofilms was at every imaging time point significantly higher than the background BLI signal measured from wt biofilms. From the period of adhesion (90 min) towards mature biofilm formation on day 2, the BLI signal increased significantly for both ACTgLuc-expressing and wt biofilms (Fig. 2, panel D). From day 2 to day 6 of biofilm maturation, the BLI signal intensity remained at the same level. These BLI results were in agreement with the cfu retrieved from each biofilm that were also significantly increased on day 2 and remained unchanged by day 6 (Fig. 2, panel D).

Taken together, our data indicate that BLI can be used as a sensitive tool to monitor in vitro biofilm development on foreign bodies, including on biofilms formed on the inside of catheter pieces.

**BLI of biofilms formed in vivo**

We evaluated the feasibility to non-invasively follow-up in vivo biofilm development in subcutaneously implanted catheters with BLI. Hereto, three catheters inoculated with wt C. albicans cells were implanted on the left side and three catheters inoculated with ACTgLuc-expressing C. albicans were implanted on the right side under the skin of the lower back of mice (n = 12). In vivo biofilm formation was visualized with repeated BLI starting on the same day of catheter implantation, and after 2 and 6 days of mature biofilm formation. Catheter pieces (n = 4 per C. albicans strain) were handled in parallel with the catheters for implantation, imaged in vitro with BLI and used for quantification of cfu at the adhesion time point to verify adhesion of equal numbers of cells on all catheters at the time of implantation. After catheter implantation (n = 12) and after 2 days (n = 12), all mice were imaged with BLI. After the 2 days’ imaging time point, half of them (n = 6) were sacrificed for catheter explantation and ex vivo quantification of biomass for validation of the BLI results. At the 6 days’ imaging time point, the remaining half of the group (n = 6) was imaged and sacrificed thereafter for catheter explantation.

At every imaging time point, a significant BLI signal above background was detected from ACTgLuc-expressing biofilms formed on the inside of catheters in vivo, while ACTgLuc-expressing and wt biofilms were verified to contain the same amount of cfu per biofilm (Fig. 3, panel A). The BLI signal could clearly be visualized and followed up over time in every individual animal (Fig. 3, panel B) and was in agreement with the amount of cfu quantified from the biofilms (Fig. 3, panel A). Interestingly, wt biofilms elicited a significant BLI signal above background at all time points, as was also observed for the in vitro biofilm BLI experiments (Fig. 2). However, at every imaging time point, the BLI signal quantified from ACTgLuc-expressing biofilms was at least one order of magnitude (i.e. log10) and significantly higher than the background signal of wt biofilms, indicating that BLI can be used for longitudinal monitoring and quantification of in vivo biofilm development on implanted catheters.

As bioluminescence images represent low-resolution 2D projections overlaid on a planar photographic image,
individual catheters might not always be clearly visible on the resulting image. Therefore, signal quantification may potentially suffer from variability due to implantation depth and/or incorrect delineation of the catheters. Also, light absorption due to scar formation or unexpected bleedings may affect BLI quantification. We have therefore acquired high-resolution magnetic resonance (MR) images while the animals were still anesthetized from BLI and fixed to the same reference frame, i.e. animal holder. Anatomical details and the position of the catheters as visualized on the MR images were used for delineation of the region of interests (ROIs) used for subsequent BLI signal quantification (Fig. 3, panel C). In order to explore the potential of MRI to improve the accuracy of BLI signal quantification, the BLI signal quantified from correctly positioned ROIs using prior knowledge from the MR images was compared with BLI signal intensities quantified from ROIs that were placed over the focus of the BLI signal spot while varying the angulation of the ROI. No significant influence from different angulations of the ROIs could be found (data not shown).
ACTgLuc to the medium (Sudbery, 2011). We incubated wt, from the yeast form to the hyphal form can be monitored (Sudbery, 2011). Next, we tested whether the transition known to be in the yeast form in serum-free medium panel A). These results are in line with HWPgLuc. The relative increase in BLI signal intensity from wt and increase in signal intensity measured from wt and ACTgLuc-expressing cells, both after 1.5 h and 4.5 h of incubation (Fig. 4, panel B). These results demonstrate that BLI can be used to quantify hyphal growth in vitro.

Furthermore, we evaluated whether the hyphal growth during in vitro biofilm formation in catheter pieces can be followed with BLI. Catheters were incubated with ACTgLuc- (n = 12) or HWPgLuc-expressing C. albicans (n = 12) in medium containing FBS, which is known to stimulate yeast-to-hyphae transition (Sudbery, 2011). After 90 min, 2 days, 6 days and 9 days of biofilm growth, three catheters of each strain were imaged with BLI, followed by cfu quantification. At each time point, the number of cfu recovered from biofilms formed by each strain was equal, confirming that differences in BLI signal intensity between the strains were not due to differences in the amount of biofilm forming cells (Fig. 4, panel C). After 90 min of C. albicans cell adhesion on the catheters, a significantly lower BLI signal intensity was detected from HWPgLuc-expressing C. albicans cells compared with ACTgLuc-expressing cells, corresponding to the lower numbers of C. albicans hyphal cells compared with yeast cells, characteristic at this stage (Blankenship and Mitchell, 2006). After 2 days of biofilm growth, the BLI signal intensities from both yeast as well as hyphal cells showed to be significantly increased due to biofilm proliferation, whereas after 6 days they remained at the same levels when the mature biofilm is maintained. That HWPgLuc-expressing C. albicans cells showed a more pronounced BLI signal intensity increase during the first 2 days of biofilm formation relative to ACTgLuc-expressing cells corresponds to hyphal growth in addition to cell proliferation during mature biofilm development. After 9 days of in vitro biofilm growth, the BLI signal intensity from both ACTgLuc- and HWPgLuc-expressing biofilms has decreased, which is in agreement with the lower cell numbers (cfu) recovered from the catheters.

In order to demonstrate the potential of BLI to non-destructively determine the yeast-to-hyphal cell ratio, we imaged HWPgLuc-expressing C. albicans cells that were either in the yeast or hyphal stage, or as mixtures at different ratios of yeast to hyphal cells. The percentage of yeast to hyphal cells could be calculated from the bioluminescence signal of the samples (Fig. S2), demonstrating that the yeast-to-hyphal ratio can be determined by using BLI.

To verify whether BLI can be used as a technique to non-invasively follow up on hyphal growth in vivo, catheters inoculated with ACTgLuc- or HWPgLuc-expressing C. albicans were implanted on the back of mice (n = 6). All mice were repeatedly monitored with BLI on the same day of catheter implantation (adhesion of C. albicans cells to the catheters) and after 2, 6 and 9 days of biofilm maturation. After catheter implantation, all mice exhibited a significantly lower BLI signal intensity

**BLI of C. albicans morphogenesis during biofilm development**

In a next step, we investigated whether the expression of gLuc under control of a morphogenesis-dependent promoter can be applied for BLI of the C. albicans yeast-to-hyphae transition during biofilm formation. For these experiments, we used the CEC971-HWPgLuc C. albicans strain (Enjalbert et al., 2009), which expresses gLuc under control of a hyphal promoter that becomes active when cells undergo yeast-to-hyphae transition (Staab et al., 1996). As a control, we used a strain that expresses gLuc under control of the constitutively active ACT1 promoter, i.e. in both the yeast and hyphal forms (CEC988-ACTgLuc).

First, we determined the BLI signal of 10-fold dilution series of CEC988-ACTgLuc, CEC971-HWPgLuc, DAY185 (wt control) C. albicans cell suspensions and CTZ alone. The BLI signal intensity from the cells expressing gLuc under control of the HWP1 promoter was equal to the background BLI signal from the controls. A specific BLI signal that correlated with the number of cells could only be detected for C. albicans expressing gLuc under control of the ACT1 promoter (R² = 0.998, Fig. 4, panel A). These results are in line with C. albicans cells known to be in the yeast form in serum-free medium (Sudbery, 2011). Next, we tested whether the transition from the yeast form to the hyphal form can be monitored with BLI, which is known to be stimulated by adding serum to the medium (Sudbery, 2011). We incubated wt, ACTgLuc- and HWPgLuc-expressing cells with and without fetal bovine serum (FBS) (10%) in the culture medium and imaged them after 1.5 h and 4.5 h with BLI. The relative increase in BLI signal intensity from HWPgLuc-expressing cells grown in medium compared with serum-free medium was significantly higher than the increase in signal intensity measured from wt and ACTgLuc-expressing cells, both after 1.5 h and 4.5 h of
from HWPgLuc-inoculated catheters (Fig. 4, panel D and E). After two days of biofilm maturation, there was a more pronounced and significant increase in the BLI signal for HWPgLuc-expressing C. albicans biofilms compared with ACTgLuc-expressing biofilms, indicating hyphal growth. When the biofilm matures further, the BLI signal from ACTgLuc-expressing biofilms showed a decrease whereas the BLI signal from HWPgLuc-expressing biofilms remained largely unchanged. This may point to the mainly hyphal content of biofilms of this age (Nett and Andes, 2006). After the last imaging time point at day nine, the mice (n = 6) were sacrificed and cfu were quantified from explanted catheters, confirming that biomass was comparable among all biofilms (Fig. 4, panel E). Taken together, the data indicate that BLI can be used to monitor hyphal growth during in vitro and in vivo biofilm formation, which is most pronounced during the earliest stage of biofilm development.
BLI of defective biofilm formation by BCR1 mutant C. albicans

In the next series of experiments, we aimed to use and validate BLI to identify biofilm-defective mutants. For these experiments, we used a C. albicans BCR1 knockout strain that was engineered to constitutively express gLuc on its cell wall in order to non-invasively image its capacity to adhere to and form biofilms inside catheters. The bioluminescence emitted by this ACTgLuc-expressing bcr1Δ− strain showed to be directly comparable to the bioluminescence of the ACTgLuc-expressing strain (SKCA23-ACTgLuc), used as wild type control for biofilm formation in the following experiments (Fig. S3, panel A).

First, we used BLI to evaluate the capacity of ACTgLuc-expressing bcr1Δ− C. albicans cells to adhere and to form biofilms on catheter pieces in vitro. After C. albicans adhesion on the catheters, a significantly lower BLI signal was detected from catheters inoculated with bcr1Δ−-ACTgLuc compared with those inoculated with SC-ACTgLuc (Fig. 5, panel A). This corresponds to the lower numbers
of bcr1- Candida cells compared with wt that were able to attach onto the catheter, which is in agreement with the known adhesion defect of this strain (Nobile et al., 2006; Říčicová et al., 2010). After 2 days and 6 days of mature biofilm formation, significantly lower BLI signal intensities were detected from catheter pieces inoculated with bcr1- -ACTgLuc compared with the SC-ACTgLuc strain (Fig. 5, panel A), indicating the inability of the bcr1 mutant strain to form normal biofilms. The lower BLI signal intensities emanating from bcr1- -ACTgLuc-inoculated catheters corresponded to significantly reduced numbers of cfu recovered from these catheters compared with the control strain, validating BLI as a technique to monitor defective adhesion and biofilm formation of the bcr1- C. albicans strain on catheters under in vitro conditions.

To evaluate the biofilm forming capability of bcr1- mutant C. albicans under in vivo conditions with BLI, catheters inoculated with ACTgLuc-expressing bcr1- and wt strains were implanted on the back of mice (n = 12). All mice were imaged with BLI after catheter implantation (n = 12) and after 2 days (n = 12). After the 2 days’ imaging time point, half of the mice (n = 6) were sacrificed for catheter explantation and ex vivo quantification of cfu. At the 6 days’ imaging time point, the remaining half of the group (n = 6) was imaged and sacrificed thereafter for catheter explantation and validation of the imaging results. Catheter pieces (n = 4 per strain) were handled in parallel with the catheters for implantation and used for quantification of cfu, to evaluate the adhesion properties of the Candida strain at the time of implantation. In vivo BLI revealed significantly lower BLI signal intensities for the bcr1- -ACTgLuc-inoculated catheters compared with SC-ACTgLuc-inoculated catheters, illustrating the hampered adhesion properties of this strain. This was confirmed by the cfu retrieved from the catheters at the time of implantation (Fig. 5, panels B and C). Also at the following in vivo imaging time points (2 and 6 days), the BLI signal intensity from bcr1- -ACTgLuc-inoculated catheters was significantly lower than the signal from ACTgLuc-expressing control biofilms, demonstrating the inability of the bcr1- strain to form normal biofilms under in vivo conditions (Fig. 5, panels B and C). This was clearly visualized for every individual animal (Fig. 5, panel C). The imaging results were at all time points in agreement with the amount of cfu quantified from the biofilms (Fig. 5, panel B) and defective in vitro and in vivo biofilm formation by the bcr1- -ACTgLuc-strain was confirmed by SEM (data not shown) as shown before (Říčicová et al., 2010).

In the context of assessing adherence properties and biofilm forming capabilities of a mutant Candida strain that is potentially defective for biofilm formation, it becomes relevant to evaluate the bioluminescence emitted by biofilm cells versus planktonic cells that are not associated with a biofilm structure. In order to evaluate to what extent planktonic cells that are not attached to mature biofilm structures contribute to the overall in vivo BLI signal, we collected the free floating cells from inside the lumen of the explanted catheters and compared them with the biofilm that remains adhered to the catheters. Comparing the BLI signal of the cells in the washing fluid with the overall in vivo BLI signal emanating from biofilms formed on implanted catheters (compare Fig. S3, panel B with Fig. 5, panel B), the BLI signal intensity is at least one order of magnitude lower, showing that the BLI signal of planktonic cells not associated with the biofilm structure form only a minor fraction and have only a marginal contribution to the overall in vivo BLI signal. Interestingly, after 2 days of biofilm formation, a significantly lower BLI signal intensity, close to background, was measured for bcr1- -ACTgLuc-inoculated catheters, due to a large fraction of catheters for which zero cfu of planktonic cells were recovered from the catheter lumen (89% for bcr1- -ACTgLuc-inoculated catheters versus 39% for ACTgLuc-expressing control biofilms). This illustrates that the overall in vivo BLI signal reflects the biomass associated with the catheter, with a negligible contribution from loose planktonic cells in the catheter lumen. Quantification of the cfu corroborated the BLI results for the washing fluid, confirming that BLI is a valid technique to conveniently assess planktonic versus biofilm-associated cells on subcutaneously implanted catheters in vivo.

Taken together, our data illustrate that BLI is a powerful technique to monitor and quantify the adhesion between C. albicans strains and the substrate, as well as further biofilm formation, both under in vitro and in vivo conditions.

**Discussion**

We have demonstrated for the first time that biofilm formation by C. albicans, the transition from yeast to hyphae morphology during biofilm formation and defects in adherence properties and biofilm formation by selective mutants can be monitored longitudinally in individual animals by using bioluminescence imaging. One of the key challenges in Candida biofilm research is the development of suitable in vivo models that account for host factors at different infection sites and that allow to evaluate the potential and efficacy of novel antifungal treatment strategies (Nett and Andes, 2006). Several in vitro C. albicans biofilm model systems are available to elucidate the architecture, developmental stages, cell phenotypes and drug resistance of biofilms (Nett and Andes, 2006). However, these models cannot account for the numerous host and infection-site variables that are important during infections in humans. Recently, in vivo central venous catheter (CVC) C. albicans biofilm models were
developed in rabbits, rats and mice (Andes et al., 2004; Schinabeck et al., 2004; Lazzell et al., 2009). Although they are extremely valuable for C. albicans biofilm research under in vivo conditions, these models have the disadvantage of being technically demanding, exposing the animals to a major surgical intervention and having a very low throughput. Říčícová et al. have addressed this issue and developed a rat in vivo C. albicans foreign body infection model in which small pieces of catheter challenged with C. albicans cells are subcutaneously implanted in rats (Říčícová et al., 2010). This model can produce similar biofilms as in the CVC models with the major advantage that up to nine catheter fragments can be implanted in one animal with only minor surgery. Although this model cannot account for factors as shear stress and nutrient flow as is observed in the blood stream, the suitability of the model to evaluate the efficacy of antifungal drugs on biofilm development was shown, allowing more rapid screening under in vivo conditions (Kucharíková et al., 2010; 2013; Bink et al., 2012).

Translating this rat biofilm model to mice further improved the cost-efficiency of in vivo screening and holds potential for biofilm research in different transgenic mouse strains that would allow for studying different host factors. Making these animal models compatible with BLI reduces the costs and number of animals needed even further. Moreover, the major advantage of BLI-compatible animal models lies in that only non-invasive imaging has the potential to provide dynamic information on the infection process and immune response in individual animals (Hutchens and Luker, 2007). In the case of bacterial pathogens, luminescent reporters based on the lux operon can be used, which have the advantage that there is no need for substrate addition as this is produced intracellularly (Gahan, 2012). For eukaryotic cells, other intracellularly expressed luciferases such as firefly, Renilla and click beetle luciferases are available and commonly used (Badr and Tannous, 2011), but here bioluminescence relies on external substrate administration and therefore, on sufficient intracellular substrate availability. The development of BLI for imaging fungal infections has to address some additional challenges, including the fungal cell wall, which was found to be an obstacle for the intracellular substrate availability for the intracellularly located firefly luciferase (Doyle et al., 2006). Engineering the naturally secreted Gaussia luciferase to be located extracellularly partially solved the problem in the sense that superficial C. albicans infections could successfully be imaged with in vivo BLI upon topical application of its substrate CTZ (Enjalbert et al., 2009). However, BLI was not successful for imaging C. albicans infection from deeper infection sites due to yet not fully understood reasons (Enjalbert et al., 2009). Contributing factors might be found in some of the disadvantages that are associated with the in vivo use of gLuc; namely the emission of blue bioluminescence which is more absorbed and scattered by the tissue than light with longer wavelengths, and the rapid clearance of its substrate CTZ from the blood (Zhao et al., 2004; Tannous et al., 2005).

The challenges of BLI to visualize fungal infections were also illustrated by the work of Donat et al. in which a bioluminescent Aspergillus fumigatus strain was developed using cell wall-exposed gLuc (Donat et al., 2012). Their attempts to image pulmonary infection of this bioluminescent strain in vivo failed. When imaging cutaneous infection, they did observe a bioluminescent signal but could not establish a correlation between fungal load and BLI signal intensity. The latter might be explained by the high degree of instability through auto-oxidation of CTZ, which resulted in a high background signal (Shimomura et al., 1993; Tannous et al., 2005).

In our study, we also use extracellular gLuc, which warrants better accessibility of the substrate and is a prerequisite for a detectable bioluminescent signal in vivo, circumventing restricted diffusion through the fungal cell wall. Although the substrate CTZ – like any nutrient – needs to diffuse through the extracellular matrix to reach the gLuc expressed at the cell wall of the Candida cells, our results show that these challenges can be overcome and that BLI proves to be a sensitive tool providing dynamic information on biofilm development using the extracellularly located gLuc reporter that was originally developed by the d’Enfert-group (Enjalbert et al., 2009).

We have shown the feasibility of sensitive and dynamic BLI of biofilms formed on foreign bodies in vitro. It has the potential to be used as a rapid, easy and cost-effective screening technique for studies of in vitro biofilm formation, validating interesting proteins of the pathogen involved in biofilm formation as shown here for Bcr1 and assessing morphogenesis and antifungal testing. The BLI technique forms a valuable non-destructive alternative next to other alternatives for quantitative analysis of in vitro biofilm growth, e.g. cfu counting, XTT reduction assay, crystal violet assay or other published techniques such as the ATP bioluminescence assay (Nikawa et al., 1996).

More importantly, we were able to show in vivo BLI signal quantification from C. albicans biofilms developed in vivo on catheters implanted on the back of mice. The BLI signal intensity was in agreement with the amount of cfu recovered from explanted biofilms for validation, showing for the first time that BLI to non-invasively monitor fungal biofilm formation in live animals is feasible.

Co-registering BLI and MR images adds information on the exact position of the catheters and on tissue thickness and composition between the catheter and the BLI camera. However, prior knowledge of the exact catheter
position from MRI does not have a significant influence on the accuracy of BLI signal quantification in our model. This brings BLI as a relatively cheap tool for imaging and quantification of in vivo biofilm formation within the reach of many labs. When translating the technology towards other models of in vivo biofilm formation that involve deeper implantation sites, we anticipate that coregistration with MRI might become more important to provide information on the exact catheter position, on the nature of the surrounding tissue types or the presence of bleedings or scar formation to potentially correct for differences in light absorption and scattering properties.

When imaging in vitro and in vivo biofilms, one might expect a limited delivery of substances, for example CTZ, to the gLuc-expressing C. albicans cells inside the dense extracellular matrix in fully developed biofilms. However, studies looking at restricted penetration as a potential factor leading to increased drug resistance of biofilms have indicated that the matrix does not form a major barrier to drug diffusion (Al-Fattani and Douglas, 2004), although for some substances penetration can be delayed (Mah and O’Toole, 2001). C. albicans itself possesses different types of efflux pumps (Ramage et al., 2002), but as gLuc is located extracellularly, these efflux pumps are unlikely to be relevant for potentially limiting the substrate availability for the bioluminescence reactions in this study.

The observed increased background BLI signal from fully developed wt biofilms indicate that an increased amount of biomass increases the chemiluminescence of CTZ. A similar phenomenon of aspecific BLI signal from wt C. albicans infection sites compared with non-infected controls has been previously observed (Enjalbert et al., 2009), but remains unexplained. For some biomolecules like albumin and insulin it was shown that they catalyse CTZ chemiluminescence (Vassel et al., 2012), but for compounds that constitute biofilms, studies on their possible influence on CTZ chemiluminescence are currently lacking. Nevertheless, this observation highlights the importance of taking the necessary controls when imaging bioluminescence using CTZ.

In the case of in vivo BLI of biofilms in implanted catheters, the formation of fibrous scar tissue around the catheters can be an additional factor in the relatively lower specific BLI signal at later time points. This scar tissue has an influence on slowing down the diffusion of CTZ towards the catheters, which is reflected in an increased time-to-peak of the BLI signal after CTZ administration (data not shown). However, our MRI data did not indicate excessive bleeding or scar formation in individual animals.

Using a morphogenesis-dependent promoter, we showed that hyphal growth during biofilm maturation can be monitored with BLI in vitro and in live animals. The change in specific BLI signal from hyphal cells was most pronounced during the first stages of mature biofilm formation, as most cells go through the transition from the yeast to the hyphal cell stage (Blankenship and Mitchell, 2006). Once a mature biofilm is formed, the differences between the specific hyphal BLI signal and the BLI signal driven by the ACT1 promoter are less pronounced. This might reflect the hyphal content of the biofilm at this stage (Nett and Andes, 2006). In this context, it is of note that the activity of the ACT1 promoter is not constant throughout the different stages of biofilm formation (Nailis et al., 2006). It would be of interest to add BLI studies on morphogenesis of biofilms using a yeast cell stage specific promoter for gLuc expression and/or mutants defective in hyphae formation i.e. that do not express the hyphal cell wall protein (Baillie and Douglas, 1999).

We also demonstrated that BLI can be used to monitor the role of a gene involved in biofilm formation. By engineering the bcr1− mutant C. albicans to be bioluminescent, we could visualize and quantify its hampered substrate adherence and inability to form normal biofilms in vitro and in vivo, which is a known effect of the double BCR1 knockout in this strain (Nobile et al., 2006; Řicícová et al., 2010). BLI shows to be a powerful tool to dynamically monitor the function of genes involved in biofilm formation, thereby opening the door towards more rapid and convenient screening and validation of genes of the pathogen playing a role in biofilm formation and dynamic follow-up of the interplay between pathogen and substrate within the living host.

Conclusion & perspectives

We have demonstrated the feasibility of using BLI to non-invasively and longitudinally monitor biofilm development and morphogenesis in subcutaneously implanted catheters in rodents. We expect this approach to be readily translatable to other biofilm models such as the CVC model as the intravenous (iv) catheter is located not too deep under the skin and iv CTZ injection might result in a favourable substrate delivery and reproducibility of BLI results. Studies are underway to evaluate the feasibility of BLI for in vivo biofilm research in the CVC model that will enable to account for factors as shear stress and nutrient flow on biofilm formation. The here described multi-temporal non-invasive imaging tool for quantifying in vitro and in vivo biofilm formation has the potential for applications in the screening and validation of antifungal drugs under in vivo conditions, for studying host–biofilm interactions in different transgenic mouse models and for studying the virulence of mutant C. albicans in this luminescent background, and hereby contribute to a better understanding of the pathogenesis of yeast infections during biofilm formation.
**Experimental procedures**

**Strains and culture media**

Wild-type *C. albicans* SC5314, a clinical isolate with a success-fully sequenced genome (Gillum et al., 1984) was used as a control strain in these studies. This strain was engineered to express *C. albicans* codon-optimized *Gaussia princeps* luciferase (gLUC) at the cell wall, under the control of a constitutive (actin, ACT1) and a hyphal growth phase-specific (HWP1) promoter. SC5314 was transformed in the RPS1 locus with the Clp10::Act1p-gLUC59 plasmid (Enjalbert et al., 2009), kindly provided to us by Prof. C. d’Enfert, in which we replaced the *URA3* transformation marker with SAT1. Positive transformants were identified by BLI and confirmed by PCR. Two bioluminescent transformants (SKCA23- ACTgLuc and SKCA43-ACTgLuc) that were normal regarding hyphal induction and growth (data not shown) were selected for both in vitro and in vivo biofilm bioluminescence assays. These strains express gLuc fused to the endogenous PGA59 gene under the control of the ACT1 promoter. The results with both strains were similar and all data presented in this paper were obtained with SKCA23-ACTgLuc.

Similarly, we transformed the mutant *C. albicans* CJN702 - bcr1::bcr1 (ura3Δ::imm434/ura3Δ::imm434 arg4::hisG/arg4:: hisG hist1::his/hist1::hisG::phHIS1 bcr1::ARG4/bcr1::URA3) (Nobile and Mitchell, 2005) with the Clp10::Act1p-gLUC59 plasmid, resulting in the bioluminescent strain further referred to as bcr1Δ::ACTgLuc.

Also used in this study were strains CEC971 (HWP1-PCA59- gLUC, morphogenesis-dependent expressing of gLuc, further referred to as CEC971-HWPgLuc), CEC988 (ACT1p-PCA59- gLUC, further referred to as CEC988-ACTgLuc) and DAY185 (wt control), kindly provided to us by Prof. C. d’Enfert (Enjalbert et al., 2009).

**In vitro biofilm formation**

Strains were grown overnight on YPD plates at 37°C, washed and resuspended in PBS. The cells were counted to prepare a *C. albicans* cell suspension in RPMI medium (RPMI-1640, R6504, Sigma-Aldrich, Diegem, Belgium; with L-glutamine and without sodium carbonate buffered with MOPS, pH 7.0). To study biofilm formation in vitro, sterile 96-well (24-well) polystyrene cell culture plates were inoculated by adding 100 μl (200 μl) per well of a 1 × 10⁷ cells ml⁻¹ *C. albicans* cell suspension in RPMI. The *C. albicans* cells were allowed to adhere to the bottom of the wells by incubating the plate for 90 min at 37°C. After washing twice with PBS, attached cells were submerged in 200 μl (96-well plate) or 1 ml (24-well plate) of fresh RPMI medium and the plate was again incubated at 37°C for the duration of the experiment to allow formation of mature biofilms. To study biofilm formation on the inside of a catheter in vitro, polyurethane triple-lumen intravenous catheters (2.4 mm diameter, Certofix Trio S730, BBraun, Diegem, Belgium) were cut into 1 cm long fragments and incubated overnight with FBS (F7524, Sigma) at 37°C. *C. albicans* cell suspension of 5 × 10⁷ cells ml⁻¹ was added to the catheters placed in a 24-well plate and incubated for 90 min at 37°C for adhesion. Catheters were washed twice with PBS, transferred to a clean plate and submerged in 1 ml of fresh RPMI medium and incubated at 37°C for the duration of the experiment.

**In vitro BLI**

To obtain in vitro bioluminescence measurements, the plates containing cell suspensions, biofilms formed on the bottom of cell culture plates or biofilms formed inside catheter pieces were placed in a BLI-camera (IVIS 100, Perkin-Elmer, Waltham, MA, USA). For all in vitro experiments, a native coelenterazine (CTZ; Nanolight Technologies, Pinetop, AZ, USA) working solution in PBS was prepared freshly from a 5 mg ml⁻¹ stock solution in acidified ethanol, prepared and stored at −80°C according to the manufacturer’s instructions. After acquisition of a background image, CTZ was added to the wells at a final concentration of 6 μM. Subsequent frames were acquired with a 5-20 s exposure time depending on the signal intensity, at medium binning. The BLI signal was quantified using Living Image software (version 2.50.1, provided by the manufacturer) and reported as photon flux per second (p s⁻¹) for a given ROI of fixed size covering the well or catheter (for more details, see Vande Velde et al., 2013). In vitro BLI data were normalized to the background signal from CTZ alone unless stated otherwise.

**Animals**

Female, 8-week-old Balb/C mice and Sprague-Dawley rats of 200 g (Janvier, Le Genest Saint-Ise, France) were used for in vivo biofilm studies. The animals were kept in individually ventilated filter top cages with free access to standard food and water *ad libitum*. All animals were immunosuppressed starting 24 h before and during the entire experiment by adding dexamethasone (0.4 mg l⁻¹; Fagron SAS, Paris, France) to their drinking water. Ampicillin (ampicillin sodium powder 0.5 g l⁻¹; Duchefa Biochemie, Haarlem, the Netherlands) was also added to the water to prevent possible bacterial infections. All aspects of animal experiments were carried out in compliance with national and European regulations and were approved by the animal ethics committee of the KU Leuven.

**In vivo biofilm model**

Catheter segments of 1 cm length were incubated overnight in PBS (100%) at 37°C. Serum-coated catheters were incubated for 90 min at 37°C in a 1 ml *C. albicans* cell suspension (5 × 10⁴ cells ml⁻¹ prepared in RPMI). After incubation, catheters were washed twice with PBS before implanting them under the skin of mice or rats as described previously, with minor adaptations (Ričícová et al., 2010). In brief, general anesthesia was performed by intraperitoneal injection of a mixture of ketamine (Ketamine1000®, Pfizer, Puurs, Belgium) and medetomidine (Domitor®, Pfizer) (45 mg kg⁻¹ ketamine and 0.6 mg kg⁻¹ medetomidine for mice, 60 mg kg⁻¹ ketamine and 0.4 mg kg⁻¹ medetomidine for rat) and local anaesthesia by application of xylocaine gel (2%, AstraZeneca, Zoetermeer, the Netherlands) on the skin. The lower back of the animals was shaved and disinfected with iodine isopropanol (1%). A small incision was made and the subcutis was carefully dissected to create two (for mice) or three (for rats) subcutaneous tunnels. In each tunnel, three catheter pieces were inserted. In total, six (for mice) or nine (for rats) catheter fragments were implanted (see Vande Velde et al., 2013 for more details). The incision was closed with surgical staples or sutured with surgical thread and disinfected.
Anesthesia was reversed with intraperitoneal injection of atipamezole [Antisedan® (Pfizer), 0.5 mg kg⁻¹ for mice, 1 mg kg⁻¹ for rats]. For catheter explantation, the animals were euthanized by cervical dislocation. The skin was disinfected with 0.5% chlorhexidine in 70% alcohol; catheter fragments were removed from under the subcutaneous tissue and washed twice with PBS for further quantification of biomass.

**Magnetic resonance imaging (MRI)**

MR images were acquired on a 9.4 Tesla Biospec small animal scanner (Bruker Biospin, Ettlingen, Germany) equipped with an actively shielded gradient set of 600 mT m⁻¹ and in combination with a 7.5 cm quadrature coil (Bruker Biospin) for radio-frequency irradiation and detection. The mice were anesthetized by inhalation of 3% isoflurane (Abbott Laboratories, Queenborough, UK) for induction and 1.5% isoflurane for maintenance in 100% O₂ at a flow rate of 600 ml min⁻¹. Breathing rate and body temperature were continuously monitored using an MR compatible physiological monitoring system (SAIL, Stony Brook, NY, USA). Localizer images were acquired first and used for proper positioning and orientation of subsequently acquired images. Whole-body images were acquired with an in-plane resolution of 200*200 μm² by using a 2D spin echo sequence (RARE) with the following parameters: RARE factor = 8, TEeff = 15.9 ms, TR = 6000 ms, 2 averages, matrix = 200*400, FOV = 40*80 mm², 50 continuous slices of 500 μm thickness; resulting in a total scanning time of 12 min. MR images were processed using Paravision 5.1 software (Bruker Biospin) and exported in Dicom format. For co-registration of BLI and MR images, the photographic image from BLI was overlaid with the whole-body MR image using in-house developed software (Carlon et al., 2010). Hereby, limb position and size were used as reference points.

**In vivo BLI**

The animals were anesthetized using a gas mixture of isoflurane in oxygen (1.5–2% for mice and 2–3% for rats) and imaged in the IVIS 100 system. Before every in vivo imaging session, the 1.2 mM CTZ working solution was prepared freshly from a 5 mg ml⁻¹ stock solution by dilution in PBS. A volume of 100 μl CTZ was topically applied simultaneously to each catheter trio by subcutaneous injection in each tunnel. Subsequently, the animal was placed in the BLI camera and consecutive frames were acquired with a field of view of 10 cm. Consecutive scans with acquisition time of 20–60 s (depending on the signal intensity) were acquired starting immediately (i.e. within 10 s) after CTZ administration until maximum signal intensity was reached. The BLI signal of each catheter trio was quantified using Living Image software and reported as photon flux per second (p s⁻¹) for a rectangular ROI of 95 cm² placed over each catheter trio. Background BLI signal was measured by using the same ROI after injecting CTZ subcutaneously on the upper back of the mouse (no catheters implanted). All in vivo experiments were repeated twice with similar results.

**Biofilm quantification**

The metabolic activity of the biofilm forming cells was quantified by using the XTT reduction assay. Biofilms formed on 96-well plates were washed twice with PBS and 100 μl of XTT-solution (1 mg ml⁻¹) supplemented with menadion (1 μM) was added in the absence of light. Plates were subsequently incubated at 37°C for 1–3 h and the colorimetric changes were measured with a spectrophotometer (SPECTRAmax plus 384, Molecular Devices, Sunnyvale, CA, USA) at 490 nm.

For fungal burden assessment by cfu quantification, biofilms formed on cell culture plates, in in vitro evaluated catheters and explanted catheters were washed twice with PBS, sonicated for 10 min at 40 000 Hz in a water bath sonicator (Branson 2210) and vortexed for 30 s in PBS. Original samples, 1:10 and 1:100 dilutions were plated on YPD agar in duplicate. Cfu were counted after incubating the plates for 2 days at 37°C.

**Statistics**

All reported experiments were repeated twice and analysed separately, yielding consistent results. Data were expressed as mean ± standard deviation (SD). Significance tests were performed using unpaired t-test and ANOVA with Tukey post-test with P-values of < 0.05 considered significant, represented as follows: *P < 0.05, **P < 0.005, ***P < 0.0005. For statistics and representation of the results we used Microsoft Office Excel 2007 (Microsoft Corporation, Seattle, WA, USA) and GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

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**Conflict of interest**

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. Phosphorescence of different common catheter types. Three different brands of triple lumen catheters (Arrow, Edwards Swan-Ganz, BBraun Certofix) were cut in 1 cm long pieces and placed in Petri dishes for image acquisition with the BLI camera. Panel A: luminescence image acquired after exposing the catheters to light by opening-closing of the scanner door, showing phosphorescence signal emitted by the triple-lumen Swan-Ganz and Arrow catheter pieces, while no phosphorescence signal was detected for the Certofix catheter pieces. Panel B is the same image as in panel A, showing the overlaid ROIs used for quantification of the background and phosphorescence signals as plotted in Fig. 1A.

Fig. S2. BLI of yeast-to-hyphal cell ratio. HWPgLuc-expressing C. albicans cells were cultured either to be in the yeast cell form (in YPD medium) or in the hyphal cell form (YP medium containing 10% fetal bovine serum). Candida yeast and/or hyphal cell suspensions of 1 × 10^7 cells ml^-1 were plated in 24-well plates, in a total volume of 1 ml medium, imaged with BLI in triplicate series as follows: HWPgLuc-expressing C. albicans hyphal/yeast cells in ratios (%): 100/0–80/20–60/40–40–60–20/80–0/100. The graph represents the BLI results and linear regression line (R^2 = 0.9559), showing near-perfect correlation of the bioluminescence signal intensity with the percentage hyphal content in the sample. Error bars are SD of triplicate samples.

Fig. S3. BLI of planktonic bcr1^-ACTgLuc cells. A. BLI of 10-fold dilutions of bcr1^-ACTgLuc and SC5314-ACTgLuc (wt control) C. albicans cells, and CTZ alone (n = 3). The BLI signal emitted from bcr1^-ACTgLuc and SC5314-ACTgLuc C. albicans was found to be equal and correlated strongly with the number of cells. (R^2 (bcr1^-ACTgLuc) = 0.957, R^2 (SC5314-ACTgLuc) = 0.999). B. BLI signal (left graph) and cfu (right graph) quantified from the planktonic cells collected from the lumen of explanted catheters at different time points of biofilm formation by bcr1^-ACTgLuc and SC-ACTgLuc C. albicans strains. The means of triplicate samples per catheter (n = 18) were plot. Error bars indicate SD of replicate samples; *P < 0.05, **P < 0.005, ***P < 0.0005.