Surface display of *Gaussia princeps* luciferase allows sensitive fungal pathogen detection during cutaneous aspergillosis

Stefanie Donat,1,2 Mike Hasenberg,3,4 Tina Schäfer,1 Matthias Gunzer,3,4 Hermann Einsele,2 Jürgen Löffler,2 Andreas Beilhack3 and Sven Krappmann5,*

1Institute of Molecular Infection Biology, Julius Maxilimilans University; Würzburg, Germany; 2Department of Internal Medicine II; University Clinic; Würzburg, Germany; 3Research Center for Infectious Diseases; Julius Maximilians University; Würzburg, Germany; 4Centre for Medical Biotechnology; University of Duisburg-Essen; Essen Duisburg, Germany; 5Research Center for Infectious Diseases, Julius Maximilians University, Würzburg, Germany

Keywords: *Aspergillus fumigatus*, bioluminescence, biophotonic imaging, antifungal therapy, cutaneous aspergillosis

Non-invasive imaging techniques in microbial disease models have delivered valuable insights in the intimate pathogen-host interplay during infection. Here we describe evaluation and validation of a transgenic bioluminescence reporter strain of the human-pathogenic mold *Aspergillus fumigatus*, one of the main fungal pathogens affecting immunocompromised individuals. Expression and surface display of the *Gaussia princeps* luciferase allowed sensitive and rapid detection of luminescence emitted from this strain after substrate addition, with photon fluxes strongly correlating to the amounts of fungal conidia or germlings. The reporter strain allowed spatio-temporal monitoring of infection in a cutaneous model of aspergillosis, where neutropenic mice maintained the fungal burden while immunocompetent ones were able to clear it entirely. Most importantly, antifungal therapy could be followed in this type of disease model making use of the bioluminescent *A. fumigatus* strain. In conclusion, combining sensitivity of the *Gaussia luciferase* with a surface display expression system in the fungal host allows longitudinal infection studies on cutaneous forms of aspergillosis, providing perspective on drug screening approaches at high-throughput.

Introduction

Fungal infections have gained increasing relevance for public health as well as in distinct clinical settings.1,2 Among the predominant fungal pathogens, the ubiquitous mold *Aspergillus fumigatus* has become of interest, based on the fact that the number of susceptible, immunocompromised patients has steadily risen over the past two decades.3,4 Hematological malignancies and prolonged neutropenia are the leading risk factors associated with Aspergillus infections, which primarily occur via the pulmonary route to possibly result in invasive or disseminated, systemic forms. Invasive aspergillosis (IA) is associated with a high mortality as it is often diagnosed late with therapeutic options being limited. Besides this prevalent type of the disease, several less frequent ones have been described, among them cutaneous forms of aspergillosis, which make up 5 to 10% of IA cases.5,6 Hematological malignancies and prolonged neutropenia are the leading risk factors associated with Aspergillus infections, which primarily occur via the pulmonary route to possibly result in invasive or disseminated, systemic forms. Invasive aspergillosis (IA) is associated with a high mortality as it is often diagnosed late with therapeutic options being limited. Besides this prevalent type of the disease, several less frequent ones have been described, among them cutaneous forms of aspergillosis, which make up 5 to 10% of IA cases.5,6 Primary cutaneous infections with *A. fumigatus* conidia may occur after skin injuries or extensive burns; also, neonatal infections by contaminated dressings have been described.6,7 Additionally, systemic forms of IA may result in cutaneous localization of fungal lesions.

Investigations on factors relevant for aspergillosis pathogenesis or drug testing experiments strictly rely on suitable disease model, most commonly established in susceptible small animals like immunocompromised guinea pigs or mice.11 For pulmonary aspergillosis, murine models have been validated extensively to cover distinct preconditioning aspects (e.g., neutropenia or corticosteroid treatment). A model for cutaneous aspergillosis, however, was just recently introduced using nude (nu/nu) mice rendered leukopenic by cyclophosphamide treatment.12 Soft tissue infections with *A. fumigatus* were performed by subcutaneous injection of a conidial suspension in the animals’ thigh. From there, a skin lesion expanded, the area of which correlated with fungal burdens as estimated by galactomannan contents. In this setting, the attenuated virulence of distinct *A. fumigatus* mutants that had been characterized to be hypovirulent in pulmonary infections, as well as a reduction in tissue lesion size during antifungal treatment could be monitored, validating this type of disease model to a reasonable extent.

Monitoring fungal infections in real time and repeatedly in an animal enables longitudinal host-pathogen interaction studies. Non-invasive imaging allows the collection of spatial and temporal data during an infection with lesser animals required. Recent improvements in live imaging techniques had been achieved by the help of luminescent reporter enzymes and the use of highly sensitive charge-coupled device cameras.13 Among
expression, and membrane anchoring. From this plasmid, (pSK478) harboring several functional elements for expression, 
expression of the according GLuc reporter. This strain allows sensitive imaging that is less dependent on the environmental context. For aspergillosis and antifungal treatment of the disease, efficient catalyzing light emission in an ATP-independent context.22 For aspergillosis and antifungal treatment of the disease, however, this particular luciferase reporter has not been evaluated to date.

In order to assess capacities of the luminescent G. princeps enzyme for monitoring infections by A. fumigatus, we here describe generation and characterization of a recombinant strain expressing a synthetic gene expression construct to result in surface display of the GLuc reporter. This strain allows sensitive and longitudinal infection studies by bioluminescence imaging, as validated in a cutaneous murine infection model of aspergillosis.

Results

Expression and surface display of Gaussia princeps luciferase in A. fumigatus. Pathogen detection via bioluminescent imaging requires proper and high-level expression of the according luciferase enzyme. Furthermore, suitable conditions for substrate turnover have to be taken into account, making the validation of a bioluminescent reporter strain a challenging task. In order to establish a recombinant A. fumigatus strain that would express the coped GLuc luciferase, we assembled a synthetic construct (pSK478) harboring several functional elements for expression, secretion, and membrane anchoring. From this plasmid, transcription of the gluc gene is driven by a truncated version of the constitutively strong A. nidulans gpdA promoter, which had been validated before to be actively transcribed under in vitro and in vivo conditions. In order to achieve targeting of the translation product into the secretory pathway, two secretion signals were fused in frame to the gluc coding sequence: the one from the A. oryzae gpdA gene plus the Photinus pyralis N28 region, which derives from a lipase-encoding gene. To express the luminescent enzyme in a surface-exposed manner and allow facilitated substrate turnover, its coding sequence was furthermore fused to a putative glucose-phosphate isomerase (GPI)-anchored protein, which serves as a carrier for cellular localization. There, the A. fumigatus ortholog of the A. oryzae cell wall protein MP1 was chosen, as this had already been successfully exploited in

A. oryzae tglA which derives from a lipase-encoding gene.23 To express the GLuc reporter, this strain was chosen, as this had already been successfully exploited in

...
imaging in neutropenic mice. Given the high sensitivity of the cyclophosphamide to result in prolonged neutropenia.28 BALB/c mice were immunosuppressed by the repetitive applica-

AfS75 in an animal model for aspergillosis. To that end, inbred interested in the bioluminescence imaging capacities of strain Gaussia luciferase reporter combined with unhampered substrate accessibility when targeted to the fungal cell wall, we were

The bioluminescence reporter strain allows non-invasive imaging in neutropenic mice. Given the high sensitivity of the Gaussia luciferase reporter combined with unhampered substrate accessibility when targeted to the fungal cell wall, we were interested in the bioluminescence imaging capacities of strain AF575 in an animal model for aspergillosis. To that end, inbred BALB/c mice were immunosuppressed by the repetitive application of cyclophosphamide to result in prolonged neutropenia. Pulmonary infections performed in these animals did, however, result in detectable photon fluxes only when high inocula of about $10^6$ conidia were used and directly after the substrate coelenterazine had been applied intravenously (data not shown). Even then, signals could merely be detected after several days post-infection when animals already suffered severely from disease progression due to the high infection doses. In consequence, the rapid decline in photon emission activity by the GLuc enzyme accompanied by instability or limited bioavailability of its substrate does not allow for convenient in vivo imaging of pulmonary infections by the transgenic strain AF575 in susceptible mice.

To test an alternative as well as relevant model of aspergillosis, cutaneous infections of neutro-

pneumonic mice were performed following the experimental layout of Ben-Ami et al.13 While these authors used nude (nu/nu) BALB/c mice infected with $A. fumigatus$ strains and followed progress-

Figure 1. Surface display of the Gaussia princeps luciferase on Aspergillus fumigatus. (A) Schematic presentation of the integrative plasmid to express a synthetic, codon-optimized version of the gluc gene in $A. fumigatus$ and to target its product to the cell surface by anchoring it to the membrane protein MP1. Functional elements of the expression cassette are the constitutively transcribed gpdA promoter (gpdA) from A. nidulans and the his2A terminator region (his2A) from A. fumigatus. For secretion, the GLuc coding sequence (gldA) is preceded by a signal sequence (ss) from the A. oryzae tglA gene and the Rhyzopus oryzae N28 terminator region (N28). In-frame fusion via a (GA)5 linker region to the coding region of the GPI-anchored MP1 protein from $A. fumigatus$ results in surface display of the bioluminescent reporter. (B) X-ray film autoradiography after addition of the GLuc substrate coelenterazine to mycelia of different transformants carrying the expression construct. Besides the wild-type recipient strain ATCC 46645, isolates carrying one, two and four copies, respectively, of the integrative plasmid pSK481 are displayed. (C) Light intensities emitted from GLuc reporter strains expressing varying doses of the bioluminescent reporter based on the indicated copy numbers. Photographs were taken before (upper panel) and after (lower panel) coelenterazine addition with a digital camera in the light and dark, respectively.

Fig. 4C). However, no significant correlation of the corresponding tissues. Photon fluxes from the AF575-infected sites of each neutropic animal varied only to a limited degree over the course of infection, being followed for seven days post-infection, which is most likely attributed to the relatively high loads of fungal inocula that might result in a saturated bioluminescence signal. Between animals, differences in signal intensities were evident, reflecting the natural variance in infection series. Fungal burdens in the infected tissues (Fig. 4B), as assessed by galactomannan immunoassays, rose over the course of infection (Fig. 4C). However, no significant correlation of
signal intensities with tissue weight-corrected galactomannan indices (cGMI) could be deduced from the bioluminescence images.

To estimate the minimal dose of infection that would result in a clear bioluminescent signal, decimal multiplicities of AfS75 conidia ranging from $10^7$ to $10^9$ were used in a further infection
series. Light emissions from infected dorsal tissues were monitored for three days post-infection to reveal that as little as $10^3$ conidia of strain AF53 resulted in a detectable and stable bioluminescent signal over the course of infection (Fig. 5A). Most lucid signals, however, could be detected from doses of $10^5$ and $10^6$ infectious propagules. A clear correlation with the infection dose and photon emission could be deduced for the early phase of infection when determining photon fluxes eight hours after infection (Fig. 5B); at later time points, this correlation was lost because of considerable variations between the monitored animals, and also no correlations to cGMI values could be deduced (data not shown).

Most importantly, we assessed the possibility to monitor antifungal drug efficiency in this aspergillosis model by in vivo imaging of bioluminescence. For that purpose, cohorts of neutropenic animals were administered two established antifungal substances with different modes of action, amphotericin B (AmB) and posaconazole, from the day of infection with $5 \times 10^5$ AfS75 conidia on, and photon fluxes were measured directly after subcutaneous substrate injection. Imaging series clearly revealed that oral treatment with the antifungal agent posaconazole resulted in effective resolution of the fungal infection within five days, while a clear bioluminescent signal could be detected for the AmB-treated animals (Fig. 6). The latter observation was most likely due to limited bioavailability of the drug at the cutaneous site of infection, as direct injection of AmB into the seat of infection resulted in rapid clearance of the bioluminescence.

Figure 3. Bioluminescent properties of the reporter strain expressing surface-exposed GLuc. (A) AfS75 displays a flash kinetic of the bioluminescent signal. Emission of photons after substrate addition to $1 \times 10^3$ germinated conidia was monitored for approximately six minutes to result in a rapid, nonlinear decay of luminescence. (B) Bioluminescence can be detected from fungal structures formed from at least 1,000 conidia as early as 4 h. Increased spore numbers or extended incubation times result in increases of signal intensities. Subtracted background values from blank medium were in a range of $1-2 \times 10^2$ photons per second.
of immune defense.14 Luminescent reporters derived from hosts, especially with respect to infection processes or mechanisms characterizing the intimate interplay between pathogens and their respective infection model system is crucial for in vivo imaging, one. Substrate availability and stability in the luciferin for the permanent emission of photons, current eukaryotic systems do require addition of the corresponding luciferase substrate, such as luciferin for the Photinus pyralis luciferase or coelenterazine for the G. princeps one. Substrate availability and stability in the respective infection model system is crucial for in vivo imaging, and limitations there might restrict the usefulness of one or the other bioluminescence system in different disease models. Here, we followed the approach of Ennulatt et al.31 that localized the highly sensitive luciferase of the copepod G. princeps, encoded by a synthetic, codon-optimized gene, to the fungal cell surface, resulting in better substrate accessibility as a strict prerequisite for luminescence. In preliminary experiments expressing the GLuc expression module integrated in the fungal genome has to be taken into account. When analyzing clonal descendants from a conidial population cultured under non-selective conditions in vitro or in vivo, we did not observe a substantial decrease in numbers of colony forming units when plating again on selective culture medium (our unpublished results). Yet, we were able to monitor infections semi-quantitatively for several days, noticing increases in fungal load and, most importantly, the complete clearance upon antifungal treatment or in immunocompetent animals. As such, our system represents a significant and consistent further development of the recently introduced, pivotal model of cutaneous aspergillosis by Kontoyiannis and co-workers.13 There, nude mice were further immunocompromised and infected in their thighs by injection of high inocula to result in skin lesions, the area of which correlated with the tissue fungal burden to a certain degree. Interestingly, we were unable to detect similar skin lesions when infecting our immunosuppressed BALB/c mice at the dorsal flank, which might relate to the differing genetic background, the alternative site of infection, or the reduced infection doses. And indeed, when inoculating neutropenic BALB/c mice with 10^5 conidia at the dorsal flank, prominent lesions could be spotted after five days, but not when applying this inoculum of 5 × 10^5 conidia to the dorsal flank (data not shown). In conclusion, non-invasive imaging is the method of choice in this type of cutaneous aspergillosis model because fungal particles are directly visualized, so it represents an advantageous experimental approach for pathogen detection and surveillance of disease progression. Of particular interest, we were demonstrating the general susceptibility of the reporter strain to antifungal treatment with amphotericin B. In essence, this demonstrates the usefulness of the bioluminescence readout from transgenic strain AT75 as an indicator for failed or successful antifungal therapy.

Discussion

Non-invasive imaging techniques have become a cornerstone in characterizing the intimate interplay between pathogens and their hosts, especially with respect to infection processes or mechanisms of immune defense.31 Luminescent reporters derived from biological systems have proven extremely useful in such purposes, as they allow sensitive and dynamic imaging in various disease models, thereby reducing the numbers of required animals in such studies. In contrast to bacterial luminescence reporters that are based on the lux operons of Photobacterium luminescens or Xenorhabdus luminescens and allow constant imaging due to the permanent emission of photons, current eukaryotic systems do require addition of the corresponding luciferase substrate, such as luciferin for the Photinus pyralis luciferase or coelenterazine for the G. princeps one. Substrate availability and stability in the respective infection model system is crucial for in vivo imaging, and limitations there might restrict the usefulness of one or the other bioluminescence system in different disease models. Here, we followed the approach of Ennulatt et al.31 that localized the highly sensitive luciferase of the copepod G. princeps, encoded by a synthetic, codon-optimized gene, to the fungal cell surface, resulting in better substrate accessibility as a strict prerequisite for luminescence. In preliminary experiments expressing the GLuc expression module integrated in the fungal genome has to be taken into account. When analyzing clonal descendants from a conidial population cultured under non-selective conditions in vitro or in vivo, we did not observe a substantial decrease in numbers of colony forming units when plating again on selective culture medium (our unpublished results). Yet, we were able to monitor infections semi-quantitatively for several days, noticing increases in fungal load and, most importantly, the complete clearance upon antifungal treatment or in immunocompetent animals. As such, our system represents a significant and consistent further development of the recently introduced, pivotal model of cutaneous aspergillosis by Kontoyiannis and co-workers.13 There, nude mice were further immunocompromised and infected in their thighs by injection of high inocula to result in skin lesions, the area of which correlated with the tissue fungal burden to a certain degree. Interestingly, we were unable to detect similar skin lesions when infecting our immunosuppressed BALB/c mice at the dorsal flank, which might relate to the differing genetic background, the alternative site of infection, or the reduced infection doses. And indeed, when inoculating neutropenic BALB/c mice with 10^5 conidia at the dorsal flank, prominent lesions could be spotted after five days, but not when applying this inoculum of 5 × 10^5 conidia to the dorsal flank (data not shown). In conclusion, non-invasive imaging is the method of choice in this type of cutaneous aspergillosis model because fungal particles are directly visualized, so it represents an advantageous experimental approach for pathogen detection and surveillance of disease progression. Of particular interest, we were...
able detect complete elimination of the fungal pathogen over time when animals were treated with the established antifungal drug posaconazole. This is in sharp contrast to the skin lesions’ readout, dimensions of which were significantly reduced but did not disappear in the course of fungicidal treatment. Accordingly, the bioluminescent reporter allows dynamic and non-invasive monitoring of cutaneous *A. fumigatus* infections and their resolution either in the course of a proper immune response or during antibiosis. As a further aspect, this particular GLuc reporter strain appears as well suited for multiplex imaging studies employing alternative bioluminescence reporters that are expressed, for instance, by transgenic cells of the host immune system.

The use of a non-invasive imaging technique as experimental approach to follow fungal infection furthermore allows screening approaches in high-throughput, requiring lower numbers of animals. Moreover, assessing the virulence of fungal mutants, which correlates to growth at the site of infection, appears feasible and convenient in the bioluminescence reporter background, allowing to assess the contribution of distinct *A. fumigatus* genes and their products to virulence of this fungal pathogen.

![Non-invasive imaging of bioluminescence with increasing amounts of fungal spores.](image-url)
Materials and Methods

Strains, media, transformations. The *Escherichia coli* strain DH5α was used for cloning procedures. Plasmid-carrying *E. coli* strains were routinely grown in at 37°C LB liquid medium (1% peptone, 0.5% yeast extract, 0.5% NaCl) under selective conditions (100 μg/ml ampicillin).

The wild-type isolate ATCC 46645 served as recipient *Aspergillus fumigatus* strain and was generally cultured in nitrate-based minimal medium at 37°C. Integration of the expression vector carrying the ptrA marker gene was selected for by the presence of pyrithiamine at a concentration of 0.1 μg/ml.

*E. coli* competent cells were prepared and transformed following the calcium chloride method. Protoplast fusion-mediated transformation of *A. fumigatus* was performed following essentially the procedure of Punt and van den Hondel.

Construction of plasmids and recombinant *A. fumigatus* strains. Standard protocols of recombinant DNA technology were performed. Phusion high-fidelity DNA polymerase was generally used in polymerase chain reactions (PCRs) and essential cloning steps were verified by sequencing.

The plasmid pSK481 was constructed by inserting a synthetic 1.53 kb DNA fragment acquired from the company GENEART AG (Regensburg, Germany) in the PmeI site of the *Aspergillus* expression vector pSK379. The coding sequence of the chimeric gene is based on the *A. oryzae* TglA signal sequence (GenBank accession number BAA92327.1), the *Rhizopus oryzae* triacylglycerol lipase preproprotein (BAG16921.1), the *G. princeps* luciferase (AAG54095.1), and the *A. fumigatus* MP1 protein (AFUA_4G03240) and was deposited at GenBank under accession number JN223418.

Luciferase assays for bioluminescence. The kinetic of bioluminescence emitted from *AfS75* was determined in a Victor Light luminescence counter (PerkinElmer) as follows: In a white 96-well flat bottom plate, 1 x 10⁶ conidia per well were incubated in Aspergillus minimal medium overnight at 30°C to allow germination. Culture supernatants were removed, germlings were washed three times with phosphate-buffered saline (PBS), 200 μl Gaussia-Glow-Juice (PJK GmbH) were added, and photon fluxes (in counts per second, cps) were determined every 5 sec for time periods of up to 6 min after substrate injection.

![Figure 6. The bioluminescent reporter strain AfS75 allows in vivo monitoring of antifungal therapy. Shown are animals from an in vivo imaging series of neutropenic mice infected with 5 x 10⁶ conidia of *AfS75* (GLuc4) and the wild-type isolate ATCC 46645 (ATCC), respectively, that had been treated with amphotericin B deoxycholate (AmB, 5 mg/kg/day i.p.) or posaconazole (40 mg/kg/day per os), demonstrating failure of antifungal treatment by AmB or complete clearance of the fungal infection by posaconazole as evident from the persistence and absence, respectively, of bioluminescence. When applying AmB directly at the seat of infection (+ AmB s.c.), bioluminescence was resolved within two days.](image-url)
Localisation studies. Cellular localization of the GLuc enzyme in \( \text{AS75} \) was assessed by indirect immunofluorescence following essentially the protocols of Momany\(^5\) and Oakley and Osman.\(^6\) In particular, 1 × 10\(^6\) conidia in minimal medium were incubated in Lab-Tek\(^7\) Chamber Slides overnight at 37°C to allow germination, washed in PEM buffer (50 mM PIPES pH 6.7, 25 mM EGTA pH 7.0, 5 mM MgSO\(_4\)), and fixed in IF fixer solution (3.7% formaldehyde, 5% DMSO, 25 mM EGTA pH 7.5, 5 mM MgSO\(_4\)) for 30 min at room temperature. After washing with PBS (0.1% BSA in PBS) and pre-incubation for 1 h at room temperature in 1% PBSA, 150 μl of a 1:200 dilution of a monoclonal anti-GLuc IgG 2a antibody (PJ3KJ4G4A) were added to 2 × 10\(^3\) conidia grown on minimal medium.

Following the approach of Ben-Ami et al., female BALB/c mice (Charles Rivers) weighing 18–20 g were rendered neutropenic by the repeated use of cyclophosphamide (150 mg/kg i.p. at days -3, -1, +2 and every other following day). Infections were performed with homogenous suspensions of freshly harvested conidia in PBS/0.005% Tween 80 by subcutaneous injection in dorsal sites after fur removal. For non-invasive imaging under ketamine/xylazine anaesthesia, 100 μl of a 15.6 mM coelenterazine solution were injected subcutaneously at the site of infection and animals were immediately put in an IVIS\(^\text{®}\) Lumina II imaging chamber (Caliper Life Sciences) for bioluminescence detection within an exposure time of one minute. Images were analyzed by the LivingImage\(^\text{®}\) 3.1 software.

Tissue fungal burden by galactomannan assay. Skin tissue with underlying muscles was excised from infected areas, weighed, and homogenized in PBS. After centrifugation, supernatants of homogenates were diluted 1:250 and assayed at the Institute of Hygiene and Microbiology, University Clinic Würzburg, Germany with an enzyme immunoassay kit (Placida Aspergillus EIA from Biorad) detecting galactomannan contents. The resulting galactomannan indices (GMI) were corrected for tissue weights.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We are indebted to Astrid Dyda, Anxelle Degu, and Michaela Dünig for excellent technical assistance and to all other members of the Institute for Molecular Infection Biology for continuous support. Werner J. Heine is thanked for providing posaconazole, Thomas Hartmann for qPCR analyses, Hilde Merkert for providing assistance with fluorescence microscopy, and Christian Erck from the HZI Braunschweig, Germany for typing the monoclonal \( \text{K} \)-GLuc antibody. Funding was received from the German José Carreras Leukaemia Foundation (Cooperation Project DJCLS 10/15), the Free State of Bavaria, and the University of Würzburg.

References

1. McNeil MM, Matt V, Naga R, Pusch M, Gans LA, Hohenberg R, et al. Trends in mortality due to invasive fungal infections in the United States, 1999-2005. Clin Infect Dis 2011; 53:664-75. PMID:2194620; http://dx.doi.org/10.1093/cid/cir430.

2. Warrenk DW. Fungal disease: an evolving public health challenge. Med Mycol 2006; 44:497-505. PMID:17117626; http://dx.doi.org/10.1080/13693780500534493.

3. Acquisti O, Rea RJ, de Pillier B, Bontem JP, Belle J, Croissant F, et al. Defining opportunistic invasive fungal infections in immune-compromised patients with cancer and hematopoietic stem cell transplants: an international consensus. Clin Infect Dis 2011; 53:357-67. PMID:21731919; http://dx.doi.org/10.1093/cid/cir533.

4. Talbot GH, Bodey J, Edwards J, Jr., Gilbert D, Schell M, Barlow JG. Bad bugs need bad bugs: an update on the development pipeline of antifungal drugs. Availability Task Force of the Infection Disease Society of America. Clin Infect Dis 2006; 42:597-606. PMID:16443711; http://dx.doi.org/10.1086/490819.

5. Warrenk DW. Trends in the epidemiology of invasive fungal infections. Nippon Sanka Fujinka Gakkai Zasshi 2007; 48:1-12. PMID:17207757; http://dx.doi.org/10.1536/cb-48-1.1.

6. Bruges A, Zarata TE, Doucet CV, Hoffmann JA, Knuppi KH, Naria I, et al. Pediatric invasive aspergillosis: a multicenter retrospective analysis of 141 consecutive cases. Pediatrics 2008; 121:e2006-96. PMID:184587; http://dx.doi.org/10.1542/peds.2007-2117.

7. Patterson TF, Kirkpatrick WR, White M, Hiemenz JW, Wingard JR, Dupont B, et al. Invasive aspergillosis: a multicenter retrospective analysis of 135 consecutive cases. Pediatrics 2008; 121:e2006-96. PMID:184587; http://dx.doi.org/10.1542/peds.2007-2117.

8. Ptak J, Holubcik J, Wiegand J, Depout R, et al. Intracranial aspergillosis: diagnosis, intracranial treatment, and outcome. J Neurosurg Neurosci Group Medico 2006; 76:290-46. PMID:16943154; http://dx.doi.org/10.1057/palgrave.smg.7500812.

9. Hennig KA, Sakhale CP, Chandrab D, Sode P, Miao J, Megl F, et al. Investigation of a cluster of cutaneous aspergillosis in a research primate colony. J Hosp Infect 2011; In press. PMID:21849804; http://dx.doi.org/10.1016/j.jhin.2011.06.012.

10. Mamik CK, Lee H, HooperH DF, Gusciuc UC, Inoue JA, Kim SH, et al. Incidence of systemic fungal infections and related mortality following seven burn units. Burns 2009; 35:1184-92. PMID:19455261; http://dx.doi.org/10.1016/j.burns.2008.06.007.

11. van Bakel J, Cohen B, Sarda JP. Cytomastitis aspergillosis. J Clin Microbiol 1998; 36:1515-21. PMID:977589.

12. Wald T. Primary cutaneous aspergillosis—an emerging infection among immunocompromised patients. Clin Infect Dis 1996; 23:473-7. PMID:977589; http://dx.doi.org/10.1093/clinids/23.4.473.

13. Zaa AK, Steinbuch T. Mammalian models of aspergillosis. In: Goldman GH, Osman MA, eds. The Aspergilli: Molecular Aspects, Pathogenicity, and Human Infections. Boca Raton: CRC Press, 2007:401-12.

14. Bachar J, Luei RE, Lazurka K, Luji JP, Koyrentzov DP. Cutaneous model of invasive aspergillosis. Acta Derm Venereol 2010; 90:984-9. PMID:2044378; http://dx.doi.org/10.1111/j.1600-0645.00648.
14. Andreu N, Luker GD. Applications of bioluminescence imaging for studies of infectious diseases. EMSH Microbiol Rev 2011; 35:86-94. PMID:22057395.

15. Conte CR, Conte PR, Smith SD, Levens DR, Rumlayan DS. Photonic detection of bacterial pathogens in living hosts. Mol Microbiol 1995; 18:593-603. PMID:7687861; http://dx.doi.org/10.1111/j.1365-2958.1995.ea.844993x.

16. Huchon M, Debay EB. Applications of bioluminescence imaging to the study of infectious diseases. Cell Microbiol 2007; 9:415-20. PMID:17719326; http://dx.doi.org/10.1111/j.1462-5822.2007.00959.x.

17. Ziao KR, Chenfier TR, Khadra AA, Kevan D, Writer C, Driver B, et al. Noninvasive bioluminescence imaging in small animals. J Invest 2008; 4:100-15. PMID:18137257.

18. D'Elia C, Visocchi A, Brumon AJ. Bioluminescent fungi for real-time monitoring of fungal infections. Viruses 2010; 2:79-86. PMID:20718406; http://dx.doi.org/10.1155/2010/5.1.11119.

19. Beck M, Jourdin G, Dore-Boisjoly D, Domage O, Nicole MA, Brunkner-Ganter O. Bioluminescent Aspergillus fumigatus, a new tool for drug efficiency testing and in vivo monitoring of invasive aspergillosis. Appl Environ Microbiol 2007; 74:7023-35. PMID:18442350; http://dx.doi.org/10.1128/AEM.01288-08.

20. Ibrahim-Granet O, Jouvion G, Hohl TM, Droin-Bergere S, Philippart F, Kim OY, et al. Quantification of Aspergillus fumigatus by a noninvasive bioluminescence reporter for live mammalian cells. J Infect Dis 2009; 200:1057-65. PMID:1977660; http://dx.doi.org/10.1086/592110.

21. Hama S, Tamalampudi S, Shindo N, Numata T, Hara M, Gutierrez-Blazquez MD, Nombela C, Gil C. Identification of cell-wall stability and full virulence. Eukaryot Cell 2008; 7:213-22. PMID:18203488; http://dx.doi.org/10.1128/EC.00375-09.

22. Yoo Ar, DeCruz E, Noyer-Weidner M, et al. Quantitative analysis reveal distinct roles for resident and recruited immune effector cells in defense against invasive fungal pathogen Candida albicans. Mol Microbiol 2008; 77:4847-58. PMID:19687206; http://dx.doi.org/10.1111/j.1365-2958.2008.06189.x.

23. Munro R, Sankaran S, Haidl K, Wang SC, Dugger K, et al. Noninvasive bioluminescence imaging in small animals. ILAR J 2008; 50:164-69. PMID:18795995; http://dx.doi.org/10.1093/ilar/ilo025.

24. Contag CH, Contag PR, Mullins JI, Spilman SD, Stevenson DK, Benaron DA. Photonic detection of fungal pathogens in living hosts. Mol Microbiol 2001; 41:1128-39. PMID:12036096; http://dx.doi.org/10.1042/9780076253-000-150-4.

25. Meade T, Inj, Kanase K, Kart M, Ishida H, Saham H, et al. Characterization of an Aspergillus fumigatus cell-surface display system using a positive G-Polypeptide reporter. Appl Microbiol Biotechnol 2008; 81:167-78. PMID:18188121; http://dx.doi.org/10.1007/s00253-008-1112-x.

26. Tannous BA, Kim DE, Fernandez JL, Weissleder R, Breakefield XO. Codon-optimized Gaussia luciferase-cDNA for mammalian gene expression in culture and in vivo. J Mol Ther 2005; 11:435-43. PMID:15727940; http://dx.doi.org/10.4161/viru.1.3.11119.

27. Tannous BA, Tamalampudi S, Hara M, Gutierrez-Blazquez MD, Nombela C, Gil C. Isolation and characterization of expression of plasmid and phage recombinant luciferase. J Mol Ther 2004; 10:499-510. PMID:15430232.

28. Smith JM, Tang CM, Van Noorden S, Holden DW. Identification of two strains of Gaussia princeps luciferase reporter for live mammalian cells. J Mol Ther 2004; 10:476-81. PMID:15126887; http://dx.doi.org/10.1162/1528002047728962.

29. Woodcock DM, Crowther PJ, Doherty J, Jefferson S, McManus J, et al. Development and testing of a new tool for drug efficiency monitoring of fungal infections. Viruses 2010; 2:79-86. PMID:20718406; http://dx.doi.org/10.1155/2010/5.1.11119.

30. Hearn VM, Mackenzie DW. Mycelial antigens from the pathogenic fungus Aspergillus fumigatus. Mol Microbiol 2002; 44:495-507. PMID:11986627; http://dx.doi.org/10.1046/j.1365-2958.2001.03185.x.

31. Käfer E. Meiotic and mitotic recombination in filamentous fungi for real-time monitoring of fungal infections. Mykosen 1980; 23:549-62. PMID:6160400; http://dx.doi.org/10.1111/j.1365-2958.1995.ea.844993x.

32. Kubodera T, Yamashita N, Nishimura A, Pyrithiamine resistance gene (pdr1) of Aspergillus fumigatus, characterization and application as a dominant selectable marker for transformation. Rev Inst Med Trop Sao Paulo 2000; 42:161-62. PMID:11192258; http://dx.doi.org/10.1016/j.ymthe.2004.10.016.

33. Hanakar D, Inomoto T, Hoshino N. Plasmid transformation of Escherichia coli and other bacteria. Methods Enzymol 1995; 248:63-113. PMID:15471876; http://dx.doi.org/10.1016/0076-6879(94)04004-0.

34. Post PJ, van den Hondel CA. Transformation of filamentous fungi based on hypoxanthine B and phlorizin resistance markers. Methods Enzymol 1992; 216:857-67. PMID:1799904; http://dx.doi.org/10.1101/med.9728180001.

35. Sandkvist J, Friman K, Mattace R, Molecular and cellular biology of filamentous fungi a practical approach. Oxford: Oxford University Press, 2001.

36. Oakley RH, Orton SA. Cell-cycle analysis using the fluorescent reporter gene for monitoring of filamentous fungi. In: Fantes P, Revell K, eds. The cell cycle: a practical approach. Oxford: Oxford University Press, 2001.

37. Oakley RH, Orton SA. Cell-cycle analysis using the fluorescent reporter gene for monitoring of filamentous fungi. In: Fantes P, Revell K, eds. The cell cycle: a practical approach. Oxford: Oxford University Press, 2001.

38. Broek M, Jourdin G, Heil TM, Dore-Boisjoly D, Philippart F, Kim OY, et al. In vivo bioluminescence imaging and histopathopathologic analysis reveal distinct roles for resident and recruited immune effector cells in defense against invasive aspergillosis. BMC Microbiol 2010; 10:105; PMID:20377900; http://dx.doi.org/10.1186/1471-2180-10-105.