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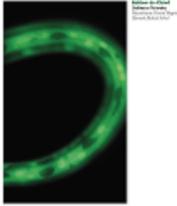
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Bioluminescent fungi for real-time monitoring of fungal infections

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Novel luciferase reporters have been developed that allow real-time monitoring of infections by the fungal pathogens *Candida albicans* and *Aspergillus fumigatus*. Although these reporters still suffer limitations in the context of invasive infections, they provide unprecedented tools to monitor superficial infections and the efficacy of antifungal drugs or vaccines. In particular, the sensitivity and ease of detection of the cell-surface *Gaussia princeps* luciferase developed for *C. albicans* should make it a powerful tool for functional genomics studies in this and other pathogenic fungi.

Real-time monitoring of microbial infections has now become integral to the study of host-pathogen interactions. Indeed, by monitoring in real-time and in a single animal the spatial and temporal progression of a microbial infection, or of the host response, it becomes possible to reveal significant variations in pathogen/host responses that can be masked by the heterogeneous behavior of individual animals, and to identify the spread of pathogens to unexpected infection sites. Importantly, real-time monitoring has significant ethical advantages because it permits statistically significant datasets to be generated with reduced numbers of animals.¹ In recent years in vivo imaging technologies have been developed that take advantage of sensitive charge-coupled device (CCD) cameras to detect low levels of light emitted from luciferase reporters in vivo. Contag et al.² in a pioneering work demonstrated that bioluminescent *Salmonella* could be localized to specific tissues in live animals, allowing

the temporal monitoring of the infection process and the efficacy of antimicrobial treatment. This approach has now been extended to numerous pathogenic bacteria, virus and parasites¹ and several luciferases are available for in vivo imaging including firefly (*Photinus pyralis*), sea pansy (*Renilla reniformis*) and *Gaussia princeps* luciferases.³⁻⁵

Only recently was this approach applied to the monitoring of fungal infections. Doyle et al.^{6,7} have used the firefly luciferase gene expressed under the control of the *C. albicans* *ENO1* promoter to image different forms of *C. albicans* infections. While this approach was successful in a vulvo-vaginal candidiasis model and allowed the efficacy of an antifungal treatment to be monitored, it suffered limitations for the analysis of disseminated candidiasis. In particular, the limited permeability of hyphal cells to the firefly luciferase substrate luciferin might be regarded as a major drawback of this reporter system given the central importance of the yeast-to-hypha transition in *C. albicans* virulence.^{8,9} In a recent report, we attempted to circumvent this limitation by targeting the naturally secreted *G. princeps* luciferase to the cell surface of *C. albicans* using the targeting signals of a *C. albicans* GPI-linked cell wall protein.¹⁰ This approach resulted in the expression of a highly sensitive reporter, referred to as gLUC59, at the *C. albicans* cell surface and allowed the detection of luciferase in intact yeast and hyphal cells even when expressed from a weak promoter. This new reporter has been used successfully to monitor different forms of superficial *C. albicans* infections whereby the gLUC59 substrate

Key words: luciferase, *Gaussia princeps*, *Candida*, candidiasis, *Aspergillus*, aspergillosis, imaging, reporter, gene fusion, host-pathogen interaction

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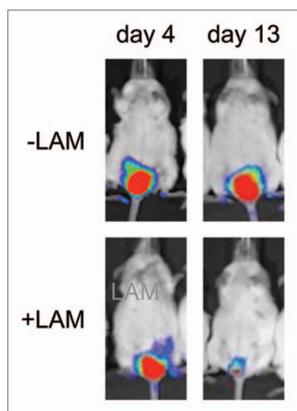


Figure 1. In vivo imaging of vaccinated mice vaginally infected with bioluminescent *Candida albicans*. Mice, previously treated with adjuvant (-LAM) or vaccinated with adjuvant plus a β -glucan-conjugate (+LAM), were infected into the vaginal lumen with a suspension of *C. albicans* cells expressing the gLUC59 cell-surface exposed luciferase. At day 4 and 13 post-infection, mice received an intravaginal injection of coelenterazine and were imaged in the IVIS-200™ imaging system (Xenogen Inc.) See Pietrella et al.¹¹ for details.

coelenterazine is delivered directly to the site of infection. Using this approach, the efficacy of antifungal treatments in combating the development of cutaneous candidiasis could be monitored.¹⁰ The efficacy of a β -glucan-conjugate vaccine and of anti- β glucan antibodies against murine vaginal candidiasis was also demonstrated (Fig. 1).¹¹ Importantly, these studies have shown that imaging superficial candidiasis with gLUC59-expressing *C. albicans* strains was more reliable than vaginal colony-forming unit (CFU) counts in assessing the extent and duration of these infections, and the consequent protection level. This undoubtedly results from the efficient detection of the gLUC59 luciferase from yeast and hyphal cells in contrast to the unreliable assessment of CFU from *C. albicans* populations that contain a mixture of these cell morphologies. Yet, monitoring systemic candidiasis using gLUC59-expressing *C. albicans* strains was unsuccessful.¹⁰ There are several possible explanations for this failure: the autoluminescence of coelenterazine; the uneven distribution of this substrate following intravenous injection into the mouse; the deep-seated location of the kidneys, the main target organ

of *C. albicans* in systemic candidiasis, which precludes efficient visualization of photons from this organ through the mouse body; and the particular, possibly hypoxic, environment generated in infected organs that may inhibit *G. princeps* luciferase activity. Thus, an imaging system that will be suitable for the real-time monitoring of systemic candidiasis remains to be developed. This might in particular involve improvements in the mode of administration of coelenterazine and its stability in vivo in order to take advantage of the exciting properties of the gLUC59 reporter. Developing *C. albicans* strains that co-express a luciferase along with enzymes necessary for the production of its substrate might represent a suitable alternative. In this regard, progress is being made on the mechanisms involved in the biosynthesis of luciferin and coelenterazine.^{12,13} It might be possible to take advantage of the *Photobacterium luminescens lux* system, as has been done in a variety of bacterial species.¹⁴ Yet all attempts so far have proved unsuccessful for imaging disseminated candidiasis, even though light production was observed in vitro (Y. Fu, personal communication). This again suggests a potential limitation of such approaches arising from the hypoxic environment generated in the infected organs.

Bioluminescent *Aspergillus fumigatus* strains have also been generated through expression of the firefly luciferase gene under the control of the constitutive *A. fumigatus gpdA* promoter.¹⁴ These strains proved useful to monitor the efficacy of different antifungals in vitro. Moreover, they could be used to image the early stages of pulmonary infection, with luciferin being provided by the intraperitoneal route. However, during later stages of infection, when fungal burden in the lung increased, the light signal obtained from live animals decreased. Again, several possible explanations were raised for the failure to image the late stages of pulmonary invasive aspergillosis: the reduced distribution of intraperitoneally-injected luciferin that may result from the clinical distresses of animals; and the environment generated in the infected lungs which might restrict oxygen availability necessary for the activity of firefly luciferase.¹⁴ Here again the luciferase/substrate

combination will have to be optimized to allow monitoring of the entire course of *Aspergillus* infection taking into account the environment that is generated as a consequence of the infection process.

Although the luciferase reporters described by Enjalbert et al.¹⁰ and Brock et al.¹⁴ were not developed primarily to study gene expression in vitro or ex vivo, they provide powerful new tools for such studies in pathogenic fungi. In particular, the cell-surface exposed gLUC59 luciferase proved a highly sensitive and easy-to-use reporter to monitor the activity of a variety of *C. albicans* promoters in multiple growth conditions using intact cells.¹⁰ Clearly, this reporter will facilitate functional dissection of specific promoter regions and their regulation in *C. albicans*. Additionally, we envision that gLUC59 could be used as a transformation marker in a program of systematic gene knock-outs in *C. albicans*, thereby providing both knock-out mutants and strains for the monitoring of promoter activity in a variety of growth conditions, including interactions with host-cells. Data obtained with such strains would not only complement those obtained by transcript profiling but also provide more subtle evaluation of promoter activity. Whether the firefly luciferase reporter developed for *A. fumigatus* has similar potential for the study of gene regulation remains to be established. Yet, the gLUC59 reporter could be adapted to any pathogenic fungus of interest. In this respect, it should be noted that this reporter was optimized for expression in *C. albicans* and while it could, in principle, be used directly in related *Candida* species, it would need additional optimization for use in more distantly related species such as *Candida glabrata* and pathogenic filamentous fungi.

In summary, recently developed luciferase reporters provide new tools for genetic and host-pathogen interaction studies in pathogenic fungi. These reporters will need further optimization in order to allow the imaging of fungal invasive infections, which appear to generate an environment that is not optimal for luciferase activity and/or substrate availability. When this goal has been achieved (as is already the case for superficial infections), it will become possible to combine the use of bioluminescent fungi with mice that express

bioluminescent reporters of the innate immune system, thus providing greater insights in the fungal-host interplay.

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