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A Luciferase Reporter for Gene Expression Studies and Dynamic Imaging of Superficial *Candida albicans* Infections

Donatella Pietrella, Brice Enjalbert, Ute Zeidler, Sadri Znaidi, Anna Rachini, Anna Vecchiarelli, and Christophe d'Enfert

Abstract

Real-time imaging of fungal infections is becoming integral to the study of host–pathogen interactions, as it allows monitoring of the spatial and temporal progression of pathogen growth or of the host response in a single animal as well as reducing the number of animals used to obtain significant data. We present different applications of a novel luciferase reporter gene constructed from the coding sequences of the *Candida albicans* *PGA59* gene, encoding a GPI-linked cell wall protein, and the *Gaussia princeps* luciferase gene. Upon addition of the coelenterazine substrate, light produced by the surface-exposed luciferase can be used to quantify gene expression from a variety of *C. albicans* promoters as well as monitoring cutaneous, subcutaneous, and vaginal infections.

Key words: Luciferase, *Gaussia princeps*, Imaging, Reporter, Gene fusion, Vulvovaginal candidiasis, Subcutaneous candidiasis, Superficial infection

1. Introduction

The study of host–pathogen interactions has recently benefited from the development of real-time imaging in small animals. Real-time imaging approaches take advantage of sensitive charge-coupled device (CCD) cameras to detect low levels of light emitted from luciferase reporters in vivo (1). They allow monitoring of the spatial and temporal progression of pathogen growth or of the host response in a single animal, making it possible to reveal the spread of pathogens to unexpected infection sites as well as significant variations in pathogen/host responses that can be masked by the heterogeneous

behavior of individual animals. Moreover, real-time monitoring allows for a reduction in the number of animals required to generate statistically significant datasets (1).

Real-time imaging of *Candida albicans* infections has been pioneered by Doyle et al. (2, 3) who used the firefly luciferase gene expressed under the control of the *C. albicans* *ENO1* promoter to image different forms of *C. albicans* infections. This approach was successful in a vulvovaginal candidiasis model and allowed monitoring of the efficacy of an antifungal treatment. However, it suffered drawbacks, such as a limited permeability of hyphal cells to the firefly luciferase substrate, luciferin, and monitoring of disseminated candidiasis was unsuccessful.

In this chapter, we present a novel luciferase reporter whereby the naturally secreted *Gaussia princeps* luciferase (4) is targeted to the cell surface of *C. albicans* using the targeting signals of the *C. albicans* GPI-linked cell wall protein Pga59 (5) (Fig. 1a). This approach resulted in a highly sensitive reporter, referred to as gLUC59, localized at the cell surface and allowing detection of luciferase in intact yeast and hyphal cells even when expressed from a weak promoter (6). gLUC59 has been used successfully to monitor different forms of superficial *C. albicans* infections where the luciferase substrate coelenterazine can be directly delivered at the site of infection (Fig. 2). The efficacy of antifungal treatments, e.g., a β -glucan-conjugate vaccine and anti- β -glucan antibodies, against the development of different superficial *C. albicans* infections could be efficiently monitored (6, 7). Importantly, these studies showed that imaging superficial candidiasis with gLUC59-expressing *C. albicans* strains was more reliable than colony-forming unit (CFU) counts in assessing the extent and duration of these infections. However, it should be noted that monitoring systemic candidiasis has so far been unsuccessful when using *C. albicans* strains expressing the gLUC59 luciferase (6, 8).

Here, we present protocols for measuring *C. albicans* promoter activity in in vitro grown cells and monitoring of superficial, namely cutaneous, subcutaneous, and vaginal, *C. albicans* infections by imaging infected animals in the IVIS imaging system.

2. Materials

2.1. Plasmids and *C. albicans* Strains

Plasmids and *C. albicans* strains of interest for imaging infections using the gLUC59 reporter are listed in Table 1 (see Note 1).

2.2. Equipment

1. IVIS-200™ imaging system (Xenogen Corporation).
2. Anesthesia chamber (Xenogen Corporation).
3. Flat-bottom black-wall 96-well plates (see Note 2).
4. Infinite M200 PRO multimode plate reader (Tecan).

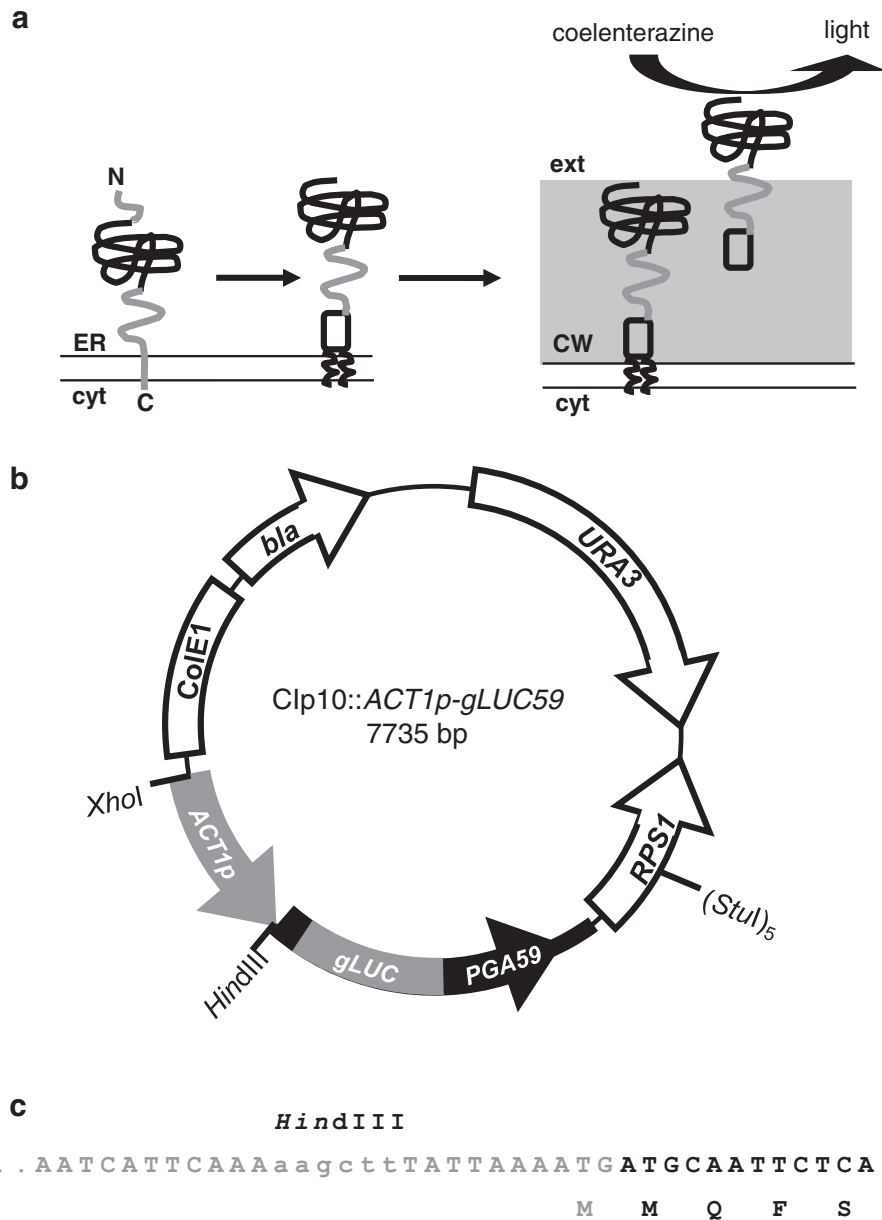


Fig. 1. The cell surface exposed gLUC59 luciferase. **(a)** gLUC59 is synthesized as a fusion between the amino-terminal signal peptide of Pga59 (gray line), the *Gaussia princeps* luciferase (black line), and the mature and carboxy-terminal hydrophobic domains of Pga59 (gray line). Upon entry into the endoplasmic reticulum (ER), the Pga59 amino-terminal signal peptide and carboxy-terminal hydrophobic domain are cleaved and the protein is modified by addition of a glycosylphosphatidylinositol (GPI) anchor. The GPI-modified protein is then shuttled to the cytoplasmic membrane where the GPI anchor is processed, allowing gLUC59 to become anchored to cell wall (CW) β -glucans and exposed at the cell surface. Owing to the cell surface exposure of gLUC59, light is produced upon addition of coelenterazine to intact cells. **(b)** The Clp10::ACT1p-gLUC59 vector is a derivative of the *C. albicans* Clp10 integrative vector (10) that harbors the *C. albicans* URA3 transformation marker (URA3; white arrow) and that, upon cleavage by *StuI*, integrates at the *C. albicans* RPS1 locus (RPS1; white arrow). A *XhoI/HindIII* fragment encompassing the ACT1 promoter (ACT1p; gray arrow) was cloned upstream of the gLUC59 luciferase reporter gene. gLUC59 is a fusion between the *C. albicans* PGA59 gene (PGA59; black arrow) and the *Gaussia princeps* luciferase gene that has been adapted for efficient expression in *C. albicans* (gLUC; gray box). Propagation of Clp10::ACT1p-gLUC59 is achieved in *E. coli* in the presence of ampicillin (bla; white arrow). The ACT1 promoter is easily exchanged by other promoter regions using a *XhoI/HindIII* digest. **(c)** Nucleotide sequence at the junction between the ACT1 promoter and the gLUC59 reporter gene. Replacement of the ACT1 promoter (gray) by another promoter should ensure that an ATG start codon does not occur upstream and out-of-frame of the gLUC59 ATG start codon (black).

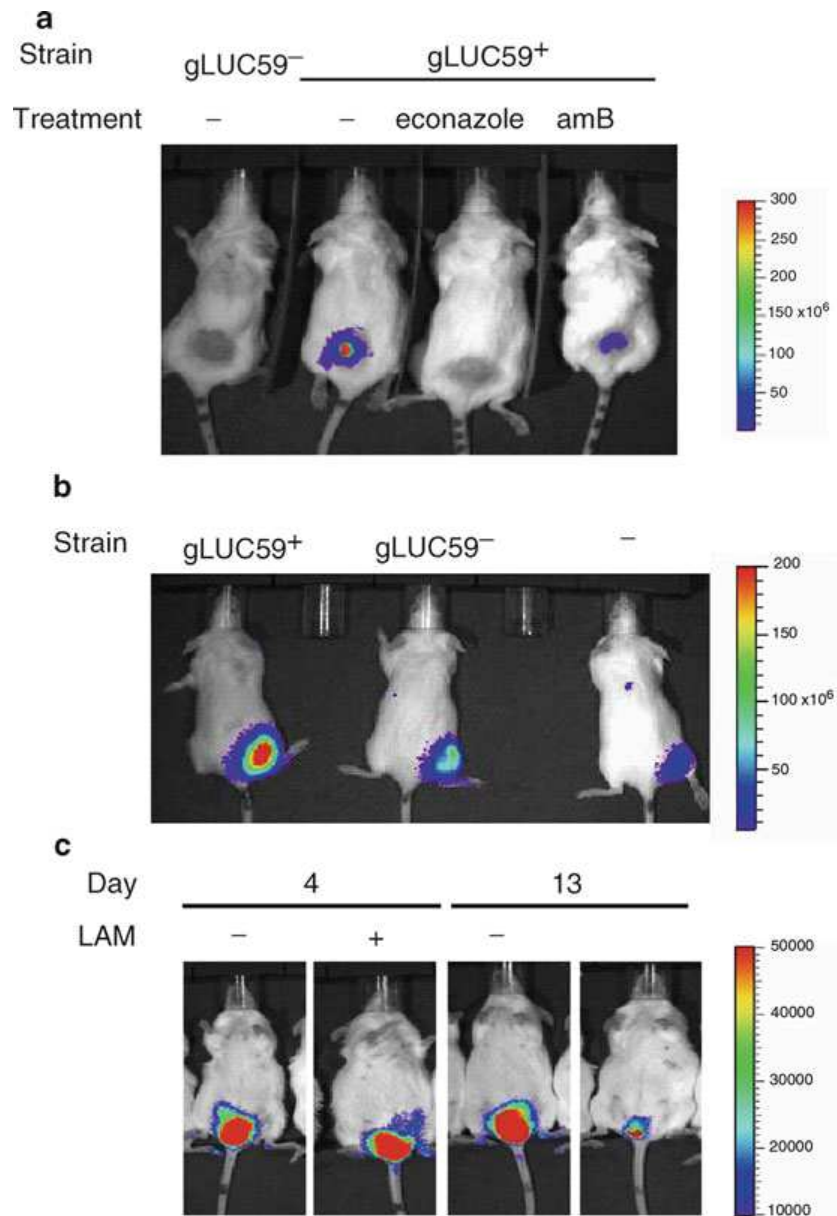


Fig. 2. In vivo imaging of mice infected with bioluminescent *Candida albicans*. **(a)** Cutaneous infection. Immunosuppressed mice were inoculated on a shaved and abraded skin area with *C. albicans* cells either expressing the gLUC59 cell surface exposed luciferase (gLUC59⁺) or not (gLUC59⁻). gLUC59⁺-infected mice were treated intraperitoneally with a placebo (-), econazole (10 mg/kg body weight), or amphotericin B (amB; 10 mg/kg) every day post infection. At day 7 post infection, coelenterazine was deposited at the site of infection and mice were imaged in the IVIS-200™ imaging system (Xenogen Inc.) (4). **(b)** Subcutaneous infection. Uninfected mice or mice inoculated subcutaneously with *C. albicans* cells expressing the gLUC59 cell surface exposed luciferase (gLUC59⁺) or not (gLUC59⁻) received a coelenterazine injection at the site of infection at 4 days post infection and imaged in the IVIS-200™ imaging system (4). Background luminescence with control strains is frequently observed especially in inflamed regions. **(c)** Vaginal infection. Mice, previously treated with adjuvant (-LAM) or vaccinated with adjuvant plus a β -glucan conjugate (+LAM) were injected into the vaginal lumen with a suspension of *C. albicans* cells expressing the gLUC59 cell surface exposed luciferase. At days 4 and 13 post infection, mice received an intravaginal injection of coelenterazine and were imaged in the IVIS-200™ imaging system (7).

Table 1
Plasmids and *C. albicans* strains

Plasmid	Promoter		<i>C. albicans</i> strain	Reference
CIp10:: <i>gLUC59</i>	–		CA1399	(6)
CIp10:: <i>gLUC59</i> , <i>ura3::SAT1</i>	–			Lab collection
CIp10:: <i>ACT1p-gLUC59</i>	<i>ACT1p</i>	Constitutive expression	CA1398	(6)
CIp10:: <i>ACT1p-gLUC59</i> , <i>ura3::SAT1</i>	<i>ACT1p</i>	Constitutive expression		Lab collection
CIp10:: <i>TDH3p-gLUC59</i>	<i>TDH3p</i>	Constitutive expression	CEC2181	Lab collection
CIp10:: <i>TDH3p-gLUC59</i> , <i>ura3::SAT1</i>	<i>TDH3p</i>	Constitutive expression		Lab collection
CIp10:: <i>ADH1p-gLUC59</i>	<i>ADH1p</i>	Constitutive expression	CEC2183	Lab collection
CIp10:: <i>TEF1p-gLUC59</i>	<i>TEF1p</i>	Constitutive expression	CEC2180	Lab collection
CIp10:: <i>EFT3p-gLUC59</i>	<i>EFT3p</i>	Constitutive expression	CEC975	(6)
CIp10:: <i>TRR1p-gLUC59</i>	<i>TRR1p</i>	Oxidative stress-induced (strong)	CA1435	(6)
CIp10:: <i>TRR1p-gLUC59</i>	<i>TRX1p</i>	Oxidative stress-induced (strong)	CA1400	(6)
CIp10:: <i>IPF9996p-gLUC59</i>	<i>IPF9996p</i>	Oxidative stress-induced (weak)	CA1434	(6)
CIp10:: <i>HWPIp-gLUC59</i>	<i>HWPIp</i>	Hyphal-specific	CEC971	(6)
CIp10:: <i>ALS3p-gLUC59</i>	<i>ALS3p</i>	Hyphal-specific	CEC939	(9)
CIp10:: <i>HSP70p-gLUC59</i>	<i>HSP70p</i>	Heat shock responsive	CEC2134	Lab collection
CIp10:: <i>MKC1p-gLUC59</i>	<i>MKC1p</i>	PKC cell integrity pathway induced (weak)	CEC2136	Lab collection
CIp10:: <i>UTR2p-gLUC59</i>	<i>UTR2p</i>	Calcineurin pathway induced	CEC2138	Lab collection
CIp10:: <i>CRZ1p-gLUC59</i>	<i>CRZ1p</i>	Calcineurin pathway induced	CEC2140	Lab collection

2.3. Media and Reagents

1. YPD: 1% yeast extract (Difco), 2% Bacto-peptone (Difco), 2% D-glucose; autoclave 10 min at 120°C; add 1.5% Bacto-agar for solid medium.
2. 1 M coelenterazine (Molecular Probes; see Note 3) in methanol.
3. 500 µg/mL coelenterazine (Synchem; see Note 3) in 1:9 methanol:PBS.

4. 1 mg/mL coelenterazine (Synchem; see Note 3) in 1:4 methanol:PBS.
5. Estradiol valerate sesame oil.
6. LA buffer: 0.5 M NaCl, 0.1 M KH_2PO_4 pH 6.7, 1 mM EDTA, 0.6 mM NaN_3 , 1 mM phenylmethylsulfonide fluoride cocktail (Boehringer).

2.4. Animals (see Note 4)

Six-week-old female CD1 mice (Harlan), housed in groups of 4–6 mice per cage, were allowed to acclimatize for 1 week prior to experimentation. Animals were maintained under specific pathogen-free (SPF) conditions that included testing sentinel animals for unwanted infections; according to the Federation of European Laboratory Animal Science Association standards, no infections were detected.

3. Methods

3.1. Measurement of Luciferase Activity from In Vitro Grown *Candida albicans* Cells Expressing *gLUC59* Constructs (see Note 5)

1. Using *C. albicans* cells from an overnight culture, inoculate two 20 mL cultures of YPD to OD 0.1.
2. Allow cells to grow for 2 h at 30°C with shaking.
3. Take a 1 mL sample from both cultures, measure the OD₆₀₀, and keep on ice.
4. If examining fungal cell responses to a compound, add the compound of interest to one of the two cultures and then continue incubation of both cultures at 30°C.
5. Collect 1 mL samples from the untreated and treated cultures at different time points, measure the OD₆₀₀, and keep on ice.
6. Centrifuge 1 mL samples for 3 min at 3,000 × *g* at 4°C in an Eppendorf centrifuge.
7. Resuspend cells in 500 μL cold LA buffer and centrifuge for 3 min at 3,000 × *g* at 4°C.
8. Resuspend cells in 250–500 μL cold LA buffer to obtain the same cell/volume concentration in all samples and keep on ice (see Note 6).
9. Put 100 μL of each sample in duplicate into a flat-bottom black-wall 96-well plate.
10. Add 100 μL of freshly made 2 μM coelenterazine in LA buffer to each well. Pipet up and down once.
11. Measure immediately in a luminometer (e.g. Infinite M200 PRO plate reader; see Note 7).

**3.2. Real-Time
Imaging of *C. albicans*
Cutaneous Infection
(see Note 8)**

1. Three days prior to infection, immunosuppress mice by intraperitoneal injection of cyclophosphamide (150 mg/kg).
2. One day prior to infection, inoculate a 5 mL YPD culture with a freshly grown colony of *C. albicans* strain CA1398 and grow at 20–30°C with shaking for 16 h.
3. On the day of infection, harvest cells, wash twice in sterile endotoxin-free physiological saline, enumerate by hemocytometer, and adjust to a final concentration of 5×10^8 CFU/mL in sterile physiological saline.
4. Immunosuppress mice by intraperitoneal injection of cyclophosphamide (150 mg/kg).
5. Anesthetize mice by intraperitoneal injection of 80 μ L pentobarbital (50 mg/kg), shave an area of 4 cm² on the lower back (Fig. 1a) to remove the fur, and then abrade the skin with sand paper until glistening (see Note 9).
6. Deposit 20 μ L of the *C. albicans* cell suspension on the abraded area.
7. Monitor infection every day for 7 days post infection by anesthetizing mice with 2.5% isoflurane and depositing 20 μ L coelenterazine (500 μ g/mL) on the abraded skin area and imaging the animal dorsal side up in the IVIS-200™ imaging system under anesthesia with 2.5% isoflurane (see Note 10).
8. For infections continuing for more than 3 days, perform a third immunosuppressive treatment by intraperitoneal injection of cyclophosphamide (50 mg/kg) on day 3 post infection.

**3.3. Real-Time
Imaging of *C. albicans*
Subcutaneous
Infection**

1. One day prior to infection, inoculate a 5 mL YPD culture with a freshly grown colony of *C. albicans* strain CA1398 and grow at 20–30°C with shaking for 16 h.
2. On the day of infection, harvest cells, wash twice in sterile endotoxin-free physiological saline, enumerate by hemocytometer, and adjust to a final concentration of 10^8 CFU/mL in sterile physiological saline.
3. Inoculate mice subcutaneously in the right thigh with 100 μ L of the *C. albicans* cell suspension.
4. After challenge, and every day post infection (see Note 11), inject mice subcutaneously with 100 μ L coelenterazine (500 μ g/mL) and image animals in the IVIS-200™ imaging system under anesthesia with 2.5% isoflurane (see Note 10).

**3.4. Real-Time
Imaging of Vaginal
Candidiasis**

1. Five days prior to infection, induce pseudo-estrus in mice by subcutaneous injection of 0.2 mg estradiol valerate in 100 μ L sesame oil. Repeat injection weekly to maintain pseudo-estrus.
2. One day prior to infection, inoculate a 5 mL YPD culture with a freshly grown colony of *C. albicans* strain CA1398 and grow at 20–30°C with shaking for 16 h.

3. On the day of infection, harvest cells, wash twice in sterile endotoxin-free physiological saline, enumerate by hemocytometer, and adjust to a final concentration of 10^9 CFU/mL in sterile physiological saline.
4. Anesthetize mice with 2.5% (v/v) isoflurane gas (see Note 12).
5. Infect mice with 10 μ L of the *C. albicans* cell suspension administered from an Eppendorf 0.5–10 μ L mechanical pipette into the vaginal lumen, close to the cervix. To encourage vaginal contact and adsorption of fungal cells, hold mice head down for 1 min following inoculation.
6. Inoculate a 5 mL YPD culture with a freshly grown colony of *C. albicans* strain CA1398 and grow at room temperature with shaking for 16 h.
7. On day 1 post infection, repeat steps 3–5.
8. Allow mice to recover for 24–48 h, during which time the *C. albicans* vaginal infection will establish.
9. From day 4, add 10 μ L coelenterazine (5 mg/mL) to the vaginal lumen and image animals in the IVIS-200™ imaging system under anesthesia with 2.5% isoflurane (see Note 10).

4. Notes

1. Methods presented in this chapter only make use of strain CA1398. However, we also provide a list of the plasmids and strains which could be used in in vitro or in vivo experiments; these strains are available upon request.
2. Alternatively, black plates with transparent bottoms can be used, allowing simultaneous OD₆₀₀ measurements.
3. Coelenterazine from different suppliers varies in quality. High-quality coelenterazine from Molecular Probes is used for in vitro experiments. Lower quality coelenterazine from Synchem is used for animal experiments. Coelenterazine derivatives with increased stability, such as ViviRen (Promega), have not proven different from standard coelenterazine in our hands.
4. Animal experiments should adhere to ethical rules in place in the laboratory/animal facility where they are performed. In our case, all animal experiments adhered to the EU Directive 86/609. Experiments were performed according to the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. (ETS No. 123). The protocol was approved by the Perugia University Ethics Committee (Comitato Universitario di Bioetica) (permit numbers 41-2005B and 34/2003-A).

5. Luciferase activity can also be measured in a tube luminometer or detected by autoradiography (4).
6. Cell concentration in the assay can vary as it has been shown, using strain CA1398 expressing *gLUC59* under the control of the *ACT1* promoter, that luciferase activity varies linearly when using cell concentrations from 10^3 to 10^8 cells/mL. The use of identical cell concentrations for a given strain exposed to different conditions will facilitate comparisons between conditions.
7. Luciferase activity is expressed in relative luciferase units/cell. There is no requirement for a standard curve.
8. Groups of 4–6 animals are generally used for each strain tested.
9. Under these anesthesia conditions, mice remain asleep for 2 h. The whole treatment—shaving, skin abrasion, and *C. albicans* cells deposition—takes a maximum of 2 min and does not require analgesia. Upon recovery from anesthesia, mice are active without signs of pain.
10. The duration for imaging varies according to the infection model. In our hands, using strain CA1398, expressing *gLUC59* under the control of the *ACT1* promoter, imaging was carried out for 10–60 s in the cutaneous model of infection, 1–5 min in the subcutaneous model of infection, and 1–2 min in the vaginal candidiasis model. The imaging duration should be such that saturation at the site of infection is not achieved.
11. For the time of the studies, no adverse effects were observed due to repeated subcutaneous injection with coelenterazine.
12. Anesthesia is required for vaginal infection in order to inject the yeast suspension close to the cervix. To encourage vaginal contact and adsorption of fungal cells, mice are held head down for 1 min following inoculation (this procedure could be uncomfortable for mice when awake).

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