

Visualizing Hypoxia in a Murine Model of *Candida albicans* Infection Using *in vivo* Biofluorescence

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[Abstract] *Candida albicans* is a leading human fungal pathogen that uses several metabolic adaptations to escape immune cells and causes systemic disease. Here, we describe a protocol for measuring one of these adaptations, the ability to thrive in hypoxic niches. Hypoxia was generated after successful subdermal infection with *C. albicans* in a murine infection model. Hypoxia was measured using a fluorescent dye for carbonic anhydrase 9, a host enzyme active under hypoxic conditions. Emitted fluorescence was subsequently quantified using an IVIS system. This protocol was optimized for the use in subdermal infection in mice but has the potential to be adapted to other models of fungal infection.

Keywords: *Candida albicans*, Subdermal infection, Hypoxia, IVIS, Mycology

[Background] Fungal colonizers of humans have evolved to sense and adapt to niches available in the host (Grahl and Cramer, 2010). Oxygen is a changing environmental parameter. Levels change in different tissues and during different stages of infection and immune activation (Carreau *et al.*, 2011; Wenger *et al.*, 2015). The ability to sustain growth and to survive in low oxygen environments has been linked to virulence in several fungi (Shepardson *et al.*, 2013; Gresnigt *et al.*, 2016; Pradhan *et al.*, 2018). We have developed a protocol to follow the generation of hypoxia in a mouse infected with *C. albicans* (Lopes *et al.*, 2018). We chose subdermal infection as a local, non-disseminated model of mycosis with acute onset which allows analysis of hypoxia in a confined space, where *C. albicans* and host cells interact (Urban *et al.*, 2009; Santus *et al.*, 2018). Thereby, natural variation of oxygen levels in other tissues can be eliminated and secondary effects from distant locations arising in systemic infection can be avoided. During infection, hypoxia is created mainly by neutrophil influx to the site of infection. Neutrophil extravasation and the activation of oxygen-consuming enzymes create environments with low oxygen levels. In turn, *C. albicans* exploits this environmental shift to avoid immune recognition by changing cell wall composition leading to masking of recognized entities (Lopes *et al.*, 2018).

Materials and Reagents

1. Pipette tips (P20, P200, P1000)
2. Inoculation loop
3. Glass or disposable round bottom sterile tubes for growing 10 ml cultures of yeast (Sarstedt, catalog number: 62.547.004)
4. Tube, 50 ml (Sarstedt, catalog number: 62.547.004)
5. 1 ml micro-fine U100 insulin syringes (BD, catalog number: 324827)
6. Latex gloves
7. *Candida albicans* wt isolate SC5314
8. Mice 6- to 8-week-old albino female mice [B6(Cg)-Tyrc-2J/J; Jackson Laboratory-USA]
9. PBS (Thermo Scientific, catalog number: 18912014)
10. Isoflurane (Orion Pharma, Abbott Laboratories Ltd., Great Britain, CAS: 26675-46-7)
11. HypoxiSense 680 Fluorescent Imaging Agent (PerkinElmer, NEV11070r)
12. Yeast nitrogen base (Difco, catalog number: 233520)
13. Ammonium sulfate (Sigma, catalog number: 31119)
14. Yeast Synthetic Drop-out Medium Supplements (Sigma, catalog number: Y1501-20g)
15. Glucose (Sigma, catalog number: 47829)
16. Synthetic complete dropout medium (SC medium) containing 2% glucose (see Recipes)
17. 1x PBS (see Recipes)

Equipment

1. Pipettes (P20, P200, P1000)
2. 30 °C shaker for growing yeast cultures (speed approximate 180 rpm) (IKA KS 4000i)
3. Centrifuge (Eppendorf, model: 5180 R)
4. Cell counter Vi-CELL Cell Viability Analyzer Beckman Coulter
5. GI-8 gas anesthesia system (Caliper Life Sciences, Inc.)
6. Electric clipper to shave mice (Moser Chromini)
7. Heating lamp
8. Xenogen IVIS Live Animal Imaging System (PerkinElmer)
9. Incubator (Binder KB 53)

Software

1. Living Image software 4.5 (PerkinElmer)

Procedure

A. Fungal growth and mouse preparation (Days 1-3)

1. Start *C. albicans* liquid cultures by inoculation frozen stocks with a loop into 10 ml SC medium broth in a 50 ml tube and incubate for 20 h at 30 °C with shaking at a speed of 180 rpm.
2. Take 10 µl of *C. albicans* culture from the previous day to 10 ml fresh SC medium in a 50 ml tube. Incubate the new mixture for an equivalent time period.
3. At Day 3, count the cells in the cell counter and prepare a fresh subculture in 10 ml SC medium with a starting cell number of 5×10^7 cells/ml and let it grow for 3 h. Centrifuge the fungi at 3,000 $\times g$, 10 min at room temperature and re-suspend in 1 ml 1x PBS and count the fungal cell number using a cell counter.
4. Adjust the fungal concentration to 5×10^8 cells/ml and keep on ice if not used immediately. Calculation example: To prepare 1 ml of yeast suspension with a concentration of 5×10^8 cells/ml from a stock suspension of 8×10^8 cells/ml, add 625 µl of the stock suspension to 375 µl PBS.
5. All mice are injected intraperitoneally with 100 µl HypoxiSense 680 dye (2 nmol/100 µl of HypoxiSense dye per mouse) 24 h before *C. albicans* subdermal infection.

B. Subdermal infection (Day 4)

1. Place the mouse in an anesthesia box and regulate the level of isoflurane gas to 2.5% on the GI-8 gas anesthesia system. Remove the mouse from the anesthesia box and gently place the mouse with its nose inside an isoflurane delivering cone.
2. With the mouse anesthetized, shave the fur off the dorsal flank using the hair trimmer (Figure 1).
3. Pull and hold the mouse skin over the shaved area with two fingers. Insert the syringe into the pulled skin and infect the mouse with 100 µl of the *C. albicans* inoculum prepared (5×10^7 cells). A small liquid bump under the animal skin will be visible for a few minutes.
4. Treat and inject control animals in a similar way using 100 µl 1x PBS.
5. Animals should be placed under a heating lamp until they are fully awake to assure their well-being.

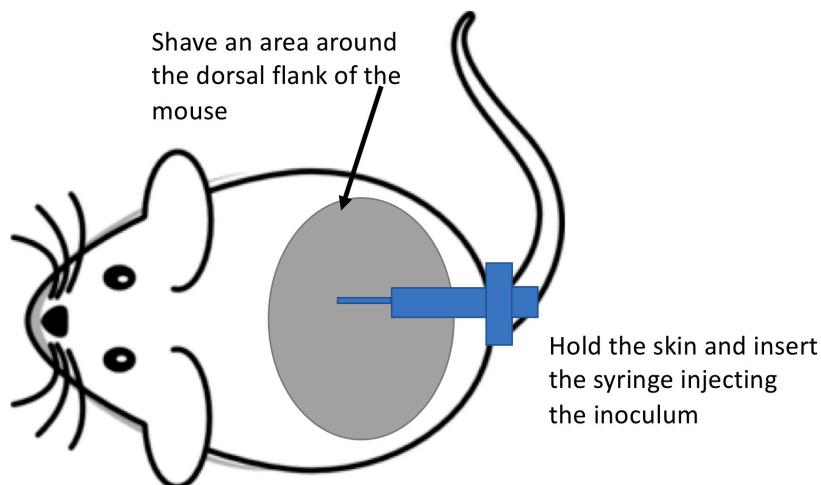


Figure 1. Schematic drawing on how to perform the subdermal infection

C. Biofluorescent imaging (Days 4-7)

1. Anesthetize the mouse in the anesthesia box using a gas flow of 2.5% isoflurane and transfer them to the camera box of the IVIS imager. Place the mouse facing downwards in the chamber with each animal's nose inside the cone that delivers the isoflurane.
2. Select the IVIS filter setting that fit the emission and excitation wavelength of the dye (675 ex/720 em).
3. Using guards to prevent signal bleeding from neighboring animals take a time-zero image of animals in both groups on Day 4 after the initial infection (Figure 2). Expose the animals for 0.5 s exposure capturing *in vivo* biofluorescence generated by the activity of HypoxiSense dye as a marker for hypoxia. In order to image 5 mice, select D field of view.
4. Since the animals might remain under anesthesia for a long period, assure that they are placed under a heating lamp to prevent excessive cooling down of the animal until they are fully awake.

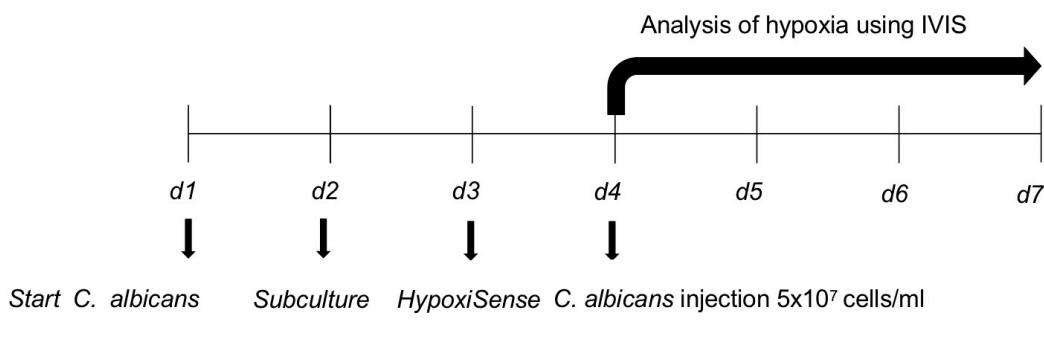


Figure 2. Schematics of the experimental procedure. d: Day.

Data analysis

Image treatment to quantify average epi-fluorescence:

1. Draw region of interested (ROI) over the areas of higher epi-fluorescence corresponding to the areas where the abscess is present. Place an additional ROI in a dark region to define a background (Figure 3).
2. Perform ROI analyses to measure average epi-fluorescence in each animal.
3. Subtract the epi-fluorescence values of the dark background from the values obtained for each animal.
4. Use these values to create a graph plot with epi-fluorescence values on the y-scale and the animal group in the x-scale. Statistical significance between the infected and control groups was performed using a two-tailed Mann-Whitney U-test.

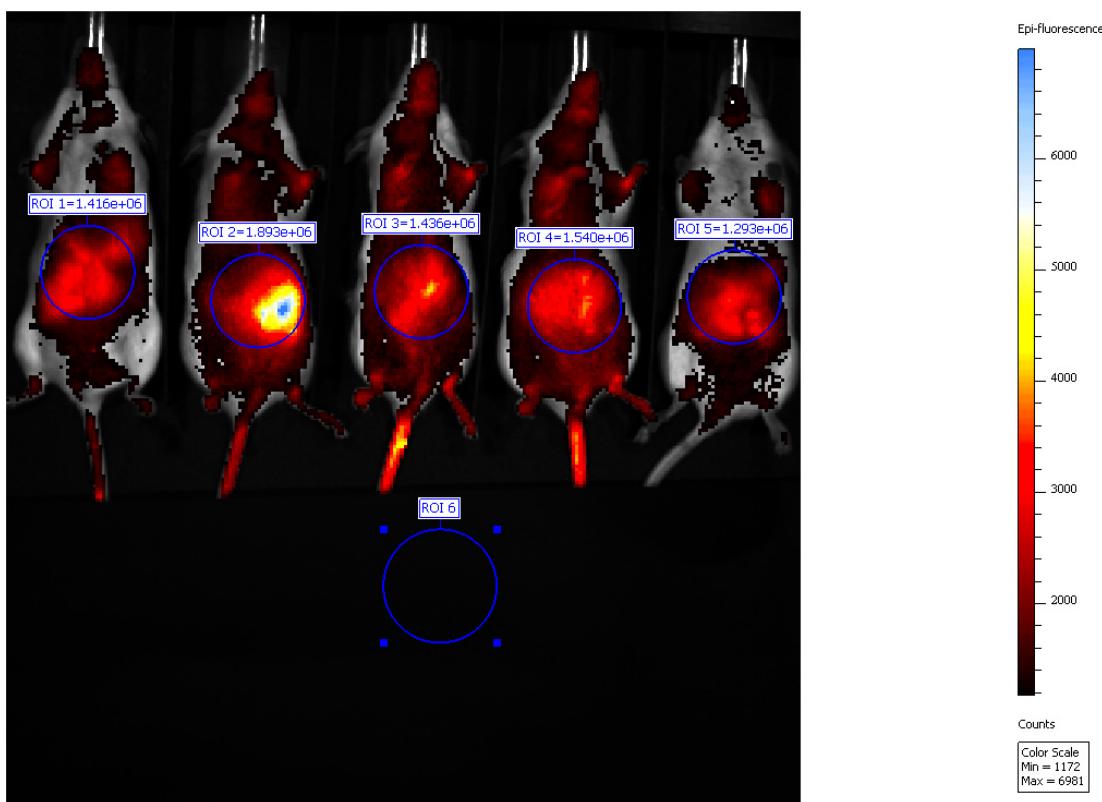


Figure 3. Biofluorescence *in vivo* imaging. Representative IVIS imaging of mice with hypoxic zones which have developed upon *C. albicans* infection.

Notes

1. This is a non-lethal infection that is self-resolving after 7 days. The fungi do not spread beyond the subdermal tissue and animals appear not be greatly perturbed in their wellbeing (Video 1).



Video 1. Mice with a subdermal infection of *C. albicans* one day after inoculation. The video depicts mice at Day 1 post infection. The mice have received a subdermal injection of *C. albicans*. The activity and wellbeing of the animals are only affected to a minor extent.

2. Animal-to-animal variability and the age of the animal might interfere with the results. Careful selection of the size of the animal groups when planning the experiment is advised. The described protocol used $n > 5$ animals per group.
3. At the end-points animals can be sacrificed and abscess excised to measure infiltration of immune cell or fungal burden.
4. Animals were kept at all times in IVC system cages with water *ad libitum* and monitored daily by the facility care-takers during their stay at the animal infrastructure and by the researchers and the responsible veterinary during the infection period.

Recipes

1. SC medium (for 1 L)

Yeast nitrogen base	3.4 g
Ammonium sulfate	10 g
Yeast Synthetic Drop-out Medium Supplements	1.92 g
Glucose	2% (w/v)
MQ water	1 L

Autoclave afterward
2. PBS 1x Phosphate-buffered saline (PBS)

Dissolve 1 tablet of PBS in 500 ml MQ water	
Autoclave afterward	

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Competing interests

All authors declare no competing interests.

Ethics

The animal experiments were conducted in accordance with animal ethical guidelines stated in permission A79-14 from the Swedish Board of Agriculture.

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