Imaging morphogenesis of *Candida albicans* during infection in a live animal

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Abstract. *Candida albicans* is an opportunistic human fungal pathogen that requires an intact host immune response to prevent disease. Thus, studying host-pathogen interactions is critical to understanding and preventing this disease. We report a new model infection system in which ongoing *C. albicans* infections can be imaged at high spatial resolution in the ears of living mice. Intradermal inoculation into mouse ears with a *C. albicans* strain expressing green fluorescent protein results in systemic *C. albicans* infection that can be imaged *in vivo* using confocal microscopy. We observed filamentous growth of the organism *in vivo* as well as formation of microabscesses. This model system will allow us to gain significant new information about *C. albicans* pathogenesis through studies of host-*C. albicans* interactions in the native environment.
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The human fungal pathogen *Candida albicans* exists as a commensal organism in individuals with an intact immune system but causes opportunistic disease in patients with defects in phagocyte function or number. *C. albicans* can exist in multiple morphologies, including hyphae, pseudohyphae, and yeast forms; the ability to transition from yeast to filamentous forms (morphogenesis) is important for virulence.[1] Traditional model systems for the study of *C. albicans* infections include *in vitro* observation of interactions between organisms and host cells and murine models of *C. albicans* disease. The most well-established animal model of *C. albicans* disease is the tail vein inoculation model. In this model, mice are injected with a large number of *C. albicans* yeast via the lateral tail vein. Outcome measurements are typically survival and kidney organism burden 48 h after infection, which correlates with disease severity.[2]

These models of *C. albicans* disease have significant limitations. *In vitro* studies of host-pathogen interactions are hampered by the artificial environment that lacks local environmental signals and interactions important to the function of leukocytes. The tail vein inoculation model relies on fairly broad outcome measures, and does not allow the researcher to study conditions in which the animal recovers from infection. The ability to image the progress of a *C. albicans* infection at high spatial resolution *in vivo* would enable the study of morphogenesis and host-pathogen interactions in the native host environment. Furthermore, such studies would allow investigation of the process through which an intact immune system successfully defends the host against *C. albicans* disease.

The recent expansion of *in vivo* imaging techniques, including confocal and multiphoton microscopy, has enabled acquisition of high resolution images of malaria[3] and leishmania[4] infections in intact hosts. Whole-mouse fluorescence imaging has been used to track the progression of green-fluorescent-protein (GFP)-expressing bacterial infections in internal organs.[5] Bioluminescence imaging provides high contrast, low resolution images of microbial infection *in vivo*, and has been used for evaluation of localized *C. albicans* infections.[6,7] Using fluorophore-conjugated antibodies to label host cells expressing major histocompatibility complex II, we recently reported confocal fluorescence images of two dendritic cell populations in the ears of living mice.[8] The ability to resolve fine dendritic cell processes at depths up to $\sim$100 μm suggested that we would be capable of detecting morphology in GFP-expressing *C. albicans in vivo* as well. One previous report describing the use of reflectance confocal microscopy to image the cornea of patients documented high contrast elongated particles in the cornea of a patient later diagnosed with *C. albicans* keratitis.[9] While the particles were presumed to be *C. albicans* pseudohyphae, there was no way to definitively identify fungal elements in the clinical images. In this work, we describe the use of a new mouse model of infection and *in vivo* confocal fluorescence microscopy to follow *C. albicans* morphogenesis during an ongoing infection. To the best of our knowledge, this is the first experimental observation of this phenomenon *in vivo* and the first high resolution images of *C. albicans* infections in a living animal.

The *C. albicans* strain used in this study, YAW3, was generously provided by Drs. Konopka and Warenda (State University of New York, Stony Brook). YAW3 was constructed by integrating a construct containing yeast EGFP[10] under control of the constitutively active ADH1 promoter[11] into the parental strain BWP17. Female BALB/c mice used in these experiments were maintained on chlorophyll-free chow to minimize endogenous fluorescence. Prior to injection of *C. albicans*, mice were anesthetized and hair on the ears was removed by chemical depilation. *C. albicans* yeast were then injected intradermally in a 30- to 50-μl volume of phosphate buffered saline. As a control, the opposite ear was injected with phosphate buffered saline alone. Mice were placed on the stage in the supine position so the ventral side of the ear was facing downward for imaging. Images were acquired using a 10×, 0.45-NA objective and a 100-μm-diam pinhole, which provides an optical section thickness of approximately 6 μm as determined by fluorescence edge response measurements.[12] GFP was excited at 488 nm, and emission was detected using a 515/30 bandpass filter.

Immediately after injection, *C. albicans yeast* could be detected in the ears of living mice with signals similar to that

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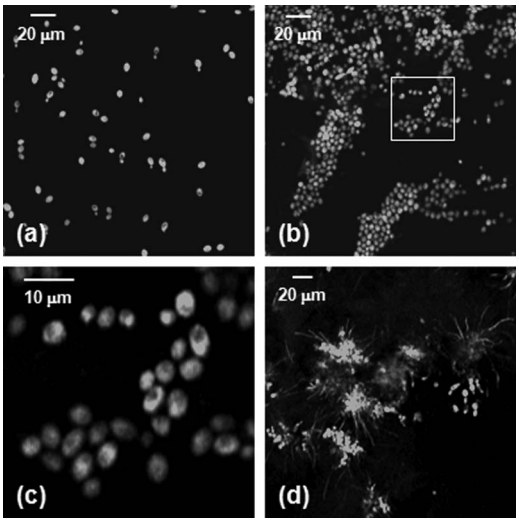

Fig. 1 Confocal images of *C. albicans* strain YAW3 *in vitro* and *in vivo*. (a) YAW3 was grown overnight, washed three times in phosphate buffered saline, placed on a microscope slide, and imaged. (b) YAW3 yeast approximately 30 min after an intradermal injection were imaged at a depth of ~50 μm in a mouse ear *in vivo*. (c) An expanded view of the region of interest indicated by the white box superimposed on the image in panel (b). (d) An image obtained 24 h after injection demonstrating *in vivo* morphogenesis [obtained as in panel (b)]. All images were acquired using 488-nm excitation and a 10×, 0.45-NA objective. The optical section thickness was 6 μm.

observed when yeast were imaged *in vitro* [Figs. 1(a) and 1(b)]. Yeast structures such as the vacuole can be readily detected *in vivo* [Fig. 1(c)]. 24 h after injection, organism replication resulted in the development of colony-like clusters with filamentous forms projecting out into the tissue [Fig. 1(d)]. One commonly suggested function of filamentous forms of *C. albicans* is that the filaments are responsible for invasion into new areas of tissue. While imaging alone cannot test such a hypothesis, this observed growth pattern is certainly consistent with a role for tissue invasion by filaments.

Intradermal injection of YAW3 resulted in a significant *C. albicans* infection. 24 h after injection, the ears injected with 10^5 or more *C. albicans* yeast/ear developed significant swelling and redness, indicating that local inflammation was occurring. In addition, the mice that received *C. albicans* injections developed ruffled fur and a hunched posture, indicating systemic illness. Mice injected with 10^6 to 10^7 yeast/ear developed systemic candidiasis, as detected by the presence of viable *C. albicans* in the kidneys after euthanasia. The development of systemic disease after intradermal injection of *Candida* demonstrates that this system successfully models dissemination of *C. albicans* infection, a critical stage in the pathogenesis of *Candida*.

The presence of *C. albicans* in the kidney after intradermal infection parallels the tail vein inoculation model of candidiasis, in which the kidney organism burden is monitored to follow the outcome of infection. Unlike the tail vein model, however, by using the intradermal injection we were able to use *in vivo* imaging to follow the progression of infection in a living animal. We observed two main patterns of infection. In the first, individual organisms were spread out throughout the tissue. In this pattern, we clearly observed the formation and growth of filamentous forms over 24 to 48 h (Fig. 2), with complex 3-D networks of filaments observed 48 h after infection. In the second pattern, we observed clusters of *C. albicans* organisms with the appearance of microabscesses [Fig. 3(a)]. These lesions increased in size over time, with some areas almost 1 mm in diameter 72 h after infection. At 72 h, some mice were euthanized and the ears were harvested for a comparative histological evaluation. Hematoxylin-and-eosin stained slides demonstrate the extensive inflammatory infiltrate in ears injected with *C. albicans* [Fig. 3(c)] in comparison to saline injected ears [Fig. 3(b)]. Silver staining demonstrates the presence of fungal elements in the lesions [Fig. 3(d)]. Thus, both the fluorescence and histological images are consistent with microabscesses identified in the kidney after tail vein inoculation of *C. albicans*.[2]

Using contemporary confocal fluorescence imaging techniques and a new model of invasive candidiasis, we have been able to microscopically follow the development of *C. albicans* infections in living mice. We observed morphogenesis and the formation of microabscesses, consistent with currently used models of infection. *In vivo* imaging is particularly relevant

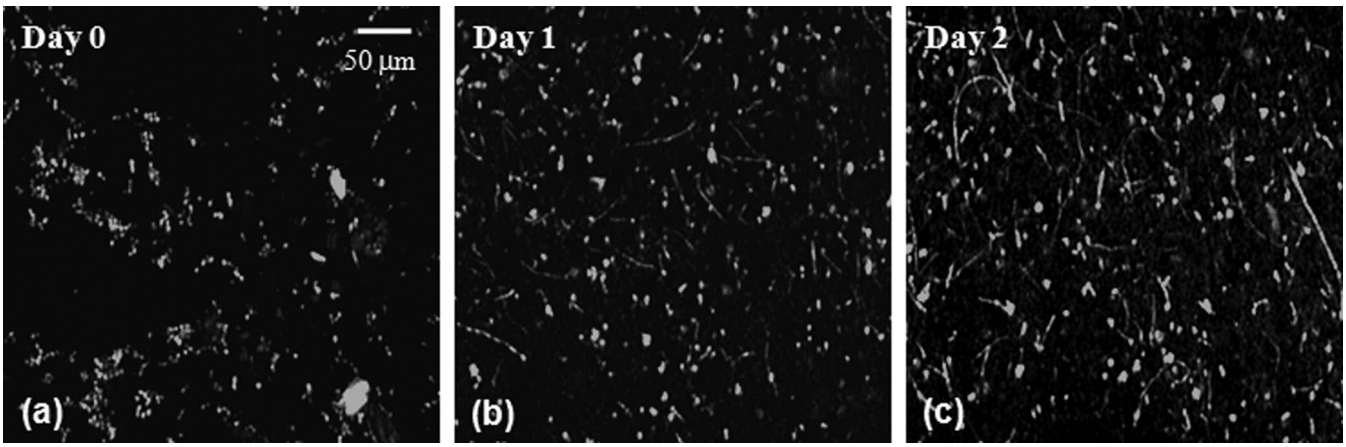

Fig. 2 Observation of *C. albicans* morphogenesis *in vivo*. Mouse ears were injected with *C. albicans* YAW3-GFP yeast on day 0 and imaged daily. Over two days, the yeast developed into a complex network of filamentous forms. Image depth and acquisition parameters were as described for Fig. 1.

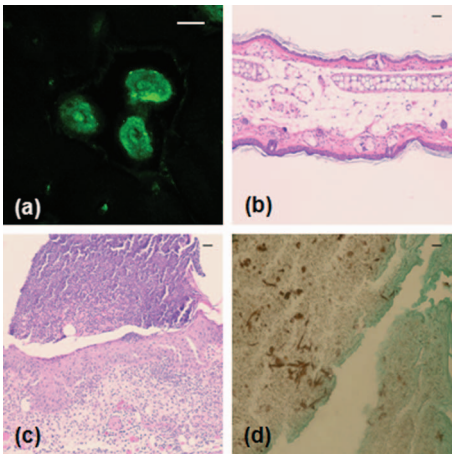

Fig. 3 Microabscess formation in infected ear tissue. 72 h after injection, mouse ears were imaged for fluorescence signal as in Figs. 1 and 2, after which the mouse was then euthanized and the ears were harvested, fixed in 10% formalin, and sectioned and stained for histological analysis. (a) Representative *in vivo* fluorescence image illustrating the formation of microabscess. The scale bar represents 100 μm. Hematoxylin-and-eosin stained sections of (b) the saline injected control ear and (c) the *C. albicans* injected ear. (d) Sections were stained with Modified Grocott's Methenamine Silver stain (Richard Allan Scientific staining kit, VWR, West Chester, Pennsylvania) to demonstrate the presence of *C. albicans* in the tissue. Bright field images shown in (b), (c), and (d) were acquired with a 40×, 0.95-NA objective and the scale bars represent 10 μm.

for the study of *C. albicans*, as this opportunistic disease is dependent on host-pathogen interactions. We expect future studies using this technique will incorporate the use of multicolor imaging to analyze host-pathogen interactions, as has been performed for Leishmania.[4] The use of bioluminescent *C. albicans* to follow infections in whole mice has been tested.[6,7] However, the luciferase imaging model was designed to acquire low resolution images at the level of the entire mouse. The model system we have described has the potential to allow us to evaluate host-pathogen interactions at high spatial resolution in the intact host environment. Thus, it should significantly further our understanding of how this opportunistic fungal pathogen causes disease and responds to host defenses and antifungal therapy.

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