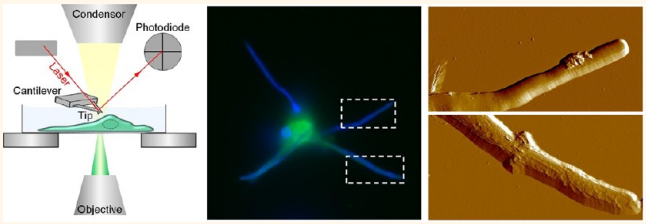
Nanoscale Imaging of the *Candida*–Macrophage Interaction Using Correlated Fluorescence-Atomic Force Microscopy

Sofiane El-Kirat-Chatel and Yves F. Dufrêne*

Institute of Life Sciences, Université catholique de Louvain, Croix du Sud, 1, bte L7.04.01., B-1348 Louvain-la-Neuve, Belgium

ABSTRACT Knowledge of the molecular bases underlying the interaction of fungal pathogens with immune cells is critical to our understanding of fungal infections and offers exciting perspectives for controlling immune responses for therapy. Although fluorescence microscopy is a valuable tool to visualize pathogen–host interactions, the spatial resolution is low, meaning the fine structural details of the interacting cells cannot be observed. Here, we demonstrate the ability of correlated fluorescence-atomic force microscopy (AFM) to image the various steps of the interaction between fungal pathogens and macrophages with nanoscale resolution. We focus on *Candida albicans*, known to grow as two morphological forms (yeast cells, filamentous hyphae) that play important roles in modulating the interaction with macrophages. We observe the main steps of macrophage infection, including initial intercellular contact, phagocytosis by internalization of yeast cells, intracellular hyphal growth leading to mechanical stretching, and piercing of the macrophage membrane resulting in pathogen escape. While fluorescence imaging clearly distinguishes fungal cells from macrophages during the various steps of the process, AFM captures nanoscale structural features of the macrophage surface that are of high biological relevance, including ruffles, lamellipodia, filopodia, membrane remnants, and phagocytic cups. As fungal pathogenesis is mainly controlled by the ability of fungi to escape from immune cells, the nanoimaging platform established here has great potential in nanomedicine for understanding and controlling fungal infections.



KEYWORDS: nanoscale imaging · AFM · fluorescence · *Candida* · fungal pathogens · immune cells

Mucosal fungal infections caused by pathogenic *Candida* species represent a serious clinical and economical problem.[1,2] While *Candida albicans* is the most frequently isolated species, non-*albicans* species such as *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, and *C. lusitaniae* are also emerging as important pathogens.[2,3] During host infection, phagocytes from the innate immunity, including monocytes, macrophages, and neutrophils, represent the main defense mechanism against fungal pathogens.[4,5] Hence, studying the molecular mechanisms by which *C. albicans* interacts with immune cells is a hot topic in current microbiological research and may help in the development of new approaches to modulate immune responses for therapeutic purposes.

The *Candida*–macrophage interaction is initiated by the specific recognition between immune receptors expressed by phagocytes (so-called pathogen recognition receptors, PRR) and fungal cell wall components (pathogen associated molecular pattern, PAMP).[4,5] This recognition step then leads to activation of pro-inflammatory pathways and internalization of the fungal cells.[6] The resulting phagosome further fuses with the subcellular compartments, such as lysosomes, to form the phagolysosome in which the yeast is exposed to the host antimicrobial arsenal.[7] Despite the hostile environment of the phagolysosome, numerous studies have demonstrated that *Candida* species have the ability to persist within macrophages and to resist antimicrobial defenses often leading to phagocyte death and yeast dissemination.[8,9] In addition, pathogenic yeasts have developed various strategies to escape macrophage killing.[8,9] Nonfilamentous species like *C. glabrata* use

* Address correspondence to yves.dufrene@uclouvain.be.

Received for review September 6, 2012 and accepted November 3, 2012.

Published online November 12, 2012
10.1021/nn304116f

simple mechanisms such as intracellular multiplication to induce macrophage burst.[10,11] By contrast, *C. albicans* takes advantage of the yeast-to-hyphae transition to facilitate piercing and escape from phagocytes.[10,12,13] Although the different steps of the *C. albicans*—macrophage interaction have been widely investigated, how the various morphogenetic forms of the fungus (yeast cells *vs* hyphae) modulate this interaction is poorly understood.

Fluorescence microscopy is traditionally used to look at the interaction between microbial pathogens and immune cells.[10,14−18] Although fluorescence techniques enable to stain specific components of the two partners, and to track the real-time dynamics of their interaction, the spatial resolution is limited to the wavelength of the light source. Also, quantitative information on cell surface nanostructures cannot be obtained. Electron microscopy techniques have allowed researchers to obtain much higher resolution views of pathogen—host interactions,[13,14,17,18] but these methods are demanding and require vacuum conditions during the analysis, meaning cells cannot be observed in aqueous solution. Here, we use correlated fluorescence-atomic force microscopy (AFM)[19,20] to track the different steps of the interaction of *C. albicans* with macrophages. While fluorescence imaging clearly distinguishes fungal cells from macrophages during the process, AFM visualizes various nanostructures of biological relevance on the macrophage surface. *C. glabrata*, which only grows as yeast cells, is found to behave very differently from *C. albicans*, confirming the crucial role of morphogenesis in fungus—macrophage interactions.

RESULTS AND DISCUSSION

Nanoscale Imaging of Noninfected Macrophages: Live Cells *vs* Gently Fixed Cells. To visualize the main steps of the *C. albicans*—macrophage interaction, we used an integrated platform combining an AFM with an inverted light microscope (Figure 1a). This setup offers several benefits, including the possibility to use optical images to precisely guide AFM imaging and force measurements, and the ability to easily generate correlated AFM and optical images. We first imaged living macrophages that were not infected. To this end, murine macrophages were plated in culture medium in a glass Petri dish, incubated overnight (37 °C, 5% CO_2), and then stained by addition of the carboxyfluorescein succinimidyl ester (CFSE) fluorescent dye. Once inside live metabolically active cells, unspecific esterases cleave the acetate groups of the nonfluorescent CFSE yielding a green, highly fluorescent carboxyfluorescein succimidyl ester. In doing so, highly contrasted differential interference contrast (DIC) and fluorescence images of individual cells were obtained. Overlapping these images (Figure 1b) enabled to simultaneously visualize the AFM cantilever and the cell. We observed

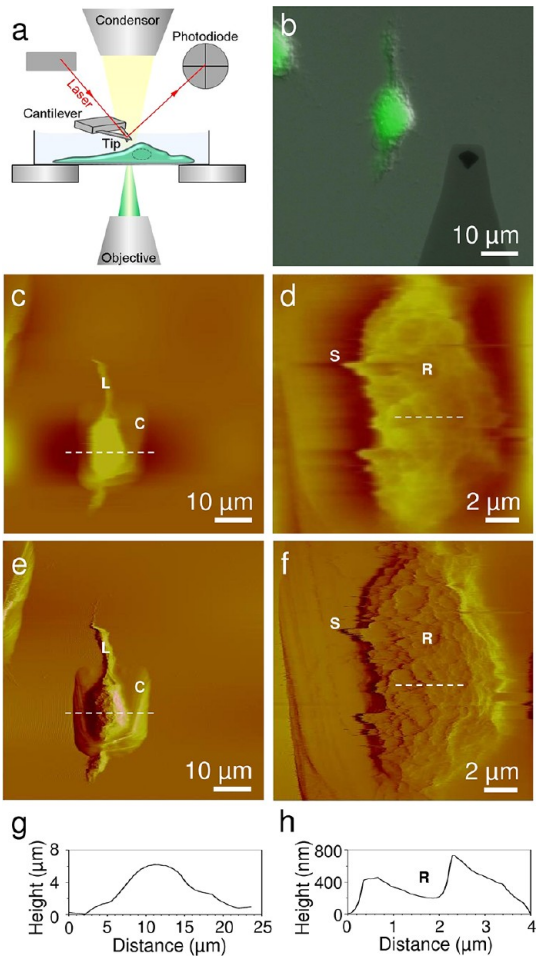


Figure 1. Nanoscale imaging of living macrophages using correlated fluorescence-atomic force microscopy. (a) Schematic of the nanoscale imaging platform combining advanced AFM capabilities with modern optical microscopy. Transmission, DIC and epifluorescence microscopy images of the cells are obtained in culture medium or buffer, together with AFM topographic images of the cell surface. (b) Overlay of DIC and fluorescence images in RPMI medium showing the AFM cantilever and a live macrophage spread on glass and stained with the green fluorescent dye carboxyfluorescein succinimidyl ester (CFSE). (c−f) AFM height (c, d) and deflection (e, f) images of the same macrophage shown in (b). Images (d) and (f) are enlarged views of the cell shown in (c) and (e). (g, h) Vertical cross sections taken in the height images along the dashed lines. Labels L and R correspond to lamellipodia and ruffles, two nanostructural features of the macrophages, while labels S and C denote streaks and tip convolution imaging artifacts.

the morphological features that are characteristic of macrophages:[21,22] (i) well-spread, flat cells, or round-shaped cells with a diameter of ∼10 μm; (ii) cellular protrusions, ∼2 μm in width and ∼15 μm in length, reflecting "lamellipodia", *i.e.*, membrane distortions enriched with a network of actin filaments and which help the cell to migrate.[21]

To obtain high-resolution views of the macrophage surface, we recorded simultaneous height (Figure 1c,d) and deflection (Figure 1e,f) images of the cells using contact mode AFM. Due to the large curvature of the

cells, AFM height mode images had fairly poor resolution, while deflection mode images were much more sensitive to the surface relief. Yet, height images were very useful in providing quantitative measurements of cell surface features through vertical cross sections (Figure 1g,h). Some cells were round, with a height of 4−8 μm (Figure 1g), while others were flat. Strikingly, we found that the cell surface was always rough and decorated with numerous dorsal "ruffles" of 100−500 nm height. These ruffles, hardly detectable by optical microscopy, have already been observed in macrophages using electron microscopy and AFM.[21−23] Such nanostructures are of biological relevance as they are believed to reflect the presence of "podosomes", *i.e.*, specialized structures found in macrophages and involved in cell−matrix interactions, matrix degradation, and migration.[22] Importantly, we found that reliable recording and interpretation of high-resolution images on live macrophages was complicated by their soft, deformable properties. The image contrast was fuzzy and showed streaks in the scanning direction, reflecting strong interactions between the tip and the soft cell membrane (Figure 1d,e). Also, most cells were detached or pushed aside during scanning.

We therefore postulated that gentle chemical fixation should enhance the quality of AFM images without modifying the morphological features of the cells.[22−25] We used a fixation protocol (0.5% glutaraldehyde) that has already been successfully applied in AFM studies,[23,24] and which is known to prevent osmotic and temperature changes, and preserve cellular structures. As expected, correlative fluorescence-AFM images of gently fixed cells revealed the same general features as in live cells, including membrane ruffles and lamellipodia, yet with a much better resolution and much less tip-induced imaging artifacts (Figure 2c,d). We note that fixation with paraformaldehyde, instead of glutaraldehyde, yielded images of poor quality. Quantitative height measurements on these cellular structures (Figure 2e,f) yielded critical dimensions that were in the same range as those measured on live cells, thus confirming they were actual features of the cell surface and not artifacts. Moreover, the images revealed structural details of the cell surface that were hardly detected on live cells, including thin filamentous structures, 50−300 nm in diameter, that we attribute to "filopodia", *i.e.*, protrusions which contain parallel bundles of actin filaments.[21] Overall, these observations show that gentle fixation not only preserves the native ultrastructure of the macrophages, but dramatically enhances the image quality, thus revealing subtle details hardly visible on live cells.

Unravelling the Early Stages of Infection. We imaged the early stages of macrophage infection, *i.e.*, initial contact of *C. albicans* yeast cells with the macrophage surface, followed by their engulfment, or "phagocytosis", which

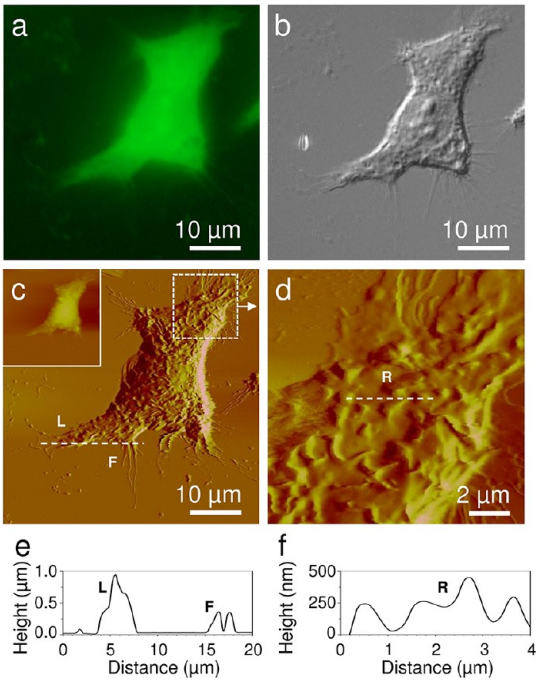


Figure 2. Nanoscale imaging of gently fixed macrophages. (a, b) Fluorescence (a) and DIC (b) images, in PBS, of a macrophage stained in green with CFSE and fixed with 0.5% glutaraldehyde. (c) Correlative AFM deflection image of the same macrophage (inset: corresponding height image). (d) Enlarged view (deflection) of the macrophage corresponding to the white dashed box shown in (c). (e, f) Vertical cross sections taken along the dashed lines in the height images corresponding to (c) and (d). Labels L, F and R correspond to lamellipodia, fillopodia and ruffles, three nanostructural features of the macrophages.

usually takes place within the first 15 min of infection.[10,15] Figure 3 shows representative sets of correlative fluorescence-AFM images recorded for macrophages that were incubated with yeast cells for 30 min. While macrophages were labeled in green using CFSE, yeast cells were specifically stained in blue using Calcofluor White (CFW). In several images, the macrophage surface was in close contact with spherical yeast cells displaying a smooth, featureless surface (roughness < 1 nm) (Figure 3e), consistent with earlier reports.[26,27] The cells were generally in close contact with lamellipodia extensions (Figure 3a,b), suggesting that macrophages projected these filaments to bind and ingest yeast cells. Another set of images showed multiple yeast cells engulfed or "internalized" inside macrophages (Figure 3c,d). In this early stage of the infection process, AFM images of the phagocytes did not show any evidence for the presence of yeast cells, confirming these were trapped inside the macrophage without altering its morphology. Also, the infected macrophages were still decorated by multiple filopodia (Figure 3d,f), suggesting they were strongly anchored to the surface. We note that while optical images alone (Figure 3a,c) were enabled to discriminate between free and internalized yeast cells, this distinction could

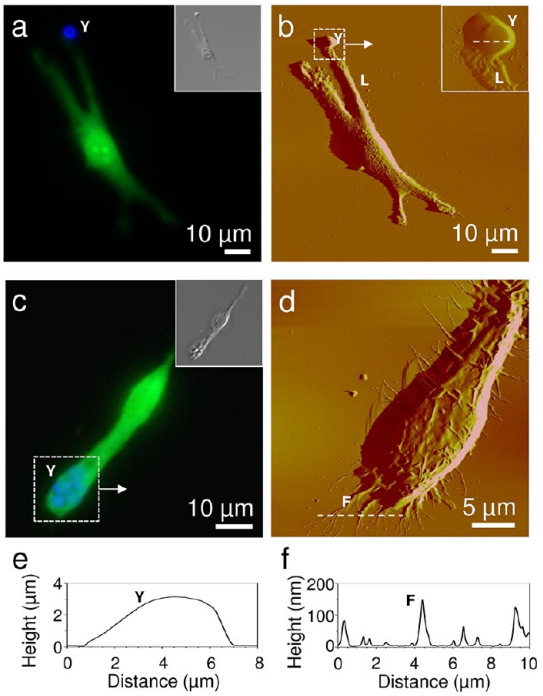


Figure 3. Fluorescence-AFM imaging captures the early stages of macrophage infection, *i.e.*, initial intercellular contact followed by internalization (phagocytosis). (a, c) Fluorescence images (insets: corresponding DIC images) and (b, d) correlative AFM deflection images, in PBS, of single macrophages incubated for 30 min with *C. albicans* yeast-form cells in DMEM medium, labeled with the fluorescent dyes CFSE (green color) and Calcofluor White (CFW, blue color specific to fungal cells), and gently fixed (0.5% glutaraldehyde). The inset in (b) is an enlarged view of the dashed area shown in the AFM image (b), while image (d) is an enlarged view of the dashed area shown in the fluorescence image (c). (e, f) Vertical cross sections taken along the dashed lines in the height images corresponding to (b, inset) and (d). Labels Y, L and F correspond to yeast cells, lamellipodia, and fillopodia.

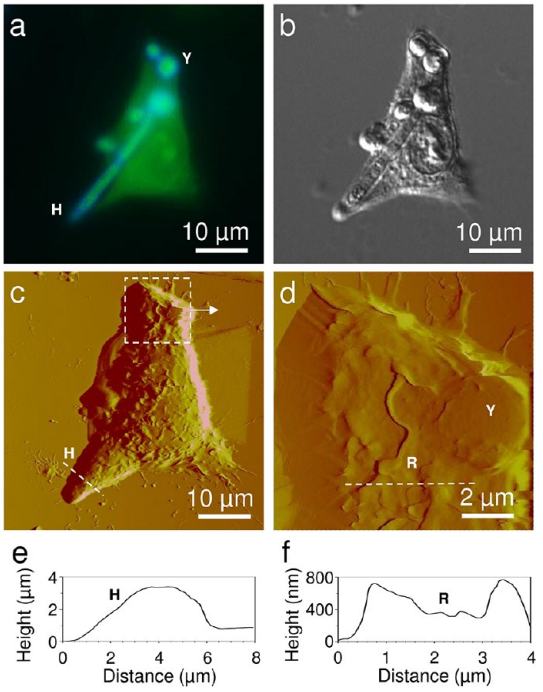


Figure 4. Hyphal growth triggers mechanical stretching of the macrophage membrane. (a, b) Fluorescence (a) and DIC (b) images, in PBS, of a single macrophage incubated for 90 min with *C. albicans* cells, labeled with CFSE and CFW, and gently fixed. (c) Correlative AFM deflection image of the same macrophage. (d) Enlarged view of the macrophage corresponding to the dashed area shown in (c). (e, f) Vertical cross sections taken along the dashed lines in the height images corresponding to (c) and (d). Labels Y, H and R correspond to yeast cells, hyphal cells and macrophage ruffles.

easily be made by AFM which revealed major differences in cell surface ultrastructure (smooth yeast surface *vs* rough macrophage surface). This demonstrates the strong complementarity of the techniques: while fluorescence provides microscale images of the whole cell volume, AFM unravels the nanoscale details of the cell surface.

Fungal Morphogenesis Induces Major Changes in Macrophage Morphology. Next, we investigated the intracellular morphogenetic switch of the fungus, which typically occurs within 90 min.[10,15] Figures 4 and 5 show representative sets of correlative fluorescence-AFM images of macrophages containing both nongerminated yeast cells and hyphae after 90 min of infection. While DIC images revealed elongated structures associated with the macrophages (Figures 4b and 5b), the corresponding fluorescence images confirmed these were indeed due to *C. albicans* hyphae (Figures 4a and 5a). Optical images could not unambiguously demonstrate that yeast and hyphal cells were internalized, whereas correlative AFM images (Figures 4c,d and 5c,d) clearly showed that this was indeed the case for most cells. Since only yeast-form cells were present at the beginning of the infection and were mostly internalized, we conclude that the observed hyphae result from an intracellular morphological switch within the hostile environment of the macrophage.[8,9,12] We note that free yeast cells in close contact with the macrophage surface were sometimes seen (Figure 5d), suggesting that phagocytes were still catching and internalizing yeast cells even when they already contained hyphae.

Remarkably, we also found that germ tube formation was accompanied by major changes in macrophage morphology (Figures 4 and 5). For several reasons, we believe this phenomenon results from the mechanical stretching of the cell membrane through hyphal growth: first, the plasma membrane perfectly contoured the hyphae; second, membrane portions covering the hyphae showed much less ruffles compared to other regions of the macrophage; third, no (very few) filopodia and lamellipodia were observed around the deformed areas. This suggests that the cell extensions resulted from hyphal growth rather than from macrophage migration. Accordingly, our correlated images demonstrate that the intracellular hyphal growth stretches the phagocyte

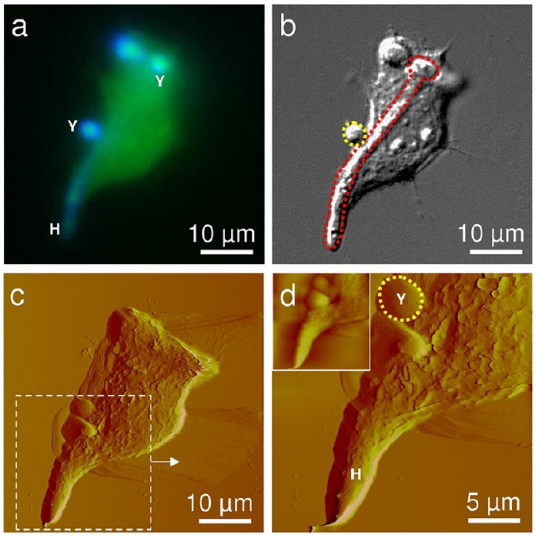


Figure 5. AFM discriminates between free and internalized fungal cells. (a, b) Fluorescence (a) and DIC (b) images, in PBS, of a single macrophage incubated for 90 min with *C. albicans* cells, labeled with CFSE and CFW, and gently fixed. (c) Correlative AFM deflection image of the same macrophage. (d) Enlarged view (inset: corresponding height image) of the macrophage corresponding to the dashed area shown in (c). Labels Y and H correspond to yeast and hyphal cells. The red and yellow dashed lines in (b) indicate an internalized hyphal cell and a free yeast cell, respectively. Only the free yeast cell is seen in the AFM image (d, see yellow dashed line).

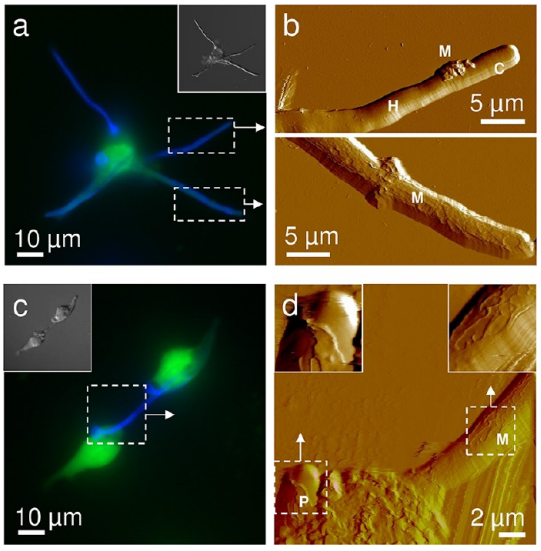


Figure 6. Unravelling macrophage piercing and fungal escape. (a, c) Fluorescence images (insets: corresponding DIC images) and (b, d) correlative AFM deflection images, in PBS, of macrophages incubated for 3 h with *C. albicans* cells labeled with CFSE and CFW, and gently fixed. The two images in (b) and the image in (d) are enlarged views of the dashed areas shown in the fluorescence images (a) and (c), respectively. Insets in image (d) are enlarged views of the dashed areas. Labels H, M, C and P correspond to hyphal cells, macrophage membrane, tip convolution artifacts, and phagocytic cup.

membrane. Our observations are fully consistent with optical video microscopy data,[10,15] but, for the first time, provide quantitative, high-resolution information on structural changes associated with the process.

Hyphal Growth Leads to Macrophage Piercing and Fungal Escape. As shown in Figures 6 and 7, we also captured the last step of the *Candida*–macrophage interaction, *i.e.*, escape of the fungus from the macrophage leading to its externalization,[8−10,13] which usually occurs within 3 h. Figure 6a shows a macrophage associated with four long hyphae with a star-like orientation. Fluorescence images provided remarkable contrast enabling to clearly distinguish between hyphae and macrophage regions, but did not provide information on cell surface nanostructures. As can be seen in Figure 6b,d, AFM complemented these data by unravelling major variations of cell surface supramolecular structure. Some hyphae (Figure 6b, upper image) showed a very smooth morphology, very similar to that reported for free *C. albicans* hyphae,[27] indicating they were "naked" hyphae that escaped macrophage phagolysis. Supporting this interpretation, we observed local patches of extracellular material, not visible in fluorescence, that we believe represent macrophage membrane remnants as these structures were never observed on free fungal cells.[27] The presence of residual macrophage components on externalized hyphae could represent a fungal strategy to mask its PAMPs to avoid recognition by the immune system.[28,29] Other hyphal

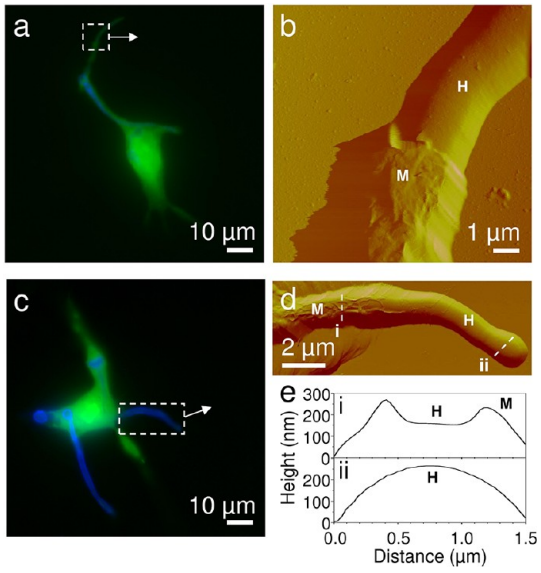


Figure 7. Capturing the externalization of hyphal cells. (a, c) Fluorescence images and (b, d) correlative AFM deflection images, in PBS, of macrophages incubated for 3 h with *C. albicans* cells labeled with CFSE and CFW, and gently fixed. Images (b) and (d) are enlarged views of the dashed areas shown in the fluorescence images (a) and (c), respectively. (e) Vertical cross sections taken along the dashed lines in the height image corresponding to (d). Labels H and M correspond to hyphal cells and macrophage membrane.

cells (Figure 6b, bottom image) showed a much rougher surface, very different from that of free hyphae, that presumably corresponds to internalized hyphae covered

with the phagocyte membrane. Figure 6c and d present a similar, yet peculiar organization, *i.e.*, two macrophages ongoing phagocytosis of the same hypha. Was this hypha inside or outside the macrophages? Again, high-resolution AFM clearly demonstrated that the hypha surface was completely covered by the macrophage membrane (Figure 6d, right inset), thus that it was internalized. In addition, macrophages sometimes presented membrane projections (Figure 6d, left inset) not seen in fluorescence, that we believe reflect phagocytic cups, *i.e.*, membrane extensions that partially encircle the phagocytic target during the early stage of phagocytosis.[21]

Figure 7 presents further evidence that hyphal growth led to mechanical piercing of the phagocyte membrane. The images show two hyphae protruding from different macrophages and that are partially externalized. While fluorescence imaging could not discriminate between internalized *vs* externalized fungal cells, AFM showed that a portion of the protruding hyphae was partially covered with the macrophage while their most external portion was completely smooth and devoid of such macrophage material. Quantitative height measurements (Figure 7e) revealed that the thickness of the macrophage material surrounding the hyphae was up to ~100 nm, thus much thicker than the membrane.

In summary, fluorescence-AFM imaging unravelled mechanical piercing of macrophages by *C. albicans* and the externalization of hyphal structures with an unprecedented level of details. Specifically, AFM could distinguish between internalized and externalized hyphal cells, visualize subtle structural details like macrophage membranes and cups, and measure their critical dimensions. As *C. albicans* pathogenesis is mainly controlled by its ability to escape from the immune system,[8−10,13] the imaging platform established here has great potential in future research to better understand the molecular details of *C. albicans* infections.

Nonfilamentous *Candida* Species Do Not Cause Macrophage Stretching and Piercing. Since the most remarkable morphological changes observed during the *C. albicans*−macrophage interaction were associated with the yeast to hypha transition, we speculated that pathogenic *Candida* species that do not show such transition should not lead to the same behavior. To test this idea, we analyzed the interaction between macrophages and *C. glabrata*, a frequently isolated pathogen that only grows as yeast.[1,30] *C. glabrata* is known to be easily phagocytosed,[31] to persist and to multiply by budding until macrophage burst.[10,32] The whole process typically takes 12−24 h, thus much longer that the time for *C. albicans* escape. Figure 8 shows representative fluorescence-AFM images obtained for this interaction after 12 h infection. Optical images (Figure 8a,b) document the round shape of the macrophages and the presence of multiple yeast cells. As a complement, AFM

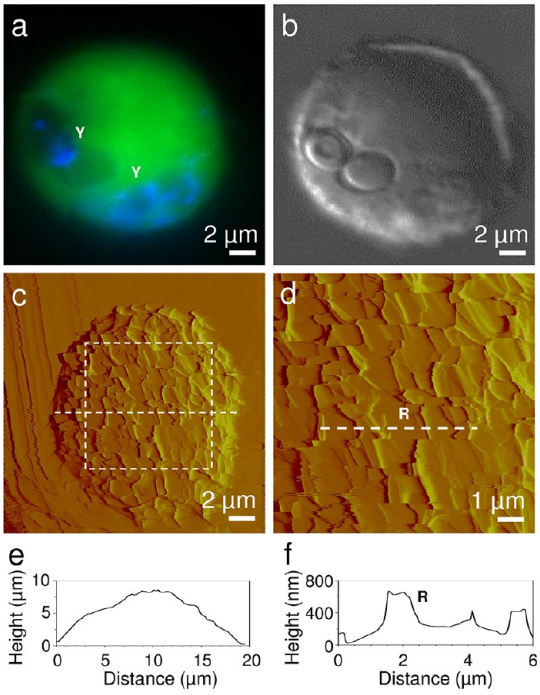


Figure 8. Nonfilamentous *C. glabrata* species multiplies inside macrophages without stretching/piercing of the membrane. (a, b) Fluorescence (a) and DIC (b) images, in PBS, of a single macrophage incubated for 12 h with *C. glabrata* cells, labeled with CFSE and CFW, and gently fixed. (c) Correlative AFM deflection image of the same infected macrophage. (d) Enlarged view of the macrophage corresponding to the dashed area shown in (c). (e, f) Vertical cross sections taken along the dashed lines in the height images corresponding to (c) and (d). Labels Y and R correspond to yeast cells and membrane ruffles.

images (Figure 8c,d) show that all yeast cells were internalized and reveal ruffle structures that were unexpectedly large, up to 500 nm in height and width, suggesting a cell stress response to the surface detachment occurring.[21,22] Also, the macrophage height (8 μm, Figure 8e) was larger than that observed with *C. albicans* (5 μm), in agreement with the notion that they were filled with yeast cells and no longer spread. We note that images obtained for the early stages of infection were similar to those recorded for *C. albicans*, while the ultimate stage, macrophage burst, could not be captured because of its very transient nature.

CONCLUSIONS

Fungal pathogens have developed impressive ways to resist immune cells. A prominent example is *C. albicans*, which uses the yeast-to-hyphae transition to escape from macrophages.[8−10,12,13] To date, the molecular details of this phenomenon remain poorly understood, mainly owing to the lack of high-resolution imaging techniques. We have shown that correlated fluorescence-AFM is a powerful platform for imaging the *Candida*−macrophage interaction on the nanoscale. Our main findings are as follows. First, imaging of gently fixed macrophages revealed

structural details of the cell surface with unprecedented resolution, including nanoscale ruffles, lamellipodia, and filopodia, all of which are of high biological relevance (migration, phagocytosis). Second, we tracked the main steps of the *C. albicans*–macrophage interaction, including initial contact between the partners, internalization of yeast cells, intracellular hyphal formation leading to mechanical stretching of the macrophage membrane, and hyphal elongation resulting in membrane piercing and escape from the macrophage. Third, we provided novel nanoscale structural information for each step, enabling us to answer pertinent questions, such as whether yeast and hyphal cells were free, internalized or in the process of being externalized, and whether macrophage nanostructures (*e.g.*, ruffles, lamellipodia, and filopodia) were associated to a given step. Fourth, we observed a different behavior for *C. glabrata*, demonstrating the important role of morphogenesis in fungus–macrophage interactions.

METHODS

Strains, Media, Growth Conditions and Infection of Macrophages. *Candida albicans* SC5314 and *Candida glabrata* ATCC 90030 wild type strains were grown in standard medium YPD (1% yeast extract, 2% bactopeptone, 2% glucose). The murine macrophage cell line J774A.1 (ATCC TIB-67) was cultured in DMEM (Gibco) containing 10% decomplemented fetal bovine serum (FBS, Gibco), 1 mM sodium pyruvate, 50 U/mL penicillin-G and 50 μg/mL streptomycin, at 37 °C in 5% CO_2. Infections of macrophages were done at a multiplicity of infection (MOI) of 1 yeast: 1 macrophage, by adding 1×10^6 *Candida* cells from stationary growth phase to 1×10^6 J774 cells plated 16 h before in a glass bottom Petri dish (Willco wells, The Netherlands) containing 2 mL of complete DMEM. The cell mixtures were incubated at 37 °C in 5% CO_2 for 30 min, 1 h 30 min and 3 h for *C. albicans*, or 12 h for *C. glabrata*. For noninfected macrophage experiments, phagocytes were treated in the same conditions without adding yeast cells.

Correlative Fluorescent-Atomic Force Microscopy. For yeast fluorescent staining, 5 μg/mL of Calcofluor White (CFW blue, excitation 350 nm/emission 460 nm, Sigma) were added at the beginning of the interaction. For staining of live cells, 10 μM of carboxyfluorescein succinimidyl ester (CFSE green, excitation 492 nm/emission 517 nm, Molecular Probes) were added 30 min before the end of interaction following the supplier recommendations. Live cells were directly imaged after replacement of the medium by prewarmed RPMI 1640 (Sigma) supplemented with 10% decomplemented FBS, 1 mM sodium pyruvate and 2 g/L sodium bicarbonate (pH 7.4). For fixed cells, 0.5% of glutaraldehyde (Sigma) was added into the medium and fixation was achieved during 1 h at 37 °C in 5% CO_2 before changing the fixation medium with PBS buffer. Correlative microscopy images were acquired with a Bioscope Catalyst microscope (Bruker corporation, Santa Barbara, CA), a fully integrated system consisting of an inverted optical microscope (Zeiss Axio Observer Z1) equipped with a Hamamatsu camera C10600 coupled with an AFM. Acquisition and analysis of fluorescence images were done using the AxioVision Rel. 4.8.2 software. AFM contact mode images were obtained using Si_3N_4 cantilevers with a nominal spring constant of ~0.01 N/m (MLCT, Bruker, Santa Barbara, CA), an applied force of ~500 pN, and a scan rate of 1 Hz. AFM images were analyzed with the Nanoscope 8.10 software (Bruker, Santa Barbara, CA). Each set of fluorescence-AFM images was obtained at least in duplicate, using independent cell preparations.

Conflict of Interest: The authors declare no competing financial interest.

Acknowledgment. Our work was supported by the National Foundation for Scientific Research (FNRS), the Université catholique de Louvain (Fonds Spéciaux de Recherche), the Région Wallonne, the Federal Office for Scientific, Technical and Cultural Affairs (Interuniversity Poles of Attraction Programme), and the Research Department of the Communauté française de Belgique (Concerted Research Action). We thank T. Noël for providing us with the *Candida* strains and macrophage cell line and B. Knoops for providing access to cell culture facility. Y.F.D. is Senior Research Associate of the FRS-FNRS.

REFERENCES AND NOTES

1. Pfaller, M. A.; Diekema, D. J. Epidemiology of Invasive Candidiasis: A Persistent Public Health Problem. *Clin. Microbiol. Rev.* **2007**, *20*, 133–163.
2. Pfaller, M. A.; Espinel-Ingroff, A.; Canton, E.; Castanheira, M.; Cuenca-Estrella, M.; Diekema, D. J.; Fothergill, A.; Fuller, J.; Ghannoum, M.; Jones, R. N.; et al. Wild-Type MIC Distributions and Epidemiological Cutoff Values for Amphotericin B, Flucytosine, and Itraconazole and *Candida* spp. as Determined by CLSI Broth Microdilution. *J. Clin. Microbiol.* **2012**, *50*, 2040–2046.
3. Roetzer, A.; Gabaldon, T.; Schuller, C. From *Saccharomyces cerevisiae* to *Candida glabrata* in a Few Easy Steps: Important Adaptations for an Opportunistic Pathogen. *FEMS Microbiol. Lett.* **2011**, *314*, 1–9.
4. Gow, N. A.; van de Veerdonk, F. L.; Brown, A. J.; Netea, M. G. *Candida albicans* Morphogenesis and Host Defence: Discriminating Invasion from Colonization. *Nat. Rev. Microbiol.* **2012**, *10*, 112–122.
5. Netea, M. G.; Brown, G. D.; Kullberg, B. J.; Gow, N. A. An Integrated Model of the Recognition of *Candida albicans* by the Innate Immune System. *Nat. Rev. Microbiol.* **2008**, *6*, 67–78.
6. Romani, L. Immunity to Fungal Infections. *Nat. Rev. Immunol.* **2011**, *11*, 275–288.
7. Haas, A. The Phagosome: Compartment with a License to Kill. *Traffic* **2007**, *8*, 311–330.
8. Chai, L. Y.; Netea, M. G.; Vonk, A. G.; Kullberg, B. J. Fungal Strategies for Overcoming Host Innate Immune Response. *Med. Mycol.* **2009**, *47*, 227–236.
9. Seider, K.; Heyken, A.; Luttich, A.; Miramon, P.; Hube, B. Interaction of Pathogenic Yeasts with Phagocytes: Survival, Persistence and Escape. *Curr. Opin. Microbiol.* **2010**, *13*, 392–400.
10. Dementhon, K.; El-Kirat-Chatel, S.; Noël, T. Development of an *in vitro* Model for the Multi-Parametric Quantification of the Cellular Interactions between *Candida* Yeasts and Phagocytes. *PLoS One* **2012**, *7*, e32621.
11. Kaur, R.; Ma, B.; Cormack, B. P. A Family of Glycosylphosphatidylinositol-Linked Aspartyl Proteases is Required for Virulence of *Candida glabrata*. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 7628–7633.
12. Ghosh, S.; Navarathna, D. H.; Roberts, D. D.; Cooper, J. T.; Atkin, A. L.; Petro, T. M.; Nickerson, K. W. Arginine-Induced Germ Tube Formation in *Candida albicans* is Essential for Escape from Murine Macrophage Line RAW 264.7. *Infect. Immun.* **2009**, *77*, 1596–1605.
13. McKenzie, C. G.; Koser, U.; Lewis, L. E.; Bain, J. M.; Mora-Montes, H. M.; Barker, R. N.; Gow, N. A.; Erwig, L. P. Contribution of *Candida albicans* Cell Wall Components to Recognition by and Escape from Murine Macrophages. *Infect. Immun.* **2010**, *78*, 1650–1658.
14. Brinkmann, V.; Reichard, U.; Goosmann, C.; Fauler, B.; Uhlemann, Y.; Weiss, D. S.; Weinrauch, Y.; Zychlinsky, A.

Neutrophil Extracellular Traps Kill Bacteria. *Science* **2004**, *303*, 1532–1535.
15. Lewis, L. E.; Bain, J. M.; Lowes, C.; Gillespie, C.; Rudkin, F. M.; Gow, N. A.; Erwig, L. P. Stage Specific Assessment of *Candida albicans* Phagocytosis by Macrophages Identifies Cell Wall Composition and Morphogenesis as Key Determinants. *PLoS Pathog.* **2012**, *8*, e1002578.
16. Lewis, L. E.; Bain, J. M.; Lowes, C.; Gow, N. A.; Erwig, L. P. *Candida albicans* Infection Inhibits Macrophage Cell Division and Proliferation. *Fungal Genet. Biol.* **2012**, *49*, 679–680.
17. Mesquita, F. S.; Thomas, M.; Sachse, M.; Santos, A. J.; Figueira, R.; Holden, D. W. The *Salmonella* Deubiquitinase SseL Inhibits Selective Autophagy of Cytosolic Aggregates. *PLoS Pathog.* **2012**, *8*, e1002743.
18. Urban, C. F.; Reichard, U.; Brinkmann, V.; Zychlinsky, A. Neutrophil Extracellular Traps Capture and Kill *Candida albicans* Yeast and Hyphal Forms. *Cell. Microbiol.* **2006**, *8*, 668–676.
19. Müller, D. J.; Dufrêne, Y. F. Atomic Force Microscopy: A Nanoscopic Window on the Cell Surface. *Trends Cell. Biol.* **2011**, *21*, 461–469.
20. Müller, D. J.; Dufrêne, Y. F. Force Nanoscopy of Living Cells. *Curr. Biol.* **2011**, *21*, R212–216.
21. Buccione, R.; Orth, J. D.; McNiven, M. A. Foot and Mouth: Podosomes, Invadopodia and Circular Dorsal Ruffles. *Nat. Rev. Mol. Cell. Biol.* **2004**, *5*, 647–657.
22. Labernadie, A.; Thibault, C.; Vieu, C.; Maridonneau-Parini, I.; Charriere, G. M. Dynamics of Podosome Stiffness Revealed by Atomic Force Microscopy. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 21016–21021.
23. Ahmad, S. F.; Chtcheglova, L. A.; Mayer, B.; Kuznetsov, S. A.; Hinterdorfer, P. Nanosensing of Fcgamma Receptors on Macrophages. *Anal. Bioanal. Chem.* **2011**, *399*, 2359–2367.
24. Chtcheglova, L. A.; Waschke, J.; Wildling, L.; Drenckhahn, D.; Hinterdorfer, P. Nano-Scale Dynamic Recognition Imaging on Vascular Endothelial Cells. *Biophys. J.* **2007**, *93*, L11–13.
25. Zeng, X.; Wang, T.; Zhu, C.; Xing, X.; Ye, Y.; Lai, X.; Song, B.; Zeng, Y. Topographical and Biological Evidence Revealed FTY720-Mediated Anergy-Polarization of Mouse Bone Marrow-Derived Dendritic Cells *in vitro*. *PLoS One* **2012**, *7*, e34830.
26. Dupres, V.; Alsteens, D.; Wilk, S.; Hansen, B.; Heinisch, J. J.; Dufrene, Y. F. The Yeast Wsc1 Cell Surface Sensor Behaves Like a Nanospring *in vivo*. *Nat. Chem. Biol.* **2009**, *5*, 857–862.
27. Heinisch, J. J.; Lipke, P. N.; Beaussart; A., El-Kirat-Chatel, S.; Dupres, V.; Alsteens, D.; Dufrêne, Y. F. Atomic Force Microscopy – Looking at Mechanosensors on the Cell Surface. *J. Cell Sci.* In Press.
28. Aimanianda, V.; Bayry, J.; Bozza, S.; Kniemeyer, O.; Perruccio, K.; Elluru, S. R.; Clavaud, C.; Paris, S.; Brakhage, A. A.; Kaveri, S. V.; et al. Surface Hydrophobin Prevents Immune Recognition of Airborne Fungal Spores. *Nature* **2009**, *460*, 1117–1121.
29. Levitz, S. M. Innate Recognition of Fungal Cell Walls. *PLoS Pathog.* **2010**, *6*, e1000758.
30. Silva, S.; Negri, M.; Henriques, M.; Oliveira, R.; Williams, D. W.; Azeredo, J. *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*: Biology, Epidemiology, Pathogenicity and Antifungal Resistance. *FEMS Microbiol. Rev.* **2012**, *36*, 288–305.
31. Keppler-Ross, S.; Douglas, L.; Konopka, J. B.; Dean, N. Recognition of Yeast by Murine Macrophages Requires Mannan but not Glucan. *Eukaryot. Cell* **2010**, *9*, 1776–1787.
32. Roetzer, A.; Gratz, N.; Kovarik, P.; Schuller, C. Autophagy Supports *Candida glabrata* Survival During Phagocytosis. *Cell. Microbiol.* **2010**, *12*, 199–216.