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Microscopy of fungal biofilms

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Fungal biofilms are heterogeneous, surface-associated colonies comprised of filamentous hyphae (chains of elongated cells), pseudohyphal cells, yeast-form cells, and various forms of extracellular matrix. When grown on a substratum under liquid culture medium, the microbial fungus *Candida albicans* forms dense biofilms that range in thickness from 100 to 600 μm. Apical hyphae in the medium and invasive hyphae in the substratum may add greatly to the thickness and complexity of the biofilm. Because of the heterogeneity of the structure, and the large refractive index differences between cell walls, cytoplasm, and medium, fungal biofilms appear optically opaque. For fixed specimens that can be transferred out of an aqueous medium, refractive index matching methods provide a high degree of clarification. Confocal scanning, 2-photon scanning, or selective-plane illumination microscopy then can be used to obtain high-quality image data spanning the full thickness of the biofilm. Using refractive index matching and confocal microscopy, we have imaged many interesting features within wild-type, mutant, and engineered biofilms, including cellular phenotypes that vary with position, the effect of growth conditions, and gene expression through reporter constructs. This approach greatly expands the range of microscopical studies, allowing researchers to observe and quantify specific phenomena within medically or industrially relevant forms of microbial growth.

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Introduction

Microbial fungi are of interest for many reasons, from fundamental cell and molecular biology to pathogenesis and commercial fermentation processes. The genus *Saccharomyces* is well known for yeast-form unicellular growth, though filamentous (hyphal) growth as chains of elongated cells can be induced [1,2]. In the genus *Candida*, the changeover between yeast-form and hyphal growth is a stress response that is much more prominent. It is of interest not only for its fundamental significance as a genetic switch regulating dimorphism and colonial growth, but also because of dramatically different behavior in the two phases. In species such as *C. albicans*, hyphae are adhesive, form highly entangled biofilms on certain types of surfaces, and can be invasive and virulent. Though it is commensal in healthy individuals, *C. albicans* is an opportunistic human pathogen accounting for a majority of mucosal and systemic yeast infections [3]. Experiments show that the ability to form a biofilm correlates with virulence [4], and that biofilm cells are more resistant to antifungal agents than planktonic cells [5]. Understanding the biology of cells within a biofilm is both fundamentally and medically relevant, so we have developed methods for microscopic viewing of cells within intact biofilms.

Optical characteristics of a biofilm

Candida albicans biofilms originate from cells that adhere to a surface, and generally consist of highly entangled hyphae (filaments of elongated cells), clusters of pseudohyphal and yeast-form cells, voids, and extracellular matrix. After 24–48 h growth on a surface submerged in liquid culture medium, dense biofilms range in thickness from 100 to 600 μm. Under 5–10× magnification, a biofilm has the appearance of very fine 'wet felt'. Long, non-entangled apical hyphae may extend an additional 500 μm into the medium, while basal hyphae may invade the substratum. These features add greatly to the thickness and complexity of the biofilm. Because of the refractive index differences between cell walls, cytoplasm, and medium, any entering light ray will be partially scattered at every boundary in its path. Therefore, much of the light that enters a biofilm is scattered so that the biofilm has a high diffuse optical density (dOD) and appears opaque. When viewed in a microscope, contrast generally is degraded for any biofilm features buried more deeply than 30 μm. To view cells within an intact biofilm therefore requires (A) a method for optical clarification, and (B) appropriate immersion optics with sufficient focusing range (working distance) and axial resolution

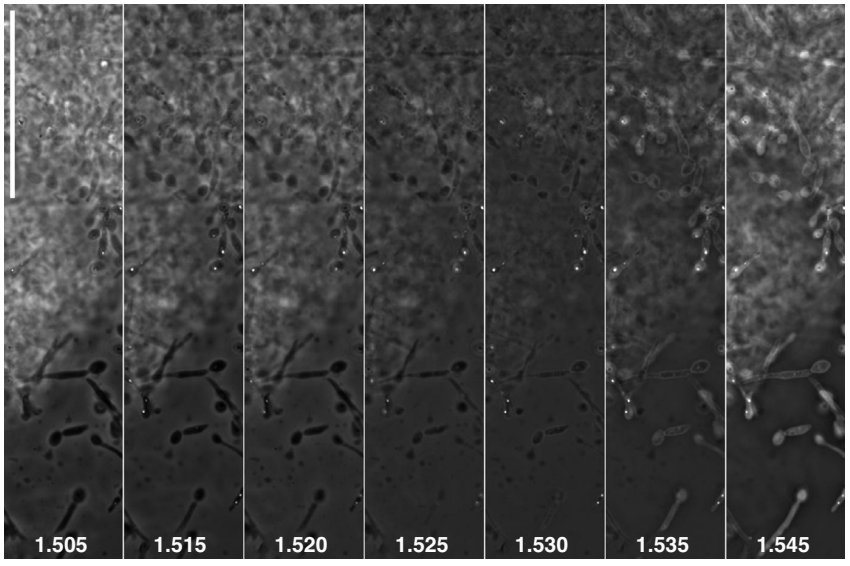
to be useful for optical sectioning microscopy. The most straightforward clarification method, applicable mainly to fixed biofilms, is index-matching the cellular structures within the biofilm by exchange of its water content for a specific organic monomer or solvent having a higher refractive index than water (n_w = 1.333). The literature on tissue clearing and mounting for microscopy is extensive, but the topic is resurgent [6•,7•].

Because *C. albicans* biofilms generally are not pigmented, perfect index matching, if it were possible, would render the biofilm invisible in transmitted light microscopy — brightfield, darkfield, phase contrast, and DIC. However, for molecular studies, fluorescence microscopy is the major imaging mode. Maximizing optical clarity makes it possible to get sharp fluorescence images from focus planes deep within the biofilm. Because biofilm thickness far exceeds the depth-of-field of the microscope, out-of-focus features would dominate any conventional image. Therefore, fluorescence imaging of intact specimens requires optical sectioning microscopy — most commonly confocal scanning, 2-photon scanning, or selective-plane illumination.

To determine the immersion refractive index that would optimize clarity, biofilms grown on coverglasses were fixed, then transferred through a series of miscible solvents (phosphate-buffered saline (PBS) to methanol to xylene (n = 1.497), followed by a series of Cargille refractive-index reference fluids [8]) to visually find a minimum in light scatter. For *C. albicans* biofilms, the minimum occurred in the index range 1.525–1.535. The measurement was refined by immersion refractometry [9] using a conventional phase contrast microscope, which showed contrast reversal of the fixed cell wall at 1.530, and of the fixed cytoplasm close to 1.535 (Figure 1). Methyl salicylate, long used in embryology and histology for tissue clearing, is slightly outside this range (n = 1.537), but was found to give excellent index-matching and has the dual advantage of moderate polarity and use as a pure solvent. It is possible to adjust 'n' downward with xylene or an alcohol, but the index then would drift if one component was more volatile than the other.

The essential technical point to emerge from index matching is that the optimal index for *Candida* biofilms is high, exceeding the index of standard immersion oil (n_D = 1.518) by approximately 8%. For aberration-free imaging of cells deep within a specimen, it is a fundamental principle of microscopy that the index of the immersion fluid should exactly match the index of the specimen to avoid focus-dependent spherical aberration [10•,11•]. Though this ideal is almost never met in practice, conventional oil-immersion optics give very good performance with the two mounting methods detailed below. A limitation is that high-NA oil-immersion objectives mostly have insufficient working distance (focusing range) for fungal biofilm specimens. In response

Figure 1



Phase contrast immersion refractometry of a fixed *C. albicans* biofilm. Refractive index standard fluids spanned the range 1.505–1.545 in 0.005 increments. The images show a minimum in biofilm visibility (maximum in transparency) for n = 1.530, corresponding mainly to the match point for the cell wall. Contrast reversal in the cytoplasm occurs at a slightly higher index. Scale bar = 50 μm.