Video Article

Clarifying and Imaging *Candida albicans* Biofilms

Frederick Lanni¹, Katherine Lagree¹, Manning Y. Huang¹, Lan Yan², Carol A. Woolford¹, Aaron P. Mitchell¹

¹Department of Biological Sciences, Carnegie Mellon University

²Department of Pharmacology, Second Military Medical University

Correspondence to: Frederick Lanni at lanni@cmu.edu

URL: <https://www.jove.com/video/60718>

DOI: [doi:10.3791/60718](https://doi.org/10.3791/60718)

Keywords: Immunology and Infection, Issue 157, *Candida albicans*, biofilm, refractive index matching, methyl salicylate, clarification, confocal microscopy

Date Published: 3/6/2020

Citation: Lanni, F., Lagree, K., Huang, M.Y., Yan, L., Woolford, C.A., Mitchell, A.P. Clarifying and Imaging *Candida albicans* Biofilms. *J. Vis. Exp.* (157), e60718, doi:10.3791/60718 (2020).

Abstract

The microbial fungus *Candida albicans* can undergo a change from commensal colonization to virulence that is strongly correlated with its ability to switch from yeast-form growth to hyphal growth. Cells initiating this process become adherent to surfaces as well as to each other, with the resulting development of a biofilm colony. This commonly occurs not only on mucosal tissue surfaces in yeast infections, but also on medical implants such as catheters. It is well known that biofilm cells are resistant to antifungal drugs, and that cells that shed from the biofilm can lead to dangerous systemic infections. Biofilms range from heavily translucent to opaque due to refractive heterogeneity. Therefore, fungal biofilms are difficult to study by optical microscopy. To visualize internal structural, cellular, and subcellular features, we clarify fixed intact biofilms by stepwise solvent exchange to a point of optimal refractive index matching. For *C. albicans* biofilms, sufficient clarification is attained with methyl salicylate ($n = 1.537$) to enable confocal microscopy from apex to base in 600 μm biofilms with little attenuation. In this visualization protocol we outline phase contrast refractometry, the growth of laboratory biofilms, fixation, staining, solvent exchange, the setup for confocal fluorescence microscopy, and representative results.

Video Link

The video component of this article can be found at <https://www.jove.com/video/60718/>

Introduction

Candida albicans is a microbial fungus that is typically commensal in humans. It is of fundamental interest to biologists because the organism has multiple morphologies. For example, in response to certain environmental cues or stressors, yeast-form budding cells will switch to filamentous growth as septating chains of highly elongated cells known as hyphae. The transition is important as an example of phenotypic expression of a changeover between a unicellular and multicellular gene expression program. Likewise, *C. albicans* is of biomedical interest because the organism is a well-known opportunistic pathogen. It is responsible for mucosal yeast infections such as thrush (oral candidiasis), genitourinary infections, and a cause of dangerous invasive or systemic infections in immunologically weakened patients.

This organism's potential for virulence is closely connected to its multiple morphotypes and other aspects of its genetic versatility^{1,2,3,4}. Germ tube extension, the first visible stage of the transition to hyphal growth, occurs with sufficient rapidity to enable engulfed *C. albicans* cells to break out of phagocytes and thereby escape an early phase of the cellular immune response of the host⁵. Additionally, filamentation is preceded and accompanied by a large increase in cell-to-cell and cell-to-surface adherence that is due to regulated expression of several classes of cell wall proteins known as adhesins^{6,7,8,9}. Under a wide variety of conditions, the combination of adherence and filamentation results in a dramatic shift from planktonic, unicellular growth to surface-associated colonial growth that forms a biofilm. *C. albicans* biofilms may develop on common implanted medical devices such as venous catheters. Disseminated infections can result when such biofilms begin to bud off yeast-form cells and shed them into circulating blood. Multiple studies have shown that biofilm cells are more resistant to antifungal drugs than planktonic cells^{10,11}, which may be due in part to lowered metabolism¹². Furthermore, the high overall adherence of a biofilm may provide the anchoring needed for efficient invasive growth of hyphae into host tissue¹.

Optical clarification of *C. albicans* biofilms by refractive index matching has had a major impact on our ability to visualize the structure of this microbial community and discover relationships between gene expression and phenotype. Biofilms grown in the laboratory on test surfaces under liquid culture medium for 48 h appear as a whitish coating that is dense enough and thick enough to be opaque (**Figure 1**). Therefore, many interesting phenotypic features of the biofilm are hidden from view. The 24 h wild type biofilms grown in YPD, RPMI-1640, or Spider medium at 37 °C range up to 300 μm thick, with 48 h biofilms often reaching 500 μm . The opacity is due to light scattering, which arises from refractive heterogeneity: the fungal cell wall is more refractive than the surrounding medium, and the cytoplasm slightly more refractive than the cell wall. The resulting high optical density of a native biofilm obscures all structures more than 30 μm deep when viewed with a conventional microscope or even a confocal scanner (**Figure 2**). However, a large degree of clarification can be achieved by infiltrating fixed biofilms with a high refractive index liquid that approximately matches the refractivity of major cellular constituents. After clarification, imaging at submicron resolution can be carried out by confocal microscopy through the entire thickness of almost any *C. albicans* biofilm¹³. In wild type specimens, it is easy to see that biofilms consist of long entangled hyphae, clusters of budded yeast-form cells or pseudohyphal cells, void spaces, and

some amount of extracellular matrix. Moreover, biofilms generally are stratified, showing spatially variant morphological differences in cell type, cell density, and the presence of budding or branching cells. Many variations in one or more of these features have been observed in biofilms developed by mutant strains^{14,15}. Additionally, reporter strains show in situ spatial differences in gene expression^{16,9,13}. The surprising degree of clarification obtainable with this relatively simple and inexpensive approach also makes it possible to see that many biofilms include apical hyphae and basal invasive hyphae extending out to millimeter distances.

In this visualization protocol, we demonstrate the fixing, labeling, clarification, and imaging of a *C. albicans* biofilm using a simple cell wall marker as a stain. The origin of the present version of the protocol is the improved clarity we observed when formaldehyde-fixed biofilms were infiltrated with 97% glycol methacrylate (GMA), which was then polymerized to embed the biofilm in a hard plastic having a moderately high refractive index ($n = 1.49$). We then used conventional phase contrast microscopy and a series of refractive index reference liquids to more accurately determine the point of contrast reversal (maximum transparency) of the biofilm (**Figure 3**). For *C. albicans* biofilms this occurred close to $n = 1.530$, which was surprisingly high given that a dense protein structure such as hair keratin is only slightly more refractive (1.54–1.55). Though it is on the upper end of the optimal range in **Figure 3**, we adopted methyl salicylate (oil of wintergreen, $n = 1.537$) in the current protocol because it has long been used as a clarifying agent for microscopy in embryology and histology, it has low toxicity and low vapor pressure, and it gave excellent results in our trials using moderate-NA oil immersion optics.

Four points should be made here:

- (1) With few exceptions, the ideal situation in microscopy is that the refractive index of the specimen should be equal to the refractive index of the objective lens immersion fluid^{17,18,13}. For three-dimensional (3D) imaging studies where deep focusing is necessary, this is always important.
- (2) Our experimental result for optimal clearing ($n = 1.525$ – 1.535) is slightly higher than standard immersion oils ($n = 1.515$, 1.518) and therefore violates point (1), and thus introduces a source of spherical aberration when using standard oil immersion optics. One solution to this problem is use of an objective designed for an immersion index in the higher range. Because of a resurgence of interest in imaging cleared specimens, specialty objectives with the needed correction are becoming available. The other solution is to adjust the index of the clarifying medium down to 1.515 or 1.518 by the addition of a small amount of butanol ($n = 1.399$) for optimal oil immersion performance, and accept the slightly degraded clarity.
- (3) Microscopy of intact biofilms (and other specimens on this scale) requires a significant working distance to accommodate the specimen thickness. This is a major practical consideration. A long working distance limits the optics to a moderate numerical aperture ($NA = 0.6$ – 1.25), which limits resolution but also limits the severity of spherical aberration.
- (4) The optimal refractive index for clarification may be different for other types of fixed specimens. Specimens should be tested accordingly using phase contrast and a series of refractive index reference liquids as shown in **Figure 3**.

Protocol

1. Growth of Biofilm Cultures

CAUTION: *Candida albicans* is a human pathogen. At some institutions, culture of this organism requires BSL-2 containment.

1. Streak out selected strain on a yeast extract-peptone-dextrose (YPD) agar plate and grow 48 h at 30 °C.
2. Select single colonies from the plate to inoculate 5 mL of YPD medium in 15 mL culture tubes for overnight aerobic growth (carousel rotator, 52–55 rpm) at 30 °C.
3. Determine cell density using a 40- to 50-fold dilution of the overnight culture. Compute the dilution factor needed to bring the cell density to 3×10^6 cell/mL for silicone substrata, or 0.6 – 1.2×10^6 cells/mL for cover glass surfaces treated with concanavalin-A (ConA) or wheat-germ agglutinin (WGA) (see Discussion).
4. For biofilm growth in a 12 well plate (**Figure 1**), dispense 2.0 mL of sterile filamentation medium into each well, and warm the plate to 37 °C in a humidified incubator. A variety of media will induce filamentation, more strongly at an elevated temperature (37 °C) and with added serum. Fetal bovine serum (FBS) is recommended. Recommended media are YPD, Spider-Mannitol, or RPMI-1640.
5. With sterile tweezers, place a prepared substratum into each well in the warmed plate and dislodge bubbles. Add the inoculum from the overnight culture to reach the cell density recommended in step 1.3 in each well. One well without inoculum serves as a visual blank and control against contamination.
6. Place the plate on a 60 rpm orbital mixer in a humidified 37 °C air incubator for 90 min to allow time for cell adhesion. After 90 min, remove the medium and unattached cells, wash with sterile medium or phosphate-buffered saline (PBS), and place inoculated substrata into culture dishes or another multiwell plate containing prewarmed filamentation medium. With multiwell plates, it is very convenient to use a second plate for the wash step and a third plate with 2.0 mL of prewarmed medium per well to receive the washed inoculated substrata. Sterilize tweezers prior to each transfer.
7. Return the plate to the 37 °C humidified ambient-air incubator and grow the biofilms up to 48 h with 60 rpm orbital mixing for aeration. There should be little planktonic growth in each culture unless the developing biofilm is shedding yeast-form cells. A biofilm grown in this way is shown in **Figure 1**.

2. Specimen Processing for Imaging

CAUTION: Aldehyde fixatives are volatile and hazardous. Prepare fixative dilutions in a fume hood, not in a biosafety cabinet. Do not put fixatives into incubators used for live cells. Work with diluted fixatives in covered dishes in a fume hood or a well-ventilated benchtop area. Dispose of fixatives and post-fix wash solutions as hazardous waste.

1. Prepare fresh fixative, 4% formaldehyde or paraformaldehyde in PBS, optionally with up to 2% glutaraldehyde. Formalin diluted to 4% may be used for routine work.
NOTE: Glutaraldehyde in particular will increase broadband autofluorescence and may attenuate the fluorescence of expressible tags such as dTomato.
2. Remove the culture medium from the specimen and replace with PBS to dilute away serum proteins, or transfer the biofilm on its substratum to PBS, for several minutes.
3. Transfer the specimen into fixative, and set the covered dish on a slow orbital mixer for 20 min. Extend the time period when processing thicker specimens (see Discussion). Sufficient fixative volume should be added to each dish to immerse all biofilm growth, including any on the inner sides of the dish.
4. Remove the fixative and refill the dish with PBS to wash out the residual fixative. Dispose of the fixative as hazardous waste. Repeat several short-term washes, then longer washes to allow time for the residual fixative to diffuse out of the biofilm or agar block. The wash period depends on the time allowed for fixation (see **Discussion**).
5. The needed quantity of stain will depend on the biofilm mass. Remove all biofilm from non-specimen areas of the culture dish prior to staining, or transfer the fixed specimen into a new dish ahead of staining. Do not allow the biofilm to drain or dry. Keep it immersed in PBS.
6. Add the stain to the dish (see Discussion) and set the covered dish on a slow orbital mixer overnight (see Discussion). Protect from light.
7. In the morning, remove the staining solution and refill the dish with PBS to dilute the unbound stain. Set the dishes on slow orbital mixer to destain for several hours.

3. Clarification Protocol: Biofilms on Impermeant Substrata

1. Clarified biofilms are hard to discern visually. Therefore, if desired, mark one side of each substratum with a scratch to designate the side for inoculation.
2. Use labeled long Pasteur pipettes for all subsequent waste and solvent transfers to avoid cross contamination of the solvents.
3. Using tweezers, transfer the fixed biofilm from PBS to 5 mL of 50:50 PBS:methanol in a 20 mL glass vial with the biofilm facing up. Manually mix with orbital motion at 1 min intervals for 5 min (see Discussion).
4. Remove the solvent to a waste bottle, being cautious to avoid contact between the pipette and biofilm, or the biofilm and vial. Gently refill with 3 mL of neat methanol. Manually mix with orbital motion at 1 min intervals for 3 min.
5. Remove the solvent to a waste bottle, and immediately refill with 5 mL of methanol. Manually mix with orbital motion at 1 min intervals for 5–10 min. Remove the solvent to the waste bottle, and immediately refill with 3 mL of methanol. Biofilms often look more opaque at this point than their initial appearance.
6. Remove the solvent to the waste bottle, and immediately refill the vial with 5 mL of 50:50 methanol:methyl salicylate. Manually mix with orbital motion at 1 min intervals for 5–10 min. The biofilm should be semitransparent in this mixed solvent.
7. Remove the solvent to a waste bottle. Gently refill the vial with 3 mL of neat methyl salicylate (MS). Manually mix with orbital motion at 1 min intervals for 3 min.
8. Remove the solvent to the waste bottle, and immediately refill with 5 mL of neat MS. Manually mix with orbital motion at 1 min intervals for 5–10 min. At this point, the biofilm should be transparent. Remove the solvent to the waste bottle, and immediately refill the vial with 3 mL of neat MS. The processed biofilm is stable in this solvent.
9. For biofilms on agar (see Discussion) extend all exchange time periods to allow for water and solvent diffusion through the agar. The necessary time periods can be estimated using a low-power dissecting microscope with darkfield illumination to view the solvent advance into the agar. The protocol achieves good clarification and no major shrinkage effects when 2% agar is used, but the clarified agar will condense if squeezed while handling with tweezers. Use of a spatula is recommended.

4. Imaging Setup for Confocal Microscopy

1. The following steps are for the use of an inverted microscope. Use a solvent-proof dish that has a cover glass bottom to hold the inverted specimen on the microscope stage (see Discussion).
2. Prepare spacers for support of the inverted specimen. Use small rectangles cut from microscope slides (1,000 μm thick), cover glasses (170 μm), or 13 mm silicone rings (330 μm , see **Table of Materials**), which may be stacked as needed. Presoak the rings in methyl salicylate for 1 h to minimize focus drift due to swelling.
3. For a biofilm on a medical grade silicone square, invert the square (i.e., turn it biofilm down) and set it on a spacer in a pool of methyl salicylate in the dish (**Figure 2**). Make sure to avoid any bubbles below the specimen.
4. Mount the dish firmly on the stage of the microscope and make oil immersed contact with the objective (see Discussion).
5. Adjust the amount of MS in the dish so that the meniscus holds the specimen firmly down on the spacer by surface tension. Place a droplet of MS on top of the inverted square substratum to reduce light scatter from the matte finish, and cover the dish with a glass plate to limit evaporation. Wait several minutes for the specimen to settle onto the spacers prior to imaging.
6. Use transmitted light to visually inspect the field of view and locate the current focus position relative to the apical and basal regions of the inverted biofilm. Set the condenser illuminating numerical aperture (INA) to a minimum (close down the condenser iris) to increase contrast, otherwise the clarified biofilm will be nearly invisible. When done, switch off the transmitted light source.
7. Switch over to confocal fluorescence and set the lower and upper limits of the serial-focus image stack needed to span the biofilm. Upward focus drive stepping, which is recommended, will first show dislodged cells that have settled onto the cover glass and very long apical hyphae that are resting on the glass. At the upper end of the sequence, the founder cells should be seen adhered to the substratum at the base of the biofilm. Set the pinhole diameter, the pixel spacing in the image plane, and the focus step increment using the general criteria outlined in the Discussion.

Representative Results

Biofilms such as that in **Figure 1** are heavily translucent to opaque, but are routinely clarified and imaged by employing the protocol above. **Figure 2** shows the strong attenuation of fluorescence with focus depth in a fixed biofilm in PBS, compared to the same biofilm after index

matching. As shown in **Figure 3**, using serially miscible solvents of increasing refractive index the maximum in clarity can be quickly estimated. This is refined by conventional phase contrast microscopy to find the point of minimum contrast (maximum transparency). It is evident that this optimum is not achieved by homogeneous index matching. This is because subcellular structures differ slightly in refractive index. In this case, contrast reversal occurred in the cell wall at a slightly lower solvent index than in the cytoplasm. For *Candida albicans* biofilms, the optimum occurs close to $n = 1.530$. The 48 h wild type and *cak1* DX mutant biofilms in **Figure 4A** were 500 μm in thickness, yet the yeast cells at the base were imaged nearly as sharply as the apical cells. Likewise, as shown in **Figure 4C**, attenuation of fluorescence was not strongly correlated with focus depth. **Figure 5** shows invasive hyphae in agar, hundreds of micrometers below a surface biofilm. Mature invasive hyphae consistently produce lateral budding yeast proximal to the septa in the hyphal chain, giving rise to subcolonies of yeast-like cells at regular intervals along an invasive hypha.

Figure 1: Biofilm cultures prior to clarification. A 24 h biofilm grown from an isolate of a complemented *efg1* Δ/Δ strain of *C. albicans* in a 12 well plate on a silicone square in RPMI-1640 liquid medium supplemented with 10% FBS. The sterile blank is in well C4. Inset shows cut cross-section of 96-hr wild-type biofilm grown in a 6-well plate on a 3.75 mm agar base in the same medium showing invasive hyphae. Both panels are to the same scale. Scale bar = 2.0 mm. [Please click here to view a larger version of this figure.](#)

Figure 2: Improvement of deep imaging by index matching. The figure shows sideview projections of confocal image stacks from a 48 h wild type biofilm that was fixed and stained with ConA-Alexafluor 594. **(A)** The specimen was imaged in PBS using a 63x 1.0 NA direct water-immersion objective. Attenuation was severe at a focus depth of 30 μm . **(B)** After clarification by protocol steps 3.3–3.8, the same biofilm was imaged in methyl salicylate with minimal attenuation using a 40x 0.85 NA oil immersion objective. After background subtraction and sideview projection of the 3D image stacks, the 40x data was spatially rescaled to match the 63x data for comparison. **(C)** Schematic diagram showing inverted mounting of the cleared specimen (from MS) by transfer to MS in a black-anodized aluminum dish with a cemented cover glass bottom (see Discussion). Scale bar = 50 μm . [Please click here to view a larger version of this figure.](#)

Figure 3: Phase contrast refractometry shows contrast reversal of cellular features in the range of optimum index matching. Fixed biofilms on cover glasses were exchanged from PBS into methanol, then into xylene ($n = 1.496$). These biofilms were then exchanged one each from xylene into a set of refractive index reference liquids (Series E, Cargille Laboratories, see **Table of Materials**) and visually examined side-by-side to determine the index range giving highest transparency. (upper panel) Phase contrast images: One biofilm was serially exchanged between xylene and a narrow set of reference liquids in that range. After each exchange, the same field was relocated and viewed through a cover glass using a 40x 0.85 NA Ph2 oil immersion phase contrast objective and Ph2 condenser. All images were recorded with the same lamp setting and camera exposure to accurately display changes. With increasing index, contrast reversal is first seen at $n = 1.530$ for the cell wall, followed by cytoplasm at $n = 1.535$. Scale bar = 10 μm . (lower panel) Graph showing the minimum in average biofilm brightness at the point of maximum transparency. Strong scattering of light within the biofilm causes the average brightness to exceed the blank field. Isolated cells appear darker than the blank field, up to contrast reversal in the range 1.530–1.535. [Please click here to view a larger version of this figure.](#)

Figure 4: Deep imaging of mutant and wild type *C. albicans* biofilms. A wild type strain (DAY185) and a cell cycle related protein kinase diminished expression strain (*cak1 DX*)¹⁴ were grown at 37 °C for 48 h on silicone substrata in liquid YPD medium. The biofilms were fixed, stained with ConA-Alexafluor 594, and clarified using the solvent exchange protocol into methyl salicylate. The 3D confocal microscopy showed that both biofilms were ~500 µm thick. Image data were converted to 32-bit format in ImageJ or Fiji (<https://imagej.nih.gov/ij/> or <http://fiji.sc>), then processed using the **Subtract Background** function with a 50-pixel rolling ball radius, thresholding to set negative pixels to zero, reslicing, and using maximum intensity projection to produce side views. **(A)** Axial and side-view projections: The wild type biofilm grew to a thickness of 502 µm as seen in the side view projection of the 558-plane confocal image stack. Scale bar = 100 µm. (left hand panels) 40-plane axial projections from apex (top) to base (bottom) show the variation in cell types in different strata of the biofilm. This example showed characteristic wild type structure: adherent cells at the base give rise to hyphae that ascend into a dense middle zone of pseudohyphal and yeast-like cells. Above the midzone, hyphae reemerged and gave rise to clusters of budding yeast. The long hyphae seen in the apical zone likely extended into the culture medium in the living biofilm, but folded over onto the surface of the biofilm during specimen processing. The *cak1 DX* biofilm grew to a thickness of 500 µm as seen in the side-view projection of the 556-plane image stack. This mutant, which is known to undergo filamentous growth in the absence of inducing stresses¹⁴, produced a dramatically different architecture. As seen in both the sideview and axial projections (right hand panels), many branching cells gave rise to radial outgrowths. **(B)** Examples of branching hyphae within the *cak1 DX* biofilm. Scale bar = 10 µm. **(C)** Fluorescence attenuation with depth in the clarified wild type biofilm was quantified by masking out background pixels, then computing the average digital signal for all non-background pixels in each image plane. With the exception of apical hyphae that are brightly tagged by the lectin, there was no strong attenuation trend with focus depth. [Please click here to view a larger version of this figure.](#)

Figure 5: Invasive hyphae in agar. *C. albicans* hyphae in a surface biofilm will penetrate an agar substratum to millimeter distances (see **Figure 1**). After clarification, invasive structures may be viewed downward through the biofilm, or upward from the bottom side of the agar. The latter demands greater working distance. Alternatively, after fixation but prior to staining and solvent exchange, the agar may be cut vertically with a scalpel or razor blade into 1–2 mm slabs that then can be turned on a side and imaged directly in side view after staining and clarification. This procedure is recommended. In this projection of a 213 μm square field of view, a rainbow color scale was used to encode axial position over a 75 μm range: blue features are near and red features are deeper into the plane of the page. This view shows that long hyphae bud off lateral yeast-form cells at semiregular intervals that give rise to subcolonies. Scale bar = 50 μm . [Please click here to view a larger version of this figure.](#)

Discussion

Advances in fluorescence microscopy in optical sectioning, resolution, speed, avoidance or compensation of aberration, multichannel acquisition, and in computing power, have caused a resurgence in imaging intact specimens. For fixed specimens, both classical and novel clarification and expansion methods have had a major impact^{19,20,21,22,23,24}. In this case, we applied a fast and simple solvent-based index matching approach to study the structure of fungal biofilms that are heavily translucent to opaque.

The preceding protocols have been tested with a range of specimens. In our laboratory *Candida albicans* biofilms are most often grown on 14 mm squares cut from a sheet of medical grade silicone rubber (see **Table of Materials**). This substratum is used because growth on PDMS (poly-dimethylsiloxane) submerged in liquid culture medium has been established as an important in vitro model for infections associated with medical implants, particularly indwelling catheters. Stressed cells initiating filamentation adhere quickly to nonpolar surfaces such as PDMS. In practice, used squares that have been cleaned and resterilized in an autoclave (dry cycle) give the most consistent biofilms for any particular strain. Biofilms also are commonly grown on cover glasses, in cover glass bottom culture dishes, in standard bacteriological grade polystyrene dishes, and on agar. Because *C. albicans* cells do not adhere well to glass, cover glasses should be treated with a lectin that will bind cell wall polysaccharides. We apply 40 μL of 1 mg/mL ConA or WGA in sterile water onto each coverslip surface. After drying, cover glasses are treated with 40 μL of 1% glutaraldehyde for 3 min to crosslink the protein into an insoluble film. They are then washed with sterile distilled water to remove the fixative and any soluble lectin and air-dried. If necessary, the treated cover glasses or dishes are resterilized under a germicidal UV lamp for 10 min.

Specimen thickness will affect the required incubation periods in the protocols. Fixative diffusion into a 300 μm biofilm requires several minutes to equilibrate. This time period increases as the square of the specimen thickness, and should be extended to an hour or more for very thick biofilms or agar block specimens that may be 2–3 mm thick. The equilibration time period for staining also depends on the specimen thickness as described for fixing and washout. However, because the lectins diffuse more slowly than the low-MW fixatives, especially within an agar gel, staining should be extended overnight. The same should be done for the washout of excess stain.

Stains of various types may be incorporated into the protocols. We use a cell wall stain for structural imaging, most commonly Calcofluor White M2R (Fluor. Brightener #28) or a dye-tagged lectin such as ConA-Alexafluor 594 or WGA-Alexafluor 594. Attention should be paid to the valency of the protein. The ConA tetramer may cause crosslinking of highly flexible hyphae in the apical region of a biofilm. This is less apparent with the WGA dimer. The canonical specificities of these markers are Calcofluor for chitin, ConA for mannose residues, and WGA for N-acetyl-D-glucosamine and sialic acid residues.

Because the solvent exchange protocol is graded from PBS or water through methanol into methyl salicylate, specimen containers must be solvent-resistant. Methyl salicylate (MS) will quickly soften polystyrene plasticware and slowly soften other plastics. The protocol steps up to the use of the neat methanol can be carried out in plasticware, but at that point in the protocol we generally switch over to standard 20 mL glass scintillation vials with solvent-resistant screw caps. The vials are convenient for biofilms on silicone square substrata, because the squares can be picked up and transferred using fine tweezers. Also, a vial can be drained and refilled with the flat square resting on the inner curved surface so that the biofilm does not contact the glass. The substratum should be biofilm-up when it is immersed in the upright vial. The vials are excellent for long-term specimen storage.

In the clarification protocol, more graded solvent exchange steps minimize the risk of specimen deformation due to solvent mixing effects. For speed and convenience in the basic protocol, there are four steps: 1) step 3.3, PBS to 50:50 PBS:methanol; 2) steps 3.4 and 3.5, PBS:methanol

to neat methanol, 3) step 3.6, neat methanol to 50:50 methanol:MS; and 4) steps 3.7 and 3.8, methanol:MS to neat MS. Methanol provides the transitional miscibility. However, because water and MS are nearly immiscible, it is essential that all water be displaced by methanol prior to the introduction of any MS. Likewise, because methanol is highly volatile, it is important to displace all methanol by MS. Otherwise, the continued evaporation of residual methanol will cause refractive index variations within the specimen. Within the four step sequence, repeating the second and fourth steps by adding another change of neat solvent, or even a third change, is advisable. For particularly fragile specimens, solvent changes could be formulated in smaller percent steps.

With agar block specimens, steps 3.4 and 3.5 (neat methanol) may cause the appearance of salt crystals within the agar due to the insolubility of residual PBS salts in the methanol. This can be avoided by using distilled water (DW) in the first mixed solvent step in place of PBS to dilute salts during the water:methanol exchange. The alternative solvent exchange sequence for fixed biofilms then consists of these steps: 1) step 3.3, transfer the specimen from PBS into 50:50 DW:methanol (allow extra time for diffusion through agar); 2) step 3.4, transfer the specimen from 50:50 DW:methanol into neat methanol (repeat 2x with methanol as in step 3.5); 3) step 3.6, transfer the specimen from methanol to 50:50 methanol:methyl salicylate; and 4) step 3.7, transfer the specimen from 50:50 methanol:MS to neat MS (repeat 2x with MS as in step 3.8).

Because methyl salicylate quickly softens polystyrene plasticware, we use an anodized aluminum dish with a cover glass bottom to hold the clarified biofilm on the stage of an inverted microscope. The cover glass is held in place using UV-curing optical cement (Norland Optical Adhesive #61, see **Table of Materials**). Provided the MS is removed at the end of the day by washing the dish with isopropanol followed by soapy water and rinsing, the cemented cover glass will serve for many months.

Objective lens selection is critically important in large-scale imaging of clarified specimens because of the importance of minimizing spherical aberration and the need for sufficient working distance. We have experience with three objectives in these studies. In the inverted microscope setup, we use a long working distance, moderate numerical aperture (NA) objective oil immersed below the cover glass with the biofilm immersed in methyl salicylate in the dish above the cover glass. Most of our work has been done with a Zeiss Universal series achromatic objective, type 461708, 40x 0.85NA Oel 160/1.5, used with a negative 160 mm focal length adapter for nominal infinite-conjugate (IC) compatibility. This objective originally was designed for oil immersed viewing through 1.5 mm microscope slides with 0.35 mm working distance²⁵. When used with a standard cover glass (0.17 mm), the working distance is much greater: $1.5 + 0.35 - 0.17 = 1.68 \text{ mm} = 1,680 \text{ }\mu\text{m}$. We also use a new Zeiss multi-immersion objective, type 420852, 25x 0.8NA LD LCI Plan-apochromat with a working distance exceeding 540 μm , with the immersion correction set to the high side of 'oil'. This objective is highly corrected and produces a superb image. On an upright microscope stand, we have used a new Nikon multi-immersion objective, type MRD71120, 10x 0.5NA CFI Plan-apochromat with a working distance exceeding 5,000 μm (5 mm), also with the immersion correction set to the high side of 'oil' ($n = 1.518$). Though this objective has a lower NA than the others, it has the advantage of being directly immersible in methyl salicylate.

To optimize data acquisition in terms of speed and resolution, confocal microscope settings ideally should meet both transverse and axial Nyquist sampling densities¹⁸. Using conventional criteria, set the confocal pinhole diameter nominally to 1 Airy unit. In magnified coordinates,

$$d_p (\mu\text{m}) = 1.22 \times \text{magnification} \times (\text{emission wavelength in } \mu\text{m}) / \text{NA}$$

Transverse sampling (pixel spacing) should not exceed $(1/2) \times \text{Abbe's resolution limit}$. In object-space coordinates,

$$\Delta x, \Delta y = (\text{emission wavelength in nm}) / (4 \text{ NA})$$

Axial sampling (focus increment) should not exceed the inverse axial bandwidth,

$$\Delta z = (\text{immersion index}) \times (\text{emission wavelength in nm}) / (\text{NA}^2)$$

The confocal optical system expands the bandwidth of the microscope and sharpens both the transverse and axial resolution by at least $1/\sqrt{2}$.

For the 40x 0.85 NA objective that we utilize most often, see **Table 1** for the results of these formulas for a 600 nm emission wavelength (Alexa Fluor 594).

	formula	$\times 1/\sqrt{2}$	set (typical)
$d_p (\mu\text{m})$	34.5 μm	-	25 or 50 μm
$\Delta x, \Delta y$	176 nm	125 nm	161 nm
Δz	1200 nm	848 nm	900 nm

Table 1: Confocal pinhole diameter, transverse sampling, and axial sampling values for a 600 nm emission wavelength.

Using a spinning-disk confocal scanner with these settings and a 1,392 x 1,040 pixel scan field, data from biofilm specimens can be acquired with reasonable speed and resolution. Typical single-color 3D image stacks run 0.35–1.55 GB.

Clarification media in wide use span a significant refractive index range. By darkfield illumination and visual inspection, we found that fixed *C. albicans* biofilms were most transparent above $n = 1.5$. This was refined by phase contrast microscopy to $n = 1.530$ – 1.535 . For a number of practical reasons, we use methyl salicylate ($n = 1.537$) as the final index matching solvent. Though solvent exchange brings the risk of specimen deformation or other artifacts, cells in fixed, clarified specimens have similar cell body dimensions, hypha diameter, and interseptal length dimensions as live specimens.

Many variations in the solvent exchange process are possible (e.g., using a different transitional solvent, or a different final solvent). Methanol was chosen for its high polarity and rapid diffusion, but ethanol was shown to better preserve red fluorescent protein (RFP) quantum yield²⁶ as a transitional solvent. Methyl salicylate was selected for its index, moderate polarity, low vapor pressure, and compatibility with many stains, dyes,

and fluorescent proteins. However, a final solvent with slightly lower index, or a mixture of methyl salicylate and a lower index solvent, such as butanol, may serve better.

Unexpectedly, the ability to see through a biofilm reveals not only its stratified internal features, but also aids in viewing the origin of extended structures such as long, unentangled apical hyphae and invasive basal hyphae on certain substrata. Opportunistic virulence in *C. albicans* depends on its genetic versatility (i.e., switchover to hyphal growth, upregulation of cell substratum and cell-cell adherence, generation of osmolytes for cell budding and elongation, and use of alternative nutrients). Hyphal extension enables breakout of individual *C. albicans* cells from phagocytic immune cells but also is essential for invasion. Biofilm adhesion and hyphal entanglement appear to provide the surface anchoring needed for hyphae to efficiently invade a substratum. When that substratum is host tissue, increased virulence may result.

Imaging intact biofilms enables a vast number of informative experiments utilizing reporter strains for gene expression, model tissue substrata, and the inclusion of other organisms such as bacteria found in natural biofilms. Even in the simplest case of purely structural imaging with a cell wall stain, in situ phenotypes are revealed that then may be quantified and genetically analyzed.

Disclosures

The authors have no competing interests to disclose.

Acknowledgments

This research was supported in part by NIH grants R01 AI067703, R21 AI100270, and R21 AI135178 to A.P. Mitchell. The authors are grateful to Greenfield 'Kip' Sluder for providing the long working-distance oil immersion objective used in this work, and to Daniel Shiwerski for confocal microscopy with direct methyl salicylate immersion.

References

- Desai, J. V. et al. Coordination of *Candida albicans* invasion and infection functions by phosphoglycerol phosphatase Rh2. *Pathogens*. **4**, 573-589 (2015).
- Miramón, P., Lorenz, M. C. A feast for *Candida*: Metabolic plasticity confers an edge for virulence. *PLoS Pathogens*. **13**(2), e1006144 (2017).
- Bonhomme, J. et al. Contribution of the glycolytic flux and hypoxia adaptation to efficient biofilm formation by *Candida albicans*. *Molecular Microbiology*. **80**, 995-1013 (2011).
- Ramírez-Zavala, B. et al. The Snf1-activating kinase Sak1 is a key regulator of metabolic adaptation and in vivo fitness of *Candida albicans*. *Molecular Microbiology*. **104**, 989-1007 (2017).
- Westman, J., Moran, G., Mogavero, S., Hube, B., Grinstein, S. *Candida albicans* hyphal expansion causes phagosomal membrane damage and luminal alkalization. *mBio*. **9**, e01226-18 (2018).
- Finkel, J. S., Mitchell, A. P. Genetic control of *Candida albicans* biofilm development. *Nature Reviews Microbiology*. **9**, 109-118 (2011).
- Finkel, J. S. et al. Portrait of *Candida albicans* adherence regulators. *PLoS Pathogens*. **8**, e1002525 (2012).
- de Groot, P. W., Bader, O., de Boer, A. D., Weig, M., Chauhan, N. Adhesins in human fungal pathogens: glue with plenty of stick. *Eukaryotic Cell*. **12**, 470-81 (2013).
- Lagree, K., Mitchell, A. P. Fungal Biofilms: Inside Out. *Microbiology Spectrum*. **5** (2017).
- Hawser, S. P., Douglas, L. J. Resistance of *Candida albicans* biofilms to antifungal agents in vitro. *Antimicrobial Agents and Chemotherapy*. **39**, 2128-2131 (1995).
- Chandra, J. et al. Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *Journal of Bacteriology*. **183**, 5385-5394 (2001).
- LaFleur, M. D., Kumamoto, C. A., Lewis, K. *Candida albicans* biofilms produce antifungal-tolerant persister cells. *Antimicrobial Agents and Chemotherapy*. **50**, 3839-3846 (2006).
- Lagree, K., Desai, J. V., Finkel, J. S., Lanni, F. Microscopy of fungal biofilms. *Current Opinion in Microbiology*. **43**, 100-107 (2018).
- Woolford, C. A. et al. Bypass of *Candida albicans* filamentation/biofilm regulators through diminished expression of protein kinase Cak1. *PLoS Genetics*. **12**, e1006487 (2016).
- Huang, M. Y., Woolford, C. A., May, G., McManus, C. J., Mitchell, A. P. Circuit diversification in a biofilm regulatory network. *PLoS Pathogens*. **15**(5), e1007787 (2019).
- Ganguly, S. et al. Zap1 control of cell-cell signaling in *Candida albicans* biofilms. *Eukaryotic Cell*. **10**, 1448-1454 (2011).
- Friskin, G. S., Lanni, F. Experimental test of an analytical model of aberration in an oil-immersion objective lens used in three-dimensional light microscopy. *Journal of the Optical Society of America*. **8**, 1601-1613 (1991). Reprinted in **9**, 154-166 (1992).
- Lanni, F., Keller, H. E. Microscope principles and optical systems. In *Imaging, A Laboratory Manual*. Edited by Yuste RM. Cold Spring Harbor Laboratory Press pp. 1-55. Chapter 1. (2011).
- Chung, K. et al. Structural and molecular interrogation of intact biological systems. *Nature*. **497**, 332-337 (CLARITY) (2013).
- Tomer, R., Ye, L., Hsueh, B., Deisseroth, K. Advanced CLARITY for rapid and high-resolution imaging of intact tissues. *Nature Protocols*. **9**, 1682-1697 (2014).
- Richardson, D. S., Lichtman, J. W. Clarifying tissue clearing. *Cell*. **162**, 246-257 (2015).
- Chen, F., Tillberg, P. W., Boyden, E. S. Expansion microscopy. *Science*. **347**, 543-548 (2015).
- Zhao, Y. et al. Nanoscale imaging of clinical specimens using pathology-optimized expansion microscopy. *Nature Biotechnology*. **35**, 757-764 (2017).
- Gao, R. et al. Cortical column and whole brain imaging with molecular contrast and nanoscale resolution. *Science*. **363**, eaau8302 (2019).
- "Optical Systems for the Microscope" publication 41-101-e (archive CZO-Mi 823, ed. 1967), CARL ZEISS. 7082 Oberkochen, West Germany page 48. (1971).

26. Oldham, M., Sakhalkar, H., Oliver, T., Johnson, G. A., Dewhirst, M. Optical clearing of unsectioned specimens for three-dimensional imaging via optical transmission and emission tomography. *Journal of Biomedical Optics*. **13**(2), 021113 (2008).