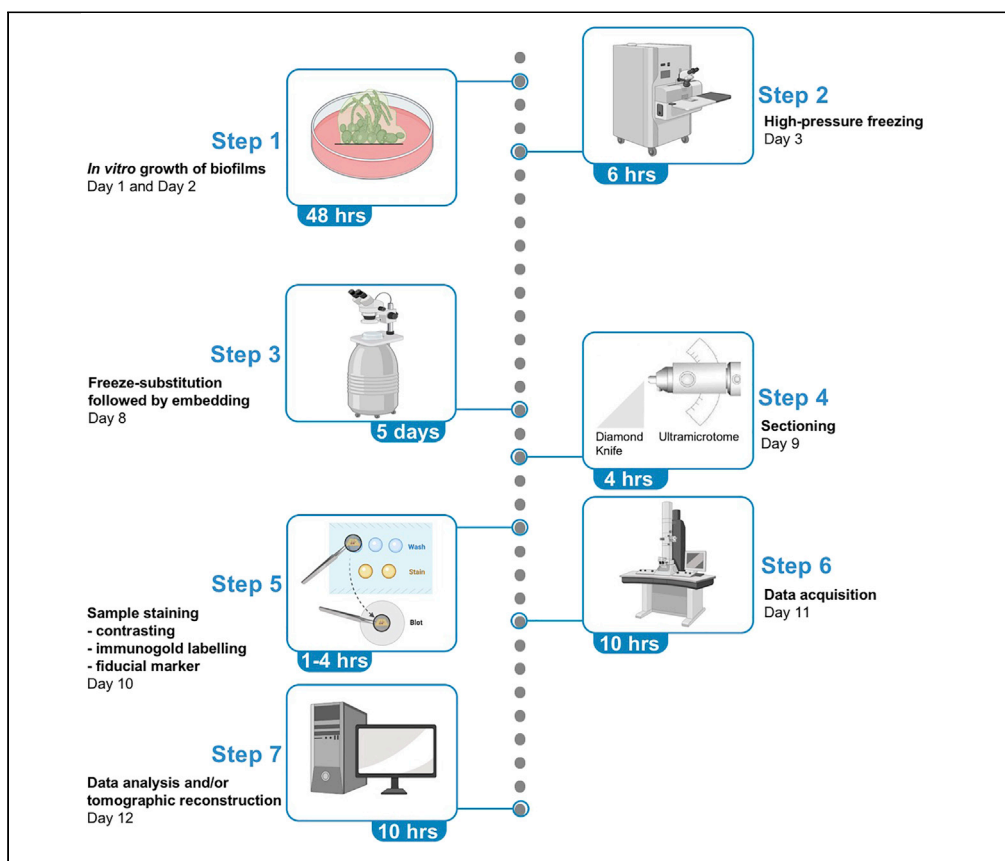


Protocol

A protocol for ultrastructural study of *Candida albicans* biofilm using transmission electron microscopy



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Highlights

A protocol to study the ultrastructure of unperturbed biofilms of *C. albicans*

Suitable for contrasting, immunogold labeling, and electron tomography

Maintain cellular junctions between fungal cells from the microbial community

The protocol may be applicable to other microorganisms able to form biofilms

This protocol describes how to analyze *C. albicans* biofilm using transmission electron microscopy. We present two approaches to observe the ultrastructure of fungal cells within unperturbed biofilms, as well as an immunogold labeling procedure. This approach maintains the architecture of the fungal biofilm close to its native state by growing *C. albicans* biofilm on a plastic surface. After the freeze substitution procedure, classical transmission electron microscopy or electron tomography will allow the ultrastructural analysis of the microbial community.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol

A protocol for ultrastructural study of *Candida albicans* biofilm using transmission electron microscopy

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SUMMARY

This protocol describes how to analyze *C. albicans* biofilm using transmission electron microscopy. We present two approaches to observe the ultrastructure of fungal cells within unperturbed biofilms, as well as an immunogold labeling procedure. This approach maintains the architecture of the fungal biofilm close to its native state by growing *C. albicans* biofilm on a plastic surface. After the freeze substitution procedure, classical transmission electron microscopy or electron tomography will allow the ultrastructural analysis of the microbial community.

BEFORE YOU BEGIN

State of art and usefulness of the protocol for the community: The human commensal fungus *Candida albicans* can, under some conditions, cross the digestive mucosa, disseminate into the bloodstream, and cause invasive candidiasis (Miranda et al., 2009). This yeast can also form structured communities, namely biofilms, on epithelia or indwelling medical devices (Lotte and Van Dijck, 2013). *C. albicans* is thus a huge burden for healthcare systems, worsened by the fact that biofilms are strongly resistant to classical antifungal drugs and can also evade the immune system (Brown et al., 2012). Electron microscopy (EM) has a strong potential to reveal the ultrastructure of *C. albicans* within biofilms and help understand how cells from these microbial communities interact with each other. Most EM studies performed so far on *C. albicans* biofilms used scanning electron microscopy (SEM) (de Barros et al., 2020), a technique that gives information about the surface of *C. albicans* cells encased within the biofilm and the extracellular matrix. However, SEM images do not provide information about the ultrastructure of cellular compartments, including the cell wall. Transmission electron microscopy has already been used to observe *C. albicans* biofilms (Taff and Andes, 2013). However, the protocol used to prepare samples in this study displayed two possible drawbacks. First, biofilm cells are mechanically separated which can lead to dramatic damages of the cell wall ultrastructure. Moreover, the physical interactions between cells are also disrupted (Taff and Andes, 2013). Second, authors used ambient-temperature dehydration that can cause severe alterations of cellular ultrastructure (Taff and Andes, 2013; Al-Amoudi et al., 2004). To circumvent these issues, we have developed a protocol that preserves contacts between fungal cells within the biofilm, as well as their cellular architecture. Indeed, here the entire *C. albicans* biofilm is frozen without any mechanical disruption. Biofilms of *C. albicans* are grown in RPMI medium on an Aclar film that is next transferred within a sample carrier to be submitted to high pressure freezing. During this step, cells are not dissociated from each other, and integrity of the biofilm is kept intact. Subsequently, water molecules are replaced from samples by a solvent containing chemical fixative at low



temperature using a freeze-substitution procedure. *C. albicans* biofilms are then embedded in a resin before being warmed up at room temperature. Finally, biofilms are sectioned in various thickness according to the observation method to be used: contrasting, immunogold labeling or electron tomography. Regarding the immunogold labeling procedure, we used a strain that expresses a GFP-tagged version of Pga62, a protein known to be a resident of the cell wall in *C. albicans* (Moreno-Ruiz et al., 2009). The present protocol is particularly well-suited to study the ultrastructure of the cell wall of *C. albicans* cells assembled as a biofilm and allows the observation of other organelles; it is thus a significant improvement to study and visualize the ultrastructure of *C. albicans* cells encased in intact biofilms using transmission electron microscopes.

Growth of *C. albicans* biofilms on an Aclar film

⌚ Timing: 3 days

Before starting sample preparation for transmission electron microscopy, fungal biofilms are grown on an Aclar film (plastic surface). *C. albicans* biofilms should be prepared as described below:

1. Inoculate a colony of *C. albicans* from a Petri dish in 4 mL of YPD medium and incubate overnight at 30°C.
2. Immerse the Aclar film in a freshly prepared 0.1% poly-L-lysine solution for 30 min at 37°C. Then, wash the Aclar film 2 times with distilled water and cut it in small squares (1 × 1 cm) that will fit in the wells of a 12-well tissue culture plate. Sterilize the pieces under UV light for 15 min.

Note: The Aclar film squares can be stored for several months under sterile conditions at room temperature.

3. Wash the preculture of *C. albicans* cells twice with sterile PBS and dilute the cells in prewarmed RPMI medium (37°C) at a cell density of 1×10^6 cells/mL.
4. Place Aclar film squares at the bottom of wells from a 12-well tissue culture plate. Fill up each well with 3 mL of RPMI medium containing *C. albicans* cells.
5. Incubate the culture plates for 1 h at 37°C under gentle shaking (110 rpm) to allow fungal cells to adhere to the Aclar film. Subsequently, wash the wells with PBS to remove non-adherent cells, and fill up with 3 mL of RPMI medium.
6. Incubate the culture plates at 37°C for 48 h under gentle shaking (110 rpm).
7. Mature *C. albicans* biofilms are fixed 1 h at room temperature with 3 mL of PHEM buffer (60 mM PIPES, 50 mM HEPES, 10 mM EGTA, 2 mM MgCl₂ pH7.2) containing 2% formaldehyde and 0.2% of glutaraldehyde.

Optional: After 48 h of cultivation, the structure of the biofilm could be verified by CLSM microscopy (Figure 1). To do so, wash the biofilms grown on the Aclar film twice with PBS and subsequently stain them with 3 mL of Concanavalin A, Alexa Fluor 594 Conjugate (50 µg/mL in PBS) at 37°C for 1 h. Then, wash the biofilms with PBS before observation under an upright confocal microscope using a dipping objective (red channel). The protocol for CLSM microscopy on *C. albicans* biofilm was described in Rai et al. (2019).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-GFP (clone B-2) Working dilution: 1/200	Santa Cruz Biotechnology	Cat#sc-9996

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Dulbecco's phosphate-buffered saline (PBS)	Fisher Scientific	Cat#11590476
Gibco™ RPMI 1640 medium	Fisher Scientific	Cat#10379144
Aclar® Embedding Film	TED PELLA, INC.	Cat#10501-10
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat#A7906-50G
Protein A-Gold 10 nm particle	Utrecht University	N/A
Ethanol absolute	Merck	Cat#1009831011
Lowicryl HM20 Embedding medium	Electron Microscopy Sciences	Cat#14330
Sodium hydroxide pellets	Sigma-Aldrich	Cat#567530-250GM
Acetone	Sigma-Aldrich	Cat#179124-500ML
Lead citrate	Delta Microscopies	Cat#11300
Uranyl acetate	Electron microscopy Sciences	Cat#22400-25G
HEPES buffer	Fisher Scientific	Cat#BP299100
PIPES	Sigma-Aldrich	Cat#P6757-25G
Glutaraldehyde solution	Sigma-Aldrich	Cat#G5882
Formaldehyde solution	Sigma-Aldrich	Cat#F8775
Poly-L-lysine solution	Sigma-Aldrich	Cat#8920
Methanol	Fisher Scientific	Cat#15263498
1-hexadecene	Sigma-Aldrich	Cat#8220640100
Concanavaline A, Alexa Fluor™ 594 Conjugate	Thermo Fisher Scientific	Cat#C11253
Toluidine blue O	Sigma-Aldrich	Cat#198161-5G
Sodium tetraborate decahydrate	Sigma-Aldrich	Cat#198161-5G
MgCl ₂	Sigma-Aldrich	Cat#208337-100G
EGTA	Sigma-Aldrich	Cat#E4378
Ammonium chloride	Sigma-Aldrich	Cat#213330-25G
Experimental models: Organisms/strains		
<i>Candida albicans</i> BWP17	Wilson et al., 1999	N/A
CEC65 (BWP17 PGA62/PGA62:::(PGA62-GFP, ARG4))	The d'Enfert Laboratory/ Institut Pasteur	Moreno-Ruiz et al., 2009
Software and algorithms		
Serial EM	Mastrorade, 2005	https://bio3d.colorado.edu/SerialEM/
IMOD version 4.9	Mastrorade and Held, 2017	https://bio3d.colorado.edu/imod/doc/etomoTutorial.html
Other		
FEI Tecnai T12 120 kV Transmission Electron Microscope equipped with a Gatan Ultrascan US4000 CCD detector	FEI Company	Cat#D2216
FEI Tecnai F20 200 kV Transmission Electron Microscope equipped with a Gatan Ultrascan US4000 CCD detector	FEI Company	Cat#D1283
Leica DMC 2900 binoculars	Leica	Cat#12730466
Leica EM ICE High Pressure Freezer	Leica	Cat#16771801
Leica EM AFS2 for Freeze-substitution and Low Temperature Embedding System for Light and Electron microscopy	Leica	Cat#16707101
Flow-through ring	Leica	Cat#16707157
6 mm sample carriers for high-pressure freezing Type A	Leica	Cat#16770181
6 mm sample carriers for high-pressure freezing Type B	Leica	Cat#16770182
Leica EM UC7 ultramicrotome	Leica	Cat#16705805
Diamond knife for 70 nm sections	Drukker International	Cat#45273
Diamond knife for 150 nm sections	DiATOME	Cat#HI 4352
Heat block at 37°C	Major Science	Cat#MD-01N
Microcentrifuge	Eppendorf	Cat#Z606235
Biological safety cabinet	TechGen	Cat# ESB-4
37°C humid chamber	Memmert	Cat#UN30
12 wells tissue culture plates TPP	Dutscher	Cat#092412B
Formvar/Carbon Supported Copper Grids size 200 mesh	Delta Microscopies	Cat#FCF200-CU-50

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Molecular sieve 4 Å	VWR	Cat#70955-01-0
Dumont tweezers type 5	Delta Microscopies	Cat#72877
Whatman filter paper grade 4	Sigma-Aldrich	Cat#WHA1004125

STEP-BY-STEP METHOD DETAILS

High-pressure freezing and freeze-substitution of *C. albicans* biofilms

⌚ Timing: 5 days

1. Place the chemically fixed biofilms of *C. albicans* in a Petri dish and cut them in 4 × 4 mm squares with a scalpel.
2. Insert the biofilm square in the 200 μm cavity of a type A sample carrier filled with PBS and cover with the type B carrier to close the sandwich. Freeze with a Leica EM ICE high pressure freezer.

Note: Biofilms of *C. albicans* cultured on Aclar films are deposited within the type A sample carrier and oriented toward the type B sample carrier. Also, coat the flat part of the 6 mm type B sample carrier with 1-hexadecene to prevent *C. albicans* from sticking (Figure 2).

3. Transfer the type A sample carrier within a cryotube and store in a liquid nitrogen dewar.

Note: the type A sample carrier contains the frozen biofilm of *C. albicans* on the Aclar film.

All steps of the freeze-substitution are performed in the tank of the Leica EM AFS2 apparatus (steps #4 to #9). See Figure 2.

Note: Plastic Pasteur pipettes are used to change buffers along the freeze-substitution procedure by using the middle hole of the flow-through ring.

4. Transfer biofilms from cryotubes to a flow-through ring that contains 10 mL of a solution of acetone/0.2% uranyl acetate/2% methanol and incubate the samples at −90°C for 2 h (Figure 3).

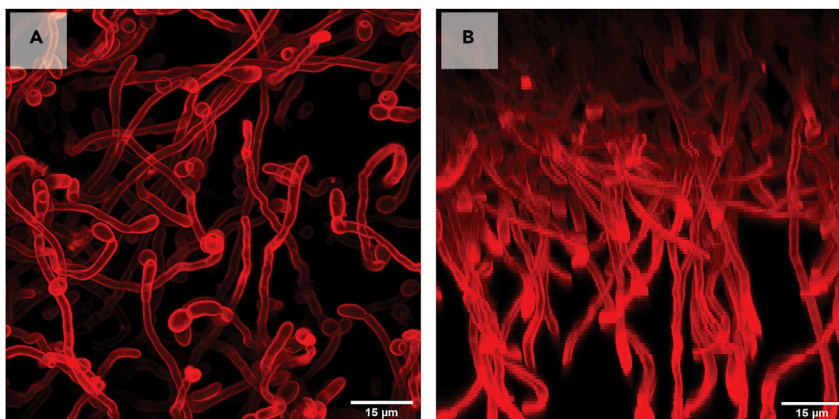


Figure 1. CLSM microscopy on *C. albicans* biofilm grown on Aclar film

A *C. albicans* biofilm was grown on an Aclar film for 48 h and subsequently treated with Concanavalin A, Alexa Fluor 594 Conjugate. Structure of the fungal biofilm was recorded by using confocal laser scanning microscopy. (A and B) Top view (A) and side view (B) of the biofilm are presented here. The scale bars are represented as white lines.

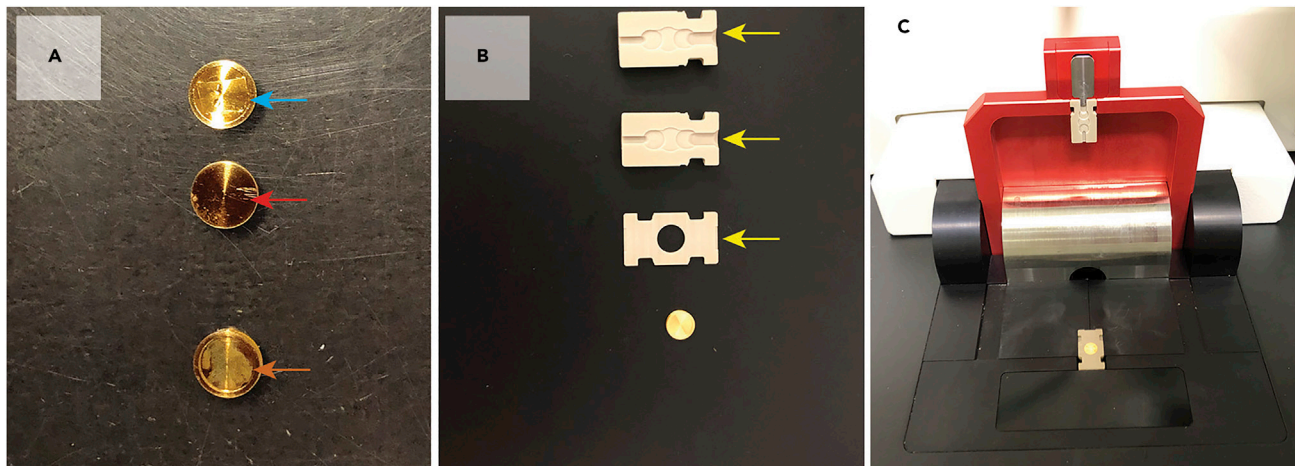


Figure 2. Pictures summarizing the different steps of high pressure freezing

(A) Type A sample carrier containing *C. albicans* biofilm on the Aclar film (blue arrow). The type B sample carrier is shown with the red arrow. The assembly of both sample carriers is pinpointed with the orange arrow.

(B) Plastic half cylinders (top and middle) and middle plate (bottom) used to transit samples in the high-pressure freezer are indicated with yellow arrows. The sample carrier is shown underneath.

(C) Picture showing the assembly of plastic half cylinders and the sample carrier on the high-pressure freezer.

Note: the tweezers as well as the solution (acetone, uranyl acetate and methanol) should be precooled at -90°C .

5. Then, heat up biofilms to -50°C (the slope is 20°C per hour). From this stage, samples are kept at -50°C until the second step of UV polymerization (step #9).
6. Wash frozen biofilms every 20 min with 100% ethanol, cooled at -50°C , for 1 h.

Note: Ethanol used for the freeze-substitution procedure (either in washing steps or to dilute the HM20 resin) needs to be treated with molecular sieves before use to remove water molecules. The presence of water molecules in ethanol would compromise the freeze-substitution, and subsequently negatively impact the ultrastructure of fungal biofilms.

7. Infiltrate samples with several solutions of Lowicryl HM20 resin following the order below:
 - a. 75% ethanol/25% HM20 for 2 h.
 - b. 50% ethanol/50% HM20 for 16 h.
 - c. 25% ethanol/75% HM20 for 2 h.
 - d. 100% HM20 for 2 h.
 - e. 100% HM20 for 2 h.
 - f. 100% HM20 for 16 h.
 - g. 100% HM20 for 4 h.

Note: The solutions of resin correspond to a mix of ethanol and HM20 that follows specific ratios over time. To ensure good infiltration, pure HM20 needs to be replaced at each step.

8. After the final incubation with 100% HM20, place samples within the AFS2 apparatus under UV light at -50°C for 44 h to allow resin polymerization.
9. During the HM20 polymerization under UV light, set up a temperature increase from -50°C to 20°C with a slope of 10°C per hour.
10. After reaching the temperature of 20°C , the HM20 polymerization could be continued under direct sunlight for several hours.

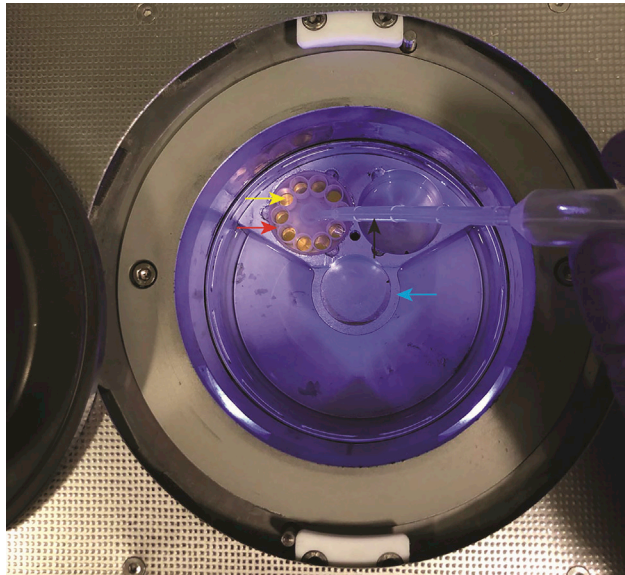


Figure 3. Picture of the AFS2 apparatus tank during the freeze-substitution procedure

The tank is partially filled with 100% ethanol. The flow-through ring (10 positions) containing the lower part of the different sample carriers is placed on a metal stand. Arrows use the following color code: blue (metal stand), red (Flow-through ring), yellow (sample carrier) and black (plastic Pasteur pipette).

⚠ **CRITICAL:** Samples must be kept at -196°C (liquid nitrogen temperature) until their transfer within the acetone solution. Any premature warming of the sample may lead to ice crystal formation and induce severe alterations of biofilm ultrastructure (step 4).

Cut samples with an ultramicrotome and arrange the sections on electron microscopy grids

⌚ Timing: 4 h

11. Remove the type B sample carrier and the Aclar film from the polymerized HM20 resin block (Figure 4A).
12. Under a binocular microscope, localize the area containing *C. albicans* cells and trim the block of HM20 as a thin triangle with a razorblade.
13. For tomography experiments, cut sections of 150 nm with an ultramicrotome. For immunogold labeling and contrasting, use sections of 70 nm.

Note: Sections of 150 nm and 70 nm are done with different diamond knives to avoid any damage to the knives.

14. Add a droplet of the toluidine blue solution stain on each section and incubate on a heat block for 5 min, then wash thoroughly with distilled water (Figure 4D).

Note: The toluidine blue staining is used to check the presence of *C. albicans* cells in the sections. To do so, place some sections on a microscope glass slide before staining them with the toluidine blue. Subsequently, observe the sections under a light microscope.

15. After checking for the presence of *C. albicans* biofilm cells, place the following sections (unstained) on a copper grid for TEM analysis.

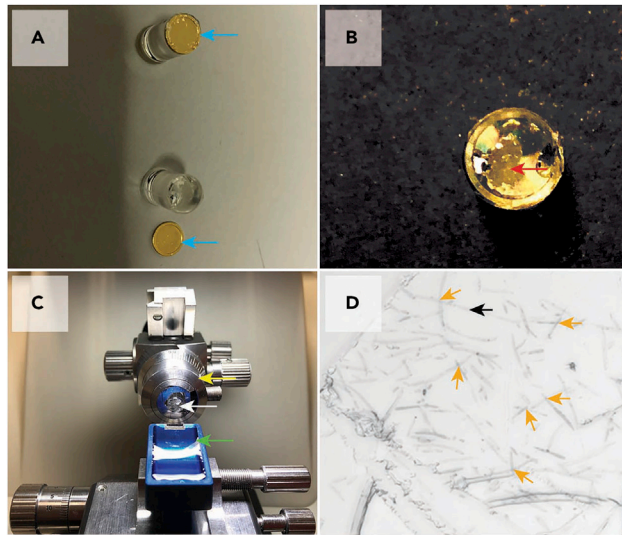


Figure 4. Steps to perform thin sections of *C. albicans* biofilm with an ultramicrotome for TEM analysis

(A) Polymerized HM20 resin block before (top) or after (bottom) removing of the type B sample carrier (blue arrow). (B) Picture showing *C. albicans* biofilm within the HM20 resin. The area containing *C. albicans* cells is indicated with a red arrow.

(C) Picture of the Leica EM UC7 ultramicrotome during the sectioning process. The diamond knife is shown with a green arrow while the ultramicrotome cutting arm is pinpointed with a yellow arrow. The sample is placed on the cutting arm as indicated by the white arrow.

(D) Toluidine blue staining of a plastic section (150 nm) which contains *C. albicans* biofilm. A fungal cell growing as a filament is indicated with a black arrow. Contacts between *C. albicans* cells are indicated with orange arrows.

△ **CRITICAL:** The area containing fungal cells within the HM20 resin looks whitish due to a very high cellular density. Sections should be made in this area. Polymerized HM20 resin without cells is translucent (step 12).

Sample preparation for TEM observations

⌚ Timing: 4 h

16. Immunogold labeling.

Immunogold labeling will be used to detect specific antigens in samples with a transmission electron microscope. The protein of interest can be fused to a tag that will be used as a target for the primary antibody. Subsequently, protein A-gold particles will specifically recognize the primary antibody to generate the complex antibody:protein A-gold particle. The gold particle will create a highly dense area toward electrons that will appear as a black dot (the size of the gold particle) on the phosphoscreen of an electron microscope. Thus, antigens of interest will be specifically pinpointed on the sections with a black dot.

All the following steps are performed at room temperature, and the droplets are deposited at the surface of sealing film (such as Parafilm®).

- For each grid, prepare 5 droplets of PBS 50 mM NH_4Cl on Parafilm®. Wash the grids in the droplets for 2 min. Repeat this step 4 times.
- Incubate each grid in a droplet of blocking solution (PBS BSA 1%) for 5 min.
- Incubate grids on 10 μL -droplets containing the primary antibody for 1 h at room temperature.

Note: For each grid, prepare a droplet of PBS BSA 1% that contains the primary antibody properly diluted. In this work we used an α -GFP antibody diluted 200 times.

- d. Wash the grids in 10 μ L-droplets of PBS BSA 0.1%. Repeat this operation 4 times.
- e. Wash the grids in 10 μ L-droplets of PBS. Repeat 4 times.
- f. Prepare one 10 μ L-droplet of PBS BSA 1% containing protein A conjugated to 10 nm gold particles (working dilution 1/50) per grid. Incubate the grids in the mixture for 20 min.

Note: Protein A-Gold particles tend to aggregate. Hence, do not incubate the sections more than 20 min with protein A-Gold particles mixture to avoid particles clustering.

- g. Wash the grids in droplets of PBS. Repeat this operation 5 times.
- h. Incubate the grids in droplets of PBS 1% glutaraldehyde for 5 min.
- i. Finally, wash the grids in droplets of water. Repeat the washes 8 times.
- j. Before being observed with a TEM microscope, the grids need to be contrasted. Contrasting should be done directly after the washes.

△ CRITICAL: To ensure immunogold labeling specificity, working dilution of the primary antibody needs to be carefully assessed (step 16.c). See [problem 2 in the troubleshooting section](#) for additional assistance.

17. Contrasting protocol.

Contrasting can also be used alone, without immunostaining, to increase contrast on biological sections. To do so, uranyl acetate and lead citrate are added onto the sections. This will result in an increased scattering of electrons from the heavy atoms absorbed from the sample, and therefore a better contrast.

- a. Incubate the grids in droplets of 4% uranyl acetate for 30 min.

Note: Tweezers used for the contrasting protocol need to be sonicated in a water bath before use. This will remove any uranyl acetate from the tweezers and avoid a contamination of the samples with uranyl acetate aggregates.

- b. Wash 5 times in droplets of water.
- c. Blot away the excess of water from the grids on a Whatman filter paper.
- d. Incubate the grids in droplets of 2% lead citrate for 2 min.

Note: Lead citrate tends to precipitate in the presence of CO₂. To avoid this precipitation, place sodium hydroxide pellets in close vicinity to lead citrate droplets. This is sufficient to remove the surrounding CO₂ from the lead citrate droplets and avoid precipitation.

- e. Wash 5 times in droplets of water.
 - f. Blot away the excess water from the grids on a grade 4 Whatman filter paper.
 - g. Allow the grids to air dry for at least 24 h on a filter paper at room temperature.
- #### 18. Sample preparation for electron tomography.
- a. Prepare a fresh aliquot of protein A-Gold particle (10 nm in diameter) diluted 30 times in water. Here, protein A-Gold particle is used as a fiducial marker.

Note: The three-dimensional tomographic volume of samples is reconstructed based on a tilt series of projection images recorded by TEM. In this case protein A-Gold particles are not used for immunostaining but rather as fiducial markers. The fiducial markers are used to accurately align/register the projection images of the tilt series before the actual tomographic reconstruction step takes place.

- b. Prepare two 100 μ L-droplets of diluted protein A-Gold particle per grid to be imaged using electron tomography.

- c. Incubate each grid in the first droplet of protein A-Gold particle with section oriented toward the mixture ("face up" toward the surface of the drop) and incubate for 10 min.

Note: The section is attached on the treated side with the formvar/carbon of the grid. This side is called "face up". The other side of the grid, that is not treated with formvar/carbon, is called "face down". The "face up" side of the grid looks dull under visible light, while the "face down" side is shiny. The fiducial marker is mandatory on both side of the grid. Hence, each side of the grid is soaked in the protein A-Gold particle mixture.

- d. Wash the grids in a water drop and blot immediately on a Whatman filter paper.
- e. Subsequently, immerse each grid in the second droplet of protein A-Gold particle with section oriented toward the air ("face down" toward surface of the drop) and incubate for 10 min.
- f. Wash the grids in a water drop and blot immediately on a Whatman filter paper.
- g. Incubate the grids with sections facing down in 4% uranyl acetate droplets for 30 min.
- h. Wash the grids 5 times in droplets of water.
- i. Incubate the grids with sections facing down in 2% lead citrate droplets for 2 min.
- j. Wash 5 times in droplets of water and blot away the excess of water with a Whatman filter paper.
- k. Let the grids dry at room temperature for 16 h before performing electron tomography.

Data acquisition with transmission electron microscopes

Here we describe the basic steps to record tilt series with a transmission electron microscope. Because the use of transmission electron microscopes is particularly challenging, especially for users who are not familiar with electron microscopy, it is strongly suggested to collaborate with TEM specialists.

19. Fill up the cryo-trap dewar of the Tecnai microscope with liquid nitrogen allowing the temperature of the cryo-trap to go down to -196°C .

Note: At -196°C , the cryo-trap will catch any sample contamination from remaining dirt within the column of the microscope.

20. Mount the grid on the sample holder.

Note: Usually sample holders for electron tomography are allowing tilt series acquisition with a tilt angle range of $\pm 70^{\circ}$.

21. Insert the sample holder in the stage of the electron microscope.

Note: Make sure that column valves are closed to not disrupt the vacuum of the microscope. Users should also verify that both the objective aperture and the cryo-box, which are 2 components of the electron microscope, are not inserted.

22. Open the SerialEM software which will provide an interface to record micrographs, as well as acquiring tilt series, of fungal biofilms.

Note: Detailed instructions on how to use SerialEM are available on the following websites:

- a. <https://bio3d.colorado.edu/SerialEM/>.
- b. https://www.youtube.com/results?search_query=SerialEM.

23. Center the condenser aperture and bring the sample to eucentric height.
24. Collect a Full Grid Map (FGM) at low magnification (150 \times) using SerialEM.

25. Insert and center the objective aperture.
26. Find an area with fungal biofilm on the FGM and collect a Middle Magnification Map (MMM) at 2500 \times .
27. Add a marker (point) on every area of interest suitable for data acquisition on the MMM using SerialEM's navigator (Add Points).

Note: An area suitable for data acquisition is characterized mainly by preserved fungal ultrastructure. Also, the contrast in this area should be sufficient to observe ultrastructures of interest and acquire data.

28. Acquire a tilt series of every area designated with a marker on the MMM.

Note: Before each data acquisition, users must perform the following routine: (i) cook the sample by sending a total dose of 1500 electrons per square Ångström, (ii) refine eucentricity, (iii) autofocus, (iv) raise the magnification to 49,000 \times and (v) launch tilt series acquisition. Repeat these 5 steps for every point of interest found on the MMM.

29. For dual axis tomography, close the valves and take the sample holder out. Rotate the grid clockwise manually through an angle of 90° and put back the sample holder in the stage of the electron microscope.
30. Redo steps 23 and 25.

Note: The second time you are inserting the grid you have to increase the stage registration in number 2 on the Navigator. Collect a new FGM in this registration and use registration points between the new and old FGM maps to shift the previous coordinates to the new stage coordinates. There is no need to redo the MMM. The shifted coordinates will be used to collect tilt series at the same area.

31. Collect new tilt series in the same areas of interest as in step 28.

Note: Tilt series acquisition could be done either one-by-one or in batch. For the batch processes, users should create an anchor map to avoid losing the field of view during the serial imaging. Subsequently, set up the routine as described in the note (step 28) and start tilt series acquisition.

△ CRITICAL: To record classical TEM micrographs (contrasting and immunogold labeling), it is not required to produce FGM, MMM and collect tilt series. User should follow the following procedure: (i) mount the grid in the sample holder, (ii) insert the sample holder in the stage of the microscope, (iii) open the valves, (iv) center condenser aperture and bring the sample to eucentric height, (v) center the objective aperture, (vi) go to the appropriate magnification and (vii) record micrographs with SerialEM.

Tomographic reconstruction

⌚ Timing: 10 h

Tomographic reconstruction is performed with the IMOD version 4.9 software (Mastrorarde and Held, 2017). The fully detailed procedure is available online at the following address: <https://bio3d.colorado.edu/imod/doc/etomoTutorial.html>.

Here we cite the major steps allowing tomographic reconstruction for dual-axis tomography performed on *C. albicans* biofilms. For dual-axis tomography, tilt series should be recorded with two perpendicular tilt axes called A- and B-axis.

Moreover, tomographic reconstruction is also a complicated process that could require the help of specialists of image processing.

32. Start the tomographic reconstruction by setting up ETomo which is the graphical user interface to the IMOD package.

Note: To do so, users should sequentially enter the following information: (i) dataset name, (ii) indicate that tilt series were performed on plastic sections, (iii) select dual axis tomography, (iv) enter the size of the fiducial marker (10 nm) and (v) tick the box “extract tilt angles from data”. Then, press the “Create Com Scripts” to open the ETomo main window. Every step used by IMOD for tomographic reconstruction is displayed under the form of clickable sections on the left part of ETomo main window.

33. Go to the pre-processing section and click sequentially on “Create Fixed Stack” and the “Use Fixed Stack” buttons.

Note: The first step to build up tomograms is the pre-processing procedure. To avoid contrast problems or artifacts in the tomograms, the pre-processing will remove extreme pixel values in the tilt series.

34. Go to the coarse alignment section and click on the “Calculate Cross-Correlation” button. Then, press the “Generate Coarse Aligned Stack” button.

Note: This step will create an alignment between consecutive images coming from the tilt series by generating a coarse-aligned stack. Pressing the “Calculate Cross-Correlation” button will launch the tiltcorr program that will use cross-correlation to calculate XY shifts between successive images of the tilt series. For cross-correlation to take place the program first stretches the image with the larger tilt angle perpendicular to the tilt axis. The resulting file consists of a list of calculated shifts which will be applied to the dataset in the next step. Xftoxg will run once users will press the “Generate Coarse Aligned Stack” button. Xftoxg will use the output of tiltcorr to compute a global set of alignments. Then, the program Newstack will apply the global set to the tilt series and generate a new image stack that the user is able to observe by pressing the “View Aligned Stack In 3dmod” button.

35. Generate a seed model on the zero-angle tilt micrograph based on gold particles positioning and then, track the gold particles on all other sections of the tilt series to generate the fiducial model.

Note: The algorithm that uses the seed model to track beads in all other sections of the tilt series can fail in cases of bad contrast or if the beads are outside the field of view in particular tilt angles. This will generate gaps in the fiducial model which can be added manually with the bead fixer. It is important to mention that an accurate fiducial model is mandatory to complete the next step of tomographic reconstruction namely the “fine alignment”.

36. Refine gold particle tracking with the help of residual errors in every section of the tilt series.

Note: The refinement of gold particles throughout all sections performed by the tiltalign program will not be perfect. Some gold particles will have to be repositioned from one section to another. Users should correct this displacement manually with the bead fixer.

37. Sample three regions of the tomogram (top, middle, and bottom parts of the tilt series) by pressing “Create Sample Tomograms”. Then add the top and bottom surfaces from every

reconstruction by pressing the “Create Boundary Models”. Subsequently, press on “Compute Z shift and Pitch Angles” button.

Note: This step is to fit the reconstruction into the smallest volume possible (Tomogram positioning section). Pressing the “Compute Z shift and Pitch Angles” button will allow tomopitch to reconstruct the flattest possible tomogram and fit it in the smallest volume possible.

38. Generate the tomogram of the axis A using Weighted Back Projection (WBP).

Because we used dual-axis tomography, users should now reconstruct the tomogram corresponding to the second axis (B axis).

39. Start the reconstruction of the B axis by doing the pre-processing and the coarse alignment on the dataset corresponding to the axis B as you did for axis A.
40. By using transferfid (press the “Transfer Fiducials From Other Axis” button), create a seed model for the B axis based on the fiducial model from the axis A.

Note: It could happen that some fiducials failed to transfer from axis A- to the B-axis. Users could correct that by adding points to the seed model.

41. Once the seed model is completed, track gold particles throughout all sections of the tilt series to generate the fiducial model linked to the B-axis. Fill the gaps with the bead fixer.
42. Repeat steps 36–38 on the dataset corresponding to the B-axis.
43. Start the process of dual-axis tomogram combination by creating command files to run different programs used for the combine procedure. Press the “Start Combine” button. Then, press the “Open Combined Volume” to visualize the final tomogram.
44. Trim the volume of the final tomogram along the X, Y and Z axis.

Note: The final tomogram displays some areas in X, Y and Z with no information regarding fungal ultrastructures. The Trimvol tool will remove those areas and the resulting tomogram will be focused on the biological material. Furthermore, the file size corresponding to the trimmed tomogram will be smaller.

45. Cleanup intermediate files.

Note: IMOD generates plenty of intermediate files that can be removed once the final tomographic volume is obtained. To save several gigabytes of space, it is strongly recommended to delete all intermediates files.

46. Repeat steps 32–45 for all tilt series acquired by dual-axis tomography on fungal biofilms.

EXPECTED OUTCOMES

Regarding the contrasting protocol, several points must be taken into account to consider the TEM images of acceptable quality. First, the ultrastructural integrity of the studied cell compartment should be preserved. Although this method can be applied to observe any organelle of the fungal cell, here we focus on the cell wall. *C. albicans* cell wall is split in two parts namely the sugar-containing inner part and the mannoprotein-containing outer part. In [Figure 5](#) both parts are clearly identifiable, where the inner part looks brighter than the dark (denser to electrons, red arrow) and thorny outer part (black arrow). Note that the cell wall remains attached to the plasma membrane and its thickness is homogeneous throughout the fungal cell. The contrast created by the uranyl acetate and lead citrate should allow to observe organelles of interest. In the context of TEM on fungal

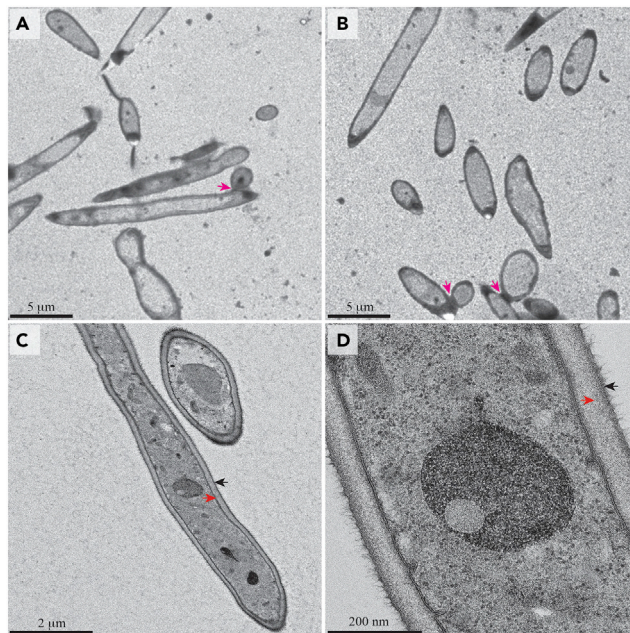


Figure 5. Contrasted images of *C. albicans* biofilms for TEM analysis

(A and B) Samples of *C. albicans* biofilm were grown, prepared for TEM and contrasted as described in the present protocol. TEM micrographs of fungal biofilms at low magnification (440 \times) showing contact points between fungal cells within biofilms (pink arrows).

(C and D) TEM pictures showing fungal cells were recorded at low (C) and high (D) magnification. The inner and outer parts of the cell wall are shown with red and black arrows respectively.

The scale bars are represented as black lines.

biofilm, we also show in [Figure 5](#) that connections between *C. albicans* are maintained within the microbial community (pink arrows).

For immunogold labeling experiments, the specificity of the antibodies is essential: no immunogold labeling should be observed in the negative control. Immunogold labeling is observed as black spherical dots originating from the gold particle (from the complex antibody:protein A-Gold particle): the gold particles will strongly scatter electrons away and the specific areas of the sample will appear as black dots. As a negative control we strongly suggest using cells that express the untagged version of the protein of interest. We were not able to observe any signal on the pictures after the immunogold labeling procedure of cells expressing the wild-type version of Pga62, a protein known to be a resident of the cell wall of *C. albicans* ([Figure 6A](#)); positive signals of immunogold labeling were observed on the cell wall of fungal cells expressing a GFP-Pga62 fusion ([Figure 6B](#)). Overall, these results show that this immunogold labeling procedure allows accurate tracing of specific fungal proteins.

For electron tomography, it is critical to choose well contrasted and informative areas for tilt series acquisition. First, the ultrastructure of the cells, as well as the contrast, should be good enough to record tilt series and ensure tomographic reconstruction. Moreover, the fiducial markers to align the recorded projection images should be evenly distributed in the field of view. Uniform distribution of the fiducial marker will increase alignment quality between projection images of the tilt series, and hence, the quality of tomograms. Tilt series are recorded with the Tecnai F20 microscope by using the SerialEM software. Once the tilt series recorded, the tomographic reconstruction is conducted with the IMOD software. IMOD will create a fiducial model by using the position of gold particles, trace them across the tilt series and finally optimize the alignment of the series. Subsequently, IMOD will generate the full-size tomogram using the weighted back-projection method. In case of optimal

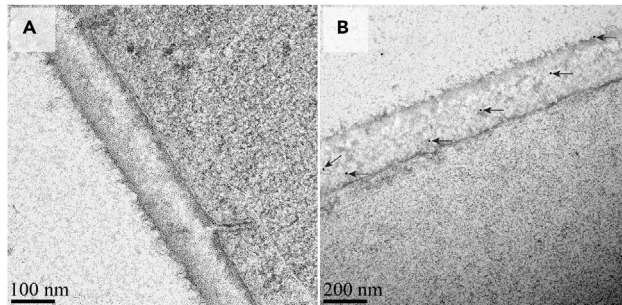


Figure 6. Immunogold labeling of the cell wall protein Pga62 on *C. albicans* biofilm

(A and B) fungal cells were grown as described in section A and subsequently labeled with α -GFP antibody following the protocol of the section D.1. *C. albicans* cells used for this experiment expressed either the untagged (A) or the GFP-tagged (B) versions of Pga62. Positive signals for immunogold labeling take the form of black dots (pinpointed with black arrows) and are visible only in the cell wall of the strain expressing the GFP-Pga62 fusion (B). The scale bars are represented as black lines.

contrast surface representations of the tomographic volume can be calculated in ChimeraX. Different features can be highlighted then with various colors using the “measure & Colorblobs” tool of ChimeraX. As an example, the inner cell wall (green), the outer cell wall (yellow) and the plasma membrane (magenta) were colored on a tomogram of fungal cells (Figure 7). Finally, dual axis tomography is strongly recommended to restrain the missing wedge of the data to only a missing pyramid.

LIMITATIONS

In the present method we used chemically fixed biofilms. Chemical fixation with formaldehyde and glutaraldehyde may induce damages of the fungal ultrastructure. In our case, chemical fixation is mandatory due to the safety rules adopted by our host institution regarding *C. albicans*. However chemical fixation is not mandatory to perform the present protocol. So, in accordance with safety rules, living biofilms of *C. albicans* can be frozen directly with high pressure freezing, hence avoiding chemical fixation. Even if glutaraldehyde is known to preserve cell ultrastructures due to its

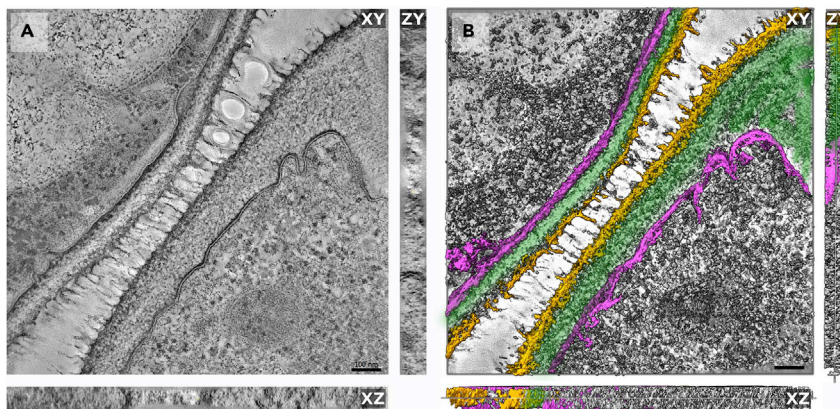


Figure 7. Electron tomography on *C. albicans* biofilms

(A and B) A tilt series has been collected at areas of interest using a Tecnai F20 microscope. Tomographic reconstructions have been performed with the IMOD software. A, Orthoslices (XY, XZ, ZY) from the middle of the volume are presented. B, Surface representation of cellular components has been highlighted with the ChimeraX program. The following color code was used: magenta (plasma membrane), green (inner cell wall) and yellow (outer cell wall). The XZ and ZY orthoslices/views are shown to demonstrate the homogeneous contrast across the entire volume. The scale bars are represented as black lines (100 nm).

crosslinking capabilities, it relies on diffusion and acts therefore slowly. High pressure freezing immobilizes the sample in milliseconds and preserves the cell wall at the same time as the interior of the cell. In addition, the cell wall is a cellular compartment that can be reshaped according to the growing conditions as well as the genetic background. Thus, under certain conditions, the cell wall may be sensitive to chemical fixation. If this situation happened, high pressure freezing without chemical fixation would possibly allow a better preservation of the cell wall ultrastructure.

To define the boundary between the plastic resin and the biological matter (the outer part of *C. albicans* cell wall) could be complicated by both the contrast and the strains used for the study. If the outer mannan layer is difficult to differentiate from the plastic resin, Alcian blue staining could be added to increase the contrast from the outer layer of *C. albicans* cell wall (Fassel et al., 1997). Indeed, Alcian blue will bind specifically to glycoproteins, which are found in the outer layer of the cell wall, and add more contrast within this area. The Alcian blue molecule owns a copper atom at its core that will generate a high electron density in TEM (Scott, 1996). If a more contrasted cell wall is required to delineate the boundaries of fungal cells within the biofilm, 0.1% Alcian blue could be added to the fixative during the chemical fixation. In theory, Alcian blue could be useful to add more contrast to the outer cell wall of fungal cells found under the biofilm form.

Depending on the fungal cell wall architecture, samples infiltration with the HM20 resin could be modified. In fact, the thickness and the composition of *C. albicans* cell wall is tightly regulated by the carbon source (Ene et al., 2012). Also, according to the genetic background of *C. albicans* cells, both chemical nature and architecture of the cell wall could be modified as compared to a wild-type strain (Moreno-Ruiz et al., 2009). Overall, due to tremendous combination of culture conditions and genotypes that can modify the composition of the fungal cell wall, the infiltration time of the HM20 resin to ensure proper embedding of the samples should be adjusted for every studied strain and culture condition.

The protocol described here will allow users to have access to ultrastructural details of *C. albicans* cells within unperturbed native biofilms. According to the biological question, the ultrastructure of fungal organelles (e.g., the cell wall) will be recorded and analyzed. However, this protocol will not allow users to have access to the tridimensional structure, as well as the thickness, of the microbial community itself. To analyze these parameters, SEM or Confocal Laser Scanning Microscopy methods should be used.

TROUBLESHOOTING

Problem 1

C. albicans strains are not able to form biofilms on the Aclar film.

Potential solution

Lack of fungal biofilms on Aclar film could be mainly explained by the fact that *C. albicans* strains are impaired for a gene essential for biofilm establishment. To achieve a fungal biofilm from planktonic cells, *C. albicans* must pass through biological processes like adhesion, morphogenesis, and the synthesis of an extracellular matrix. Those processes are tightly regulated at the transcriptional level and lack of genes encoding key transcription factors will block biofilm development (e.g., *EFG1*, *NDT80*, *ROB1*). To study genes essential for fungal biofilm formation, users can control genes expression with a conditional promoter (e.g., Tet promoter). Alternatively, gain-of-function mutations can be achieved by governing the gene expression with a strong constitutive promoter (e.g., P_{TDH3}). Both strategies will modulate expression of this specific gene and allow to assess its biological impact on fungal biofilms by electron microscopy (step 2).

Problem 2

The immunogold labeling staining is not specific for the targeted antigen.

Potential solution

The working dilution needs to be determined experimentally for each antibody. The best working dilution will be the one yielding the strongest signal possible from immunogold labeling with no or little background signal in the negative control. In case of nonspecific immunogold labeling, other blocking buffers could be used (PBS with milk powder, blocking buffer with Tween 20) (step 16c).

Problem 3

The immunogold labeling staining is not working on your biological samples.

Potential solution

According to the epitope used for the experiment, chemical fixation could be a major issue. In our protocol we used the GFP epitope that is very tolerant to chemical fixation using formaldehyde and glutaraldehyde. However other epitopes, namely HA- or Myc-tag, are sensitive to fixatives meaning that chemical fixation could disrupt the epitope making recognition by the antibody impossible. To avoid this phenomenon, reduce the incubation time of the samples with the fixatives. This incubation time should be optimized for each fusion protein. Another alternative is to change the fixative and to use 70% ethanol. Finally, if the immunogold labeling staining is still not working, another option is to change the molecular tag of the studied protein (step 16).

Problem 4

The ultrastructure of fungal cells is damaged.

Potential solution

If users observe any damage on the ultrastructure of their samples, some points of the method can be adjusted. First, infiltration time with the 100% HM20 resin can be increased leading to harder samples after resin polymerization. Subsequently, sectioning with diamond knives will be more efficient by avoiding ultrastructure damages induced by mechanical compression of biofilms.

Also, according to *C. albicans* strains used by the experimenters, chemical fixation could lead to dramatic alterations of organelles ultrastructure. To overcome this issue, users should either reduce incubation time with fixatives or lower both formaldehyde and glutaraldehyde content in the fixing buffer (steps 16–18).

Problem 5

The fiducial marker is not evenly distributed on sections which makes tomographic reconstruction complicated.

Potential solution

Protein A-Gold particles tend to aggregate. To make sure that protein-A Gold particles are monomeric before use and will therefore be evenly distributed all over the sections, sonicate them in a water bath for 5 min (step 18a).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sophie Bachellier-Bassi (sophie.bachellier-bassi@pasteur.fr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets or code.

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AUTHOR CONTRIBUTIONS

T.M. designed the study and performed experiments. T.M., A.D.G., M.S., and C.d.E. analyzed the data. A.D.G. and M.S. provide expertise and feedback. T.M., A.D.G., and S.B.-B. wrote the manuscript. C.d.E. edited the text and obtained funds for the study. The authors reviewed this method and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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