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Streamlined chemical fixation method for morphological investigation of *Candida albicans* with scanning electron microscopy

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ABSTRACT

This method describes the preparation of *Candida albicans* specimen for scanning electron microscopy (SEM), starting from specimen collection to obtaining SEM images. Our approach involves preserving cellular structure and preventing cell degradation through chemical fixation and post-fixation without Osmium tetroxide (OsO_4), followed by air dried specimen placed onto Si substrate. Additionally, the method outlines the steps for mounting the prepared specimen onto SEM sample holder aluminum stubs, followed by coating with an ultrathin gold layer via thermal evaporation to enhance specimen/substrate/stub conductivity. The specimens are then mounted in the SEM sample holder. This step-by-step procedure demonstrates successful morphological analysis of *C. albicans*, providing a comprehensive and effective specimen preparation for SEM analysis.

- Effective preparation of *Candida albicans* specimens for SEM analysis, preserving cellular structure.
- Step-by-step specimen mounting and gold coating for enhanced detailed SEM imaging.
- Chemical fixation of yeast cells for electron microscopy visualization.

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Specifications table

Subject area:	*Scanning Electron Microscopy (SEM)*
More specific subject area:	*Biological specimen preparation for scanning electron microscopy*
Name of your method:	*Facile and viable Candida albicans chemical fixation method for morphological study using scanning electron microscopy*
Name and reference of original method:	*Transmission electron microscopy of yeast DOI* https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/10.1002/1097-0029(20001215)51:6%3C496::AID-JEMT2%3E3.0.CO;2-9 https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/epdf/10.1002/1097-0029%2820001215%2951%3A6%3C496%3A%3AAID-JEMT2%3E3.0.CO%3B2-9 ***CTRL + CLICK THE LINK***
Resource availability:	*All the resources used in this method are available commercially*

Background

Scanning electron microscopy (SEM) serves as a pivotal tool in the study of *Candida albicans* (*C. albicans*) due to its polymorphic nature and its potential to cause infections in the human body. *C. albicans* exhibits various morphological forms, including yeast, pseudo-hyphal, and hyphal forms. This technique allows researchers to capture high-resolution images, providing detailed insights into the diverse morphological states of *C. albicans*. These observations are essential for understanding how the fungus adapts and transforms within the host, which is crucial for assessing its pathogenic potential and its role in infectious diseases [1,2]. SEM analysis comes with inherent challenges, including potential specimen damage from electron bombardment and the vacuum environment in the SEM chamber. To address these challenges, meticulous sample preparation is essential to protect the specimen and enable detailed SEM analysis [3].The preparation procedure for fixing biological samples is a series of steps aimed at preserving and stabilizing specimens; the fixation process, serves to immobilize cellular structures and maintain their integrity. There are different reports in the literature to choose between various fixation procedures, including chemical fixation with agents like formalin, glutaraldehyde, paraformaldehyde, or even freezing and cryofixation [4], which involves rapid freezing using liquid nitrogen or cryoprotectants, depending on their specific analytical goals; the focus of all these preparation techniques is to maintain the native structure of the sample [5]. We describe in detail a method required to ensure the *C. albicans* is collected and prepared for optimal specimen to perform SEM analysis. The method consists of three major procedures: first, provide a facile and viable fixation, post-fixation, and dehydration of *C. albicans* cell. Second, desiccation and specimen coating, and final is specimen pre-SEM analysis procedure.

Method details

Preliminary notes

Culture of *C. albicans:* in order to prepare the fungal cells for SEM fixing protocol, we used purified *C.albicans* strain, which was previously isolated from a 62-year-old female patient diagnosed with chronic anthropic oral denture candidiasis, as reported [6,7],the working cell suspension was prepared by inoculation discrete colonies of freshly grown *C.albicans* into newly prepared Sabouraud dextrose broth (SDB, Becton Dikinson, USA) and incubated overnight at 37 ˚C under standard aerobic conditions. Then, the *C. albicans* inoculum was diluted in warm SDB to approximately 1×10^7 colony forming units (CFU)/mL, corresponding to an optical density (O. D.) of 1 at 590 nm [8]

Preparation of all necessary solutions, media, and materials in advance will streamline the process.

All specimen processing procedures were performed at room temperature (RT) (~ 23 ˚C).

Solutions and reagents, initially stored under refrigeration (~ 4 ˚C), were carefully brought to RT before use.

Careful collection and handling of *C. albicans* broth, ensuring minimal damage is advised.

The first procedure encompassing the sequence of wash, fixation, and dehydration, is divided into three distinct stages for enhanced efficiency and clarity, as describe below:

Stage A. *C. albicans* biomass separation is followed by a washing process from the initial broth medium.

Stage B. Fixation and post-fixation.

Stage C. Dehydration, dry and desiccation.

The second procedure encompasses transfer and placing of a freshly dehydrated sampling drops onto the substrate.

Third procedure encompassing sample mounting and gold coating process to ensure the readiness of the sample to be analyzed by SEM.

First procedure

Fixation, post fixation and dehydration of the C. albicans *cells from broth*

The first and one of the most important steps in scanning electron sample preparation is fixation. This was achieved chemically (another alternative is freezing). Chemical fixation is carried on by aldehydes used to cross-link proteins. Post-fixation was performed by glutaraldehyde 3 % instead of the commonly reported, Osmium tetroxide (OsO_4) used post-fixative and contrasting agent due to

its strong affinity for cell membranes, influencing acyl chain formation in lipid membranes. It's a heavy metal that can stiffen cells, essential for preserving the macromolecular structure in biological specimens for detailed SEM analysis [9,10]. However, OsO_4 has limitations: it can only penetrate tissue to a depth of 1–2 mm, it's highly toxic, allergenic, carcinogenic, and flammable [11]. In our study, we intentionally omitted OsO_4 to the scope of our work focusing on morphologic, topographic of *C. albicans* by SEM. We successfully obtained SEM images without OsO_4, simplifying specimen preparation process and enhancing safety.

Dehydration: The presence of water causes samples to collapse under vacuum in electron microscope, so it should be replaced with an organic solvent such as acetone, isopropyl alcohol, ethanol, among others. A fundamental limitation is probable alteration of molecular structures inside the cells during the dehydration process [12]. To mitigate negative impacts, we opt for low-temperature absolute ethanol as a gentle dehydration agent. This choice is accompanied by the implementation of gradual dehydration steps, enabling cells to acclimate to the changing environment. Additionally, we reduce the dehydration time to avoid over-dehydration, which can lead to specimen collapse.

Stage A. step-by-step *C. albicans* biomass separation is followed by a washing process from the initial broth [13] medium schematic is depicted in Fig. 1a and b. During the preparation process, samples were centrifuged at each step of the protocol, 10 min at 4 000 rpm.

1. Initially, 1 mL of overnight cultivated *C. albicans* broth is centrifuged at 4000 rpm for 10 min to separate *C. albicans* cells from the mother broth.
2. The resulting pellet is resuspended in 1 mL of *C. albicans* broth, followed by a second centrifugation to obtain a pellet with sufficient biomass. The supernatants (approximately 750 μL each) will be discarded according to laboratory hygiene and safety protocols. This step is repeated as necessary until an ample biomass is obtained.
3. Subsequently, the pellet is resuspended in 1 mL of 0.2 M phosphate buffer (pH 7.3) at RT inside a 1.5 mL Eppendorf tube.
4. Washing with sodium phosphate (PBS) is performed by centrifugation and resuspension of the *C. albicans* pellet three times.

Stage B. Fixation and post-fixation schematic is depicted in Fig. 2a.

5. Fixation, the washed pellet is gently resuspended in 1 mL of a 3 % glutaraldehyde solution and left for 2 h at RT.
6. After impregnation, the specimens are washed once in 0.1 M sodium phosphate buffer, following the procedure outlined in step 4.
7. Post-fixation, carefully resuspend the washed pellet in 3 % glutaraldehyde in 0.1 M sodium phosphate buffer overnight at 4 °C.
8. After overnight fixation, remove the glutaraldehyde fixative and wash off the samples in the phosphate buffer solution (0.2 M, pH 7.3) as outlined in step 4.
9. Remove the buffer residues, wash off the samples with deionized water (step 4, once).

Stage C. Dehydration and desiccation. schematic is depicted in Fig. 2b.

10. Dehydration, was carried out by immersing the specimens in a pressive series of ethanol concentrations: 15 %, 40 %, 60 %, 80 % and 100 % during 2 h at RT. At each step, specimens were centrifuged and resuspended in the next ethanol concentration in the sequence. Ethanol was pre-chilled at 4 °C.

NOTES: After fixed, specimens were washed to remove excess fixative, then placed into a phosphate buffer solution to maintain a stable pH and prevent further cellular degradation. During dehydration step, gradually replaces water in the specimen with ethanol an organic solvent, to prepare the specimen for air dry on a Si wafer, as describe in next section in Second Procedure. Afterwards specimens are mounted and metallic coated prior introducing it into the SEM chamber process expounded upon in the third procedure.

Second procedure

In the second procedure, involves the transfer and placement of freshly dehydrated specimen drops onto the Si substrate.

11. Resuspend the precipitates right after finishing the total sequence of ethanol dehydration. Utilize a micropipette (0.1–2 μL) to extract a drop of the specimen.
12. Position the drop over a clean Si substrate (see next section for more information regarding substrate cleaning) then allow it to dry overnight in a dry environment at RT using a desiccator.

Third procedure

The third procedure encompasses gold coating and sample mounting to ensure the preparedness of the sample to be analyzed by SEM. The specimens are stored in a desiccator overnight at RT.

Sample coating process

Non-conductive dried biological specimens are susceptible to damage from electron bombardment and vacuum evaporation during SEM analysis. To meet these challenges, an ultrathin metallic coating is applied. This protective conductive coating reduces the electric charge-up phenomenon while enhancing contrast and resolution during SEM analysis. Deposition parameters were fine-tune for Au thermal evaporation, with a focus on depositing the thinnest and finest coating to minimize gold granularity. This optimization is crucial as the quality of the coating film directly influences the limit of resolution for the finest details that can be resolved by SEM.

Substrate cleaning notes: The Si substrates used are brand new, so a simple cleaning was performed before the specimen drop deposition of the dehydrated sample. This process takes ~30 min to complete.

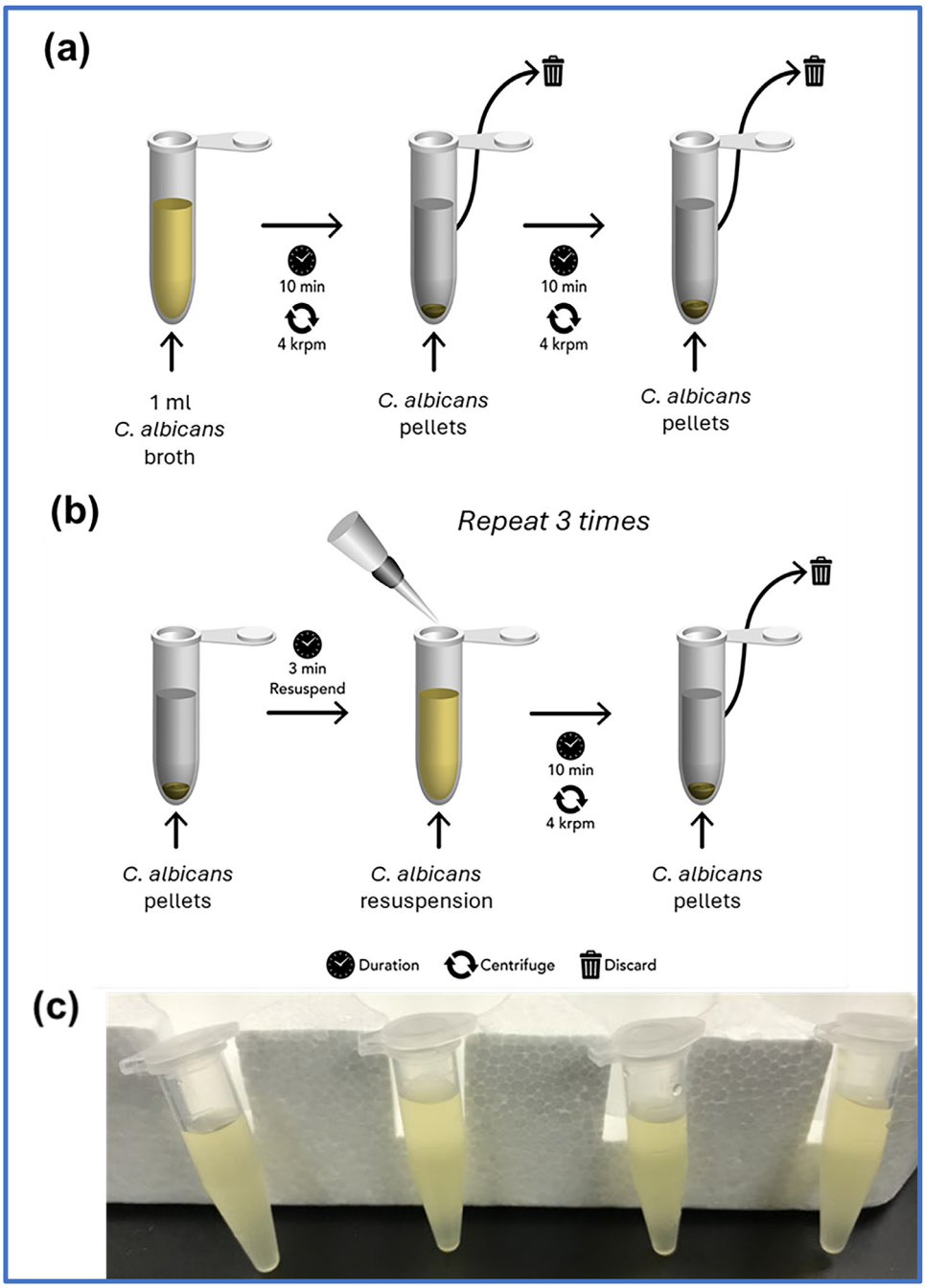

Fig. 1. Schematic step-by-step (a) biomass collection procedure, (b) washing process and (c) different stages of biomass collection.

1. Cut Si substrate 5 × 5 mm using a pen with diamond tip.
2. Ultrasonic the substrate during 5 min in three subsequent baths of: deionized water, acetone and ethanol.
3. Pour the ethanol into a 50 mL beaker.
4. Pour the acetone into a 50 mL beaker.
5. Place the acetone container onto a hot plate and warm up (do not exceed 55 °C) and turn off the hot plate.
6. Place the Silicon substrate in the warm acetone bath for 5 min.
7. Remove the Si substrate from the acetone and place it into the ethanol for 5 min in an ultrasonic bath at RT.
8. Remove the Si Substrate and rinse in deionized water and blow dry using an Ar flux.
9. Place the cleaned Si substrate on a clean working surface.

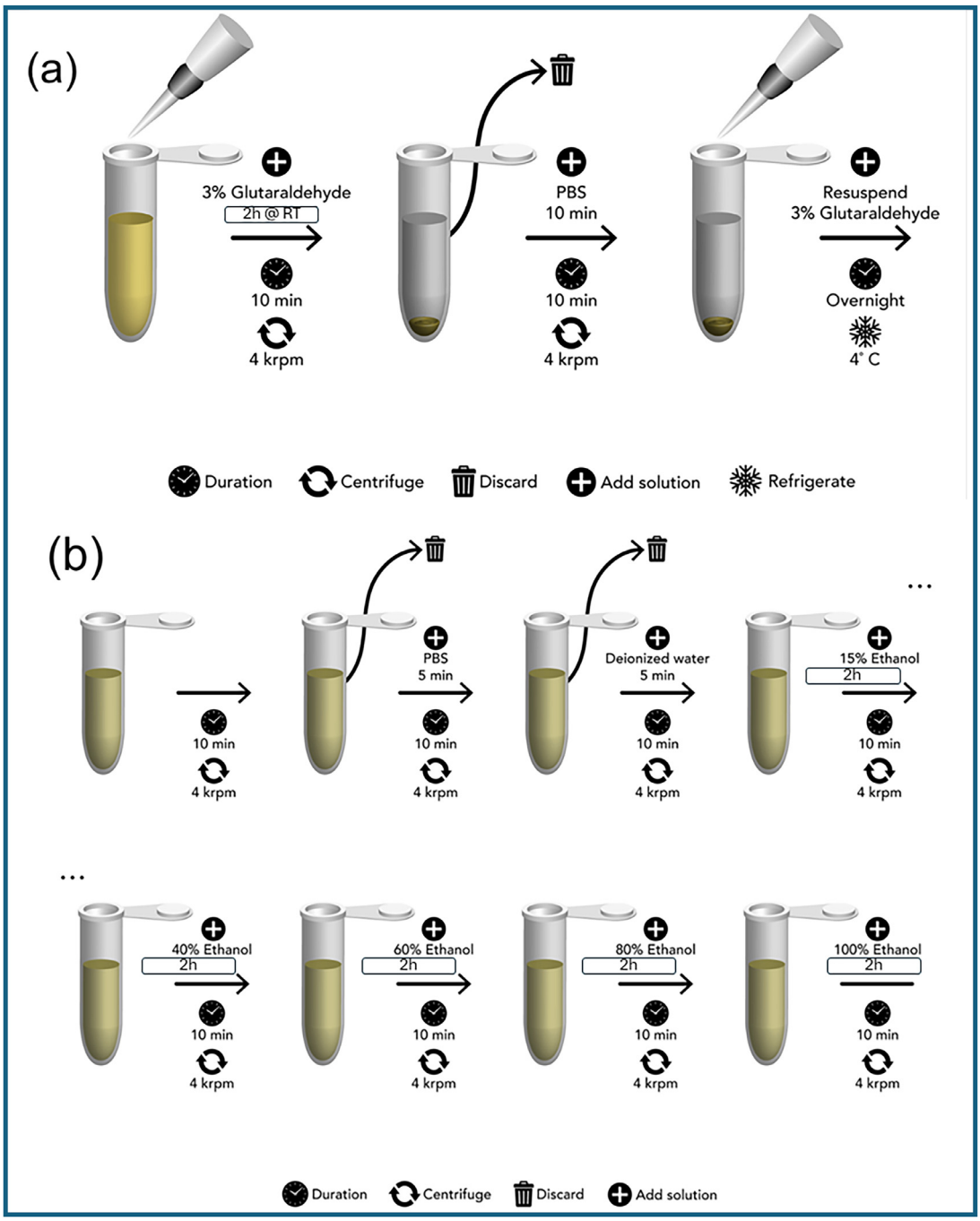

Fig. 2. Schematic of step-by-step (a) fixation, (b and c) dehydration procedures ethanol increase concentration.

Once the Si substrate is cleaned, place a drop of the prepare specimen and store it in a desiccator for 24 h before proceeding to mount the sample.

To assure conductivity, the sample's mounting is performed in the following way.

1. Cut a square of 4 × 4 mm and two strips of 10 × 2 mm of double-sided carbon conductive tape (C-tape).
2. Stick the bare side of the C-tape in the center of the cylindrical pin (9 mm diameter).
3. Take off the protective paper of the other side of the C-tape.
4. Place the Si substrate onto the C-tape being careful that most of the unpolished area is totally stuck to C-tape. (see Fig. 3)

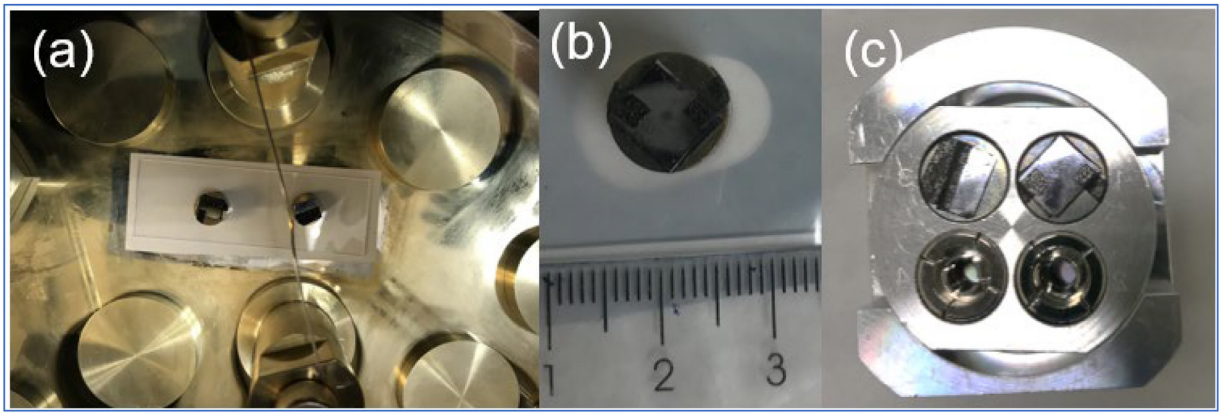

Fig. 3. Images taken of (a) specimens inside the thermal evaporation chamber, (b) Au/specimen/stub after au coated and carbon tape secured and (c) Au/specimen/stub mounted on the SEM sample holder. SEM sample holder.

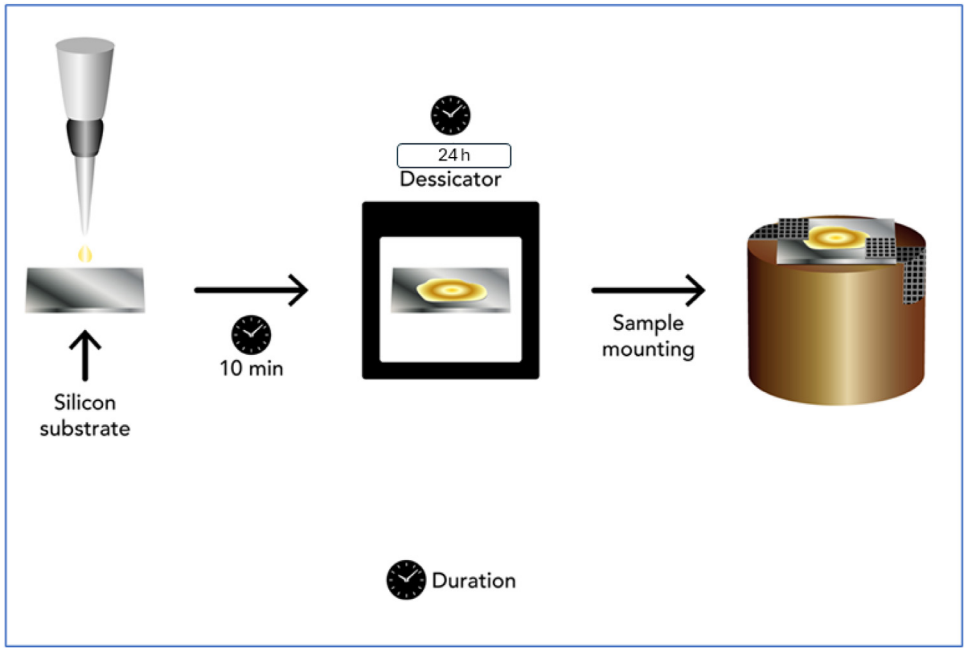

Fig. 4. Schematic of sample mounting for subsequent SEM analysis.

5. Place the strips of the C-tape making sure that there is a continuity from the sample surface, the width of the substrate and the pin (see Fig. 3b schematic for better understanding of the process).

Now, the prepared sample is ready for coating. It was mounted on SEM cylindrical pins and coated with a thin layer of gold using the thermal evaporation technique INTERCOVAMEX TE12 (See Fig. 3a). The deposition process was carried out at a rate of 0.5 Å / *sec* for a duration of 30 s, at a deposition pressure of 5×10^{-5} Torr, resulting in a 20 nm thin film covering the treated specimen. Finally, specimens are ready for morphological analysis. The sample remained under vacuum until it was transferred to the SEM sample chamber (Fig. 4,Table 1).

Method validation

The morphological images, as depicted in Fig. 5, were captured using a JEOL JSM-6390 SEM instrument. Fig. 5.Typical SEM image at different magnification(a) 270 X, (b)1000 X and 7000 X showing *C. albicans* of ~3 µm (Fig. 6).

Our proposed method emphasizes the use of glutaraldehyde (GA) chemical fixation to preserve the native morphology of Candida albicans for high-resolution SEM imaging. It ensures that the samples are adequately fixed, dehydrated, coated, and prepared to withstand the vacuum environment of the SEM chamber, thereby facilitating detailed analysis of the fungus's polymorphic forms and structural characteristics. Each method has specific advantages and disadvantages depending on the application and desired outcome.

Table 1
Comparison of the Proposed Method of Chemical Fixation Using Glutaraldehyde (GA) and other published methods.

	Method	Advantages	Disadvantages
1	Cryofixation [15,16]	-Offers Instantaneous fixation at near native state. -Widely held to be superior to chemical fixation for well-preserved morphology and antigenicity.	-Low contrast. -Potential for ice crystal formation, which can cause artifacts. -In some cases, penetration of cryoprotectants can be limited, potentially affecting the preservation of larger or denser tissues. -Involves more complex and technically demanding procedures compared to chemical fixation. -Requires specialized equipment such as liquid nitrogen and plunge freezing apparatus, which may not be readily available in all laboratories. - Requires trained personnel to perform the procedure correctly and handle the equipment safely. -Higher Costs.
2	Our proposed method of chemical fixation using Glutaraldehyde (GA) [17,18]	**- GA: Irreversible fixation of proteins.as well as slow penetration through the cell wall.** **Offering good preservation of antigenicity.** **-Effective at cross-linking proteins to preserve cell structure.** **-Easier for laboratory personnel to perform with standard training.** **-Fixatives can penetrate tissues more uniformly.** **- Requires standard laboratory equipment and fixatives.** **-Less expensive, using readily available fixatives like formalin, glutaraldehyde.** **- Fixatives like glutaraldehyde also penetrate well, ensuring good overall fixation.**	**-GA can cause volume changes, transformation of protein gels into reticulated structures, spatial changes due to cross-linking of proteins.** **- Changes in molecular bonds, i.e. creation of new bonds between macromolecules can lead to reactive site misinterpretation during labeling** **-May not preserve membrane structures and fine details as effectively as OsO_4 or cryofixation.** **-May cause distortion and artifacts in cell morphology.**
	Chemical fixation Paraformaldehyde (PFA)[1] [19]	- Fast penetration through the cell wall. - Preserves antigenicity better than GA.	- Causes fixation artifacts: volume change, denatured components lead to texture changes, transformation of protein gels into a reticulated structure, spatial changes due to cross-linking of proteins.
3	Chemical fixation using OsO_4 [20,21]	- Provides good, sometimes excellent preservation of cell membranes and fine cellular details. - penetrates tissues well, ensuring uniform fixation.	-Chemical fixation can introduce artifacts, such as shrinkage or cross-linking artifacts. -May not preserve cytoskeletal elements as well as cryofixation. -May cause distortion and artifacts in cell morphology. -OsO_4 is expensive and requires stringent safety measures for handling due to its high toxicity and volatility.
4	Other methods [22]	Improve imaging.	Complex methodology Requires specialized equipment Suitable for Transmission Electron Microscopy

Equipment, materials, and solution• Eppendorf 5453 Minispin Plus Centrifuge.

- Digital Hotplate Stirrer
- Branson 5800.Ultrasonic cleaner with digital timer plus digital heat control
- Bel-Art F42400-4011. Vacuum Desiccator, 35 L
- Evaporation chamber, INTERCOVAMEX TE12.
- Scanning Electron Microscopy, JEOL JSM 6390
- Eppendorf micropipette and single use tips
- 1.5 ml Eppendorf tubes
- 50 mL beakers
- Hazardous waste containers
- Parafilm and tape
- Distilled water (dH_2O)
- 0.2 M phosphate buffer (pH 7.3)
- 3 % glutaraldehyde in 0.1 M
- Silicon wafer ⟨111⟩ diameter 5", SSP.
- Microscope specific stage mount
- SEM aluminum stubs
- carbon conductive tape
- Fine-tip tweezers
- SEM stub tweezers
- Diamond tip pen
- Fine-tipped forceps

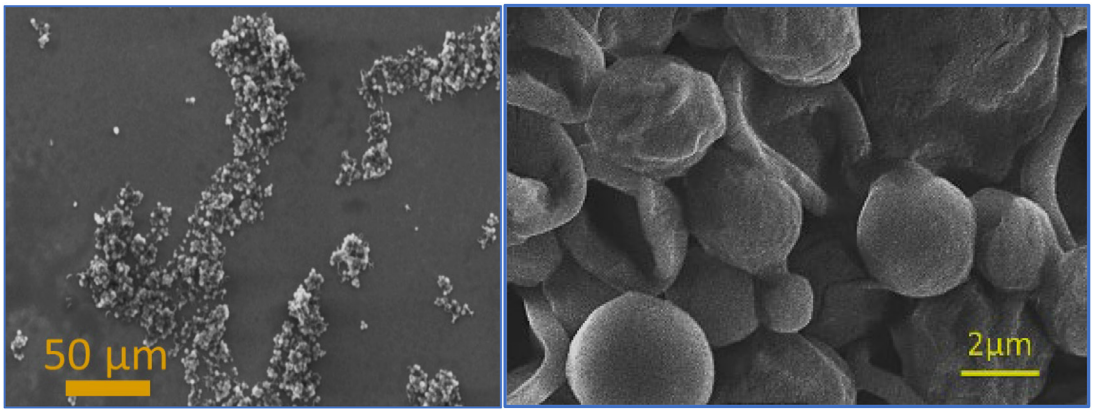

Fig. 5. Typical SEM image. *C. albicans* of (a) show a general view Magnification 270 X. (b) *C. albicans* were the main cell ~5 µm and the budding cells ~3 µm, ~ 400 nm. Magnification 9 kX.

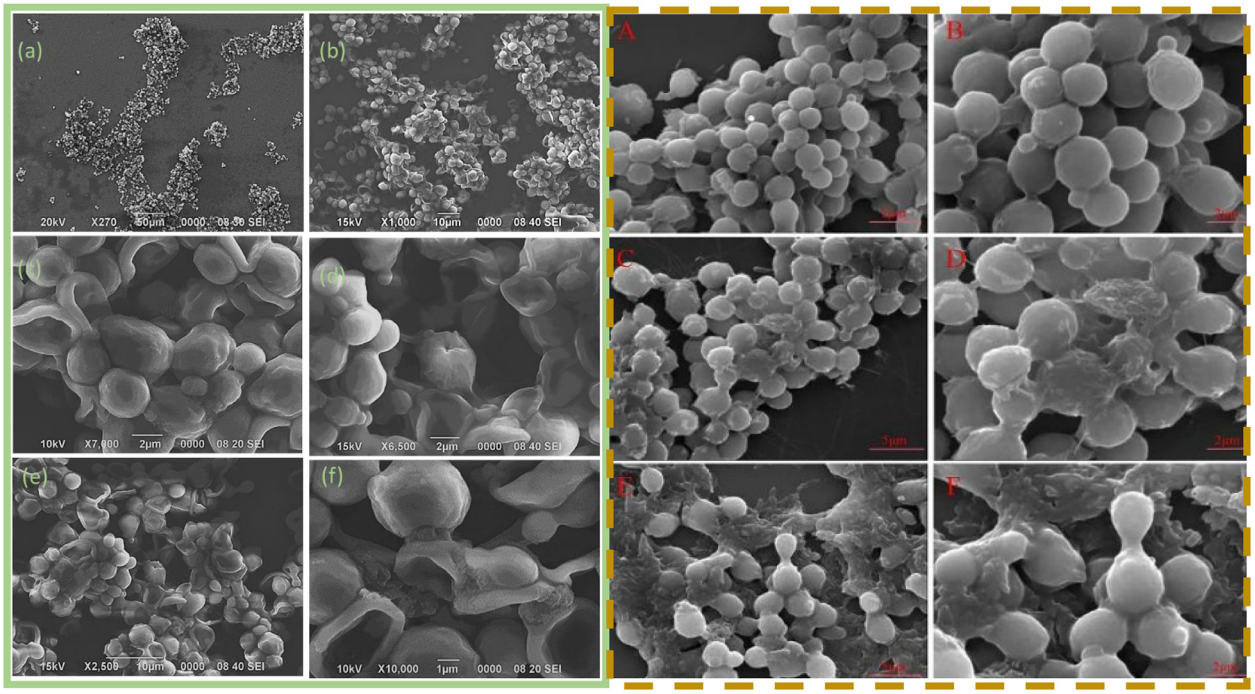

Fig. 6. Scanning electron micrographs of C. albicans. Left side (continuous green line) images were obtained using our chemical fixation method, images from (a to f) were taken in the same area at different magnification. Right side (dash yellow line) exhibits images obtained from Cui Peng et al. (2022) [14]. both *C. albicans* specimens (left and right) were chemically fixated with glutaraldehyde but different method.

- Gold wire 0.5 mm diameter / 99.995 %, ChemPur, 009166, 150 cm

Limitations

In our work we did not encounter of morphologic changes nor degradation of the *C. albicans* structural provoke by the protocol of fixation here propose, even thou we recommend validation thru different means of visualization if further study is required.

Ethics statements

Our method did not involve human subjects, nor animal experiments neither no data was collected from social media platforms.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Mayra Cecilia Ramirez-Camacho: Conceptualization, Methodology, Investigation, Formal analysis, Validation, Writing – original draft, Writing – review & editing. **Ernesto Alonzo Beltran-Partida:** Conceptualization, Methodology, Resources, Validation, Writing – review & editing, Funding acquisition. **Benjamín Valdez-Salas:** Resources, Writing – review & editing, Funding acquisition. **Mario Alberto Curiel Alvarez:** Resources, Writing – review & editing, Funding acquisition.

Data availability

Data will be made available on request.

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