

A Method to Evaluate the Ultrastructure of Free Cells by Scanning Electron Microscopy

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Abstract: The aim of this study was to develop an easy method for the analysis of the surface ultrastructure in prokaryotic and eukaryotic free cells. The procedure to prepare three types of free cells, the adhesion to poly-L-lysine (PLL)-coated glass substrate, fixation, washing, dehydration in increasing ethanol concentrations, critical point drying, mounting, coating, and observation by scanning electron microscopy (SEM) are described. Cell suspensions of peripheral blood mononuclear cells, yeast (*Saccharomyces cerevisiae*), and actinomycete were prepared. Each sample was layered on PLL-coated cover glasses, incubated under physiological conditions, washed with phosphate buffer, and fixed with glutaraldehyde. The observation was performed using an Inspect™ (FEI) SEM. This method allowed the acquisition of high-quality images, which showed the ultrastructure of the three types of samples evaluated here and could be applied to other samples with similar characteristics.

Keywords: Scanning Electron Microscopy, Eukaryotic Cells, Prokaryotic Cells, Ultrastructure, Yeast.

1. INTRODUCTION

Scanning electron microscopy (SEM) is a technique used for the study of cell and tissue surfaces. As part of the procedure, a metal coating of the biological samples is recommended to protect their surfaces from electron interaction and bouncing as well as to favor the acquisition of high-resolution images and relevant information [1, 2]. One of the main challenges in SEM and transmission electron microscopy is the preparation of free cell samples. In 1910, Harrison described the use of coverslips as substrate for the study of nerve cells [3], but this application was indicated for optical microscopy only. These challenges motivated the use of cationic polymers such as poly-L-lysine (PLL) to facilitate adherence of the cells to substrates. However, the type of cells that can adhere to PLL-coated substrates must be evaluated [4]. Therefore, methodologies need to be adapted to the samples and supplies available in each laboratory [5].

Considering the multiple steps in the sample preparation process, it is important to maintain cell viability and adherence to the substrate [6]. Furthermore, the fixation and dehydration steps should not cause cell collapse [5]. SEM methodologies are variable as they involve the use of distinct reagents and material to facilitate cell adherence, most commonly poly-L-lysine and poly-D-lysine. Different types of substrates have been also proposed, from highly expensive options to the most commonly available in every laboratory. Regardless of the materials chosen, it is crucial to ensure adherence and retention of the sample throughout the entire process until the visualization by SEM [7, 8]. The aim of this study was to develop a simple methodology for the analysis of the surface ultrastructure of eukaryotic as well as prokaryotic free cells.

2. MATERIALS AND METHODS

2.1. Preparation of Substrates Coated with Poly-L-Lysin

Glass microscope slides of 75 x 26 mm and ~1mm thickness were used as starting material for the elaboration of substrates. The slides were cut into small rectangles of ~25 mm² using a glass diamond cutter. One of the corners

of the rectangle was bevelled and used as an orientation mark to easily identify the sample-containing face. The resulting small glass pieces were used as sample substrates (SS). SS were degreased in 75% ethanol overnight. Then, with the aid of fine forceps, these SS were placed in a Petri dish and let to dry. Next, 20 μL of 0.1% poly-L-lysine (PLL, Sigma-Aldrich P-1274) diluted in double distilled water (ddH₂O) was evenly spread on the surface of the SS followed by a short incubation of 1 min at room temperature. The SS were then washed twice (30 s each time) by immersion in ddH₂O and placed in a Petri dish to dry at room temperature overnight.

2.2. Sample Loading on PLL-SS

Peripheral blood mononuclear cells (PBMCs), *Saccharomyces cerevisiae* yeast, and actinomycete bacteria were used as samples.

PBMCs were first isolated by density gradient using Ficoll-Paque and then cultured in RPMI medium containing 10% fetal bovine serum, antibiotics, and antimycotic for 24 h, 37 °C, 5% CO₂, and 95% relative humidity. After incubation, cell viability was assessed using trypan blue. Cells were washed with 0.1 M phosphate buffer (FB) pH 7.2, the pellet was resuspended in the same buffer, and the concentration was adjusted to 10% in a total volume of 100 μL FB. For the adherence step, the PLL-SS were placed in a 24-well plate (Corning®) (1 PLL-SS per well), then 20 μL of the PBMC suspension was layered on top of each substrate, trying to spread the sample from the center with slow and gentle swirls. The plates with samples were incubated for 15 min at 37 °C, 5% CO₂, and 95% relative humidity.

In the case of *S. cerevisiae* samples, yeast was first cultured in Sabouraud dextrose broth for 24 h, 37 °C. Cultures were centrifuged and the pellet was washed as described above for the PBMCs. Gram staining was performed to verify the absence of contamination. Cells were counted in a Neubauer chamber, and the concentration was adjusted to 10⁴ cells/mL in FB. Then samples were layered on PLL-SS following the same procedure described for PBMCs. For the actinomycete sample, a culture in marine broth was used (48 h, 20°C). The cell concentration was adjusted to 10% before layering them on the PLL-SS as described before.

2.3. Samples Processing

After cell adherence, each sample-loaded PLL-SS (n=3 per sample type) was washed twice by immersion in 0.1 M FB (30 s each wash). Samples were then submerged in 2% glutaraldehyde (G5882, Sigma-Aldrich) in 0.1 M FB to allow fixation for 3 h at room temperature. After fixation, samples were washed by immersion 4 times (30 s each) with 0.2 M FB and twice (10 min each) with ddH₂O. For the dehydration step, Petri dishes containing ethanol at the increasing concentrations of 50%, 70%, 90%, 100%, and 100% were prepared and samples were sequentially submerged in each of them for 5 min in the respective concentration. Samples were maintained in the last 100% ethanol dish until the critical point drying step. Substrates without cells or PLL were used as control.

Samples were placed in a K850 (Quorum Technologies, UK) equipment to perform the critical point drying. Through this process, the liquid from the dehydration step is replaced by CO₂. After critical point drying, the PLL-SS-samples were mounted on aluminium stubs using conductive double-sided adhesive carbon tape. Then, the stubs were placed in a SPI-MODULE sputter coater (11430E-AX, USA) and coated with a thin layer (~15 nm) of gold for 4 min. Samples were analyzed immediately after or stored at room temperature in a chamber containing silica gel and protected from light.

In the case of actinomycete, fixation was performed for 2.5 h followed by a wash step for 10 min in 0.2 M FB. The last two steps of dehydration (100% ethanol) were performed for 10 and 15 min, respectively.

2.4. Sample Analysis

Samples were observed using a FEI Inspect™ S50 scanning electron microscope (Netherlands). Images of each sample were acquired at different magnifications.

3. RESULTS

The SS prepared in this study was compatible with PLL, allowing the adherence of free cells and ensuring cell integrity throughout the process. Differences in size and morphology of lymphocytes and monocytes were detectable using this method (Figure 1). Particularly, a great amount of membrane projections was notorious in monocytes and, to a lesser extent, in lymphocytes, which is likely related to the activation of the former. The contrast and definition of these pictures were comparable to images of the same sample type in published works, demonstrating a good quality procedure. The washing steps as well as the concentration adjustment favored the even distribution of the cells on the PLL-SS.

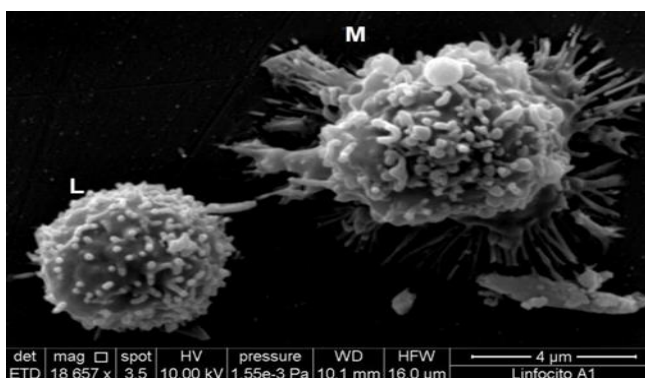


Figure 1. Lymphocytes and monocytes isolated from human peripheral blood. The lymphocyte (L) is smaller and shows less membrane projections than the monocyte (M). 18,657 x, 10.00 kV, ETD: Everhart–Thornley detector (a secondary electron and back-scattered electron detector).

In the case of *S. cerevisiae* sample (Figure 2A), a relatively low magnification (2,000 x) allowed the detection of yeast at initial and final stages of budding. At a 37,963 x magnification, budding scars were clearly distinguished in the parent yeast cells as well as in the bud or daughter cells (Figure 2B). Finally, at 80,000 x, small bumps were evident on the surface of parent cells but absent on buds (Figure 2C), allowing the distinction between aged and newly formed yeast cells.

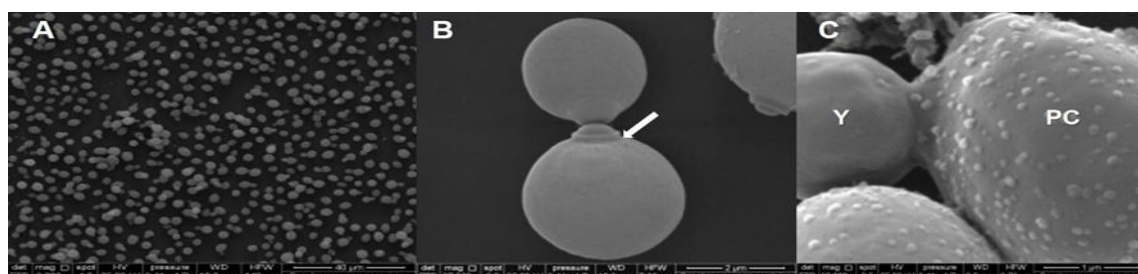


Figure 2. *Saccharomyces cerevisiae* yeast. (A) Field view (2,000 x, 25.00 kV). (B) Budding scars are observed as bumps on the cell wall (arrow, 37,963 x, 25.00 kV). (C) budding process will leave the characteristic scar on both, the parent cell (PC) and the daughter cell (Y) (birth scar). Bumpy surfaces of mature yeast cells are observed (80,000 x, 25.00 kV). ETD: Everhart–Thornley detector (a secondary electron and back-scattered electron detector).

In actinomycete sample, the continuous of filaments were observed as well as lightly prominent zones, which may be indicative of incipient formation of spores (Figure 3).

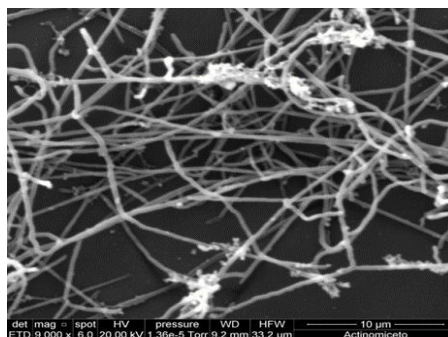


Figure 3. Vegetative mycelium of actinomycetes (aerobic bacteria). The continuous, filiform aggregates can be observed (9,000 x, 20.00 kV). ETD: Everhart–Thornley detector (a secondary electron and back-scattered electron detector).

Among the factors influencing the quality of the result is the sample dilution, which allowed the observation of evenly distributed individual cells. In addition, PLL was suitable for the adherence of free cells at the suggested time points, ensuring sample persistence throughout the washing steps and the rest of the process. In control SS that lack PLL coating, a considerable fewer number of cells were able to attach, showing also uneven distribution and lower image quality.

4. DISCUSSION AND CONCLUSION

The method developed here is efficient and affordable considering the use of glass microscope slides. Unlike coverslips, microscope slides are more resistant and endured the entire preparation process. Moreover, compared to methods described before for the analysis of similar samples, the procedure described here requires less time. Importantly, despite the omission of OsO_4 treatment, the resulted images showed good contrast (Figures 1, 2A, 2B, 2C, 3).

Several approaches have been proposed for the adherence free cells to substrates and analysis by SEM. Among the most conventional substrates are the carbon-coated cellulose acetate membranes (Millipore) [9], Formvar resin applied directly on the stubs [10], freeze-fractured agarose in 50% dimethyl sulfoxide followed by maceration with OsO_4 [11], copper grids covered with a Formvar film [12], polycarbonate membranes (Nucleopore) [13], direct sample mounting on stubs [14], etc. Clearly, methodologies are in constant revision, and it is important to determine whether the substrate is biologically inactive or if it can affect cell structure and function [15].

A recent study by Stil *et al.* (2023) using diverse cell lines and primary neurons has shown that surface coating with Poly-D-lysine (PDL) and PLL does not affect cell proliferation or differentiation [7], suggesting that the use of these reagents does not entail a significant risk of obtaining erroneous information. They also demonstrated that PDL or PLL-coated surfaces are biologically neutral and do not introduce contaminants to the cell cultures [7], which could occur when using natural polymers. Previous reports indicate the use of other reagents such as polyethylenimine for the adherence of actinomycetes, since PLL, polyethylene glycol, and dextran did not yield satisfactory results [16]. However, the method developed here allowed the efficient adherence of actinomycete without altering their integrity throughout the process.

The fixation process in the method proposed here is performed in a single step of 3 h incubation with 2% glutaraldehyde. Other researchers such as Hernández-Chavarría *et al.* (2003) performed two-step fixation for LLC-MK2 cells (adhered on borosilicate coverslips) using 2.5% glutaraldehyde for 2 h, followed by another 2 h in 2%

paraformaldehyde, and included a post-fixation step in 1% OsO₄ for 1 h [17]. These steps prolong the duration of the procedure, but cell morphology is maintained. Similarly, Koga *et al.* (2012) used 0.5% paraformaldehyde and 0.5% glutaraldehyde as fixatives to observe HeLa cells and leukocytes [11].

Several protocols have also been described for the dehydration process. For instance, to analyze human osteosarcoma cells and adipose tissue-derived mesenchymal stem cells, Minuti *et al.* (2023) used a sequence of seven concentrations of ethanol (10%, 25%, 40%, 50%, 70%, 90%, and 100%) [18]. In the current method, a series of five ethanol concentrations (50%, 70%, 90%, 100%, and 100%) were used and the dehydration time (5 min) was the same in each concentration. This ethanol sequence has been previously used by Kuehlmann *et al.* (2021) [14].

Regarding the critical point drying, there are also variations in the procedure, which mainly depend on the equipment available in each laboratory (generally CO₂ and t-butyl alcohol are used). Minuti *et al.* (2023) replaced the need of a critical point dryer by placing the sample-loaded silicon wafer substrates in a biosafety hood for 3 to 10 h to air dry, followed by vacuum drying [18]. It is noteworthy that before these steps, they applied a treatment with OsO₄ followed by three washes with deionized water (15 min each) [18]. Although this method makes up for the lack of a critical point dryer, the time invested is considerably long. Overall, reports indicate that the methodologies for the evaluation of free cells using SEM will continue to innovate and adapt to the needs and limitations of each investigation.

In conclusion, the method implemented here is practical, economical, and allowed the observation of the ultrastructure of PBMCs, *S. cerevisiae* and actinomycetes. The described procedure can be used for SEM studies to evaluate the ultrastructure of free cells from organisms, cultures, and microorganisms.

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