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SAMPLE PREPARATIVE PROCEDURE FOR *PSEUDOMONAS AERUGINOSA* OBSERVATION UNDER SCANNING ELECTRON MICROSCOPE

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SUMMARY

Scanning electron microscope (SEM) is a popular tool used for observing bacteria surface and morphology. Using *Pseudomonas aeruginosa* as a model, this work aimed to show a SEM preparative procedure that is simple and economical but does not result in considerable data loss. This was accomplished via testing fixing ability of 10% formalin versus 2.5% glutaraldehyde, efficiency of air drying versus t-butyl alcohol drying method. Following that, polypropylene, dialysis tubing and agar were also assessed for their ability to serve as a supporting material for cell adhesion in preparing sample for SEM. Consequently, obtained data showed that the procedure using 24-hour 10% formalin fixation and t-butyl alcohol drying preserved well bacterial morphology. With this procedure, little cell or membrane damage was seen while extracellular structures were clearly observed. Furthermore, when this procedure was applied with different types of substrates including polypropylene, dialysis tubing, and agar, it showed that sample fixed on polypropylene maintained well extracellular structures meanwhile sample fixed on agar presented well bacterial morphology. In conclusion, our data suggested that coating samples on polypropylene, followed by 24-hour 10% formalin fixation and t-butyl alcohol drying was appropriate for observing bacteria under SEM.

Keywords: formalin, glutaraldehyde, *Pseudomonas aeruginosa*, bacterial sample preparation, scanning electron microscope, SEM, t-butyl alcohol, TBA

INTRODUCTION

Scanning electron microscope (SEM) is a useful tool for observing biological samples' morphology and topography. However, hydrated biological samples cannot be observed directly by conventional SEM because the high vacuum conditions in the SEM machine make the surface and sub-surface water quickly evaporate, resulting in sample collapse or destruction. Therefore, a clear SEM image with well-preserved morphology requires a careful sample preparation method.

In general, a bacterial sample preparation consists of fixation, dehydration, drying and sputter coating steps. Before the fixation, the bacteria can be grown on an agar plate or in

suspension with or without adherent substrates such as plastics, aclar films, coverslips, silicon chips for bacterial attachment. Different types of substrate material provide different backgrounds and disperse charge properties for imaging. Then, the fixation process will help prevent decay and autolysis, preserve and stabilize the cell structures and size in a live-like state as long as possible for further preparation steps. The fixation usually uses osmium tetroxide and aldehyde fixatives (Russell, Daghlian, 1985). Dehydration with organic solvents is then required. Usually, ethanol is recommended to replace water in the cells and preserve the fixed structures. In the next step to efficiently dry the sample, different drying methods have been used including air-drying, freeze-drying, critical point drying (CPD) and chemical drying (hexamethyldisilazane (HMDS-drying) or t-butyl alcohol (TBA-drying). The latter one using t-butyl alcohol (Koon *et al.,* 2019) has become popular recently due to its low cost, simplicity and ability to produce desirable results with preserved cellular morphology when compared to CPD, the most common method for drying. Finally, the samples are coated to be observed under SEM. Because bacterial sample is non-conductive, it requires sputter coating with conductive materials such as gold, gold/palladium alloy, or platinum to avoid generating image artifacts.

In this study, *Pseudomonas aeruginosa*, a Gram-negative, rod-shaped and ubiquitous bacterium was used as a model sample. The goal of this study was to assess different *P. aeruginosa* sample preparation procedures to provide a protocol that is economical, straightforward with no major data loss. Substrates for bacterial attachment, fixatives and fixation duration as well as different drying methods were evaluated.

MATERIALS AND METHODS

Bacterial strain

Pseudomonas aeruginosa ATCC 9027 was stored in Luria-Bertani (LB) broth (HiMedia, India) with 30% glycerol (LB:glycerol 7:3 v/v) at -80^{o}C. Before doing any experiments, *P. aeruginosa* ATCC 9027 from stocks was inoculated on cetrimide (CE) agar (HiMedia, India).

Substrate preparation

The polypropylene plastics (Corning DeckWorks Pipet Tips box, USA) and the dialysis tubing (Serva, Germany) was cut into small squares with the side length of 5-6 mm and autoclaved in distilled water before use. Agar (HiMedia, India) was prepared in LB at 1.5%, autoclaved, and settled at 2-3 mm thickness.

Sample preparation for SEM

The sample preparation procedure was modified from a previously published protocol (Fischer *et al.,* 2012) in which the cells were grown on solid substrates. A preculture of *P. aeruginosa* in LB broth was prepared from the colony on CE agar and adjusted to OD600nm of 0.08 - 0.10, then incubated the culture at 37^{o}C for 18-24 hours with plastic pieces inside the broth.

The plastics were taken out and briefly washed with 1X PBS buffer twice at room temperature for 10 minutes each time. To avoid drying off the sample and adversely affecting the ultrastructure, the liquid was not removed completely. After being washed, the specimens were fixed in fixative at room temperature for either 30, 60 or 120 minutes in 2.5% glutaraldehyde (GA) or for either 24 or 48 hours in 10% formalin (FA). The fixed samples were washed again three times with PBS 1X at room temperature for 10 minutes each. After that, the samples were dehydrated via submerging in a series of graded ethanol from 25, 50, 70, 80, 90, 95 to 100% for 10 minutes each, in which the final submerging in 100% ethanol was repeated 3 times. Finally, the samples were dried by either air drying or chemical drying. *For air drying*: the samples were allowed to dry by air at room temperature for 24 hours. *For chemical drying using t-butyl alcohol (TBA)* (Koon *et al.,* 2019): the 100% ethanol solution was first replaced with a 1:1 solution of TBA/100% ethanol for 20 minutes, followed by two times drying with 100% TBA for 20 minutes. The solution was kept at 37°C so that TBA did not freeze. The samples in TBA were then transferred

into a disposable aluminum weighing dish. Once in the aluminum weighing dish, TBA was removed and replaced with just enough fresh 100% TBA to cover the sample. The samples in TBA were put into the fridge at 4°C for 10 minutes and transferred to a desiccator with frozen gel packs to keep TBA frozen. The samples were allowed to be dried overnight.

When agar was used instead of plastics as substrate, the colony from CE agar was taken out and streaked on LB agar plate and incubated at 37ºC for 18-24 hours. A scalpel or razor blade was used to excise blocks of agar with colony; the blocks should have a side length of approximately 5-6 mm. The blocks were placed into a 24-well plate with the colony side put upward. A volume of fixative was added to wet the agar block without allowing the fluid to reach the colonies. The samples were covered and let stand before continuing with dehydration and drying. When dialysis tubing was used as substrate, a preculture of *P. aeruginosa* in LB broth was prepared from the colony on CE agar and adjusted to OD600nm of 0.08 - 0.10. Then the culture was incubated at 37ºC for 18-24 hours with the dialysis pieces inside the broth.

SEM imaging

Images were collected using a scanning electron microscope (JEOL JSM-IT100, InTouchScope™, Tokyo, Japan). The samples were sputtered-coated with gold for one minute and observed at an accelerating voltage of 10 kV to avoid membrane degradation, and a working distance of 9 mm at room temperature.

RESULTS AND DISCUSSION

From the obtained SEM images, we observed that the level of cell damage varied depending on the sample preparation procedure. The formation of lysis transmembrane tunnels, which are tiny indents on the cell surface, was one of the most apparent damages that appeared in most of samples. Membrane clumping was another unfavorable characteristic, which was observed in all samples except in the FA-treated sample followed by TBA-drying.

Effect of fixation using glutaraldehyde (GA) and formalin/formaldehyde (FA) and fixation duration on SEM images of *P. aeruginosa*

Our results suggested that fixation using FA provided better SEM images of bacterial cell morphology compared to using GA. The FA-fixed image showed lower damage levels with less cell bursting and deformation (Figure 1A, 1B). Cell clumping and surface dents were problems seen in both FA and GA samples. In addition, GA samples even showed cell shrank or wrinkled surfaces (Figure 1C, 1D, 1E).

GA possesses greater potential for cross-linking and fast reaction with protein, usually in minutes, as it can occur through both the -CHO groups and over variable distances. GA has been previously claimed to be a good fixative but greatly reduces extracellular polymeric substances (Chao, Yang, 2011). On the other hand, FA is a small molecule which exists as low polymer in aqueous solution. FA rapidly dissolves and can penetrate the cell or biological molecules better than GA (Kiernan, 2000; Al Shehadat *et al.,* 2018) thus it has strong potential to be used in fixing biological samples like bacteria. A research on periapical bacterial plaques also revealed the SEM of samples fixed with 10% formalin, displaying bacteria embedded in or held together by great amounts of extracellular material (Tronstad *et al.,* 1990).

Our data showed that 24 hours of FA fixation was sufficient. Cell morphology appeared to be well preserved in samples fixed at 24 hours compared to 48 hours which showed more indents on the surface (Figure 1B). For GA-treated samples, cell morphology preservation is greatest with 60 min fixation (Figure 1D) whereas 30 minutes seemed not adequate, and 120 minutes were too excessive as SEM micrographs showed outflowed bacterial cytoplasm and serious deformation with convoluted, flattened cells (Figure 1C, 1E).

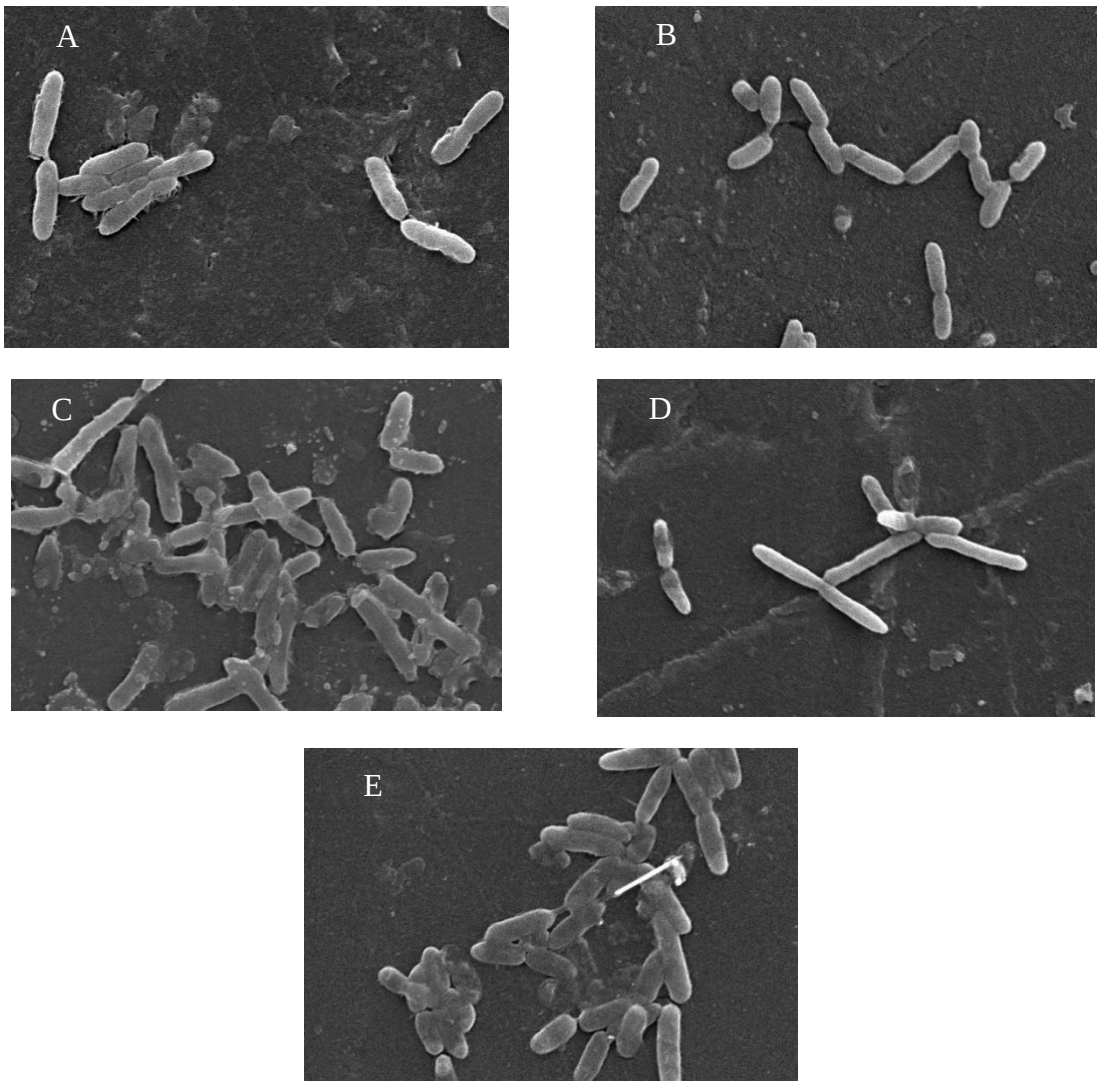

Figure 1. SEM images at 10000X magnification of *Pseudomonas aeruginosa* on polypropylene under FA fixation for 24-hour (A) and 48-hour (B) and GA fixation for 30 min (C), 60 min (D) and 120 min (E). The samples were air-dried.

For bacterial cells, glutaraldehyde fixation can take 1 to 48 hours, whereas formaldehyde fixation can take at least 24 hours and occasionally several days to complete (Czerwińska-Główka, Krukiewicz, 2021). However, most preparative procedures only applied glutaraldehyde fixation for one to two hours to get well-preserved bacterium morphology (Allan-Wojtas *et al.*, 2008; Forge *et al.*, 1992; Fratesi *et al.*, 2004). In the case of formaldehyde, 24 hours of fixing is sufficient in the usual process (Williams, Bloebaum, 2010; Afrikian *et al.*, 1973). The regularly used fixative 10% formalin is a low-cost, widely accessible fixative that does not induce significant shrinkage or cellular structural deformation (Thavarajah *et al.*, 2012).

Effect of drying using air and t-butyl alcohol (TBA) on SEM images of *P. aeruginosa*

The air-dried cells in formalin-fixed samples were more impaired than those in TBA-fixed

samples, and the former were clumping together with traces of extracellular layer remnants (Figure 2A). The cell-cell connection was disrupted since fixed cells were spread across the surface of a whole sample as single cells and there is little or no detection of extracellular structures between them except for the sample treated with FA followed by TBA-drying (Figure 2B).

For most types of samples, air drying at room temperature is the most basic and easy technique. Air drying was considered to be an adequate pre-treatment in several studies (Tronstad *et al.,* 1990; Hynninen *et al.,* 2018). However, it is not recommended for biological specimens because the surface tension at the air-water interface disturbs the cells and causes cell lysis (Bennett *et al.,* 2006). Most images of air-dried samples in our study revealed specimen deformation, shrinkage, and collapse. Furthermore, as compared to TBA drying, our results showed that air drying did not conserve any extracellular structures.

CPD is widely considered as a universal method for drying samples due to its superiority in preserving delicate structures by avoiding the liquid-gas interface, which occurs in the air-drying method and disrupts surface tension. The t-butanol freeze-drying method has recently been reported to provide equivalent or even better results to CPD (Baskin *et al.,* 2014). In the original protocol (Inoué, Osatake, 1988) or any protocol that uses TBA as solvent for drying (Baskin *et al.,* 2014; El Sharaby *et al.,* 2012; Kaneko *et al.,* 1990), the vacuum with a rotary pump or a freeze-drying device is needed to allow the sample to dry by vacuum sublimation of frozen TBA, causing it to transition from the solid to the gas phase and thereby avoiding a liquid–gas interface. Nevertheless, in our experiment, this instrument was not available so the samples in this study were only put in a bell jar desiccator overnight with frozen gel packs to keep TBA frozen. Surprisingly, the 24-hour FA-treated sample (Figure 2B) still showed a clear image of *P. aeruginosa* morphology and extracellular substances with the least damage compared to other methods. The advantages of this drying method can be mentioned as follows. First, this method is very simple and affordable that requires neither a costly apparatus nor liquid carbon dioxide for CPD except a desiccator. Second, the t-butyl alcohol used in this study can be easily frozen in a refrigerator without using liquid nitrogen or vacuum pumping. Third, many specimens can be dried at the same time in a bell jar.

Based on the results in the table, it was clearly shown that the best-preserved and most easily observable morphology and extracellular polymeric substances was the sample treated with FA fixation for 24 hours followed by TBA-drying.

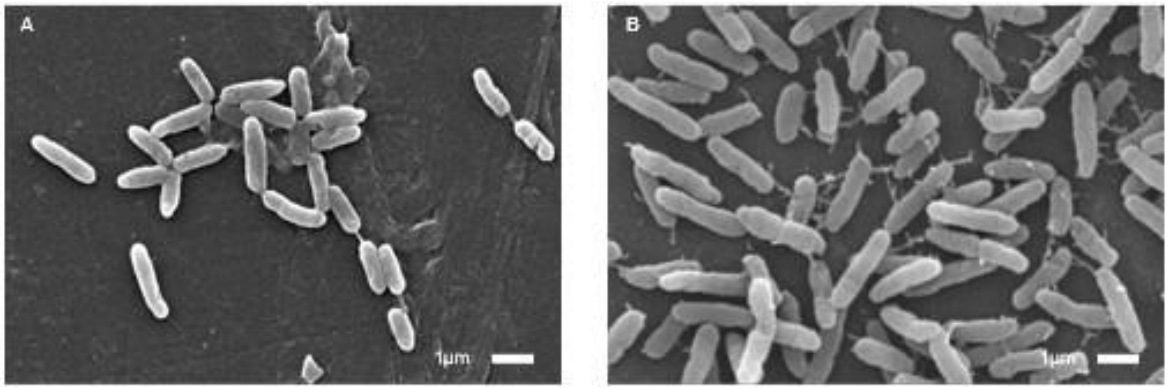

Figure 2. SEM images at 10000X magnification of *Pseudomonas aeruginosa* on polypropylene under formalin fixation at 24 hours, dried using air-drying (A) and TBA-drying (B).

Table 1. Fixation and drying treatment optimization assessment.

Drying method	Fixation	Criteria				
		3D shape	**Extracellular structures**	***Membrane damage***	***Membrane clumping***	***Cell rupture***
Air drying	FA24h	+++	0	++	++	0
	FA48h	+++	0	++	+++	0
	GA30min	0	0	+++	X	+++
	GA60min	+++	0	0	+	++
	GA120min	0	0	+++	+++	+++
TBA-drying	FA24h	+++	+++	+	0	0
	FA48h	+	+	+++	+	0
	GA30min	0	0	+++	X	+++
	GA60min	+	0	+	+++	+
	GA120min	0	0	+++	X	+++

***+++**: clearly observable; **++**: adequately observable; **+**: hardly observable.*
***0**: not observable; **X**: cannot be identified*

Effect of substrate materials on SEM images of *P. aeruginosa*

Applying the afore-mentioned optimized condition, which is 24-hour 10% formalin fixation followed by TBA-drying to *P. aeruginosa* sample on other substrate materials, it was concluded that agar is the most appropriate material for morphology observation. All of the cells uniformly have normal rod shape, smooth surface with no visible signs of damage or cell membrane ruptures (Figure 3A). For the sample fixed on polypropylene, although the cell morphology was clearly shown, the cell membrane was slightly ruptured and extracellular structures were only detected in this type of sample. The cells fixed on the dialysis tubing were the most severely damaged with obvious collapsed cells, incomplete morphology and many signs of cell membrane impairment.

Cell-substrate interaction is important for less cell removal from the surface while treating with chemicals. Dialysis tubing was reported to be an excellent material since it supports bacterial growth, readily available, easily handled, sterilizable, withstand chemical fixation, drying, vacuum pressure, and remain undistorted under electron bombardment in the scanning microscope (Afrikian *et al.*, 1973). Dialysis tubing was used to study the growth of an epiphytic diatom and bacterial community on its surface and the SEM results revealed clear bacteria morphology and biofilms (Vargo *et al.*, 1975). Polypropylene is another versatile material that has been widely employed in a variety of industries. Because of its unique rigidity, chemical solvent resistance, low cost, and susceptibility to bacterial surface colonization and biofilm formation (Kayes *et al.*, 2018), it is an ideal substrate for bacteria adhesion. Compared to these two materials, agar is harder to handle due to its fragility and cells easily washed off during the changing of treatment solutions. However, in this study, agar had shown its advantages as there was no detection of damage to cell morphology observed on agar samples. This was most likely due to the fact that when the agar sample was treated during the preparation procedure, the chemicals did not come into direct contact or touch with the cells, but rather penetrated through the agar matrix to reach the cells (Fischer *et al.*, 2012).

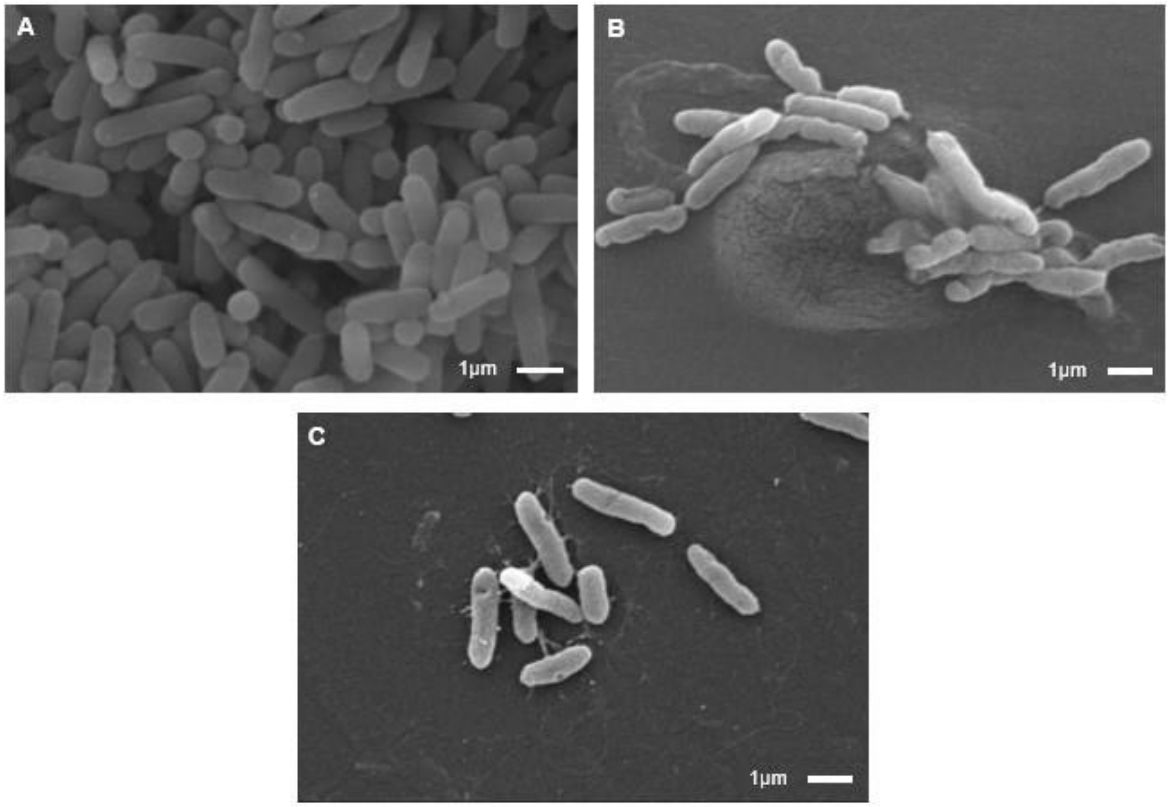

Figure 3. SEM images at 15000X magnification of *Pseudomonas aeruginosa* on different substrate materials: agar (A), dialysis membrane (B), polypropylene (control) (C).

Comparison between the standard and the optimized protocol

The standard protocol (Fischer *et al.*, 2012) used glutaraldehyde with the addition of alcian blue-lysine and osmium tetroxide as primary and post-fixatives to retain the extracellular structures. Since using GA alone as a primary fixative, the biofilm was virtually undetectable (data not shown in the article). But when using the alcian blue/lysine fixative mixture, the extracellular substances were apparent (Fischer *et al.*, 2012). Meanwhile, our study used formalin only as the fixative and the extracellular structures were still observable with preserved morphology. On the other hand, TBA-drying method used in the optimized protocol is also more simple, affordable and convenient than using a critical point dryer in the standard protocol for the reasons mentioned above. Therefore, as compared to the standard protocol, the optimized protocol in this study is less costly, simpler, and safer due to the use of TBA-drying and the absence of OsO_4 in the fixation.

Optimized *P. aeruginosa* sample preparation for SEM observation

After the evaluation of the results from the optimization tests and depending on the observation objective, two protocols are suggested in the figure below (Figure 4).

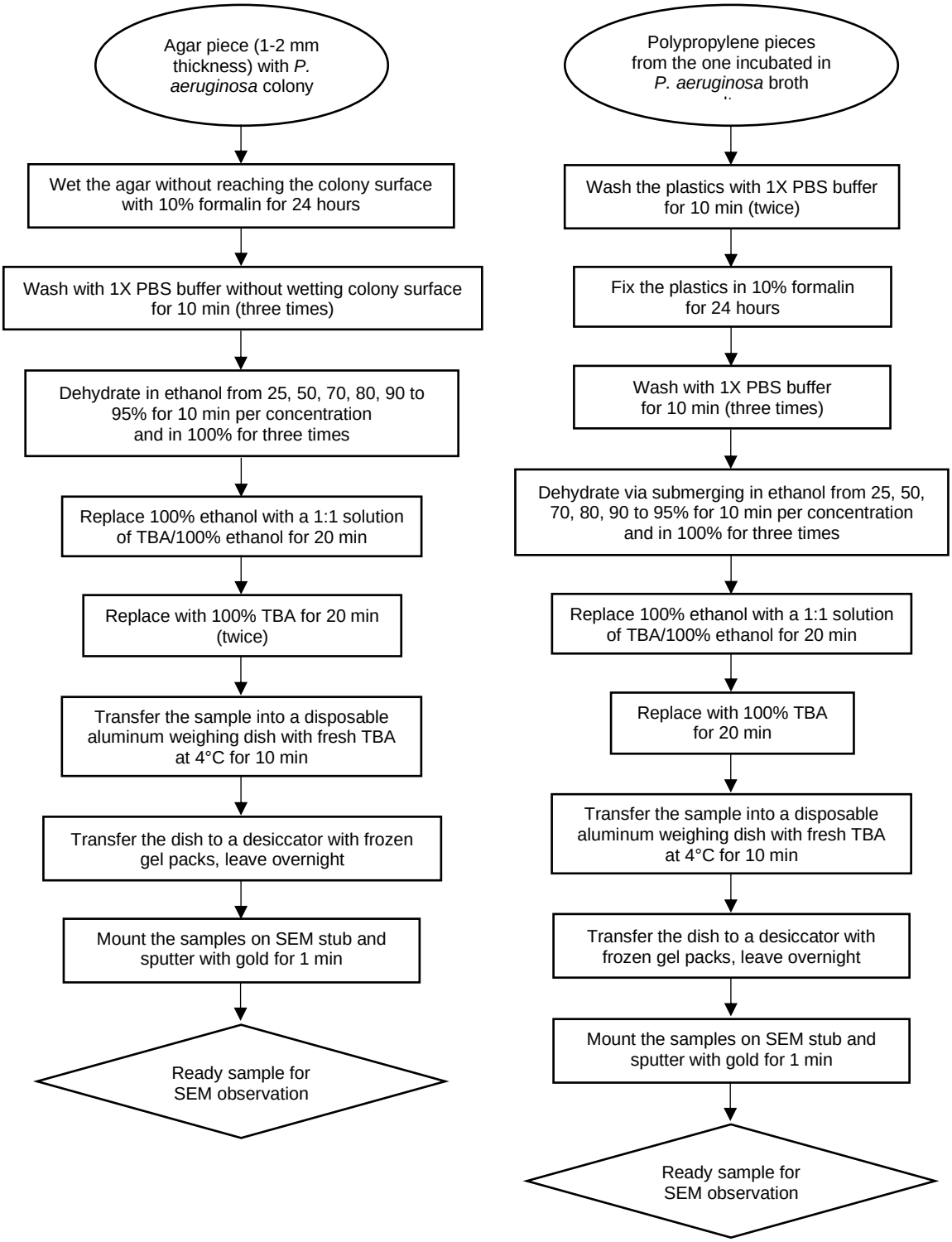

Figure 4. Optimized SEM protocol for extracellular structures observation (left) and cell morphology observation (right). During manipulation, the replacement of chemicals should be gently performed to avoid causing the loosely attached cells to detach from the surface and get misplaced or rinsed out. Min: minutes.

CONCLUSION

In conclusion, our study has provided cheap but sufficient protocols for observing bacterial samples under SEM. Depending on the purpose of research, observation of either bacterial surface or extracellular structures, agar and polypropylene can be chosen as supporting substrates respectively.

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