Bulgarian Chemical Communications, Volume 45, Number 4 (pp. 510–515) 2013

A simple and rapid scanning electron microscope preparative technique for observation of biological samples: application on bacteria and DNA samples

I. Piroeva[1], S. Atanassova-Vladimirova[1], L. Dimowa[2], H. Sbirkova[2], G. Radoslavov[3], P. Hristov[3], B. L. Shivachev[2]*

[1] *Academician Rostislav Kaishev Institute of Physical Chemistry, Acad. Georgi Bonchev str., building 11, Sofia 1113, Bulgaria*
[2] *Institute of Mineralogy and Crystallography, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bl. 107, 1113 Sofia, Bulgaria*
[3] *Institute of Biodiversity and Ecosystem Research , Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bl. 25, 1113 Sofia, Bulgaria*

Received February, 2013; Revised May, 2013

This protocol details a rapid preparative procedure enabling the visualization of biological samples like DNA and bacteria by scanning electron microscopy (SEM). This involves the fixation of the samples, followed by subsequent dehydration and the processing for electron microscopy observation. The fixation step is performed by allowing the samples to embed into an agar matrix. The dehydration of the samples ensures that they will not disrupt when processing for SEM observation or in the SEM vacuum camera. The whole process may be achieved for 6–24 hours and has been optimized for the visualization of bacteria, DNA. Samples once processed for SEM can be stored mild vacuum for weeks, allowing sufficient time for image acquisition.

Key words: scanning electron microscopy, SEM, *Esherichia Coli, Thermobispora bispora*.

INTRODUCTION

Biological samples e.g. bacteria, DNA, proteins, contain significant amounts of water and exhibit low conductivity. In their natural state, these samples cannot be observed directly by conventional scanning electron microscope (SEM) because the surface and subsurface water quickly evaporates under the high vacuum conditions necessary for electron microscopy observation [1, 2]. As a result usually the sample distorts collapses or destructs. In addition, water vapor from the specimen chamber decreases the vacuum and contaminates the detectors and column. The low conductivity of the biological samples also results in electrical charging that interferes with the electron beam and the secondary electron (SE) emission from the sample. Therefore, biological samples must be fixed, dehydrated, critical-point dried and coated before they can be observed in a conventional high-vacuum SEM [3]. Currently used protocols enabling electron microscopy observation are focused for obtaining high-quality data and involve successive series of procedures. While high quality data is achieved the protocols often include hazardous materials (OsO_4 or glutaraldehyde), are long-lasted and in some cases may extend for more than 7–8 days [4]. Our aim has been to adapt a protocol that will reduce significantly the processing time of such samples without significant loss of data collection quality. The protocol described hereafter provides a fast alternative for biological samples preparation [5, 6]. It can be used for rapid assessment of samples shapes, structural morphology etc.

MATERIALS AND METHODS

Although that there are many conditions to be met for "ideal" sample preparation for electron microscopy (EM) observation the most important and necessary condition is to maintain the integrity of the biological sample during sample preparation and subsequent work in the electron microscope [7]. Thus for biological samples the first difficulty lies in

* To whom all correspondence should be sent:
E-mail: blshivachev@gmail.com

the stable fixation of the samples onto a suitable surface as not to violate their integrity. The second difficulty is related to the high water content present in such samples. Unfortunately EM requires relatively high vacuum (10^{-6} to 10^{-7} torr) and if the samples are subject to such harsh conditions the water dehydration process of the samples is accompanied by the distortion, collapse or destruction. Fortunately, this drawback of EM can be circumvent by dehydration of the biological samples [8]. The last but not always the least difficulty is related to the low contrast provided by the samples build up predominantly by amino acids or nucleic acids e.g. C, N and O atoms [9]. Fortunately negative and positive staining may be used for enhancing the contrast – having in mind that DNA backbone is negatively charged [10].

Specific safety measures

- Because of the toxicity of the reagents and because possible biological contamination cannot be entirely excluded it is highly recommended to wear gloves during this procedure.
- NB! Staining with osmium should be done wearing gloves and under a fume hood.
- All waste materials generated during this procedure, such as the sheets of Whatman filter paper should be appropriately disposed (e.g. autoclavable waste container).

Materials

- Distilled water, sterile (dH_2O);
- Agar, biology grade;
- Optional: contrast enhancement (OsO_4, $UO_2(CH_3COO)_2$, WO_3).

Small laboratory material

- Small (60 mm diameter) or large (120 mm diameter) polyethylene petri dishes;
- Sterile centrifuge tubes (1.5 or 2 ml);
- sterile filter (<0.44 μm);
- Microbiology laboratory slides (18×18) – suitable for EM holder;
- Beaker glass, 50 ml,
- Filter paper (Whatman, 54 hardened);
- Forceps to manipulate laboratory slides;
- Permanent, waterproof marker (Staedler Lumocolor);
- Ball point to indicate references on filter paper.

Equipment

- Scanning electron microscope (Jeol, JSM 6390);
- Ultraviolet lamp;
- Laboratory oven;
- Centrifuge (~5000 rpm);
- Pipettes.

Sample processing preparation

Growth of bacteria and genomic DNA isolation

Two bacterial strains were employed: *Escherichia coli* (*E. coli*) *DH5α* strain and *Thermobispora bispora* (DSMZ 43038) [11]. *DH5α* is nowadays the most frequently used *E. coli* strain for routine cloning applications. It grows easily at 37 °C and its exponential growth can be controlled by spectrophotometric technique. *Thermobispora bispora* is a thermophilic strain that grows at 55 °C and in addition slowly than *E. coli*.

For the DNA investigation we isolated genomic *E. coli* DNA by standard procedure (PureLink, Invitrogen). The successful isolation was monitored electrophoretically (1% agarose gel, stained with 5 μl ethidium bromide, 10 mg ml^{-1}).

Harvesting of the samples

DNA elution was performed with 20 μl PCR water in order to avoid the presence of additional parasite salts from buffers.

The *E. coli* and *Thermobispora bispora* samples were collected by centrifugation at 3500 rpm for 5 min from the growth cultures (from 2 ml or more). The supernatant was discarded and the procedure was repeated two times with the addition of distilled water. The two additional steps are required in order to dissolves the salts and remains from the growth media. Finally 5–20 μl of dH_2O are added and the pellet is carefully homogenized thus ready for fixation for EM observation

Preparation for fixation and embedding of biological samples

1. 0.8% wt Agar-water solution: 400 mg agar were dissolved in 50 ml of distilled water by heating in a microwave oven without allowing the solution to boil.

2. Clean cover slips 18×18 mm^2 are sterilized by UV irradiation 10 for minutes. After what the slides are dipped in the agar solution (the 0.8% wt Agar solution is kept at ~ 50–60 °C in order to remain liquid) and left horizontally allowing a thin agar film to materialize. NB!!! Agar polymerization starts with temperature drop however it takes more than 30 min to obtain a sufficient degree of cross linking of the matrix.

3. The biological sample (bacteria, DNA) is carefully placed (pipetted) on the agar film and spread if

necessary. The fixation/embedding of the samples is achieved with agar cross-linking after approximately 30–45 min.

4. The agar is dehydrated in an oven at 37 °C for 12 hours (if necessary a low vacuum 0.8 atm may be additionally employed).

5. Dehydration of the samples: we choose to utilize a classical dehydration processing of the fixed samples by successive immersion in ethanol solutions starting from low to high concentration: 10, 25, 50, 75, 96 and absolute 99.99%). The samples were maintained in each ethanol solution for at least 30 minutes. Finally a drying at 37 °C for about 1 hour is performed.

6. The prepared samples are coated with a thin gold film (<10 nm).

Contrast enhancement by negative or positive staining can be optionally performed (OsO_4, $UO_2(CH_3COO)_2$, WO_3 etc.) of the samples before the first ethanol dehydration steps or immediately after the dehydration process depending on the samples.

RESULTS

SEM analyses were performed on a JSM 6390 electron microscope (JEOL, Japan) in conjunction with energy dispersive X-ray spectroscopy (EDS, Oxford INCA Energy 350) equipped with ultrahigh resolution scanning system (ASID-3D) in regimes of secondary electron image (SEI). The sample (cover slip) is mounted on a double coated conductive carbon tape that holds the sample firmly to the stage surface and can be used as a ground strap from the sample surface to sample holder. The samples were gold coated (time of coating ~30 s). This thickness of gold layer resulted in decent image quality without causing any electric charging. With thinner gold films (decrease of coating time below 30 s) electric charging was observed [12, 13]. When the coating time was longer (more than 40 s), the gold layer was thicker but no improvement of image quality was observed. The accelerating voltage was adjusted to 15 kV, I ~ 65 mA. Lower voltages (e.g. 10 kV) resulted in loss of contrast while higher voltages lead to rapid degradation of the imaging [14–16]. The pressure was of the order of 10^{-4} Pa [17].

The production of cover slips and embedding of the samples into the "agar" layer was achieved without difficulty. One should adjust carefully the required amounts of sample (not as on Fig. 1 were we intentionally overestimates the amounts for better visualization) as to have a "horizontal" (flat) surface allowing facile focusing of EM observation.

Figure 2 shows the observation of *E. coli* cells. As one can see the centrifugation and subsequent spreading of the water-cell solution over the cover slip allows better separation of individual cells. If a single colony of the same cells is directly embedded onto the cover slip the surface roughness can be observed. Interestingly, the average size of the cells is different (single colony shows that the cells are smaller). This is probably due to the centrifugation as the process collects more easily bigger ones. Thus an adjustment of centrifugation speed will be necessary in function of the size and consistency of the sample.

The results from the *Thermobispora bispora* observation are shown on Fig. 3. As one can see the quality of the visualization is reasonable. The concentration of the sample (Fig. 3a) leads to a some-

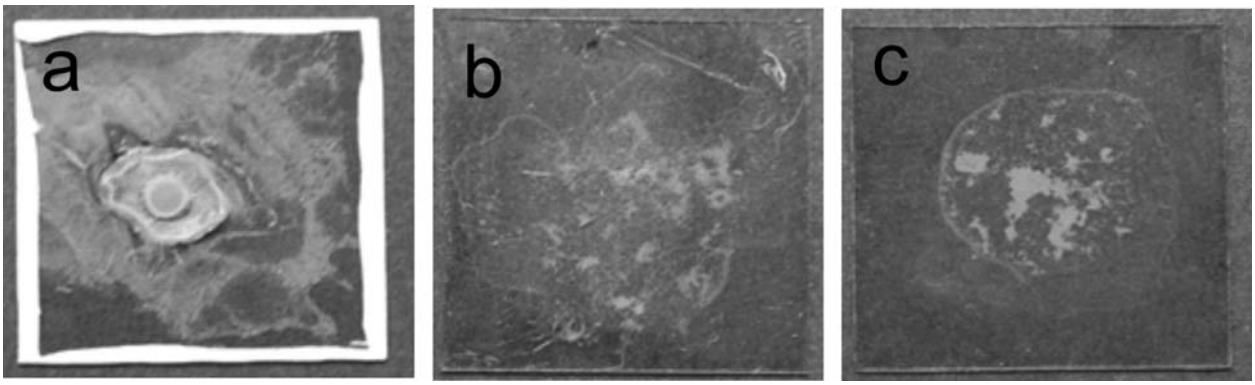

Fig. 1. Covers slips 18 mm^2 with embedded samples that were subject to dehydration and covering with gold layer a) Single *E. coli* colony from agar plate b) *E. coli* collected by centrifugation and spread over and c) *Thermobispora bispora* collected by centrifugation from media contaminated with TiO_2 (nano)particles. The amounts of samples here were intentionally overestimated

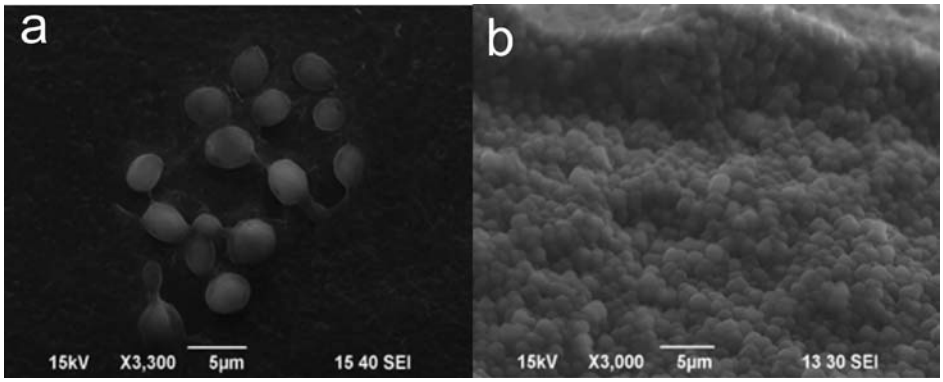

Fig. 2. SEM images of *E. coli* a) spreading after centrifugation b) single colony taken directly from plate (solid media)

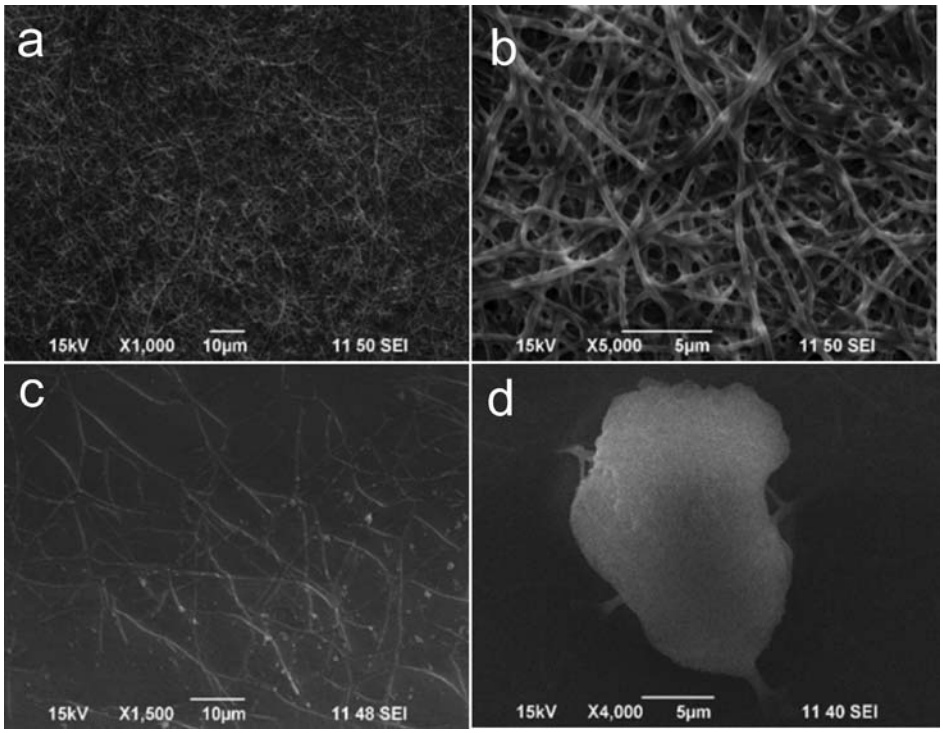

Fig. 3. SEM images of *Thermobispora bispora* with different concentration a) and b) centrifugation of 3×1.5 ml and c) and d) centrifugation of 1.5 ml

what higher contrast of the images – probably due to the fact that the "denser" surface allows a more uniform coverage of the gold layer and thus better conductance. The lower concentration of the sample, as for *E. coli*, gives better individual resolution and is able to discern bigger aggregates.

In order to estimate the use of the protocol in relatively unfriendly conditions *Thermobispora bispora* was grown in the presence of TiO_2 nanoparticles (Fig. 4). The TiO_2 particle cannot dissolve in the used water and thus bigger aggregates of them will be present after the centrifugation. Figure 4a shows that the TiO_2 particles are positioned "on top" of the bacteria. Of course this observation can be an artifact due to the employed centrifugation or to the pipetting or spreading of the samples. Nevertheless the bacterial network is clearly visible (Fig. 4b).

Finally the protocol has been used for DNA observation (Fig. 5). The DNA backbone features a negative charge and thus cations (Na, K, Zn, Cu etc.)

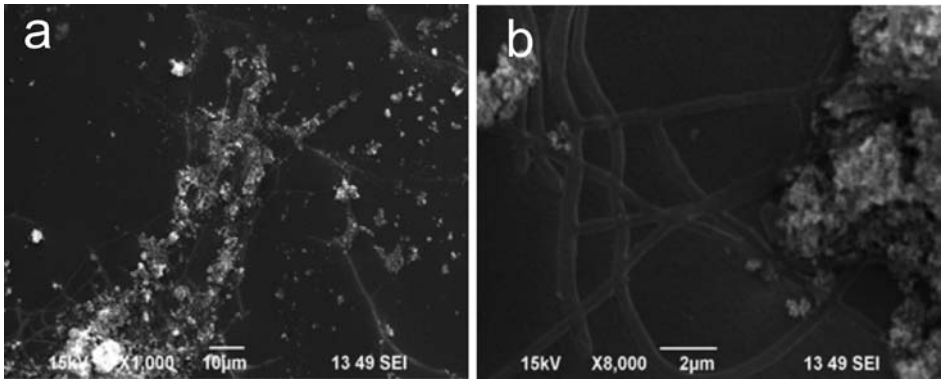

Fig. 4. SEM images of *Thermobispora bispora* grown with TiO_2 contamination

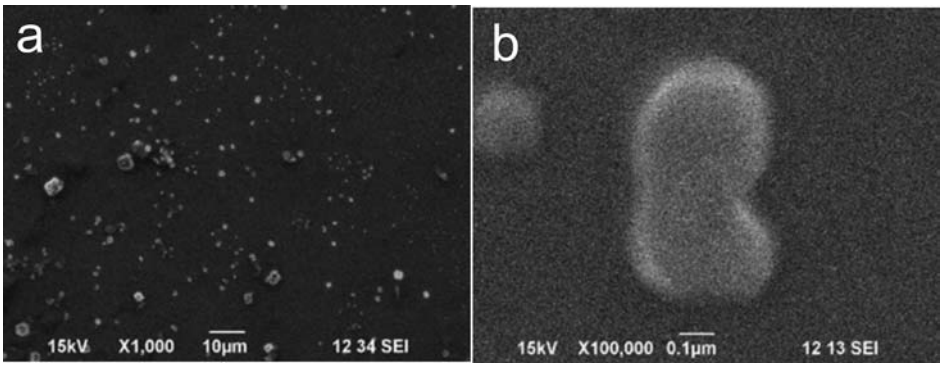

Fig. 5. SEM images of a) NaCl, $MgCl_2$ salts and b) genomic DNA

are needed to balance its charge. Thus the elimination of salts is more difficult than in the case of uncharged samples. Although the elution of DNA yields sufficient quantities for molecular biology work its concentration is typically ng/mL to µg/mL and thus very limited. More over DNA tends to compact tightly and thus its primary aggregate size is smaller – usually below 1 µm. Thus the routine SEM observations of DNA are more difficult than bacterial ones. As one can see from Fig. 5 the presence of salt is easily observable while the imaging of the DNA and its contrast are not as good as for the bigger biological samples.

CONCLUSIONS

We have adapted a rapid preparative procedure for preparing biological samples for SEM visualization. The processing is relatively rapid and may be performed for one day. The SEM visualization and data quality allows sufficient detail for samples that are bigger than 1 µm while for smaller size a contrast enhancement is suited. Compared to classical procedures the developed one is cost and time effective with minimal loss of data quality (mainly contrast lost).

Acknowledgments: *The authors are grateful for the financial support of Project BG051 PO 001-3.3.06-0038 funded by OP Human Resources Development 2007–2013 of EU Structural Funds and of the Bulgarian National Science Fund through grant DRNF 02/1.*

REFERENCES

1. T. Ogura, *Biochem. Biophys. Res. Comm.*, **391** (1), 198 (2010).
2. F. Iwata, Y. Mizuguchi, H. Ko, T. Ushiki, *J.* El. Microscopy, **60** (6), 359 (2011).
3. Ye J., X. Zhang, C. Young, X. Zhao, Q. Hao, L. Cheng, O. Jensen. *J. Proteome Res.*, **9** (7), 3561 (2010).
4. A. Dwiranti, L. Lin, E. Mochizuki, S. Kuwabata, A. Takaoka, S. Uchiyama, K. Fukui, *Microscopy Res. Tech.*, **75** (8), 1113 (2012).

5. J. Castillo, M. Dimaki, W. E. Svendsen. *Integr. Biology*, 1, 30 (2009).
6. T. Miyazaki, T. Nomoto, Y. Shimada, T. Tanaka, K. *Watanabe patent* No. US 8243266 B2 (2012).
7. S. Susan, W. Tai, M. Tang, *Journal of Scanning Microscopies*, 23, 267 (2001).
8. H. Vlčková, D. Solichová, M. Bláha, P. Solich, L. Nováková, *J. Pharm. Biomed. Anal.*, **55(**2), 301 (2011).
9. D. J. Billingsley, J. Kirkham, W. Bonass, N. Thomson, *Phys. Chem. Chem. Phys*., 12, 14727 (2010).
10. M. Kircher, J. Kelso, *Bio Essays*, **32(**6), 524 (2010).
11. K. Liolios, J. Sikorski, M. Jando, A. Lapidus, A. Copeland, Tijana Glavina Del Rio, M. Nolan, S. Lucas, H. Tice, Jan-Fang Cheng, Cliff Han, T. Woyke, L. Goodwin, S. Pitluck, N. Ivanova, K. Mavromatis, N. Mikhailova, O. Chertkov, Ch. Kuske, Amy Chen, Krishna Palaniappan, M. Land, L. Hauser, Yun-Juan Chang, C. D. Jeffries, J. C. Detter, T. Brettin, M. Rohde, M. Göker, J. Bristow, J. A. Eisen, V. Markowitz, P. Hugenholtz, H.-P. Klenk, N. C. Kyrpides, *Stand. Genomic Sci.*, **6** (2), 265 (2010).
12. M. Ohring, *Mater. Sci. Thin Films*, **42**, 794 (2002).
13. D. C. Bell, N. Erdman (eds), Low Voltage Electron Microscopy: Principles and Applications, 2012, p. 238.
14. L. Reimer, Image Formation in Low-voltage Scanning Electron Microscopy, 1993.
15. J. Bozzola, L. Russell, Electron Microscopy: Principles and Techniques for Biologists, 1998.
16. Scanning Electron Microscopy for the Life Sciences, H. Schatten (ed), 2012.
17. P. Mestres, N. Pütz, S. de las Heras, E. Poblete, A. Morguet, M. Laue, *Annals of Anatomy – Anatomischer Anzeiger*, **193** (3), 197 (2011).

БЪРЗА И ОПРОСТЕНА ПРЕПАРАТИВНА ПРОЦЕДУРА НА БИОЛОГИЧНИ ПРОБИ, ПОЗВОЛЯВАЩА ПОСЛЕДВАЩА ВИЗУАЛИЗАЦИЯ ЧРЕЗ СКАНИРАЩА ЕЛЕКТРОННА МИКРОСКОПИЯ

И. Пироева[1], С. Атанасова-Владимирова[1], Л. Т. Димова[2], Х. И. Сбиркова[2], Г. Радославов[3], П. Христов[3], Б. Л. Шивачев[2]

[1] Институт по физикохимия, Българска академия на науките, ул. „Акад. Георги Бончев“, бл. 11, 1113 София, България
[2] Институт по минералогия и кристалография „Акад. Иван Костов“, Българска академия на науките, ул. „Акад. Георги Бончев“, бл. 107, 1113 София, България
[3] Институт по биоразнообразие и екосистемни изследвания, Българска академия на науките, ул. „Акад. Георги Бончев“, бл. 25, 1113 София, България

Постъпила февруари, 2013 г.; приета май, 2013 г.

(Резюме)

Настоящият протокол описва бърза препаративна методика за биологични проби, като ДНК и бактерии, позволяваща последваща визуализация чрез сканираща електронна микроскопия (СЕМ). Това включва закрепването на пробите върху агарозна подложка, последвано от дехидратация на биологичните образци и нанасяне на тънко проводимо покритие за наблюдение чрез електронна микроскопия. Обезводняване на пробите гарантира, че те няма да деструктират във вакуумната камера на СЕМ. Нанасянето на тънък слой допълнително фиксира пробите и подобрява контраста. Процесът на пробоподготовка се извършва от 6 до 24 часа и е оптимизиран за визуализация на бактерии и ДНК. Пробите могат да се съхраняват във вакуум в продължение на седмица, което дава достатъчно време за получаване на СЕМ изображения. Протоколът не включва токсични, канцерогенни или други вредни химикали и реактиви.