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Method Article

An easy, fast and inexpensive method of preparing a biological specimen for scanning electron microscopy (SEM)

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A B S T R A C T

Biological samples usually require cumbersome preparation steps before SEM imaging. Here we propose a simple, fast and inexpensive method to prepare and visualize biological cell culture samples in a few easy steps. We have tested this method with success on several adherent breast cancer and non-adherent leukemia cell lines. This method gives results comparable to other well-established techniques, and it can be convenient in day-to-day biological sample preparation for SEM imaging.

- An easy and rapid method to visualize biological specimens under SEM.
- Cells are grown on carbon tapes and gold coated.
- Air drying without compromising the image quality.

A R T I C L E I N F O

Method name: Fast and Easy method of preparing biological specimen for SEM
Keywords: Scanning electron microscopy, Cancer cells, SEM preparation, HDMS, Biological samples
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Specifications table

Subject Area:	Scanning Electron Microscopy (SEM)
More Specific Subject Area:	SEM imaging of Biological Samples
Method Name:	Fast and Easy method of preparing biological specimen for SEM
Name and Reference of Original Method:	Parts of this method are adapted from: A.M. Kashi, K. Tahermanesh, S. Chaichian, M.T. Joghataei, How to Prepare Biological Samples and Live, 3 (2014) 63–80. F. Braet, R. De Zanger, E. Wisse, Drying cells for SEM, AFM and TEM by hexamethyldisilazane: A study on hepatic endothelial cells, J. Microsc. 186 (1997) 84–87. doi:10.1046/j.1365-2818.1997.1940755.x. H.P. Ting-Beall, D. V Zhelev, R.M. Hochmuth, Comparison of different drying procedures for scanning electron microscopy using human leukocytes, Microsc. Res. Tech. 32 (1995) 357–361. https://doi.org/10.1002/jemt.1070320409.
Resource Availability:	All the resources used in this method are available commercially

Background

SEM is a well-established technique to visualize a given sample's surface at a very high-resolution using a focused beam of electrons [1,2]. The resolution of SEM is approximately ten times higher than that of light microscopes. Therefore, it is used to study ultra-micro structures by producing a scanning electron micrograph of biological and non-biological specimens' surfaces. Biological sample preparation for SEM is the most essential and critical process to preserve the sample's ultra-structure. For example, biological samples usually are grown on a suitable substrate, chemically fixed, dehydrated, and dried before SEM [3]. It is typically a lengthy and time-consuming procedure, and it can also introduce artifacts during the processing [4–6]. Therefore, researchers are always on the hunt for a more affordable and more comfortable use method to prepare a biological specimen.

SEM operates in a vacuum that can irreversibly affect the structure of a sample. Therefore, samples placed in SEM should be dry and free of organic contaminants to avoid any gas molecules interfering with the SEM system's electron-dense environment [3]. As fluids are an essential component of biological samples and water constituent a significant part of it; therefore, dehydration becomes a necessary procedure to remove all the fluids from a biological specimen before imaging. Traditionally biological samples are dried using a critical point drying (CPD) process, which dehydrates samples with minimal alteration to their native state [7]. Another alternative is to use Hexamethyldisilazane (HMDS) [8–10]. CPD is an expensive and lengthy procedure. HMDS is a highly toxic and flammable alternative requiring a safety fume hood to perform sample drying procedures, although less costly and results in minimal preparation-related artifacts [11–14]. An easier and faster alternative to CPD and HMDS methods is to air dry the samples [15]. Air drying has its pitfalls, and if not performed carefully, then it can introduce artifacts. Artifacts due to cellular constituents' shrinkage during SEM sample preparation are critical during drying processes [14,16]. The shorter drying time duration for biological samples is recommended to avoid cellular artifacts [4]. In our experience, careful preparation and handling of samples can considerably reduce the artifacts introduced during air drying, avoiding time-consuming CPD and risky HMDS procedures.

This work describes a simple, fast and direct method for the growth of living cells followed by fixation, dehydration, and air drying. Our approach uses commercially available carbon tapes to culture, grow, and treat biological cell samples.

Materials and Methods

Carbon tapes 12mm (Ted Pella, Inc. USA) were placed in a 12 well plate (Greiner bio-one Cell Star®) followed by UV sterilization for 30 minutes under a cell culture hood. Adherent breast cancer cells KAIMRC1 [17], MCF-7 (ATCC, USA), and primary breast cancer cells were seeded at a density of 1×10^4 cells per well directly onto the sterilized carbon tapes. Primary fibroblasts were isolated from breast cancer patient samples at KAIMRC under the Institutional Review Board (IRB). All the adherent cells, including the primary fibroblasts, were maintained in advanced Dulbecco's Modified

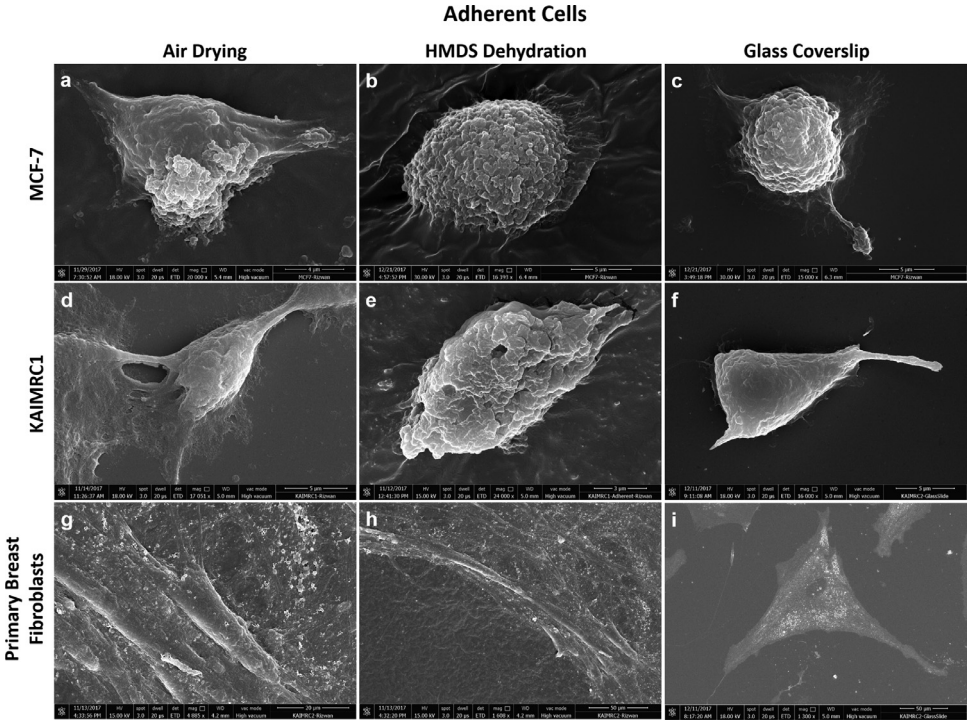

Fig. 1. Comparative SEM images of adherent cells. (a, b, d, e, g & h) Adherent breast cancer cells were grown on carbon tapes and (c, f, & i) on glass slides. Images a, d, g c, f & i present the air-drying method, and images c, f & i presents HMDS dehydration. All the photos show cells with intact cellular structure and protrusions. Primary breast fibroblasts are usually large and very flat. That is why it isn't easy to visualize them clearly on carbon tapes.

Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 50 units/ ml penicillin, 50 μg/ml streptomycin (Gibco), and 2 mM L -glutamine (Gibco) at 37°C and 5% CO_2 for 24-48 h depending on the cell growth rate. Non- adherent human erythroleukemia cell line, K562, derived from a female chronic myelogenous leukemia patient, human monocytic acute leukemia cell line, ThP-1, and human promyelocytic leukemia cell line, HL-60, derived from the peripheral blood of a female with AML FAB M2, were maintained in RPMI 1640 medium supplemented with 10% FBS, 50 units/ ml penicillin (Gibco), 50 μg/ml streptomycin (Gibco) and 2 mM L -glutamine (Gibco) at 37°C and 5% CO_2 for 24-48 h depending on the cell growth rate. Peripheral Blood Mononuclear Cells (PBMCs) sample was collected at KAIMRC under the approval of IRB. PBMCs were then isolated using a ficoll-plaque gradient as described previously. Briefly, 10 ml of drawn blood was diluted 3-fold in dilution buffer (PBS saline, 2 mM EDTA), layered carefully over ficoll-plaque, and centrifuged at 4500 rpm for 20 min. The layer corresponding to PBMCs was isolated, transferred to 45 ml of dilution buffer, centrifuged twice at 3000 rpm for 20 minutes and the resulting pellet re-suspended in 10 ml growth media. Post cell adhesion to carbon tapes, cell culture media was aspirated, and cells were washed three times with PBS (Gibco). Cells were then fixed with 2% Gluteraldehyde (Sigma-Aldrich) for 20 minutes at 4°C. After fixation, cells were washed three times with cold PBS at room temperature for 1 minute each. Cells were immediately subjected to gradient dehydration using 30%, 50%, 70%, 80%, 90%, and twice with 100% ethanol (Sigma-Aldrich) for 3 minutes at each concentration. The processed carbon tapes were transferred to aluminum stubs 12.7 mm x 8 mm (Ted Pella, Inc.) and left for air drying under a fume hood for 15-20 minutes. In case of HMDS dehydration, samples were incubated in HMDS for 15 minutes and then air dried. The samples were sputter-coated (Quorum Q300TD) with a thin layer

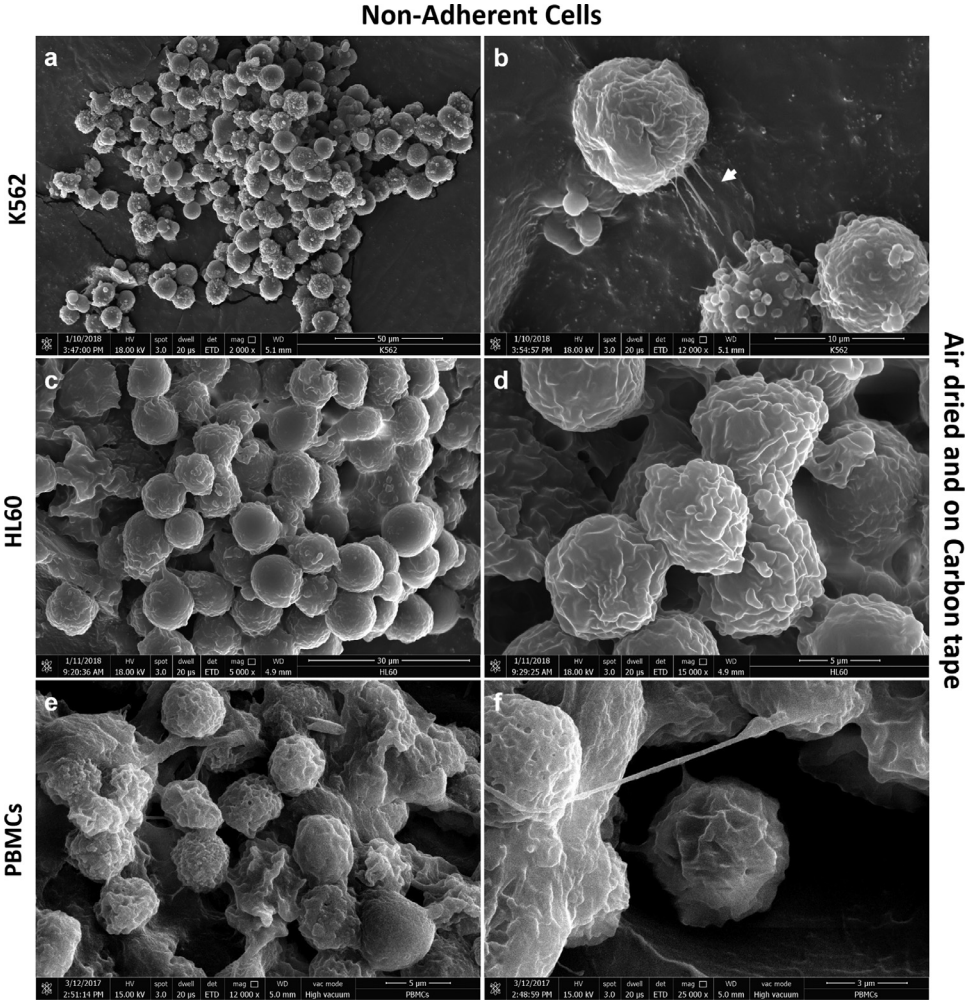

Fig. 2. SEM images of Non-adherent cells. (a & b) K562, (c & d) HL60, and (e & f) PBMCs. Non-adherent cells were grown, fixed, dehydrated, air dried, and mounted on carbon tapes. Cellular protrusions are visible in K562 and PBMCs.

of gold approximately 12.5 nm for 50 sec with sputter current of 20 mA. Images were acquired using Nova NanoSEM 450 microscope system (FEI) at 15-30 keV with Everhart-Thornley Detector (ETD).

Results and Discussion

SEM is an excellent tool for investigating biological specimen and their ultra-structures [2]. Several types of ultra-structural protrusions exist on the cell surface. These self-assembled structures are in a size range that is not usually visible by light microscopy. These structures play a crucial role in the motility and migration of the cells. Especially in cancer cell biology, these protrusions become very important in understanding the mechanism of cell-cell interaction and communication. For example, the filopodia, an extension of the cell cytoplasm, can be visualized by a light microscope only at its broader end that protrudes out of the plasma membrane. The further parts of a filopodium are usually not visible by light microscopy [18]. Other structures such as coated pits, microvilli, and caveolae are also present on the outer surface of the cytoplasm that can only be accurately visualized by SEM.

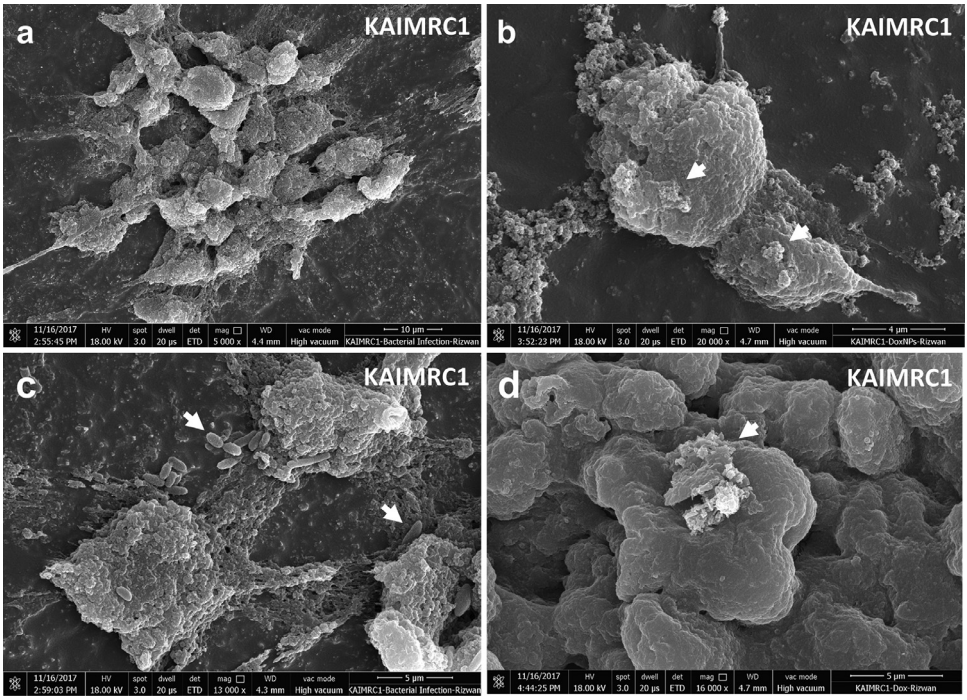

Fig. 3. SEM images of KAIMRC1 cells grown on carbon tapes. Cells were grown directly on carbon tapes for 24 h and then treated with ccdB strain of bacteria, doxorubicin-loaded nanoparticles, and Doxorubicin alone. (a) KAIMRC1 cells without treatment. (b) KAIMRC1 cells treated with doxorubicin (c) KAIMRC1 cells with a bacterial infection. White Arrows show bacteria invading cells. (d) KAIMRC1 cells were treated with 1μM Doxorubicin.

Here we report a simple method to prepare and visualize adherent and non-adherent biological cell samples for SEM. The schematic design of the proposed method is shown in the graphical abstract.

Briefly, adherent cells were grown on UV sterilized carbon tapes placed in a 12 well cell culture plate. Cells were incubated at 37°C and 5% CO_2 for 24h. Later cell culture media was aspirated, and cells were washed three times with PBS. In the next step cells were fixed in 2% gluteraldehyde, washed, and gradually dehydrated using 30%, 50%, 70%, 80%, 90%, and 100% ethanol. The processed carbon tape with cells on it was then transferred to a metallic stub and left for air drying under a hood. The sample was sputter gold coated and visualized with SEM.

Air drying of samples is easy and resulted in comparable results to other well-established methods, provided it is performed quickly. Carbon tapes with processed samples were then mounted on metallic stubs and gold sputter-coated before SEM imaging.

Various substrates are commercially available to grow adherent cells, including coverslips, aclar membrane filters, or silicon chips [19,20]. Coverslips are the most widely used substrate. A suitable substrate usually requires appropriate dimensions, efficient charge dispersal, and minimal background electron emission [13]. Specimens in a conventional SEM must be electrically grounded, and glass coverslips are not conductive; therefore, they cannot properly ground the sample. In this protocol, we grew adherent cells directly on the carbon tapes without another substrate's need. Carbon tape has the advantage of providing both excellent adhesion and conductivity [21]. Hence growing the cells directly on the carbon tape increases the conductivity of the specimen.

We used three types of adherent and three types of non-adherent cells to validate our method. Adherent cells were seeded on carbon tapes in multi-well cell culture plates, whereas non-adherent cells were chemically fixed, dehydrated, and mounted directly on the carbon tapes. Fig. 1 shows images of adherent KAIMRC1, MCF-7, and primary breast fibroblast cells. It compares our carbon

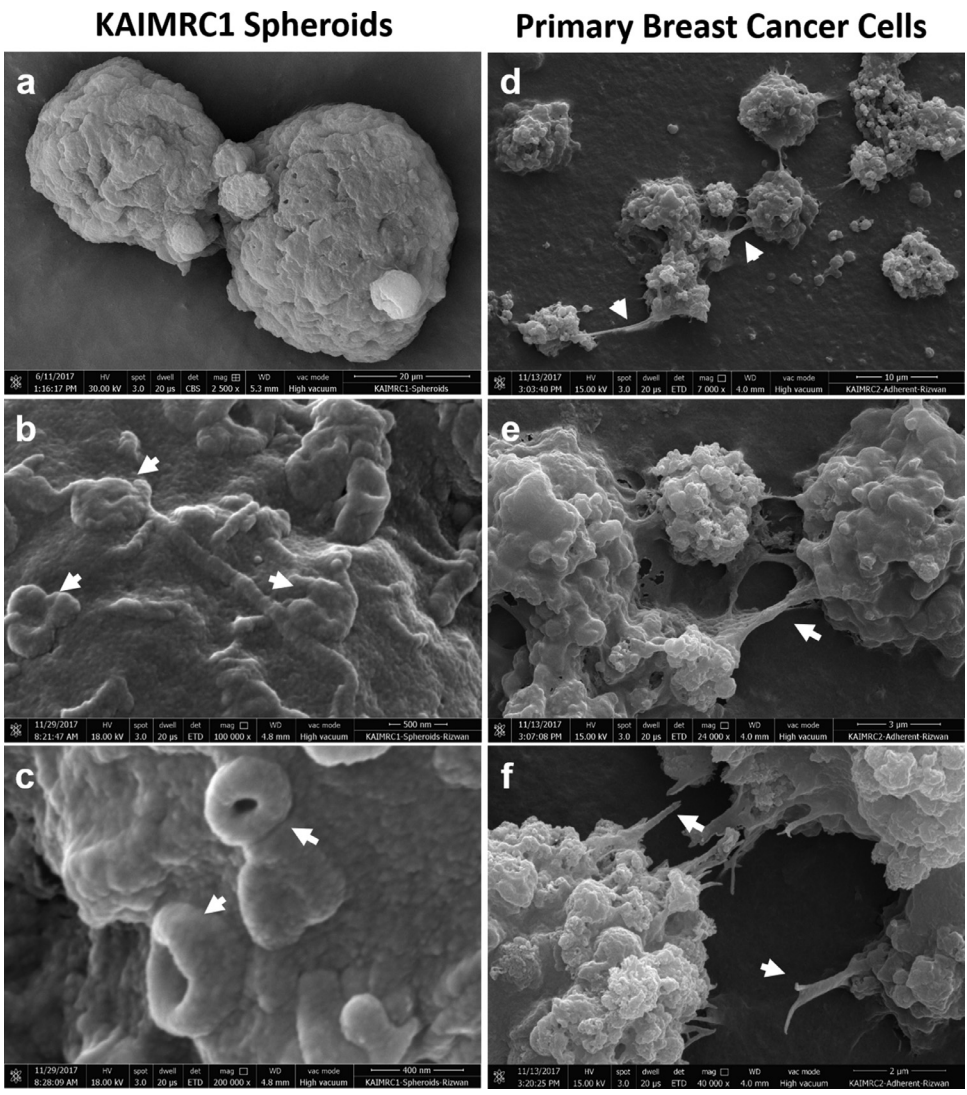

Fig. 4. SEM images of breast cancer cells. Cells were grown directly on carbon tapes for 24 h and air-dried. (a-c) KAIMRC1 cell spheroid. Increasing magnification of spheroid cells showcases in great detail the caveolae present on individual cells. (d-f) Primary breast cancer cells grown on carbon tapes and air-dried. Images show the interaction between various cells in great detail. Images e and f show the movement of cells towards each other. Filopodia are visible protruding from one cell towards another cell. These filopodia are the sites of actin polymerization and responsible for cell movement and communication.

tape and air-drying method to the HMDS dehydration method. Acquired pictures of our approach are similar to the HMDS method. We have also tried the CPD method, which was time-consuming and resulted in the loss of many cells during processing. In contrast, our method was faster with very acceptable image quality.

Fig. 2 showcases non-adherent HL60, K562, and PBMCs fixed, dehydrated, air-dried, and mounted on carbon tapes. All the acquired electron micrographs showed cells with intact ultrastructure and comparable image quality to the ones deposited on glass slides.

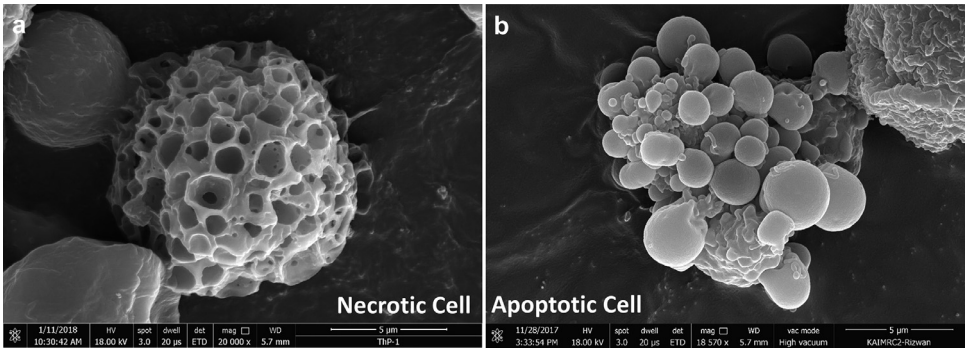

Fig. 5. SEM images of an (a) necrotic and a (b) apoptotic cell. Cells were grown directly on carbon tapes for 24 h and air-dried after graded dehydration. These 2 images are an excellent example of our simple and easy approach resulting in high quality and resolution images of biological samples.

Next, to visualize cells' interaction with foreign agents, we treated the KAIMRC1 cell line with bacteria, NPs, and drugs. SEM images of treated KAIMRC1 cells are shown in Fig. 3. Cells were grown directly on carbon tapes for 24 h and then exposed to E. coli bacterial strain resulting in bacterial infection of these cells. White arrows in Fig. 3 c show bacteria invading KAIMRC1 cells. Doxorubicin is an anthracycline type of chemotherapy, and it works by slowing down or killing the cancer cells. We treated KAIMRC1 cells with a 1μM concentration of Doxorubicin alone or encapsulated in the nanoparticles. As visible in Fig. 3 b, post-treatment to doxorubicin-loaded nanoparticles (Dox-NPs) [22], cells were imaged using SEM, and Dox-NPs were found on the surface of the cell cytoplasm (white arrows) as well as in the periphery. Similar results were obtained when cells were treated with Doxorubicin alone.

In Fig. 4, KAIMRC1 cell plasma membranes exhibit complicated folding and seem to be studded with many pinocytotic vesicles or caveolae-like structures (white arrows). These structures are commonly associated with sites of mitochondria [23]. These small pores in Fig. 4 b and c that are found sporadically spread all over the cell membranes may also be microvilli bases [24]. Primary breast cancer cells grown on carbon tapes are also showcasing the interaction between each other in great detail. Fig. 4 e and f show the movement of these cells towards each other. Filopodia are also visible protruding from one cell to another cell. These filopodia are actin polymerization sites [25] and responsible for cell movement and communication.

Last but not least, Fig. 5 display a necrotic and an apoptotic cell. A necrotic cell is typically represented by a damaged plasma membrane with large holes, whereas bleb formation on a cell's surface is a hallmark of apoptosis. These two images are an excellent example of our simple and straightforward approach resulting in high quality and resolution images of biological samples.

Conclusion

In conclusion, we have presented a simple, fast, and easy procedure to prepare adherent and non-adherent biological samples for SEM imaging. This protocol can be followed easily on a routine basis by cell/molecular biologists interested in studying ultra-structural changes in the cells.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Authors' contributions

RA conceived the idea, designed and performed the experiments. RA wrote the manuscript. KHB provided the NPs. MB provided the access to the facility. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

Privacy and confidentiality were completely protected, no identifiers or personal information was collected or stored including the patient's name, IDs, and others. Ethical approval and consent form was approved by the institutional review board (IRB) of the King Abdullah International Medical Research Center (KAIMRC).

References

[1] A. Bogner, P.H. Jouneau, G. Thollet, D. Basset, C. Gauthier, A history of scanning electron microscopy developments: Towards "wet-STEM" imaging, Micron (2007), doi:10.1016/j.micron.2006.06.008.
[2] S. Amelinckx, D. Van Dyck, J. Van Landuyt, G. Van Tendeloo, Electron Microscopy: Principles and Fundamentals, 2007, doi:10.1002/9783527614561.
[3] A.M. Kashi, K. Tahermanesh, S. Chaichian, M.T. Joghataei, How to Prepare Biological Samples and Live, 3 (2014) 63–80.
[4] A. Katsen-Globa, N. Puetz, M.M. Gepp, J.C. Neubauer, H. Zimmermann, Study of SEM preparation artefacts with correlative microscopy: cell shrinkage of adherent cells by HMDS-drying, Scanning 38 (2016) 625–633, doi:10.1002/sca.21310.
[5] A.K. Pathan, J. Bond, R.E. Gaskin, Sample preparation for SEM of plant surfaces, Mater. Today (2009), doi:10.1016/S1369-7021(10)70143-7.
[6] M. Kalab, Artefacts in Conventional Scanning Electron Microscopy of Some Milk Products, J. Food Struct. 3 (1984) 95–111 http://digitalcommons.usu.edu/foodmicrostructure/vol3/iss2/2.
[7] Y. Jusman, S.C. Ng, N. Azuan, A. Osman, Investigation ofCPD and HMDS samplepreparation techniques for cervical cells indeveloping computer aided screening system based on FE-SEM /EDX, 2014 (2014).
[8] F. Braet, R. De Zanger, E. Wisse, Drying cells for SEM, AFM and TEM by hexamethyldisilazane: a study on hepatic endothelial cells, J. Microsc. 186 (1997) 84–87, doi:10.1046/j.1365-2818.1997.1940755.x.
[9] J.L. Nation, A new method using hexamethyldisilazane for preparation of soft insect tissues for scanning electron microscopy, Biotech. Histochem. (1983), doi:10.3109/10520298309066811.
[10] A.I. Teixeira, Epithelial contact guidance on well-defined micro- and nanostructured substrates, J. Cell Sci. (2003), doi:10.1242/jcs.00383.
[11] J.C. Araujo, F.C. Téran, R.A. Oliveira, E.A.A. Nour, M.A.P. Montenegro, J.R. Campos, R.F. Vazoller, Comparison of hexamethyldisilazane and critical point drying treatments for SEM analysis of anaerobic biofilms and granular sludge, J. Electron Microsc. (Tokyo) (2003), doi:10.1093/jmicro/52.4.429.
[12] D.N. Leonard, G.W. Chandler, S. Seraphin, Scanning Electron Microscopy, Charact. Mater. (2012), doi:10.1002/0471266965.com081.pub2.
[13] E.R. Fischer, B.T. Hansen, V. Nair, F.H. Hoyt, D.W. Dorward, Scanning electron microscopy, Curr. Protoc. Microbiol. (2012), doi:10.1002/9780471729259.mc02b02s25.
[14] J. R., BG Nordestgaard, Critical-point drying versus freeze drying for scanning electron microscopy: a quantitative and qualitative study on isolated hepatocytes, J. Microsc. 135 (1985) 189–207.
[15] H.P. Ting-Beall, D.V. Zhelev, R.M. Hochmuth, Comparison of different drying procedures for scanning electron microscopy using human leukocytes, Microsc. Res. Tech. 32 (1995) 357–361 https://doi.org/https://doi.org/, doi:10.1002/jemt.1070320409.
[16] A. Osahor, K. Deekonda, C.-W. Lee, E.U.-H. Sim, A. Radu, K. Narayanan, Rapid preparation of adherent mammalian cells for basic scanning electron microscopy (SEM) analysis, Anal. Biochem. 534 (2017) 46–48, doi:10.1016/j.ab.2017.07.008.
[17] R. Ali, N. Samman, H. Al Zahrani, A. Nehdi, S. Rahman, A.L. Khan, M. Al Balwi, L.A. Alriyees, M. Alzaid, A. Al Askar, M. Boudjelal, Isolation and characterization of a new naturally immortalized human breast carcinoma cell line, KAIMRC1, BMC Cancer 17 (2017), doi:10.1186/s12885-017-3812-5.
[18] C. Heckman, S. Kanagasundaram, M. Cayer, J. Paige, Preparation of cultured cells for scanning electron microscope, Protoc. Exch. (2007), doi:10.1038/nprot.2007.504.
[19] C.G. Golding, L.L. Lamboo, D.R. Beniac, T.F. Booth, The scanning electron microscope in microbiology and diagnosis of infectious disease, Sci. Rep. 6 (2016) 1–8, doi:10.1038/srep26516.

[20] J. Goldstein, D.E. Newbury, D.C. Joy, C.E. Lyman, P. Echlin, E. Lifshin, L. Sawyer, J.R. Michael, Scanning Electron Microscopy and X-ray Microanalysis, 2003. doi:10.1007/978-1-4615-0215-9.
[21] W.F. Chissoe, E.L. Vezey, J.H. Skvarla, Removal of Double-Stick Carbon Tape from Scanning Electron Microscope Specimen Holders, Proc. Okla. Acad. Sci. 83 (2003) 79–81.
[22] K. El-Boubbou, R. Ali, H.M. Bahhari, K.O. Alsaad, A. Nehdi, M. Boudjelal, A. Alkushi, Magnetic Fluorescent Nanoformulation for Intracellular Drug Delivery to Human Breast Cancer, Primary Tumors, and Tumor Biopsies: Beyond Targeting Expectations, Bioconjug. Chem. (2016), doi:10.1021/acs.bioconjchem.6b00257.
[23] R.A.B. Burton, E.A. Rog-Zielinska, A.D. Corbett, R. Peyronnet, I. Bodi, M. Fink, J. Sheldon, A. Hoenger, S.C. Calaghan, G. Bub, P. Kohl, Caveolae in Rabbit Ventricular Myocytes: Distribution and Dynamic Diminution after Cell Isolation, Biophys. J. 113 (2017) 1047–1059, doi:10.1016/j.bpj.2017.07.026.
[24] H. Fukudome, K. Tanaka, a_method_for_observing_intracellular_structures_of_free_cells_by_SEM.pdf, 141 (1986) 171–178.
[25] M. Nemethova, S. Auinger, J.V. Small, Building the actin cytoskeleton: Filopodia contribute to the construction of contractile bundles in the lamella, J. Cell Biol. (2008), doi:10.1083/jcb.200709134.