

# Applications of a Novel SEM Technique for the Analysis of Hydrated Samples

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## BIOGRAPHY

Vered Behar specializes in molecular and cellular biology research. She holds a PhD in medical sciences from Harvard University and carried out her post-doctoral training at Wyeth-Ayerst in Boston, MA. At QuantomiX, Dr Behar is the Director of Scientific Application Development and she manages the research of new applications in a wide array of life science fields.



## ABSTRACT

This article describes a novel electron microscope methodology that enables high-resolution imaging of fully hydrated samples. This technique involves separation of the sample from the vacuum by an electron-transparent yet robust membrane. Imaging is based on detection of backscattered electrons resulting in the capability to see a defined sample volume proximal to the membrane. Consequently, unsectioned samples can be used, allowing simultaneous viewing of global and local structures. As there is no requirement for sectioning, sample preparation is both simple and rapid, making this technique suitable for economic, high-throughput research in the materials, medical and biological sciences. Examples of medical and biological applications of this technology are described here.

## KEYWORDS

scanning electron microscopy, wet samples, cell biology, gold immunolabelling, brain tumour, Crohn's disease

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## INTRODUCTION

Electron microscopy (EM) of fully hydrated samples has been an elusive goal. Imaging of wet tissues at high resolution is necessary to facilitate and advance studies in the materials, medical and biological sciences.

We describe here an innovative method to view wet samples using a scanning electron microscope whereby a thin membranous partition separates the sample from the vacuum. This membrane has two crucial properties; it is practically transparent to the electron beam and yet is tough enough to withstand atmospheric pressure on one side and high vacuum on the other.

We have developed novel products and protocols for SEM of fully hydrated samples based on this approach. Imaging is performed using a standard scanning electron microscope combined with a backscattered electron detector (Fig 1). It is possible to detect readily contrast between materials of low atomic number, such as carbon and oxygen. However, staining samples with electron-dense stains or nanoparticle gold labels improves image resolution. Detailed evaluation of the membrane properties and of the resolution capabilities of our technique have been described elsewhere [2, 3]. In this article we summarize applications of this novel technology (WETSEM™) in the life sciences.

Our investigations into applications of the wet SEM technique have highlighted several general advantages [2-7]. The imaged volume is limited to that in close proximity with the membrane, typically a few micrometres deep. This is due to our method of imaging, based on detection of backscattered electrons (BSE), which result only from interactions of the elec-

tron beam with a thin membrane-proximal layer. Accordingly, any material beyond the layer of beam penetration is effectively invisible. Consequently, samples a few millimeters thick, for example tissue specimens, can be imaged without any time-consuming, costly processing such as thin sectioning, embedding or freezing.

The ability to view unsectioned tissue specimens in this way, unlike transmission electron microscopy (TEM) which samples only very thin and sometimes arbitrary sections, means this technique has the potential to reveal new detailed structural information concerning the connectivity and organization of cells and extracellular structures. Therefore, in contrast to secondary electron detection employed in traditional SEM that limits visualization to the sample surface, BSE detection in the wet SEM technique enables rapid, informative examination of defined sample depth. Furthermore, the physical position of the imaged area makes this technique ideal for the inspection of objects that are stuck to the membrane surface, such as adherent biological cells. In particular, it permits analysis of whole cells, giving information on organelles and internal structure. To our surprise, characterization of the optimum resolution of this technique exceeded calculated expectations and can be in the order of 10 nm, approximately 20 times that is possible with light microscopy.

In summary, the new wet SEM technology combines the simple, rapid sample preparation of light microscopy with the high resolution capability of EMs, and provides an ideal solution for immediate high-resolution imaging of wet samples without dehydration artifacts.

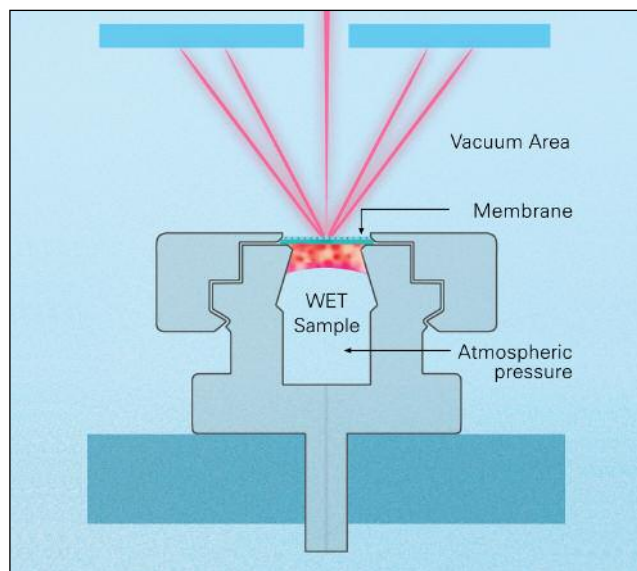


Figure 1:  
Schematic representation of the  
WETSEM™ system.

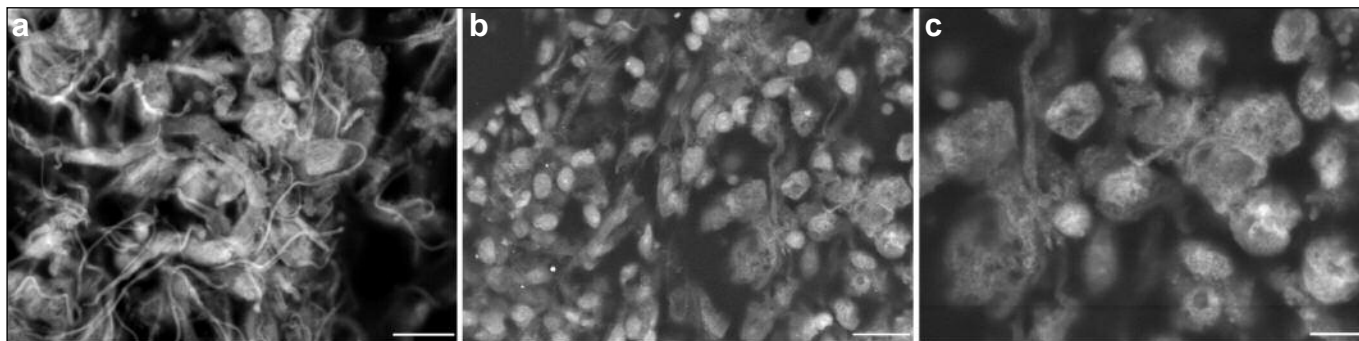


Figure 2: Wet SEM of brain tumours. (a) Fibrillary astrocytoma. Scale bar: 10  $\mu\text{m}$ . (b,c) Ependymoma. Scale bars: 20  $\mu\text{m}$  (b); 10  $\mu\text{m}$  (c). All samples were fixed in 10% buffered formalin and stained with 0.1% uranyl acetate for 10 min.

## RESULTS AND DISCUSSION

### Medical science

At present, EM is used to characterize definitively a small but significant proportion of clinical presentations (3-8%), especially of cancer and non-neoplastic renal disease [8,9]. These numbers probably underestimate the potential contribution of EM, since its usage is limited not only by lack of utility in many clinical situations, but also by cost, time required to produce results and relatively low output compared to histological methods. We propose that the wet SEM technique has the potential to resolve the latter three issues, as it is particularly amenable to immediate, simple, economic tissue specimen preparation and imaging. Two examples of diseases where this technology may improve diagnoses and/or drug development are described below.

### Brain tumours

EM is an essential tool in modern diagnostic neuropathology [9]. In particular, intraoperative differentiation between astrocytic and ependymal tumours is crucial because diagnosis of the former prompts termination of the procedure, while in the case of ependymal tumours, total excision is the only chance of a cure. Differentiation using frozen sections can be difficult, therefore a complementary, yet alternative technique for rapid pathological examination is warranted.

We carried out a study to compare the pathological features of three central nervous system (CNS) tumours using light microscopy, TEM and the wet SEM technique [7]. We found

that our technique did indeed reveal distinguishing features of each CNS tumour: numerous fibrillary processes were evident in fibrillary astrocytoma (Fig. 2a); rosette formation (Fig. 2b) and intracytoplasmic lumens (Fig. 2c) were observed in ependymoma. Evaluation of a larger series of specimens is required to optimize sample preparation and to verify that wet SEM enables differentiation between the CNS tumours.

### Crohn's disease

Crohn's disease is a particular type of inflammatory bowel disease (IBD). Traditional SEM has been used to characterize biopsied tissues for early pathological changes that are not visible macroscopically (for example see [10]). We propose that the wet SEM technique, due to its rapidity, simplicity and high resolution, may prove to be significantly more useful in clinical and pre-clinical studies of IBD. In support of this premise, a preliminary study comparing three normal large bowel specimens to three taken from patients diagnosed with Crohn's disease revealed easily-identifiable, distinctive changes in the diseased samples. Marked irregularity of cell borders with elongation of the crypt orifices were observed [6].

### Biological science

The need to dehydrate and lengthily prepare samples before viewing in an EM has long been a serious limitation of cell research. Our technique provides the solution to this problem and opens the door to many biological studies. As explained above, detailed visualization of both intercellular and intracellular

structures is now possible without dehydration artifacts. We have chosen four examples that highlight different advantages of this imaging method.

In Figure 3 micrographs of uranyl acetate stained pancreatic tissue are shown. A blood vessel is visible in the centre of the image; its wall and erythrocytes in its lumen are readily identifiable. The wet SEM technique allows both an overview of the tissue structure (Fig. 3a), as well as detailed examination of specific features (Figs 3b and 3c). An alternative staining method that does not require uranyl acetate is phosphotungstic acid (PTA) staining. In Figure 4 PTA staining is used to reveal intracellular structure of C2C12 cells. The tubular network and pleomorphic forms of mitochondria are clearly seen. PTA staining is also used to stain the HeLa cells in the images shown in Figure 5. The focus of these micrographs is intercellular structures, filopodia, that are mediators of cell-cell contact. In addition to viewing the connections between several HeLa cells at low magnification (Fig. 5a), it is easy to concentrate on and trace particular contacts at higher magnifications (Figs 5b and 5c). Finally, Figure 6 exemplifies the biological details that can be seen when using gold nanoparticle labelling in combination with the wet SEM technique. Epidermal growth factor receptors (EGFRs) of intact A431 cancer cells have been visualized using 40-nm gold-labelled monoclonal antibodies that recognize only EGFRs. The cells are also counterstained with uranyl acetate, which enables alignment of the labeled receptors with the general structure of the cell. The high contrast and uni-

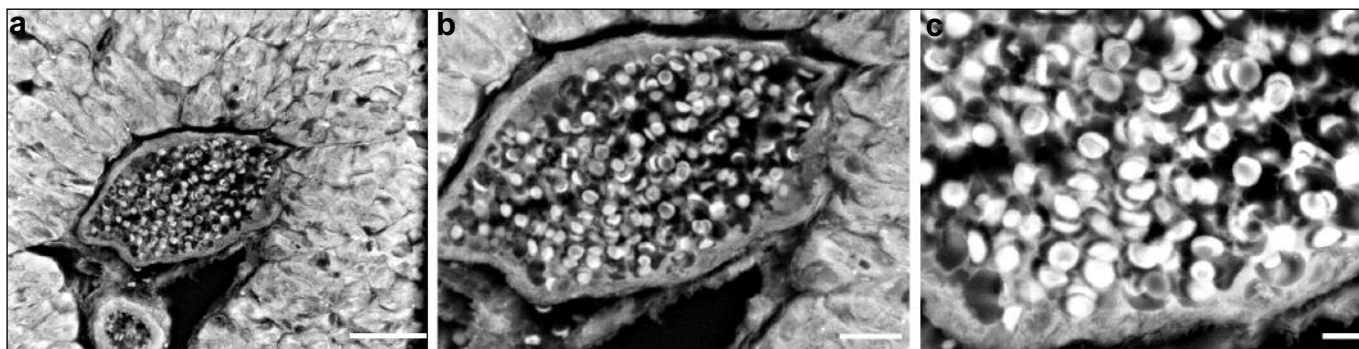


Figure 3: Wet SEM of pancreatic tissue; three magnifications of the same area are shown. Scale bars: (a) 50  $\mu\text{m}$ , (b) 20  $\mu\text{m}$ , (c) 10  $\mu\text{m}$ . All samples were fixed in 10% buffered formalin and stained with 0.1% uranyl acetate for 10 min.

form size of the nanoparticles ensure unambiguous identification and localization at the level of single nanoparticles. Importantly, unlike TEM, which samples only very thin and sometimes arbitrary sections, our technique allows quick switching between the local (Fig. 6b) and global view (Fig. 6a) of nanoparticle labelling all over the cell. Thus, in a way that has not been possible before, the wet SEM methodology facilitates imaging research aimed at understanding cellular events at the molecular level.

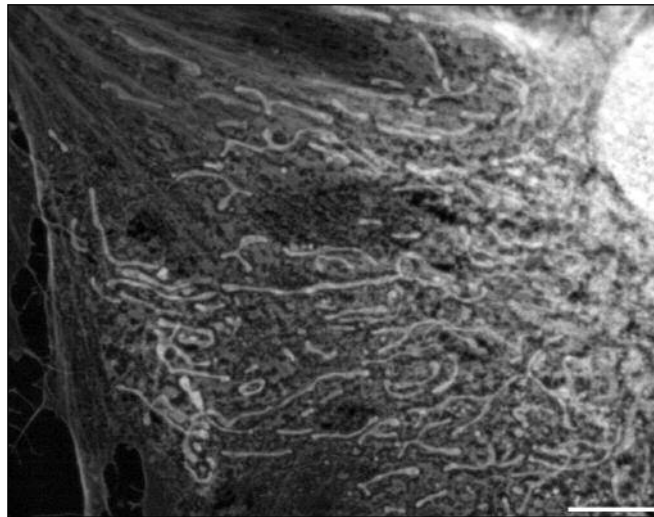
## CONCLUSIONS

The advantages of the new technique include the quality or extent of the resulting information, and a reduction in time, complexity and expense involved. The wet SEM methodology offers each of these advantages as demonstrated in this brief review: it is an innovative technology that facilitates economic, simple, rapid, high-resolution imaging of fully hydrated samples in a way that has not been possible before. Due to the ability to use unsectioned samples, it is possible to image simultaneously both the global and local structures of tissue specimens or cells. Applications for the life sciences are now being realized.

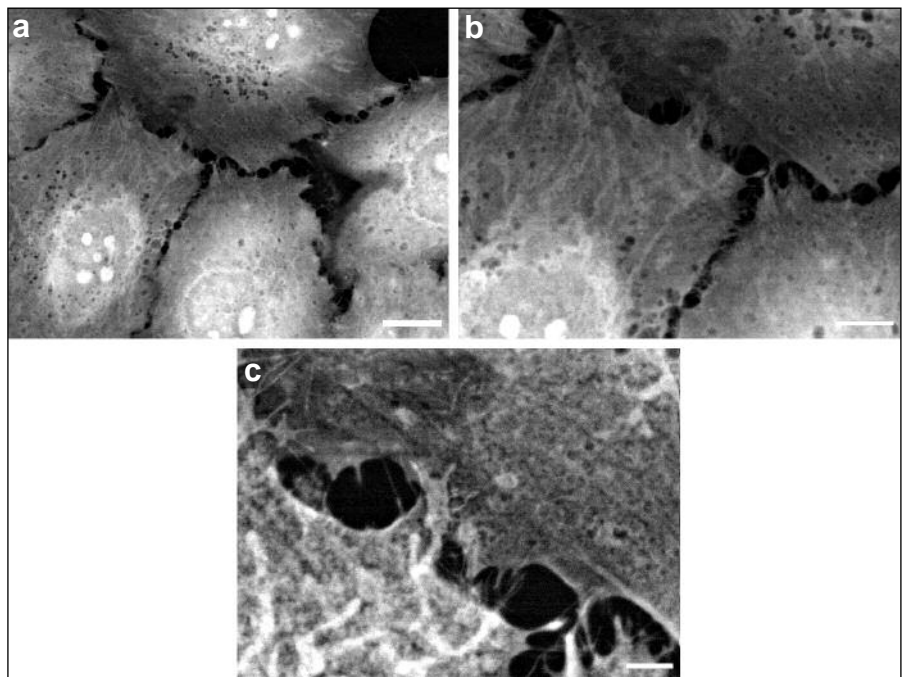
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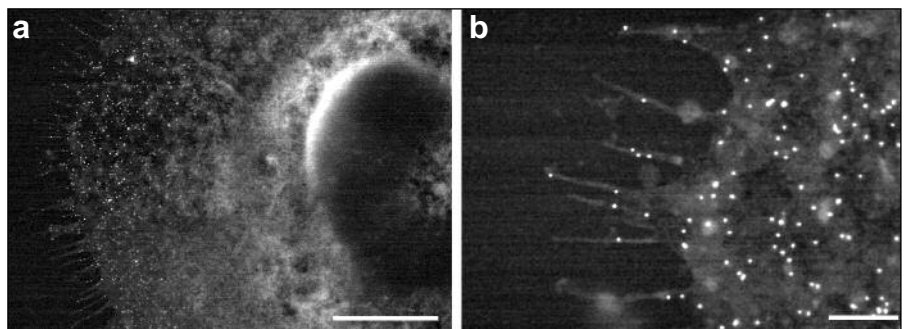
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**Figure 4:**  
Wet SEM of C2C12 cell. Cells were plated on the capsules and grown at 37°C in 5% CO<sub>2</sub> overnight. Cells were washed with PBS, fixed with 2% glutaraldehyde and stained with 2% phosphotungstic acid for 30 minutes. Scale bar: 5 μm.



**Figure 5:**  
Wet SEM of HeLa cells; three magnifications of the same area are shown. Scale bars: (a) 10 μm (b) 5 μm (c) 2 μm. HeLa cells were plated on the capsules and grown at 37°C in 5% CO<sub>2</sub> overnight. Cells were washed with PBS, fixed with 2% glutaraldehyde and stained with 2% phosphotungstic acid for 30 minutes.



**Figure 6:**  
Wet SEM of A431 cells showing distribution of epidermal growth factor receptors. Scale bars: (a) 10 μm (b) 2 μm. A431 cells were plated on the capsules and grown at 37°C in 5% CO<sub>2</sub> overnight. Cells were washed with PBS, fixed with 4% formaldehyde and immunolabelled with epithelial growth factor receptor monoclonal antibody. After washing with BSA-PBS the cells were incubated with 40 nm gold-conjugated antibody, and stained with uranyl acetate.