Variable Pressure-SEM: a versatile tool for visualization of hydrated and non-conductive specimens

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Variable Pressure Scanning Electron Microscopy (VP-SEM) provides a valuable tool for novel applications in life sciences, medicine, material sciences and engineering. VP-SEM can solve challenges in visualization of in situ conditions, precious devices, specimens used in correlative imaging, and evaluation of new tools and 3-dimensional SEM applications. Since no sample preparation is required, and specimens can be imaged without a conductive coating, VP-SEM has found niche applications, often in conjunction with conventional SEM, or combined with other imaging modalities. In hydrated samples, VP-SEM applications range from biomaterial scaffolds in bioengineering and regenerative surgery, to microbial biofilms in environmental and medical microbiology. Since VP-SEM provides charge neutralization in non-conductive specimens, dry samples with limited conductivity may be imaged without a conductive metal coating. This is especially beneficial to experimentation in geophysical sciences, nanotechnology, correlative microscopy using different imaging modalities, novel applications in volume SEM, and atmosphere sensitive samples.

Keywords: Variable Pressure-SEM; Scanning Electron Microscopy; hydrogels; non-conductive specimens

1. Introduction and Background

Very early after the birth of scanning electron microscopy in the 1930s and 40s, researchers started experimenting with alternative techniques to overcome the preparation requirements for biological specimens, while at the same time retaining superb high resolution imaging with short-wave electrons, and large depth of field, as well as additional analytical signals typical for SEM [1]. Differentially pumped, aperture-limited environmental chambers and thin-film electron-transparent windows were designed [2, 3], while environmental control stages [4] and the use of various gases (hydrogen, oxygen, nitrogen and noble gases) and high pressures in the specimen chamber were demonstrated, until stable imaging of liquid water was accomplished - albeit under fairly low magnifications (100 - 2,000x) [5-9]. Since the 1990s commercially available systems coined the terms ‘Variable Pressure’, ‘Extended Pressure’ and ‘Environmental’ SEM. Though these terms are not strictly applied, ‘Variable Pressure’ (and Extended Pressures) mostly implies imaging in a gas environment for charge control, while ‘Environmental’ includes imaging with water and hydrated samples, often with a temperature-controlled stage. Various approaches have been followed for VP-SEM signal detection, ranging from ions, gas luminescence, gas-amplified secondary electrons, and conventional Everhart-Thornley, Secondary Electrons (ET-SE) and Back-Scattered Electron (BSE) detection [10-15].

Due to this development, today’s commercially available VP-SEM has a differential pumping system that allows imaging under extended pressures and moisture inside the specimen chamber, has charge stabilization for non-conductive samples, and further allows for thermodynamic stabilization of hydrated samples. It can both be described as a ‘Lab in a Chamber’ for in situ experimentation under hydrated conditions and a versatile tool for visualization of specimens from both materials and biological sciences. Through innovative applications, VP-SEM has become integrated into various cutting edge imaging technologies in volume electron microscopy, automated imaging and correlative microscopies.

2. Practical Methods and Principles

2.1 Specimen preparation:

Little or no specimen preparation is generally needed for VP-SEM application, and fully hydrated and out-gassing samples can be visualized since the vacuum environment in the specimen chamber is separated from the high-vacuum environment in the upper column by a differential pumping system, with pressure limiting apertures and turbo-isolation valves [16]. Non-conductive biological specimens may be prepared as for conventional SEM [17,18] using aldehyde primary fixatives and critical point drying (CPD) or chemical drying with hexamethyldisilazane (HMDS). After mounting no additional conductive coating is needed, and the typical Gold/Palladium sputter-coating (or alternatively Iridium, Platinum, Silver) or Carbon evaporation, is not required since charge neutralization is provided by ionization of gas inside the specimen chamber. This has a wide range of benefits in experimental applications (see Section 3). When post-fixation with osmiumtetroxide (OsO₄) is included, some conductivity may be provided in situ by the included heavy metal.
Heavy-metal infiltration, or natural inclusion, also renders specimens more conductive for dry VP-SEM application, where specimens with limited conductivity are mounted in their natural state and without initial preparation, onto standard aluminium stubs for VP-SEM imaging without traditional metal coatings.

### 2.2: Specimen mounting and SEM operation for hydrated samples:

VP-SEM combined with Peltier coolstage control optimally allow high-resolution imaging of hydrated specimens under varying pressure (typically 50-60Pa) conditions, by limiting moisture loss through control of stage temperature. Samples are mounted in a thin film of water onto a 10mm cup-shaped stub fitting a cooling stage, which is connected to a Peltier pump. Conditions are then controlled to limit water-loss while optimizing resolution [18,19]. Initial stage temperature is set at 4°C, and pressure and temperature comparatively decreased to 60Pa/-25°C. Decreasing temperature too rapidly may result in the formation of obstructive ice crystals. Salts from retained buffer may have a similar effect when dehydration occurs if pressure is decreased too rapidly. Where BackScattered Electrons (BSE) provide the required signal for VP-SEM imaging, high accelerating voltages are used (typically 15kV for Hitachi S3400N VP-SEM) when dehydration occurs if pressure is decreased too rapidly. Where BackScattered Electrons (BSE) provide the required signal for VP-SEM imaging, high accelerating voltages are used (typically 15kV for Hitachi S3400N VP-SEM) and even though the filament can be turned on at higher pressures (270Pa), SNR (Signal to Noise Ratio) is poor under these conditions, and by limiting gas in the specimen chamber through lower pressures, one can significantly enhance SNR and thereby improve resolution. The optimal working distance with hydrated samples is often lower than for dry conditions, since escaping air-bubbles from the moist environment, as well as beam interaction with the specimen, may cause the sample to inflate, and increase in height. This may contaminate the BSE detector which is generally mounted below the pole piece and directly above the specimen. A practical precaution is to raise the stage only after evacuation of chamber to the preferred imaging conditions (60Pa/-25°C).

### 2.3 Staining of hydrated samples:

VP-SEM instruments generally include BSE detection in commercial applications, and with the signal consequently related to the atomic weight of the specimen, the researcher can benefit by exploring various heavy metals to infiltrate the specimen before mounting and imaging. Inclusion of EM applied metal stains, such as osmium (as OsO₄), ruthenium (as Ruthenium Red and RuO₄) or uranium (as Uranyl Acetate) in VP applications therefore not only will provide stabilization (fixation) of biological components, but also improve contrast and resolution. Considering the specificity of various metal components to bind with different molecules, enhanced characterization of sample composition can additionally be accomplished.

Osmiumtetroxide (OsO₄): OsO₄ is used in conventional EM to provide excellent preservation of fine features. It binds specifically to lipids [18], thus improving contrast and resolution especially in phospholipid bilayers of cell membranes and cell organelles, in addition to lipid-containing inclusions and particles. In VP applications, OsO₄ has been used with great success to enhance contrast in cells, which enables localization of cells against a hydrogel scaffold (see Fig.2B) [19-21]. It was also recently described as an excellent fixative and contrasting agent in hydrated fungal biofilms [22] where it highlights inclusion of lipid droplets and improves contrast and resolution (see Fig.1B). Specimens may be initially fixed in aldehydes (paraformaldehyde and glutaraldehyde) to cross-link proteins, whereafter 30min to 1hr incubation in 1% OsO₄ will provide the required preservation of delicate features and contrasting enhancement. It is very important to rinse samples well (5x3mins) in deionized water to remove all traces of OsO₄, which is highly toxic. Always stain in a fume hood and dispose of heavy metal reagents as required.

Ruthenium Red: Ruthenium Red has been used over decades as a cell wall stain in EM [23]. Recently it was described as contrasting agent for VP-SEM imaging of bacterial [24] as well as fungal biofilms [22] (Fig.1). It is a cation that specifically binds to polyanionic constituents of the extracellular matrix [25, 26], and has been described to bind not only to phospholipid membranes, but also associates with Ca²⁺-binding proteins. It therefore provides excellent staining of both ECM and hyphal components of Aspergillus fumigatus biofilms (Fig. 1A) due to its ability to bind to both the lipids and protein constituents of the biofilm. Since the composition of A. fumigatus biofilms were recently determined with solid-state MNR [27], researchers could purposely apply heavy metal stains to enhance and characterize these biofilm components. Similar to OsO₄, Ruthenium Red staining is done in an aqueous solution (0.01% Ruthenium Red), followed by repeated rinsing before SEM imaging. Ruthenium Red staining can be followed by OsO₄ staining [22, 24] with increased contrast and stability of delicate features (Fig.1B).

Ruthenium Tetroxide (RuO₄): This metal contrasting agent is closely related to OsO₄, fixes membranes and polymeric materials and was recently applied for VP-SEM of hydrated specimens [22] (Fig.1C). It has been described as more vigorous than OsO₄, reacting more strongly with polar lipids and staining proteins, glycopen and monosaccharides [27,28]. RuO₄ is a metal stain that has not been as frequently used as other contrasting agents, but reviving it from the archives it provides a valuable addition to the variety of stains available to enhance contrast of hydrated specimens for VP-SEM. Not only does it stain a much wider variety of cellular and non-cellular constituents, as well as abiologic polymeric substances, it is also not as toxic as OsO₄ and more ‘user-friendly’. RuO₄ staining is done in an aqueous solution (0.5% RuO₄), followed by repeated rinsing before SEM imaging.
Other stains: Alcian Blue, Safranin O, L-lysine, [27,30,31], Uranyl Acetate, Phosphotungstic Acid, Tannic Acid [32,33] and similar ‘trusted’ stains of both light and electron microscopy can be applied for different applications in VP-SEM. Especially where the chemical composition of a specimen is known, fixatives and contrasting agents can be applied aimed at preservation and characterization of specific chemical constituents. In VP-SEM both the chemistry of the specimen and the physics of the imaging environment are of equal importance to optimize imaging conditions and resolution.

Fig 1: Staining of hydrated fungal (Aspergillus fumigatus) biofilms with (A) Ruthenium Red, (B) Ruthenium Red with OsO4 and (C) RuO4, illustrate enhancement of contrast and resolution with metal reagents, resulting in increased stability of delicate features and characterization of biofilm constituents. Long arrows = apparent extracellular matrix components; Short arrow = lipid inclusions.

3. Applications

3.1 Hydrated Specimens:

3.1.1 Hydrogels (scaffolds with and without cells):

Hydrogels are cross-linked, water-swollen polymeric structures that contain either covalent bonds produced by the simple reaction of co-monomers, physical cross-links from entanglements, association bonds, or strong van der Waals interactions [34, 35]. Hydrogel biomaterials show exceptional promise in biomedical applications and are used in reconstructive surgery, as bioadhesives and as controlled drug release devices where they do not cause the typical immunological reactions that occur with organ transplants [36-38]. Since the physical behaviour of hydrogels (including solute diffusion coefficient, surface and optical properties) depends on their equilibrium and dynamic swelling behaviour in water, visualization in a moisture-saturated environment is of utmost importance to reveal their natural in situ characteristics. Since conventional SEM requires specimens to be completely dry (using ethanol dehydration, CPD, HMDS or freeze-drying) SEM had limited application for hydrogel analysis. VP-SEM therefore provides a promising technology to characterize hydrogels in their natural state. Features that are generally analysed include porosity, alignment, 3D morphology and distribution of included nanoparticles in protein scaffolds [19] (Fig. 2A). Cell proliferation on fully hydrated hydrogel scaffolds has been thoroughly explored using VP-SEM [20, 21, 39, 40] with surface properties, cell junctions, structural porosity and alignment, as well as 3D proliferation of various cell types characterized. Hydrogel scaffolds include protein and 3D polystyrene scaffolds, collagen fibres, gelatine ribbons, Matrigel, PCL (polycaprolactone) and PDMS (polydimethylsiloxane), as well as carbohydrate scaffolds of pullulan and chitosan. HESC (human embryonic stem cells), ASCs (adipose tissue-derived stem cells), MSCs (bone-marrow-derived mesenchymal stem cells), cardiomyocytes, cochlear stereocilia, endothelial cells, fibroblasts and neurons are some of the cell types that were already successfully cultured on hydrogel scaffolds for tissue-engineering application. To stabilize cells that are growing on hydrogel scaffolds, primary fixation with aldehydes (PFA and Glutaraldehyde) is recommended to cross-link proteins and other bio-active moieties before SEM visualization. Additional post-fixation with heavy-metal stains (see Section 2.3) can enhance detection and identification of cells on hydrogel scaffolds. Since the heavy-metal reagent will bind specifically to cellular constituents (e.g. lipids with OsO4), a stronger BSE signal (an increased number of BSE) will be associated with cells due to the higher atomic weight of the metal reagent, which
results in cells being brightly contrasted on the scaffold surface (Fig. 2B). Comparison of VP-SEM with specimens prepared using conventional SEM techniques may provide additional valuable information on drying effects. Correlating VP-SEM imaging with FM (fluorescence microscopy) and AFM (atomic force microscopy) can strengthen results and provide additional quantitative data on viability and physical properties of cells on hydrogels.

![Image](https://example.com/image.jpg)

**Fig. 2:** (A) Hydrogel scaffolds without cells, and (B) seeded with cells which were contrasted with OsO₄ before VP-SEM imaging. (C) Nanotechnology template with cultured neurons illustrate contrast enhancement by heavy metal (OsO₄) staining

### 3.1.2 Environmental applications/Biofilms:

Microbial biofilms are communities of bacteria and fungi, characterized by their hydrated, self-produced extracellular matrix which provides a ‘house’ for microbes in their attached lifestyle [41]. They have been widely implicated in biotechnological and medical applications, including biofouling, soil and wastewater remediation, biocatalysis, biogeochemical cycling, persistent infections, antimicrobial resistance and complex food webs [42-45]. In their biofilm mode of growth, microbes exhibit emergent properties that differ substantially from their free-living counterparts. Since the biofilm matrix plays a fundamental role in establishing biofilm properties, and consists of up to 97% water, it is important to characterize microbial biofilms in their natural hydrated state. Conventional high-vacuum SEM requires desiccation of specimens, which cause a collapse of EPS (Extracellular Polymeric Substance) matrices, resulting in deformed biofilm architecture and appearance. VP-SEM has recently been described as a valuable high-resolution alternative to characterize biofilms in their in situ hydrated state [22, 24, 43]. Since the EPS matrix has limited electron density, appropriate staining using various heavy metals can enhance contrast and resolution, and provide additional information on matrix constituents. Bacterial [24, 46-48] and fungal [22] biofilms have recently been characterized using VP-SEM after staining of cellular and extracellular matrix components with OsO₄, RuO₄ and Ruthenium Red (see Section 2.3). Additional cationic (alcian blue, safranin) and anionic dyes (Ponceau 2R, orange G, Biebrick scarlet) have been suggested to stain the glycocalyx (extracellular matrix) of microorganisms [26]. Since the extracellular matrix if often poorly characterized, it may be necessary to vary time and dye concentrations for application to different microbes – suggesting a ‘trial-and-error-approach’ in staining the biofilm matrix. Biofilms may be fixed with aldehydes (PFA and Glutaraldehyde) to cross-link proteins before contrasting with various stains. When the composition of the extracellular matrix has been characterized [27], staining can be done to highlight specific matrix constituents.

### 3.1.3 Cells on nanotechnological and valuable devices:

Cells grown on novel nanotechnological devices that are developed as molecular tools, or for cellular and tissue applications, can be imaged fully hydrated and without conventional processing and heavy-metal coating, which might render the device unsuitable for further experimentation. Structures that are evaluated with great success using VP-SEM, include retinal implants, physiological probes, nanocones and nanopillars, or nanostraws supporting or penetrating cells [49]. Cells may be fixed in aldehydes, and contrasted with heavy metals (see Section 2.3) to enhance contrast for localization of cells on the substrate (Fig.2C). Where the substrate includes higher atomic weight elements, which is frequently the case in electronic devices containing silicon, nickel, lead, copper, zinc or chromium, conductivity and subsequently SNR is enhanced to increase resolution in VP-mode imaging. Cells can be sonicated or enzymatically removed from the substrate before further experimentation using the same device.

### 3.2 Non-conductive Dry Specimens

#### 3.2.1 Repeated imaging of ROIs during experimentation (Geophysics application):

In experiments where the effect of treatment needs to be evaluated repeatedly using the same region of interest (ROI), VP-SEM provides a valuable tool for high resolution imaging of non-conductive samples, without adding an interfering conductive coating between experimental runs. In a prominent case study, evaluation of changes in microstructure in carbonate rock and gas shale provides valuable evidence of the chemical and physical effects resulting from gas and liquid injection [50,51]. Porosity, particle size, dissolution of micrite and pitting of surfaces are linked to quantitative data of permeability, elastic stiffness, mass transfer, etc. Since repeated imaging of the same ROIs needs to be done
before and after injection of carbonated water, metal sputter-coating of samples is prohibited and VP-SEM provide the ultimate technology for high-resolution imaging of microgeometry before and after experimental treatment.

### 3.2.2 Atmosphere sensitive samples:

Imaging of atmosphere sensitive samples is challenging and mostly unattainable using surface sensitive techniques such as SEM, AFM or other microprobes. Characterization of hygroscopic deliquescent materials, such as those developed as scintillator detectors, demands special handling to preserve the surface after preparation. Since VP-SEM does not require specimen preparation, air-sensitive materials need only be transferred into the specimen chamber in an airtight container, which then has to open inside the SEM chamber during the pumping cycle. VP-mode surface scanning can start as soon as the specimen chamber has been evacuated to low pressures (10Pa). When technology allows, the specimen chamber can also be purged with nitrogen or argon to limit even brief exposure to air. In a specially designed air-tight container that consists of a metal canister, covered by a disposable 500 micron latex membrane and laterally positioned sharp needle, the microstructure of SrI2 ceramics was recently characterized using VP-SEM [52]. The latex membrane gradually inflates during controlled evacuation, which allows the needle to puncture the membrane seal. The stage can be raised to optimal working distance after inflation, piercing, rupture and retraction of the membrane. Once retracted, the ceramic surface is exposed to the electron beam under high voltages, suitable for VP-SEM imaging, with no pre-exposure to air.

### 3.2.3 Correlative imaging:

In correlative imaging (i.e., using different imaging modalities in sequence to correlate data from identical ROIs) great caution has to be taken when applying analytical techniques after conventional SEM, which includes heavy metal treatment and coating. For instance, in technologies that include XRF (X-ray fluorescence), EDS (Energy-dispersive X-ray spectroscopy) and EELS (Electron Energy loss spectroscopy), a sputter-coated conductive layer will interfere with the analytical (X-ray) signal and render data inaccurate. Non-conductive biological samples can however be evaluated in VP-SEM, with limited processing and no conductive coating. Samples can be removed from the SEM mount and directly used in X-ray applications. A recent case study applying both immunogold localization and synchrotron micro-XRF analysis to characterize micronutrients in the stomach ulcer bacterium *Helicobacter pylori*, highlights the precision and elegance of this approach [53].

### 3.3 Volume (3D) SEM applications:

Novel EM techniques have recently been developed for visualization of large 3-dimensional (3D) volumes of specimens at ultrastructural resolution [54]. In addition to advances in specimen preparation techniques, instrument development and automation, as well as computational tools are rapidly evolving. SBF (Serial BlockFace)-SEM, FIB (Focused Ion Beam)-SEM and AT (Array Tomography)-SEM or ss-SEM (serial section SEM) with associated ATUM (Automatic Tape-collecting UltraMicrotome) are some of the recent innovative techniques that emerged in SEM visualization [55-59]. In all these techniques specimens have to be embedded in resin after efficient fixation to stabilize all cellular constituents, and contrasting the cell content with a cocktail of heavy metal reagents [33, 57]. In SBF-SEM, post-staining techniques using Reynold’s Lead Citrate and Uranyl Acetate are typically used to enhance contrast, especially where metal stains were initially avoided to retain antigenicity in the specimen. Substrate and specimen conductivity can also be enhanced using carbon evaporation after sectioning and staining [33, 57].

Despite these precautions, large open areas inside the specimen (intra- and extracellular) result in regions that are less conductive than dense areas that reacted with heavy metal reagents to provide contrast and conductivity to the specimen. For example, blood vessels, pulmonary alveoli, vacuoles in plant cells and fungi, regions between embedded cells (or unicellular organisms), as well as ‘empty’ regions above cells that are growing on a substrate, are all examples of challenging non-conductive ROIs. These resin-filled insulating areas are particularly susceptible to imaging artefacts that may arise from the accumulation of local negative charge, especially where high-voltage imaging is required for BSE detection. Such charging artefacts can be reduced by working in a variable pressure environment, and introducing water vapour or nitrogen inside the SEM chamber. Where high vacuum operation increases electron-dose related artefacts of image distortion, shift and sectioning artefacts, VP-SEM operation may enable continued sectioning, imaging and 3D reconstruction, albeit at slower scan times (increased pixel dwell times) or potentially lower SNR.

Optimal specimen preparation for volume EM remains a lengthy process, and exhaustive testing of variables is necessary to find the best approach for each sample type [54]. In all these technologies, the sensitivity of the final block to the electron beam is of utmost importance for uninterrupted and large volume reconstruction without imaging artefacts. Fixation, contrasting and resin selection will primarily influence specimen composition and conductivity, and
designing more conductive resins will provide a valuable alternative to in situ (in chamber) 3D-SEM techniques. Currently the benefits of VP-SEM surpass all other options, and most commercially available 3D-SEM tools are now provided with high-resolution variable pressure functionality.

4. Conclusions

VP-SEM imaging has rightly been called ‘A Lab in a Chamber’ [1]. Since no strict protocols can be followed, and limited guidelines have until recently been provided, a trial-and-error approach is needed to solve research challenges with VP-SEM. In hydrated samples, VP-SEM application ranges from hydrated biomaterial scaffolds in bioengineering and regenerative surgery, to microbial biofilms in environmental and medical microbiology. Where conventional SEM requires desiccation of specimens, with associated deformation of surface features and 3D morphology of hydrated scaffolds, VP-SEM enables characterization of acellular biomaterial scaffolds as well as cell-seeded substrates in their in situ microenvironment. In hydrogels for tissue engineering, features of porosity, fibre alignment and cell proliferation are generally explored. The ultrastructure of scaffolds containing proteins may be preserved by aldehyde fixatives, which cross-links proteins and other bio-active moieties. Where scaffolds are seeded with cells, samples may be postfixed with OsO4 and other heavy metal reagents which not only stabilizes lipids, but also other cell moieties to enhance contrast and enable localization of cells on polymer scaffolds and nanotechnological devices. Since VP-SEM provides charge compensation in non-conductive specimens, dry samples may be imaged without sputter-coating with a conductive metal layer.

VP-SEM provides a niche modality for high resolution imaging in challenging experimental conditions. In the rapidly evolving area of correlative imaging, consecutive imaging modalities may not be mutually compatible with a conductive coating required by conventional SEM. Rapid and easy imaging by VP-SEM will allow acquisition of a data set to be correlated with fluorescence, X-ray or other imaging modalities. In novel 3D SEM applications non-conductive areas in cells and tissues that interfere with repeated scanning and slicing can be imaged under extended pressures in the specimen chamber. VP-SEM thus provides a superior tool for high resolution imaging at the interface of biological and engineering sciences, as well as a valuable technology to bridge the divide between basic, applied and clinical sciences. The future for electron microscopy and visualization sciences indeed appears bright when considering emerging technologies such as VP-SEM.

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References


