

# Comparison of environmental scanning electron microscopy in low vacuum or wet mode for the investigation of cell biomaterial interactions

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**Summary.** *Aim:* The aim of the present study was to investigate the efficacy of environmental scanning electron microscopy (ESEM), in low vacuum mode (LV-ESEM) and in wet mode (wet-ESEM) in the assessment of cell-material interactions. *Methods:* Mouse calvaria MC3T3 cells (ATCC) were seeded on commercially pure machined titanium discs of 10 mm diameter in Dulbecco modified MEM, 10% Fetal Bovine Serum, 1% Penicillin and Streptomycin and 1% Glutamine. Samples were then processed for microscope observation by rinse in Phosphate Buffer saline and fixation in 4.5% Glutaraldehyde. Samples were then rinsed in Sodium Cacodylate buffer and observed or dehydrated in alcohol prior to LV-ESEM observation. Fresh samples in 0.9% NaCl solution were observed in wet- ESEM. *Results:* No significant loss of detail was observed when dehydrated or non dehydrated samples were analysed at LV-ESEM. The observation of fresh samples in wet-ESEM however proved difficult for the need to eliminate water which forms a layer covering the sample, thus hiding cell surface details. When reducing the vapor pressure in the chamber, the layer evaporated and NaCl immediately started to precipitate and cells collapsed, thus no further investigation was possible. *Conclusions:* The use of low vacuum-ESEM after cell fixation, but without dehydration or gold sputter coating proved a viable alternative to traditional high vacuum SEM observation. ([www.actabiomedica.it](http://www.actabiomedica.it))

**Key words:** ESEM, titanium, biomaterials

## Introduction

Endosseous implants are medical devices that are inserted in bone with the purpose of anchoring a prosthesis to replace lost teeth or bone (1). To function, they require a sound integration with the surrounding tissue. Host cells must thus colonise the device and deposit new bone (2). It is therefore important to

create cell-supportive devices, and to this purpose, a deeper understanding of cell-biomaterial interactions is necessary. Morphological analysis of cells growing on biomaterial surfaces is often the first step to evaluate how they respond to the substrate, and this is usually performed by means of microscopic observations, which rely on techniques such as Scanning Electron Microscopy (SEM) (3-6). SEM observation has sig-

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nificant advantages over other methods, by allowing for the assessment of the surface characteristics of biomaterials, without the need to slice the sample as with conventional or electronic transmission microscopy (7). Traditional, high vacuum SEMs however require the application of a cumbersome sample preparation protocol, which involves the use of additional instruments: samples must be fixed, dehydrated in alcohol and sputter coated with gold or palladium. This processing, beside being lengthy and costly, can lead to artefacts that bias the evaluation of cell morphology. The introduction of more recent instruments, such as Environmental Scanning Electron Microscope (ESEM), able to operate in low vacuum mode (LV-ESEM) and in wet mode (wet-ESEM) permits the investigation of nonconductive and hydrated samples without the need of time consuming sample preparation and of additional processing steps (8).

The main goals of this study are to compare the morphology of murine osteoblasts on machined titanium surfaces for endosseous implants observed by LV-ESEM prepared according to two alternative processing protocols including fixation with or without alcohol dehydration and direct observation of samples in wet-ESEM mode without fixation.

## Materials and Methods

### *Titanium surfaces*

Commercially pure, grade 4 (ISO5832/2) titanium disks of 8 mm diameter and 1 mm thickness were kindly provided by Sweden&Martina® (Due Carrare, PD, Italy). The surfaces were machined and provided as sterile discs.

### *Cell cultures*

MC3T3-E1 cells were obtained from the American Type Culture Collection (LGC Standards S.R.L., Sesto S.Giovanni, MI, Italy). They were grown in Dulbecco modified MEM (DMEM, PAA, GE Healthcare, Uppsala, Sweden), 10% Fetal Bovine Serum (FBS, Gibco, ThermoFisher, Waltham, MA, USA), 1% Penicillin and Streptomycin (Penstrep, Sigma-Sl-

drich, St.Louis, MO; USA) and 1% Glutamine (Sigma-Sldrich, St.Louis, MO; USA). For SEM analysis, 20000 MC3T3 cells were plated on titanium discs in 1 ml of complete medium in 24 well plates (Nunc, ThermoFisher, Waltham, MA, USA), in triplicate and assayed 24 hours after plating.

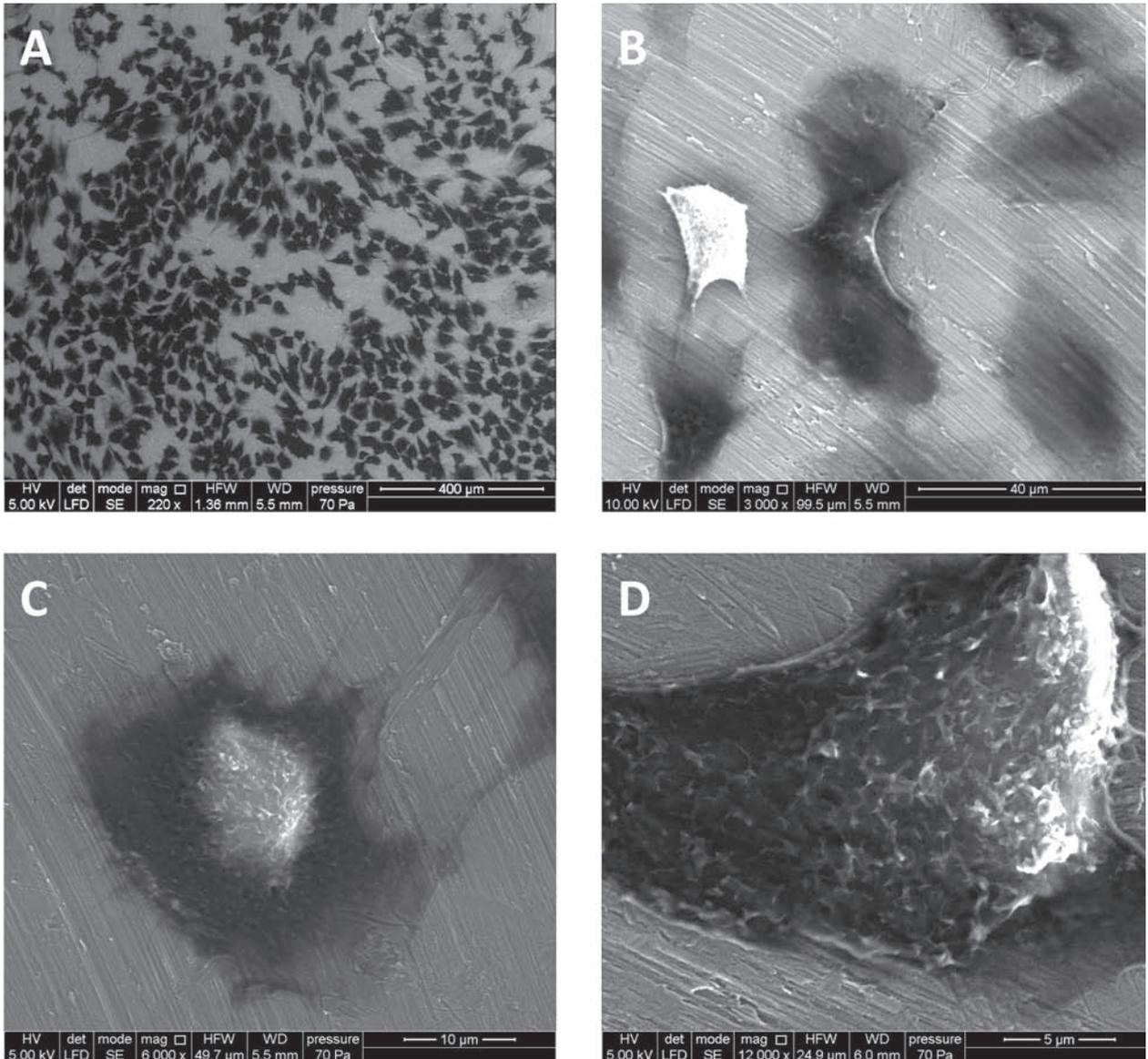
### *Environmental Scanning Electron Microscopy analysis*

Samples were processed for microscope observation by rinse in Phosphate Buffer Saline (PBS, Sigma-Sldrich, St.Louis, MO; USA) and fixation in 4.5% Glutaraldehyde (Sigma-Sldrich, St.Louis, MO; USA) in Sodium Cacodylate buffer (Sigma-Sldrich, St.Louis, MO; USA) at pH 7. Samples were then either rinsed in Sodium Cacodylate buffer and observed at low vacuum scanning electron microscopy (LV-ESEM, Group 1) or dehydrated in 35°-50°-75° alcohol for 10 minutes each and maintained in 75° alcohol prior to LV-ESEM observation (Group 2). Alternatively, samples were washed in PBS and maintained in 0.9 NaCl solution (Sigma-Sldrich, St.Louis, MO; USA) prior to observation in wet mode (wet-ESEM, Group 3). All samples were observed with Quanta™ 250 FEG environmental scanning electron microscope (FEI, Hillsboro, OR, USA), operating in low-vacuum mode (LV-ESEM) at 70 Pa for fixed and dehydrated cells and at 100 Pa for only fixed cells. In the case of wet-ESEM, the humidity was initially set to 100% (at 3°C), then it was slowly decreased.

## Results

### *Fixed-dehydrated cells in LV-ESEM*

We first investigated the morphology of murine calvaria osteoblastic cells on commercially pure machined titanium surfaces after fixation in Glutaraldehyde and dehydration in alcohol. The pressure was decreased to 70 Pa and it was possible to visualise cell morphology at different magnifications (Figure 1A-D). The titanium substrate was mostly smooth, although marked by parallel and concentric grooves with few irregularities, as a result of the machining process. Cells were quite homogeneously dispersed



**Figure 1.** Microphotographs of MC3T3-E1 cells in LV-ESEM on machined commercially pure titanium at 220 X (A), 3000 X (B), 6000 X (C) and 12000 X (D) magnification. Cells were fixed with 4.5% Glutaraldehyde, dehydrated in alcohol and maintained in Cacodylate buffer prior to observation

on the surface, although their distribution roughly followed the concentric pattern of the surface grooves (Figure 1A). Individual cells appeared flat, widespread, with a thicker central nuclear area surrounded by thin cytoplasm, through which the features of the underlying surface were clearly visible (Figure 1B,C), consistently with previous HVSEM observations (data not shown). Higher magnifications (Figure 1C-D) al-

lowed for visualisation of finer details of cell surface. Little extroflexions contributed to cell anchoring on titanium (Figure 1C).

#### *Fixed, non dehydrated cells in LV-ESEM*

Cells on titanium were alternatively fixed, washed with Cacodylate buffer and observed at LV-ESEM

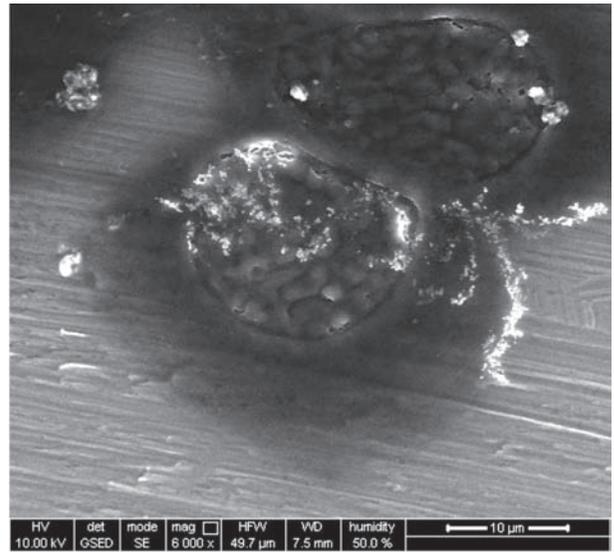
(Figure 2) without prior dehydration. Cell morphology was easily visualised at 100 Pa pressure, and even cytoplasmic projections such were preserved. Cell shape was comparable to what observed in dehydrated samples, and no apparent signs of cell distortion was visible.

### ESEM

When MC3T3 cells were observed in wet mode, they were first washed with NaCl saline solution and placed in a Peltier chamber. To gently remove the aqueous film of saline on cells and allow for microscopic visualisation, humidity was gently lowered up to 50%. At these conditions, however, salts started to precipitate, forming bright crystals (Figure 3) and cells started to display signs of cytoplasmic distortion around the nuclei.

### Discussion

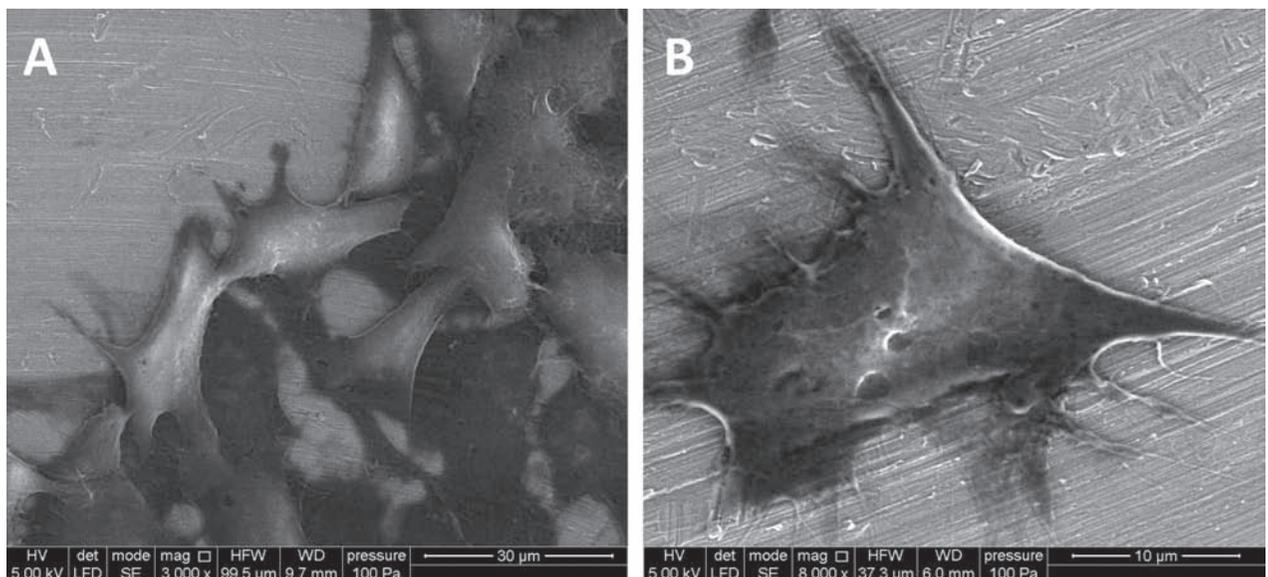
The preliminary results of the present study demonstrate that conventional sample preparation protocols for electron microscopy are not required to observe



**Figure 3.** Microphotographs of MC3T3-E1 cells in wet mode (wet-ESEM) on machined commercially pure titanium at 6000 X magnification. Cells were maintained in saline solution prior to observation. Salt precipitation on samples as a result of chamber humidity decrease is visible as white crystals

the morphology of bone cells plated on biomaterials such as titanium when LV-ESEM is exploited.

Theoretically, observation of fresh cells would be preferable over prepared samples because this would



**Figure 2.** Microphotographs of MC3T3-E1 cells in LV-ESEM on machined commercially pure titanium at 3000 X (A), 8000 X (B) magnification. Cells were fixed with 4.5% Glutaraldehyde and maintained in Cacodylate buffer prior to observation without dehydration

allow for unbiased assessment of cell-biomaterial interactions. This is possible with alternative microscopy techniques, such as inverted transmission microscopy or fluorescence microscopy, whereby living cells are observed with the use of inverted microscopes, if cells grow on a transparent substrate, or with the use of fluorescent markers, such as GFP-labelled proteins. However, these techniques are not adequate to correctly visualise the surface of biomaterials, either because they are often opaque to light or because labelling does not reveal their structure or surface features. SEM is hitherto the best instrument to observe both biomaterial surfaces and cell surface. However, it requires a lengthy sample processing, which implies additional costs and jeopardises the artefact-free evaluation of samples (8, 9). ESEM is a recent class of microscopes that allows also fresh sample observation under environmental and wet conditions (10-13). This proves however extremely challenging for animal cells, because they are in osmotic balance with the surrounding environment, and variations in humidity can affect cytoplasmic volume (14-16). Moreover, cells must be maintained in saline solution, to avoid swelling, and this can cause salt precipitations, when a certain humidity threshold is reached. This poses even greater risks for artefacts than convention SEM processing, and even special temperature conditions are not a safeguard against cellular shape changes. Low vacuum mode has fewer limitations, although cells had to be fixed prior to observation. In our study we compared fixation alone and fixation and dehydration by alcohols. The main endpoint of this study was cell morphology, that is the evaluation of cell shape and how cells adhere to the substrate. No significant differences were observed with both conditions, cytoplasmic projections and podosomes were clearly preserved, and the cell-titanium interactions were maintained, with similar results to what obtained with conventional high vacuum SEM and previously published in several reports.

This would indicate that LV-ESEM is a viable alternative to conventional HVSEM, which would avoid the need for metal coat-sputtering. More specifically, no need for sample dehydration was found in our preliminary study.

Taken together our data suggests that osteoblastic cells growing on machined titanium surfaces could

be effectively visualised after simple fixation with 4.5% Glutaraldehyde. No additional benefit could be achieved by subsequent alcohol dehydration. This appears as an effective, simple sample preparation protocol for evaluation of cell-biomaterial interaction using LV-ESEM. In this particular case, wet-ESEM did not prove as a reliable approach to assess cell morphology on biomaterials.

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