

Handbook of instrumental techniques from CCiTUB

Biomedical and Biological Applications of Scanning Electron Microscopy

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Núria Cortadellas, Eva Fernández, and Almudena Garcia

Unitat de Microscòpia Electrònica (Casanova), CCiT-UB, Universitat de Barcelona. Facultat de Medicina. Casanova, 143, 6^a pl. Ala Nord. 08036 Barcelona.

email: nuriac@ccit.ub.edu

Abstract. This article summarizes the basic principles of scanning electron microscopy and the capabilities of the technique with different examples of applications in biomedical and biological research.

1. Introduction

The scanning electron microscope (SEM) (see Fig. 1) uses electrons to form an image. A beam of electrons is produced at the top of the microscope (electron gun) and follows a vertical path through the column of the microscope, it makes its way through electromagnetic lenses which focus and direct the beam down towards the sample. The beam passes through pairs of scanning coils or pairs of deflector plates in the electron column, typically in the final lens, which deflect the beam in the x and y axes so that it scans over a rectangular area of the sample surface (see Fig. 2) [1,9].



Figure 1. Zeiss DSM 940A SEM electron microscope at the CCiT-UB (Medical school)

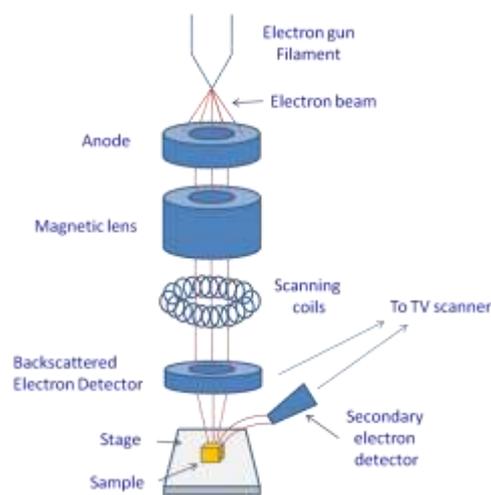


Figure. 2. Diagram of a Scanning electron microscope

The focused beam of high-energy electrons generates a variety of signals at the surface of solid specimens. The signals that derive from electron-sample interactions reveal information about the sample including external morphology or surface topography, chemical composition, and others properties such as electrical conductivity (see Fig. 3).

Different detectors collect the signals, and convert them into another signals that are sent to a viewing screen similar to the one in an ordinary television, producing an image. This image is then digitally captured and displayed in a computer monitor. Magnification in a SEM can be controlled over a range of about 10 to 500,000 times or more.

The spatial resolution of the SEM depends on the size of the electron spot, which in turn depends on both the wavelength of the electrons and the electron-optical system which produces the scanning beam. Depending on the instrument, the resolution ranges between 1 and 20 nm.

The signals result from interactions of the electron beam with the atoms at or near the surface of the sample. The type of signals produced by a SEM include secondary electrons, back-scattered electrons (BSE), characteristic X-rays, light (cathodoluminescence), specimen current and transmitted electrons (see Fig. 3) [5,9].

Secondary electrons. The most common imaging mode collects low-energy (<50 eV) secondary electrons that are ejected from the k-orbitals of the specimen atoms by inelastic scattering interactions with the beam electrons. Due to their low energy, these electrons originate within a few nanometers from the sample surface. Secondary electrons can produce very high-resolution images of a sample surface, revealing details about less than 1 to 5 nm in size. Due to the very narrow electron beam, SEM micrographs have a large depth of field yielding a characteristic three-dimensional appearance useful for understanding the surface structure of a sample.

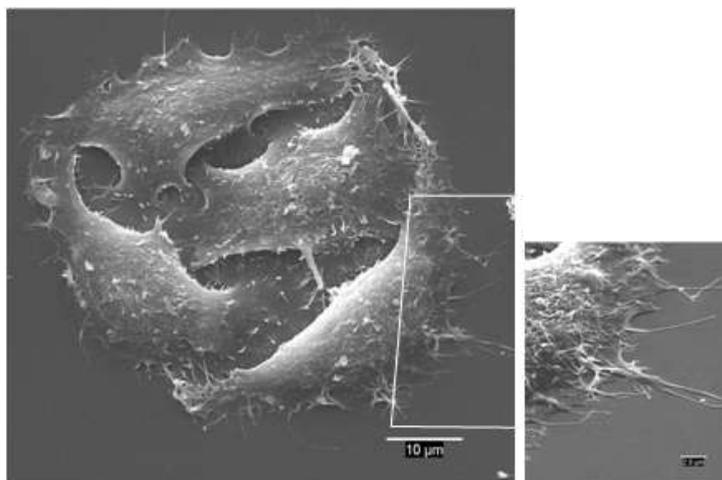


Figure 2. HeLa Cells on glass coverslips substrat. Detail: tilt image (35°).
(Courtesy of Carles Enrich, University of Barcelona).

Backscattered electrons (BSE). Back-scattered electrons (BSE) are beam electrons that are reflected from the sample by elastic scattering. As a result, the intensity of the BSE signal is strongly related to the mean atomic number (Z) of the specimen, and BSE images can provide information about the distribution of different elements in the sample. Since heavy elements (high atomic number) backscatter electrons more strongly than light elements (low atomic number), and thus appear brighter in the image, BSE are used to detect contrast between areas with different chemical compositions. For the same reason, BSE imaging can image colloidal gold immuno-labels of 5 or 10 nm in diameter which would otherwise be difficult or impossible to detect in secondary electron images in biological specimens.

Characteristic X-rays. Characteristic X-rays are emitted when the electron beam removes an inner shell electron from the sample, causing a higher energy electron to fill the shell and release energy. These characteristic X-rays are used to identify the composition and measure the abundance of elements in the sample.

Cathodoluminescence. The highly focused beam of electrons impinges on a sample and induces it to emit light from a localized area by scanning the microscope's beam in an X-Y pattern and measuring the light emitted with the beam at each point, a map of the optical activity of the specimen can be obtained.

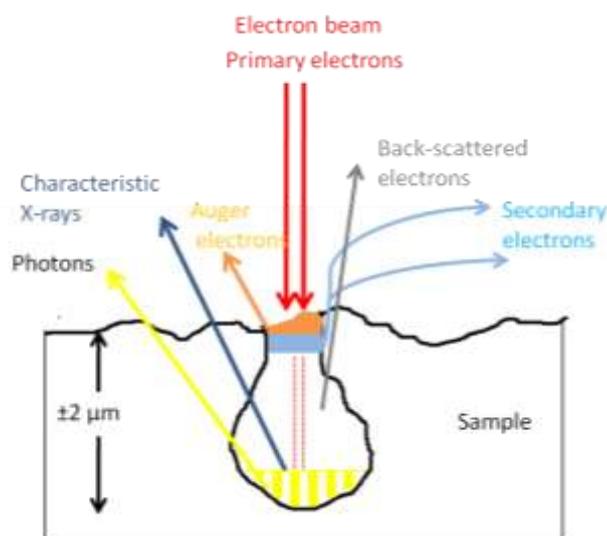


Figure 3. Electron beam interaction and signals

2. Methodology

For SEM, a specimen is normally required to be completely dry, since the specimen chamber is at high vacuum. For conventional imaging in the SEM, specimens must be electrically conductive at least at the surface, and electrically grounded to prevent the accumulation of electrostatic charge at the surface. All samples must also be of an appropriate size to fit in the specimen chamber and are generally mounted rigidly on a specimen holder called a specimen stub [1,2,6,8].

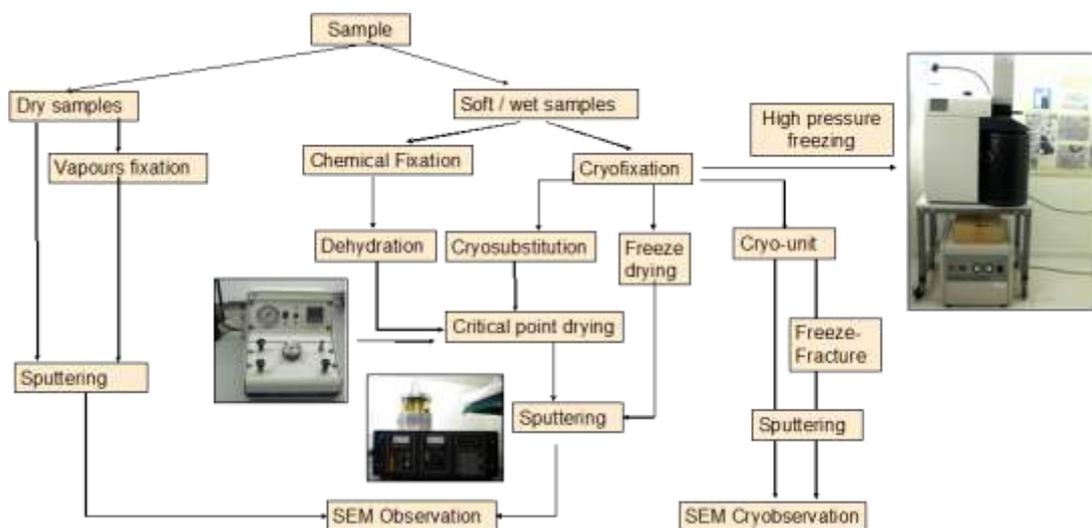


Figure 4. Schematic diagram of the sample preparation methods for the observation with the scanning electron microscope

Hard, dry materials such as bone, wood, dried Insects, seeds or teeth can be examined with little further treatment or directly. Living cells and tissues, soft-bodied organisms usually require fixation to preserve and stabilize their structure (see Fig. 4) [1,4,5,6,8]. Fixation is usually performed by incubation in a solution of a buffered chemical fixative or by cryofixation [1,2]. The fixed cell or tissue is then dehydrated or cryosubstituted. Because air-drying causes collapse and shrinkage, this is commonly achieved by critical point drying. Critical point drying involves replacement of organic solvents such as ethanol or acetone, and replacement of these solvents in turn with a transitional fluid such as liquid carbon dioxide at high pressure. The carbon dioxide is finally removed while in a supercritical state, so that no gas-liquid interface is present within the sample during drying. The dry specimen is usually mounted on a specimen stub using colloidal silver and sputter coated with gold, carbon or gold/palladium alloy before examination in the microscope [1,2,4,6].

If the SEM is equipped with a cold stage for cryo-microscopy, cryo-fixation may be used and low-temperature scanning electron microscopy performed on the cryogenically fixed specimens. Cryo-fixed specimens may be cryo-fractured under vacuum in a special apparatus to reveal internal structure, sputter coated and transferred onto the SEM cryo-stage while still frozen [9].

3. Application Examples

3.1. Parasite study by scanning electron microscopy

SEM allows us to visualize external morphological characteristics and is a very useful tool for obtaining data on systematic and taxonomic studies of parasites in general. The parasitological studies of these hosts allow us to learn the composition of their communities and thus the parasite biodiversity, providing as well interesting information as to the complex life cycles of these parasites (see Figs. 5, 6 and 7).

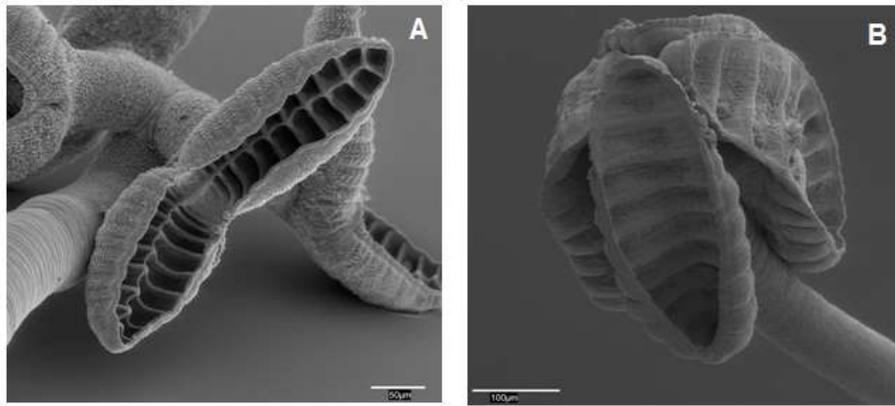


Figure 5. A. *Rhinebothrium* sp., cestode of the order Rhinebothriidea: detail of one of the bothridia in which the morphology of the loculi can be seen. B. *Scalithrium minimum*, cestode of the order Tetrathyllidea: general view of the scolex with its 4 bothridia. (Courtesy of Jordi Miquel, University of Barcelona).

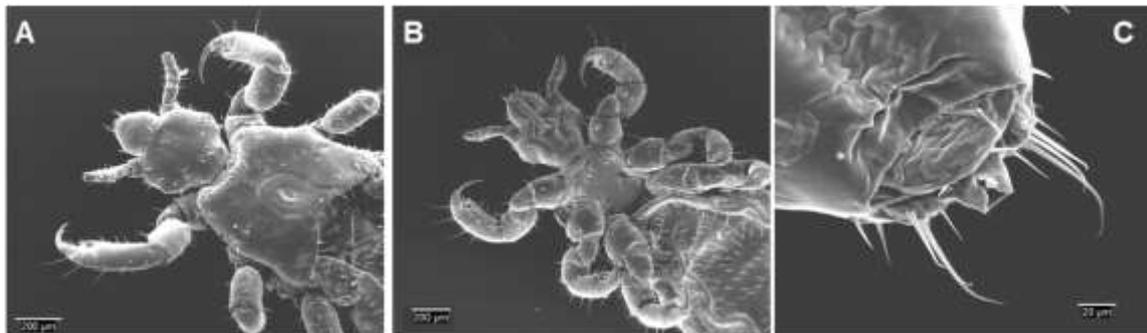


Figure 6: A. *Pediculus humanus capitis* head and thorax dorsal view. B. Ventral view. C: Female's abdomen detail.

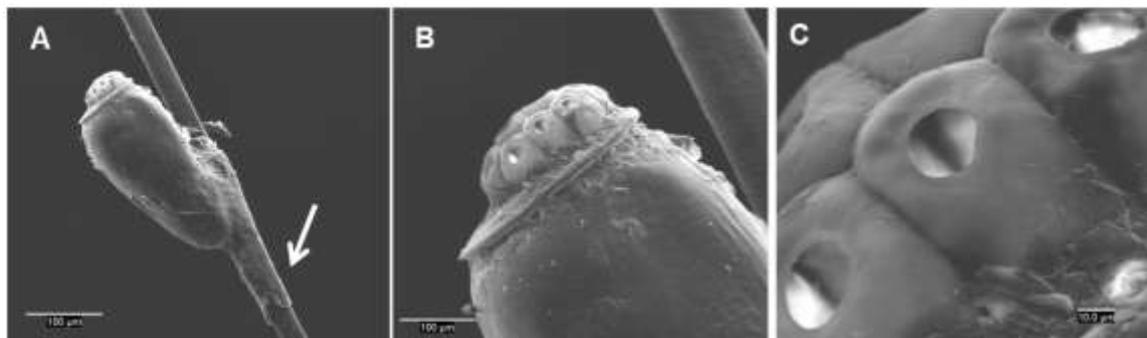


Figure7: A. Nit general view, hair (→). B. Operculum detail with aerial cameras. C. Aerial cameras detail.

3.2. Alveolar-capillary barrier

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are clinical manifestations of acute and constant respiratory failure caused by lung inflammation and the disruption of the alveolar-capillary barrier with a subsequent infiltration of protein-rich oedema fluid and inflammatory cells into the alveolar space. Some inflammatory and coagulation mediators overexpressed in these diseases may modulate alveolar-capillary barrier integrity (see Fig. 8).

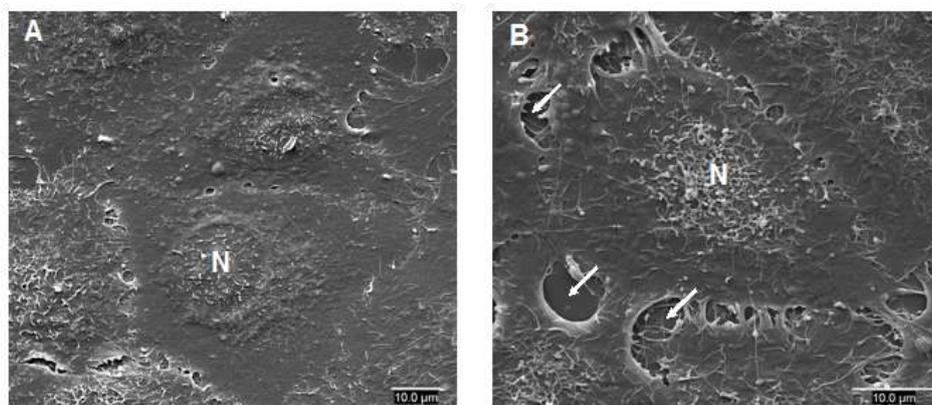


Figure 8. A. Alveolar epithelial monolayer. Nucleus (N). B. Alveolar epithelial cells challenge by Thrombin, a pro-coagulant serine protease that forms intercellular gaps (→), Nucleus (N). (Courtesy of F. Puig, G. Fuster, J. Tijero, L. Blanch, and A. Artigas, Sabadell Hospital, and R. Nieto, D. Navajas and R. Farré, University of Barcelona).

3.3. Pro-inflammatory stimuli activate dendritic cells, important players in the battle against diseases

Dendritic cells (DCs) are the sentinels of the body, playing a key role in the initiation and regulation of the immune and inflammatory responses. Under a pro-inflammatory stimulus or pathogen, immature DCs (iDCs) experience a profound molecular and morphological transformation toward a “mature” state (mDCs). Scanning electron microscopy (SEM) is a powerful tool to visualize such morphological variations occurring on the DC cell surface upon activation, such as extended dendrite formation and clustering, allowing efficient antigen presentation.

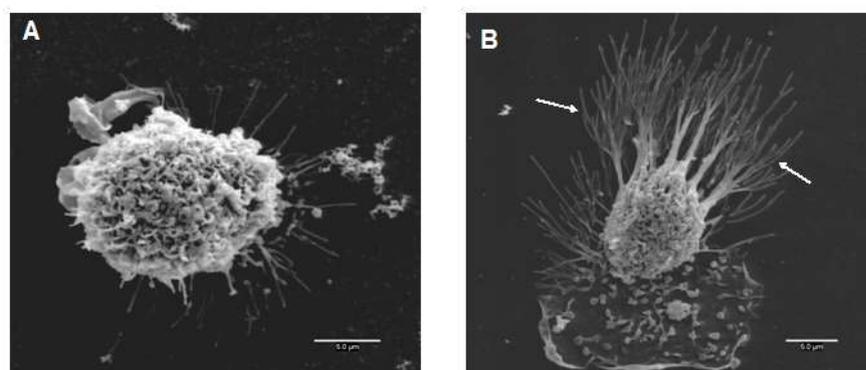


Figure 9. The images shown are SEM micrographs of monocyte-derived human dendritic cells (DCs) either untreated (iDCs) (A) or treated for 48 h with 5 µg/ml of bacterial lipopolysaccharide (LPS) (pro-inflammatory stimulus) (mDCs) (B). Note the increased and polarized dendrites (→) in mDCs stimulated with LPS. (Courtesy of Rut Olivar and Josep M. Aran. IDIBELL).

3.4. Intestinal epithelium

The main objective of the project is to study the effect of different natural substances on the control of the prevalence of *Salmonella* in chicken intestine.

3.5. Biofilm formation in endotracheal tubes of mechanically ventilated pigs with pneumonia

Biofilms are bacterial communities that adhere to a surface and grow in a self-produced polymeric matrix difficult to disrupt. Bacteria in biofilm increase their ability to survive, usually altering their resistance pattern.

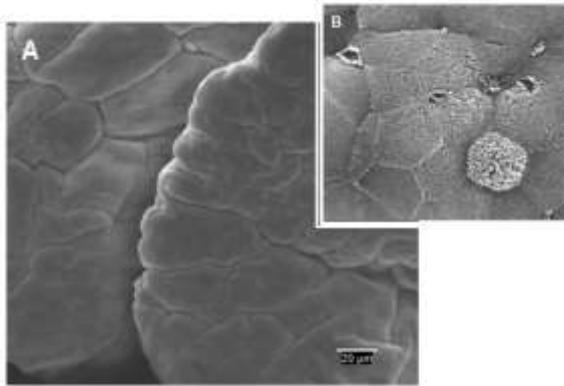


Fig. 10. Intestinal epithelium of a 21-day old chicken: A. villi, and B. surface of the enterocytes and goblet cells (mucus secreting cells). (Courtesy of M.T. Brufau, , A. Guerrero-Zamora, R. Martín-Venegas. and R. Ferrer, R-, University of Barcelona, and B. Vila, and J. Brufau, IRTA).

In tracheally intubated patients, biofilm, which is universally present in endotracheal tubes, comprises bacteria, bacterial polysaccharides, host cellular detritus and respiratory secretions. With the ventilatory flow, biofilm particles can be dislodged into the lungs and develop into responsible for persistent lung infection

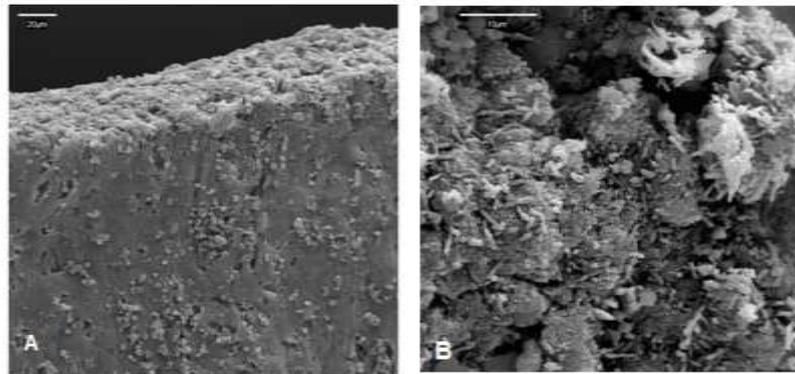


Figure 11. SEM micrographs showing the morphology (A) and bacteria-like structures (B) of a biofilm from an animal model (Courtesy of Laia Fernández-Barat, Miquel Ferrer, Gianluigi Li Bassi, and Antoni Torres. Hospital Clínic-IDIBAPS and University of Barcelona).

3.6. Human Embryo Development by Scanning Electron Microscopy.

Figure 12 shows a SEM of a of a lower limb of a human embryo after 10 weeks' development. The presence of a tactile metatarsal prominence can be seen (→) on the distal tips of the fingers. In addition, one can see how, at this stage of development, the fingers are already independent of each other.

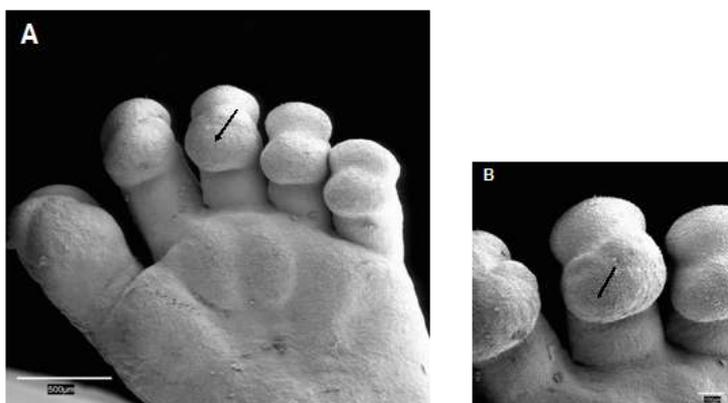


Figure 12. A. SEM micrograph of a lower limb of a human embryo after 10 weeks' development. B. Detail. Plantar view. (Courtesy of Rosa Artells, Alfons Navarro, Tània Díaz, Ruth Tejero, Victor Ciria, Dolors Fuster, Carmen Muñoz, and Mariano Monzó, University of Barcelona)

3.7. Plants diseases

Sclerotinia sclerotiorum is a plant pathogenic fungus that cause a disease called cotton rot or watery soft rot in several crops, like lettuce. This pathogen has the ability to produce a black resting structure known as sclerotia.

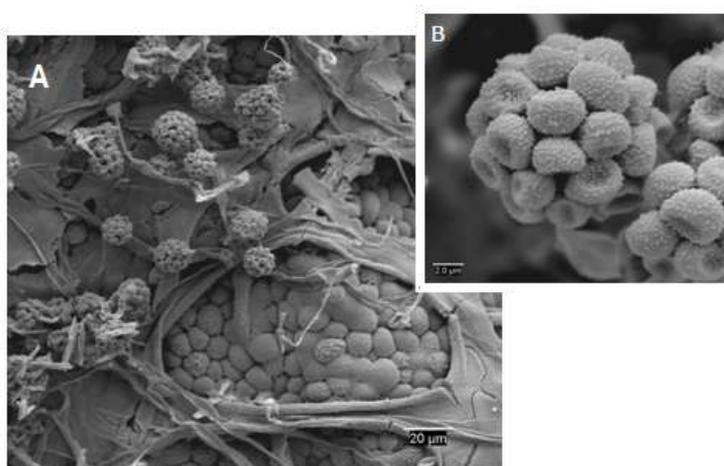


Figure 13. SEM micrographs of sclerotia of *Sclerotinia sclerotiorum* parasitized by another fungus (*Trichoderma asperellum*, strain T34) inhibiting the pathogen growth and the development of the disease (A). Detail of the conidia of the beneficial fungi (*Trichoderma asperellum*, strain T34) (B). (Courtesy of Maribel Trillas, University of Barcelona).

3.8. Leukocyte-Endothelium Interactions during the Inflammatory Response

Single layer of human endothelium treated with proinflammatory stimuli in which human lymphocytes and monocytes from peripheral blood have been perfused with a physiological flow ($1,8 \text{ din/cm}^2$). Several non-polarized leucocytes have established contact with the endothelium and have been captured during the process of rolling (see Fig. 14).

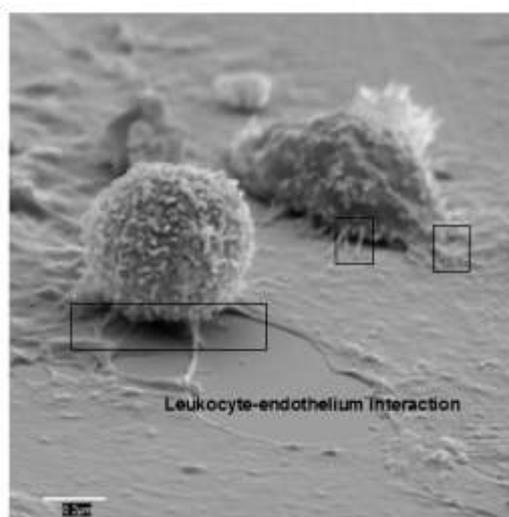


Figure 14. Interaction of the lymphocytes with the apical membrane of endothelial cells subject to flow. (Courtesy of Olga Barreiro, Universidad Autónoma de Madrid and Francisco Sánchez-Madrid, Centro Nacional de Investigaciones Cardiovasculares)

3.9. Erythrocytes morphology with amino acid-based surfactants

The erythrocytes present a characteristic morphology (discocytes, see Fig. 15 A), which enables their function of transporting oxygen. Any alteration in this morphology will cause a reduction in their capacity to transport oxygen. The surfactants interact with the erythrocytes membrane causing

changes in the structure of membrane proteins and lipids, which can induce some alterations in the external surface of the cells (see Fig. 15B).

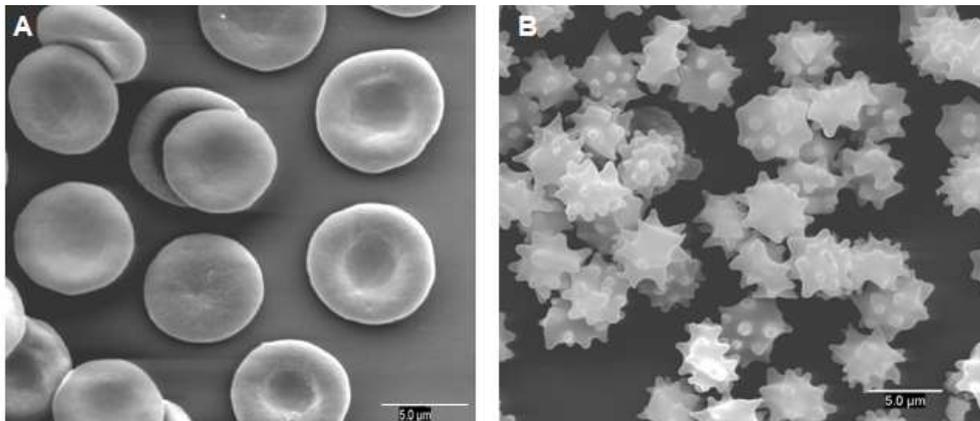


Figure 15. SEM micrograph showing the typical form of the erythrocytes (discocytes) (A), and alterations induced by the presence of surfactants at a physiological pH (echinocytes) (B) (Courtesy of Daniele Rubert, Montse Mitjans and Pilar Vinardell, University of Barcelona).

3.10. Characterization of an In Vitro Model of Mucosal and Nasal Polyp Epithelial Cells Cultured in the ALI System (Air-Liquid Interface)

In the dedifferentiation process the epithelial cells arising from the mucosa and nasal polyp undergo on being cultured in the ALI system of culture (Air-Liquid Interface). In this system, cell culture is developed on a porous support called insert or transwell that allows the cells to be exposed to air at their apical zone whilst remaining in contact with the culture medium at their base. On day 0, one can observe a single layer of dedifferentiated cells, without ciliated cells. However, ciliated cells can be seen after 14 days of culture (see Fig. 16-A) and after 28 days these cells can be seen in greater numbers and with a greater development of cilia (see Fig. 16-B). The different degrees of ciliogenesis observed at different times, indicates that the epithelial cells are becoming dedifferentiated during culture.

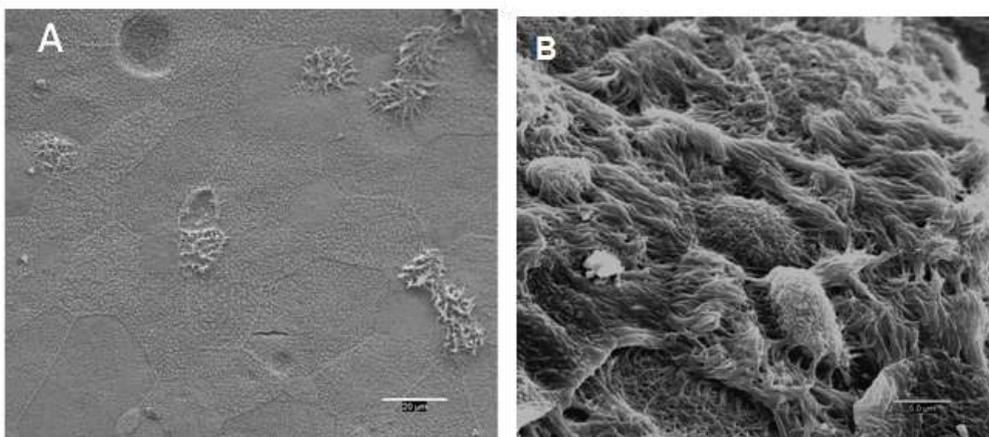


Figure 16. These images contribute, in part, to the characterization that has been carried out of the in vitro model of epithelial cells of the mucosa and nasal polyp cultured in the ALI system. SEM micrographs corresponding to days 14 (A) and 28 (B) of a cell culture under ALI conditions. (Courtesy of Francisco de Borja Callejas, Asunción Martínez-Antón, César Picado, and Joaquín Mulloll, IDIBAPS, and Eva Donet, Instituto Carlos III).

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