

# Working with Uncoated Biological Specimens

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## Application

Imaging biological material without conductive coatings in order to get information that is a step closer to in-vivo conditions.

## ORION® PLUS Capabilities

High resolution imaging, charge neutralization, surface sensitivity, depth of field.

## Background

Imaging is key in the biological sciences for understanding structure and hence function of cells and tissues. Since many of these functions are enabled in organisms by structures with nanometer length scales, high resolution charged particle microscopy is an important tool.

## Challenge

There is obviously much value in seeing structural details under conditions as close to in-vivo as possible. Samples are usually dried to facilitate insertion into the high vacuum environment of the high resolution charged particle optical tool. While critical point drying can minimize artifacts induced by the desiccation process, it presents the microscopist with samples that are electrically insulating, usually requiring the application of conductive coatings before imaging can be done. However, even a few nanometer thick coating can distort or obscure features to be studied. Therefore it is desirable to image such structures without coatings, revealing truer surfaces and more accurately measured features. Variable Pressure SEM offers charge neutralization, but at the cost of resolution, for the gas environment causes scattering in the primary beam. Low energy SEM can reduce charging, but it is difficult and sometimes not even possible to find a stable beam energy at which all the materials and surfaces are in charge balance. In addition, low energy operation sacrifices resolution. Neither of these electron beam-based technologies are good candidates for high resolution imaging of these sample types.

## ORION® PLUS Solution

The helium ion microscope (HIM) is capable of charge neutralization through the use of a low energy electron flood gun. This device can maintain charge balance on the sample during the imaging process. This procedure is possible because of consistent positive charging: when imaging with an ion beam, the incoming particles are positively charged and the outgoing secondary electron (SE) signal is negative. One can reliably use an electron flood beam to reduce surface potential variations, thus



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obtaining consistent image intensity. When this capability is combined with the demonstrated sub-nanometer probe size capability of the microscope, high magnification imaging is enabled and reduced sample preparation is allowed.

Charge neutralization is a balance of three contributing current flows: the ion beam, the escaping SE's, and the flood beam itself. There are large disparities in the magnitude, energy, and density of these flows. Consider that the imaging field of view might be less than a micrometer, while the flood beam typically has a diameter of about 1 mm. The outgoing SE have extremely low energy in HIM, meaning that they are easily suppressed. The operator has the option of several adjustments to the flood neutralization scheme in order to accommodate a variety of imaging conditions. We do not go into a tutorial on this topic here. Users of the microscope are trained in the operation of this feature. There is provided at the end of this note, in Table 1, an example set of conditions used for high magnification imaging of biological samples. We focus instead on three examples – bacterial pathogenesis, toxicity studies, and cellular biomechanics – that illustrate what can be accomplished in imaging studies on uncoated samples. The work contained herein was carried out by Dr. Daniel Pickard and co-workers at the National University of Singapore. Samples were prepared from tissues by critical point drying, but no conductive coatings of any type were applied.

Pathogenesis is the study of the onset and progression of disease. In microscopy studies, this entails observing samples captured at different stages of an advancing disease in order to observe the evolving structural characteristics. In this first application, a study was initiated into the invasion of *Pseudomonas* bacteria into epithelial cells. This bacterium is commonly found in the lungs of cystic fibrosis patients and is sometimes proves fatal for them. Thus there is a strong motivation to understand more about the invasion process. Figure 1 shows a colony of *Pseudomonas* bacteria. This overview image shows clearly the surfaces of the tissue and the bacteria, as well as the pili which extend from one bacteria to the next, communicating functions – which

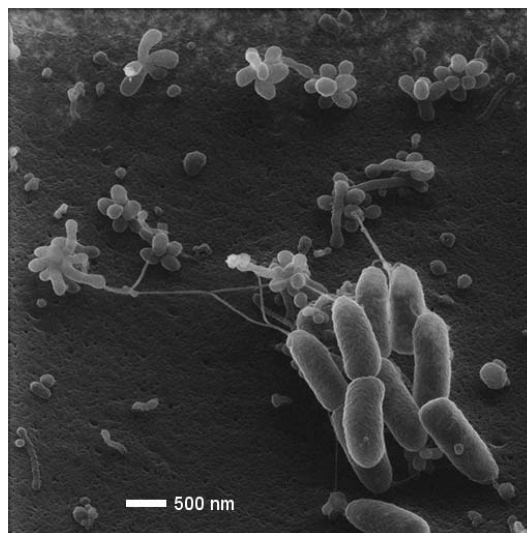


Figure 1. A colony of *Pseudomonas* bacteria.

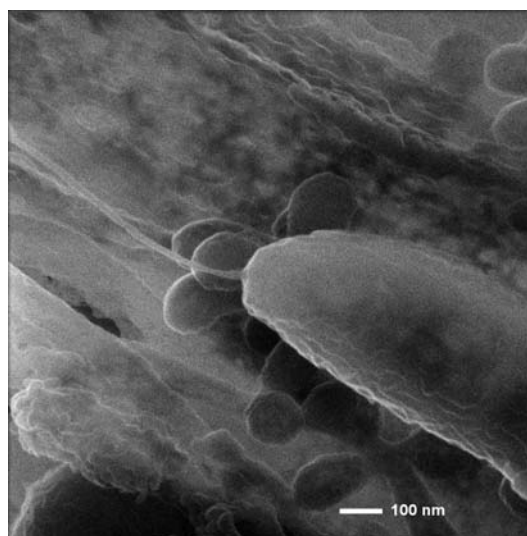


Figure 2. High magnification view of one end of a *Pseudomonas* bacterium, showing the pilus extending from the tip.

can include medically dangerous information such as antibiotic resistance. The HIM imaging has stable and even contrast, and resolution is maintained even for this charging sample. Fine detail of the surfaces are clearly visible. The communication of the larger bacteria with their smaller neighbors is captured. The long depth of focus possible in the microscope allows for all the activity in the field of view to be captured crisply. Figure 2 shows a higher magnification view centered on just one bacterium. There are obvious changes in surface roughness and contrast across this specimen, features which would be very difficult to capture if the sample had been coated,

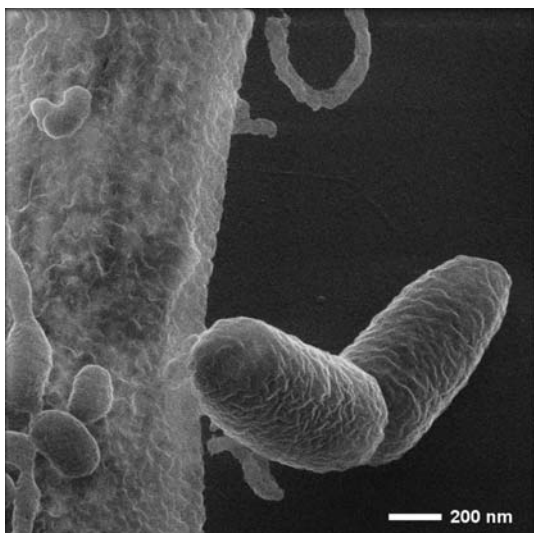


Figure 3. *Pseudomonas* bacterium attacking a lung fibroblast cell.

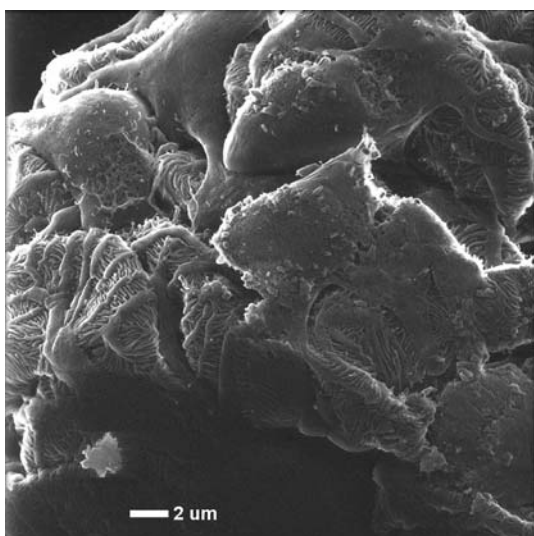


Figure 4. Kidney of a rodent exposed to nanoparticles.

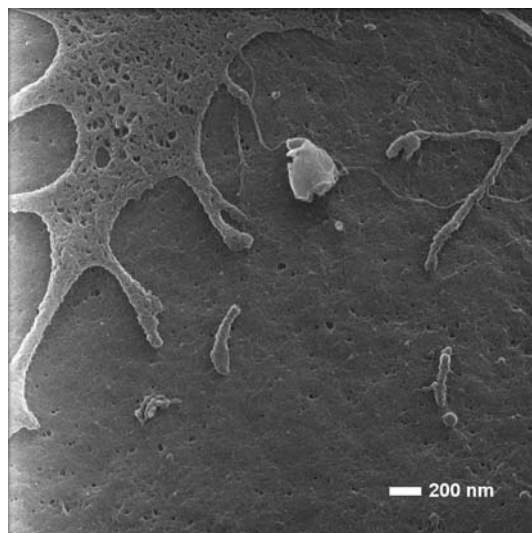


Figure 5. Rodent kidney imaged at 38 kX magnification, showing the filtration surface.

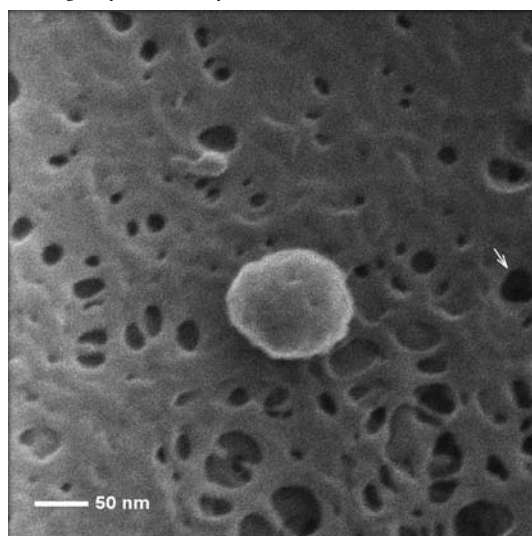


Figure 6. Rodent kidney filtration surface at 229 kX magnification, showing a captured nanoparticle.

for there would be no voltage contrast to reveal such variations and small ridges would be smoothed out. The pilus extending out to the left is also prominently visible, measuring about 12 nm in diameter near its beginning. This value would be falsely increased if a conductive coating had to be placed over the sample. Figure 3 shows *Pseudomonas* attacking a lung fibroblast cell. The attachment point to the cell is marked by a depression in the surface, which is quite evident in the image. None of the detail is blurred by charging or obscured by coatings, thus allowing for close study of this interaction.

Our second example comes from the study of nanoparticle toxicity. Serious concerns are raised in public debate about the possible health hazards of these man-made particles and where they lodge in human tissue. There is in the U.S. for example a major thrust in the National Nanotechnology Initiative on this issue. This is being done through the Nanotechnology Environmental Health Implications (NEHI) Working Group. In the study at hand, rodents were exposed to nanoparticles through either inhalation, ingestion, or injection. Organs were then studied to determine where the particles had taken

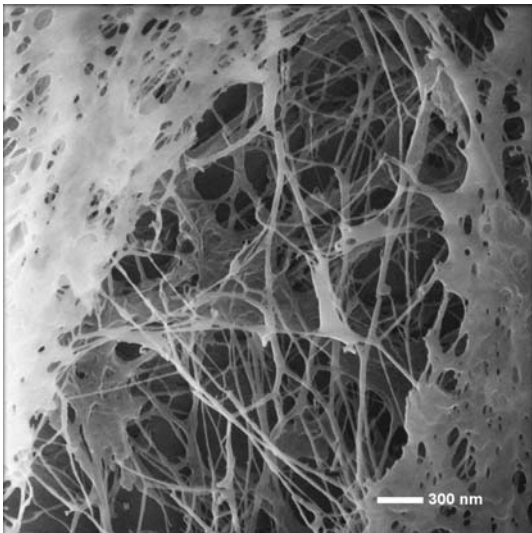


Figure 7. Image of cytoskeleton at 36kX magnification.

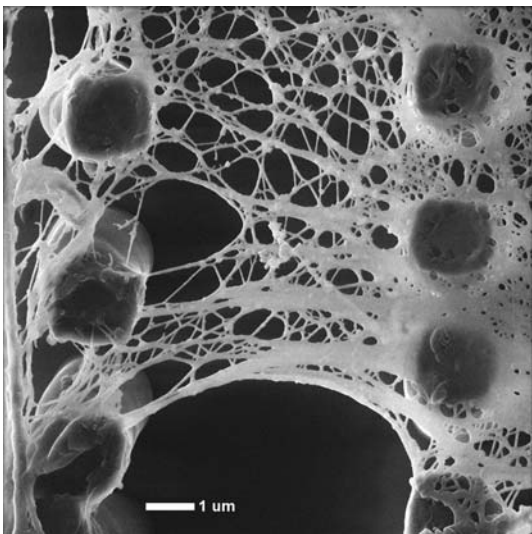


Figure 8. Image of cytoskeleton stretched out over polymer pillars.

residence. Figure 4 shows an overview image of a rodent kidney, capturing several of the blood-filtering glomeruli. This image shows excellent charge control, with the contrast well balanced over most of the surface. Figure 5 shows a 38kX magnification image of a filtering surface. The topology, including the nano-pores in the surface, can be seen quite readily. To gain even more information about the surface and the nanoparticle uptake in this organ, even higher magnification is necessary, however. This is provided in Figure 6, which shows a 229kX (500nm field of view) image. There is a nanoparticle of approximately 100 nm diameter captured here. Even with

the charging inherent to this sample, image resolution down to 1 nm can be measured (see arrow). Pores sizes down to 5 nm are observed. A structure this small would be completely obscured if a conductive coating had been applied to the sample. Gas-based (variable pressure) charge compensation techniques in scanning electron microscopes suffer from resolution-degradation, making the visibility of this type of fine structure difficult if not impossible.

Our final example comes from the study of cell motility. Cells are structurally supported by the cytoskeleton. Actin filaments, a component of the cytoskeleton, push out the periphery of the cell membrane. The moving cell anchors itself to the surrounding environment, allowing it to move forward. Samples were prepared by placing cells on a surface on or near an area where an array of pillars had been patterned. These were made from a polymer of known elastic modulus in order to measure deflection forces. After time had elapsed for the cells to interact with this surface, the nuclei were removed to expose the cytoskeleton and its connections. Figure 7 shows a 36kX magnification image of such a structure. HIM provides excellent contrast and depth of field, so that the entire network of filament and fibers can be visualized. The high imaging resolution in this and other pictures of the sample allows measurement of filament diameter down to the smallest size occurring, about 5 nm. Figure 8 shows the cytoskeleton over an area of the pillar array. The tension in the filaments as they stretch from one pillar to the next is evident. It is also observed that the cell has moved across the tops of the pillars without going down to the substrate. The long depth of field in the image helps to make this clear, for the sidewalls of the pillars can be inspected in good focus all the way down to the substrate. Note also that the top surface of three of the pillars (on the right) can be observed even though they are substantially covered by cellular material. This subsurface feature contrast in the microscope arises from the different electrical potentials that exist when there is an intimate connection to backing material, in this case the pillar, on the back side of the thin skeletal material. Finally, we see in Figure 9 the result of the lateral force that the actin filaments exerts in attempting to grow laterally. The deflection of this pillar can be ascertained

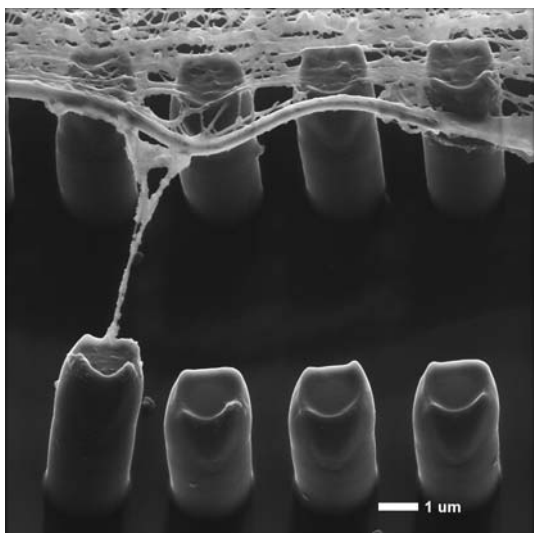


Figure 9. Filament extending from cytoskeleton, providing lateral force to the polymer pillar.

by top-down imaging in order to measure the force. The pillars that are already underneath the cell don't appear to have deflection, but top-down pictures, utilizing the subsurface imaging capability referred to earlier, can be used to find the position of the pillar tops and ascertain the way that they relax as the cell expands beyond them.

In conclusion, the ability to image uncoated samples can be applied to a variety of biological imaging applications. The ORION® PLUS can enable imaging of structures, cellular interactions at high resolution, and three-dimensional features with long depth of field. All of the data gathered is one step closer to in-vivo conditions since conductive coating can be omitted. This also reduces complexity of sample preparation.

We thank Dr. Daniel Pickard and his colleagues at the National University of Singapore for the permission to use these images.

Imaging	
Field of view	2 μm
Beam energy	28 keV
Beam current	0.25 pA
Dwell time	1 μsec
Averaging mode	Line, 128 averages per line
Imaging mode	Secondary electron
Image size	1024 × 1024 pixels
Sample bias	0 V
Working distance	8.4 mm
Flood gun settings	
Mode	Line multiplexed (i.e. a burst of charge neutralization after each imaging line)
Flood time per line	1000 μsec
Flood energy	1000 eV
Line retrace delay	20 μsec (applied after neutralization burst)

Table 1. Flood gun settings for imaging biological samples uncoated.

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