Keysight Technologies
A Comparative Microscopy Imaging Study on Tissue Specimens

Application Note
Introduction

Biological samples span an incredible range of size scales, composition, structures, and morphology. Over the last few decades, special microscopy techniques for biological specimens have moved to the forefront such as confocal laser scanning microscopy, scanning electron microscopy (SEM) and atomic force microscopy (AFM). With respect to the SEM, many biological specimens share a similar set of issues, namely the low conductivity of the biosamples and the typical insulating glass substrates.

Generally, samples examined in the SEM need to be electrically conducting in order to minimize charge build-up on the sample surface induced by the incident electron beam. Charge build-up can severely degrade the resultant image data. Advances in SEM to address a wider range of samples have led to brighter sources which are field emission filaments, low vacuum SEM, environmental SEM (eSEM), and low voltage SEM (LV-SEM). Generally, three approaches can be employed to minimize charging from insulating specimens. First, one can coat the sample with an inert metal like gold. The second option is to increase the pressure in the sample chamber so that the gas molecules balance the charge. Third, one can decrease the electron beam voltage (LV-SEM) so that the beam energy is at the charge equilibrium point. LV-SEM has been demonstrated as a successful approach for imaging energy-sensitive specimens, such as polymers and biosamples.[1]

Biological samples typically have fragile structures with low conductivities, and therefore they are subject to charge build-up and electron beam damage. It has been well documented that the usage of a LV-SEM helps to mitigate the charge build-up and localize the damage if it occurs. However, the low beam voltage operation of the SEM normally results in low resolution images, mainly caused by its huge chromatic aberration. In order to improve resolution and contrast in the SEM, increasing the source brightness and decreasing the initial probe size by using a field emission electron gun is a good solution.

The Keysight Technologies, Inc. 8500 FE-SEM (the precedent model name: Agilent 8500) is a low voltage, field emission SEM which employs a novel all-electrostatic lens design. Comparing with conventional FE-SEMs, Keysight 8500 allows a much faster image acquisition with an ideal resolution and attention to surface details. Furthermore, Keysight 8500 works at a low voltage range, from 500V to 2000V, which fits the requirement for biosample imaging. Due to the low beam voltage working in Keysight 8500 FE-SEMs, beam preparation into specimens is minimized as no metal coating is necessary. It can therefore be a perfect complement to other high resolution microscopy techniques like the atomic force microscopy.

In this work, we demonstrate the easy and fast assessment of biological samples with the FE-SEM by introducing CARDIO-AFM, a morphological study of human heart muscle tissue by means of atomic force microscopy. Moreover, imaging of other human tissues, like skeletal muscle and skin, are included as well.
Morphological diagnostics of heart muscle by means of microscopies

Alarming statistics draw attention to the growing incidence of cardiovascular diseases (CVD) as presented in a recent study by the European Heart Network [2]. The project “CARDIO-AFM” aims to analyze human heart muscle tissue in terms of an important risk factor for CVD, the left ventricular hypertrophy: Due to long-term elevated work load of the heart (i.e. caused by high blood pressure), the initial compensatory mechanism of the heart becomes pathological: as cardiomyocytes (cells of the heart muscle) grow, alterations in cytoskeleton appear. The sarcomeres, the smallest morphological subunits of striated muscle, become disorganized, cells die and are replaced by fibrotic tissue that enhances the stiffness of the whole ventricle and aggravates its contractility.

By means of high resolution microscopies the sarcomere lengths of human cardiomyocytes obtained from healthy and hypertrophic hearts are analyzed. The aim is to identify characteristic differences that allow determination of key parameters. Preliminary results are very encouraging to get a better insight into physiology and pathology of cardiomuscular tissue.

1. Sample preparation

The tissue sections are prepared at the neuropathic hospital Wagner-Jauregg following the daily routine protocols: Post-mortem tissue is excised, fixed with 4% formaldehyde in PBS, dehydrated by alcohol, embedded in paraffin and sectioned with a microtome into 3-4 µm thin sections. Subsequently, deparaffining is performed and the sections are put on object slides (Figure 1).

2. Imaging of human heart muscle tissue by AFM and FE-SEM - a comparative study

The tools that were used in this work are Keysight 5400 (AFM) and Keysight 8500 (FE-SEM). For AFM analysis, the images of the unstained sections were acquired at room temperature in contact mode in air with a scan rate of 1 Hz and a resolution of 512 x 512 pixels. For measurements triangular MSCT tips (Bruker AFM probes) with a nominal spring constant of 0.01 N/m were used. The AFM was controlled by PicoView while Pico Image was used for data analysis. For SEM imaging, the same heart muscle tissue samples were imaged without any coating. To avoid any charging, the backscattered electron imaging mode (BSE) was selected. The beam voltage was set at 1000V throughout this work. The heart muscle is a striated muscle, showing characteristic striation pattern when observed in light microscopy. The smallest morphological subunits of cardiomyocytes are sarcomeres which contain the contractile actin and myosin fibers. They are terminated by so-called Z discs, indicated as white arrows in Figure 2, which can be observed by atomic force microscopy as well as electron microscopy.

Figure 1. Histological tissue sections obtained from the neuropathic hospital Wagner-Jauregg. The sections on the left object slide are stained with HE (haematoxylin and eosin) for light microscopic analysis; the sections on the right are unstained and used for AFM analysis.

Figure 2. Microscopy images of human heart muscle. a) AFM contact mode image of human heart muscle; b) a close up AFM image; c) FE-SEM image of human heart muscle; d) a close up SEM image.
Imaging of human skeletal muscle by means of microscopies

Like the human heart muscle specimens, the same sample preparation and imaging conditions were employed for the human skeletal muscle sample. The skeletal muscle is also a striated muscle which makes up ~40% of the total body mass. It shows however a much more straight and organized structure compared to the heart. As can be seen in Figure 3, these muscle cells are branched and very large (average length of 3 centimeters, diameter between 10 and 100 µm). The Z discs are clearly depicted. In the pictures on the right-hand side the characteristic striation pattern brought about by Z discs that terminate the individual sarcomeres can be seen. FE-SEM images additionally show broken fibers (artifacts coming from the process of tissue sectioning).

Imaging of human skin tissue by means of microscopies

As an example of non-muscular origin we analyzed human skin tissue. The skin is our largest organ which protects against potential hazard from the external environment. The outermost layer is the epidermis consisting of several layers including the cornified layer (layer of dead keratinocytes) and the stratum granulosum (flattened keratinocytes with granules). Figure 4 is AFM and SEM micrographs of the human epidermis.

Discussion

The modern bioapplications demand microscopies with high resolution to resolve the morphologic features at nanoscale in biological specimens. Both AFM and FE-SEM are able to offer imaging resolution down to nanometers. Comparing with AFM however, FE-SEM has several advantages in imaging biological specimens:

Broad magnifications with various field of view

Modern scanning electron microscopes normally have a pretty wide magnification range. The field of view can be nanometers up to millimeters. Such a broad magnification range is ideal for imaging biological specimens.

Large depth of field in imaging

Optical microscopes suffer from the very shallow depth of field in imaging. For most AFMs it is not easy to image rough samples with height differences larger than tens of micrometers. Fortunately, due to the inherent electron beam’s feature, SEMs have a much larger depth of field which is very convenient to observe rough biological samples.
Quick and simple imaging procedure
Over the last two decades, FE-SEMs have been developed for not only high resolution, but also ease of use. The modern digital imaging technology also enables a much simpler instrument operation than the old analog ones. As a result, the imaging process for FE-SEM is quick enough to meet the throughput requirement.

The advantages of the AFM over FE-SEM on imaging biological specimens include: 1) higher spatial resolution 2) ability of imaging in liquid 3) 3rd dimension measurement and 4) almost non-destructive imaging. Therefore, FE-SEM indeed is a complementary imaging technique to AFM for versatile bioapplications.

Summary
Because of their high imaging resolutions, both AFM and FE-SEM have been used to observe biological specimens. The emerging low voltage FE-SEM offers attractive capabilities such as high resolution imaging, less charging effect, enhanced contrast as well as more surface details. Examples were given to demonstrate the imaging performance of Keysight 8500 FE-SEM working at low voltages for human heart muscle, human skeletal muscle and skin tissue. For comparison purpose, AFM images were presented. These two complementary microscopy techniques are both very well suited for tissue imaging.

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References

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