New Analytical Capabilities in Drug Discovery – Digital 3-D Microscopy

a report by

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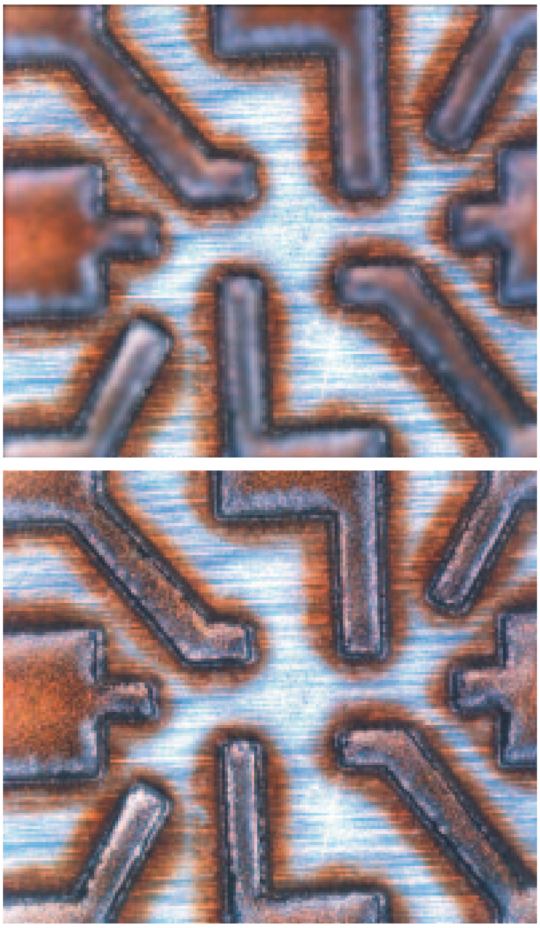
Introduction

Traditionally, microscopists have used their microscopes for two-dimensional viewing and imaging three-dimensional (3-D) objects. There are countless uses for the microscope to examine the surfaces of specimen, but the challenge has always been the microscope's inability to measure (and even image) in 3-D. With conventional light microscopy, the optic's depth of focus typically is shallower than the specimen's full depth, thereby leaving only a portion of the specimen's surface sharply focused. In scanning electron microscopy (SEM), the depth of focus is much greater, however no direct 3-D measurements also are not possible throughout this extended depth of focus. Consequently, when quantitative 3-D surface analysis is desired, an alternative device is needed to supplement the microscope, or other solutions have to be applied such as specimen sectioning. This simple limitation has posed an extreme challenge to scientists and their constraints of time, money and results.

Figure 1: Original light microscope image of a nanostructure with limited depth of field (below left); InfiniteFocus© image with up to 1,000 times increased depth of focus (bottom left); and 3-D model (below right).

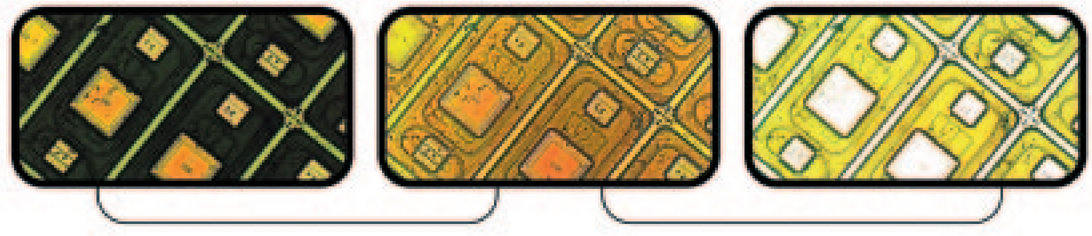


This article presents new 3-D analysis capabilities. The approaches are feasible in both SEM and light microscopy (LM). These conventional microscopes, both SEMs and LMs, can be transformed into true 3-D measurement devices with full analytical capability to generate profile, roughness, area and even volumetric measurements.

A Method to Produce 3-D Images and Measurements in Conventional LM

The InfiniteFocus© technology developed by Alicona Imaging GmbH captures a series of images at different focal planes of the specimen surface. Depending on the shape of the observed specimen and the selected magnification, the optimal number of image planes required is calculated automatically. Digital images are captured at each of these focal planes, thereby producing a 'cube' of digital spatial and image data. Highly efficient image-processing algorithms sift each image focal plane constituting this data cube to determine which pixels are optimally in focus. The result is combined into a single sharply focused image with (up to) 1,000 times increased depth of focus. *Figure 1* shows the calculation of sharp images and 3-D data.

Figure 2: From multiple, differently illuminated images (two samples at the left and right) a single, well-illuminated image is calculated (middle).

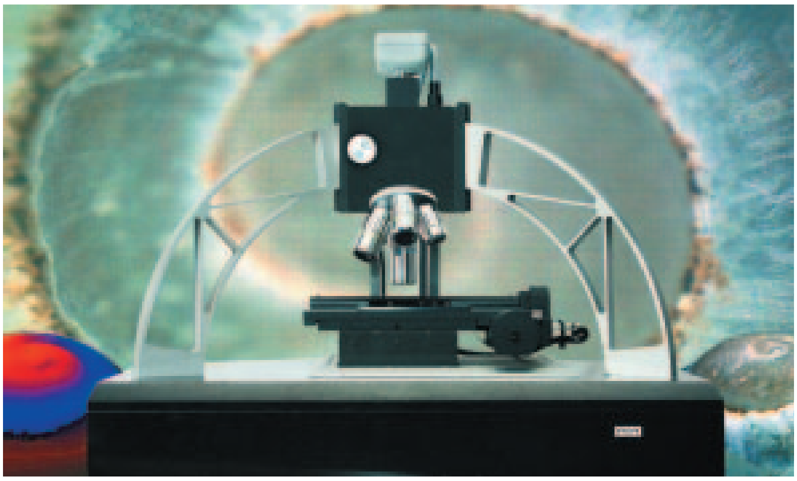


Additionally, the 3-D data (*x*, *y*, *z*) co-ordinates for each pixel comprising the optimally focused surface is derived by the algorithms. Within that surface data set, discrete operator-determined profile, area and volumetric computations can be performed directly on the recombined image. This analysis is described in *3-D Analysis Capability for Both Light and Electron Microscopy*.

Another challenge for conventional LM has been the inability to consistently illuminate all regions of the sample surface. Due to the limited radiometric resolution present in all charge-coupled device cameras, there are often areas that are under-illuminated (see *Figure 2*, left) or over-illuminated (see *Figure 2*, right). This problem cannot be solved by one setting of the illumination source. Embedded in the InfiniteFocus© technology are additional algorithms that control the illumination source. By varying the illumination at each image plane as the images are captured, additional calculations are performed to yield those regions of the image that are optimally illuminated. Out of a series of differently illuminated image planes, a single image with synthetically increased radiometric resolution is obtained. The result is shown in the centre image of *Figure 2* flanked by the two images that contain information captured at different illumination settings.

Additionally, with these radiometric routines, the InfiniteFocus© technology gains the ability to reconstruct and measure surfaces having nearly 90 degrees of steepness to the optical imaging axis.

Figure 3: Fully automatic and digital microscope InfiniteFocus© IFM1000 manufactured by Alicona.



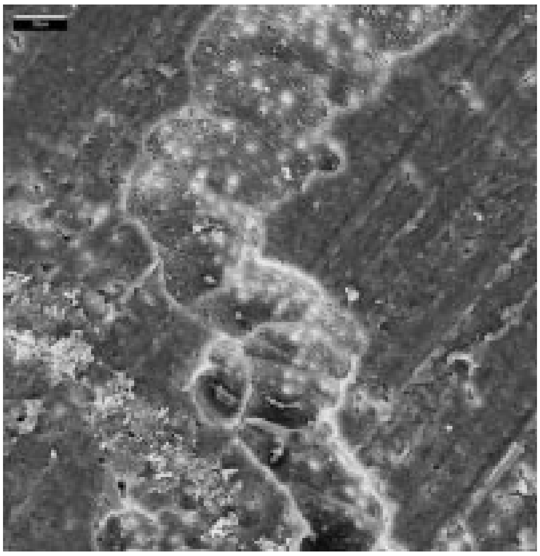
A notable advantage of this InfiniteFocus© technology is that it can be retrofit as an upgrade to most common microscopes, thereby extending the versatility and practicality of existing optical instruments. The technology is also available in a fully automatic stand-alone instrument. *Figure 3* shows the Alicona InfiniteFocus© microscope IFM1000. This system is able to capture surfaces with a vertical resolution of 0.1µm and 0.25µm lateral resolution. Total processing time is less than 100 seconds.

A Method to Determine 3-D Information Utilising SEM

Unlike the light microscope the scanning electron microscope has a very large depth of focus. Similarly, no real 3-D analysis is available. This limitation is circumvented with the stand-alone software package MeX© from Alicona. The software calculates surface data from stereoscopic SEM images. The microscope operator captures these images by a simple eucentric tilting of the specimen holder. Conventional stereoscopic approaches require accurate tilting in order to achieve acceptable results.

Developments by Alicona greatly reduce the requirements on the tilting because an automatic offset calculation and geometric correction are performed. This calculation produces highly accurate and robust 3-D surface reconstructions. Among others, the MeX© technology has been evaluated by the independent institutions of the German Society for Materials Science (DGM) and the German steel producer ThyssenKrupp AG. The relative height accuracy is better than 3% and a fully automatic calculation can be performed in less than 100 seconds. The software can be applied to any SEM images independent of the manufacturer. All surface analysis capabilities present in the InfiniteFocus© are also available in the MeX© software from Alicona. *Figure 4* shows the SEM image of a bone structure (left) and the reconstructed 3-D model (right).

Figure 4: SEM image of a bone structure (left) and resulting 3-D reconstruction (right).



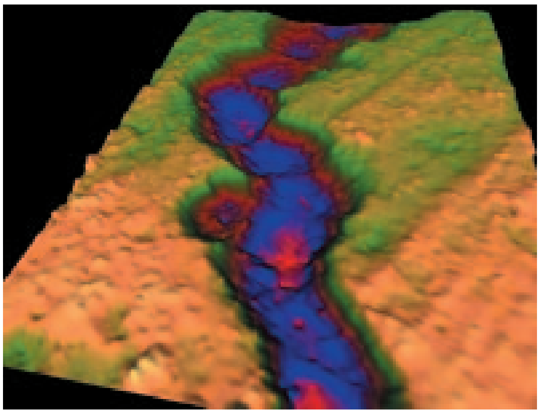
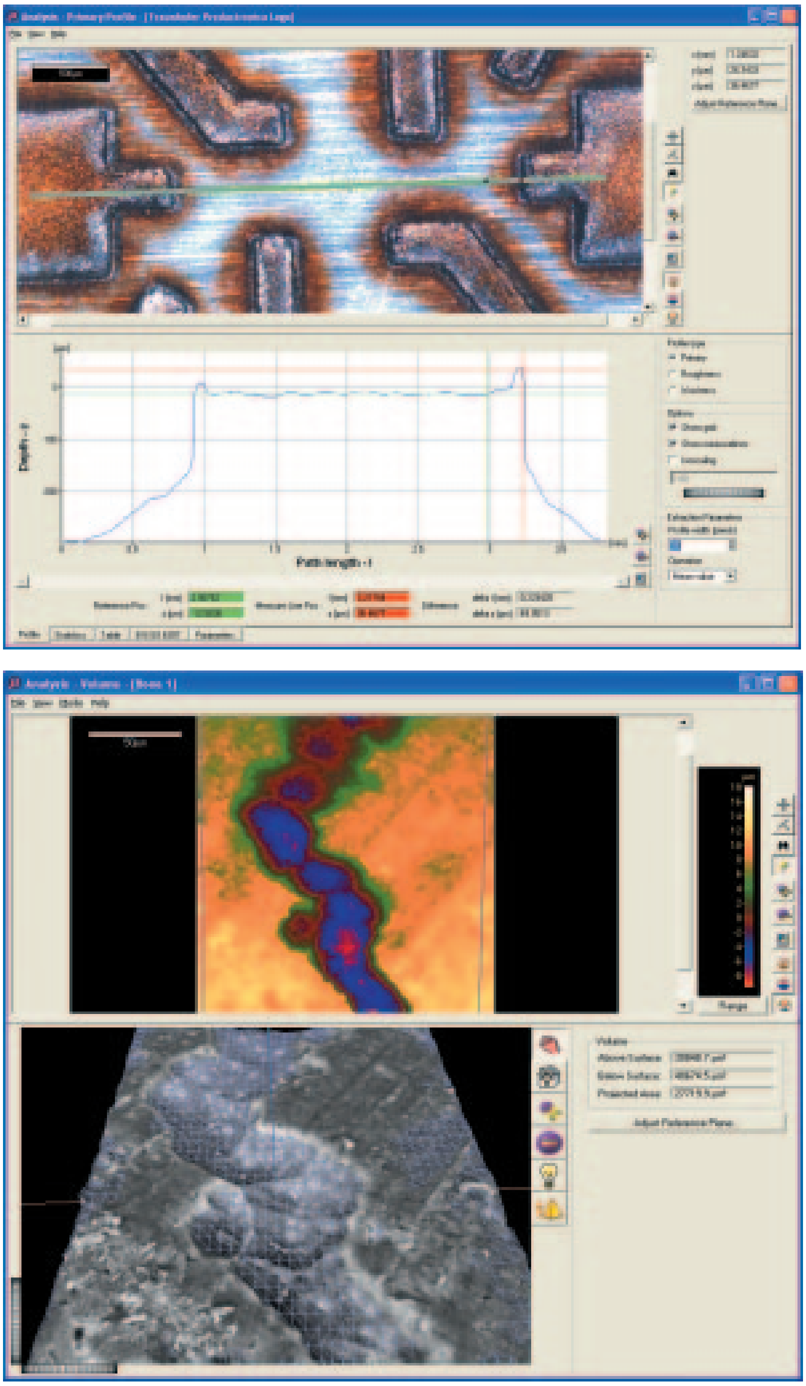


Figure 5: Profile analysis of nanostructure as shown in Figure 1 (left) and volume analysis of bone structure as shown in Figure 4.



3-D Analysis Capability for Both Light and Electron Microscopy

In either microscope technology, once the surface information of the object is determined, the 3-D data can be visualised and analysed. The possible analytical capabilities compromise numerous calculations where the description of such would go beyond the limits of this article. The profile analysis module permits the extraction of height profile along user-defined paths and can be used to calculate relative height measurements as shown in *Figure 5* (left). All established roughness, profile, and waviness measurements, conforming to European Standard (EN)/International Organization for Standardization (ISO) norm 4287 can be performed as well as various statistical calculations. The area analysis module similarly calculates parameters such as the ratio of true-to-projected area, also termed the bearing area curve. The volume analysis module as shown in *Figure 5* (right) enables direct volumetric measurements relative to freely definable polygon lines.

All analysis routines are designed in a modular, intuitive manner, and are supported with full windows reporting conventions.

Applications

The technology presented in this article can be applied in any field of study where the surface structure of a specimen is important. This may include documentation of the roughness of microscopic particles for affinity calculations, the analysis of fractured surfaces from mechanical parts, or the volumes of impressions and uprisings of formed surfaces. The special advantage of this innovative technology is the ability to establish, for the first time in microscopy, a direct link between the microscope image and the 3-D measurement capability. There is no longer the need to struggle finding the area of interest in another device, the image and 3-D surface data co-exist.

Summary

Advances in image processing permit measurement of 3-D surface parameters directly in microscopy images. Profile, area and even volumetric calculations can be performed in reconstructed images containing greatly increased depth of field and optimal illumination characteristics. Therefore, the specimen does not need be examined in other 3-D measurement devices; rather, all analysis can be performed in existing microscopes retrofitted with the Alicona technology. This reduces investment costs while dramatically improving laboratory performance. This proven technology by Alicona Imaging GmbH can be applied in LM as well as in SEM, and is quickly becoming a laboratory standard in research and development practices. ■

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