COMPUTATIONAL PROCESSING OF EXPERIMENTAL DATA FROM LASER SCANNING CONFOCAL MICROSCOPY

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Abstract
The contribution introduces basics of laser scanning confocal microscopy and its applications as metrological and topographical tool. It shows evaluation of typical surface parameters implemented in commercial computer program installed with microscope such dimensional measurements, roughness measurement and volume of material/void determination using bearing area ratio. Despite of rather broad spectra of evaluated parameters user tailored applications could be treated only with difficulties. First attempt to visualize measured data with third-party application is introduced with outlook to implement semiautomatic or automatic approaches to determine topographical parameters at complex surface patterns such as spherical spurs. Visualisation in grid-like mode using Matlab was performed on experimentally collected data. These were exported from ZEN2009 in Cartesian coordinates. Further aim is to implement semiautomatic or automatic approaches to evaluation of roughness or 3D dimensional objects with implementation of ISO standards where applicable.

Keywords: microscopy, laser scanning confocal microscopy, topography, profile roughness, topographic roughness

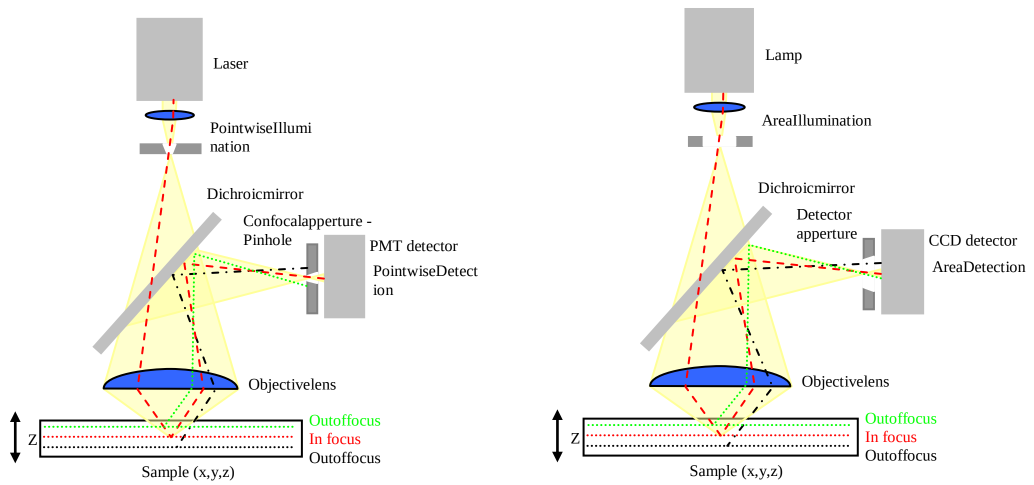
1 Introduction

Original design of the confocal microscope was proposed and patented by Marvin Minski in 1961. Despite of the ingenious and advanced solution, the idea of the microscope left unnoticed for almost 15 years. Development of light sources along with computer technology led to wide spread interest in this technique [1]. Mostly oriented on biology and biochemistry, recent design of laser confocal microscopes allows application also in the field of materials science [2].

Laser scanning confocal microscope is a light microscopy system which uses a laser light passing through the confocal optical path for image formation. In conventional light microscopy the image is formed simultaneously for all observed points of entire field of view. In contrast, system of a laser scanning confocal microscope forms image in step-wise mode, pixel by pixel.

2 Principle

Monochromatic laser light emitted from the laser diode source with power approx. 0.5W is focused on the sample surface by the optical system of the condenser lenses (objective).



a) Confocal laser scanning microscope *b) Conventional optical microscope*

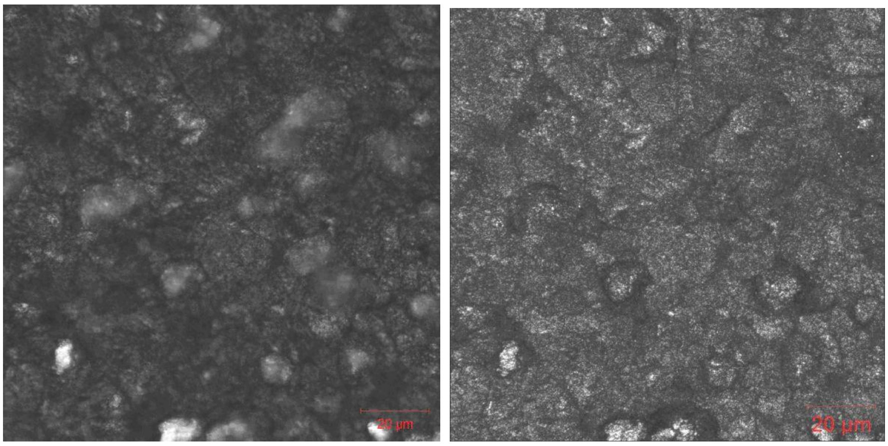
Fig. 1 *Illumination and image formation in confocal and conventional light microscope*

Light scattered by the irradiated spot of the sample surface enters successively the objective, tube lenses and through the confocal aperture (pinhole) interacts with photo multiplier (PMT).
Important role in image formation is played by the pinhole (Fig. 1) [1].

Generally, disadvantage of the conventional optical system is a fact that also light rays scattered by sample surface being not perfectly in focus contribute to image formation (green and black light rays in Fig. 1). This is suppressed in confocal system by filtering the scattered light using the pinhole aperture. Its adjustable orifice and exact position allows only focused rays (red ray in Fig. 1a) to enter the PMT and consequently contribute to image formation. The aperture is localized at the intermediate image plane in front of a photo multiplier (PMT) and is optically conjugated to a sample plane.

3 Image formation

Images taken using confocal light microscope in confocal regime and regime of conventional optical microscope is shown in fig. 2, where improved quality and sharpness of the image taken in confocal regime could be appreciated.



a) b)

Fig. 2 Image of the surface area of coated metallic substrate: a) formed using conventional light microscope, b) formed using confocal microscope using 1 Airy unit aperture of the pinhole using 405 nm laser source

The precision of the entire image formation system is so high that light entering the pinhole and the PMT detector simultaneously, originates from a light section of the sample – optical slice – with thickness limited to hundreds of nanometers. The slice thickness $FWHM_{axial}$ could be calculated using equation (1).

$$FWHM_{axial} = \sqrt{\left(\frac{0.88 \cdot \lambda_{em}}{n - \sqrt{n^2 - NA^2}}\right)^2 + \left(\frac{\sqrt{2} \cdot n \cdot PH}{NA}\right)^2}\ , \qquad [3] \quad (1)$$

where, λ_{em} is emission wavelength, n is refractive index, NA is numerical aperture and PH is pinhole diameter.

An example of the optical slice taken in confocal regime of the microscope is shown in fig. 3. In this particular case, the section is 800 nm thick with possibility of further reduction.

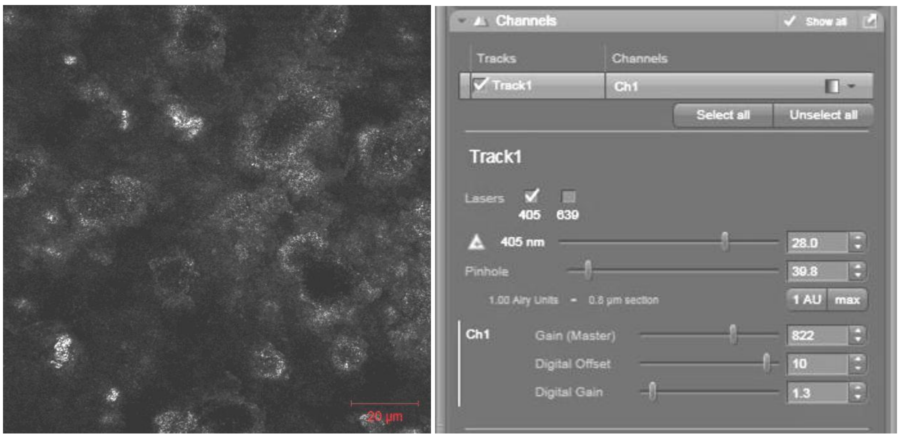


Fig. 3 *Image of the optical slice formed using Pinhole size 1 Airy unit with optical slice thickness 0.8 µm*

The capability of the confocal system to capture thin optical slice on nanometer scale plays an important role in collecting image with significantly improved depth of field in respect to conventional light microscopy. Pinhole coupled with z-axis motorized stage of the microscope allows collecting the stack of slices into the range of millimeters using focus drives with extremely small 10nm increment. Figure 4 shows series of images of 46 individual slices in total thickness of 10.21μm taken in confocal regime of the microscope.

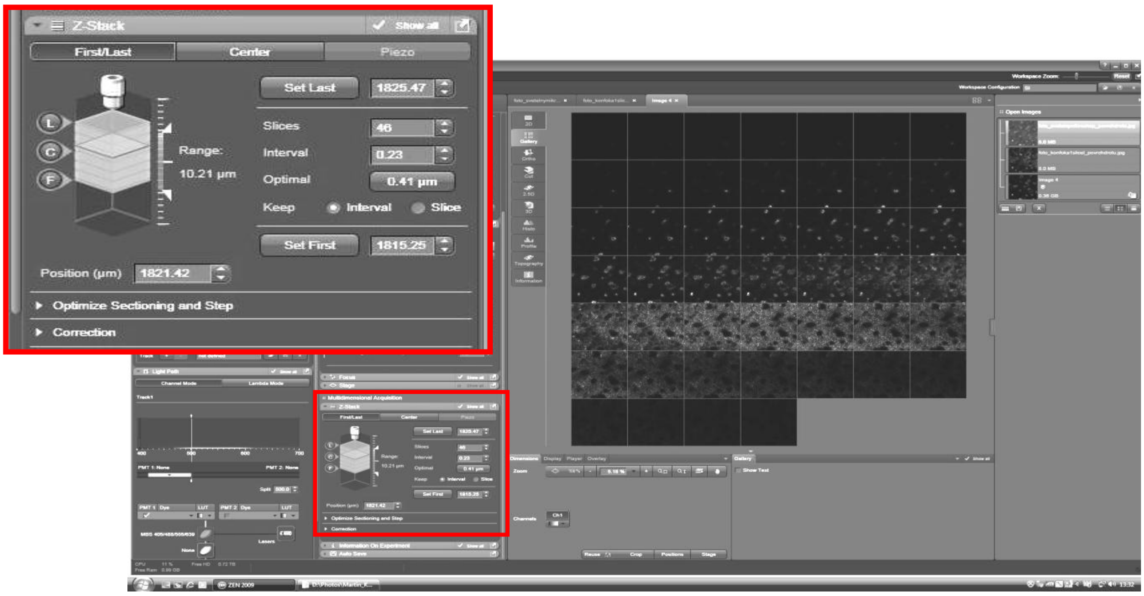


Fig. 4 *Series of images of individual optical slices defined using z-stack tool. Detail shows definition of z-stack with range 10.21 μm, optical slice thickness 0.8 μm, slice interval 0.23 μm and total number 46 slices over examined range of the z-stack*

In combination with motorize x-y axis of the stage, stacks of optical slices could be arranged in a multiple array. This provides possibility to display, review and navigate across large areas of the sample exceeding single field of view.

4 Image processing

Formation of a final 2D or 3D image is performed using a computer program. Contrary to conventional light microscopy, high computation power and special purpose computer program for microscopy remote control, data acquisition and data processing is indispensable. Amount of collected data per one final 3D image could range from MBs up to 1GB or even more. In case of Zeiss LSM700 the computer program used is ZEN2009.

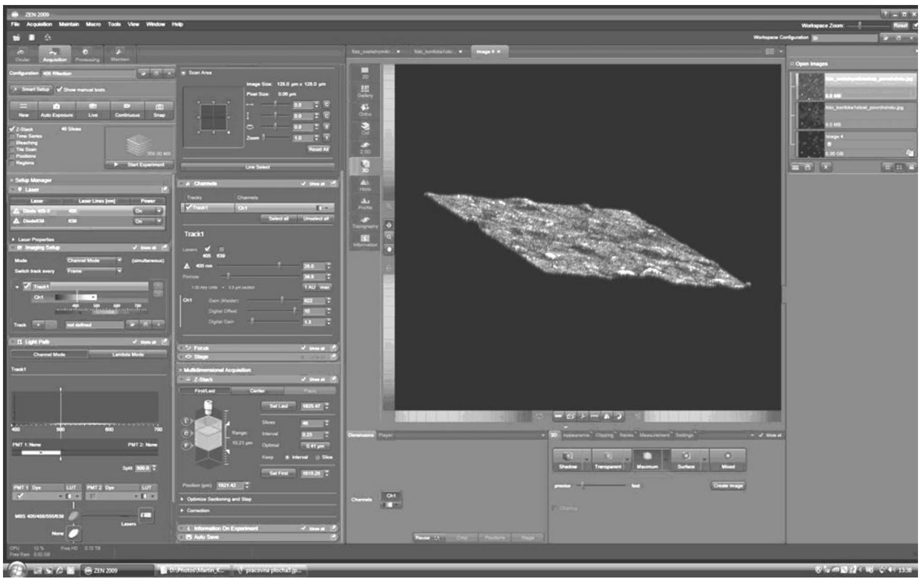


Fig. 5 *View of the finalized 3D image composed from individual optical slices*

Independently collected optical slices with defined vertical and horizontal coordinates are stitched into 3D image of the observed sample. Stitching procedure obeys user defined rules in automatic mode. Advanced computer programs allow optimization of position of stitched optical slices also manually [4].

Final 3D image could be visualized in different 3D models (wire or dot, maximum intensity model, transparent model, etc.). Magnification, rotation or tilting of the 3D model is available (Fig. 5).

Apart from basic review of the 3D model, further analysis could be performed due to the fact, that every pixel in 3D model has its unique x,y,z coordinates. This provides all prerequisites for precise, non-contact and non-destructive surface analysis in topography module. Here, the sample surface is described at a sub-micrometer range and could be used to determine real surface line profile (Fig. 6), bearing area ratio (Fig. 7) and line or area surface roughness (Fig. 8).

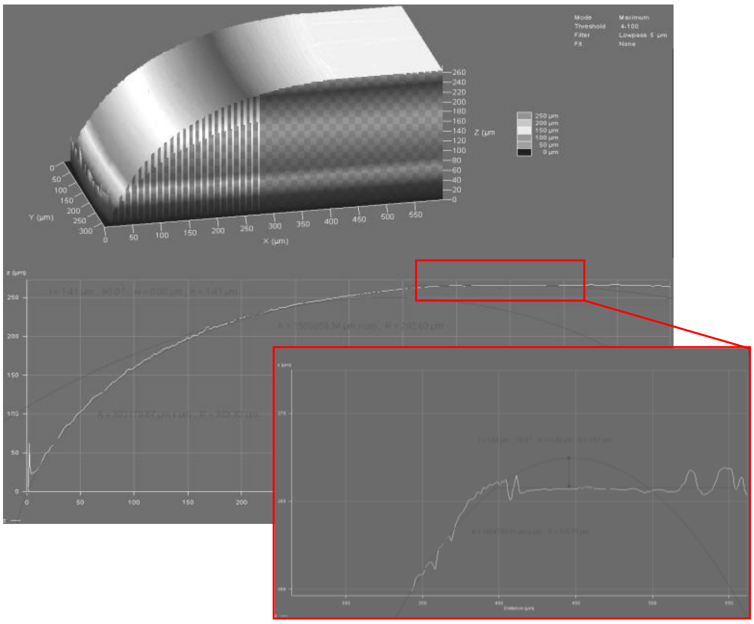


Fig. 6 *Real surface line profile*

Real surface line profile could be effectively used for example on determination of smooth transition from via radius from vertical to horizontal plane. Analysis in figure 6 shown that radius 725µm was above the horizontal plane that is the center was shifted about 1.6 µm above its ideal position.

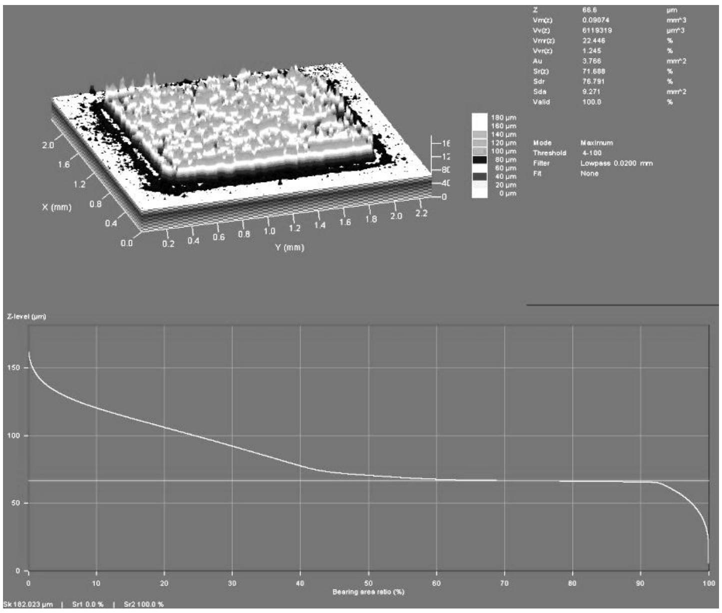


Fig. 7 *Bearing area ratio*

The real surface reconstruction could lead to effective determination of bearing area ratio as it is shown in Fig. 7. Further values determined are volume of material V_m or voids V_v above and below selected horizontal plane. Real surface area could be quantified using S_{da} value and compared with projected surface area A_u. The application could be found for example in distinguishing between materials on the bases of different fracture surfaces where real surface area could be used as a measure of materials toughness.

Further parameters such as profile roughness or topography roughness could be determined also from the real surface. Example is shown in Fig. 8. Advantage of laser scanning confocal microscopy over typical contact profilers is in ability to scan over relatively large areas and determine roughness even at surfaces where positioning of measuring stylus tip is difficult – such as thin wires or narrow facets. However, direct comparison between roughness determined from laser scanning confocal microscope and contact profilers is not straight forward.

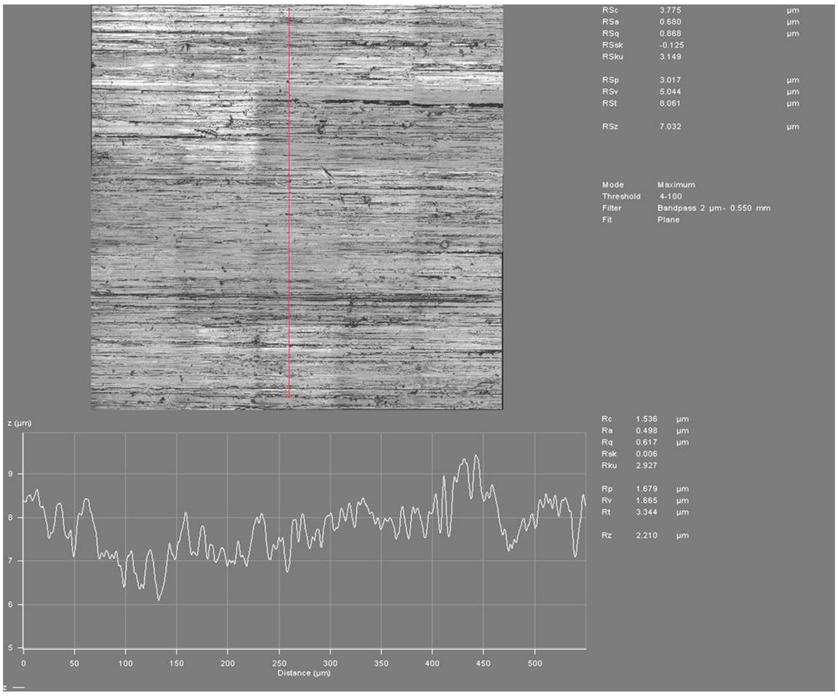


Fig. 8 *Profile and topography roughness*

5 Application

Dedicated microscope software tool is able to draw 3D surface and perform basic operations such as rotation, zooming etc. Above mentioned image analysis performed with software delivered with microscope is however limited and further user tailored analysis are however not possible. For surface analysis purposes it is necessary to implement additional functions. Delivered software tool is able to export processed image as text file consisting of Cartesian coordinates of every scanned surface point. This option allows to post-process scanned surface in third-party software tools. Our goal is to create customizable software tool, which will allow to automatically process source surface information. The core of the application should be able to render basic surface and apply different filters to eliminate inaccuracies. All other required functions will be separate modules called from main application. This ensures the ability of adding new functions and modules into existing program. For mathematical purposes it can use external software tools like Matlab. In the beginning we start development of modules:

a) Automatic surface pattern recognition

In some cases it is necessary to verify dimensions of surface patterns, e.g. spherical spurs (Fig. 9).

By using local extreme search foothills can be found. Next task is to determine dimensions of spurs based on the profile changes. For evaluation of the results statistical techniques can be used, e.g. average, minimal or maximal spur dimensions. First data processing is shown in Fig. 10.

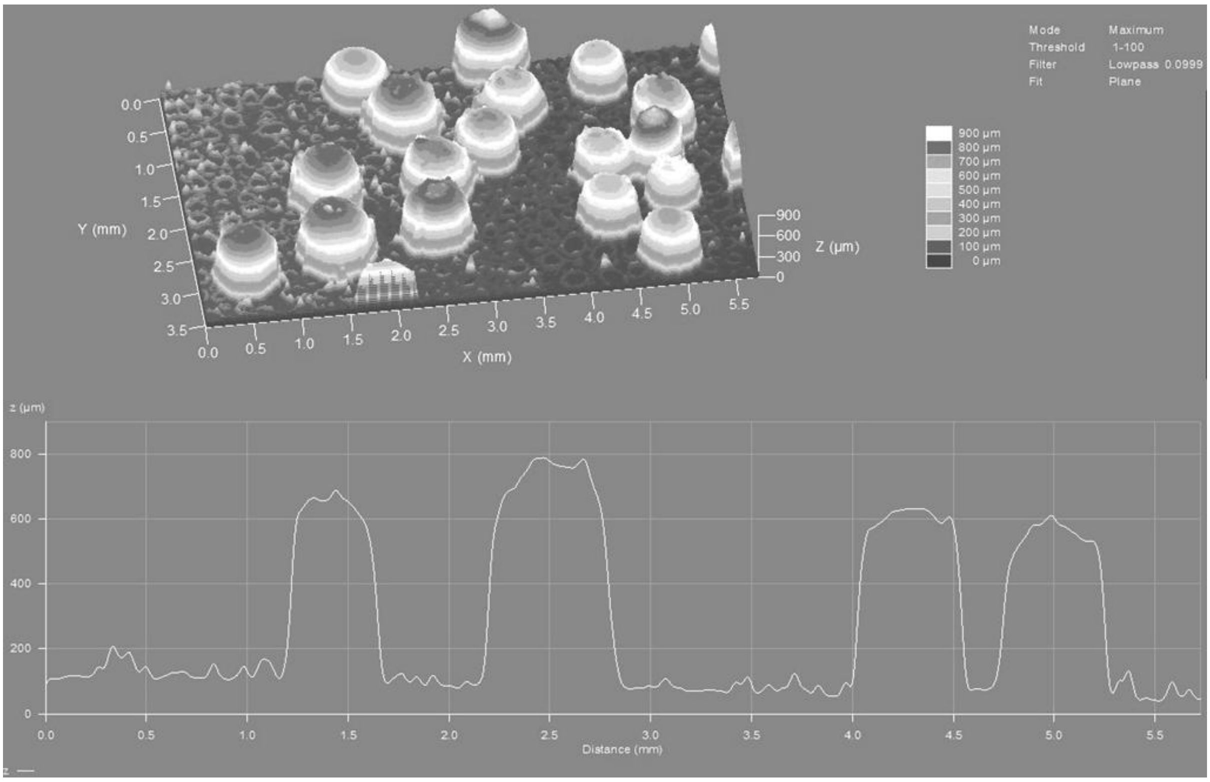


Fig. 9 *Surface scanned by microscope*

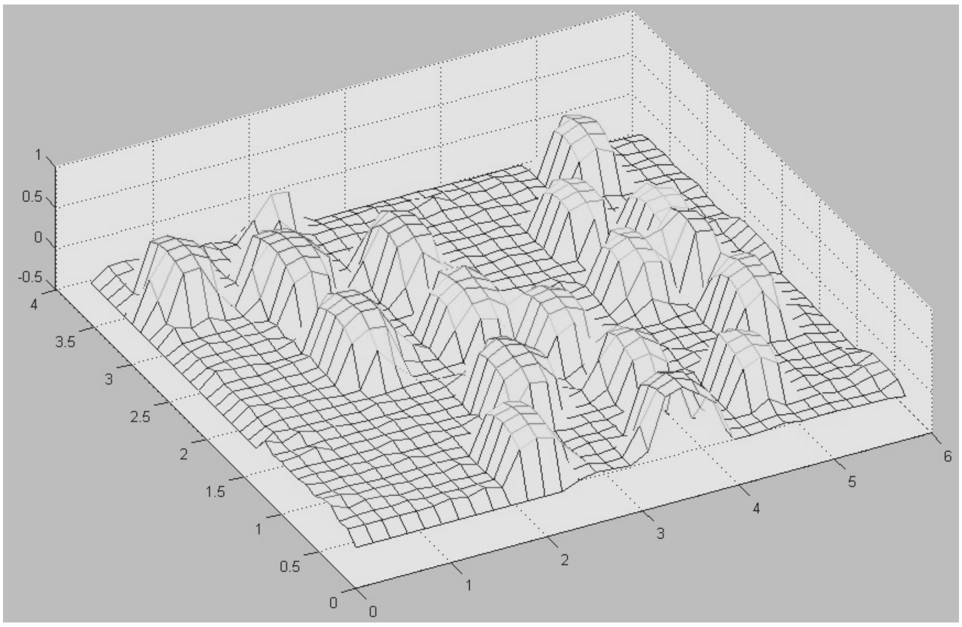


Fig. 10 *Processed surface output file*

b) Automatic detection on surface roughness

Any surface has a certain roughness caused by the machining. Another function of the software is the ability of automatic identification of the scanned surface roughness. It could be used to verify the quality of surface machining, etc.

Contrary to already implemented roughness analysis in ZEN2009 the aim of the development is the implementation of ISO standards for profile and topography roughness evaluation and user friendly application allowing selection of optimal scanning parameters to achieve direct comparison of laser scanning confocal microscopy roughness measurements with those obtained from contact profilers (Fig. 11).

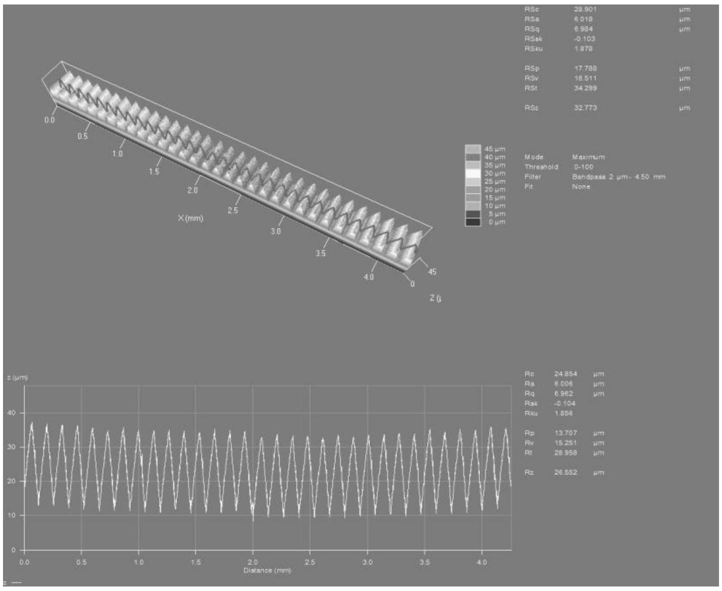


Fig. 11 *Scanned surface roughness*

6 Summary

Laser scanning confocal microscopy with its general computer program is advanced analytical tool allowing determining the real surface parameters via topography analysis. The Zeiss LSM700 in combination with ZEN2009 with Topography package allows calculating the parameters, such real surface area, volume of voids or material, roughness or dimensions of objects. Its functionalities are however limited and further advanced data processing is not possible. Raw data collected with the instrument are therefore exported in Cartesian coordinates and supplied for further processing using tailored computer program. The first attempt was made to redraw measured spurs in grid-like visualization. Further data processing will be aimed at automatic spurs localization and its size determination in x,y as well as z axis. Experience gained with the equipment as well as computer program further indicates that roughness determination is important part of data analysis. Further development will be therefore aimed at development of modules allowing complete roughness analysis in compliance with ISO standards.

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