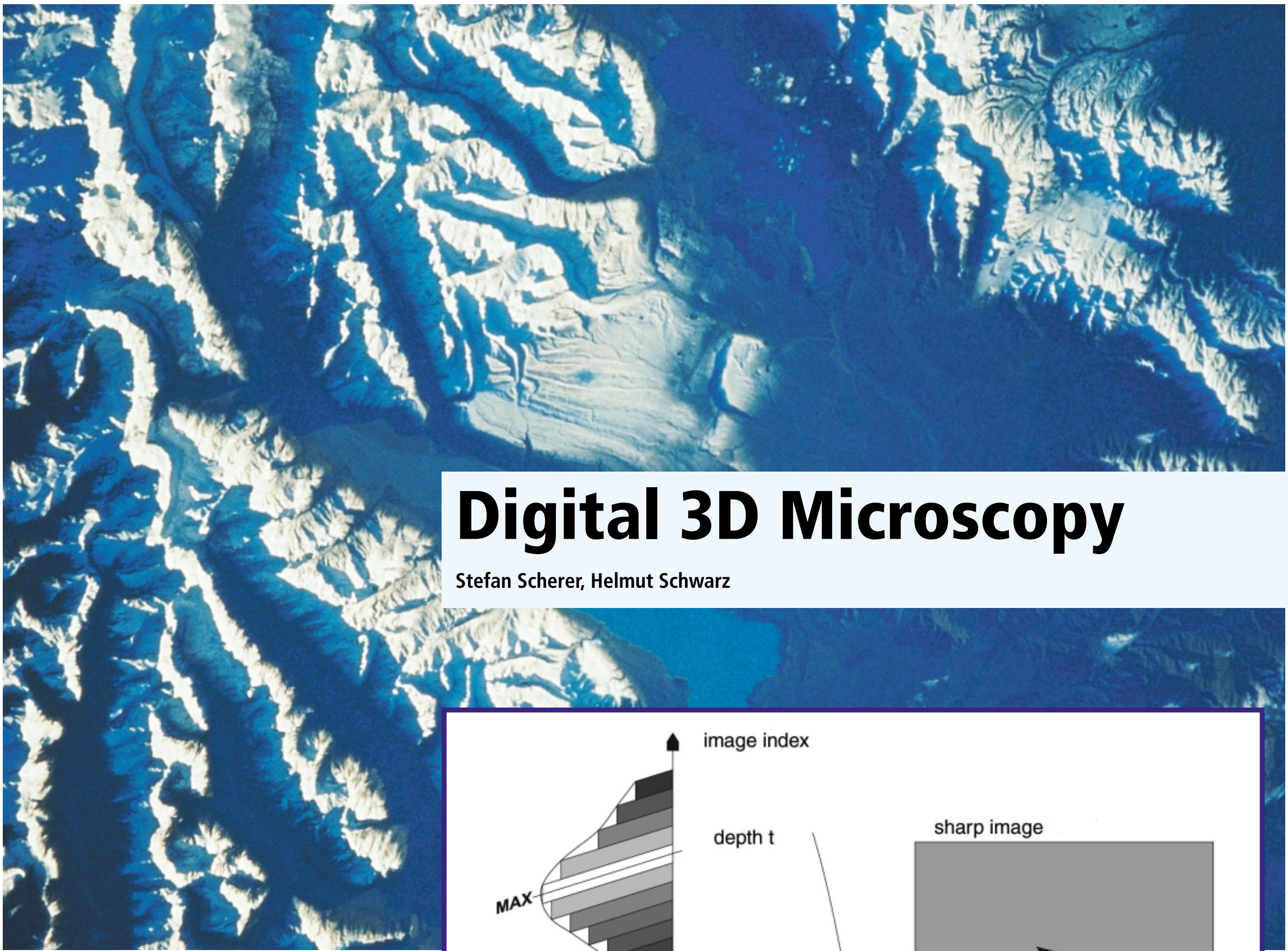

# Digital 3D Microscopy

Stefan Scherer, Helmut Schwarz

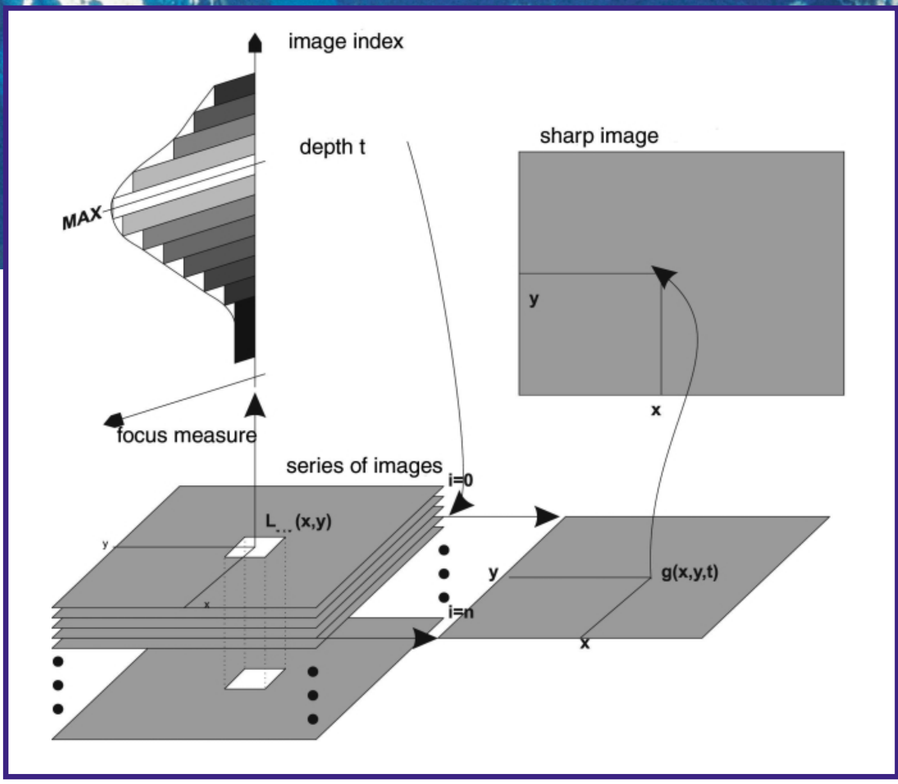


Fig. 1: Function of Shape-from-Focus technique. In every picture the sharpness, as a measure of focus, in every region L is computed. The maximum in combination with the picture index i gives the depth t. In addition, a picture with extended depth of focus can be calculated.

## The conventional light microscope and its weaknesses

**The light microscope is standard in most labs nowadays, and allows to observe objects with a magnification factor of appr. 1000. The depth of focus thereby reduces to about 1 µm. Given this, a meaningful analysis of three-dimensional objects is not possible. Another drawback lies in the fact that microscopists often get fatigued by constantly looking throught the ocular. Last not least in reflective microscopy one frequently is confronted with the problem of "optimal" illumination – if there are changes in the surface properties of the specimen, or if the angle between specimen and illumination is changed, usually the intensity has to be (manually) adjusted. It is exactly these three problems – depth of focus, illumination and observation through the ocular – which this article covers.**

## Extended Focus or Shape-from-Focus

The rapid development of electronic data processing and digital photography lead to new dimensions in microscopy. For example, new methods resulting in a wider depth of focus were presented – given that a digital camera is used for image acquisition. The specimen is moved in direction of the optical axes, and a series of pictures with varying sharpness is taken; subsequently in every region of every picture the sharpness is computed (Fig. 1).

Usually, the sharpness in a region L is defined by the variance of the grey levels.

**Fig 2: a) Picture conventionally acquired. The poor depth of focus and asymmetrical illumination are easy to be seen. b) Picture taken with the fully digital microscope of Alicona. The image is consistently sharp and illuminated over the complete depth of focus. c) The robust and accurate results allow for topographic analysis. Deeper regions are presented by darker grey levels.**

This approach however has two disadvantages: The relation between sharpness and variance of grey values is a heuristic one, and – in practice – local extrema are not identical with the real maximum. In consequence, the computed image contains flaws, which are tolerated by the human eye, but the analytical quantification of the topography can be unsatisfactory.

Alicona Imaging GmbH developed a methodology which minimizes those two drawbacks. Sharpness is modelled as a diffusion equation. The solution of the partial differential equation delivers three parameters; the subsequent determination of their extremes is not based on local maxima, but on the absolute maximum of all calculated sharpness values.

These new, robust algorithms of digital image processing resulted in the development a fully digital microscope featuring 3D quantification. The advanced high resolution CMOS camera eliminates the fatiguing look through an ocular. Based on the FireWire standard, 14 live pictures/sec can observed with a resolution of 1280 x 1024 pixels. Since CMOS-sensors allow direct pixel addressing, subsampled images (640 x 480) can be transferred with a rate of 30 frames/sec.

By using a digitally controlled light source in combination with advanced image processing tools, an optimal illumination of every region of the image is achieved. This means that not only the depth of focus, but also the depth of radiometry is substantially increased.

Fig. 2a demonstrates out-of-focus and unsatisfactory illumination on a specimen with fixed distance to the objective and constant light source. The region shown is 3.3 x 2.6 mm in size, giving a resolution of 2.5 mm. In Fig. 2b the methodology presented was applied. A depth of focus of some 1.5 mm is achieved, whereas the objective used only has one of 0.015 mm!

The topography picture shown in Fig. 2c demonstrates the robustness of the algorithms; lighter grey levels stand for higher "altitudes" than darker ones. Taking advantage of programs similarly used in electron microscopy, now profiles and variables like roughness, surface area and even volumes can be calculated.

The impressive range of possible magnifications (10 to 1000) is achieved by simply changing the objective – the associated resolutions are 6 µm and 0.7 µm, respectively. The possible extended range of focus covers 30 mm at low magnification up to 0.8 mm at the highest one.

## Summary

The application of the most modern algorithms of digital image processing allows to build fully digital microscopes. Besides the reconstruction of images with extended focus, real 3D measurements are possible. Based on latest CMOS technology, flicker-free image acquisition is possible and tiring "staring" trough an ocular are avoided.

**Dr Stefan Scherer, Dr Helmut Schwarz**
Alicona Imaging GmbH
E-mail: info@alicona.com
www.alicona.com