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'Flatten plus': a recent implementation in WSxM for biological research

Alejandro Gimeno[1,2], Pablo Ares[1], Ignacio Horcas[2,*], Adriana Gil[3], José M. Gómez-Rodríguez[1], Jaime Colchero[4] and Julio Gómez-Herrero[1]

[1]Laboratorio de Nuevas Microscopías, Departamento de Física de la Materia Condensada, Universidad Autónoma de Madrid, E-28049 Madrid, [2]WSxM solutions, C Júpiter, 7, E-28042 Madrid, [3]Nanotec Electrónica S.L., Ronda de Poniente 12, E-28760 Tres Cantos, Madrid and [4]Departamento de Física, Facultad de Química. Universidad de Murcia, E-30100 Murcia, Spain

*To whom correspondence should be addressed

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Abstract

Summary: Scanning probe microscopy (SPM) is already a relevant tool in biological research at the nanoscale. We present 'Flatten plus', a recent and helpful implementation in the well-known WSxM free software package. 'Flatten plus' allows reducing low-frequency noise in SPM images in a semi-automated way preventing the appearance of typical artifacts associated with such filters.
Availability and implementation: WSxM is a free software implemented in C++ supported on MS Windows, but it can also be run under Mac or Linux using emulators such as Wine or Parallels. WSxM can be downloaded from http://www.wsxmsolutions.com/.
Contact: ignacio.horcas@wsxmsolutions.com

1 Introduction

Since the introduction of scanning tunneling microscopy (STM) by Binnig *et al.* (1982) and later atomic force microscopy (AFM) (Binnig *et al.*, 1986), Scanning Probe Microscopy (SPM), the general denomination for these techniques, has become a very relevant tool for the life sciences research. SPM can be used to obtain atomically resolved images in different media including ultrahigh vacuum, ambient air and liquids. SPM allows the possibility to study biological systems (Baro *et al.*, 1985; Gould *et al.*, 1988) even in their native environment (Hansma *et al.*, 1992) with unprecedented results. SPM is best known for its ability to acquire images; but in reality SPM also allows many other features, as for instance manipulation of matter at the atomic/nanometer scale (Eigler and Schweizer, 1990), spectroscopy measurement of tip–sample interactions (Burnham *et al.*, 1993) or the characterization of mechanical properties of relevant biological entities including proteins (Rief *et al.*, 1997), cells (Radmacher *et al.*, 1996) and virus particles (Ivanovska *et al.*, 2004).

SPM images are acquired by scanning a very sharp tip on a region of interest. The distance between the tip and the sample surface is in the nanometer range. As in many other techniques there are a number of artifacts that are commonly present even in the best acquired images: noise due to different sources (mechanical, electrical, environmental, etc.), visible slopes in the main plane of an image or tip–sample dilations (Villarrubia, 1997). Thus, the development and improvement of the available processing tools becomes of great importance to ease the visualization and interpretation of SPM images.

Biological samples studied by SPM typically consist of the biological specimens immobilized to an atomically flat, texture-free surface (Wagner, 1998). The way an SPM is operated can make variations from scan line-to-scan line difficult to be avoided (changes in the average height, tilts or low-frequency noise) (Fogarty *et al.*, 2006; Schouterden *et al.*, 1996). Thus, processing filters such as plane and flatten filters are very common when analyzing SPM images. Plane filters fit the raw data image to a general plane or parabolic surface, which is then subtracted from the original image. However, some issues in SPM images such as vertical scanner drift, scanner non-linearities, areas with different slopes or very different heights, etc., cannot be adequately corrected by plane filters. Flatten

works by subtracting a function from each scan line in the raw data image. The simplest function is a zero-order function: the average of each line. Other common possibilities are a first-order function (a straight line) or a second-order function (a parabola). Flatten filters can be used to eliminate slopes, bows and/or bands in the images coming from low-frequency noise.

A raw data image presents different features, and therefore, different heights. If a line is basically flat, and its average is subtracted, the whole line will change the same way, therefore, it will remain uniform (for example, area within the gray rectangle in Fig. 1b). When a line contains very different heights and the average is subtracted, the originally lower parts will be even lower. This causes 'artificial' negative contrast regions (shadowing effects) around the higher parts of the filtered image (area within the black rectangle in Fig. 1b). There are some standard tools to avoid these effects: discard regions, draw master paths, etc. However, these processes can be tedious for images with a large number of features or are hard to put to practical use. There are a good number of high-impact journal publications where, without discussing here the quality of the results there presented, some of the published images show these shadowing effects. Some very recent examples can be found in the following references (Endo *et al.*, 2014; Fisicaro *et al.*, 2014; Jaksa *et al.*, 2014; Kim *et al.*, 2014; Marchi *et al.*, 2014; Miles and Frankel 2014; Moon *et al.*, 2014; Uprety *et al.*, 2014; Wang *et al.*, 2014; Yamamoto *et al.*, 2014).

WSxM is a free software devoted to the control of SPM systems and processing of images and data acquired with such microscopes. It was initially created at the New Microscopies Lab, in the Universidad Autonoma de Madrid (UAM). Then it continued growing at Nanotec Electronica SL, and now it is maintained and developed by WSxM solutions (www.wsxmsolutions.com). A detailed view of WSxM can be found in Horcas *et al.* (2007). Here, we explain in some detail the features of 'Flatten plus', an option recently developed in WSxM that is particularly beneficial for biological data, enabling in a semi-automated way to remove typical artifacts when applying common filters to process SPM images.

2 Implementation

Consider the unfiltered image shown in Figure 1b (left). Just by visual inspection one can easily find the general background. As usual, this simple task is very difficult to identify by a computer. Conventional flatten algorithms consider the lower points of an image as the flat substrate. 'Flatten plus' uses successive approximation algorithms using the information provided by the user through the *Substrate area (%)* parameter. This information can be just an ocular estimation of the percentage of background area as a share of total image area or can include regions to discard. Successive approximation algorithms discard the highest points in the image and apply a flatten process. This is similar to the method of *discarding regions* defined by the user but in this case the regions are automatically defined and they are free in shape and in number. Each iteration discards additional points over the results of the previous step and the remaining points are considered as the flat substrate. This iterative process is repeated until the *Substrate area* value is reached. Figure 1a presents a flow chart of the various steps of the 'Flatten plus' routine. Figure 1b and c shows an example of the difference between usual flatten filters (Fig. 1b right) and 'Flatten plus' (Fig. 1c right) when applied to a typical DNA image. Whereas filtered image in Figure 1b presents shadowing effects around the higher parts, they are not present in Figure 1c.

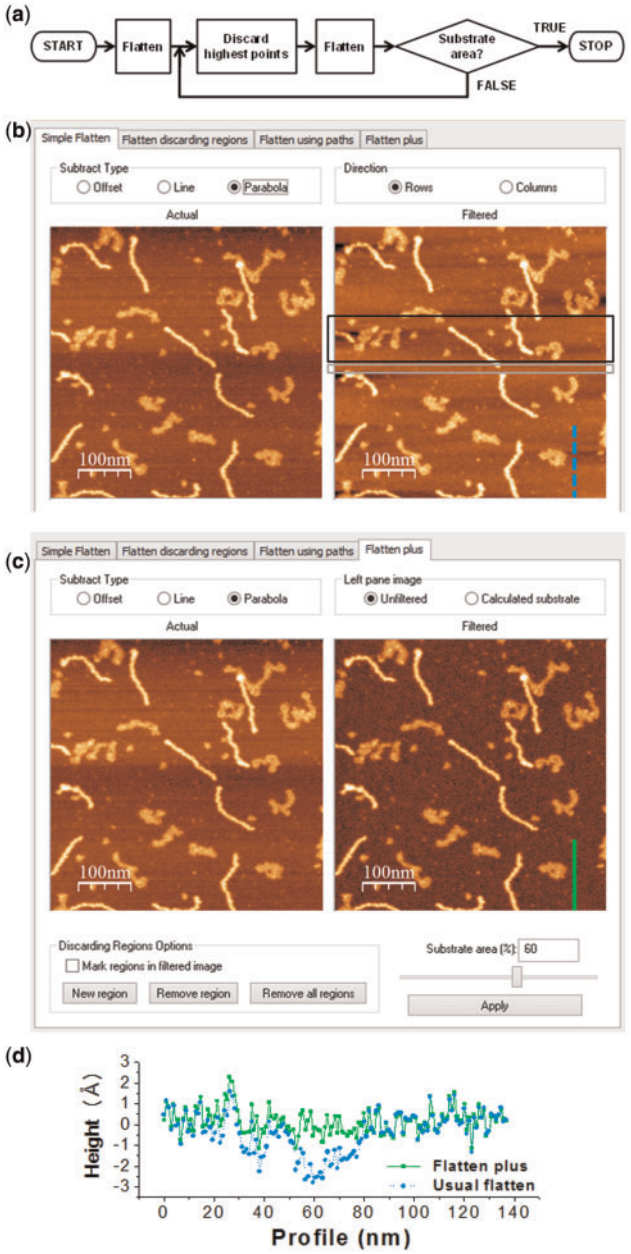

Fig. 1. 'Flatten plus' operation. (**a**) Routine flow chart. (**b**) Usual flatten filter on SMC protein particles co-adsorbed with DNA molecules on a mica substrate. Low-frequency noise of the original image (left) is eliminated after filtering (right). Typical shadowing artifacts are induced. Black rectangle: different heights are present causing shadowing effects. Gray rectangle: region without relevant height differences. Conventional flatten works correctly. (**c**) 'Flatten plus' filter. Low-frequency noise is eliminated with no shadowing effects even in the presence of relevant height differences. (**d**) Height profiles along vertical lines in (b) and (c)

Figure 1d shows height profiles after usual flatten (dashed blue line in Fig. 1b) and 'Flatten plus' (solid green line in Fig. 1c). The background of both images shows the same roughness, but in the center part of the usual flatten profile it can be clearly distinguished a depression coming from an 'artificial' dark region in Figure 1b. Since mica is atomically smooth over relatively large (often micrometer sized) areas, these dark regions correspond to artifacts.

3 Conclusion

We have introduced a relevant implementation in software techniques for SPM. 'Flatten plus' provides a semi-automated filter to eliminate low-frequency noise reflected as slopes, bows and/or bands in images preventing the appearance of undesired shadowing effects which is common in conventional flatten procedures.

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