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# Robust classification of biological samples in atomic force microscopy images via multiple filtering cooperation

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ABSTRACT

The morphological changes induced by engineered nanomaterials (ENMs) in cellular samples are a key clue to evaluate the impact of these materials on human health. The investigation of the complexity of the interaction among nanoparticles and cellular molecules requires cutting-edge instrumentation and dedicated procedures. To this regard, atomic force microscopy (AFM) is a leading imaging technique that has the peculiarities of high resolution and direct relationship with 3D cellular morphology. Expert human operators, however, are still required to manage most of the AFM-based analysis, thus introducing subjective bias and allowing a limited number of trials. The modality of interaction and the resulting topographic differences are often not a priori known. Also, the presence of artefacts in AFM images may affect the derived conclusions. In this work, we propose a robust and flexible strategy to analyse AFM topography images with single click-select actions by the human operator. The proposed system allows for not only morphological studies and quantification of the changes occurring in cellular samples in the presence of nanomaterials, but also for the investigation of diversified experiments in more flexible application domains. As a proof of concepts, samples of human EA. hy926 endothelial cells exposed to carbon nanotubes are used to demonstrate the effectiveness of the proposed solution. The system is also tested against various AFM-artefact and noise scenarios and the robustness of its discrimination capability is verified.



## 1. Introduction

The recent understanding that nanomaterials (NMs) possess new or enhanced properties in comparison to traditional materials has fostered the development of nanotechnology. The NMs are produced with controlled physical-chemical characteristics to be advantageous for novel industrial applications, i.e., engineered nanomaterials (ENMs). However, they might possess properties that are detrimental for both the biological systems and the environment [1]. At present, the assessment of possible adverse effects of ENMs on human health is investigated by means of in vitro and in vivo studies in which, after challenging of cells or organisms with ENMs, morphological and/or functional alterations are evaluated [2]. Bridging the gap between mathematical, biological models, and clinical applications is considered as one of the new challenges of medical image analysis over the last years [3,4,5]. In the field of cellular biology, for example, attempts have been made to distinguish cancer cells from normal ones based on hardness variations of biological materials, or studies have been performed to evaluate the interaction between a specific cell and its neighbouring cells in a competitive culture system, have been made [6].

Atomic Force Microscopy (AFM) is a powerful tool used, in nano-toxicological studies, as a complement to fluorescence and electron microscopy [7,8]. AFM technology provides a three dimensional image of the surface of the test sample, with a resolution of a few nanometers in the plane, and about 0.1 nm in the vertical scale [9]. Unlike standard microscopy, AFM offers insight into the supramolecular organization of cell surfaces (with no need of

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vacuum environment for proper operation or any special treatments such as metal/carbon coatings) [10,11]. The complex interactions between the ENMs and the biological structures are observed, characterized, and interpreted by the operator [12]. Statistical analysis has been used by researchers to explore the characteristics of organic and inorganic materials in AFM images [13] via measurements of surface roughness [14,15,16], grain size distribution [17], and spectral analysis [18]. A wavelet-based methodology [19] has also been proposed for obtaining quantitative measurements of surface topography at different frequency scales. Also, two-dimensional maps of the sample elasticity have been derived to quantify mechanical properties of living cells, such as migration or division, after drug injection [20]. Interactive tools are available to the scientific community to gather the quantitative information from AFM samples [21, 22]. Some of them have been used, for example, for direct measurement of the average end-to-end distance of DNA molecules [23] or to quantify the structural changes induced by magnetic nanoparticles on rod-like liquid crystals [24]. However, most of the analysis of the topography, of the ultrastructure, or of the morphological changes occurring in the cells after exposure to ENMs is performed manually by expert observers [25,26]. It is the case, for example, of recent work showing, for the first time, biomechanical alterations in epithelial cells exposed to carbon nanotubes [27,28]. Although conceptually very promising, AFM analysis often suffers a subjective and time-consuming judgment that strongly limits its reliability and application, and it is therefore labelled as a low throughput procedure.

Computerized methods can facilitate automated AFM image evaluation. However, at our knowledge, a few studies proposed algorithmic solutions to decision problems in AFM images [29,30]. This is due to the difficulty in identifying and quantifying "unique" biomechanical characteristics, and correlate those to the large variety of cell morphology encountered, both in normal and pathological conditions. An additional obstacle to adoption and implementation of computerized systems for AFM is the presence of diverse image artefacts that often are not obvious and that can easily lead to misinterpretation [31]. In particular, distortions of the images may occur due to the AFM scanning apparatus; their peculiarity is being present in different regions of the topography depending on the source of the artefact [32]. Noise contributions randomly distributed on the topography may also occur [33]. Both kinds of image degradation may alter the performance of computerized systems for image analysis.

In the present work, we intend to overcome the limitations stated above by proposing a novel algorithmic solution to a decision problem in AFM images which has never been addressed by previous studies in the literature. With respect to the other existing automated approaches, the present algorithm has been designed specifically to automatically analyse the information encoded within the AFM image topography that are related to the biomechanical alterations of endothelial cells exposed to single-wall carbon nanotubes (SWCNTs). Moreover, our method is capable of automatically providing the assessment of the sample, even in the presence of image artefacts, thus reducing the intervention of the human operator, the subjective bias, and the time requirement. Such aspects have never been addressed prior to this work. The following aspects convey, more specifically, the relevance of the study. 1) For the first time, the AFM topographic imaging is recognized as a major source of knowledge about alterations due to SWCNTs exposure in endothelial cells. 2) The relevant information is automatically extracted from the images and interpreted, without requiring the continuous and laborious intervention of an expert operator. 3) The proposed system has been designed for being robust to various image degradations related to typical AFM artefacts and noise. 4) The analysis strategy is based on a cooperative architecture [34,35] composed by multiple pattern recognition blocks. The pattern recognition blocks are independent analysis systems that receive as input a diverse 2D representation of the recorded AFM topography. The various representations, or filtering maps, are generated by applying different filtering algorithms to the original image in order to convey the information being searched in a diversified and comprehensive way. Once the information is extracted within each pattern recognition block, it is used to train a classification model for the recognition of morphological alterations in the cell. A set of classification scores is hence obtained and a cooperative decision is made via a weighted majority criterion.

First, details regarding the proposed methodology for the analysis of AFM images are provided. Secondly, information on the experimental set-up and data analysis procedures for the study of the effects of SWCNTs in Cell Line EA.hy926 via AFM are given. Lastly, the obtained experimental results are reported, including the results related to the analysis of the system response to various image degradations. To this regard, the benefits of the proposed cooperative architecture will be demonstrated via a series of numerical simulations that synthesize the presence of different sources of artefacts and image degradations due to noise.

## 2. Methods

In Fig. 1 we provide a graphical illustration of the entire system. After the collection of the AFM image and one-click selection of the cell, four fundamental algorithmic steps are performed on the image. 1) *Preprocessing* to speed up the algorithm and to enhance the local properties of the selected cell; 2) Segmentation refinement to confine the region for subsequent analysis. 3) *Parallel filtering* to exploit different representations of the sample under investigation and derive a set of filtering maps to be analyzed. 4) *Morphological characterization* to extract and quantify relevant features from each computed map. 5) *Classification and Cooperative aggregation* to recognize automatically the altered ultrastructure of the exposed cells.

### 2.1. Pre-processing

The AFM images have a spatial resolution of 10 nm/pixel on the xy grid. During digitalization, the images are converted to 8-bit grayscale images with a resolution of 10 nm on the z-direction. The images are then down-sampled on the xy grid at a spatial resolution of 40 nm/pixels to speed up the overall system. A standard bicubic interpolation technique [36] was used to produce a low-order interpolant to the xy data. The bicubic interpolation algorithm was chosen over the bilinear and the nearest neighbour algorithms because it preserves fine details better. A background subtraction procedure is also applied using a 2D surface fitting to emphasize the cell bodies and facilitate cell localization and segmentation (see Fig. 1) [37]. The image is smoothed by applying Gaussian filtering with zero mean and standard deviation $\sigma = 20$ pixels to eliminate high frequency image components that alter the localization of the cell nucleus [36]. The value of $\sigma$ corresponds to 800 nm and it was chosen to approximately bind the region around each cell nucleus in a circular portion of radius equal to $3\sigma$ (see Fig. 2b). Visual inspection of the obtained smoothed images confirmed that the patterns needed for detection of the cellular core regions were captured, while leaving out other fine-scale structures. It is important to note that the preliminary steps needed for cell localization and segmentation refinement are not applied to the subsequent stage of characterization, thus not influencing the derived descriptors.

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