



Scale-independent roughness value of cell membranes studied by means of AFM technique

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ABSTRACT

The roughness of cell membrane is a very interesting indicator of cell's health state. Atomic Force Microscopy allows us to investigate the roughness of cell membrane in great detail, but the obtained roughness value is scale-dependent, i.e. it strongly depends on measurement parameters, as scanning area and step size. The scale-dependence of the roughness value can be reduced by means of data filtration techniques, that are not standardized at nanometric scale, especially as far as biological data are concerned. In this work, a new method, based on the changes of values of some roughness parameter (root mean square roughness and skewness) as a function of filtration frequencies, has been implemented to optimize data filtering procedure in the calculation of cell membrane roughness. In this way, a root mean square roughness value independent of cell shape, membrane micro-irregularities and measurement parameters can be obtained. Moreover, different filtration frequencies selected with this method allow us to discriminate different surface regimes (nominal form, waviness and roughness) belonging to the raw cell profile, each one related to different features of the cell surface.

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1. Introduction

The roughness of plasma membrane is an important cytological parameter, because it is involved in several cellular mechanisms as motility, adhesion, intracellular contact, etc. [1–3] and consequently, it is a sensitive indicator of the cell's health state. Indeed, it has been used to monitor the damages caused to the erythrocytes by artificial organs [4], the action of antimicrobial peptide PGLa on bacteria [5], the changes induced in cardiomyocytes by aldosterone [6] and the red blood cell aging [7].

The use of the membrane roughness as cytological parameter is related to the capability to get high resolution topography of cell's surface. This can be mainly obtained by the Atomic Force Microscopy (AFM) technique, which provides images of cellular surface with a lateral resolution of a few nanometers and a vertical sensitivity of 1 Å without damaging the cellular structure [8–11].

Despite of this, the use of roughness values to characterize cell membrane is not widespread, because of the large variability of roughness value calculated from the height data of cell profiles. In fact, it is well-known that the roughness value is dependent on scanning parameters [12–15]. In particular, it strongly increases with the scanning area, i.e. it is 'scale-dependent'. Indeed, the resolution of the measurement (defined by scan step and probe size), as well as the scan length, might cause the inclusion or exclusion of some surface features. So, the value of roughness parameter in cytological

field is rarely used because of the ambiguity about which surface features it is related to, of its poor reproducibility (for the variability of the cell structure) and of its scale-dependence.

In surface metrology, such problems have been overcome by standardizing as ISO norms the parameters, procedures and instrumentations of roughness measurement [16–18]. In particular, these norms point out a filtration procedure of the height data in order to cut-off the data related to long wavelength (low frequency) sampling and to obtain a profile containing only high frequency data, from which it's possible to calculate the roughness parameters. This means that, using the same cut-off frequency for the filtration procedure, the calculated roughness would be the same at all scan lengths (if the same resolution has been preserved). Unfortunately, the standards defined by ISO norms concern the milli- and micro-scale roughness and no indication exists for the nano-roughness domain. Even if some efforts have been made in the analysis of roughness for inorganic surfaces to adapt the standard methods to the nanoscale regimes [19], no application of this procedure is known in the cytological field yet. In this work, we investigate the possibility to extend the standard method for the calculation of roughness parameters of inorganic surfaces to cellular surfaces, although the variability of structure, size and surface features according to the type of cell prevent a real standardization of membrane roughness measurements. For these reasons, a new procedure for the selection of scanning and filtering parameters is proposed and applied to two different kinds of cell. The obtained results demonstrate that not only the proposed method strongly reduces the dependence of roughness value on the scale length, but it is also useful to characterize different-size features of the cell surface.

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2. Roughness computation theory

In this section, the main items about one-dimensional roughness theory are reported: general definition, computation with and without filtration methods and scale-dependence issues.

2.1. Roughness parameters

The roughness of a real surface is evaluated from the deviations of the two-dimensional measured profile (z values measured by means of the AFM technique on the surface) with respect to an ideal surface. Such deviations can be classified in three groups (which hereinafter will be named 'regimes'), depending on the value of the irregularity steps [20], as shown in Fig. 1, where the total profile (Fig. 1a) includes the contributions of:

- *Nominal form*: corresponding to macroscopic deviations from the mean line profile (nominal shape), cleaned out of the irregularities and characterized by a big sampling step P_f (Fig. 1b);
- *Waviness*: irregularities with large amplitude and step P_w (Fig. 1c);
- *Roughness*: irregularities with small amplitude and step P_r (Fig. 1d).

Several parameters have been defined to characterize the irregularities, all described by the ISO norm 4287 [16]. The most interesting one is the Root Mean Square Roughness R_{RMS} , as it is very sensitive to isolated deviations from a regular profile. It is calculated from the following relation:

$$R_{RMS} = \sqrt{\frac{1}{n} \sum_{i=1}^n |z_i^2|} \quad (1)$$

where n is the number of data points and z_i is the height deviation of i -th point from a mean line, defined so that arithmetic sum of all z_i is equal to zero.

Another useful roughness parameter is the Skewness (R_{sk}), a statistical parameter that measures the asymmetry of the Amplitude Distribution Function, that is the probability density of the profile heights. The skewness R_{sk} is defined according to the following equation:

$$R_{sk} = \frac{1}{(R_{RMS})^3} \frac{1}{n} \sum_{i=1}^n z_i^3. \quad (2)$$

The skewness characterizes very well surfaces with different structures, which could even have the same arithmetic roughness values [21]. In particular, a negative R_{sk} value means that a larger number of valleys with respect to peaks are present on the surface profile, while a positive R_{sk} value means that the presence of peaks is prevailing [20].

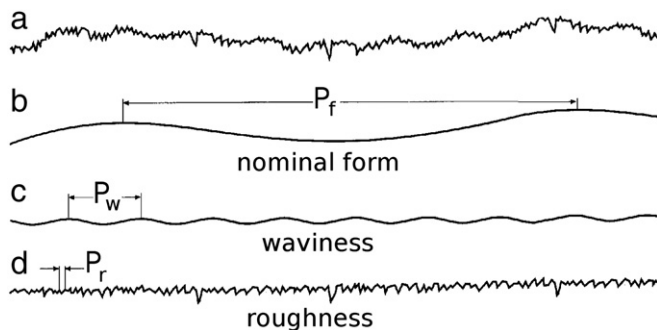


Fig. 1. An example of a graph describing a generic line profile (a) consisting of the overlapping of (b) the nominal form, (c) the waviness and (d) the roughness. In b), c) and d) the sampling step is indicated as P_f , P_w and P_r , respectively.

2.2. Digital filtering theory applied to roughness computation

The calculation of the roughness parameters from the height data measured by means of the AFM technique (raw data) provides values containing information of all kinds of irregularities discussed above. A proper application of digital filtering helps to separate the features related to the nominal form (low frequency information) from those of the waviness and roughness (medium and high frequency information) respectively. To operate a proper data filtration, it is particularly important to choose a suitable cut-off frequency f_c .

The general filtering procedure used in surface metrology is based on the use of Discrete Fourier Transform (DFT), which is applied to the M data points measured along a scan line of length L (with a scan step $\Delta x = L/M$). The transfer function $H(\omega)$, which contains all the signal frequency components, is expressed in terms of the normalized frequency ω_p (with $p = 0, \dots, M-1$; $\omega_p = p/(\Delta x M)$) in the following way:

$$H(\omega_p) = \sum_{k=0}^{M-1} h(k) \exp(-j2\pi \left[\frac{p}{M} k \right]). \quad (3)$$

The transfer function is designed to form high-pass, low-pass, band-pass and band-stop filters with appropriate cut-off frequencies, just depending on the desired frequency components.

The function $h(k)$, which is the inverse DFT of $H(\omega)$, is a filter impulse response function.

$$h(k) = \sum_{p=0}^{M-1} H(\omega_p) \exp(j2\pi \left[\frac{p}{M} k \right]) \quad (4)$$

From the convolution of the impulse response with the original data matrix $z(x)$, it is possible to obtain the final filtered profile $f_{filtered}(x)$, from which the roughness parameters can be calculated.

$$z_{filtered}(x_i) = z(x_i) \times h(x_i) = \sum_{k=(-\frac{M}{2}+1)}^{\frac{M}{2}-1} h(k) z(x_{i+k}) \quad (5)$$

with $i = 0, \dots, M-1$ [19,20].

A critical point for evaluating the roughness parameters is a proper choice of the scanning parameters, as scanning length and sampling step, in order to measure the desired features.

The scanning parameters are also important in the filtering procedure since, in spatial frequency components, the extent of spectral range of the Fourier space is defined by Δx and L . In fact, the low and high frequency limits of the spectral range are [15] $\omega_l = 1/M\Delta x$ and $\omega_h = m^{-1}/2\Delta x$ (Nyquist limit), respectively, where m is the frequency index varying between 0 and $M/2$.

The low frequency limit, ω_l , is determined by the length of the profile $L = M\Delta x$ and it plays a fundamental role in the problem of the scale-dependence. In fact, the longer the scanning length, the more the features included in the length profile, the larger the computed R_{RMS} value. For each analysed profile, there will be a length value that will include all the bigger surface features: for length values larger than this one, the R_{RMS} will remain quite constant. This is just the result obtained when R_{RMS} values of raw unfiltered data are calculated [13,14]: in this case the R_{RMS} includes contribution from all the three surface regimes, among which the larger ones (that is waviness and nominal shape) dominate.

On the other hand, the high frequency limit, ω_h , is related to scan step value Δx , which depends on the instrumental resolution. In fact, it is quite obvious that the AFM microscope has to be calibrated to resolve the scan step size. So, if the curve radius of the tip is larger than Δx , the tip's geometry acts as a low pass filter by cutting out the high spatial frequencies of the surface morphology [15].

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