A new automatic contact point detection algorithm for AFM force curves

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Abstract

A new method for estimating the contact point in AFM force curves, based on a local regression algorithm, is presented. The main advantage of this method is that can be easily implemented as a computer algorithm and used for a fully automatic detection of the contact points in the approach force curves on living cells. The estimated contact points have been compared to those obtained by other published methods, which were applied either for materials with an elastic response to indentation forces or for experiments at high loading rates. We have found that the differences in the values of the contact points estimated with three different methods were not statistically significant and thus the algorithm is reliable. Also, we test the convenience of the algorithm for batch-processing by computing the contact points of a force curve map of 625 (25 × 25) curves.

1 Introduction

Atomic force microscopy (AFM) is one of the experimental methods that are currently used to measure mechanical properties of biomaterials and forces between surfaces or molecules. AFM is a very versatile experimental tool. It allows the modification of the AFM tips or colloidal probes; it can work in air or in different solvents, and permits to vary parameters such as loading rate or the applied force for adhesion and viscoelastic experiments.

However, when we compare AFM with other experimental methods used to measure surface-surface interactions based on interferometry, like the thin film balance or the surface force apparatus, the AFM presents a major disadvantage: it does not measure
absolute distances. In other words, the separation between the AFM-tip and the surface of interest has to be derived from the position, $z$, of a moving piezoelectric scanner attached to either the AFM-tip or the surface. Therefore, we do not know “a priori” where the contact point or zero distance (i.e. the surface) is.

Since force measurements can provide different information like surface charge [5], polymer elasticity [6], molecular unbinding forces [7] or cell viscoelasticity [8], researchers and companies [9] are still proposing and programming algorithms to extract as much information as possible from force measurements. For example, Kuhn et al. [10] report on an algorithm, written in C++, that implements polymer models that are directly applied on transmembrane proteins. The algorithm detects different unfolding pathways and also classifies erratic points. In a similar topic, Baumgartner et al. [11] propose an algorithm to quantify the interaction between the AFM-tip and mica as well as the extent of cadherin unbinding events taking into account possible artifacts and the signal-to-noise ratio. More recently, Crick and Yin [12] have addressed the problem of the contact point (taking into account the noise contribution) with the goal to implement the information about mechanical properties of linear elastic materials of different Young moduli. Concerning the contact point determination in soft materials (polymer brushes), Melzak et al. [13] proposed two different methods, one based on the so called “eye inspection” assuming that the sample behaves elastically (PE), and the other based on an extrapolation to zero load of the sample deformation (ED).

The spread of the use of the AFM as a tool for determining mechanical properties of the sample has led to the development of new techniques that need a large amount of measurements to be analyzed (e.g. mapping of mechanical properties of a surface [14]). Such large amount of force-curves is very hard to be studied one by one and developing algorithms for batch processing is thus a necessity.

In this paper, we present a method for detecting the contact point in force curves that can be easily implemented in a computer algorithm allowing us to automatize the force curves analysis. This method, which can be regarded as a mathematical description of the “eye inspection” method, is a combination of local regression fits of the force curve and a threshold analysis (“double alarm” method) and, as we will see below, the results obtained are very promising.

2 Materials and methods

2.1 Cell preparation

MCF-7 cells were grown at 37°C and 5% CO$_2$ in Dulbecco’s modified eagle medium (DMEM, Sigma) supplemented with 8% fetal bovine serum (FBS, Sigma), 2% 200 mM L-glutamine, 0.4% penicillin/streptomycin (PEN/STREP, Sigma). For the individual force measurements, the cells were subcultured on borosilicate glass coverslips (24 mm in diameter and 0.16 mm thick) at a density of 14K and 25.4K cells/ml for three days; for the force map, the cells were seeded on a similar coverslip at a density of 25K cells/ml and incubated for one day. The glass coverslips were previously sonicated in an aqueous solution (2%) of Hellmanex® (Hellma, Germany), plasma cleaned in Argon (Harrick, USA) and either heat sterilized (2 hours at 180°C) or immersed in absolute ethanol in the case of the force map. Just before the experiments, the cells were immersed in CO$_2$-independent cell medium (Leibowitz medium, L15, Sigma) and thermalized at 37°C.

2.2 Force measurements

Force measurements were carried out in L15 medium and at 37°C with a Nanowizard II (JPK Instruments, Germany), coupled with the CellHesion module® (JPK Instruments, Germany) and a transmission optical microscope (Axio Observer D1, Zeiss, Germany). We employed gold backside-coated SiN cantilevers with a nominal spring constant of 0.006 N/m (MLCT,
Veeco Instr. - now Bruker -, USA). Prior to the force measurements, the cantilevers were cleaned either in ethanol and acetone or alternatively in Argon plasma (Harrick, USA) and their spring constants were evaluated by the thermal method. The cell-coated coverslips were mounted in a low-volume cell incubator (Biocell®, JPK Instruments, Germany) with approximately 400 µl of L15 medium. Individual force curves were then performed on ten different MCF-7 cells from two coverslips at a constant approach-withdrawal rate of 5 m/s and at different maximum loads in the range of 0.2-4 nN.

2.3 Force curve map

The area enclosing two MCF-7 cells (53.8 x 53.8 mm²) was divided into 25 x 25 pixels and force curves were performed at each pixel. The maximum load as well as the approach-withdrawal rate were kept constant for all curves and set to 2 nN and 5 µm/s, respectively.

2.4 Data processing

The local regression algorithm for the contact point detection was implemented in a custom Matlab® code (Mathworks Inc.). The statistical analysis and the contact point maps figure (Figure 3) were done with the R free statistical software (www.r-project.org). All computations were performed on an Intel Core i5® personal computer.

3 Local regression and contact point detection algorithm

In theory, an SPM force curve is a function, \( F = f(z) \), that gives the force measured by an SPM microscope, \( F \), as a function of the vertical position, \( z \), of either a moving tip or a moving sample. In practice, however, we can only sample the curve at a fixed frequency, and thus we always work with a finite sequence of \( n \) values, \( F_i \) and \( z_i \). The force is in addition perturbed with some noise, \( \epsilon_i \):

\[
F_i = f(z_i) + \epsilon_i, \quad i = 1, \ldots, n. \tag{1}
\]

An AFM experiment is divided into two distinct stages, each one with a different characteristic force curve. The first is the “approach curve”, in which the tip and the sample are approaching and make contact and the second one is the “retract curve”, in which the tip withdraws from the sample and adhesion forces may appear.

The objective is to detect the contact point in the approach curve. Such contact point is the \( z \) value at which the tip reaches the surface of the sample. Beyond the contact point, the force increases and therefore the slope of the force curve is higher. Therefore, a significant change in the curve should be observed at the contact point, so we expect to detect a significant variation (i.e. a variation that cannot be attributed to the noise) in the signal or in its slope. To this aim we adapt a local polynomial regression algorithm developed by Qiu and Yandell [15], which was applied to measure jumps in the atmospheric sea pressure level in a time series between the years 1921 and 1992 [16].

From the force signal, another discrete signal \( b \) is generated. The magnitude \( b_i \) is the slope of the regression line that fits the \( F - z \) data in a window of radius \( l \) centered in \( z_i \) (see Figure 1). That is, at every \( z_i \) where it is possible to define such window (i.e. for \( l + 1 \leq i \leq n - l \)), we construct a local set of data \( N(z_i) := \{z_{i-l}, \ldots, z_i, \ldots, z_{i+l}\} \), and fit a line, \( f = b_i z + a_i \), to the corresponding \( F - z \) values. For the sake of simplicity, we will consider that the slope signal \( b \) remains constant at those values where it is not possible to define the data window. That is, we take \( b_i = b_{i+l} \) for \( i \leq l \) and \( b_i = b_{n-l} \) for \( i \geq n - l + 1 \).

*This method should not be confused with the well-known LOESS/LOWESS method for local regression and scatterplot smoothing used for curve fitting (see [16]).
Figure 1: Scheme of the Local Regression method. (a) Given a force curve, for each \( z_i \) a line is fitted in the data window \([z_{i-l}, z_{i+l}]\) (in the figure \( l = 5 \)). (b) A signal \( b_i \) is defined as the slope of the line fitted in the window centered at \( z_i \). (c) The signal \( \Delta_i \) is defined as the differences of two consecutive values of the slope signal \( (\Delta_i = b_i - b_{i-1}) \). Note that the first and the last \( l \) values of signals \( b_i \) and \( \Delta_i \) are constant and zero, respectively. This is because if the center \( z_i \) is within these ranges of values, there is not enough data to build the whole local regression window. These values are thus not considered in the determination of the contact point.

To detect sudden changes in the signal we need to detect significant variations in the sequence \( \{b_i\}_{i=1}^n \) (Figure 1b). For this purpose we define the difference sequence \( \{\Delta_i\}_{i=1}^n \), as

\[
\Delta_i = b_i - b_{i-1}, \quad i = 2, \ldots, n, \quad \Delta_1 = 0.
\]

If the force curve has a constant slope then \( \Delta_i \) is always zero, and any variation in the slope will be reflected in a nonzero value of \( \Delta_i \), (see Figure 1c). We thus need to determine whether a nonzero \( \Delta_i \) value is caused by a significant change in the slope or it is just the effect of the noise of the signal.

We will then use this local regression to construct an algorithm that let us determine the contact point of a force curve. The latter must fulfill three requirements: the first one is that the contact point is unique so it is given by the first significant change in \( \Delta_i \). The second one is that the contact point is not located near the beginning of the data sequence; the first \( M \) data points of the sequence are considered to belong to the so-called baseline, where the tip and the sample are not in contact and the force is zero \(^2\) (within the experimental noise). The third one is that standard deviation of \( \Delta \) in this \( M \) first values, \( \sigma \), gives us an estimation of the experimental noise. Thus, \( M \) should be large enough for \( \sigma \) to be a good estimate of such experimental noise.

The algorithm is based on a double alarm method. Two thresholds \( \tau_1 \) and \( \tau_2 \), are set in the \( \Delta \) signal, \( 0 \leq \tau_1 < \tau_2 \), both proportional to \( \sigma \) (see Figure 2). If \( \Delta_{i_2} \) is the first value of the sequence \( \Delta \) such that \( |\Delta_{i_2}| > \tau_2 \), then a significant change in \( \Delta \) has already occurred. Note that from the shape of the approximation curve we can infer that \( \Delta_{i_2} \) for an appropriate (large enough) \( \tau_2 \), and so, condition \( \Delta_{i_2}^2 > \tau_2 \) is in fact \( \Delta_{i_2} < -\tau_2 \).

\(^1\)More sophisticated difference-type operators could be used (see [15]), but for the case of AFM curves, after analyzing more than 700 curves, experience shows that the simpler choice given in (2) offers good results.

\(^2\)This requirement implies that there are no non-contact interactions.
Figure 2: The “double alarm” algorithm. The plot above shows the signal $\Delta$ in the whole range of $z$ for a typical case example. The plot below is a zoom of the previous showing the signal in the region of interest. The first threshold alarm $\tau_1$ is set to coincide with the noise amplitude $\sigma$, and the second threshold alarm $\tau_2$ is $10\sigma$. Once the signal $\Delta$ reaches the second threshold alarm, $z_{i1}$ is determined as the last previous point where $\Delta$ overcomes $\tau_1$. The arrow indicates the direction in which the data is processed by the algorithm.
At this stage we need to determine the value of $z$ at which such significant change happened. To this aim we have to find the last previous point where the signal $-\Delta$ overcomes $\tau_1$. Let us define $i_1$ as an index verifying the following conditions (see Figure 2):

\[
\begin{align*}
  i_1 &\leq i_2, \\
  \Delta_i &\leq -\tau_1, \quad i_1 \leq i \leq i_2 \\
  \Delta_{i_1} &> -\tau_1.
\end{align*}
\]

Once determined the index $i_1$, the contact point is estimated by $z_{cp} = z_{i_1} + l$.

As a matter of fact, the contact point is considered as the value of $z$ where the signal $\Delta$ takes exactly the value $\tau_1$, but it is highly unlikely that this value should coincide with an experimental point. Thus the determination of the contact point can be refined by interpolating the values of the signal $\Delta$ between the points $\Delta_{i_1} - 1$ and $\Delta_{i_1}$, and computing the value of $\lambda$, with $0 < \lambda \leq 1$, for which $\lambda \Delta_{i_1} + (1 - \lambda) \Delta_{i_1} - 1 = -\tau_1$, i.e. $\lambda = (\tau_1 + \Delta_{i_1} - 1) / (\Delta_{i_1} - 1 - \Delta_{i_1})$. The contact point is then the interpolation value $z_{cp} = \lambda z_{i_1} + l + (1 - \lambda) z_{i_1} + l - 1$.

4 Results and discussion

4.1 Tuning the parameters

As mentioned before, the method has three main parameters that need to be properly set for a correct estimation of the contact point: $l$, $\tau_1$ and $\tau_2$. To illustrate the effect of these parameters in the computation of the contact point, we determine the contact points in a set of 25 x 25 approach curves within an area enclosing two cells. Figure 3 shows four maps of contact points obtained with different values of $l$ and $\tau_1$. The parameter $\tau_1$, the first alarm threshold, is crucial to allocate the position of the contact point. The effect of $\tau_1$ is reflected in the appearance of the map. If this parameter is set too small, the sensitivity of the method increases to the point of detecting non-significant variations in the signal, and thus the cell surface appears rougher (Figures 3a and 3c), while for large values of the threshold $\tau_1$, the surface looks smoother (Figures 3b and 3d). Our experience on data analysis of this type of force curves (see footnote †, page 4), shows that a good choice of $\tau_1$ is $\sigma \leq \tau_1 \leq 4\sigma$, although in cases when the noise of the $\Delta$ signal is small (e.g. the noise of the original force curve is small or the radius $l$ is big), smaller values of $\tau_1$ can be considered, even $\tau_1 = 0$.

Recalling that the parameter $l$ is the radius of the moving window in which local regressions are calculated, we have that for small values of $l$, the image sharpness increases (Figures 3a and 3b), while as $l$ increases, the image sharpness decreases (Figures 3c and 3d). The bigger $l$ is, the less noise the $\Delta$ signal has, but the algorithm performance will be less sensitive to the changes in the force signal. Thus, if the force varies smoothly across the contact point, then the radius does not need to be very big. The parameter $\tau_2$ is the second alarm threshold and its effect on the determination of the contact point is very small. It must be large enough to be unaffected by the $\Delta$ signal noise, but not so big that the contact point remains undetected. While $\tau_2$ is kept within this range, the value of the contact point will not be affected. As an example, $\tau_2$ was set to $15\sigma$ for the curves of the map (Figure 3) and it was set to $10\sigma$ for the individual force curves (Figure 6).

4.2 Comparison with other methods

To test the validity of our method, the results of the Local Regression algorithm (LR) here reported are compared with two other methods previously described in [13]. These methods are based on different aspects of the mechanical behavior of the sample. The first method, Pure Elastic (PE), is based on the assumption that the sample behaves elastically beyond
Figure 3: Effect of the parameters in the contact point determination. Four 25 × 25 pixel contact point maps for the same two-cell cluster with different values of the parameters \( l \) and \( \tau_1 \) are pictured. The four maps have been drawn using a contour level plot with the same color scale for comparison purposes. For small values of the window radius \( l \), the edges of the image are sharper (plots (a) and (b)), while as increases, the image is more blurred (plots (c) and (d)). The effect of \( \tau_1 \) is reflected in the noise of the signal. For small values of \( \tau_1 \) (plots (a) and (c)) the cell surface looks rougher, while for large values of the threshold \( \tau_1 \), the surface is smoother (plots (b) and (d)). The vertical color bar represents both the contact point (right) and the height of the cells adhered to the substrate (left).
Figure 4: Another plot about the effect of the parameters in the contact point determination, in the same case of Figure 3.
the contact point, so a proper choice of this point provides the best linear fit of the force versus the square of the deformation. The second method, Extrapolated Deformation (ED), is based on an extrapolation to zero load of the sample deformation relative to an experimental value. Figure 5 depicts the three methods (see [13] for a detailed description of PE and ED methods).

As experimental data we consider a total of 89 approach curves performed on ten different cells at various maximal loads ranging from 0.06 to 3.96 nN. For each curve the contact point was determined with the three methods (PE, LR, ED). For the sake of comparison and since the experimental loads are not identical for all cells, the maximum loads were factorized in 0-1, 1-2, 2-3 and 3-4 nN classes, and represented by the class midpoint, that is, 0.5, 1.5, 2.5 and 3.5 nN, respectively. The contact points were then averaged for each one of the load classes and the resulting averages numerically plotted as shown in Figure 6.

To test if the differences between the three methods were statistically different, a two-way ANOVA was performed, being the contact point the dependent variable and the method and maximum load, the factors (for a detailed description of the two-way ANOVA please refer to [17, p. 763]). The analysis showed no interactions between the factors and no significant differences between either the method ($F = 0.003$, $P > 0.997$, $df = 2$) or the maximum load ($F = 0.696$, $P > 0.555$, $df = 3$).

5 Conclusions

The Local Regression (LR) method presented in this paper is fast and reliable. The number of operations needed to compute the $\Delta$ signal is of order $O(nl^3)$, and since $l$ is much smaller than $n$, for typical force curves on living cells, the overall number of operations is of order $O(n^2)$. As an example, for the curves used in obtaining Figure 3, the average number of points in each curve was about 850, and it took an average of 0.2 seconds to obtain each contact point with the computer described in Section 2. The “double alarm” method used for obtaining the contact point has proven to be accurate, providing good contact point estimates in force - displacement curves in the absence of non-contact interactions. This method is an algorithmic description of the “eye inspection” and thus can be incorporated in AFM data analysis software to batch-process large amounts of curves. Also, this method can be used as an initialization of the Pure Elastic (PE) method, which requires an initial estimation, usually given by “eye inspection”. Therefore, the LR method introduced here provides also a straightforward way to automatize the PE method. Moreover, it is worth to note out that the LR method is more versatile since it can be applied to both elastic and non-elastic samples.

Finally, as an outlook for future work, we should mention that the algorithm presented here is a useful tool to detect not only contact points, but also any significant changes in the shape of the force curve, and so it can be used to automatically detect other important features of the AFM force curves like baselines calibrations, protein unfolding events, and adhesion areas in the retract curves.

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§ $F$ is the so called $F$ statistic whose value is compared to the $F$-distribution. The $P$ value is the probability of obtaining a test statistic at least as extreme as the one actually observed. $df$ stands for degrees of freedom.
Figure 5: Schematic representation of the three methods. ED (top plot) uses several measurements at different maximum loads and extrapolate the results to zero value. PE (center plot) considers a pure elastic response $F$ vs $(\text{Indentation})^2$ and finds the point for which there is a best fit (maximum $R^2$). LR (bottom plot) finds the point in which the slope of the force curve begins to increase overcoming some thresholds.
Figure 6: Results of the comparison of the three methods used for estimating the contact point. The plots show $z_{cp}$ as a function of the load for ten different cells. The three methods (ED, LR and PE) showed no statistically significant differences (see text) in spite of some punctual discrepancies (i.e. particularly noticeable in cell number 5).

References


